Krebs, H. A. (1942). *Biochem. J.* **36**, 303.
Lardy, H. A. (1954). *Brookhaven Symp. Biol.* **7**, 90. Cited in Wolff, J. & Wolff, E. C. (1957), *Biochim. biophys. Acta*, **26**, 387.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mahler, H. R. & Elowe, D. G. (1954). *J. biol. Chem.* **210**, 165.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
Patwardhan, M. V. (1958). *Nature, Lond.*, **181**, 187.
Sall, J., Richards, H. K., Harrison, E. & Myerson, R. M. (1957). *J. Lab. clin. Med.* **50**, 297.
Speck, J. F. (1949). *J. biol. Chem.* **178**, 315.
Srinivasan, M. (1957). *Lancet*, ii, 317.
Tonhazy, N. E., White, N. G. & Umbreit, W. W. (1950). *Arch. Biochem.* **28**, 36.
Tulpule, P. G. & Patwardhan, V. N. (1952). *Nature, Lond.*, **169**, 671.
Wijesundera, S. & Woods, D. D. (1953). *J. gen. Microbiol.* **9**, iii.
Williams, V. R. & Nielands, J. B. (1954). *Arch. Biochem. Biophys.* **53**, 56.
Wolff, J. & Wolff, E. C. (1957). *Biochim. biophys. Acta*, **26**, 387.
Yanofsky, C. (1952). *J. biol. Chem.* **198**, 343.

Biochem. J. (1960) **75**, 408

Regulation of Glucose Uptake by Muscle

4. THE SPECIFICITY OF MONOSACCHARIDE-TRANSPORT SYSTEMS IN RAT-DIAPHRAGM MUSCLE*

BY F. C. BATTAGLIA†‡ AND P. J. RANDLE
Department of Biochemistry, University of Cambridge

(*Received* 16 *November* 1959)

In previous papers (Randle & Smith, 1958*a*, *b*) evidence was presented which showed that the transport of D-glucose and D-xylose across the muscle-cell membrane of isolated rat diaphragm is accelerated by insulin, anoxia and substances which inhibit oxidative phosphorylation (transport is defined as a process in which substances move across the cell membrane in combination with a specific constituent of the cell). These factors were also shown to accelerate transport of D-glucose and L-arabinose in the perfused isolated rat heart (Morgan, Randle & Regen, 1959). The conclusion was drawn by these workers that the transport of sugars across the muscle-cell membrane is inhibited by an energy-rich phosphate compound; that factors such as anoxia, which inhibit oxidative phosphorylation, accelerate transport by lowering intracellular levels of high-energy phosphate compounds; and that insulin accelerates transport by interfering with a reaction between high-energy phosphate and the transport system (Randle & Smith, 1958*a*, *b*).

In the present study the specificity of monosaccharide-transport systems in rat diaphragm has been defined by investigating the extent to which different sugars compete for transport. Evidence has been sought for the nature of the transport system by investigating the effect of some enzyme inhibitors on the transport of sugars. Finally we have attempted to determine whether sugars may pass directly from the transport system into muscle polysaccharides and whether the effect of insulin on sugar transport is secondary to an effect of the hormone on the formation of maltose and glycogen.

* Part 3: Morgan, Randle & Regen (1959).

† U.S. Public Health Service Postdoctoral Fellow.

‡ Present address: Department of Physiology, Yale University, New Haven, Connecticut, U.S.A.

METHODS AND PROCEDURE

Incubation and perfusion media. Diaphragms were incubated in the bicarbonate-buffered medium of Gey & Gey (1936) and hearts were perfused with the bicarbonate-buffered medium of Krebs & Henseleit (1932). D-Glucose, D-galactose, D-xylose, D-arabinose, L-arabinose and raffinose were obtained from T. Kerfoot and Co. Ltd., D-mannose, D-fructose and α-methyl-D-glucoside from British Drug Houses Ltd., D-lyxose from California Corporation for Biochemical Research, Los Angeles, U.S.A. and D-3-*O*-methylglucose from Ayerst, McKenna and Harrison, N.Y., U.S.A. These compounds were used without further purification. D-3-*O*-Methylglucose was subjected to chromatography (15 μl. of 1%) on Whatman no. 1 paper with pyridine–ethyl acetate–water (1:2:2) (Jermyn & Isherwood, 1949). Only one substance was detected when the chromatogram was developed with aniline hydrogen phthalate reagent (Partridge, 1949). β-Methyl-D-glucoside was prepared in this Laboratory by Mr B. R. Slater by the Koenig–Knorr reaction.

D-[$^{14}C_6$]Glucose (5 mc/m-mole), D-[I-^{14}C]mannose (1 mc/m-mole) and D-[$^{14}C_6$]sorbitol (6·2 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Crystalline insulin (Boots Pure Drug Co. Ltd. or Novo Terapeutisk Laboratories, Copenhagen, Denmark) 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 or female albino Wistar rats of 100–150 g. wt. and heart muscle from animals of 200–250 g. wt. The animals were fed with a stock laboratory diet (Bruce & Parkes, 1949) and food was withheld for 18–20 hr. before each experiment. The procedure for removal and incubation of intact diaphragms and hemidiaphragms and for perfusion of heart muscle has been described elsewhere (Randle & Smith, 1958*a*, *b*; Morgan *et al.* 1959).

Analytical methods

Sugars and D-[^{14}C]*sorbitol.* Analyses of muscle for these substances were made on extracts prepared by boiling the muscle in 2–4 ml. of water for 15 min. and deproteinizing with $Zn(OH)_2$ (Somogyi, 1945). This procedure extracts these substances completely. Samples of medium after incubation were suitably diluted and deproteinized in the same way. Pentoses were estimated photometrically by the *p*-bromoaniline method (Roe & Rice, 1948); raffinose was estimated photometrically after hydrolysis and reaction with resorcinol (Roe, Epstein & Goldstein, 1949). Galactose and 3-*O*-methylglucose were estimated photometrically by the Somogyi (1945) modification of Nelson's method, glucose being removed when present by fermentation with baker's yeast. The blank reduction by extracts of muscle under these conditions was so small that it could safely be neglected. [^{14}C]Sorbitol was assayed by methods described elsewhere (Morgan *et al.* 1959).

[^{14}C]*Sugars in muscle.* For identification of [^{14}C]maltose in diaphragm-muscle extracts were prepared and deproteinized as described above. The extract was freed of lipid by ether extraction and desalted by addition of ethanol to 70% (v/v). The extract was dried *in vacuo* and the residue dissolved in 2% maltose + 1% glucose or mannose. Two 5 μl. samples were then chromatographed on Whatman no. 1 paper with either pyridine–ethyl acetate–water (1:2:2) or (where mannose was to be separated from glucose) ethyl acetate–acetic acid–water (3:1:3) (Jermyn & Isherwood, 1949). The chromatogram was developed with aniline hydrogen phthalate reagent and the spots so defined were cut out with a cork borer (internal diam. 2·8 cm.). Radioactivity was assayed directly by placing the disk of paper beneath a thin end-window Geiger–Müller tube. At least 1000 counts were recorded for each sample and replicate determinations under these conditions agreed to within 8%.

Glycogen. Muscle was dissolved in 30% KOH at 60° and glycogen precipitated with the addition of 2% Na_2SO_4 (2:5, v/v) and ethanol to 70% (v/v). The crude glycogen was assayed photometrically with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

For assay of radioactivity in glycogen the crude polysaccharide was prepared as described above and purified by reprecipitation (twice). The glycogen was then dissolved in water and transferred to a planchet containing a disk of lens tissue and a drop of a saturated aqueous solution of polyvinyl alcohol. The samples were dried under an infrared lamp and counted immediately, at least 1000 counts being recorded.

To determine whether [^{14}C]glucose or [^{14}C]mannose could be detected in glycogen the polysaccharide (prepared as described above except that 25% NaOH was used instead of 30% KOH) was hydrolysed with N-HCl at 100° for 3 hr. The hydrolysate was neutralized with N-NaOH (phenol red as internal indicator), desalted by addition of ethanol to 70% (v/v) and dried *in vacuo*. The residue was dissolved in 1% glucose and mannose and two 5 μl. samples were subjected to chromatography (method of assay of radioactivity was as described for [^{14}C]sugars in muscle, above).

Design of experiments

Membrane transport of sugars in diaphragm muscle has been studied by measuring their intracellular accumulation. This has been calculated as the intracellular-sugar space, obtained by subtracting the volume of extracellular water (measured as the sorbitol space) from the sugar space of the tissue (for definition of the concept of space see Randle & Smith, 1958*b*). Intracellular accumulation has also been expressed as the ratio of intracellular- to extracellular-sugar concentration × 100% and calculated from

$$\frac{\text{intracellular-sugar space}}{\text{intracellular-fluid volume}} \times 100\%.$$

In studying competition we have measured the volumes of intracellular water (total water − extracellular water), extracellular water and sugar space in the presence and the absence of the competing sugar. The period of incubation was 30–45 min. and this period was chosen because accumulation of sugars such as D-xylose is not complete in this time (Randle & Smith, 1958*b*). Competition for membrane transport is deemed to have taken place if the second sugar decreases the intracellular accumulation of the first sugar.

Intact diaphragms (weighing 3–4 g.) were incubated in 3–15 ml. of medium. The smaller volume of medium was used in some experiments in order to conserve radioactive sorbitol. Nevertheless, the tissue appeared to be in a satisfactory condition after incubation in such a small volume and furthermore the spaces obtained were similar to those obtained with larger volumes of incubation fluid.

RESULTS

Changes in the volumes of intra- and extra-cellular water

In investigating competition between sugars for membrane transport, D-sorbitol was added to the medium in the control series in place of the second sugar, in order to maintain the total solute concentrations of the medium. However, it became apparent during the course of these investigations that substances such as D-sorbitol which do not penetrate cells do not exert the same effect on the volumes of intra- and extra-cellular water as substances which do penetrate. The results of experiments which demonstrate this are summarized in Table 1. Thus although sorbitol, mannose and urea were added to the extracellular fluid at the same concentration (approx. 100 m-osmoles/kg. of water) their effect on the intracellular-fluid volume was

very different. Sorbitol, to which the cell membrane is impermeable, brought about the largest change in intracellular-water volume, and urea, which enters most rapidly, brought about the least change.

Galactose is a sugar which enters the skeletal-muscle cell in appreciable amounts only in the presence of insulin. Hence it was interesting that the effect of a given galactose concentration upon the intracellular-water volume could be substantially decreased by the addition of insulin.

Effect of other sugars and of D-*sorbitol on the transport of* D-*xylose*

The results of these experiments are recorded in Table 2 and Fig. 1. In the absence of insulin, D-glucose and D-3-*O*-methylglucose at a concentration ratio (to xylose) of 3:1 decreased the intracellular accumulation of xylose. In the presence of the hormone xylose accumulation was diminished by D-glucose (about 30%), D-mannose (about 25%) and D-3-*O*-methylglucose (about 15%). D-Galactose, D-fructose, α-methyl-D-glucoside and maltose did not diminish intracellular accumulation of D-xylose. The effect of D-glucose on the accumulation of D-xylose by the diaphragm at 10, 20, 30 and 40 min. of incubation is shown in Fig. 1. Xylose accumulation was found to be inhibited at all periods of incubation.

Effect of other sugars on the transport of L-*arabinose*

The results of these experiments are shown in Table 3. In the presence of insulin and at a concentration ratio (to arabinose) of 3:1 arabinose accumulation was inhibited by D-glucose (about 60%), D-mannose (about 45%) and D-3-*O*-methylglucose. D-Fructose, D-galactose, α-methyl-D-glucoside and β-methyl-D-glucoside did not inhibit accumulation of arabinose.

Effect of D-*glucose on the transport of* D-*galactose and* D-*arabinose*

The results of these experiments are shown in Tables 4 and 5. In the presence of insulin, glucose at a concentration ratio (to galactose) of 1:1 did not inhibit the accumulation of galactose. The

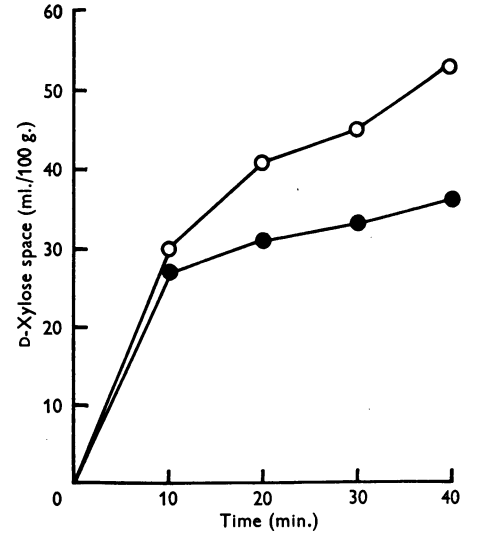

Fig. 1. Effect of D-glucose on the rate of accumulation of D-xylose by the intact diaphragm preparation. Insulin was present at a concentration of 0·1 unit/ml. Each value is the mean of two observations. ○, D-Xylose only (8·3 mM); ●, D-xylose (8·3 mM) + D-glucose (26 mM).

Table 1. *Effect of* D-*sorbitol,* D-*mannose, urea and* D-*galactose on the volumes of total water and extracellular and intracellular water of the intact diaphragm preparation*

Concentration of insulin was 0·1 unit/ml. The volume of extracellular water was measured with D-[$^{14}C_6$]sorbitol. Differences marked with an asterisk are not significant.

Experiment	Concn. (mM)	Insulin	Space (mean ± S.E.M. ml./100 g.) Total water	Extracellular water	Intracellular water	Intracellular water / Total water × 100	No. of observations
Control	—	–	78±0·8	16±1	62±1·7	79·6	6
D-Sorbitol	55	–	75±0·6	23±1·5	52±1·1	69·4	6
Difference			−3±1	7±1·8	−10±2	−10·2	
D-Mannose	100	–	73±0·5	25±0·7	48±1·1	65·6	6
D-Sorbitol	100	–	75±0·4	32±1·1	43±1·7	57·3	6
Difference			2±0·7	7±1·3	−5±2	−8·3	
Urea	100	–	77±0·7	22±2·2	55±2	71·5	6
D-Sorbitol	100	–	75±0·3	35±1·2	40±1·2	53·3	6
Difference			−2±0·8	13±2·5	−15±2·3	−18·2	
D-Galactose	26	–	78±0·9	27±2·7	51±2·2	65·3	4
D-Galactose	26	+	78±0·9	18±0·7	60±0·9	77·1	4
Difference			0±1·3*	−9±2·8	9±2·3	11·8	

Table 2. *Effect of other sugars and of* D-*sorbitol on the accumulation of* D-*xylose by the intact diaphragm preparation*

Differences given are deviations from control values given at the head of the table in parentheses. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]Sorbitol (0·12 μc/ml.) was added to all media. ICXC, ECXC: intra- and extra-cellular xylose concentrations. Differences marked with an asterisk are not significant ($P > 0·05$).

Present in addition to D-xylose (8·3 mM)	Insulin	Difference from control (D-xylose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water	Intracellular water	Xylose space	Intracellular xylose space	ICXC/ECXC (%)	No. of observations
	−	(23)	.	(30)	(7)	.	.
	+	(20)	(55)	(44)	(24)	(44)	.
D-Glucose (26 mM)	−	−5±1·2	.	−8±1·4	−3±1	.	12
D-Glucose (26 mM)	+	−6±1·1	5±1	−12±1·3	−6±1·7	−13±4	12
D-Mannose (26 mM)	+	−2±1·4*	0±1·4*	−9±1·4	−7±1·3	−11±1·8	6
D-3-*O*-Methylglucose (26 mM)	−	−2±0·9*	1±0·8*	−5±1·4	−2±1·0*	−4±1·5	6
D-3-*O*-Methylglucose (26 mM)	+	−3±0·7	3±0·7	−6±0·8	−3±1·1	−7±1·8	16
D-Galactose (26 mM)	+	−4±1·2	6±2·3	−3±1·2	1±1·3*	8±1·9	12
D-Fructose (26 mM)	+	.	.	−1±3·1*	.	.	4
D-Sorbitol (55 mM)†	+	7±1·8	−10±2	3±2·3*	−3±2·8*	0±4·4*	6
α-Methyl-D-glucoside (26 mM)	+	−2±1·7*	.	−2±2·4*	0±1·4*	.	5
Maltose (26 mM)	+	−2±1·7*	.	−1±3*	1±1·4*	.	5

† In this experiment the control contained only D-xylose.

Table 3. *Effect of other sugars on the accumulation of* L-*arabinose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICAC, ECAC: intra- and extra-cellular arabinose concentrations. Differences marked with an asterisk are not significant ($P > 0·05$).

Present in addition to L-arabinose (8·3 mM)	Insulin	Difference from control (L-arabinose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water (23)	Intracellular water (54)	Arabinose space (44)	Intracellular arabinose space (21)	ICAC/ECAC (%) (39)	No. of observations
D-Glucose (26 mM)	+	1±0·8*	−1±0·8*	−13±1·8	−12±2	−23±2	4
D-Mannose (26 mM)	+	−1±2·0*	.	−11±1·9	−11±1·9	.	4
D-Galactose (26 mM)	+	−4±2·1*	5±1·8	−1±2·7*	3±3·4*	2±3·4	5
D-3-*O*-Methylglucose (26 mM)	+	.	.	−12±1·7	.	.	5
D-Fructose (26 mM)	+	.	.	−3±2·2*	.	.	5
α-Methyl-D-glucoside (26 mM)	+	−1±0·8*	.	1±2·6*	2±2·3*	.	.
β-Methyl-D-glucoside (26 mM)	+	3±1·6*	.	2±1·5*	−1±2·4*	.	5

Table 4. *Effect of* D-*glucose on the accumulation of* D-*galactose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICGC, ECGC: intra- and extra-cellular galactose concentrations. Differences marked with an asterisk are not significant ($P > 0·05$).

Present in addition to D-galactose (14 mM)	Insulin	Difference from control (D-galactose, 14 mM + D-[^{12}C]sorbitol, 5·5 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water (24)	Intracellular water (51)	Galactose space (54)	Intracellular galactose space (30)	$\frac{ICGC}{ECGC}$ (%) (60)	No. of observations
D-Glucose (14 mM)	+	−6±1·9	4±2*	2±4·7*	8±5·6*	9±4	6

Table 5. *Effect of* D-*glucose on the accumulation of* D-*arabinose by the intact diaphragm preparation*

Differences given are deviations from control values given in parentheses at the head of the table. Concentration of insulin was 0·1 unit/ml. D-[$^{14}C_6$]-Sorbitol (0·12 μc/ml.) was added to all media. ICAC, ECAC: intra- and extra-cellular arabinose concentration. Differences marked with an asterisk are not significant ($P > 0·05$).

Present in addition to D-arabinose (8·3 mM)	Insulin	Difference from control (D-arabinose, 8·3 mM + D-[^{12}C]sorbitol, 26 mM) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water (21)	Intracellular water (55)	Arabinose space (31)	Intracellular arabinose space (10)	$\frac{ICAC}{ECAC}$ (%) (18)	No. of observations
D-Glucose (26 mM)	+	−4±1·4	3±1·2	−7±2·1	−3±1·1	−5±2·2	12

Table 6. *Effect of phlorrhizin,* N-*ethylmaleimide,* p-*chloromercuribenzoate and diisopropyl phosphorofluoridate on the accumulation of* D-*xylose and* D-*galactose by the intact diaphragm preparation*

Concentration of insulin was 0·1 unit/ml. Extracellular water was measured with D-[$^{14}C_6$]sorbitol (0·12 μc/ml.) in Expts. 1, 4 and 5 and separately with raffinose (20 mM) in Expts. 2 and 3. ICSC, ECSC: intra- and extra-cellular xylose or galactose concentrations. Differences marked with an asterisk are not significant ($P > 0·05$).

Expt. no.	Present in addition to D-xylose (8·3 mM, Expts. 1–4) or D-galactose (26 mM, Expt. 5)	Insulin	Difference from control (xylose or galactose alone) (mean ± S.E.M.; Δ ml./100 g. of wet muscle) Extracellular water	Intracellular water	Xylose or galactose space	Intracellular xylose or galactose space	$\frac{ICSC}{ECSC}$ (%)	No. of observations
1	Phlorrhizin (3 mM)	+	−1±1·6*	1±1·3*	−15±3·8	−14±3	−23±3	6
2	*N*-Ethylmaleimide (mM)	+	12±1·3	−10±1·7	−8±1·1	−20±1·7	−35±2·2	6
3	*p*-Chloromercuribenzoate (10 μM)	+	−1±0·8*	3±1	−5±1	−4±1·3	−9±2	6
4	Di*iso*propyl phosphorofluoridate (20 mM)	+	2±1·8*	.	1±3·3	−1±3·1*	.	6
5	Phlorrhizin (3 mM)	+	3±1·4	−3±1·8*	−9±3·5	−12±3·5	−20±6·3	6

accumulation of D-arabinose (glucose:arabinose concentration ratio 3:1) was inhibited (about 30%) by glucose.

Effect of D-3-O-*methylglucose on the transport of* D-*lyxose*

The results of this experiment are recorded in Fig. 2. D-3-*O*-Methylglucose at a concentration ratio (to D-lyxose) of 3:1 inhibited lyxose accumulation at 10, 20, 30 and 40 min. of incubation.

Effect of phlorrhizin, N-ethylmaleimide, p-chloromercuribenzoate and diisopropyl phosphorofluoridate on the transport of D-*xylose and* D-*galactose*

The results of these experiments are shown in Table 6. Phlorrhizin (3 mM), *N*-ethylmaleimide (mM) and *p*-chloromercuribenzoate (10 μM) inhibited accumulation of xylose (about 50, 80 and 20% respectively). Di*iso*propyl phosphorofluoridate (20 μM) did not affect the accumulation of xylose. These experiments were made in the presence of insulin. Phlorrhizin (3 mM) also diminished the accumulation of D-galactose in the presence of insulin by about 40%.

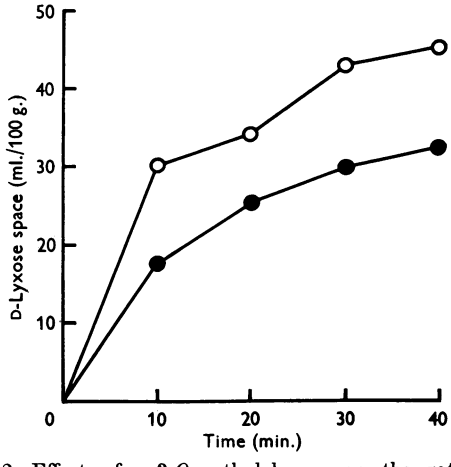

Fig. 2. Effect of D-3-*O*-methylglucose on the rate of accumulation of D-lyxose by the intact diaphragm preparation. Insulin was present at a concentration of 0·1 unit/ml. Each value is the mean of two observations. ○, D-Lyxose only (8·3 mM); ●, D-xylose (8·3 mM) + D-3-*O*-methylglucose (26 mM).

Effect of glucose on the uptake and on the incorporation of ^{14}C *from* [^{14}C]*mannose into glycogen and maltose in the diaphragm*

Since D-mannose is metabolized by diaphragm muscle the extent to which it accumulates in the muscle does not provide a measure of its transport. The uptake of the sugar and its incorporation into glycogen and maltose in isolated diaphragm have therefore been chosen as parameters of the rate of transport. The reasons for accepting rates of utilization as a measure of the rate of transport have been summarized by Randle & Smith (1958*a*).

Beloff-Chain *et al.* (1955) first showed that ^{14}C from [^{14}C]glucose is incorporated into maltose in isolated rat diaphragm and that the extent of this incorporation is enhanced by insulin. This we have confirmed in experiments in which eight hemidiaphragms were incubated for 10 min. at 37° in 3 ml. of bicarbonate medium containing [^{12}C]-glucose (5·75 mg./ml.) and [$^{14}C_6$] glucose (3 μC/ml.). Maltose was isolated from the muscle by chromatography (see Methods and Procedure section) and the radioactivity in maltose was 9400 counts/min./g. of wet muscle/hr. in the presence of insulin and 3520 in the absence of the hormone (an increase of 2·7-fold). The radioactivity in glucose was 26 200 in the presence and 24 500 in the absence of the hormone.

The results of experiments with [^{14}C]mannose are shown in Table 7. The uptake of [^{14}C]mannose from the medium by diaphragm was increased by insulin (2·8-fold) but depressed by [^{12}C]glucose (44%; ratio of concentrations glucose:mannose, 10:1). The incorporation of ^{14}C from [^{14}C]mannose into glycogen and maltose in the presence of insulin was depressed by [^{12}C]glucose (in glycogen 41%, in maltose 49%). Analysis of glycogen

Table 7. *Effect of insulin* (0·1 *unit/ml.*) *and of* [^{12}C]*glucose* (3 *mg./ml.*) *on the uptake and incorporation into maltose and glycogen in isolated rat diaphragm of* ^{14}C *from* D-[1-^{14}C]*mannose* (1·3 μC/*ml.*) *and* D-[^{12}C]*mannose* (0·3 *mg./ml.*)

Measurements were made on four hemidiaphragms incubated together in 3 ml. of medium for 20 min. at 37°

Addition	Insulin	Radioactivity (counts/min./g. of wet diaphragm/hr. of incubation) Uptake of mannose	In glycogen	In maltose
Mannose	−	273 800	.	.
	+	767 000	.	.
Mannose + glucose	+	430 140	.	.
Mannose	+	.	486 714	1880
Mannose + glucose	+	.	288 267	956

formed during incubation with [^{14}C]mannose showed that all the radioactivity was present as glucose residues and no radioactive mannose could be detected in a hydrolysate.

Effect of insulin on glycogen breakdown in diaphragm and on the incorporation of ^{14}C from [^{14}C]glucose into glycogen of rat heart under anaerobic conditions

The effect of insulin on glycogen breakdown in diaphragm muscle was investigated by incubating paired hemidiaphragms with and without insulin under aerobic conditions in medium free of glucose for 1 hr. at 37°. The final concentrations of glycogen (mg. of glycogen glucose/g. of wet diaphragm) were: without insulin, 1·7 + 0·25, and with insulin, 1·7 ± 0·25.

The effect of insulin on the incorporation of ^{14}C from [^{14}C]glucose into the glycogen of isolated rat heart in a 15 min. period of perfusion under anaerobic conditions is shown in Table 8. Under these conditions insulin did not increase the incorporation of ^{14}C into glycogen at either of the concentrations of glucose employed and indeed insulin appeared to effect a small reduction, although the differences observed were not significant. On the basis of earlier experiments (Morgan *et al.* 1959) the uptake of glucose under these conditions would be about 4·5 mg. in the presence of insulin and about 2·5 mg. in the absence of the hormone. The volume of perfusate in the present experiments was 10 ml., so that in the presence of insulin almost all of the glucose would be utilized at the lower concentration (0·5 mg./ml.) and about 25 % utilized at the higher concentration (2·0 mg./ml.).

DISCUSSION

The results of these studies of competition are summarized together with the structural features of the sugars in Table 9. We conclude from them that there is more than one monosaccharide-transport system in isolated diaphragm; that one system transports the competing sugars D-glucose, D-mannose, D-3-*O*-methylglucose, D-xylose, D-arabinose, D-lyxose and L-arabinose and that the sugars which do not compete with members of this group (D-galactose and D-fructose) are transported by different systems. α-Methyl-D-glucoside, β-methyl-D-glucose, maltose and D-sorbitol neither compete with D-xylose or L-arabinose, or both, for transport nor do they (unlike the sugars listed above) enter the muscle cell in the presence of insulin. Kipnis & Cori (1959) in similar studies with isolated diaphragm were able to detect competition between D-glucose, D-mannose and D-2-deoxyglucose, but not between D-glucose and D-xylose or D-2-deoxy-

Table 8. *Effect of insulin* (0·1 *unit/ml.*) *on incorporation of* ^{14}C *from* D-[$^{14}C_6$]*glucose into glycogen of perfused isolated rat heart under anaerobic conditions*

Differences marked with an asterisk are not significant. Numbers of observations are given in parentheses.

Concn. [^{12}C]Glucose (mg./ml.)	Concn. [^{14}C]Glucose (μc/ml.)	Insulin	Radioactivity (mean ± S.E.M.)	Counts/min. in glycogen/g. of wet heart/hr. of incubation (mean difference ± S.E.M.)
0·5	0·1	−	184 ± 25 (7)	−61 ± 35*
		+	123 ± 25 (7)	
2	0·4	−	1810 ± 41 (2)	−155 ± 160*
		+	1655 ± 156 (2)	

Table 9. *Configuration of sugars and related compounds which do or do not compete for transport in isolated diaphragm*

Sugar or related compound	Competition demonstrated with	No competition with	Position of −OH group above (+) or below (−) plane of pyranose ring C-2	C-3	C-4	C-5	C-6
1. D-Glucose	.	.	−	+	−	.	+
2. D-Mannose	1	.	+	+	−	.	+
3. D-3-*O*-Methylglucose	.	.	−	+($O.CH_3$)	−	.	+
4. D-Xylose	1, 2, 3	8, 9, 10, 12, 13	−	+	−	.	.
5. D-Arabinose	1	.	+	−	−	.	.
6. D-Lyxose	3	.	+	+	−	.	.
7. L-Arabinose	1, 2, 3	8, 9, 10, 11	−	+	+	.	.
8. D-Fructose	.	.	±	+	−	−	.
9. D-Galactose	.	1	−	+	+	.	+
10. α-Methyl-D-glucoside	.	.	−	+	−	.	+
11. β-Methyl-D-glucoside	.	.	−	+	−	.	+
12. D-Sorbitol	.	.	.	.	.	.	.
13. Maltose	.	.	.	.	.	.	.

glucose and D-galactose and D-fructose. They conclude that there may be more than one monosaccharide-transport system in diaphragm muscle. Although our observations confirm this suggestion we are unable to confirm that there is no competition between glucose and xylose. Our findings in diaphragm in this connexion are in agreement with the earlier observations of Park, Reinwein, Henderson, Cadenas & Morgan (1959), who have made similar studies in the perfused isolated heart.

In the present study all the monosaccharides which are transported by the same system as glucose are aldose sugars; the only ketose tested (fructose) is not transported by this system. Since pentoses are transported by the same system as hexoses the presence of a methoxy group on C-5 of the pyranose ring is not determinant. Our observations with 3-methylglucose and those of Kipnis & Cori with 2-deoxyglucose show that a hydroxyl group on C-2 or C-3 of the pyranose ring is not essential. Furthermore, our findings show also that the position of hydroxyl groups on C-2, C-3, C-4 or C-6 in relation to the plane of the pyranose ring is not determinant. On the other hand, the lack of competition between α-methyl-D-glucoside or β-methyl-D-glucoside and D-xylose or L-arabinose suggests that a hydroxyl group on C-1 of aldose sugars is essential; an alternative possibility is that a methyl group in this position interferes with transport. D-Galactose, which is not transported by the same system as glucose and other members of this group, differs only in having hydroxyl groups on C-4 and C-6 on the same side of the plane of the pyranose ring. Galactose is unique amongst sugars tested in this respect and it is important to note that L-arabinose, which has hydroxyl groups on the same side of the plane of the pyranose ring as galactose, on C-2, C-3 and C-4, is transported not by the system which transports galactose but by that which transports glucose. We conclude that a free hydroxyl group on C-1 of aldose sugars is necessary for transport, that aldose sugars are transported by a different system from the ketose sugar D-fructose and that the relative positions of hydroxyl groups on C-4 and C-6 in relation to the plane of the pyranose ring may determine whether an aldopyranose is transported by one system or another.

The transport of D-xylose and D-galactose in diaphragm was substantially reduced by phlorrhizin and this is in keeping with effects of phlorrhizin on sugar transport in other mammalian cells and tissues. Keller & Lotspeich (1959) find that phlorrhizin inhibits oxidative phosphorylation in mitochondrial preparations from rat kidney and liver. In view of this our finding that phlorrhizin inhibits sugar transport in diaphragm might appear anomalous, because it has been shown previously that substances such as salicylate or 2:4-dinitrophenol which inhibit oxidative phosphorylation accelerate transport of sugars in diaphragm (Randle & Smith, 1958*b*). We attribute this apparently anomalous effect of phlorrhizin to its inability to penetrate the muscle cell significantly. Thus the phlorrhizin space of isolated diaphragm estimated by the method of Jenner & Smyth (1959) was found to be very similar to the extracellular-fluid volume measured with sorbitol (phlorrhizin space 28 ml./100 g.; sorbitol space 24 ml./100 g.). Furthermore phlorrhizin does not (as would be expected if it penetrated muscle cells and inhibited oxidative phosphorylation therein) induce rigor in isolated diaphragm or alter the contractile activity of perfused isolated rat heart.

The transport of D-xylose was also inhibited by the –SH-group poisons *p*-chloromercuribenzoate and *N*-ethylmaleimide (cf. LeFevre, 1948), but not by di*iso*propyl phosphorofluoridate (thus excluding participation of enzymes or amino acid sequences reacting with this substance; for review see Dixon & Webb, 1958). This would suggest that the –SH group is involved in transport in some way, and if so there appear to be three possibilities in this connexion; (i) that transport involves reaction between the –SH group (possibly on a protein) and a group on a sugar, possibly the –OH group on C-1 of aldose sugars; (ii) that transport involves reaction between a –SH compound and a monosaccharide but that the –SH group does not participate directly in the reaction; (iii) that transport involves reaction between a monosaccharide and a cell constituent under the influence of a –SH enzyme. The third possibility is perhaps less likely than the other two because of the very low specificity of the transport system for D-xylose. Whichever of these explanations is the correct one, it is clear that transport involves reaction of more than one group on monosaccharides because both a free hydroxyl group on C-1 and a particular configuration of hydroxyl groups on C-4 and C-6 appear to be of importance in relation to the transport system for D-xylose.

Beloff-Chain *et al.* (1955) have emphasized that one of the major effects of insulin on carbohydrate metabolism in diaphragm is an enhanced formation of glycogen and of oligosaccharides such as maltose from [^{14}C]glucose. This we have confirmed. They have concluded from their observations that this is a primary site of action of the hormone and that the synthesis of glycogen and oligosaccharides may not involve phosphorylation of glucose by hexokinase. It is to be inferred from these conclusions that an effect of the hormone on sugar transport would be secondary to an effect on the synthesis of glycogen and oligosaccharides. Our findings do not support this view. Thus although insulin approximately doubles uptake of glucose by the perfused

isolated rat heart under anaerobic conditions (Morgan *et al.* 1959) it does not enhance incorporation of glucose into glycogen under these conditions. This would suggest that effects of the hormone on glucose transport are not dependent upon an increased rate of glycogen synthesis. Moreover, if insulin accelerated the formation of glycogen from some intermediate such as glucose 1-phosphate the hormone might also be expected to impede glycogen breakdown by increasing the rate at which glucose 1-phosphate derived from the breakdown of glycogen is incorporated again into the polysaccharide. Insulin did not impede glycogen breakdown in our experiments. Furthermore if the rate of glycogen synthesis in diaphragm does not depend upon transport or proceed through the hexokinase reaction it is difficult to see why glucose should inhibit the incorporation of mannose into glycogen glucose. The reactions involved in the transport and phosphorylation of glucose and mannose are the only points at which competition for entry into glycogen can be visualized as taking place at the present time. We have considered the possibility that glucose and mannose might be transferred from the transport form into glycogen without phosphorylation by hexokinase but this appears to be excluded by our finding that ^{14}C from [^{14}C]-mannose is found in glycogen only as glucose residues. Our observations are in every way consistent with the view first put forward by Levine & Goldstein (1955) and Park, Bornstein & Post (1955) that the rate-limiting step in the uptake of glucose by muscle is transport of the sugar across the cell membrane and that the major effect of insulin is to accelerate transport of the sugar.

SUMMARY

1. The specificity of monosaccharide transport in isolated diaphragm has been investigated by measuring the effect of other sugars on the intracellular accumulations of D-xylose, D-arabinose, D-lyxose, D-galactose and L-arabinose.

2. Competition for transport could be demonstrated between a group of sugars comprising D-glucose, D-mannose, D-3-*O*-methylglucose, D-xylose, D-arabinose, D-lyxose and L-arabinose, but not between members of this group and D-galactose, D-fructose, maltose, α-methyl-D-glucoside, β-methyl-D-glucoside or D-sorbitol.

3. Transport of D-xylose was inhibited by phlorrhizin and the –SH poisons *p*-chloromercuribenzoate and *N*-ethylmaleimide.

4. We conclude that there is more than one monosaccharide-transport system in muscle and that a –SH compound, possibly a –SH protein, is involved in monosaccharide transport. The factors of importance to the specificity of monosaccharide transport in diaphragm are discussed.

5. Insulin did not increase the incorporation of ^{14}C from [^{14}C]glucose into glycogen of perfused isolated rat heart under anaerobic conditions although it increases glucose uptake under these conditions. We conclude that an effect of the hormone on transport of glucose does not depend upon increased formation of glycogen.

6. [^{12}C]Glucose was found to decrease the incorporation of ^{14}C from [^{14}C]mannose into maltose and glycogen in isolated rat diaphragm. ^{14}C from [^{14}C]mannose was present only in glucose residues in glycogen; no [^{14}C]mannose could be detected in the polysaccharide. We conclude that the formation of glycogen from mannose follows transport and phosphorylation of the sugar and that sugars do not pass directly from the transport form into polysaccharide.

We wish to thank Professor F. G. Young, F.R.S., for his continued interest and encouragement. We are grateful to Mr B. R. Slater for a sample of β-methyl-D-glucoside. Mrs W. Thorne is thanked for skilled technical assistance. The cost of these investigations was defrayed in part by grants for expenses from the Medical Research Council (to P.J.R.) and from the U.S. Public Health Service (to F.C.B.).

REFERENCES

Beloff-Chain, A., Catamzarro, R., Beloff-Chain, E., Masi, I., Pocchiari, F. & Rossi, C. (1955). *Proc. Roy. Soc.* B, **143**, 481.

Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **47**, 201.

Dixon, M. & Webb, E. C. (1958). *Enzymes.* London: Longmans, Green and Co.

Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.

Jenner, F. A. & Smyth, D. H. (1959). *Biochem. J.* **71**, 138.

Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.

Keller, D. M. & Lotspeich, W. D. (1959). *J. biol. Chem.* **234**, 987.

Kipnis, D. M. & Cori, C. F. (1959). *J. biol. Chem.* **234**, 171.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.

LeFevre, P. G. (1948). *J. gen. Physiol.* **31**, 505.

Levine, R. & Goldstein, M. S. (1955). *Recent Progr. Hormone Res.* **11**, 343.

Morgan, H. E., Randle, P. J. & Regen, D. M. (1959). *Biochem. J.* **73**, 573.

Park, C. R., Bornstein, J. & Post, R. L. (1955). *Amer. J. Physiol.* **182**, 12.

Park, C. R., Reinwein, D., Henderson, M. J., Cadenas, E. & Morgan, H. E. (1959). *Amer. J. Med.* **26**, 674.

Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.

Randle, P. J. & Smith, G. H. (1958*a*). *Biochem. J.* **70**, 490.

Randle, P. J. & Smith, G. H. (1958*b*). *Biochem. J.* **70**, 501.

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.

Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.