Permeability of Rat Diaphragm Muscle to some Methyl Glycosides and its Relationship to Monosaccharide Transport

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1. Studies *in vitro* have shown that rat diaphragm muscle is freely permeable to α - and β -methyl D-xylosides and β -methyl L-arabinoside but not to α - or β -methyl D-glucoside or α -methyl D-mannoside. 2. No competition for entry between methyl pentosides and D-xylose, L-arabinose, D-glucose or 3-O-methyl-D-glucose could be demonstrated and α -methyl xyloside entry was not inhibited by phlorrhizin. 3. It is concluded that the monosaccharide transport system is unlikely to contribute significantly to the entry of the methyl pentosides in this tissue.

In rat diaphragm muscle α - and β -methyl D-glucosides do not interfere with the entry of a number of hexoses and pentoses which appear to penetrate the muscle through a common transport system. The latter include D-glucose, D-xylose and L-arabinose. The suggestion was made that the C-1 hydroxyl group of these sugars may be involved in the transport mechanism (Battaglia & Randle, 1960). Subsequently preliminary experiments suggested that rat diaphragm muscle is freely permeable to β -methyl D-xyloside and β -methyl L-arabinoside (E. A. Newsholme & P. J. Randle, unpublished observations).

The permeability of rat diaphragm to a number of methyl glycosides and their ability to compete with D-xylose or L-arabinose has now been investigated by following their intracellular accumulation in diaphragm muscle *in vitro*.

MATERIALS

Rats. Diaphragms were obtained from male albino Wistar rats, 100–150 g., starved for 18–20 hr.

Chemicals. D-Xylose, L-arabinose and D-sorbitol were obtained from T. Kerfoot and Co. Ltd., D-glucose and α -methyl D-glucoside from British Drug Houses Ltd., α -methyl D-xyloside, β -methyl D-xyloside, β -methyl D-glucoside and α -methyl D-mannoside from Pfanstiehl Laboratories Inc., Ill., U.S.A.; 3-O-methyl-D-glucose was from Ayerst, McKenna and Harrison Inc., N.Y., U.S.A., and β -methyl L-arabinoside from K and K Laboratories, N.Y., U.S.A. All methyl glycosides were in the pyranose form. D-[U-¹⁴C]Sorbitol (6.4 mc/m-mole) and α -methyl D-[U-¹⁴C]glucoside (2.14 mc/m-mole) were from The Radiochemical Centre, Amersham, Bucks. Crystalline insulin (a gift of Boots Pure Drug Co. Ltd., Nottingham, or Burroughs Wellcome Ltd.), was dissolved in 3M-N-HCl to give a stock solution of 20 units/ml. which was further

diluted with incubation medium as required. Dowex 50 (H form) (AG 50W-X2) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A., and Amberlite IR-4B (OH-) form from British Drug Houses Ltd.

Media. Diaphragms were incubated in bicarbonate-buffered medium (Krebs & Henseleit, 1932) with additions as given in text or Tables. The gas phase was O₂ + CO₂ (95:5).

PROCEDURE

Diaphragms. For measurement of tissue spaces intact diaphragms were incubated in 10–15 ml. of medium in 50 ml. Erlenmeyer flasks as described by Randle & Smith (1958). The periods of incubation are given in text, Tables or Figures. At the end of incubation the lateral masses of diaphragm muscle were excised from the rib cage, weighed to the nearest milligram on a torsion balance and sugars, methyl glycosides or sorbitol were extracted with water at 100° for 15 min. and deproteinized with Zn(OH)₂ as described by Battaglia & Randle (1960).

For measurement of incorporation of ¹⁴C into carbon dioxide and glycogen from α -methyl D-[U-¹⁴C]glucoside, pairs of hemidiaphragms were incubated for 40 min. in 5 ml. of medium containing α -methyl D-[U-¹⁴C]glucoside (2.5 mg./ml.; 1 μ C/ml.) in 50 ml. conical flasks with a centre well containing a 1.2 cm. $\times$ 5 cm. glass tube and sealed with a skirted rubber cap. At the end of incubation 0.5 ml. of M-Hyamine in methanol was injected into the glass tube and 1.2 ml. of 8N-H₂SO₄ into the outer annulus containing the muscle. Incubation was then continued for a further 90 min. Radioactivity in carbon dioxide was then assayed in the Hyamine as described by Garland, Newsholme & Randle (1964). The hemidiaphragms were removed, blotted, and digested with 30% KOH and glycogen was precipitated with 70% (v/v) ethanol at 70°. The crude glycogen was washed with cold 70% ethanol and then dissolved in water and reprecipitated twice with 70% ethanol. It was then dissolved in water and assayed for radioactivity after plating on planchets as described below.

Pentoses and methyl pentosides. D-Xylose and L-arabinose were assayed spectrophotometrically in incubation media or muscle extracts by the *p*-bromoaniline method of Roe & Rice (1948). α -Methyl D-xyloside, β -methyl D-xyloside and

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Table 1. *Incorporation of ^{14}C from α -methyl D-[U- ^{14}C]glucoside into carbon dioxide and glycogen in rat hemidiaphragms*

Two hemidiaphragms (wt. about 250 mg.) were incubated for 40 min. in 5 ml. of medium containing α -methyl D-[U- ^{14}C]glucoside (2.5 mg./ml.; 1 μC /ml.). Radioactivity in incubation medium and muscle glycogen was assayed by gas flow counting and in carbon dioxide by scintillation counting as described in the Procedure section. The values have been corrected to 100% efficiency assuming 30% efficiency for gas flow counting and 80% efficiency for scintillation counting. The intracellular α -methyl D-[U- ^{14}C]glucoside was calculated from an intracellular space of 40 ml./g. of wet muscle (see Table 2). The values for radioactivity in carbon dioxide were corrected for radioactivity released to Hyamine by acid treatment of the initial incubation medium.

Parameter	Radioactivity
α -Methyl D-[U- ^{14}C]glucoside in incubation medium (counts/min./ml.)	1 700 000
Intracellular α -methyl D-[U- ^{14}C]glucoside after 40 min. (counts/min./g. of wet muscle)	68 000
Carbon dioxide (counts/min./g. of wet muscle)	2 400
Glycogen (counts/min./g. of wet muscle)	2 000

β -methyl L-arabinoside were assayed as xylose or arabinose after heating at 100° for 3 hr. with HCl-acetic acid mixture (0.3 ml. of aq. solution containing 1.5 N-HCl and 0.9 N-acetic acid was added to 1 ml. of pentose solution). Hydrolysis of methyl pentosides was shown to be complete under these conditions.

3-O-Methyl-D-glucose. 3-O-Methyl-D-glucose was assayed spectrophotometrically by copper reduction with the Somogyi (1945) modification of Nelson's method. Muscle extracts were treated before assay with moist ion-exchange resins, Dowex 50 (H^+ form) and Amberlite IR-4B (OH-form) to diminish copper reduction by other substances. A correction for the latter (about 20% of the methylglucose concentration) was applied by measurements made with extracts of diaphragms incubated with sorbitol but without sugar.

α -Methyl D-mannoside. α -Methyl D-mannoside was assayed as mannose with the Somogyi (1945) method as described for 3-methylglucose, after hydrolysis with N-H₂SO₄ for 3 hr. at 100° and neutralization with N-NaOH. The blank correction was about 80% of the α -methyl D-mannoside concentration.

α - and β -methyl D-glucosides. These were assayed as glucose with the glucose oxidase method of Huggett & Nixon (1957). Hydrolysis was carried out as described for α -methyl D-mannoside. Glucose from other sources (estimated with diaphragms incubated without methyl glucosides) was about 20% of that from α - or β -methyl glucosides.

Table 2. *Penetration of intact rat diaphragm in vitro by methyl glycosides and sugars in the absence and in the presence of insulin (0.1 unit/ml.)*

Intact diaphragms were incubated for the time stated in medium containing sugars or their derivatives as given (2.5 mg./ml.) and D-[U- ^{14}C]sorbitol (0.12 mg./ml., 0.05 μC /ml.). Other details are as given in the Procedure section. The number of diaphragms incubated in each group is given in parentheses. * $P < 0.01$ vs. appropriate control. ** $P < 0.05$ vs. appropriate control. For other differences $P > 0.05$.

Sugar or derivative	Insulin (0.1 unit/ml.)	Intracellular space (ml./100 g. of muscle) (mean $\pm$ S.E.M.)		
		Time (min.)		
		10	20	40
β -Methyl L-arabinoside (6)	—	18 $\pm$ 1.1	31 $\pm$ 0.7	44 $\pm$ 4.0
β -Methyl L-arabinoside (4)	+	24** $\pm$ 2.4	35 $\pm$ 3.1	45 $\pm$ 4.0
β -Methyl D-xyloside (36)	—	17 $\pm$ 1.3	28 $\pm$ 1.7	36 $\pm$ 0.6
β -Methyl D-xyloside (6)	+	21** $\pm$ 1.5	32 $\pm$ 3.3	38 $\pm$ 1.8
α -Methyl D-xyloside (2)	—	27 $\pm$ 2.1	36 $\pm$ 3.5	46 $\pm$ 0.7
L-Arabinose (8)	—	6 $\pm$ 1.6	10 $\pm$ 3.3	11 $\pm$ 1.6
L-Arabinose (8)	+	10 $\pm$ 1.6	23* $\pm$ 1.0	31* $\pm$ 0
D-Xylose (24)	—	—	—	9 $\pm$ 0.2
D-Xylose (6)	+	8 $\pm$ 2.1	22 $\pm$ 2.0	32* $\pm$ 2.9
α -Methyl D-glucoside† (6)	—	1 $\pm$ 1.7	1 $\pm$ 1.4	4 $\pm$ 1.2
α -Methyl D-glucoside† (6)	+	1 $\pm$ 1.4	2 $\pm$ 1.5	7 $\pm$ 1.2
α -Methyl D-glucoside (6)	—	1 $\pm$ 1.5	1 $\pm$ 1.4	5 $\pm$ 1.4
α -Methyl D-glucoside (6)	+	2 $\pm$ 1.0	5 $\pm$ 1.4	8 $\pm$ 0.6
β -Methyl D-glucoside (6)	—	4 $\pm$ 1.4	5 $\pm$ 1.3	9 $\pm$ 1.4
β -Methyl D-glucoside (6)	+	4 $\pm$ 0.9	6 $\pm$ 1.1	9 $\pm$ 0.8
3-O-Methyl-D-glucose (6)	—	7 $\pm$ 4.4	9 $\pm$ 3.2	11 $\pm$ 2.9
3-O-Methyl-D-glucose (6)	+	10 $\pm$ 9.3	22** $\pm$ 4.4	32* $\pm$ 3.2
α -Methyl D-mannoside (6)	—	—	—	3 $\pm$ 2.0
α -Methyl D-mannoside (6)	+	—	—	3 $\pm$ 2.2

† Measurements made with α -methyl D-[U- ^{14}C]glucoside (0.05 μC ml.) with sucrose as extracellular marker in place of sorbitol.

Sucrose. Sucrose was assayed spectrophotometrically with the method of Roe, Epstein & Goldstein (1949).

D-[U-¹⁴C]Sorbitol; α -methyl D-[U-¹⁴C]glucoside; [¹⁴C]-glycogen. Radioactivity was assayed in these substances in a Nuclear-Chicago Gas Flow Counter after plating on aluminium planchets freed of grease with diethyl ether and ringed with silicone grease, addition of a drop of aq. 1% Teepol and drying with an infrared lamp.

Calculations. Tissue spaces for sugars, methyl glycosides and sorbitol were calculated from the expression:

space (ml./100 g. of wet muscle)

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

The medium concentration of sugars, methyl glycosides and sorbitol were assayed on the initial incubation medium. Control experiments showed that no loss of any of these compounds could be detected during incubation with diaphragm under the conditions used. The intracellular space of sugars or methyl glycosides which has been used as an index of rate of penetration was calculated as tissue space - extracellular volume (measured as sorbitol or sucrose space).

RESULTS

Metabolism of β -methyl D-xyloside and α -methyl D-[U-¹⁴C]glucoside by rat diaphragm muscle. To test whether or not β -methyl D-xyloside was hydrolysed, intact diaphragms were incubated for 10 and 20 min. in medium containing 2.5 mg. of the xyloside/ml. No xylose could be detected by the spectrophotometric method in extracts of the muscle. When pairs of hemidiaphragms were incubated for 40 min. in 5 ml. of medium containing β -methyl D-xyloside (2.5 mg./ml.) no loss of xyloside from the medium was detected (penetration of the muscle by xyloside would be expected to lead to a loss of less than 1%). It would appear that β -methyl D-xyloside is not appreciably metabolized by rat diaphragm muscle and we shall assume that the same is true for α -methyl D-xyloside and β -methyl L-arabinoside.

The possible metabolism of α -methyl D-glucoside by hemidiaphragms has been explored with ¹⁴C-labelled glucoside. As the results in Table 1 show, the incorporation of radioactivity into carbon dioxide or glycogen amounted to no more than 5% of the radioactivity taken up when hemidiaphragms were incubated for 40 min. with α -methyl D-[¹⁴C]glucoside.

Penetration of rat diaphragm by methyl glycosides. These results are shown in Table 2 and Fig. 1. The penetration of diaphragm muscle by α -methyl D-xyloside, β -methyl D-xyloside and β -methyl L-arabinoside was rapid and the intracellular space after 40 min. of incubation was between 36 and 46 ml./100 g. In further experiments (not shown) with β -methyl D-xyloside in which the period of incubation was extended to 60, 90 and 120 min. no further increase in the intracellular space was

Fig. 1. Time-course of intracellular accumulation of β -methyl L-arabinoside and α -methyl D-glucoside by intact rat diaphragm. ●, β -Methyl L-arabinoside; ○, α -methyl D-glucoside. Intact diaphragms were incubated for the time shown in medium containing D-[¹⁴C]sorbitol (0.12 mg./ml., 0.05 μ C/ml.) and either β -methyl L-arabinoside or α -methyl D-glucoside at a concentration of 2.5 mg./ml. Insulin (0.1 unit/ml.) was present in the experiment with α -methyl D-glucoside. Other details are as given in the Procedure section. There were six observations for each point.

detected. It would appear therefore that penetration is substantially complete at 40 min. Since the intracellular volume of rat diaphragm is about 50 ml./100 g. (calculated from total water-sorbitol space) it would appear that about 80% of intracellular water is available for penetration by methyl pentosides. The rate of penetration of diaphragm by α - and β -methyl D-xylosides and by β -methyl L-arabinoside is substantially greater than that of D-xylose or L-arabinose even when the latter has been accelerated by insulin. The penetration of diaphragm muscle by α -methyl D-glucoside, β -methyl D-glucoside and α -methyl D-mannoside was slow and for the most part not statistically significant until 20–40 min. of incubation. By contrast the penetration by 3-O-methylglucose was much more rapid, especially when accelerated by insulin.

The effects of insulin are shown in Table 2. The hormone had no effect on the rate of penetration of α - and β -methyl D-glucosides or α -methyl D-mannoside under conditions where penetration by 3-O-methyl-D-glucose was markedly accelerated. Insulin produced a small but statistically significant increase in the rate of penetration of β -methyl L-arabinoside and β -methyl D-xyloside. The effects were small by comparison with its effects on the rate of penetration of L-arabinose and D-xylose.

Studies of competition and effects of phlorrhizin.

Table 3. *Penetration of intact diaphragm in vitro by methyl glycosides and pentose: studies of composition and effect of phlorrhizin*

Intact diaphragms were incubated for the time stated in medium containing D-¹⁴C sorbitol (0.12 mg./ml.; 0.05 μ C/ml.) and other compounds at concentrations given in the Table. Other details are as given in the Procedure section. Six diaphragms were incubated in each group. ** $P < 0.05$ vs. appropriate control. For other differences $P > 0.05$.

Intracellular space measured for	Present in addition	Insulin (0.1 unit/ml.)	Intracellular space (ml./100g. of muscle) (mean $\pm$ S.E.M.)		
			Time (min.)		
			10	20	40
D-Xylose (2.5 mg./ml.)	—	+	8 $\pm$ 2.1	22 $\pm$ 2.0	32 $\pm$ 2.9
	β -Methyl D-xyloside (10 mg./ml.)	+	14 $\pm$ 2.1	22 $\pm$ 1.1	32 $\pm$ 1.9
D-Xylose (1.25 mg./ml.)	—	+	—	15 $\pm$ 2.8	—
	β -Methyl D-xyloside (20 mg./ml.)	+	—	15 $\pm$ 1.5	—
L-Arabinose (2.5 mg./ml.)	—	+	12 $\pm$ 0.9	24 $\pm$ 1.6	31 $\pm$ 1.8
	β -Methyl L-arabinose (10 mg./ml.)	+	11 $\pm$ 0.8	23 $\pm$ 1.0	30 $\pm$ 0.9
L-Arabinose (1.25 mg./ml.)	—	+	—	18 $\pm$ 1.1	—
	β -Methyl L-arabinose (20 mg./ml.)	+	—	17 $\pm$ 1.4	—
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	17 $\pm$ 1.9	32 $\pm$ 2.6	37 $\pm$ 1.6
	D-Xylose (10 mg./ml.)	—	23** $\pm$ 1.3	32 $\pm$ 2.3	43** $\pm$ 1.3
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	14 $\pm$ 1.5	26 $\pm$ 1.7	36 $\pm$ 1.4
	D-Glucose (10 mg./ml.)	—	16 $\pm$ 0.6	26 $\pm$ 1.4	38 $\pm$ 1.4
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	22 $\pm$ 1.3	30 $\pm$ 2.4	39 $\pm$ 1.7
	3-O-Methyl-D-glucose (10 mg./ml.)	—	23 $\pm$ 1.4	33 $\pm$ 1.7	42 $\pm$ 2.3
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	15 $\pm$ 1.2	26 $\pm$ 1.4	36 $\pm$ 2.6
	α -Methyl D-glucoside (10 mg./ml.)	—	19** $\pm$ 0.5	29 $\pm$ 1.7	36 $\pm$ 1.2
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	16 $\pm$ 1.1	26 $\pm$ 0.4	34 $\pm$ 1.7
	α -Methyl D-mannoside (10 mg./ml.)	—	16 $\pm$ 1.4	24 $\pm$ 1.7	37 $\pm$ 1.8
β -Methyl D-xyloside (2.5 mg./ml.)	—	—	23 $\pm$ 1.4	36 $\pm$ 2.8	38 $\pm$ 1.5
	Phlorrhizin (1.2 mg./ml.)	—	21 $\pm$ 2.0	34 $\pm$ 2.1	38 $\pm$ 1.9

These results are given in Table 3. The penetration of diaphragm by D-xylose and L-arabinose was not influenced by β -methyl D-xyloside or β -methyl L-arabinoside with concentration ratios pentoside: pentose as high as 16:1. The penetration of diaphragm by β -methyl D-xyloside was not diminished by D-xylose, D-glucose, 3-O-methyl-D-glucose, α -methyl D-glucoside or α -methyl D-mannoside at concentration ratios pentoside: hexoside or sugar 1:4. Phlorrhizin (3 mm) did not inhibit the penetration of diaphragm by β -methyl D-xyloside.

Effect of temperature on penetration of diaphragm by β -methyl D-xyloside. These results are shown in Fig. 2. The intracellular accumulation of β -methyl D-xyloside in diaphragm was substantially greater at 37° than at 25° or 15°. After 10 min. of incubation the intracellular space at 37° was approximately twice that at 15°.

DISCUSSION

The present studies have shown that α - and β -methyl D-xylosides penetrate rat diaphragm muscle rapidly and that α -methyl D-glucoside and α -methyl D-mannoside penetrate slowly. There was no evidence for competition for entry between α -methyl D-xyloside and D-xylose, D-glucose or 3-O-methyl-D-glucose, or between β -methyl L-arabinoside and L-arabinose; and phlorrhizin failed to inhibit penetration of β -methyl D-xyloside. It thus seems unlikely that the transport system for glucose, 3-methylglucose, xylose or arabinose contributes significantly to the entry of these methyl pentosides. These findings are consistent with earlier observations with methyl glucosides and with the suggestion that the C-1 hydroxyl group of glucose, 3-methylglucose, xylose and arabinose may

Fig. 2. Effect of temperature on the intracellular accumulation of β -methyl D-xyloside by intact rat diaphragm. ●, 37°; ○, 25°; △, 15°. Intact diaphragms were incubated for the time shown in medium containing D-[U-¹⁴C]sorbitol (0.12 mg./ml., 0.05 μ C/ml.) and β -methyl D-xyloside (2.5 mg./ml.). Other details are given in the Procedure section. There were six observations for each point.

participate in their transport. The observation that insulin can accelerate entry of β -methyl L-arabino- and β -methyl D-xyloside in diaphragm is not inconsistent with the proposal that they do not enter by the monosaccharide transport system. Insulin is known to affect the uptake of a number of chemically unrelated substances by muscle including (apart from monosaccharides) amino acids, phosphate, ³H-labelled water and Na⁺.

Entry of methyl glycosides into a number of other cells has been described. Rabbit erythrocytes are permeable to α -methyl D-glucoside (Hellman, Landau & Ashmore, 1959) and to β -methyl D-xyloside (unpublished observations of present authors). Human erythrocytes are permeable to α -methyl D-glucoside and α -methyl D-galactoside (Wilbrandt, 1961). Active transport of α -methyl D-glucoside has been observed in the small intestine, but the specificity of the intestinal system is known to differ from that of muscle in other respects, for example in its inability to transport mannose or 2-deoxyglucose (e.g. Crane, Miller & Bihler, 1961). Permeability to α -methyl D-glucoside but not to α -methyl D-galactoside has been noted in baker's yeast by Cirillo (1961). The presence or absence of competition for entry between methyl glycosides and hexoses or pentoses was not reported in these studies. In a strain of *Escherichia coli* Cohen & Monod (1957) noted that accumulation of α -methyl glucoside (which was not metabolized) was strongly

inhibited by glucose. Horecker, Osborn, McLellan, Avigad & Asensio (1961) found on the other hand that entry of galactose in a strain of *E. coli* was not inhibited by β -methyl D-galactoside.

The results of the present study leave open the question whether methyl xylosides and methyl arabinoside enter muscle cells by transport or diffusion. Studies of the initial rate of entry as a function of methyl pentoside concentration gradient could resolve this question. Accurate measurement of initial rates of penetration and concentration gradients in diaphragm have not been possible because the rate of extracellular penetration is not high in relation to the rate of intracellular penetration. Attempts to make kinetic measurements in rabbit erythrocytes by direct analyses of pentoside accumulation have also been unrewarding because of the rapidity of penetration even at low temperatures. The marked temperature dependence of α -methyl D-xyloside penetration in diaphragm noted in the present studies might suggest that a transport process is involved but this observation alone may not be conclusive.

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