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STUDIES ON CARBOHYDRATE-METABOLISING ENZYMES

PART XVII* THE ENZYMIC SYNTHESIS OF SOME DISACCHARIDES CONTAINING α-D-GLUCOSYL RESIDUES

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ABSTRACT

By the action of an enzyme preparation from *Tetrahymena pyriformis*, α-D-glucosyl residues may be transferred from phenyl α-D-glucopyranoside to various carbohydrates By this means, the following disaccharides have been prepared and characterised 6-*O*-α-D-glucopyranosyl-D-galactose and -D-mannose, 4-*O*-α-D-glucopyranosyl-L-xylose, 1-*O*-α-D-glucopyranosylribitol, 1-*O*-α-D-glucopyranosylerythritol, 1-*O*-α-D-glucopyranosyl-D-mannitol, and a mixture of 1-*O*- and 6-*O*-α-D-glucopyranosyl-D-glucitol These results show the preferential transfer of α-D-glucosyl residues to primary alcohol groups in carbohydrate acceptor substrates

INTRODUCTION

Previous studies[2,3] have shown that extracts of the ciliate *Tetrahymena pyriformis* show hydrolytic activity towards α-D-glucosides, and can also transfer an α-D-glucosyl residue from a suitable donor substrate (*e g*, maltose or phenyl α-D-glucopyranoside) to other carbohydrates For example, transfer to D-glucose and to D-xylose yields isomaltose[2] and 4-*O*-α-D-glucopyranosyl-D-xylose[3], respectively, whereas transfer to D-lyxose gives a mixture of 2-*O*-, 3-*O*-, and 4-*O*-α-D-glucopyranosyl-D-lyxose

The enzyme system shows a distinct specificity towards the acceptor substrates Preliminary studies[3] have shown that D-fructose, D-glucuronic acid, sucrose, and methyl α-D-glucopyranoside did not serve as acceptors, even though their chemical structures are not very different from that of D-glucose By contrast, other monosaccharides (*e g*, D-galactose and D-mannose) and several alditols (*e g*, erythritol and glycerol) were acceptors, and the enzymic transfer resulted in the formation of a new disaccharide. We now report on the characterisation of these products A preliminary account of this work has been given elsewhere[4]

*For Part XVI, see Ref 1

MATERIALS AND METHODS

Materials — Freeze-dried preparations of *Tetrahymena pyriformis* cells (Extracts *B* and *C*), kindly provided by Dr J F Ryley, were used as the source of enzyme Extract *C* was prepared in 1966 and showed[3] a similar but slightly stronger activity than extract *B*.

Phenyl α-D-glucopyranoside was synthesised as described previously[3] D-Mannose, D-galactose, D-glucitol, and L-xylose were commercial or laboratory samples which were purified, as required, by preparative, paper chromatography Ribitol was prepared by the borohydride reduction of D-ribose, and freed from borate by repeated evaporation of methanol at 40°

General methods — The conditions used for the partial, acid hydrolysis of disaccharides, before and after reduction with potassium borohydride, and for the periodate oxidation and micro-scale methylation of disaccharides were those used previously[3] Gas–liquid partition chromatography of methyl glycosides was carried out with a Pye 104 Chromatograph, according to the procedure of Aspinall[5]

Paper chromatography and electrophoresis — The following solvent systems were used for paper chromatograms (*A*) ethyl acetate–pyridine–water (10 4 3), (*B*) ethyl acetate–acetic acid–formic acid–water (18 3 1 4), (*C*) butyl alcohol–acetic acid–water (4 1 5 upper layer)

Electrophoresis of disaccharides was effected by using a small-scale Shandon apparatus, with 0 05M borate buffer (pH 10 0), results are expressed as M_G values (D-glucose = 1 00) For non-reducing disaccharides or for borohydride-reduced disaccharides, a molybdate buffer (pH 5 0) was used, results are expressed[3] as M_s (D-glucitol = 1 00)

The following spray reagents were used, aniline oxalate, silver nitrate, aniline–diphenylamine (a specific reagent[6] for oligosaccharides containing a 4-*O*-substituted aldohexose reducing end-group), periodate–*p*-rosaniline (a specific reagent[7] for carbohydrates, *e g*, 3-*O*-substituted hexoses and hexitols, which on periodate oxidation, give a malonaldehyde derivative)

Preparation and isolation of disaccharides — Digests containing phenyl α-D-glucopyranoside (5 mg), acceptor (5 mg), and extract *B* (1 mg) in water (0 5 ml) were incubated under toluene for 48 h at 37° Paper-chromatographic analysis (solvent *A*) showed the presence of the acceptor, glucose, isomaltose (R_G 0 46), and a new carbohydrate, which had R_G 0 57, 0 59, 0 65, 0 39, and 0 34 when D-mannose, ribitol, L-xylose, D-glucitol, and D-galactose, respectively, was the acceptor Acid hydrolysis of all of the new carbohydrates gave a mixture of equal amounts (visual estimation) of glucose and the acceptor, showing that the latter had all functioned as acceptors for trans-α-D-glucosylation

The rate of formation of the new carbohydrates was examined by using digests containing phenyl α-D-glucopyranoside (10 mg), acceptor (10 mg), and extract *B* (2 mg) in water (0 3 ml), which were incubated at 37° and examined at intervals by paper chromatography (solvent *A*, silver nitrate spray) The results showed that the

alditols, ribitol and D-glucitol, were the most efficient acceptors, since the glucosyl-alditols were produced in preference to isomaltose L-Xylose appeared to be equally as efficient an acceptor as D-glucose, and a considerably more efficient acceptor than either D-galactose or D-mannose

Except for the synthesis of α-D-glucosyl-L-xylose, large-scale digests [containing phenyl α-D-glucopyranoside (4 5 g), carbohydrate acceptor (5 0 g), extract *B* or *C* (1 0 g), and water (100 ml)] were incubated at 37° under toluene for a period of time (1 to 6 days) which would give the maximal amount of new disaccharide with minimal formation of isomaltose The disaccharide was then isolated from the enzyme digests (which had been heated for 20 min at 100° to inactivate the enzymes, cooled, centrifuged, and concentrated) by an appropriate combination of charcoal–Celite column chromatography or preparative, paper chromatography with Whatman 3 MM paper and solvent *A*

RESULTS

Properties of α-D-*glucosyl*-D-*mannose* — The chromatographic and electrophoretic properties of α-D-glucosyl-D-mannose (yield 85 mg) are given in Table I The disaccharide gave a brown colour with aniline–diphenylamine [absence of a (1→4)-linkage] and a blue colour with periodate–*p*-rosaniline [absence of a (1→3)-linkage] These conclusions are supported by the high M_s value of the borohydride-reduced disaccharide, which indicated the presence of at least four adjacent hydroxyl groups[8]

TABLE I

PROPERTIES OF α-D-GLUCOSYL DISACCHARIDES

Property	*Glucosyl-mannose*	*Glucosyl-galactose*	*Glucosyl-ribitol*	*Glucosyl-glucitol*	*Glucosyl-erythritol*	*Glucosyl mannitol*
$[\alpha]_D^{20}$, water (degrees)	+91	+124	+84	+76	+115	+101
R_G, solvent *A*	0 57	0 34	0 59	0 39	0 76	0 45
R_G, solvent *C*	0 42	0 31	0 53	0 38	0 75	—
M_s, molybdate	0 69[a]	0 69[a]	0 60	0 68	—	0 70
Colour with aniline oxalate	brown	brown	none	none	none	none
Colour with periodate–*p*-rosaniline	blue	blue	purple	purple	purple	purple
Periodate oxidation						
(*a*) formaldehyde released (mol)	—	—	0 96	0 99	—	0 94
(*b*) periodate reduced (mol)	—	—	2 8	4 0	—	—

[a]After borohydride reduction

The borohydride-reduced disaccharide gave a mixture of glucose and mannitol on acid hydrolysis The disaccharide was methylated[3], and after methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-

mannose were detected by g l c These results indicate a (1→6)-linkage, and since the disaccharide had $[\alpha]_D$ +91° (*c* 1 02, water), an α-D configuration is inferred The disaccharide is therefore 6-*O*-α-D-glucopyranosyl-D-mannopyranose

Properties of α-D-*glucosyl*-D-*galactose* — This disaccharide (yield 21 mg) gave a blue coloration with periodate–*p*-rosaniline and a brown colour with aniline–diphenylamine, and the reduced disaccharide had a high electrophoretic mobility (see Table I, *cf* α-D-glucosyl-D-mannose) Methanolysis of the methylated disaccharide gave the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-galactose Since the disaccharide had $[\alpha]_D$ +124° (*c* 0 98, water), these results characterise it as 6-*O*-α-D-glucopyranosyl-D-galactose

Properties of α-D-*glucosyl*-L-*xylose* — A digest containing phenyl α-D-glucopyranoside (0 9 g), L-xylose (1 03 g), and extract *C* (0 22 g) in water (20 ml) was incubated for 48 h Glucosylxylose (40 mg) was isolated from the digest, the chromatographic and other properties are given in Table II Both before and after borohydride reduction, the disaccharide gave a blue colour with the periodate–*p*-rosaniline reagent, suggesting the absence of a (1→2)- or a (1→3)-linkage After methylation and subsequent methanolysis, the methyl glycosides of 2,3,4,6-tetra-*O*-methyl-D-glucose and a carbohydrate having retention times similar to those of 2,3-di-*O*-methyl-D-xylose were detected by g l c , indicating a (1→4)-xylopyranoside or (1→5)-xylofuranoside structure On oxidation with 15mM sodium metaperiodate at 18°, the reduction of periodate was 3 1, 3 6, 3 8, and 3 9 (constant) mol after 1 5, 5 5, 19, and 23 h A (1→4)- or (1→5)-linked glucosylxylose would reduce 4 0 mol The presence of a (1→4)-linkage was finally indicated by the M_s value of the reduced disaccharide, which was similar to that for the product from 4-*O*-α-D-glucopyranosyl-D-xylose (see Table II), and lower than the value expected for a (1→5)-linked structure[3]

TABLE II

PROPERTIES OF α-D-GLUCOSYLXYLOSES

	α-D-*Glucosyl*-L-xylose	α-D-*Glucosyl*-D-xylose[3]
$[\alpha]_D$, water (degrees)	+62	+97
R_G value, solvent *A*	0 65	0 68
Colour with aniline oxalate	pink	pink
Colour with periodate–*p*-rosaniline reagent	blue	blue
M_G value, borate	0 31	0 28
M_s value, molybdate	0 35	0 30
Periodate reduction, mol	3 9	3 9

Properties of α-D-*glucosylribitol* — The α-D-glucosylribitol readily crystallised from ethanol (yield 160 mg) and was homogeneous by chromatography in solvents *A* and *C* It did not react with aniline oxalate, but gave an immediate purple colour with the periodate–*p*-rosaniline reagent, indicating the release of formaldehyde On oxidation at 2° with 2mM sodium metaperiodate, 0 83, 0 96, and 0 96 mol of formaldehyde were released after 5, 19, and 26 h On oxidation at 18° with the dilute

solution of metaperiodate, the periodate reduction was 2 7, 3 3, 3 5, and 3 9 mol after 2.5, 21 5, 28, and 48 h. When the amount of periodate reduced was plotted against time, extrapolation gave an initial periodate reduction of 2 8 mol Since a 3-*O*-substituted ribitol would have released 2 mol of formaldehyde, and a 2-*O*- or 4-*O*-substituted ribitol would have reduced 2 mol. of periodate under these experimental conditions (in which the linear ribitol residue would be preferentially oxidised, *cf* Ref 9), the results show the new disaccharide to be a 1-*O*-substituted ribitol This conclusion is supported by the high M_s value, which indicates the presence of at least four adjacent hydroxyl groups[8]

Properties of α-D-*glucosyl*-D-*glucitol* — This disaccharide was crystallised from ethanol (yield 178 mg), was chromatographically homogeneous, and had $[\alpha]_D$ +76° (*c* 0 37, water) The reaction with aniline oxalate and periodate–*p*-rosaniline reagents was the same as for glucosylribitol On oxidation in dilute solution at 2° with 2mM sodium metaperiodate, 0 92, 0 97, and 0 99 mol. of formaldehyde were released after 6, 24, and 27 h On oxidation at 18°, the periodate reduction was 4 1, 4 6, 4 8, 5 1, and 5 3 mol after 2 5, 21 5, 28, 45, and 52 h These results correspond to an initial reduction of 4 0 mol of periodate, and show the presence of a 1-*O*- or 6-*O*-substituted D-glucitol.

Glucosylglucitol (*ca* 5 mg) was oxidised at 2° by mM sodium metaperiodate (20 ml) for 24 h Excess periodate was then reduced by the addition of M-sodium sulphite (0 5 ml) After a further 30 min at 2°, the solution was deionised with mixed Amberlite IR-120 and IR-45 resins and then evaporated to dryness Complete acid hydrolysis of the residue produced a mixture of glucose, with smaller but approximately equal amounts of xylose and arabinose, as shown by paper chromatography Since the limited oxidation of 1-*O*-D-glucosyl-D-glucitol would give a derivative of L-xylose, and 6-*O*-D-glucosyl-D-glucitol would similarly yield a derivative of D-arabinose, these results show that a mixture of two isomeric disaccharides had been produced

Properties of α-D-*glucosylerythritol* — The purified disaccharide (yield 150 mg) was chromatographically homogeneous in three solvents, and on total hydrolysis with acid, both before and after borohydride reduction, gave a mixture of approximately equal amounts of glucose and erythritol The disaccharide, which had $[\alpha]_D$ +115° (*c* 2 72, water), was not hydrolysed by a β-glucosidase preparation from sweet almonds

Glucosylerythritol (20 mg) was oxidised with 1 4mM sodium metaperiodate solution (100 ml) for 8 h at 18° The solution was deionised with Amberlite IR-120 and IR-45 resins, before the addition of potassium borohydride (20 mg) After 12 h at 18°, the solution was deionised, and hydrolysed with acid The products were glucose and ethylene glycol, thus establishing that the α-D-glucosyl residue was attached to a primary alcohol group in erythritol Attachment to a secondary alcohol group would yield a mixture of glucose and glycerol

Properties of α-D-*glucosyl*-D-*mannitol* — From a digest of phenyl α-D-glucopyranoside and D-mannitol, a carbohydrate was isolated which, on acid hydrolysis, gave glucose and mannitol In solvent *A*, a single sugar (R_G 0 45) was present, but

in solvent *B*, a trace of isomaltose was indicated in addition to the main non-reducing sugar. The glucosylmannitol was therefore further purified by preparative, paper chromatography with solvent *B* The final product, yield 150 mg, crystallised from ethanol and had $[\alpha]_D$ +101° (*c* 0 93, water), it was not attacked by almond β-glucosidase, whereas an authentic sample of 1-*O*-β-D-glucosyl-D-mannitol (isolated from a partial, acid hydrolysate of laminarin[10] by Dr D H Hutson) was readily hydrolysed This latter disaccharide had the same M_s value in molybdate buffer as the enzymically synthesised carbohydrate, suggesting the presence of four adjacent hydroxyl groups, *i e*, the mannitol was substituted[8] at either C-1 or C-2

On oxidation with 2mM sodium metaperiodate at 2°, the release of formaldehyde was 0 94 mol (constant) after 12, 18, and 24 h In a similar experiment with 1-*O*-β-D-glucosyl-D-mannitol from laminarin, the yield was 0 96 mol α-D-Glucosyl-D-mannitol (20 mg) was oxidised with mM sodium metaperiodate (100 ml) for 24 h at 18°. The solution was deionised and concentrated By paper chromatography (solvent *A*, aniline oxalate spray), a minor, pink spot (R_G 0 88) and a major, yellow streak were formed The minor component (R_G 0 88) was isolated by preparative, paper chromatography On acid hydrolysis, glucose and arabinose were formed, on reduction, followed by electrophoresis in molybdate buffer, the M_s value of the alcohol was 0 45 This sugar is therefore probably 5-*O*-α-D-glucosyl-D-arabinofuranose, (*cf* Table I of Ref 3) The zone which gave a yellow colour with aniline oxalate was isolated and reduced The major component (*GM1*) had R_G 0 92 in solvent *A*, and gave glucose and glycerol on acid hydrolysis Further periodate oxidation of *GM1*, followed by reduction, gave a second sugar (*GM2*) having R_G 1 20 in solvent *A*, which, on acid hydrolysis, gave glucose and ethylene glycol These results indicate that the original disaccharide is 1-*O*-α-D-glucopyranosyl-D-mannitol, and that on

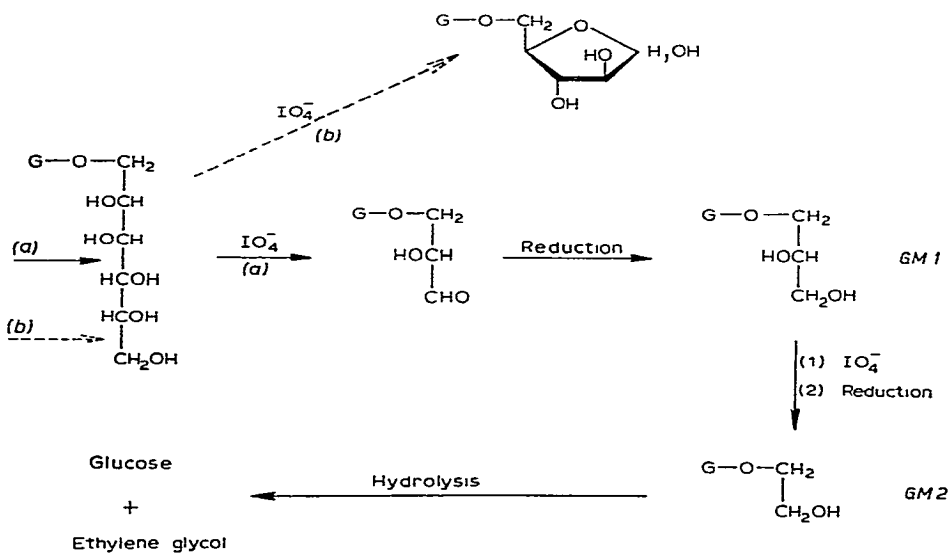

Fig 1

periodate oxidation, there is a preferential oxidation of the bond between C-3 and C-4 of mannitol, with a slower oxidation of the C-5–C-6 bond to give 5-*O*-α-D-glucosyl-D-arabinose (Fig 1) These observations are fully in agreement with the steric effects reported by Schwarz[11] on the periodate oxidation of hexitols

DISCUSSION

The present study shows clearly that the enzyme system in *Tetrahymena pyriformis* transfers α-D-glucosyl residues from phenyl α-D-glucopyranoside to various carbohydrates with retention of the anomeric configuration of the glucosyl residue This contrasts with the enzyme system in another protozoon (*Astasia ocellata*), which was examined in the previous paper[1], and found to transfer D-glucosyl residues from α-D-glucosyl phosphate to other carbohydrates with inversion of configuration

In contrast to the enzyme system in *A ocellata*, which can only use certain secondary alcohol groups as acceptor sites[1], the *T pyriformis* enzyme system shows a high preference for transfer to primary alcohol groups In the cases of D-glucose, D-mannose, and D-galactose, (1→6)-linked disaccharides are formed, and there was no evidence for the synthesis of significant amounts of disaccharides containing other types of linkage With alditols, there was also a clear requirement for primary alcohol groups However, since alditols contain two such groups, it was of interest to examine the products of enzyme action in some detail, to see if there was preferential utilisation of one of the two groups Those alditols which have a symmetrical structure, *e g*, D-mannitol and ribitol, gave, as might be expected, only one product However, D-glucitol gave a mixture of approximately equal parts of two isomeric disaccharides This mixture could not be separated by paper chromatography in two solvents or by electrophoresis The specific rotation reported in Table I therefore represents an average value and not a characteristic physical constant

When monosaccharides which do not contain a primary alcohol group (*e g*, aldopentopyranoses) are used as acceptors, transfer to the equatorial OH group at C-4 predominates[3] This situation has been observed with D-ribose, D-lyxose, and D-xylose With the availability of L-xylose, which exists in the *1C* conformation having an equatorial OH group at C-4, this specificity still holds, since the sole product was 4-*O*-α-D-glucopyranosyl-L-xylose

The enzyme system of *T pyriformis* provides a convenient method for the synthesis of a limited range of disaccharides Of the disaccharides described in Tables I and II, 6-*O*-α-D-glucopyranosyl-D-galactose ($[\alpha]_D$ +125°) has been synthesised chemically by Goldstein and Whelan[12], and isomaltitol (6-*O*-α-D-glucopyranosyl-D-glucitol), one of the two isomeric disaccharides isolated from the digest containing D-glucitol, has been prepared by the catalytic reduction of isomaltose, and had[13] $[\alpha]_D$ +89° The remainder appear to be new compounds whose synthesis is not described in two recent comprehensive publications[14,15]. The yields obtained in the present work are regarded as an order of magnitude only, since they represent the results of a compromise between the relative rate of synthesis of the new disaccharide

and of isomaltose, and of their ease of separation and isolation. In addition, there were certain unavoidable losses due to overlapping of fractions during chromatography. The new disaccharides provide a useful range of substrates for current studies on the specificity of α-glucosidases from various sources

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