FUNGAL CELLULASES

XIX.* POLYHYDROXYLIC ACCEPTORS FOR THE β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

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Summary

Transfer to polyols appears to take place principally to the terminal primary hydroxyl group. Transfer to the penultimate secondary hydroxyl group in any amount was only demonstrated for the pentitols, adonitol, and L-arabitol. Where the concentrations of the acceptor and the initial transfer product are of the same order of magnitude, there is measurable transfer to the initial transfer product to give molecules containing two glucosyl residues. Where all hydroxyls are secondary, as in *myo*inositol, no one transfer product predominates.

The various forms of methyl D-fructoside show widely different interactions with β-glucosidase, indicating that the exact architecture of the molecule controls the possibility of interaction with donor or acceptor centres and the possibility of presenting a hydroxyl group in suitable orientation for transfer to it to take place.

I. INTRODUCTION

The acceptors studied in detail in the preceding papers (Jermyn 1966*b*, 1966*c*, 1966*d*) have been confined to those having either one hydroxyl group or the two equivalent hydroxyl groups of hexane-1,6-diol. Nevertheless, in Part XV (Jermyn 1966*a*) values of T_{50} were derived for a considerable number of hydroxylic substances containing non-equivalent hydroxyls. If only one hydroxyl group is acting as an effective acceptor then T_{50} and the kinetic data would refer meaningfully to this single hydroxyl, otherwise they are merely averaged observations concerning a number of simultaneous reactions. Such questions must be answered, not by the accumulation of kinetic data, but by the direct inspection of products.

Carrying out the enzymic reaction under conditions where sufficient quantities and concentrations of products are formed for effective handling by techniques such as chromatography leads to further difficulties. The primary product of transfer still contains hydroxylic groups available for reaction, and it may conceivably be a far more efficient acceptor than the original one. Moreover, many glucosides are acceptors for the enzyme, where glucose is not, thus opening up even more possibilities for acceptor action. Interference by secondary transfer has been minimized in kinetic experiments by studying initial rates of reaction only. However, it cannot be avoided in preparative experiments; where two (or more) transfer products appear in incubation mixtures it must be decided whether they are all primary products of transfer or whether one or more of them are secondary products.

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Finally, the sugars raise in an acute form the question of acceptor specificity. Part XV shows that some sugar derivatives are effective acceptors and other closely related molecules are not. The fortunate chance that this specificity happens to exclude glucose for the *Stachybotrys atra* enzyme has made the whole of these investigations possible. Presumably quite small modifications in the relatively rigid sugar structures make it impossible for certain molecules to occupy the acceptor centre in such a way that a hydroxyl group is presented for reaction. Since benzyl β-fructopyranoside appeared to block the acceptor position without itself acting as an acceptor, a number of potential acceptors related in structure to this molecule have been investigated.

II. Materials and Methods

(*a*) *Polyols and their Glucosides*

Commercial samples of polyols were used as supplied. Sorbitol from a number of sources was tested, but in all samples traces of reducing sugar could be detected on paper chromatography. It was found to be simpler to allow for these than to attempt to remove them by recrystallization.

The basic incubation mixture was a 1% solution of phenyl β-D-glucopyranoside in McIlvaine buffer (diluted 1 in 25, pH 5) to which enough of a solid partly purified (100–200 units/mg) preparation of *S. atra* β-glucosidase was added to give 90% hydrolysis of the glucoside at 28°C in 4–6 hr. This mixture was modified by the addition of the required concentration of an acceptor. Most of the acceptors increased the rate of enzymic breakdown several fold, and in general little unreacted phenyl glucoside remained. Where incubation mixtures had to be held for more than a few hours for any reason, thymol was added as an antiseptic.

Descending paper chromatography was carried out using the butan-1-ol–dimethylformamide–water (2 : 1 : 1 v/v) solvent of Cramer and Steinle (1955) on Whatman No. 1 paper. This solvent was the most satisfactory of those tried for separating mixtures of polyolglucosides and, in addition, its relatively low volatility made it very suitable when irrigation had to be continued for long periods (up to 72 hr) to separate slow-moving mixtures.

The dried papers were treated with silver nitrate in acetone followed by spraying with 0·5N NaOH (Smith 1960). Reducing sugars showed up at once followed by glycosides and glycitols in that order. Certain glycosides have been classified as "alkali-labile" because they showed up almost immediately with the alkaline silver nitrate spray. The conditions of the experiments hardly seem to allow the formation of any compounds with free reducing groups other than glucose itself.

The salts in the reaction mixture interfered to a certain extent with the development of the sprayed papers. This was sometimes of importance for the slower-moving components and in such cases the salts and protein were removed by passing the incubation mixture through a column of mixed-bed ion-exchanger (Bio-deminrolite). Air-drying of the papers and careful heating after spraying allowed glycerol to give a clearly defined spot; ethylene glycol was either too volatile or too unreactive or both for the method to succeed.

The technique for the isolation of glycitol glucosides was exactly as described earlier for the preparation of 1-glyceryl β-glucoside (Jermyn 1958) using chromatography on a charcoal column in a concentration gradient of aqueous ethanol.

Periodate oxidations were carried out according to the directions of Jackson (1944).

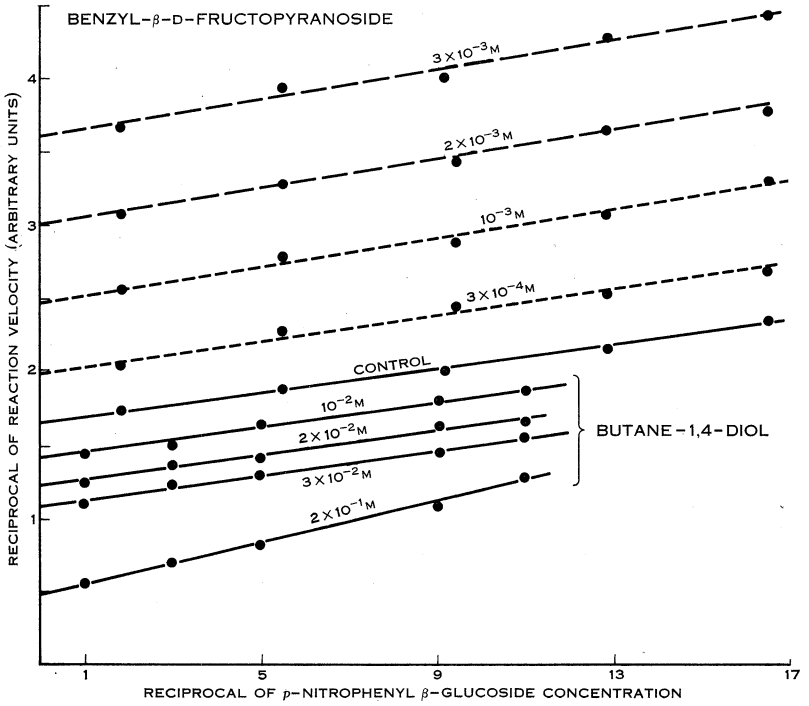

Fig. 1.—Effect of adding various concentrations of benzyl β-D-fructopyranoside or butane-1, 4-diol to incubation mixtures containing *p*-nitrophenyl β-D-glucopyranoside in pH 5 McIlvaine buffer at 28°C together with *S. atra* β-glucosidase. Unit concentration of the glucoside in the Lineweaver–Burk plots = 10^{-3}M.

(*b*) *Glycosides as Acceptors and Inhibitors*

N-Phenylfructosylamine was prepared according to the boric acid catalysis method of Knotz (1957). Benzyl β-fructopyranoside was prepared according to Purves and Hudson (1939). It was recrystallized from methanol until the test for reducing sugar became negative.

A little crystalline methyl α-fructopyranoside was prepared by the chromatography of a mixture of isomeric methyl fructosides according to Augestad, Berner, and Weigner (1953). It was not possible to repeat the reported separation of the other methyl fructosides in any purity.

Chromotography of a mixture of methyl fructosides according to Matsushima and Miyazaki (1964) gave crystalline methyl β-fructopyranoside as well as α-

and β-fructofuranosides as pure syrups showing a single component on paper chromotography and devoid of reducing power.

Methyl 6-*O*-methyl-β-D-glucopyranoside was prepared by the catalytic deacetylation of its triacetate, itself prepared from 6-*O*-methyl-D-glucose according to Helferich and Günther (1931). The material agreed in all respects with the description of Helferich and Himmer (1929).

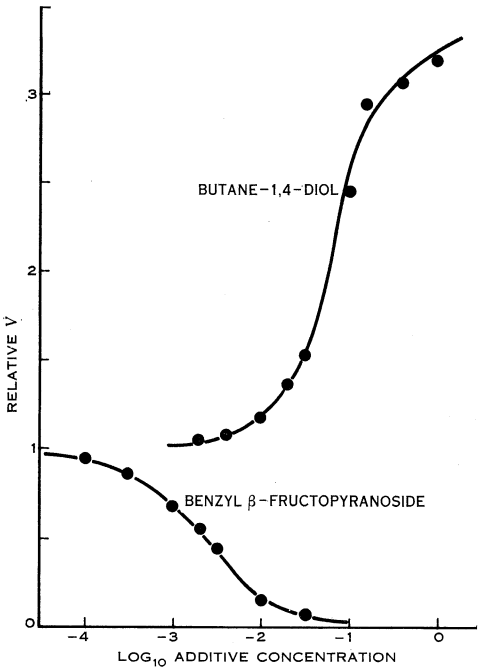

Fig. 2.—Values of V derived from Figure 1 plotted against the concentration of the two additives.

III. Results and Discussion

(a) *Monosaccharides and their Derivatives as Acceptors*

The technique for measuring transfer ratios based on the enzymic breakdown of phenyl β-glucoside is not adapted to handling acceptors that are, or act as if they were, reducing sugars. For this reason the comparison of various fructosides with fructose has been carried out using *p*-nitrophenyl β-glucoside as substrate and the analysis of Lineweaver–Burk plots as method.

A situation in which little ambiguity of interpretation is possible is presented in Figure 1. Butane-1, 4-diol is known from the work with phenyl β-glucoside to be a potent acceptor for β-glucosidase, and benzyl β-fructopyranoside not to show any signs of acceptor behaviour. The data of Figure 2 are obtained by plotting the relative values of V obtained from experiments of the type of those set out in Figure 1 against the logarithm of additive concentration. The symmetry of the behaviour of the two additives and the fact that benzyl β-fructopyranoside shows no trace of

competitive inhibitor behaviour judged by the Lineweaver–Burk plots lead at once to the simple interpretation that benzyl β-fructopyranoside has a high affinity for the acceptor centre but an efficiency as an acceptor not experimentally distinguishable from zero. If this explanation is accepted then the acceptor centre is 50% saturated at about 2×10^{-3}M.

Any discussion of the effect of fructose itself as the additive is complicated by the uncertainties about the nature of the species present in solutions of D-fructose. The comparison of data such as that of Figure 3 with other data for fructosides of fixed structure is thus difficult since it is impossible to know which of the molecular species is the active one, or indeed whether more than one is comparably active.

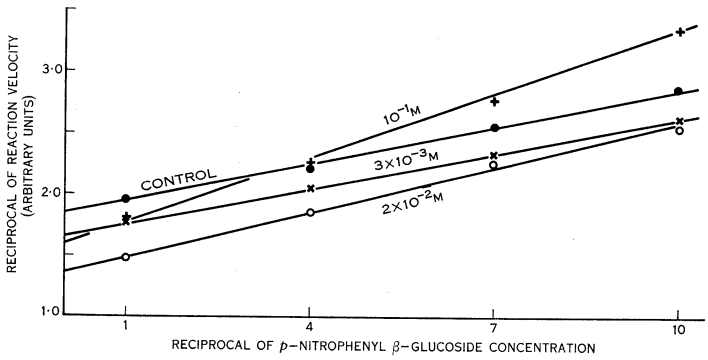

Fig. 3.—Effect of adding various concentrations of D-fructose to incubation mixtures containing *p*-nitrophenyl β-D-glucopyranoside in pH 5 McIlvaine buffer at 28°C together with *S. atra* β-glucosidase. Unit concentration of the glucoside in the Lineweaver–Burk plots = 10^{-3}M.

Figure 3 has been inserted to point out the difficulties of interpretation that arise from such data. In earlier work on the enzyme (Jermyn 1955, 1958) the data for 0·1M fructose would have been interpreted as showing a combination of an activation and a competitive inhibition due to binding at the donor centre. It could now be interpreted as activation due to the acceptor function with a change in Michaelis constant as one reaction pathway takes over from the other. The data at 3×10^{-3}M are typical of those expected of a reasonably efficient acceptor. Paper chromatography of incubation mixtures containing *p*-nitrophenyl β-glucoside and fructose show an additional unknown spot, reducing and ketose-positive, in the position expected for a disaccharide. This can hardly be anything but a glucosyl fructose formed by transfer.

None the less, the overall impression of the data in Figure 3 is that, opposed to the overall activation produced by the attachment of fructose as an effective acceptor to the acceptor centre, there is a second effect, not significant at the lowest fructose concentrations. This could well be an inefficient blocking of the donor centre.

Kinetic data can only exclude impossible mechanisms, not decide between possible ones, of which more than one can always be advanced to cover a given set

of facts. If the above explanation is accepted for the time being merely as the simplest compatible with the known behaviour of the enzyme, then the behaviour of fructose stands in sharp contrast to that of glucose. It was shown in earlier papers that glucose was a highly effective competitive inhibitor to which also appeared to show a general depression of enzyme activity when the effect of high concentrations of glucose on V was considered. No transfer to glucose has ever been demonstrated. This may be explained by supposing that glucose has a high affinity for the donor centre and a low affinity for the acceptor centre which it blocks.

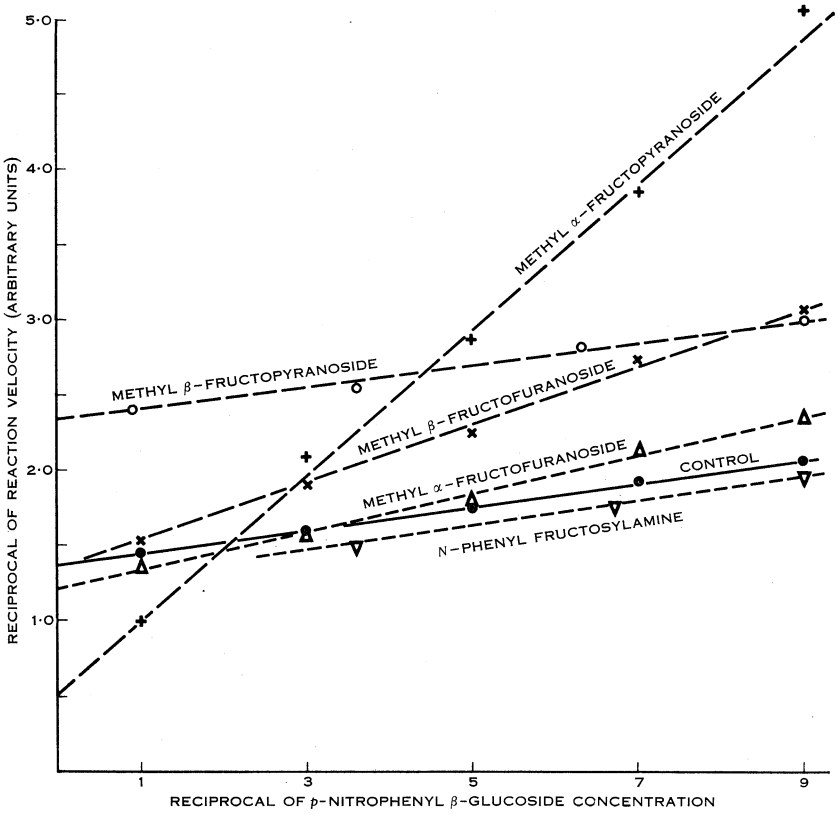

Fig. 4.—Effect of four methyl D-fructosides and N-phenyl D-fructosylamine, all at 3×10^{-3}M, on incubation mixtures containing p-nitrophenyl β-D-glucopyranoside in pH 5 McIlvaine buffer at 28°C together with *S. atra* β-glucosidase. Unit concentration of the glucoside in the Lineweaver–Burk plots = 10^{-3}M.

The data for the methyl D-fructosides at a common concentration are shown in Figure 4. In terms of the explanations that have been developed earlier, methyl β-fructopyranoside (an "anticompetitive" inhibitor) blocks the acceptor centre, methyl β-fructofuranoside (a "competitive" inhibitor) blocks the donor centre, methyl α-fructopyranoside is a potent acceptor, and methyl α-fructofuranoside possibly shows some sort of mixed behaviour, possibly is merely a relatively poor

acceptor. In agreement with these observations, methyl α-fructopyranoside was the only one of these compounds for which the formation of new components by transfer in incubation mixtures could be demonstrated.

For the present the different behaviour of the methyl fructosides must be taken as an observed fact for which no explanation can be attempted. It is proposed to discuss it further in a general way in connection with cellobiase; the results to be presented there show very different relative behaviour, and the behaviour set out here is a function of the enzyme not of the fructosides as such.

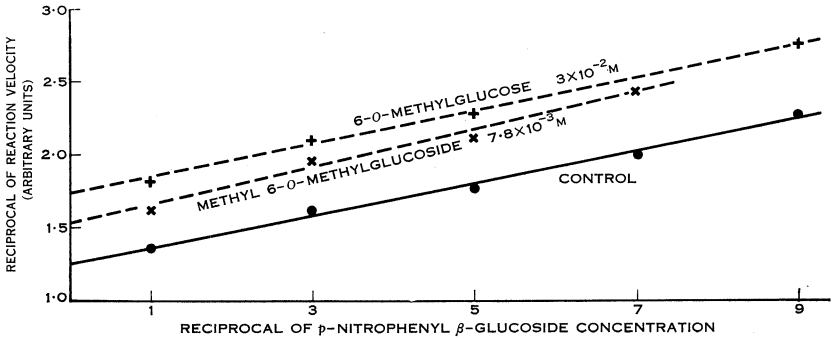

Fig. 5.—Effect of 6-*O*-methyl-D-glucose and methyl 6-*O*-methyl-β-D-glucopyranoside on incubation mixtures containing *p*-nitrophenyl β-D-glucopyranoside in pH 5 McIlvaine buffers at 28°C. Unit concentration of the glucoside in the Lineweaver–Burk plot = 10^{-3}M.

From the observed initial optical rotation in methanolic solution, it may be inferred that crystalline *N*-phenyl fructosylamine has a β-D-pyranose structure. The observed mutarotation (Kahl and Stzark 1963) demonstrates a rapid change to an equilibrium mixture with other anomers in methanolic solution. There are no data for aqueous solution; something of the nature of the equilibrium may be inferred from the fact that the fructosylamine mimics fructose closely (Fig. 4 and other data not presented graphically). The equilibrium may thus be not dissimilar for the two substances.

It was demonstrated earlier in this series (Jermyn 1955) that *p*-nitrophenyl 6-*O*-methyl-β-D-glucopyranoside is a substrate for the β-glucosidase of *S. atra*, but that the kinetics of the hydrolysis were anomalous; beyond a certain point increasing concentrations of the substrate brought about an increase in reaction rate that was much greater than Michaelis–Menten kinetics would allow. Figure 5 shows the effect of the presence of methyl 6-*O*-methyl-β-D-glucopyranoside or the parent sugar on the enzymic decomposition of *p*-nitrophenyl β-D-glucopyranoside. It is apparent that these 6-*O*-methyl compounds are "anticompetitive" inhibitors and, presumptively, block the acceptor site. In incubation mixtures with high concentrations of both enzyme and 6-*O*-methyl-D-glucose, a little transfer to 6-*O*-methyl-D-glucose could be demonstrated, so that the sugar is a very inefficient acceptor rather than a

complete blocking agent. The outlines of a qualitative interpretation of the kinetics of the breakdown of *p*-nitrophenyl 6-*O*-methyl-β-D-glucoside in terms of an interaction with both centres are clear, without it being possible to fill in quantitative details.

(*b*) *Transfer from Phenyl β-Glucoside to Glycitols*

The detailed analysis of the results which will be discussed in this section required the inspection of a large number of paper chromatograms which it is impracticable to present as such. The conclusions of this analysis will therefore be presented in tabular form (Table 1), after which individual polyols will be discussed and finally an attempt made to draw some general conclusions.

TABLE 1

COMPOUNDS (OTHER THAN GLUCOSE AND THE INITIAL GLUCOSIDE AND ACCEPTOR) APPEARING ON PAPER CHROMATOGRAMS OF MIXTURES OF PHENYL β-D-GLUCOPYRANOSIDE AND CERTAIN POLYOLS INCUBATED IN SOLUTION IN pH 5 BUFFER AT 28°C WITH *S. ATRA* β-GLUCOSIDASE

Where intensities are assigned to diglucoside spots, these are for an acceptor concentration of 0·1M

Polyol	R_G*	Comments	Polyol	R_G*	Comments
Ethylene glycol	1·22	Main component (+++++)	Galactitol	0·46	Trace (±)
	0·93	Trace (±)		0·30	Main component, alkali-sensitive (+++++)
	0·21	Diglucoside			
Glycerol	0·79	Main component		0·15	Diglucoside, trace (±)
	0·21	Diglucoside		0·09	Diglucoside (+)
Erythritol	0·70	Main component (+++++)	D-Glucitol	0·36	+
	0·40	++		0·23	Main component (+++++)
	0·30	+		0·12	Diglucoside (++)
L-Arabitinol	0·62	+		0·09	Diglucoside (++)
	0·45	Main component (+++++)	D-Mannitol	0·44	Trace (±)
	0·34	Alkali-sensitive (+++)		0·26	Main component (+++++)
	0·15	? Diglucoside (++)		0·13	Diglucoside (++)
Ribitol	0·63	+		0·09	Diglucoside (++)
	0·48	Main component (+++++)	*myo*Inositol	0·67	++
	0·32	Alkali-sensitive (++)		0·47	Alkali-sensitive (++)
	0·19	Diglucoside (++)		0·47	++
				0·33	++
				0·16	++

* Rate of travel relative to glucose in butanol–dimethylformamide–water.

(i) *Ethylene Glycol*

The main compound formed in incubation mixtures containing ethylene glycol was eluted as a single peak from the charcoal column. The fractions containing this compound were evaporated *in vacuo* and finally dried in a vacuum desiccator to give a non-crystalline glass. This procedure has been adopted for all the compounds discussed in this section. No attempt has been made to isolate a crystalline product even where incipient crystallization of the glass was seen. Glucose estimation by the anthrone method (Jermyn 1956) gave 79% ($C_6H_{11}O_5.OC_2H_4OH$ requires 80·3%). Acetylation with excess acetic anhydride containing sodium acetate gave white crystals, m.p. 54°C, on recrystallization from methanol and $[\alpha]_D^{20}$ −14° (*c*, 5 in

$CHCl_3$ [Lindberg (1949) quotes 53·5—54·5°C and —14·3 for 2-acetoxyethyl 2, 3, 4, 6-tetra-*O*-acetyl-β-D-glucopyranoside]. The principal transfer product is thus without doubt 2-hydroxyethyl β-D-glucopyranoside.

The trace component ($R_G = 0·93$) has not been identified. It did not appear in all experiments and may have arisen from condensation products in the ethylene glycol.

The slow-moving component ($R_G = 0·21$) was eluted from the column as a single peak and dried down to a glass. It has a glucose content of 93% ($C_6H_{11}O_5.OC_2H_4O.C_6H_{15}O_5$ requires 93·3%). Acetylation gave a compound, recrystallized from ethanol, m.p. 118°C, $[\alpha]_D^{20}$ —28° (*c*, 5 in $CHCl_3$). Helferich and Hiltmann (1937) found m.p. 117·5–119°C and $[\alpha]_D^{20}$ —28·7° for 1,2-bis(2′,3′,4′, 6′-tetra-*O*-acetyl-β-D-glucopyranosyloxy)ethane. Hence the slow-moving compound is the product of the transfer of two glucose residues to ethylene glycol, 1,2,-bis-(β-D-glucopyranosyloxy)ethane.

The structure of these compounds was investigated in detail, not only because the physical constants of their derivatives were already in the literature, but also because their identification made it possible to interpret the results of experiments in which acceptor concentrations were varied. At the end of an incubation in which ethylene glycol concentration had initially been 0·1M, the slow-moving compound was present at roughly 20% of the amount of the fast moving compound. As the glycol concentration rose to 3M the amount of the slow compound fell, till at the highest concentration it was invisible on the chromatogram. It appeared that the glycol and its first transfer product were competing for the acceptor centre of the enzyme.

The behaviour found for the ethylene glycol glucosides was used to identify primary and secondary transfer products in cases where their isolation and characterization was impractical. Any spot which weakened and disappeared as acceptor concentration was raised has been taken as that of a diglucoside.

(ii) *Glycerol*

The principal product has already been identified as 2,3-dihydroxypropyl β-D-glucopyranoside (Jermyn 1958). The slow-moving product, isolated from the column as a glass, had glucose content 87% [$C_3H_6O_3.(C_6H_{11}O_5)_2$ requires 86·5%] and, assuming a formula weight of 416, had 3·88 glycol groups per molecule by periodate oxidation (4 required). The analysis does not distinguish between the 1,2- and 1,3-diglycosides. The preference of the enzyme for primary hydroxyl groups that will be established leads to the probability of the 1,3-structure but this is not proved.

(iii) *Erythritol*

The calculated glucose content for a monoglucoside of erythritol ($C_4H_9O_3.O.C_6H_{11}O_5$) is 63·4%; a 1-erythrityl glucoside will have 4 glycol groups per molecule and 2-erythrityl glucoside 3 groups per molecule. Component 1 ($R_G = 0·70$) gave 62% glucose and 3·74 glycol groups, assuming a molecular weight of 284; component 2 ($R_G = 0·40$) gave 64% and 4·08 glycol groups. Both thus appear to be 1-erythrityl glucosides; since substitution on the primary hydroxyl

group of the internally compensated erythritol gives rise to two non-enantiomorphic isomers (++−+−−− and ++−+−++, reading, on the Fischer convention, from carbon 5 of glucose) this result is intelligible. For glycerol the stereochemistry of the enzymic reaction appears to favour one of the two possible isomers over the other so overwhelmingly that only a single primary transfer product has been detected (*cf.* the full discussion in Jermyn 1958). This is obviously not true for erythritol; although there is preference for one isomer over the other, this preference is far from exclusive.

The slowest component ($R_G = 0{\cdot}30$) has not been chemically investigated; it does not change in amount with changing erythritol concentration and is presumably one of the 2-erythrityl glucosides.

(iv) *Ribitol,* L-*Arabitinol*

The behaviour of the two pentitols, the optically active L-arabitinol and the internally compensated ribitol is sufficiently alike for them to be considered together. A 1-pentityl glucoside should have 57·3% glucose and 5 glycol groups per molecular weight of 314. For ribitol the main component had 57% glucose and 4·8 glycol groups; for L-arabitinol 56% glucose and 4·9 glycol groups. These compounds are thus 1-pentityl glucosides.

The minor fast-running component in both cases was not investigated chemically; extrapolation from the erythritol data suggests that it would be a second 1-pentityl-glucoside.

The slower component showed 59% glucose and 3·7 glycol groups for ribitol, 56% glucose, and 3·9 groups for L-arabitinol; it thus is probably a 2-pentityl glucoside in both cases. The 3-pentityl structure cannot be excluded on analytical grounds; it is, however, sterically most unlikely (Jermyn 1966*a*). No simple explanation can be advanced why these two glucosides, plus the 1-galactityl and one of the *myo*inosityl glucosides should be alkali-sensitive and reduce the silver reagent immediately.

(v) *Galactitol,* D-*Mannitol,* D-*Glucitol*

The three hexitols, like the pentitols, show a nearly identical pattern. The trace fast-running component in each case is presumably a 1-hexityl glucoside. The main peak is certainly such a glucoside in each case (galactitol, 52% glucose and 5·8 glycol groups; D-mannitol, 50% glucose and 6·1 glycol groups; D-glucitol, 53% glucose and 5·7 glycol groups; calculated for 1-hexityl glucoside: 52·3% glucose and 6 glycol groups per molecular weight of 344).

There are no signs of any spots on paper chromatograms that might be attributable to 2- or 3-hexityl glucosides, but a new phenomenon appears at the hexitol level—a pair of diglucoside spots (diagnosed by the concentration-dependence test), one of which is very weak in the case of galactitol. The trend of results would lead to their identification as 1,6- or 1,5-diglucosides.

(vi) *myoInositol*

The appearance of the chromatogram of incubation mixtures involving *myo*-inositol is quite different from that seen with the acyclic polyols. There are no primary

hydroxyl groups in *myo*inositol; instead there are five nearly functionally equivalent equatorial and one axial secondary hydroxyl groups (Angyal and Anderson 1959). It is tempting to equate the five components revealed in nearly equal amounts by paper chromatography with the five equatorial hydroxyl groups. However, it must be noted that substitution of the optically active β-D-glucosyl residues for any one of the hydroxyl groups in the internally compensated *myo*inositol potentially leads to more than one non-enantiomorphic isomer. None of the observed spots seem to be secondary transfer products since they were not concentration-sensitive.

(vii) *Conclusions*

Transfer to acyclic polyols is principally to the primary hydroxyl groups. Moreover except in the case of erythritol this transfer is highly stereospecific, i.e. one of the possible 1-glycityl β-D-glucosides is formed to a much greater degree than the other. Transfer to secondary hydroxyl groups is not inhibited as such, as shown by the minor transfer products for erythritol and the pentitols as well as the situation with *myo*inositol. But it is obvious that it is the stereochemistry of the acceptor as a whole, or rather of the enzyme–acceptor complex, that is important and that this normally prevents much transfer to secondary hydroxyls.

The complete structural determination of all the compounds produced by transfer to the polyols studied is beyond the scope of the task, no more than ascertaining the point to which transfer takes place, that has been attempted here. It will, however, be an obviously profitable task to attempt, at some later time, when enough is known of the active site in the enzyme, to correlate the nature of the products with the stereochemistry of the active complex.

Note.—Some of the glycityl glucosides isolated in this study were used as inducers of the β-glucosidase and cellobiase of *S. atra* in another study (Jermyn 1965). The β-glucosides used were those identified as "main components" i.e. the 1-glycityl β-glucoside (specifically for erythritol 1-erythrityl β-glucoside with $R_G = 0{\cdot}70$). For the *myo*inosityl β-glucosides, which do not resolve well on charcoal column chromatography the fraction labelled "inositol 3,4" was the $R_G = 0{\cdot}67$ and $0{\cdot}47$ components, that labelled "inositol 5,6" was the $R_G = 0{\cdot}41$ and $0{\cdot}33$ components, and "inositol 7" was the $R_G = 0{\cdot}16$ component.

IV. Summarizing Discussion

In default of the discovery of a solvent for β-glucosidase which is not a substrate for the enzyme, the amount of information that can be gained about the kinetics of the enzyme is limited by the ineluctable presence of water. Ideally, for a two-substrate enzyme, to extract the maximum information, one should be able to hold the concentration of either substrate independently at any desired value and for many enzymes this is indeed possible. Parts XV—XVIII of this series (Jermyn 1966*a*, 1966*b*, 1966*c*, 1966*d*) have shown how far the general conclusions deducible for these enzymes must be limited in the case of the β-glucosidase.

However, the general nature of the action of the β-glucosidase is now clear. It binds donor and acceptor molecules into a ternary complex that breaks down to give products. There is no necessary connection between the affinity of the enzyme for

these substrates and their effect on the rate of breakdown of the complex. In the limit the action of the enzyme can be inhibited by the blocking of either binding centre by unreactive molecules. There is a very narrow specificity for those molecules that can act as effective donors and a much wider specificity for effective acceptors. For the donor centre the specificity for "effective donors" is only a selection from the much wider specificity for substances that will bind at the centre. Binding at the donor centre therefore does not guarantee that the glycosidic linkage will be presented in an orientation suitable for fission to take place. For the acceptor centre it seems that except for certain molecules of complex structure, binding at the centre normally leads to acceptor activity; the exceptions, perhaps, are cases where the stereochemistry of a rigid structure leads to no hydroxyl group being presented in a suitable orientation for the transfer reaction.

All the numerical values of the parameters of enzyme action are subject to the effects of pH, ionic strength, and the presence of solutes that do not take part in the enzyme action. However, none of these effects seem to alter the general nature of the reaction.

However precise the numerical values may be that are calculated for the parameters of the enzyme reaction, in the absence of any detailed knowledge of the acceptor centre they can only be incorporated in a general description of the above type. It is not even at all clear what further questions it would be meaningful to ask. The elucidation of the structure of the β-glucosidase as a protein and the characterization of its active centre are obviously the steps that ought to be taken before more enzymological questions are devised and put.

V. Acknowledgment

I am indebted to Miss Carol May for some of the experimental work included in this paper.

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ADDENDUM: A CORRECTION

It was stated in an earlier paper (Jermyn 1955) that *o*-nitrophenyl β-glucoside is not a substrate of the enzyme. This statement has now been found to be a mistake due to mislabelling of samples. The true values of the enzymic parameters for this glucoside are $K_m = 2{\cdot}1 \times 10^{-4}$M, and V (relative to phenyl β-glucoside) $= 0{\cdot}18$. These values are more in line with the general relations between enzyme and donor than the extreme manifestation of the *ortho* effect involved in the apparent total unavailability of the *o*-nitrophenyl glucoside as a substrate.