Regulation of Glucose Uptake by Muscle

2. THE EFFECTS OF INSULIN, ANAEROBIOSIS AND CELL POISONS ON THE PENETRATION OF ISOLATED RAT DIAPHRAGM BY SUGARS*

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The utilization of glucose by muscle is thought to be limited by the rate at which glucose enters the muscle cell and insulin is believed to stimulate glucose uptake by speeding its entry (Levine & Goldstein, 1955; Park, Bornstein & Post, 1955; Park & Johnson, 1955). We have shown that the uptake of glucose by isolated rat diaphragm incubated in a bicarbonate-buffered medium is increased by anoxia and by substances which inhibit oxidative phosphorylation, as well as by insulin (Randle & Smith, 1957, 1958). These observations led us to suggest that the entry of sugars such as glucose into the muscle cell is restrained under basal conditions by a process dependent upon a supply of energy-rich phosphate. We have now attempted to obtain more direct evidence in support of this suggestion by studying the effects of insulin, anaerobiosis and substances which inhibit oxidative phosphorylation on the accumulation of glucose and xylose in isolated diaphragm.

We will express the amount of glucose or xylose accumulating in diaphragm as a space, the glucose or xylose space being that fraction of the volume of the tissue which appears to contain fluid of the same specific gravity and glucose or xylose content as the incubation medium. Interpretation of these results in terms of the distribution of sugar between extracellular and intracellular water involves comparing the glucose or xylose space with the volume of extracellular fluid held by the tissue. Glucose or xylose are deemed to be present in intracellular water only if the glucose or xylose space exceeds the volume of extracellular fluid. The volume of extracellular fluid may be estimated by measuring the space occupied by substances which are believed not to enter the cells under normal conditions. We have used both inulin and the thiosulphate ion for this purpose, though we have found, as others have (Kipnis & Cori, 1957), that thiosulphate yields higher values for the volume of extracellular fluid than does inulin. The reliance that can be placed on either the thiosulphate space or the inulin space alone is therefore uncertain and the conclusions which we draw are, for the most part, based on both estimates of the volume of extracellular fluid. In most of the experiments we have used a diaphragm preparation, first described by Kipnis & Cori (1957), in which no muscle fibres are cut. The advantage of this preparation is that its spaces are apparently similar to those of the muscle *in vivo* (Kipnis & Cori, 1957).

* Part 1: Randle & Smith, 1958.

METHODS AND PROCEDURE

Incubation media. The composition of the basal media is given in Table 1. A.R.-grade chemicals were used if commercially available. Inulin and D-xylose were obtained from T. Kerfoot and Co. Ltd.

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

Media containing bicarbonate were gassed with O_2+CO_2 or N_2+CO_2 (95:5); pH 7·4 at 37°. Phosphate medium was gassed with O_2 or N_2; pH 7·4 at 37°. When glucose or thiosulphate space was measured D-glucose was added (2·5 mg./ml.); for xylose space, D-xylose (1·5 or 5 mg./ml.) and D-glucose (1 mg./ml.) were added; inulin space was measured in the presence of inulin (2 or 4 mg./ml.) and D-glucose (1 or 2·5 mg./ml.). 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	Cl	HCO_3	PO_4*	SO_4	S_2O_3	
Bicarbonate‡	124·8	5·2	2·7	2·6	105·7	27·2	1·8	0·6	—	281
Bicarbonate–thiosulphate I	134·8	5·2	2·7	2·6	75·7	27·2	1·8	0·6	40	281
Bicarbonate–thiosulphate II	151·5	5·2	2·7	2·6	92·4	27·2	1·8	0·6	40	314
Phosphate	143·2	5·1	2·7	2·6	132·8	—	18·2	0·6	—	309
Phosphate–thiosulphate	169·9	5·1	2·7	2·6	119·5	—	18·2	0·6	40	342
High phosphate–bicarbonate	133·0	5·2	2·7	2·6	77·9	27·2	37·8	0·6	—	281

* Assuming valency of 1·8 at pH 7·4.
† Calculated for media containing 2·5 mg. of glucose/ml. or its equivalent.
‡ Gey & Gey (1936).

Cut-diaphragm preparation. Hemidiaphragms were prepared and incubated as described in the preceding paper (Randle & Smith, 1958). After incubation each hemidiaphragm was removed from the flask, and lightly blotted, and weighed on a torsion balance and frozen immediately on an aluminium planchet resting on solid carbon dioxide.

Intact-diaphragm preparation. Animals were killed by decapitation and exsanguinated. The skin and fascia were dissected from the thoraco-abdominal wall, and the ribs and abdominal wall on either side of the diaphragm were divided, and the viscera separated from the diaphragm and the vertebral column transected above and below the diaphragm. The diaphragm, with its attachments to spine, ribs, xiphisternum and costal cartilages intact, was then washed in the appropriate incubation medium with continuous gassing. Each diaphragm was then slid into a 50 ml. conical flask containing 10 or 20 ml. of medium. The flasks were gassed with the appropriate gas or gas mixture, sealed with rubber stoppers and incubated with shaking in a water bath at 37°. Incubation was for 1 hr. unless otherwise stated. The tissue was then removed from the flask, the diaphragm (excepting the posterior portion) excised from the rib cage and blotted, and weighed and frozen as described for the cut preparation. If more than one analysis was made on the same diaphragm, the muscle was divided along the central tendon and the two hemidiaphragms were weighed and frozen separately.

Analytical methods

Glucose, xylose or inulin. Analyses of muscle for these substances were made on extracts prepared by grinding the frozen muscle rapidly to a paste with sand in a mortar, and adding 3 ml. of water and deproteinizing with $Zn(OH)_2$ (Somogyi, 1945). Samples of medium after incubation were suitably diluted and deproteinized in the same way. Glucose was determined photometrically with glucose oxidase, peroxidase and *o*-dianisidine (see Randle & Smith, 1958), xylose photometrically by the *p*-bromoaniline method (Roe & Rice, 1948) and inulin photometrically after hydrolysis and reaction with resorcinol (Roe, Epstein & Goldstein, 1949).

Total water and thiosulphate. These were determined in the same sample of muscle. The sample was weighed wet, and freeze-dried and reweighed. The dried tissue was then ground to a powder with sand in a mortar and deproteinized by addition of 5 ml. of tungstic acid (Van Slyke & Hawkins, 1928). Samples of medium after incubation were similarly deproteinized. Thiosulphate in the extract was then assayed iodometrically (Newman, Gilman & Philips, 1946):

$$\text{Space (ml./100 g.)} = \frac{\text{muscle content (g./100 g. of wet muscle)}}{\text{medium concentration (g./ml.)}}.$$

$$\text{Total water (ml./100 g.)} = \frac{\text{wet wt.} - \text{dry wt.}}{\text{wet wt.}} \times 100.$$

Other experimental details and the statistical methods have been given in the preceding paper (Randle & Smith, 1958).

RESULTS

Parameters of isolated diaphragm

(1) *Total water content.* The intact preparation always contained 74 ml. of water/100 g. of wet tissue, whether incubated in bicarbonate medium or bicarbonate–thiosulphate medium I or II (Table 2) or in phosphate–thiosulphate medium (Table 4). The cut preparation incubated in bicarbonate–thiosulphate medium II also contained 74 ml. of water/100 g. The total water content of the cut preparation incubated in bicarbonate medium was not determined in these experiments, though the same value of 74 ml./100 g. had previously been found by drying at 110° (Randle, 1956). Values of 77–78 ml./100 g. were obtained by Creese (1954) and Kipnis & Cori (1957) by drying at 100° or 105°.

(2) *Extracellular volume (thiosulphate and inulin spaces).* The thiosulphate space of intact diaphragm was 21–25 ml./100 g. (Tables 2 and 3). The inulin

Table 2. *Parameters of isolated diaphragm*

For composition of media see Table 1. The number of observations is given in parentheses.

Medium	Parameter	Space. Mean ± S.E.M. (ml./100 g. of wet diaphragm)	
		Cut diaphragm	Intact diaphragm
Bicarbonate	Total water	(74*)	74±0·7 (6)
	Inulin space	30±1·1 (5)	12±0·7 (11)
	Glucose space	—	13±0·7 (5)
	Xylose space	52±0·7 (3)	25±0·3 (5)
Bicarbonate–thiosulphate I	Total water	—	74±0·6 (6)
	Inulin space	—	12±0·7 (5)
	Xylose space	—	28±1·0 (6)
Bicarbonate–thiosulphate II	Total water	74±0·6 (12)	74±0·2 (51)
	Thiosulphate space	37±0·9 (12)	23±0·3 (39)
	Inulin space	—	14±0·5 (6)
	Glucose space	29±0·8 (12)	14±0·2 (5)
	Xylose space	—	34±0·7 (6)
High phosphate–bicarbonate	Xylose space	—	32±1·4 (5)

* Determined by drying at 110° by Randle (1956).

Table 3. *Influence of insulin, anaerobiosis or cell poisons on the parameters of intact diaphragm incubated in bicarbonate-buffered medium*

Diaphragms were incubated in bicarbonate–thiosulphate medium II (for composition see Table 1). Glucose uptake, total water and thiosulphate and glucose spaces were measured in the same experiment; inulin spaces were measured separately. The number of observations is given in parentheses.

Expt. no.	Addition	Space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)				Glucose uptake. Mean ± S.E.M. (mg. of glucose/hr. of incubation)
		Total water	Thiosulphate	Glucose	Inulin	
1	None (aerobic control)	74 ± 0·92 (5)	21 ± 1·05 (5)	16 ± 0·97 (5)	14 ± 0·48 (6)	4·4 ± 0·37 (5)
	Insulin (0·1 unit/ml.)	75 ± 0·53 (5)	17 ± 1·18 (5)	14 ± 1·41 (5)	12 ± 0·0 (3)	6·2 ± 0·50 (5)
	Significance of difference (P)	<0·4>0·3	<0·05>0·02	<0·3>0·2	<0·01>0·001	<0·02>0·01
2	None (aerobic control)	74 ± 0·30 (18)	25 ± 0·47 (18)	17 ± 0·73 (18)	14 ± 0·48 (6)	2·5 ± 0·21 (18)
	None (anaerobic)	76 ± 0·25 (18)	22 ± 0·81 (18)	11 ± 0·45 (18)	10 ± 0·42 (3)	3·1 ± 0·14 (18)
	Significance of difference (P)	<0·001	<0·01>0·001	<0·001	<0·001	<0·05>0·01
3	None (aerobic control)	73 ± 0·31 (5)	21 ± 0·71 (5)	15 ± 0·84 (5)	14 ± 0·48 (6)	2·6 ± 0·14 (5)
	2:4-Dinitrophenol (0·25 mM)	77 ± 0·71 (5)	24 ± 1·20 (5)	30 ± 0·50 (5)	13 ± 1·21 (3)	3·2 ± 0·15 (5)
	Significance of difference (P)	<0·001	<0·1>0·05	<0·001	<0·5>0·4	<0·02>0·01
4	None (aerobic control)	73 ± 0·32 (6)	24 ± 0·61 (6)	14 ± 0·88 (6)	—	2·9 ± 0·29 (6)
	2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	76 ± 0·36 (6)	27 ± 1·81 (6)	53 ± 2·90 (6)	—	2·0 ± 0·16 (6)
	Significance of difference (P)	<0·001	<0·2>0·1	<0·001	—	<0·05>0·02
5	None (aerobic control)	75 ± 0·59 (5)	21 ± 1·38 (5)	15 ± 1·05 (5)	14 ± 0·48 (6)	2·3 ± 0·64 (5)
	Sodium arsenite (mM)	78 ± 0·32 (5)	34 ± 2·14 (5)	36 ± 2·04 (5)	19 ± 0·71 (3)	5·5 ± 0·60 (5)
	Significance of difference (P)	<0·01>0·001	<0·001	<0·001	<0·001	<0·01>0·001
6	None (aerobic control)	73 ± 1·00 (6)	23 ± 0·92 (6)	19 ± 1·79 (6)	14 ± 0·48 (6)	1·7 ± 0·35 (6)
	Sodium cyanide (mM)	75 ± 0·17 (6)	28 ± 1·39 (6)	19 ± 0·91 (6)	19 ± 1·21 (6)	3·8 ± 0·24 (6)
	Significance of difference (P)	<0·1>0·05	<0·02>0·01	—	<0·01>0·001	<0·001
7	None (aerobic control)	74 ± 0·48 (6)	22 ± 1·32 (6)	17 ± 2·01 (6)	14 ± 0·48 (6)	1·8 ± 0·29 (6)
	Sodium salicylate (5 mM)	75 ± 0·63 (6)	25 ± 1·30 (6)	18 ± 2·20 (6)	11 ± 0·39 (6)	3·8 ± 0·24 (6)
	Significance of difference (P)	<0·3>0·2	<0·2>0·1	<0·8>0·7	<0·01>0·001	<0·001

Table 4. *Influence of insulin, anaerobiosis or cell poisons on the parameters of intact diaphragm incubated in phosphate-buffered medium*

Diaphragms were incubated in phosphate–thiosulphate medium (for composition see Table 1). The number of observations is given in parentheses.

Expt. no.	Addition	Space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)			Glucose uptake. Mean ± S.E.M. (mg. of glucose/hr. of incubation)
		Total water	Thiosulphate	Glucose	
1	None (aerobic control)	74 ± 0·63 (6)	25 ± 0·73 (6)	17 ± 1·11 (6)	2·8 ± 0·13 (6)
	None (anaerobic)	77 ± 0·18 (6)	25 ± 0·66 (6)	15 ± 0·52 (6)	3·4 ± 0·31 (6)
	Significance of difference (P)	<0·01>0·001	—	<0·2>0·1	<0·1>0·05
2	None (aerobic control)	73 ± 0·69 (6)	25 ± 0·81 (6)	18 ± 0·63 (6)	2·6 ± 0·13 (6)
	2:4-Dinitrophenol (0·25 mM)	76 ± 0·55 (6)	34 ± 1·11 (6)	37 ± 2·50 (6)	2·2 ± 0·16 (6)
	Significance of difference (P)	<0·01>0·001	<0·001	<0·001	<0·1>0·05
3	None (aerobic control)	—	—	27 ± 1·12 (6)	3·5 ± 0·22 (6)
	Sodium salicylate (5 mM)	—	—	29 ± 2·09 (6)	3·8 ± 0·41 (6)
	Significance of difference (P)	—	—	<0·5>0·4	<0·6>0·5

space was 12–14 ml./100 g. whether determined in the presence or in the absence of thiosulphate (Tables 2 and 3). The thiosulphate and inulin spaces of the cut preparation were both very much higher (37 and 30 ml./100 g. respectively), though a similar discrepancy was apparent as Kipnis & Cori (1957) also found. It seems unlikely that this disparity between the two estimates of extracellular water is due to incomplete equilibration of the tissue with inulin during the period of incubation (1 hr.) as Creese has shown that the inulin space attains a maximum value within this time. Thiosulphate must therefore be in equilibrium with a larger fraction of the tissue than inulin.

(3) *Glucose and xylose spaces.* The glucose spaces of the intact and cut preparations incubated in bicarbonate-buffered media (13–14 and 28 ml./100 g. respectively; Tables 2 and 3) were very similar to the corresponding inulin spaces though considerably smaller than the corresponding thiosulphate spaces; but by either estimate of the volume of extracellular fluid there was apparently no free glucose in intracellular water. Essentially similar results were obtained with the intact preparation in phosphate-buffered medium (Table 4). On the other hand, the xylose space of either intact or cut diaphragm in bicarbonate medium (26 and 52 ml./100 g. respectively; Table 2) exceeded both estimates of the volume of extracellular fluid. Some xylose therefore entered intracellular water under basal conditions. Kipnis & Cori (1957) obtained higher figures for the xylose spaces of both intact and cut preparations but they used medium containing thiosulphate and a high concentration of phosphate. Phosphate in high concentration (20 mM) increases the xylose space of intact diaphragm and so to a smaller extent does thiosulphate (20 mM) (Table 2). The accumulation of xylose in either diaphragm preparation was found to be rapid, being 80–85% complete after incubation for 5–10 min. in bicarbonate medium (Figs. 1–3). For a reason which is not clear, penetration of the intact diaphragm by xylose was considerably faster, though ultimately less extensive, than in the experiments of Kipnis & Cori (1957).

(4) *Other effects of thiosulphate.* In view of the effect of thiosulphate on the xylose space just noted and because it also increases the uptake of glucose by cut diaphragm (Randle & Smith, unpublished observations) it was important to show whether thiosulphate affected any other parameters of diaphragm. The comparisons given in Table 2 indicate that thiosulphate does not influence the inulin or glucose spaces or the total water of intact diaphragm incubated in bicarbonate-buffered medium. In separate experiments it was also shown that thiosulphate did not appreciably modify the influence of 2:4-dinitrophenol, sodium cyanide or sodium salicylate on the glucose space of the intact diaphragm incubated in bicarbonate-buffered medium.

Effects of insulin, anaerobiosis and cell poisons on parameters of intact diaphragm

In studying the effects of insulin, anaerobiosis or cell poisons, the glucose space, thiosulphate space and total water were measured in the same experiment. The inulin space was determined in separate experiments but in the presence of thiosulphate to validate comparison with the thiosulphate and glucose spaces. When the xylose space was measured thiosulphate was excluded from the medium because of its effect on the xylose space and inulin was also omitted because it interferes with the estimation of xylose.

(1) *Glucose space in bicarbonate-buffered medium.* The results in Table 3 show that insulin (0·1 unit/ml.) or anaerobiosis reduced the glucose space, but that this change was associated with a similar fall

in the volume of extracellular fluid as measured with both inulin and thiosulphate. Neither sodium cyanide (mM) nor sodium salicylate (5 mM) affected the glucose space, though small changes in the extracellular volume occurred when these substances were present. Hence it may be concluded that none of these agents caused free glucose to accumulate in intracellular water. On the other hand 2:4-dinitrophenol (0·25 mM) increased the glucose space to a value exceeding both the inulin and the thiosulphate spaces and with both 2:4-dinitrophenol (0·25 mM) and sodium fluoride (10 mM) the glucose space increased still further. Free glucose was therefore present in the intracellular water under these conditions. The effect of sodium arsenite (mM) was anomalous in that although it more than doubled the glucose space the thiosulphate space also rose to a comparable value. The inulin space was much smaller than the glucose space or the thiosulphate space in the presence of arsenite. If the inulin space is to be regarded as a more reliable estimate of the volume of extracellular fluid then free glucose accumulated in intracellular water in the presence of arsenite.

(2) *Glucose uptake in bicarbonate-buffered medium.* The uptake of glucose from the medium by intact diaphragm was increased by insulin, anaerobiosis, 2:4-dinitrophenol, sodium arsenite, sodium cyanide and sodium salicylate (Table 3). These factors thus had effects on the uptake of glucose by the intact diaphragm which were similar to those obtained with the cut preparation (Randle & Smith, 1958). It should, of course, be borne in mind that with the intact preparation structures other than the diaphragm itself presumably take up glucose from the medium. The absolute values of the glucose uptakes with the two preparations may not therefore be strictly comparable. If sodium fluoride was

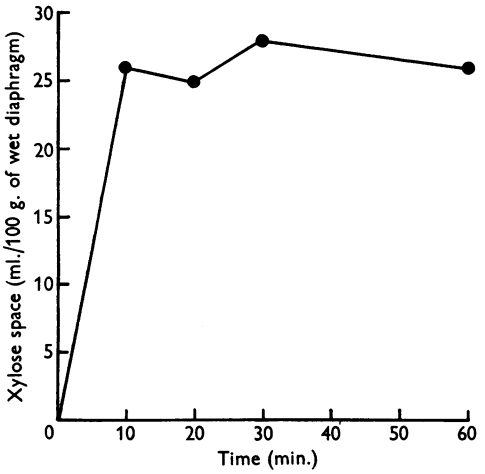

Fig. 1. Accumulation of xylose in intact diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.).

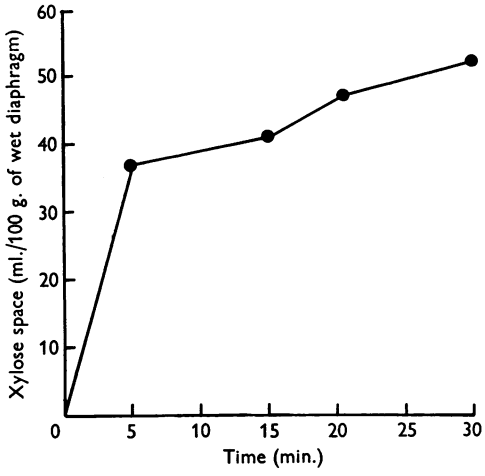

Fig. 2. Accumulation of xylose in cut diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.).

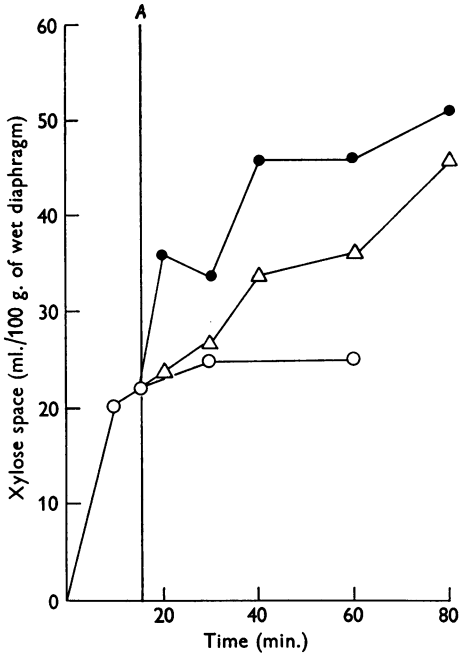

Fig. 3. Effect of insulin and salicylate on accumulation of xylose in intact diaphragm. Incubations were made in bicarbonate medium (for composition see Table 1) to which was added D-xylose (5 mg./ml.) and glucose (1 mg./ml.). ○, Aerobic control; △, salicylate added at *A* (to 5 mM); ●, insulin added at *A* (to 0·1 unit/ml.).

present in addition to the 2:4-dinitrophenol the glucose uptake was lower than in the control. Similar experiments with the cut diaphragm showed that the actual utilization of glucose (as distinct from the disappearance of glucose from the medium) in the presence of 2:4-dinitrophenol plus sodium fluoride was very much lower than that in the control. Much of the glucose which disappeared from the medium under these conditions accumulated in the muscle (Table 5).

(3) *Glucose space and glucose uptake in phosphate-buffered medium.* The effects of anaerobiosis, 2:4-dinitrophenol or sodium salicylate on the glucose space of intact diaphragm incubated in phosphate-buffered medium (Table 4) did not differ appreciably from their effects in bicarbonate-buffered medium (Table 3). On the other hand, dinitrophenol and salicylate failed to increase glucose uptake by intact diaphragm in phosphate-buffered medium (Table 4), in agreement with the results obtained previously with cut diaphragm (Randle & Smith, 1958).

(4) *Xylose space in bicarbonate or phosphate-buffered media.* The xylose space of the intact diaphragm preparation was expanded by insulin, 2:4-dinitrophenol, sodium arsenite, sodium cyanide or sodium salicylate or by exclusion of oxygen (Table 6). Of these factors arsenite and cyanide increased the xylose space to the greatest extent. They were also the only factors to increase the inulin and thiosulphate spaces (Table 3); the two effects may be associated. Moreover, insulin, 2:4-dinitrophenol and sodium salicylate increased the xylose space in phosphate medium (Table 6). Thus in every instance insulin, anaerobiosis and cell poisons expanded the xylose space to a value greatly in excess of the volume of extracellular fluid as estimated with either inulin or thiosulphate. It must therefore be concluded that each of these agents promotes the entry of xylose into intracellular water.

The rate of xylose penetration under the influence of insulin or salicylate was studied also. In this experiment intact diaphragms were first incubated for 10 min. in bicarbonate medium without insulin or salicylate to allow basal penetration to occur. Insulin or salicylate was then added and further estimations of xylose space were made at intervals over a total period of 1 hr. Fig. 3 shows that additional xylose immediately entered the

Table 5. *Effect of 2:4-dinitrophenol plus sodium fluoride on the parameters of cut diaphragm*

Hemidiaphragms were incubated in bicarbonate–thiosulphate medium II (for composition see Table 1). Glucose utilization was calculated by correcting the glucose uptake for free glucose present in the muscle.

Addition to medium	Space. Mean ± S.E.M. (ml./100 g. of wet diaphragm)			Glucose uptake and utilization. Mean ± S.E.M. (mg. of glucose/g. of wet diaphragm/hr. of incubation)		No. of observations
	Total water	Thiosulphate	Glucose	Uptake	Utilization	
None (aerobic control)	74 ± 0·52	32 ± 0·91	27 ± 1·41	3·7 ± 0·32	3·7 ± 0·32	6
2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	78 ± 0·41	45 ± 8·09	61 ± 0·95	1·7 ± 0·28	0·9 ± 0·28	6
Significance of difference (*P*)	<0·001	<0·3>0·2	<0·001	<0·001	<0·001	

Table 6. *Influence of insulin, anaerobiosis or cell poisons on the xylose space of intact diaphragm*

For composition of media see Table 1. The number of observations is given in parentheses.

Medium	Addition	Xylose space. Mean ± S.E.M. (ml. of water/100 g. of wet diaphragm)	*Difference (from control) ± S.E. of difference
Bicarbonate	None (aerobic control)	25 ± 0·4 (16)	—
	Insulin (0·1 unit/ml.)	48 ± 1 (4)	23 ± 1
	None (anaerobic)	44 ± 1 (3)	19 ± 1
	2:4-Dinitrophenol (0·25 mM)	48 ± 1 (3)	23 ± 1
	2:4-Dinitrophenol (0·25 mM) + sodium fluoride (10 mM)	44 ± 1 (4)	19 ± 1
	Sodium arsenite (mM)	66 ± 2 (3)	41 ± 2
	Sodium cyanide (mM)	52 ± 2 (4)	27 ± 2
	Sodium salicylate (5 mM)	44 ± 3 (3)	19 ± 3
Phosphate	None (aerobic control)	26 ± 2 (3)	—
	Insulin (0·1 unit/ml.)	46 ± 1 (3)	20 ± 3
	2:4-Dinitrophenol (0·25 mM)	49 ± 0·6 (3)	23 ± 2
	Sodium salicylate (5 mM)	50 ± 2 (3)	24 ± 3

* In every case the significance (*P*) of the difference is <0·001.

tissue in both cases, the rate of penetration being greater with insulin than with salicylate. The values ultimately attained after 1 hr. of incubation were, however, similar in the two cases.

DISCUSSION

We showed in the preceding paper that insulin, anaerobiosis and a number of cell poisons all enhance the uptake of glucose by isolated rat diaphragm (Randle & Smith, 1958). Levine & Goldstein (1955) and Park *et al.* (1955) believe that the uptake of glucose by muscle such as diaphragm occurs in two stages: (i) penetration of the muscle cell by glucose, and (ii) metabolism of glucose within the cell; and that it is the first of these which is normally rate-limiting. We therefore concluded that insulin, anaerobiosis and cell poisons such as 2:4-dinitrophenol or salicylate all act by stimulating the entry of glucose into the muscle cell, possibly by inhibiting a process which restrains the penetration of glucose under basal conditions. The results recorded here give further support for this view.

If intracellular utilization of glucose can proceed under basal conditions at a faster rate than entry, then little or no free glucose would be expected to accumulate within the cells. This was indeed shown to be the case with the cut-diaphragm preparation by Park *et al.* (1955) and the present investigation has demonstrated that the same is true for the intact diaphragm. Furthermore, the distribution of free glucose remained extracellular under anaerobic conditions or in the presence of insulin, cyanide or salicylate, even though these factors markedly enhanced glucose uptake. Hence either the potential rate of intracellular utilization of glucose must greatly exceed the rate of entry under these conditions or, alternatively, glucose does not enter the cell as such. Free glucose was detected within the cell, however, when diaphragm was incubated in the presence of 2:4-dinitrophenol, especially when sodium fluoride was also present. Thus unless the mechanism of entry of glucose in the presence of these substances is abnormal it seems likely that glucose does enter intracellular water as the free sugar, accumulation occurring only when utilization is inhibited to such an extent that it becomes rate-limiting. The results of measurements of glucose uptake and glucose space of intact diaphragm are therefore compatible with the view that insulin, anaerobiosis or substances which inhibit oxidative phosphorylation promote glucose uptake by accelerating the entry of glucose into the cell.

This view is strengthened by the results of experiments with xylose. D-Xylose does not appear to be utilized by isolated rat diaphragm (Kipnis & Cori, 1957). Consequently the xylose space may be used to measure the rate and extent of penetration of diaphragm by this sugar. Under basal conditions xylose rapidly enters a small volume (10–15%) of intracellular water; thereafter further penetration is very slow. This observation suggests that a barrier within the cell confines xylose to a small fraction of intracellular water under basal conditions (cf. Helmreich & Cori, 1957). Insulin, lack of oxygen and the cell poisons 2:4-dinitrophenol, arsenite, cyanide and salicylate all increase the fraction of intracellular water which xylose penetrates to 40–80%. Each of these agents except insulin has the property of inhibiting oxidative phosphorylation. It would therefore appear that the processes which confine xylose to a small fraction of intracellular water under basal conditions are dependent upon a supply of energy-rich phosphate compounds. Insulin, on the other hand, does not inhibit oxidative phosphorylation (Stadie, 1954), so the mechanism of its effect on glucose uptake and xylose entry presumably differs from that of factors which do inhibit oxidative phosphorylation. One possibility is that insulin may augment glucose or xylose penetration by restricting the access of energy-rich phosphate to a process concerned with the regulation of glucose or xylose entry (Randle & Smith, 1957, 1958). The observation that all of these factors increase both glucose uptake and entry of xylose in diaphragm incubated in a bicarbonate-buffered medium is compatible with the following views: (i) that glucose and xylose enter the muscle cell by similar processes, and (ii) that uptake of glucose in most instances is limited by the rate of its entry into the cell. Nevertheless the rates of glucose uptake elicited by these various factors are generally different in bicarbonate medium (Randle & Smith, 1958). If the views just outlined are correct then the rates of penetration of xylose with each of these factors should differ. This was shown to be the case for insulin and salicylate; insulin more markedly stimulated glucose uptake than salicylate (Randle & Smith, 1958) and also promoted a more rapid accumulation of xylose (Fig. 3). On the other hand, in phosphate medium 2:4-dinitrophenol and salicylate augment xylose accumulation without increasing glucose uptake. The failure of dinitrophenol and salicylate to increase glucose uptake in phosphate medium cannot result from a diminution in the utilization of glucose within the cell to such a level that it has become rate-limiting, for free glucose does not accumulate within the cell under these conditions. One must therefore conclude either that the processes which govern the entry of glucose and xylose into the muscle cell are different or else that glucose uptake does not necessarily reflect glucose entry, even when there

is no intracellular accumulation of glucose. One explanation of these anomalous findings in phosphate medium, which has been discussed more fully in the preceding paper (Randle & Smith, 1958), is provided by the suggestion of Shaw & Stadie (1957) that glucose may be utilized partly within the cell and partly at the cell surface. Thus increased entry of glucose into intracellular water would not be associated with increased glucose uptake if at the same time there was a more or less proportionate reduction in the rate of utilization of glucose at the surface of the cell.

Although the present investigations have provided further support for the view that it is the penetration of the muscle cell by glucose which is the rate-limiting step in the uptake of glucose by isolated diaphragm and that insulin or factors which inhibit oxidative phosphorylation augment glucose uptake by increasing penetration, there are, nevertheless, at least two unsatisfactory features in the evidence for this hypothesis. One is the assumption that the same processes govern the entry of glucose and non-utilizable sugars, such as xylose, into the muscle cell. The other is the assumption that glucose utilization in muscle is entirely intracellular. Clearly, further information about the process by which glucose enters the muscle cell and about the localization in muscle of the enzymes concerned with the metabolism of glucose is prerequisite for any explanation of the mechanism of action of insulin or of the Pasteur effect in this tissue.

SUMMARY

1. The accumulation of glucose or xylose in muscle has been studied *in vitro* with an isolated rat-diaphragm preparation in which none of the muscle fibres is cut.

2. The glucose space of this preparation does not exceed the volume of extracellular fluid when diaphragm is incubated aerobically in a bicarbonate-buffered medium. On the other hand, xylose appears to accumulate in about 10–15% of the intracellular water under these conditions.

3. Glucose remains extracellular in distribution when diaphragm is incubated anaerobically or in the presence of insulin (0·1 unit/ml.), sodium cyanide (mM) or sodium salicylate (5 mM), but accumulates within the cells in the presence of sodium arsenite (mM), 2:4-dinitrophenol (0·25 mM) or dinitrophenol (0·25 mM) plus fluoride (10 mM). Each of these factors causes a further accumulation of xylose within the muscle cell.

4. These results provide further support for the views that the entry of glucose into the muscle cell is normally the rate-limiting step in its metabolism; that the glucose entry is restrained by a process dependent on a supply of a substance generated during oxidative phosphorylation; and that insulin may promote glucose uptake by diaphragm by preventing access of this substance to the process regulating glucose entry.

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REFERENCES

Creese, R. (1954). *Proc. Roy. Soc.* B, **142**, 497.
Gamble, J. L. (1950). *Chemical Anatomy, Physiology and Pathology of Extracellular Fluid*, 5th ed. Cambridge, Mass., U.S.A.: Harvard University Press.
Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Helmreich, E. & Cori, C. F. (1957). *J. biol. Chem.* **224**, 663.
Kipnis, D. M. & Cori, C. F. (1957). *J. biol. Chem.* **224**, 681.
Levine, R. & Goldstein, M. S. (1955). *Recent. Progr. Hormone Res.* **11**, 343.
Newman, E. V., Gilman, A. & Philips, F. S. (1946). *Johns Hopk. Hosp. Bull.* **79**, 229.
Park, C. R., Bornstein, J. & Post, R. L. (1955). *Amer. J. Physiol.* **182**, 12.
Park, C. R. & Johnson, L. H. (1955). *Amer. J. Physiol.* **182**, 17.
Randle, P. J. (1956). *J. Endocrin.* **14**, 82.
Randle, P. J. & Smith, G. H. (1957). *Biochem. biophys. Acta*, **25**, 442.
Randle, P. J. & Smith, G. H. (1958). *Biochem. J.* **70**, 490.
Roe, J. H., Epstein, J. H. & Goldstein, N. P. (1949). *J. biol. Chem.* **178**, 839.
Roe, J. H. & Rice, E. W. (1948). *J. biol. Chem.* **173**, 507.
Shaw, W. N. & Stadie, W. C. (1957). *J. biol. Chem.* **227**, 115.
Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
Stadie, W. C. (1954). *Physiol. Rev.* **34**, 52.
Van Slyke, D. D. & Hawkins, J. A. (1928). *J. biol. Chem.* **79**, 739.