FUNGAL CELLULASES

VIII. FURTHER OBSERVATIONS ON THE β-GLUCOSIDASE OF STACHYBOTRYS ATRA

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Summary

A number of experiments have been carried out which were designed to throw further light on the specificities of the "substrate" and "acceptor" centres of the β-glucosidase of *S. atra*. The type 2 activator, glycerol, has been shown to be an acceptor for a glucosyl residue transferred from *p*-nitrophenyl β-glucoside. *p*-Nitrophenyl 6-*O*-*p*-toluenesulphonyl-, 6-*O*-methyl-, and 3-*O*-methyl-β-D-glucopyranosides are all substrates for the β-glucosidase. The implications of the observations for the theory of the action of the enzyme are discussed.

I. INTRODUCTION

The sixth paper of this series (Jermyn 1955*b*) left a number of questions about the specificity of the β-glucosidase of *Stachybotrys atra* unanswered and this paper records some attempts to produce the required answers. The material here presented does not form a logical whole but, since the author is unlikely to return again to the study of this enzyme in the near future, he feels it best to publish the results of a number of small investigations under a single heading. Some of the experimental results obtained, especially those concerned with the products of hydrolysis in the presence of various inhibitors, have already been discussed at length elsewhere (Jermyn 1957*a*), and the relevant portions of the present paper may be looked on as providing experimental justification for the statements made there.

II. METHODS AND MATERIALS

The methods used in this work do not differ materially from those used previously (Jermyn 1955*a*, 1955*b*). Any small deviations will be noted at the appropriate point.

Many of the substances used as potential inhibitors or substrates have been synthesized for the first time and their synthesis is recorded elsewhere (Jermyn 1957*b*). D-Threose and D-erythrose were synthesized according to Perlin and Brice (1955), 2-*O*-methylglucose according to Hodge and Rist (1952), and 6-*O*-methylglucose according to Bell (1936). 3-*O*-methylglucose, 2-deoxyglucose and D-rhamnose were commercial samples.

III. HYDROLYSIS OF THIOGLUCOSIDES

The β-glucosidase of *S. atra* appears to be unique amongst those so far studied in hydrolysing phenyl β-thioglucoside, which is also a competitive inhibitor of the enzymic hydrolysis of *p*-nitrophenyl β-glucoside. However, the activity against the thioglucoside is low and the results show great experimental scatter, perhaps owing to erratic oxidation of the liberated mercaptan in dilute solution. The hypothesis that

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the hydrolysis of the *O*- and *S*-glucosides was due to the same enzyme was checked by showing that the Michaelis constant (K_m) of the *S*-glucoside as a substrate was approximately the same as its inhibitor constant (K_i) as a competitive inhibitor. For the reason given above the determination of K_m involved a long and uncertain

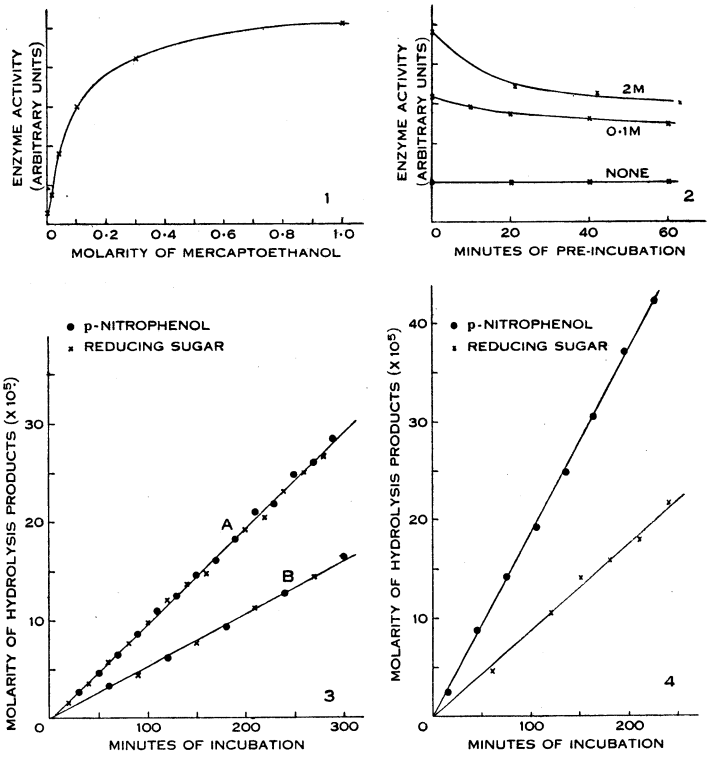


Fig. 1.—Effect of mercaptoethanol concentration on the hydrolysis of *p*-nitrophenyl β-D-glucoside (10^{-3}M) by the *S. atra* β-glucosidase for 20 min at 28°C in McIlvaine sodium phosphate–citric acid buffer (pH 5·0).

Fig. 2.—Effect of pre-incubation of *S. atra* β-glucosidase at 28°C with the indicated concentrations of mercaptoethanol on the enzymic activity determined under the conditions of Figure 1.

Fig. 3.—Hydrolysis of *p*-nitrophenyl β-D-glucoside (10^{-3}M) by the *S. atra* β-glucosidase at 28°C and pH 5·0, *A*, alone and *B*, in the presence of 10^{-2}M phenyl α-cellobioside. Both *p*-nitrophenol and reducing sugar concentrations are indicated.

Fig. 4.—Hydrolysis of *p*-nitrophenyl β-D-glucoside (10^{-3}M) by the *S. atra* β-glucosidase at 28°C and pH 5·0 in the presence of 10^{-1}M glycerol. Both *p*-nitrophenol and reducing sugar concentrations are indicated.

extrapolation and it therefore seemed advisable to cross-check the hypothesis by studying the reverse case—inhibition of the hydrolysis of *p*-nitrophenyl β-thioglucoside by phenyl β-glucoside.

p-Nitrophenyl β-thioglucoside on synthesis proved to be very difficult to use as a substrate. In solution at pH 5–9 it decomposed rapidly and rough experiments showed that at pH 5 and 28°C it broke down by the "alkaline hydrolysis" mechanism

10^4–10^6 times as fast as the *O*-glucoside. The blanks for the experiments with the enzyme were thus very high, and although this difficulty could be minimized by using freshly prepared solutions and suitably diluting them, only fairly high enzymic activities would have been demonstrable. A further complication appeared when an attempt was made to demonstrate the actual existence of such activities. The incubation mixture containing enzyme and substrate was found to have a lower optical density than the blank containing substrate alone.

The obvious hypothesis to account for this observation was to suppose that the *p*-nitrothiophenol originally present in the solution was reacting with the enzyme protein. Youatt (personal communication) informs me that he observed a similar "negative activity" in the reaction between trypsin and diethyl-*S*-*p*-nitrophenyl phosphorothiolate which should phosphorylate trypsin with the liberation of *p*-nitrothiophenol. If such reaction with proteins is a general property of *p*-nitrothiophenol it should be inhibited by the addition of sufficient quantities of other thiol compounds.

Additions of cysteine, glutathione, and thioglycollic acid failed to affect the *p*-nitrothiophenol–protein reaction at any concentration. However, concentrations of mercaptoethanol higher than about 0·5M completely inhibited this reaction, but in the system enzyme–substrate–1M mercaptoethanol it was impossible to demonstrate any enzymic activity. That this observation was not due to inhibition of the enzyme by mercaptoethanol but to an inherently low enzyme activity was demonstrated by the observations set forth in Section IV.

The negative result of the present work leaves the earlier observations (Jermyn 1955*b*) still the only evidence for the hydrolysis of thioglucosides by the *S. atra* β-glucosidase.

IV. Effects of Mercaptoethanol

Mercaptoethanol, tested for its effect on the enzymic hydrolysis of *p*-nitrophenyl β-glucoside under the standard conditions, where active agent and substrate were mixed together in solution and the enzyme added, was found to be an activator (Fig. 1).

Since activation by thiols is often time-dependent the enzyme and the mercaptoethanol were mixed and incubated together for varying periods before addition to the substrate (Fig. 2). The results of this experiment seem to show an immediate activation, followed by a slow inactivation, the rate of which not only increases with increasing mercaptoethanol concentration but also falls off with time. However, since the enzyme activity in the presence of mercaptoethanol at zero time is really measured after 20 min further incubation in the presence of mercaptoethanol at one-fifth of the original concentration, another experiment was devised to eliminate any effects due to this further incubation. Enzyme at a suitable dilution was incubated alone or with mercaptoethanol for 10 min. These enzyme samples were then added to standard substrate mixtures with and without mercaptoethanol respectively and incubated for the standard period (20 min). The results are set out in Table 1. The independence of the activating and inactivating effects and the increase in the rate of the latter with mercaptoethanol concentration are clearly demonstrated.

The activation with mercaptoethanol is apparently an ordinary type 2 activation (Jermyn 1955*b*, 1957*a*) in which mercaptoethanol, like glycerol, acts as a more efficient

acceptor for the glucosyl radical than water. The time-dependent inactivation can be most readily explained as due to the reduction of one or more disulphide bonds essential to the integrity of the enzyme. As with rhodanese (Sörbo 1953), the alternative explanation of thiohemiacetal formation from an essential carbonyl group may be excluded by the ineffectiveness of other carbonyl reagents, e.g. phenylhydrazine, as inhibitors (Jermyn 1955*a*). Combining the present results with the earlier ones on inhibition by heavy metals, it appears that both a disulphide bond or bonds and a thiol group or groups are necessary for full enzyme activity.

V. Effects of Type 2 Activators and Inhibitors

Type 2 effects in the *S. atra* β-glucosidase were originally diagnosed (Jermyn 1955*b*) by the fact that plots of the reciprocal of reaction velocity (Lineweaver and Burk 1934) for the affected reactions gave straight lines parallel to those for the unaffected reaction. Later (Jermyn 1957*a*) reasons were given for supposing that these

Table 1

EFFECTS OF 10 MINUTES PRE-INCUBATION WITH MERCAPTOETHANOL ON THE ENZYMIC ACTIVITY OF S. ATRA β-GLUCOSIDASE

Mercaptoethanol Concn. (M)		Measured Enzyme Activity (arbitrary units)	Mercaptoethanol Concn. (M)		Measured Enzyme Activity (arbitrary units)
plus Enzyme, Pre-incubate for 10 min	Incubate with Substrate and Enzyme for 20 min		plus Enzyme, Pre-incubate for 10 min	Incubate with Substrate and Enzyme for 20 min	
5·0	1·0	139	0	1·0	639
1·0	0·2	630	0	0·2	695
0·2	0·04	415	0	0·04	443
0·04	0·008	376	0	0·008	395

effects were due to interactions of the active agents with the acceptor centre of the enzyme. If type 2 activators act by attaching themselves to the acceptor centre and act as more efficient acceptors than water, then some of the glucose which would have been liberated in the non-activated reaction should, in the presence of activator, now appear as a β-glucoside of the activating molecule. Type 2 inhibitors, which merely block the acceptor centre, should leave the ratio liberated glucose/liberated aglucone unchanged. This point is readily checked by following the liberation of both *p*-nitrophenyl and glucose from the enzymic hydrolysis of *p*-nitrophenyl β-glucoside in both the presence and absence of the inhibitors or activators. Glycerol (non-reducing and showing pure type 2 behaviour) was an obvious choice as an activator but the choice of the non-reducing phenyl α-cellobioside as the inhibitor was attended by some disadvantages, since its solubility did not allow it to be used at a concentration showing profound depression of activity. The results of these experiments are set out in Figures 3 and 4. These accord with the hypothesis, but it was obviously desirable to

confirm the postulated mechanism further by the direct isloation of a glyceryl glucoside from the enzymic digest. The following procedure was therefore adopted.

p-Nitrophenyl β-glucoside (500 mg), *S. atra* β-glucosidase (50 units in concentrated solution), glycerol (3·5 ml), and McIlvaine buffer (10 ml, pH 5·0) were made up to 50 ml and the whole incubated for 24 hr at 28°C. The product was deionized by passage through a column of "Bio-Deminrolit" (more than 99 per cent. of the *p*-nitrophenol removed) and the effluent concentrated to small volume. The concentrate was absorbed on a carbon–cellulose column and eluted by an ethanol gradient. A first carbohydrate peak which was eluted by water alone and gave tests for reducing sugar was obviously glucose. This was followed by a second peak of non-reducing carbohydrate at 7·5 per cent. ethanol (using the "small column" with 100 per cent. ethanol feed under the conditions already described in detail in Jermyn 1957*c*). The fractions containing this material were evaporated *in vacuo* but the residue (190 mg of syrup, $[\alpha]_D^{20} - 26{\cdot}2°$ (*c*, 5 in H_2O)) could not be induced to crystallize. A sample was dried at 80°C under high vacuum and submitted to the C.S.I.R.O. Microanalytical Laboratory. (Found: C, 42·1; H, 7·2; O, 50·2 %. Calc. for $C_9H_{18}O_8$: C, 42·5; H, 7·1; O, 50·4%.) Periodate oxidation according to Jackson (1944) gave an HIO_4 uptake of 2·94 moles per mole of $C_9H_{18}O_8$; 1-glyceryl-β-D-glucoside contains three glycol groups per mol.

1-Glyceryl-β-D-glucoside has been synthesized biochemically by the action of emulsin on glycerol plus glucose (Bourquelot, Bridel, and Aubry 1915; $[\alpha]_D^{20} -27{\cdot}25°$) and chemically (Karrer and Hurwitz 1922; $[\alpha]_D^{18} -27{\cdot}7°$), in both cases as a non-crystalline syrup. The optical activity is no guide to purity since a fresh asymmetric centre is created at the 2-position of glycerol and the two isomers will be formed in unknown proportions. Indeed, Bourquelot *et al.* give good reasons for supposing that their product was a mixture of two substances. In the circumstances the properties of the present product agree as well as can be expected with those already reported, and it is equally probably a mixture.

Like all alkyl β-glucosides the 1-glyceryl compound is highly resistant to the *S. atra* β-glucosidase and the glyceryl glucoside resulting from carrying out the hydrolysis under the conditions indicated in Figure 4 itself remained unhydrolysed in the presence of active enzyme for an indefinite period. It may therefore be concluded that there is convincing evidence of the transfer of a glucosyl residue to glycerol but not to phenyl α-cellobioside. An interesting point illustrated by Figure 3 is that there is virtually no transfer of the glucose residue to the product of hydrolysis (glucose) so that the amounts of liberated glucose and aglucone coincide at any time. This is in marked contrast to the behaviour of the *Aspergillus oryzae* β-glucosidase (Jermyn and Thomas 1953) and agrees with the fact that glucose does not show type 2 effects except at very high concentrations (Jermyn 1955*b*).

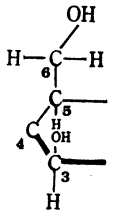
VI. Inhibitors of β-Glucosidase

(*a*) *Threose and Erythrose*

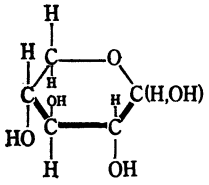
An interesting test of a hypothesis about competitive inhibitors of the *S. atra* β-glucosidase can be made by using sugars that cannot assume the pyranose ring form. The simplest cases that can be considered are those of the two tetroses, D-threose and

D-erythrose, which cannot exist in a ring form larger than the five-membered furanose ring.

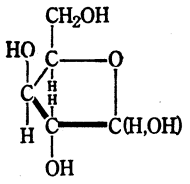
It has been postulated (Jermyn 1955*b*) that the essential configuration for a molecule to act as a competitive inhibitor of the glucosidase is given by the portion of the D-glucose molecule namely:



and thus D-xylose can have the configuration of a competitive inhibitor in neither its

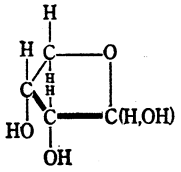


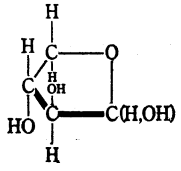
D-Xylose (pyranose)

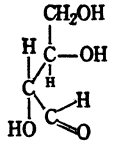


D-Xylose (furanose)

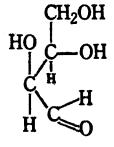
pyranose nor furanose forms and is not, in fact, a competitive inhibitor. We may write the furanose and straight-chain forms of D-threose and D-erythrose as shown and







D-Erythrose



D-Threose

make the further prediction that none of these molecules will be effective competitive inhibitors. The actual experimental results are set forth in Figure 5.

Both sugars appear to be pure type 2 activators and, indeed, D-erythrose is the most powerful of these yet investigated. There is no trace of activity as competitive inhibitors at the concentrations tested.

(*b*) *Deoxy Sugars*

It has been found (Jermyn 1955*b*) that both D-glucose and D-mannose are competitive inhibitors of the *S. atra* β-glucosidase and hence surmised that the steric configuration about carbon atom 2 (C_2) of the D-glucopyranose ring is not very important in deciding affinity for the substrate centre of the enzyme. 2-Deoxy-D-glucose, which has no hydroxyl group on C_2 supplies an interesting test of this hypothesis. Figure 6 demonstrates that this sugar is indeed a pure competitive inhibitor with $K_i = 126 \times 10^{-5}$M at both concentrations. A comparison with D-glucose ($K_i = 19 \times 10^{-5}$M) and D-mannose (43×10^{-5}M) shows that both the steric configuration and the addenda at C_2 have an effect on the affinity for the substrate centre. Some significance that cannot at present be assessed must also be attached to the fact that the absence of a hydroxyl group at C_2 seems to abolish all affinity for the acceptor centre.

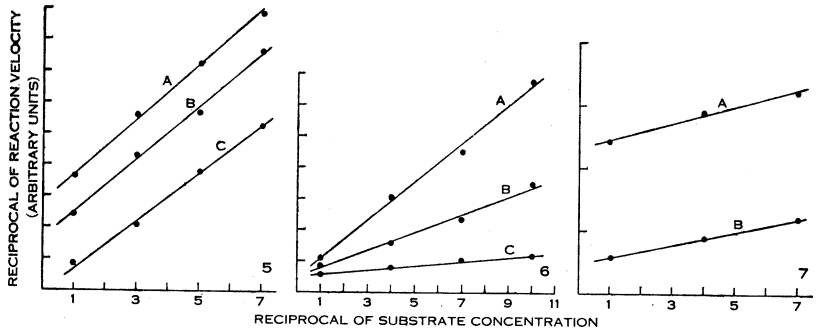


Fig. 5.—Effect of D-erythrose and D-threose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition; *B*, D-threose, $2{\cdot}5 \times 10^{-2}$M; *C*, D-erythrose, 7×10^{-3}M. Unit substrate concentration, 10^{-3}M.

Fig. 6.—Effect of 2-deoxy-D-glucose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition; *B*, 4×10^{-3}M; *C*, 10^{-2}M. Unit substrate concentration, 10^{-3}M.

Fig. 7.—Effect of D-rhamnose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, no addition: *B*, D-rhamnose, 10^{-1}M. Unit substrate concentration, 10^{-3}M.

It was surmised at the same time that the polarity of the substituent on C_6, rather than the nature of this substituent or the configuration about C_5, was essential for effectiveness as a competitive inhibitor. This hypothesis can be partly checked by testing the behaviour of D-rhamnose (D-mannose with the hydroxyl group on C_6 removed). Figure 7 shows that D-rhamnose is a pure type 2 inhibitor and that there appears to be no affinity for the substrate centre. Replacement of the $-CH_2OH$ substituent on C_5 by both H (D-xylose) and CH_3 (D-rhamnose) appears to abolish this affinity.

(*c*) *Monomethylated Glucoses*

Some idea of the nature of the interaction between D-glucose and the substrate centre of the enzyme can be gained by using compounds in which the hydroxyl groups of D-glucose have been replaced by methoxyl groups. The substituent remains polar but the possibility of forming hydrogen bonds through the hydroxyl group has been

destroyed. The simplest cases that can be considered are those of the six *O*-monomethyl derivatives of glucose. A consideration of their interaction with the enzyme allows the influence of the five hydroxyls of glucose to be evaluated separately.

The six sets of experimental results in Figure 8 show the results for inhibitor concentrations selected to demonstrate particularly clearly the general effect of each

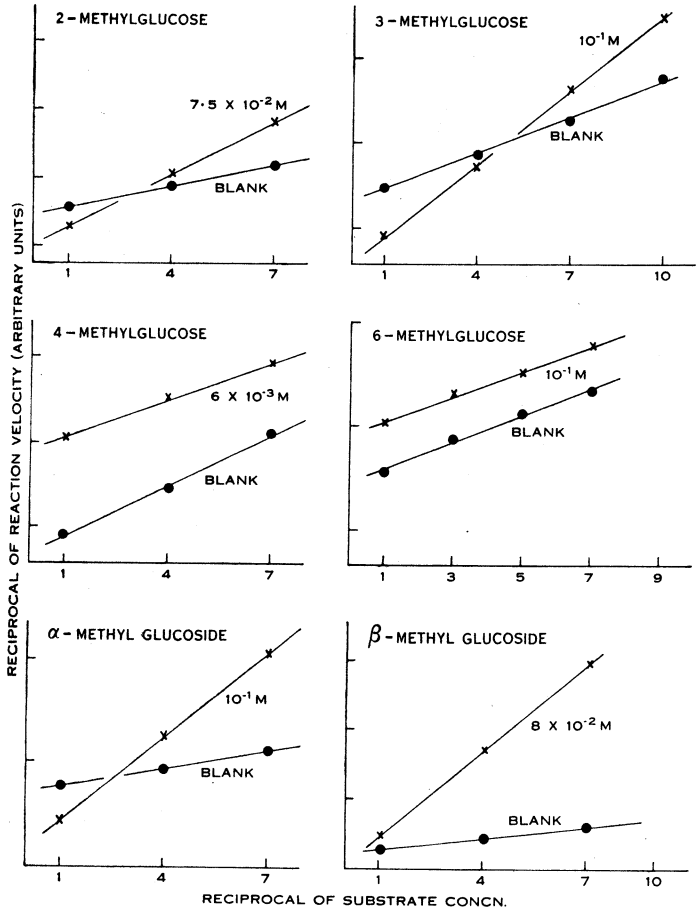


Fig. 8.—Effect of the six mono-*O*-methyl derivatives of D-glucopyranose on the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions. Unit substrate concentration, 10^{-3}M throughout.

inhibitor. However, the same tendencies and comparable values of K_i were observed with a number of inhibitor concentrations in each case. The observations of this and the preceding subsection are combined with some from a previous paper (Jermyn 1955*b*) in Table 2.

The results suggest the following summation of the requirements that molecules related to D-glucose should have *some* affinity for the substrate centre of the β-glucosidase of *S. atra*. Conclusions arrived at earlier (Jermyn 1955*b*) are incorporated in brackets. The nature and configurations of the groups attached to C_1 and

C_2 of D-glucopyranose (or even the existence of C_1 and C_2) are immaterial; there must be a polar but not necessarily a hydroxyl group on C_3 (and the configuration about C_3 must be that of D-glucose); there must be a hydroxyl group on C_4 (but the configuration about C_4 is immaterial); the grouping attached to C_5 must be $-C(6)H_2OH$ (but the configuration about C_5 is immaterial). The magnitude of the affinity (here taken as the numerical value of K_i) is then determined by further more detailed influences of the geometry of the molecule.

There seems at present to be no clue that will allow a general statement about whether a given molecule can be bound at the acceptor centre or whether it will act as an activator (acceptor) or inhibitor.

TABLE 2

EFFECTS OF SOME ALTERATIONS TO THE STRUCTURE OF THE D-GLUCOSE MOLECULE ON EFFECTIVENESS AS AN INHIBITOR OF S. ATRA β-GLUCOSIDASE

Substance	Alteration to the D-Glucose Molecule	K_i as Competitive Inhibitor ($\times 10^{-5}$M)	Type 2 Effects
D-Glucose		19	Slight inhibitor
D-Gluconolactone	=O for H+OH on C_1	0·32	
Methyl α-D-glucoside	$-OCH_3$ for –OH on C_1	2700	Powerful activator
Methyl β-D-glucoside	$-OCH_3$ for –OH on C_1	1200	Slight activator
Aryl β-D-glucosides	–OAr for –OH on C_1	2–600	Some are slight inhibitors
D-Mannose	Inversion of –OH on C_2	43	None
2-Deoxy-D-glucose	–H for –OH on C_2	126	None
2-*O*-Methyl-D-glucose	$-OCH_3$ for –OH on C_2	4000	Activator
3-*O*-Methyl-D-glucose	$-OCH_3$ for –OH on C_3	9000	Activator
D-Galactose	Inversion of –OH on C_4	1000	Very slight inhibitor
4-*O*-Methyl-D-glucose	$-OCH_3$ for –OH on C_4	Non-competitive	Powerful inhibitor
6-*O*-Methyl-D-glucose	$-OCH_3$ for –OH on C_6	Non-competitive	Inhibitor
D-Rhamnose	–H for –OH on C_6	Non-competitive	Inhibitor

VII. Enzymatic Hydrolysis of Substituted *p*-Nitrophenyl β-Glucosides

Various elements of the structure of an aryl β-glucoside will allow a molecule to be bound at the substrate centre of the *S. atra* β-glucosidase, but it earlier appeared that the complete structure was necessary for this molecule to be a substrate (Jermyn 1955*b*). The use of monosubstituted *p*-nitrophenyl β-glucosides appears to be a way of checking this in more detail.

Certain 6-substituted glucosides are readily prepared but the solubility of *p*-nitrophenyl 6-benzoyl-β-glucoside in water was too low ($<10^{-4}$M at 28°C) and that of the 6-*p*-toluensulphonyl derivative (5×10^{-4}M at 28°C) barely high enough for any results to be obtained. The results with the latter compound are included in Table 3 but it should be realized that the forced use of low concentrations of substrate, together with the very high enzyme concentrations necessary to demonstrate very low activities may make these uncertain with in an order of magnitude. The other four compounds recorded are the four monomethyl ethers of *p*-nitrophenyl-β-D-glucopyranoside. The negative results for the 2- and 4-substituted compounds held for

the highest enzyme and substrate concentrations that could be obtained and it is certain that in both cases the rate of enzymic hydrolysis at 28°C is less than 10^{-6} of that of the unsubstituted glucoside. For the 3-substituted compounds all that can be stated is that there is positive evidence of enzymic hydrolysis but that the combined effects of low solubility, limited stability, and small available quantities of the substrate make it impossible to assign accurate numerical values as yet.

Before the significance of the results in Table 3 can be assessed two further questions must be answered. Are the 6-substituted derivatives actually hydrolysed by the same enzyme as the unsubstituted glucoside? Are the 2- and 4-methyl derivatives bound to the enzyme and not hydrolysed or are they not bound at all? The first question can be answered by finding out whether the enzymic hydrolysis of the 6-methyl derivative is competitively inhibited by the same substances, with the same K_i values, as that of the unsubstituted glucoside. The second by examining whether the 2- and 4-methyl derivatives will themselves act as competitive inhibitors.

TABLE 3

SUBSTITUTED *p*-NITROPHENYL β-D-GLUCOSIDES AS SUBSTRATES FOR β-GLUCOSIDASE OF S. ATRA

Substituent	Behaviour	K_m ($\times 10^{-5}$M)	$\frac{k_3 \text{ (unsubstituted)}}{k_3 \text{ (substituted)}}$
2-*O*-Methyl-	Non-substrate	—	—
3-*O*-Methyl-	Substrate	*c.* 10,000	Too high for accurate measurement. Relative reaction rate *c.* 380 at 10^{-3}M
4-*O*-Methyl-	Non-substrate	—	—
6-*O*-Methyl-	Substrate	360	3·4
6-*O*-Tosyl-	Substrate	29	130
None	Substrate	5	1

It was found that both phenyl β-D-glucoside and D-glucose were competitive inhibitors of the enzymic hydrolysis of *p*-nitrophenyl 6-*O*-methyl-β-D-glucoside. When it is remembered (Jermyn 1955*b*) that it was impossible to make K_m and K_i agree more than approximately for a large number of aryl β-glucosides tested with the *S. atra* enzyme it is apparent that the results set out in Table 4 agree as nearly as can be expected with the hypothesis that the unsubstituted and 6-methyl glucosides are being hydrolysed by the same enzyme. The opposite trends in the value of K_i with increasing phenyl β-glucoside concentration seems to be linked with the experimental fact that the deviation from Michaelis-Menten kinetics is negative at high substrate concentration (i.e. excess substrate inhibits) for *p*-nitrophenyl β-glucoside and positive (excess substrate activates) for the 6-methyl derivative.

The concentrations of the 2- and 4-methyl derivatives used as inhibitors could not be raised above 5×10^{-3}M because of difficulties with excessively large blanks and the fact that the 4-methyl derivative is readily salted out of solution. However, there concentrations were enough to show that the 2-methyl derivative is certainly not

bound and the 4-methyl compound only weakly bound (K_i = c. 300×10^{-5}M) at the acceptor centre (Fig. 9) and that these compounds are to be classed as type 2 inhibitors with practically identical affinities for the acceptor centre (identical $V_{max.}$ in Fig. 9). The conclusion therefore follows that no prediction can be made about the behaviour of the substituted glucoside from that of the substituted sugar. Table 5 shows that, in fact, the behaviour of the two groups tends to be opposite.

TABLE 4

COMPETITIVE INHIBITION OF S. ATRA β-GLUCOSIDASE BY D-GLUCOSE AND PHENYL β-D-GLUCOSIDE

Substrate	K_i ($\times 10^{-5}$M) for D-Glucose at Indicated Concentration as Inhibitor			K_i ($\times 10^{-5}$M) for Phenyl β-D-glucoside at Indicated Concentration as Inhibitor		
	10^{-4}M	3×10^{-4}M	10^{-3}M	10^{-4}M	3×10^{-4}M	10^{-3}M
p-Nitrophenyl 6-*O*-methyl-β-D-glucoside	21	43	60	32	34	33
p-Nitrophenyl β-D-glucoside	27	21	17	21	19	20

Since both the 4-methyl and 6-methyl compounds have about the same affinity for the substrate centre, the conclusion may be drawn that 4-methyl substitution in *p*-nitrophenyl β-glucoside produces a molecule that will not act as a substrate. If the

TABLE 5

INTERACTION OF METHYL-SUBSTITUTED D-GLUCOSES AND *p*-NITROPHENYL β-D-GLUCOSIDES WITH THE SUBSTRATE AND ACCEPTOR CENTRES OF S. ATRA β-GLUCOSIDASE

	2-*O*-Methyl-		3-*O*-Methyl-		4-*O*-Methyl-		6-*O*-Methyl-	
	Sugar	Glucoside	Sugar	Glucoside	Sugar	Glucoside	Sugar	Glucoside
Substrate centre	Blocked	No interaction	Blocked	Substrate	No interaction	Blocked	No interaction	Substrate
Acceptor centre	Activated	Blocked	Activated	?	Blocked	Blocked	Blocked	Activated (?)

binding is primarily due to the β-glucosidic linkage, then perhaps 4-methyl substitution blocks some vital attachment necessary for enzymic action, since the same substitution abolishes the affinity of D-glucose for the substrate centre.

There may well be some correlation between the non-interaction of *p*-nitrophenyl 2-*O*-methyl-β-D-glucoside with the substrate centre and the fact that this

2-methyl substitution virtually makes it impossible for electrons to be withdrawn from the C(1)—O bond as demonstrated by the extreme resistance of this substance to alkaline hydrolysis. This matter will be discussed in detail elsewhere.

VIII. General Discussion

The general principles on which the experimental results here presented have been interpreted have been given by Gottschalk (1950) and discussed elsewhere by Jermyn (1957*a*) but there seem to be some general points that are worth emphasizing. Consideration of the ideas of Dodgson, Spencer, and Williams (1956) and Ebersole, Gutentag, and Wilson (1944) showed the author that the interpretation placed on the existence of "type 2" activation and inhibition (see Part VI of this series (Jermyn

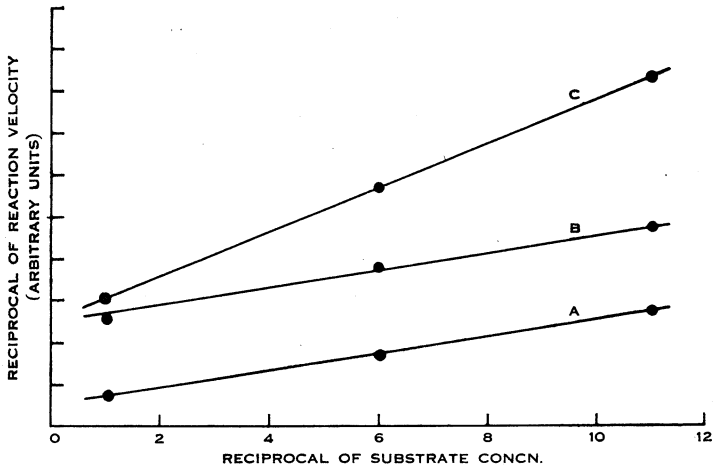


Fig. 9.—Inhibition by *p*-nitrophenyl 4-*O*-methyl- and 2-*O*-methyl-β-D-glucosides of the enzymic hydrolysis of *p*-nitrophenyl β-D-glucoside by the *S. atra* β-glucosidase under the standard conditions: *A*, 5×10^{-3}M *p*-nitrophenyl 4-*O*-methyl-β-D-glucoside; *B*, 5×10^{-3}M *p*-nitrophenyl 2-*O*-methyl-β-D-glucoside; *C*, no addition. Unit substrate concn., 10^{-3}M.

1955*b*)) was too narrow. The conclusion drawn there that these kinetic effects depended on combination at an "acceptor" centre was correct, but there was no need for the special relationship between K_i, k_2, and k_3 that had been deduced. Abandonment of this special relationship makes it possible to explain rationally a great deal of the kinetics of the *S. atra* β-glucosidase and, by implication, of many other carbohydrases, as due to the existence and independent specifications of a pair of active centres—the "substrate" and "acceptor" centres.

Examples of the typical Lineweaver-Burk plots given by what the author had called type 2 inhibition and Dodgson *et al.* call "anticompetitive" inhibition can be found scattered throughout the biochemical literature. One case is quoted by Jermyn (1955*b*); Dodgson *et al.* quote several others and show how the interpretation to be placed on this phenomenon has generally been overlooked. Levvy in a series of papers has shown that there are two active centres in β-glucuronidase and that one of them

can be "blocked" by excess substrate (Levvy and Karunairatnam 1951). The interpretation of "competitive" and anticompetitive inhibition as due to the blocking of substrate and acceptor centres thus provides an attractive explanation of a wide range of experimental facts. None the less, the exact interpretation to be placed on the existence of these two centres is extremely uncertain. Thus D-glucose reacts primarily with the substrate centre as an inhibitor and secondarily also as an inhibitor with the acceptor centre, and *p*-nitrophenyl β-D-glucoside primarily with the substrate centre as a substrate and secondarily with the acceptor centre as an inhibitor; on the other hand, 6-*O*-methyl-D-glucose reacts only with the acceptor centre as an inhibitor but *p*-nitrophenyl 6-*O*-methyl-β-D-glucoside primarily with the substrate centre as a substrate and secondarily with the acceptor centre as an activator. Facts like these suggest that the substrate and acceptor centres may be only two aspects of the same site but even the "double displacement" mechanism of Koshland (1953) which seems to be supported by the kinetics of the *S. atra* β-glucosidase (Jermyn 1957*a*) also seems to involve two neighbouring non-identical sites. It must be admitted that no fully meaningful picture of the mechanisms involved can be put forward at the present time. Nor can the reaction of complex sugar derivatives with the acceptor site be harmonized in a simple theory with that of simple molecules such as water, glycerol, and mercaptoethanol.

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