FUNGAL CELLULASES

VI. SUBSTRATE AND INHIBITOR SPECIFICITY OF THE β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

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[*Manuscript received May* 18, 1955]

Summary

A large number of compounds with glycosidic linkages have been tested as substrates for the β-glucosidase of *Stachybotrys atra*. The enzyme appears to be specific for β-glucosides and all configurational alterations to the D-glucopyranose ring or substitutions in it lead to non-substrates; phenyl-β-thioglucoside is a substrate, however. Aryl-β-glucosides have a higher affinity for the enzyme than alkyl-β-glucosides and no hydrolysis of cellobiose by the enzyme can be demonstrated. *ortho*-Substitution in aryl-β-glucosides leads to a marked decrease in the affinity between enzyme and substrate.

All substrates of the enzyme are competitive inhibitors of its hydrolysis of *p*-nitrophenyl-β-glucoside as are also glucose and a number of other sugars and sugar acids. All polyhydroxy compounds tested affect β-glucosidase activity, many raising it slightly at moderately high concentrations and depressing it as the concentration is raised further. These compounds do not appear to act as orthodox non-competitive inhibitors. The combination of the two effects complicates the behaviour of the less efficient competitive inhibitors.

In general, the Michaelis constant for the hydrolysis of substrate is not identical with its inhibitor constant for the competitive inhibition of the hydrodysis of *p*-nitrophenyl-β-glucoside. This result is contrary to the predictions of the Michaelis-Menten theory.

It appears that there must be at least two types of binding centre in the enzyme molecule and the nature of the complex between enzyme, substrate, and third molecular species is discussed.

I. INTRODUCTION

Beta-glucosidase has been the classic subject for studying enzyme specificity, perhaps because of the ease with which a wide variety of substrates for the enzyme can be synthesized. It has been known since the work of Willstätter and Kuhn (1924) that the specificities of β-glucosidases even from closely-related plant species are different.

Many of the enzyme preparations used were not highly purified and even in the case of the much-studied kernel emulsins of *Prunus* species it is doubtful if the behaviour of a single enzyme species has been studied in any case. Thus Miwa and Tanaka (1949) have shown that the β-glucosidase, β-galactosidase, and β-xylosidase of apricot emulsin can be at least partially separated although they are often thought to be activities of a single enzyme. For fungal preparations the separation of glycosidase activities is often somewhat easier, e.g. β-xylosidase from β-glucosidase (Morita 1952), β-glucosidase from β-galactosidase (Niwa 1951) in *Aspergillus niger* preparations, and β-galactosidase from β-glucosidase in taka-diastase (Nishizawa 1942).

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The homogeneity of the β-glucosidase itself has not been shown in many cases and demonstrations have depended on showing the constancy of the ratios of the activity towards various substrates both in different preparations and in the same preparation at different stages of purification. Nishizawa and Wakabayashi (1951) have used a varying ratio of activities in different preparations to show that malt β-glucosidase is probably the sum of at least two enzymes, one a "cellobiase." Jermyn (1952) found that *A. oryzae* preparations contained a number of β-glucosidases and obtained evidence that their specificities were different. Only one of these enzyme components was active against *p*-nitrophenyl-β-glucoside although the others hydrolysed a wide variety of β-glucosides. Similarly Hattori and Kurihara (1950) obtained a β-glucosidase from *Aucuba japonica* ("aucubinase") that specifically attacked the β-glucoside aucubin. The isolation of distinct narrow-specificity α-glucosidases from a yeast strain has been claimed by Hestrin and Lindegren (1950). Levvy and Marsh (1954) have shown that claims for narrow-specificity enzymes must be treated critically, taking as an instance the β-glucuronidase from *Scutellaria baicalensis* (Miwa 1932) where a substrate of high affinity for the enzyme, thus acting as a powerful competitive inhibitor of the hydrolysis of other potential substrates, is a persistent impurity in supposedly purified enzyme preparations.

Parts IV and V of this series (Jermyn 1955*a*, 1955*b*) have described the isolation of a highly purified β-glucosidase from culture filtrates of *Stachybotrys atra*. This enzyme was readily separated during the course of purification from cellobiase as well as other enzyme activities which have often been attributed to β-glucosidases. This preparation appeared to be ideally suited for determining the specificities of a purified enzyme.

It was shown (Jermyn 1955*b*) that the S. *atra* β-glucosidase was inhibited by the glucose produced in the hydrolysis of aryl-β-glucosides but not by the aglycone. The absence of the aglycone inhibition that must be allowed for with some β-glucosidases (Veibel and Lillelund 1940), makes the task of determining the constants of competitive inhibitors of the enzyme much easier.

It has been emphasized by Levvy and Marsh (1954) that the problem of determining whether a given glycoside is a substrate of a certain glycosidase can be solved by observing whether it is a competitive inhibitor of the hydrolysis of a chromogenic substrate. This method has been successfully applied by Lederberg (1950) and Levvy and Marsh (1952). Ezaki (1940) considered the cross-inhibition of β-galactosidase by β-glucosides and β-glucosidase by β-galactosides in almond emulsin to prove them to be identical enzymes.

Ability to act as a competitive inhibitor is not confined to potential substrates, however; β-galactosidases are inhibited by galactonate or gluconate (Nishizawa 1942), taka-diastase β-glucosidase by gluconates or glucono-1,4-lactone (Ezaki 1940), emulsin by the 1,4-lactone only (Horikoshi 1942) and rumen β-glucosidase by glucono-1,4- and -1,5-lactones (Conchie 1954).

The S. *atra* enzyme shares the property of being inhibited by glucose with many other β-glucosidases (Veibel and Eriksen 1940). The further compli-

cation of inhibition by excess substrate is also not uncommon in glycosidases and Nishizawa (1951) has figured a large number of examples for β-galactosidases. Hence the common simplified versions of the Michaelis-Menten theory of enzyme-substrate-inhibitor relationships must be applied to the glycosidases with considerable reserve.

II. Materials

The glycosides required for this investigation were synthesized by suitable methods. Where they had been synthesized previously, their physical constants agreed with those in the literature. Jermyn (1955*c*) has described the synthesis and properties of those glycosides that were previously unreported.

The sugar acids required for inhibition experiments were synthesized as the potassium or calcium salts by the method of Moore and Link (1940).

The *N*-aryl-D-glucosylamines (*N*-glucosides) were recrystallized from water to give the monohydrate (*o*-toluidine, *p*-toluidine, 2 naphthylamine derivatives), or dihydrate (*p*-nitroaniline derivative) of the β-form.

The *Pseudomonas tumefaciens* polysaccharide was prepared using the method of Hodgson, Rijker, and Peterson (1945) and a culture of the bacterium given by Professor A. J. Rijker. The laminarin was a gift from Professor T. Dillon, and D-altrose and the heptoses were a gift from the late Professor C. S. Hudson.

The enzyme samples used, unless otherwise stated, were lead-precipitated fractions (Jermyn 1955*a*) with activity of at least 2000 units/mg N. It was found that the shape of the pH-activity curve (Jermyn 1955*b*), the Michaelis constants for the hydrolysis of salicin and *p*-nitrophenyl-β-glucoside, and the ratio of the hydrolysis rates of the two substrates were constant from one preparation to another.

III. Methods

(*a*) *Michaelis Constants*

It was shown for four aryl-β-glucosides (phenyl-, *p*-nitrophenyl-, *o*-cresyl-, 2-naphthyl-) that K_m at pH 5 (citric acid-sodium phosphate buffer) and 28°C was independent of enzyme concentration. The concentrations of substrate and enzyme were therefore chosen to give a suitable optical density by the methods of determining phenol liberation previously described (Jermyn 1955*a*) as well as a marked dependence of enzyme activity on substrate concentration. For alkyl glucosides the liberated glucose was determined colorimetrically by the Somogyi-Nelson method (Nelson 1944). The lower limit of practical substrate concentration was taken as 2×10^{-4}M; below this point substrate concentration would change sufficiently during incubation to affect the results. Since excess substrate did not significantly inhibit at concentrations less than about $K_m \times 10^2$, this left a usable range of substrate concentrations for determining even the lowest K_m values.

The Michaelis constant was determined graphically according to the method of Lineweaver and Burk (1934), the slope of the lines being determined by the method of least squares.

(*b*) *Determination of* V_{max}

From the Lineweaver-Burk plot it is possible to calculate V_{max}, the theoretical velocity at infinite substrate concentration. This is in fact fictitious where excess substrate inhibits, but its value at a standard enzyme concentration can be used as a measure of k_3 in the fundamental equation for enzyme action

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P. \qquad (1)$$

No attempt has been made to determine absolute values for k_3 and the various values of V_{max} were recalculated to moles of aglycone liberated during identical reaction periods at identical enzyme concentrations and expressed on a comparative basis with V_{max} for phenyl-β-glucoside as unity. The values are thus in fact though not in form comparable with the k_3 values of Veibel and Lillelund (1940).

(*c*) *Determination of Inhibitor Constants*

For a suitable concentration of inhibitor and enzyme the velocity of the inhibited and uninhibited hydrolysis of *p*-nitrophenyl-β-glucoside was determined over the substrate range 2×10^{-3}-2×10^{-4}M and the results expressed as Lineweaver-Burk plots.

Where inspection of the graphs showed that the inhibition was of simple competitive type, K_i (inhibitor constant) was determined according to the principles set out by Massart (1950).

(*d*) *Determination of Absolute Specificity*

If 5 or 10 ml of a solution of an aryl-glycoside is incubated for 24 hr with 10 units of enzyme at 28°C, a rate of decomposition 10^{-4} times that of *p*-nitrophenyl-β-glucoside should give a marked liberation of the corresponding phenol, and a rate of 10^{-5} can be readily detected by sufficient attention to detail. No glycoside has been classified as a non-substrate if hydrolysis could be detected by this test. Since low reaction velocity is usually due to low affinity (as shown by a high K_m) rather than a low value of k_3, the substrate concentration was usually kept at the highest level allowed by its solubility and this concentration is given with the listed non-substrates.

IV. Absolute Specificity of the Enzyme

Table 1 lists a number of glycosides which have been tested and found not to be substrates for the enzyme. Only compounds of types which have been claimed from time to time to be hydrolysed by β-glucosidases (cf. the review of Veibel 1950) were investigated. D-Glucofuranosides and L-glucosides have not been investigated in view of the results of Tsou and Seligman (1952)

with β-glucuronidase and 2-naphthyl-β-D-glucopyruronoside and β-D-glucofururonoside and the stereo-specificity of sugars and sugar acids as inhibitors of the *S. atra* β-glucosidase.

TABLE 1

NON-SUBSTRATES OF THE β-GLUCOSIDASE OF *S. ATRA*

Potential Substrate	Maximum Concentration Tested	Linkage (all pyranoside unless stated)
p-Nitrophenyl-α-D-glucoside	5×10^{-2}M	α-Glucoside
p-Nitrophenyl-α-D-mannoside	5×10^{-3}M	α-Mannoside
p-Nitrophenyl-β-D-mannoside	3×10^{-2}M	β-Mannoside
Phenyl-α-D-galactoside	3×10^{-2}M	α-Galactoside
p-Nitrophenyl-β-D-galactoside	5×10^{-2}M	β-Galactoside
Menthyl-β-D-glucuronide	5×10^{-2}M	β-Glucuronide
Maltose	2×10^{-3}M	α-Glucoside
Trehalose	10^{-1}M	α-Glucoside
Turanose	2×10^{-3}M	α-Glucoside
Sucrose	10^{-1}M	α-Glucoside β-Fructofuranoside
Cellobiose	2×10^{-3}M	β-Glucoside
Gentiobiose	2×10^{-3}M	β-Glucoside
Lactose	2×10^{-3}M	β-Galactoside
Melibiose	2×10^{-3}M	α-Galactoside
p-Nitrophenyl-β-cellobioside	5×10^{-2}M	2× β-Glucoside
p-Nitrophenyl-β-maltoside	5×10^{-2}M	α-Glucoside β-Glucoside
p-Nitrophenyl-β-lactoside	5×10^{-2}M	β-Glucoside β-Galactoside
Phenyl-α-cellobioside	3×10^{-2}M	α-Glucoside β-Glucoside
Phenyl-α-lactoside	5×10^{-2}M	α-Glucoside β-Galactoside
Phenyl-α-maltoside	5×10^{-2}M	2× α-Glucoside
Cellodextrins	1%	Polymeric β-glucoside (1, 4)
Laminarin	1%	Polymeric β-glucoside (1, 3)
Pseudomonas tumefaciens polysaccharide	1%	Polymeric β-glucoside (1, 2)

V. INHIBITION OF THE ENZYME

(*a*) *Screening for Effective Inhibitors*

A number of lines of evidence showed that the enzyme is sensitive to the presence of aliphatic polyhydroxy compounds. For most of the substances tested the effect takes the form of a slight activation rising to a peak at intermediate concentrations and giving way to inhibition at lower concentrations (Fig. 1).

After studying a number of cases it was decided to introduce an empirical criterion in testing a large number of possible inhibitors for their effectiveness.

TABLE 2

EFFECT OF VARIOUS SUBSTANCES ON THE HYDROLYSIS OF 10^{-4}M *p*-NITROPHENYL-β-GLUCOSIDE BY THE *S. ATRA* β-GLUCOSIDASE (pH 5, 28°C)

The limit of the experimental variation is about ±3 per cent. All additions of ionic compounds have been accompanied by readjustments to the correct pH

Reagent	Percentage of Original Activity at Indicated Concentration of Reagent	
	10^{-2}M	10^{-1}M
Inhibitors		
D-Glucose	6	
Methyl-α-D-glucoside	93	58
Methyl-β-D-glucoside	87	29
2-Chloroethyl-β-D-glucoside	48	
t-Butyl-β-D-glucoside	25	
*cyclo*Hexyl-β-D-glucoside	12	
Phenyl-β-D-glucoside	96	
p-Nitrophenyl-α-D-glucoside	87	80
o-Hydroxymethylphenyl-β-D-glucoside (salicin)	40	
p-Hydroxyphenyl-β-D-glucoside (arbutin)	22	
D-Gluconate	16	
D-Glucono-1,5-lactone	0	
D-Mannose	23	
p-Nitrophenyl-α-D-mannoside	95	
D-Mannonate	69	
D-Galactose	89	35
2-Naphthyl-β-D-galactoside	95	
D-Galactonate	93	
D-Idose	91	
D-*gala*-L-*manno*Heptose	90	
D-*gala*-L-*gluco*Heptose	80	
D-*gulo*-L-*gala*Heptose	93	
D-Fructose	95	67
L-Sorbose	55	33
L-Rhamnose	92	
L-Rhamnonate	82	
L-Arabinose	65	19
D-Arabinose	90	40
D-Arabonate	32	
D-Arabono-1,4-lactone	40	
p-Nitrophenyl-β-D-xyloside	45	
D-Ribonate	92	
Maltose	87	
Phenyl-α-maltoside	66	
Phenyl-β-maltoside	68	
Maltobionate	69	
Cellobiose	85	
Phenyl-α-cellobioside	67	42
Cellobionate	58	
Lactobiono-δ-lactone	58	
Turanose	42	
Melezitose	90	55

TABLE 2 (*Continued*)

Reagent	Percentage of Original Activity at Indicated Concentration of Reagent	
	10^{-2}M	10^{-1}M
L-Menthyl-β-D-glucuronide	70	
Ascorbic acid	78	
i-Erythritol	93	
Ineffective		
L-Xylose	103	
D-Ribose	98	
Trehalose	102	
Dulcitol	98	111
Sorbitol	98	
Mannitol	99	
Methyl-α-D-mannoside	97	99
D-Glucuronate	99	
D-Glucurone	98	
D-Galactonate	101	
D-Galactono-1,4-lactone	101	
L-Arabonate	101	
D-Xylonate	101	
D-Lyxonate	103	
D-Ribono-1,4-lactone	102	
D-Saccharate	99	
Mucate	99	
Lactobionate	97	
α-Glucoheptonate	98	
α-Glucoheptono-1,5-lactone	100	
Activators		
D-Xylose	108	
D-Lyxose	104	
Phenyl-β-lactoside	111	
Phenyl-α-lactoside	137	
p-Nitrophenyl-β-cellobioside	117	
Raffinose	108	
i-Inositol	114	
Ribitol (adonitol)	117	
Glycerol	125	
D-Altrose	114	
D-Glucosamine	120	
N-Acetyl-D-glucosamine	113	
D-*gulo*-L-*talo*Heptose	118	
D-*gluco*-D-*gulo*Heptose	120	
D-*manno*-D-*gala*Heptose	117	
Phenyl-β-D-mannoside	118	
Phenyl-α-D-mannoside	123	
Phenyl-α-D-galactoside	117	
Melibiose	112	
Lactose	112	
Sucrose	107	90
p-Nitrophenyl-β-lactoside	121	

Conditions were so chosen that about 30-40 per cent. of a 10^{-4}M solution of *p*-nitrophenyl-β-glucoside was hydrolysed, and a parallel experiment was run

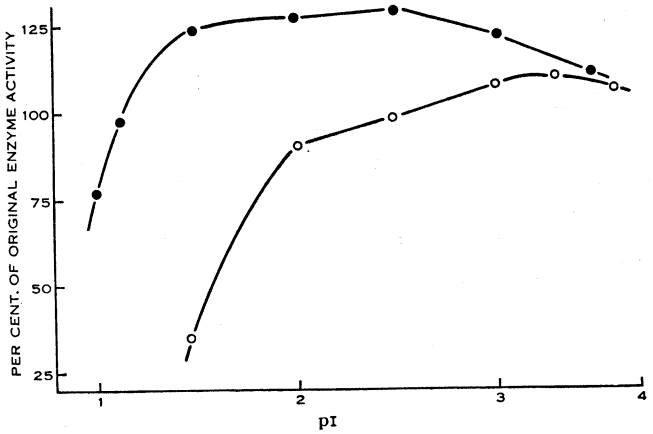


Fig. 1.—Effect of high concentrations of ○ sucrose and ● glycerol on the hydrolysis of 10^{-3}M *p*-nitrophenyl-β-glucoside at 28°C and pH 5·0 by S. *atra* β-glucosidase.

in which the solution was also 10^{-2}M in the compound under investigation. The results are set out in Table 2.

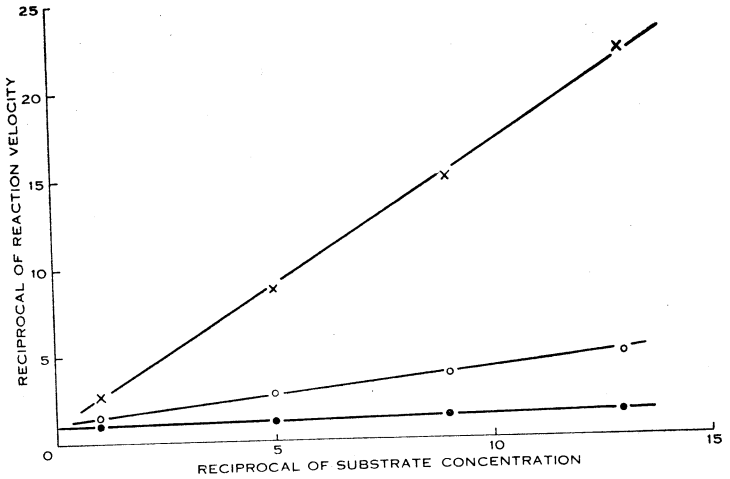


Fig. 2.—Arbutin (*p*-hydroxyphenyl-β-glucoside) and D-mannose as competitive inhibitors of the hydrolysis of *p*-nitrophenyl-β-glucoside by S. *atra* β-glucosidase. ● No addition. ○ 10^{-2}M Mannose. × 10^{-2}M Arbutin. Unit substrate concentration = 2×10^{-3}M.

Since it was not possible to examine in detail all substances with an effect on the enzymic reaction, a certain number of selected cases have been used in an attempt to elucidate the mechanism of inhibition.

(*b*) *Competitive* (*Type* 1) *Inhibitors*

The sugars D-glucose, D-mannose, and D-arabinose, the sugar acids D-gluconic and D-arabonic, and the lactones D-glucono-1,5-lactone, D-arabono-1,4-lactone, and lactobiono-δ-lactone are sufficiently effective competitive inhibitors of the hydrolysis of *p*-nitrophenyl-β-D-glucoside for the competitive effect to be studied in isolation from non-competitive effects. Non-chromogenic β-glucosides as well as certain other glycosides which are not substrates but have the same ring configuration as β-D-glucosides (*p*-nitrophenyl-β-D-xyloside and menthyl-β-D-glucuronide) are also competitive inhibitors.

Figure 2 shows that both D-mannose and the β-glucoside arbutin give the typical Lineweaver-Burk plots to be expected for competitive inhibition.

(*c*) *Type* 2 *Inhibition*

The plots of enzyme activity against inhibitor concentration for D-glucose (competitive inhibitor) and turanose are almost indistinguishable (Fig. 3). Yet when a Lineweaver-Burk plot is constructed for turanose inhibition (Fig. 4) it is apparent that it does not follow the criteria usually laid down for either competitive or non-competitive inhibition (cf. Massart 1950). For competitive inhibition $1/V_{max}$ remains constant and the slope of the line and hence the apparent K_m are increased by the factor $(1 + [I]/K_i)$, where $[I]$ is the concentration of inhibitor; for non-competitive inhibition $1/V_{max}$ and the slope of the line are increased by $(1 + [I]/K_i)$ and the apparent K_m remains constant. In type 2 inhibition, to the first approximation, $1/V_{max}$ and the apparent K_m are both increased by the same factor and the slope of the line remains constant.

In terms of the constants of equation (**1**) it can be shown that

$$K_m = \frac{k_2 + k_3}{k_1}. \qquad .. \quad .. \quad .. \quad (2)$$

The usual treatment of inhibition is based on the assumption that $k_2 >> k_3$ and that k_3 can in practice be neglected. But if, on the other hand, $k_3 >> k_2$ and the effect of a non-competitive inhibitor bound to the enzyme molecule is to block the stage

$$ES \xrightarrow{k_3} E + P,$$

reducing the true k_3 to a new apparent value k'_3 for a given concentration of inhibitor, we have

$$K_m = \frac{k_2 + k_3}{k_1} \simeq \frac{k_3}{k_1},$$

and

$$K'_m = \frac{k_2 + k'_3}{k_1} \simeq \frac{k'_3}{k_1}.$$

This would lead to V_{max} and apparent K_m decreasing in approximately the same ratio. Any discrepancy can be used to calculate the ratio of k_3 to k_2; for the hydrolysis of *p*-nitrophenyl-β-D-glucoside the ratio k_3/k_2 is at least 50. The likeness of the two curves in Figure 3 may therefore be interpreted as being due to the fact that they are both dissociation curves, one for the binding of

glucose at the enzymatically active centre, and one for the binding of turanose at a second centre. The affinity constant for turanose is about 5×10^{-3}M. Since the dissociation curve tends asymptotically to 100 per cent. inhibition at hig

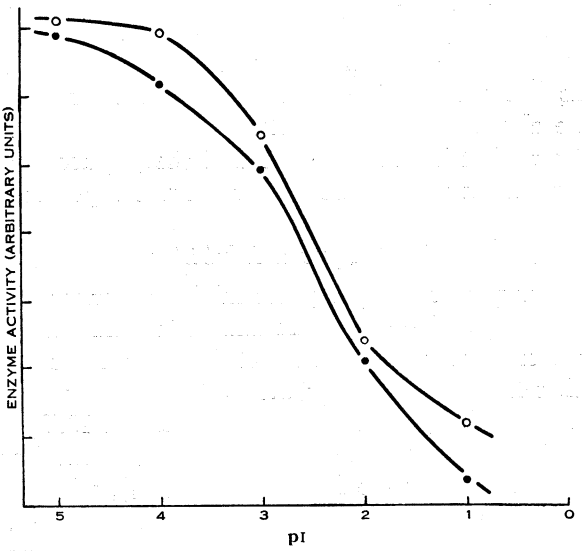


Fig. 3.—Effect of ○ turanose and ●glucose on the enzymic hydrolysis of 10^{-3}M *p*-nitrophenyl-β-glucoside by S. *atra* β-glucosidase.

turanose concentrations, it appears that the reaction $ES \rightarrow P$ is totally blocked by turanose binding and not depressed to a lower rate.

TABLE 3

DETERMINATION OF K_i FOR GLUCOSE FROM THE DATA OF FIGURE 3

$[I]$	Slope	$\frac{\text{Slope of Inhibited Reaction}}{\text{Slope of Non-Inhibited Reaction}}$	$\frac{K_i}{[I]}$	K_i
Nil	0·096			
10^{-4}M	0·149	1·55	0·55	$1{\cdot}8 \times 10^{-4}$M
10^{-3}M	0·61	6·3	5·3	$1{\cdot}9 \times 10^{-4}$M
10^{-2}M	5·8	60	59	$1{\cdot}7 \times 10^{-4}$M
$10^{-1{\cdot}5}$M	13·7	142	141	$2{\cdot}2 \times 10^{-4}$M
10^{-1}M	53·7	558	557	$1{\cdot}8 \times 10^{-4}$M

Phenyl-α-cellobioside, methyl-α-D-glucoside, and L-arabinose give results similar to those for turanose.

It appears that inhibition by excess substrate is due to binding at the

second centre. This hypothesis can be tested by observing the consequences of counteracting high concentrations of a competitive inhibitor by raising the substrate concentration. The results for glucose and *p*-nitrophenyl-β-glucoside are shown in Figure 5. If the fact that all the lines do not converge to a common value of $1/V_{max}$ is neglected and K_i is calculated from the ratio of slopes, the results tabulated in Table 3 are obtained. The constancy of K_i shows that the competition of glucose and *p*-nitrophenyl-β-glucoside for the enzymatically active centre is not affected by the action of excess substrate in depressing the activity.

Lineweaver-Burk plots corresponding to the atypical non-competitive inhibition here called type 2 have often been observed (e.g. for the inhibition of adenosinetriphosphatase by ethylurethane (Gross 1954)) without comment on the fact that they imply unusual relationships among the rate constants.

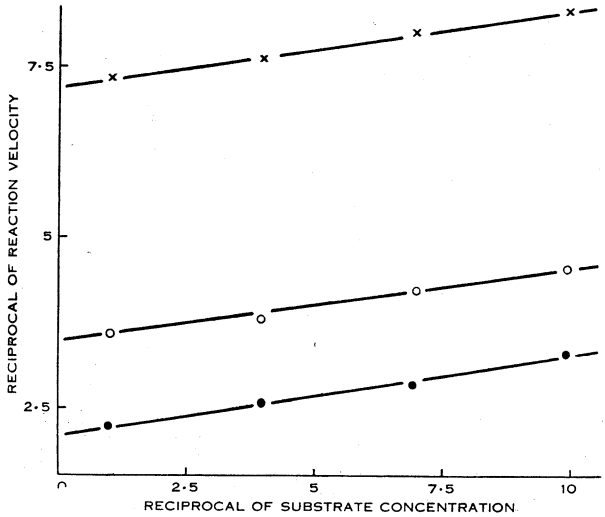


Fig. 4.—Effect of turanose on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. ● No addition. ○ 2×10^{-3}M Turanose. × 10^{-2}M Turanose. Unit substrate concentration 10^{-3}M.

(*d*) *Mixed Type* 1 *and Type* 2 *Inhibition*

If a substance has affinity for both binding centres on the enzyme molecule it can be expected to show behaviour intermediate between those of type 1 and type 2 inhibitors. D-Galactose gives Lineweaver-Burk plots that can be explained on this hypothesis (Fig. 6). The calculated K_i for galactose is found to have a constant value of 10^{-2}M with changing galactose concentration. The affinity constant for the second centre is about 5×10^{-1}M. This behaviour should be shown most clearly where, as for galactose, both constants are relatively high. Cellobiose behaves similarly to galactose with an even higher

value of K_i, a number of effective β-glucosidic competitive inhibitors show a slight affinity for the second centre.

(*e*) *Type* 3 *Inhibition*

Substances giving curves such as those shown in Figure 1 are classified as type 3 inhibitors. It can readily be shown from a study of Lineweaver-Burk plots constructed for various concentrations of the reagent that the activating effect is the primary one, and that at higher reagent concentrations a competitive inhibition is superimposed on the activation. Glycerol was most intensively studied as a type 3 inhibitor but very similar results are given by sucrose. Figure 7 shows the effect of increasing concentration of glycerol, and it can be calculated that K_i for glycerol is approximately 2×10^{-1}M. For substances having rather smaller values of K_i, the results of Figure 8 are obtained.

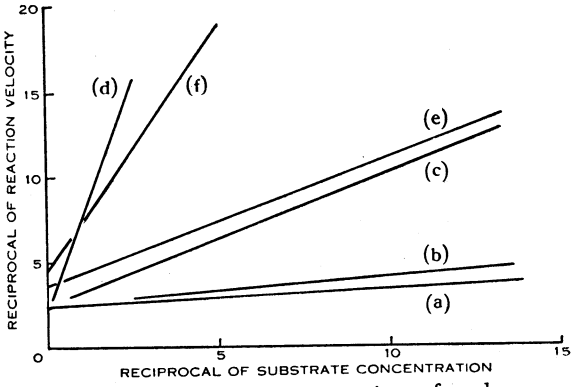


Fig. 5.—Effect of various concentrations of D-glucose on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. (*a*) No addition. (*b*) 10^{-4}M Glucose. (*c*) 10^{-3}M Glucose. (*d*) 10^{-2}M Glucose. (*e*) $10^{-1.5}$M Glucose. (*f*) 10^{-1}M Glucose. Enzyme concentration constant. Unit substrate concentration $= 10^{-3}$M for (*a*)-(*d*); 2×10^{-2}M for (*e*), (*f*).

Increasing the concentration of glycerol eventually leads to Lineweaver-Burk plots analogous to those of Figure 8, but there is a region of anomalous results between 0·5M and 1·65M. Here the degree of activation apparently falls as the glycerol concentration rises, leading finally for M and 1·5M glycerol to what may be described as "competitive activation" (Fig. 9). There is then a sharp change between 1·5M and 1·65M to the expected type of line, and calculation from the lines for 1·75M and 2M shows that the K_i (2×10^{-1}M) is the same as for 10^{-1}M glycerol. In this range the dependence on concentration is so critical that it was impossible to reproduce the results exactly in successive experiments with glycerol solutions that had been made up separately. No hypothesis explaining these phenomena can be offered at present; it may be that the identity of K_i at high and low glycerol concentrations is illusory.

(*f*) *Identity of Type* 2 *and Type* 3 *Effects*

The Lineweaver-Burk lines for 2×10^{-3}M turanose (Fig. 4) and 10^{-2}M glycerol (Fig. 7) are identical except that one is above and one below the null line. There is nothing to contradict the assumption that both effects are due to binding at the same centre, one reagent blocking the process $ES \rightarrow P$ and the other facilitating it. If this is true, V_{max} should increase with increasing concentration of a type 3 reagent and this is found to be the case. Since the theoretical value of k_3 for complete saturation of the enzyme is unknown it is not possible to use such data to construct dissociation curves.

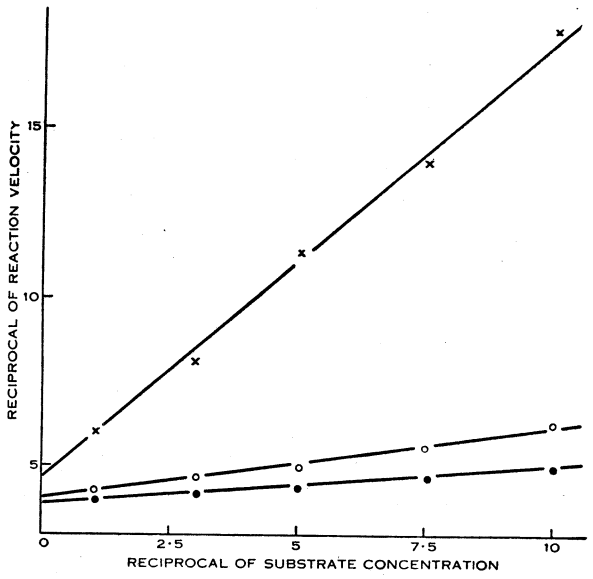


Fig. 6.—Effect of D-galactose on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. ● No addition. ○ 10^{-2} Galactose. × 10^{-1}M Galactose. Unit substrate concentration 10^{-3}M.

Most substances tested that are not competitive inhibitors show type 3 effects. Type 2 effects are only shown by substances with α-glucosidic linkages (turanose, phenyl-α-cellobioside, and methyl-α-glucoside), the sterically related D-galactose and L-arabinose (differing only by a $-CH_2OH$ attached to C_5 of the pyranose ring), and some β-glucosides.

VI. Relation of β-Glucosidase, β-Xylosidase, and β-Cellobiosidase

The non-identity of these three activities was demonstrated by the existence of batches of enzyme with only β-glucosidase activity, and by the variation in the ratio between the activities in batches containing all three (Table 4). Nevertheless, the activities must have belonged to very similar enzyme proteins

TABLE 4

COMPARISON OF THE ACTIVITY OF THREE PREPARATIONS OF *S. ATRA* β-GLUCOSIDASE IN HYDROLYSING 10^{-3}M *p*-NITROPHENYL-β-GLYCOSIDES AT 28°C AND pH 5 (CITRATE-PHOSPHATE BUFFER)

Batch No.	Rate of Hydrolysis of *p*-Nitrophenyl-β-cellobioside (β-xylosidase = 1)	Rate of Hydrolysis of *p*-Nitrophenyl-β-glucoside (β-xylosidase = 1)
2 (lead fractionated)	7·9	133
4 (lead fractionated)	1·6	107
5 (dialysed ethanol concentrate)	22·8	104
5 (after lead fractionation)	28·3	126
9*	1·7	157

* Fraction 3 (Jermyn 1955*a*).

since the lead-fractionation procedure which sufficed to separate all other enzymes from the β-glucosidase did not much affect the activity ratios.

Inspection of the pH-activity curves for the three shows them to be rather unlike (Fig. 10) although similar variation in the pH-activity curves for a single enzyme against different substrates has often passed without comment.

A number of possible inhibitors were then tried with the results shown in Table 5. From the results recorded in the last section, it appears that they

TABLE 5

COMPARISON OF CERTAIN SUBSTANCES AS INHIBITORS OF THE β-GLUCOSIDASE, β-CELLOBIOSIDASE, AND β-XYLOSIDASE IN A SINGLE BATCH OF *S. ATRA* ENZYME (LEAD-FRACTIONATED BATCH 5 OF TABLE 4). INHIBITORS, 10^{-3}M; *p*-NITROPHENYL-β-GLYCOSIDE SUBSTRATE, 10^{-4}M

Inhibitor	Per Cent. of Uninhibited β-Glucosidase	Per Cent. of Uninhibited β-Cellobiosidase	Per Cent. of Uninhibited β-Xylosidase
D-Glucose	10	21	4
Phenyl-α-D-glucoside	96	120	
p-Nitrophenyl-α-D-glucoside	89	113	
Arbutin	13	10	33
D-Mannose	34	25	
Phenyl-α-D-galactoside	117	116	
2-Naphthyl-β-D-galactoside	95	120	
D-Xylose	109	122	81
p-Nitrophenyl-β-D-xyloside	45	85	
Cellobiose	86	9	
Cellobionate	58	7	
Phenyl-α-cellobioside	72	29	
p-Nitrophenyl-β-lactoside	121	144	
Lactobionate	97	94	
Maltobionate	69	29	

would be unlikely to be conclusive without detailed investigation of inhibitor type and the relative affinities of substrates and competitive inhibitors. Taken together the three lines of evidence (Table 4, Fig. 10, Table 5) would suggest that there were three separate enzymes in the purified preparation from batch 5, but fall far short of proof.

In batch 6 there was no detectable β-xylosidase or β-cellobiosidase, thus proving the β-glucosidase to be a separate entity.

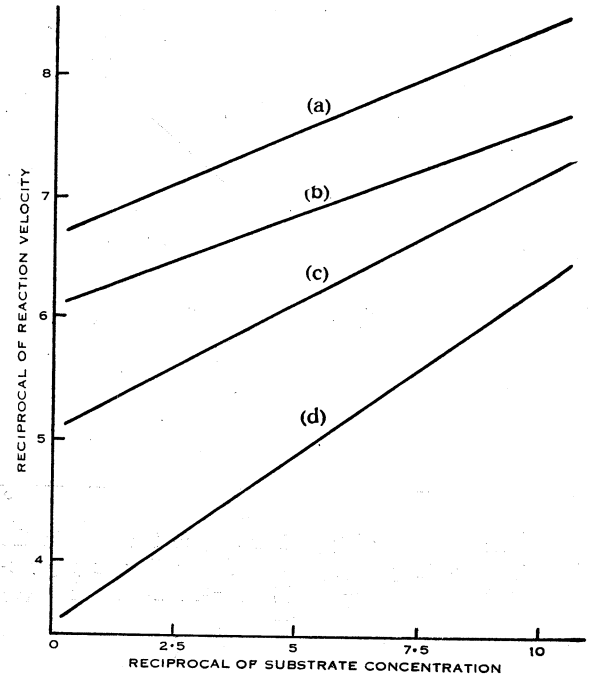


Fig. 7.—Effect of various concentrations of glycerol on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. (*a*) No addition. (*b*) 10^{-2}M Glycerol. (*c*) $10^{-1.5}$M Glycerol. (*d*) 10^{-1}M Glycerol. Unit substrate concentration 10^{-3}M.

VII. Relative Specificity of the Enzyme

A large number of β-glucosides was examined as substrates for the enzyme and competitive inhibitors of the hydrolysis of *p*-nitrophenyl-β-D-glucoside. The values of K_m, relative V_{max}, and K_i are shown in Table 6, together with K_i values for a number of those non-substrates that were shown to be competitive inhibitors.

The thiophenol liberated from phenyl-β-thioglucoside could be readily estimated by the Folin-Ciocalteau method and this glucoside shown to be a substrate as well as an inhibitor. Although the aryl-*N*-glucosides were effective

competitive inhibitors it was not possible to show whether or not they were substrates because of the high rate of spontaneous hydrolysis. With *N*-*p*-nitrophenyl-β-D-glucosylamine, which is much less absorptive than *p*-nitroaniline in the range 410-450 mμ, the increase in optical density at 435 mμ (Beckmann spectrophotometer) was used as a measure of the hydrolysis of the *N*-glucoside. It was found that the rate of enzymic hydrolysis of this substrate at 10^{-3}M could not exceed 10^{-4} times that of *p*-nitrophenyl-β-D-glucoside.

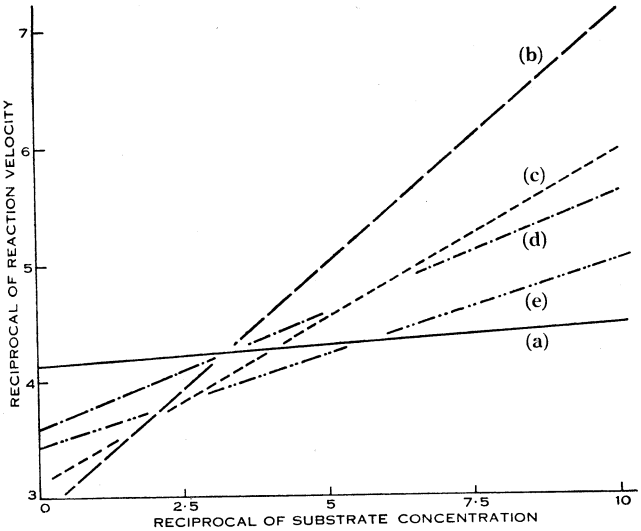


Fig. 8.—Combination of type 1 and type 3 effects in the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. (*a*) No addition. (*b*) 10^{-2}M L-Sorbose. (*c*) 10^{-1}M D-Fructose. (*d*) 10^{-1}M Ascorbic acid. (*e*) 10^{-1}M Sucrose. Unit substrate concentration 2×10^{-3}M.

It is a consequence of the hypothesis developed to explain the behaviour of type 2 and 3 inhibitors that K_m and V_{max} might be expected to show parallel variation, since the former is strongly influenced and the latter wholly determined by the value of k_3. Since the relationship is

$$K_m \simeq \frac{k_3}{k_1},$$

and the relative effects of different substrates on k_1 and k_3 are unknown, strict proportionality is not to be expected. Figure 11 shows that the trend is in fact in the opposite direction to that predicted and the substrate effect on k_1 must outweigh that on k_3.

When L-arabinose, a type 2 inhibitor, was tested for its effect on the hydrolysis of a number of β-glucosides of high K_m, it was found that Lineweaver-Burk plots were obtained identical in form with those of Figure 4. It appears that $k_3 >> k_2$ here also, and that the kinetics of all substrates investigated can be explained in terms of k_3 and k_1 only. It would be expected from the explana-

tion advanced for type 2 inhibition, that V_{max} would be diminished in the same proportion for all substrates at a given concentration of L-arabinose, and this was found to be the case.

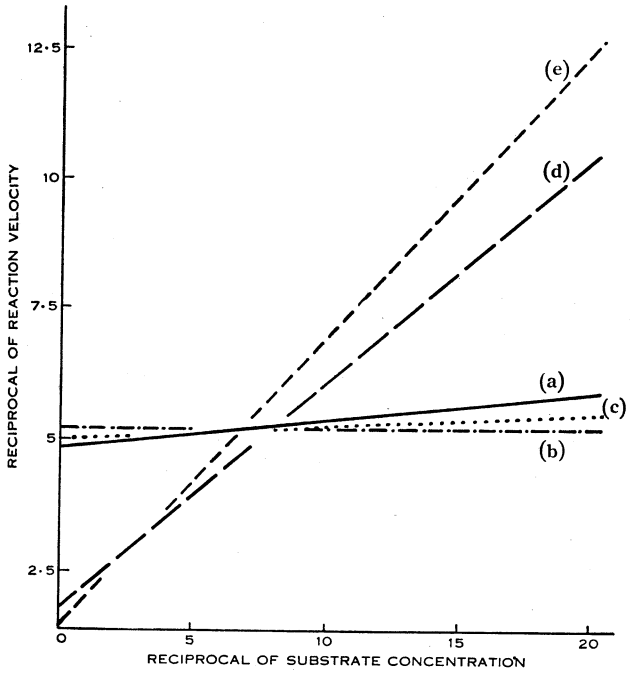
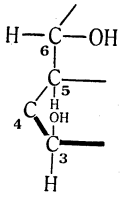


Fig. 9.—Effect of higher concentrations of glycerol on the hydrolysis of *p*-nitrophenyl-β-glucoside by *S. atra* β-glucosidase. (*a*) No addition. (*b*) 1·0M Glycerol. (*c*) 1·5M Glycerol. (*d*) 1·75M Glycerol. (*e*) 2·0M Glycerol. Unit substrate concentration 2×10^{-3}M.

VIII. Discussion

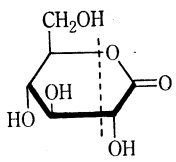
(*a*) *Absolute Specificity of the Enzyme*

One portion of the specificity of the *S. atra* β-glucosidase appears to be directed towards the configuration about carbon atoms 3, 5, and 6 of glucose and the required structure appears to be given by

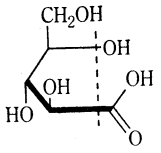


All those competitive inhibitors of the enzyme which do not contain β-glucosidic linkages appear to fit into this pattern. The most important single element of this structure appears to be that the configuration at C_3 should be

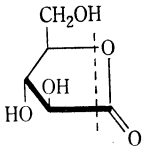
the same as that in D-glucopyranose and no sign of activity as a competitive inhibitor has been found in any substance with the opposite configuration. Gottschalk (1950) predicted from a survey of the relevant data that one point of specific attachment of β-glucosides to all β-glucosidases would prove to be the —OH group at C_3. The pyranose or any other ring structure does not seem to be necessary for activity; thus, although the activity of D-gluconic acid may be explained by the small amount of D-gluconolactone in equilibrium with it at pH 5 (cf. the results of Conchie 1954) this can hardly be the case with D-arabonic acid and D-arabonolactone. Some examples of inhibitors with the full D-glucose structure on the critical carbon atoms are given below.



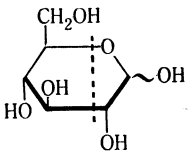
D-Glucono-1,5-lactone
$K_i = 0{\cdot}32 \times 10^{-5}$M.



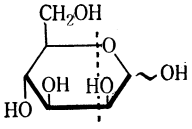
D-Arabonic acid
$K_i = 4{\cdot}6 \times 10^{-5}$M.



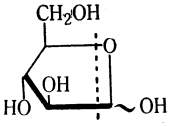
D-Arabono-1,4-lactone
$K_i = 27 \times 10^{-5}$M.



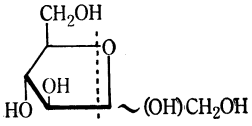
D-Glucopyranose
$K_i = 19 \times 10^{-5}$M.



D-Mannopyranose
$K_i = 43 \times 10^{-5}$M.



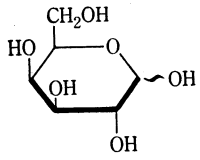
D-Arabinofuranose
$K_i = 580 \times 10^{-5}$M.



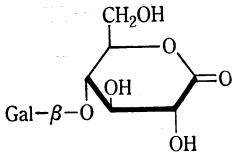
D-Fructofuranose
$K_i = 1000 \times 10^{-5}$M.

In two cases (D-fructose, D-arabinose) the equilibrium in solution is against the forms with the structure required for activity, and the K_i values found experimentally are not of the same order as those of the true inhibiting species. Similarly there is no indication from the data whether D-arabonic acid or the D-arabonate ion is the inhibiting species and the true K_i will again be different from the calculated value.

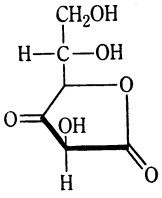
The configuration at C_4 does not appear to be critical and if the rest of the structure is correct variations at this centre still result in competitive inhibitors:



D-Galactopyranose
$K_i = 1000 \times 10^{-5}$M.



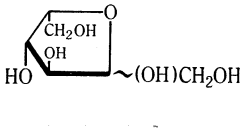
Lactobiono-δ-lactone
$K_i = 87 \times 10^{-5}$M.



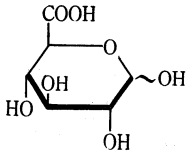
D-Ascorbic acid
(one keto form; not favoured by equilibrium)
$K_i = 140 \times 10^{-5}$M.

The insertion of a β-galactopyranosyl group at C_4 in the very active D-glucono-1,4-lactone molecule gives a fairly active inhibitor; in the less active D-glucose molecule it leads to a disaccharide (lactose) without detectable activity.

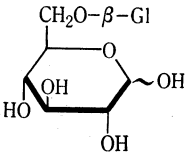
It appears that the nature rather than the configuration of the groups around C_5 is important, thus L-sorbose is a competitive inhibitor, while D-glucuronic acid, D-glucurone, and gentiobiose (6-β-D-glucopyranosyl-D-glucose) are not.



L-Sorbofuranose
$K_i = 87 \times 10^{-5}$M.

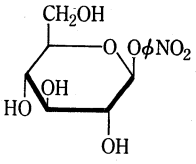


D-Glucuronic acid
(pyranose form)
non-competitive.

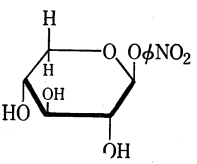


Gentiobiose
non-competitive.

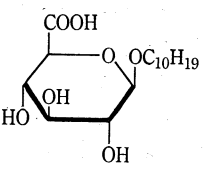
A second determinant of a specific attachment between the enzyme and the active molecule appears to be the configuration about C_1 (cf. the discussion in Gottschalk 1950). Neither D-glucuronic acid nor D-xylose are competitive inhibitors of the enzyme, but *p*-nitrophenyl-β-D-xylopyranoside is an inhibitor comparable with aryl-β-D-glucosides, and menthyl-β-D-glucopyruronoside is comparable with alkyl-β-D-glucosides. The specificity does not appear to require that the β-linkage shall be through oxygen; phenyl-β-D-thioglucoside is both a substrate and an inhibitor assuming that all the observed effects are due to a single enzyme. All other β-glucosidases which have so far been tested are unable to hydrolyse phenyl-β-thioglucoside (Veibel 1950), although Conchie (1954) showed it to be a slight inhibitor, not established as competitive, of rumen β-glucosidase. A linkage through nitrogen appears to allow attachment at the specific centre and the aryl-*N*-β-glucosides are competitive inhibitors, although it is doubtful whether they are substrates.



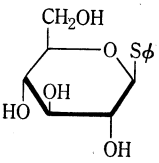
p-Nitrophenyl-β-D-glucopyranoside
$K_m = 5 \times 10^{-5}$M.
Substrate.



p-Nitrophenyl-β-D-xylopyranoside
$K_i = 3{\cdot}7 \times 10^{-5}$M.
Non-substrate.



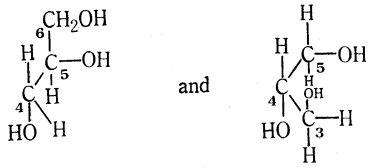
Menthyl-β-D-glucopyruronoside
$K_i = 1200 \times 10^{-5}$M.
Non-substrate.



Phenyl-β-D-thioglucopyranoside
$K_i = 153 \times 10^{-5}$M.
Substrate.

This requirement appears to involve the space relationship between the aglycone residue and the hydroxyl groups on C_2 and C_4 since neither mannosides nor galactosides are competitive inhibitors.

It would appear that while it is sufficient for a competitive inhibitor to meet one of the stereo-specificity requirements, a substrate must meet them both. There must also be a limit to the detection of any specific effects set by the apparent general affinity for polyhydroxyl compounds (K_i for glycerol, $20{,}000 \times 10^{-5}$M). Although glycerol can be written in such forms as



and

it is impossible to believe that its affinity for the enzyme can be any more specific than that of the many other polyhydroxyl compounds in which similar portions of the molecule occur. This effect does not appear to be limited to the S. *atra* β-glucosidase,or even to β-glucosidases in general, for de Grandchamp-Chaudun and Moreau (1953) found that besides the competitive inhibition of various sucrases by such sugars as glucose, fructose, and galactose with structures possibly related to the substrate, there was also a similar effect from glycerol at rather higher concentrations.

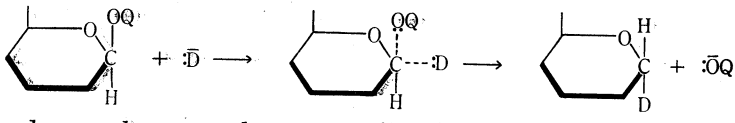
The specificity of the β-glucosidase of S. *atra* is much narrower than that of most β-glucosidases previously studied (Veibel 1950; Gottschalk 1950). If affinity for the enzymically active centre is considered without regard to whether the bound substance is a substrate or a competitive inhibitor, then the specificity of the enzyme resembles most closely that of brain hexokinase as reported by Sols and Crane (1954) whose specificity is directed towards the hydroxyl groups on C_1, C_3, C_4, and C_6 of glucose. The most obvious differences are the lack of requirement for an intact pyranose ring, the actually enhanced affinity arising from replacement of CHOH by CO at C_1 (D-glucono-1,5-lactone is inactive for hexokinase) and C_4, and the ineffectiveness of a nitrogen atom on C_2 (the glucosaminium ion and *N*-acetylglucosamine are not competitive inhibitors).

Crane and Sols (1954) also showed that there was a second binding centre on the brain hexokinase molecule leading to non-competitive inhibition of the enzymic reaction. Some elements of the specificity of the second binding centre of the β-glucosidase, at any rate for molecules blocking $ES \rightarrow E + P$, may be discerned in its affinity for α-glucosides and D-galactose $(+--+)$ and L-arabinose $(--+)$. It may be speculated whether further research might not reveal it to be a second enzymically active centre with substances with affinity for the first centre acting as type 2 inhibitors.

(*b*) *Relative Specificity of the Enzyme*

Koshland (1954) and Koshland and Stein (1954) have developed and presented experimental evidence for a theory by which enzymes showing a high specificity for the *R* portion of the *R*-O-*Q* molecule and acting as *R*-transferases catalyse the fission of the *R*-O bond. The glucotransferase action of β-glucosidases is well known, and the S. *atra* β-glucosidase is highly specific for the glucose portion of the molecule, so that it is reasonable to follow Koshland and

Stein and write (the apparent absence of inversion about C_1 in the finally liberated hydrolysis product being actually a double inversion (Koshland 1953))



for the fundamental step in the action of β-glucosidase.

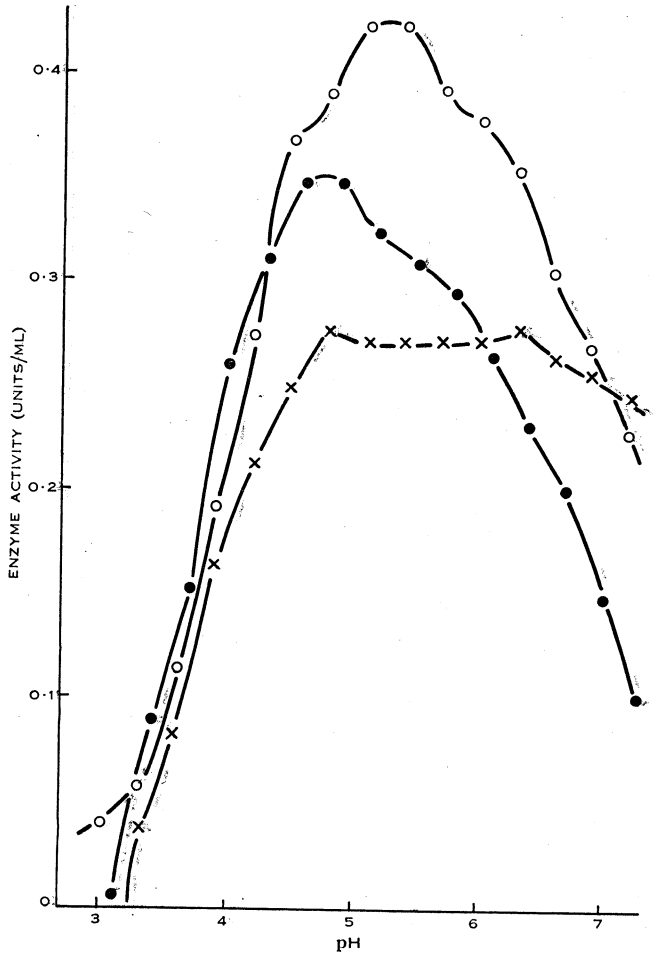


Fig. 10.—pH-Activity curves in citric acid-sodium phosphate (McIlvaine) buffers for two other enzyme activities in a preparation of *S. atra* β-glucosidase. All activities measured against 10^{-3}M *p*-nitrophenyl-β-glycoside at 28°C. ○ β-Cellobiosidase. × β-Xylosidase. ● β-Glucosidase from a preparation without β-xylosidase or β-cellobiosidase activity.

The primary nucleophilic reagent $:\bar{D}$ is unspecified. The nature of Q can affect the rate of the reaction either by altering the electronic structure of the *R*-O bond, or through steric influences on the approach of electrophilic reagents

TABLE 6

RELATIVE SPECIFICITY OF THE β-GLUCOSIDASE OF *S. ATRA* FOR VARIOUS SUBSTRATES AND INHIBITORS

Reagent	K_m ($M \times 10^{-5}$)	Relative V_{max} (phenyl-β-glucoside = 1)	K_i for the Hydrolysis of *p*-Nitrophenyl-β-D-glucoside ($M \times 10^{-5}$)
β-D-Glucosides			
Phenyl-	7·2	1·00	33
p-Hydroxyphenyl-			7·6
p-Nitrophenyl-	5·0	1·05	
m-Nitrophenyl-	36	0·88	
o-Nitrophenyl-	*		
p-Methylphenyl-	9·5	0·52	17·0
m-Methylphenyl-	6·7	1·24	27·2
o-Methylphenyl-	66	0·32	217
o,o'-Dimethylphenyl-	*c.* 680	0·002	*
o-Hydroxymethylphenyl-	122	0·37	216
2-*iso*Propyl-4-methylphenyl-	*c.* 700	0·022	*
o-Methoxyphenyl-	9·2	1·36	43·9
m-Methoxyphenyl-	8·2	0·83	4·3
p-Methoxyphenyl-	5·1	0·74	4·4
o-Chlorophenyl-	34	0·38	22·8
p-Chlorophenyl-	4·3	1·23	9·6
2,4-Dichlorophenyl-	7·0	1·03	8·8
o-Iodophenyl-	33	0·04	28
p-Iodophenyl-	3·1	0·13	3·0
2,4-Di-iodo-6-methylphenyl-	*		
p-Phenylphenyl-	12	116	2·3
1-Naphthyl-	178	0·13	5·8
2-Naphthyl-	5·2	0·88	4·5
1-Methyl-2-naphthyl-	21	0·12	23·5
1-Bromo-2-naphthyl-	5·0	0·25	5·7
6-Bromo-2-naphthyl-	2·2	0·72	3·1
8-Quinolinyl-	*		
*cyclo*hexyl-	63	0·096	164
sec.-Butyl-	84	0·12	
*iso*Propyl-	1300	0·055	
tert.-Butyl-†		0·015	
2-Chloroethyl-†		0·016	
Methyl-†		0·035	10,000
N-β-D-Glucosides			
N-*o*-Tolyl-β-D-glucosylamine			105
N-*p*-Tolyl-β-D-glucosylamine			34
N-2-Naphthyl-β-D-glucosylamine			27
Phenyl-β-D-thioglucoside	200	0·35	153
Sugars			
D-Glucose			19
D-Mannose			43
D-Galactose			*c.* 1,000

* Activity too low for accurate measurement.

† Non-linear Lineweaver-Burk plots at the substrate concentrations necessary for measurable activity; reaction velocities at 10^{-2}M have been used for comparison.

TABLE 6 (*Continued*)

Reagent	K_m ($M \times 10^{-5}$)	Relative V_{max} (phenyl-β-glucoside = 1)	K_i for the Hydrolysis of *p*-Nitrophenyl-β-D-glucoside ($M \times 10^{-5}$)
D-Fructose			*c.* 1,000
L-Sorbose			58
D-Arabinose			580
Sucrose			600
Cellobiose			5,900
Sugar acids and lactones			
D-Gluconic acid			140
D-Glucono-1,5-lactone			0·32
D-Arabonic acid			4·6
D-Arabono-1,4-lactone			27
4-(β-D-galactopyranosyl)-D-Glucono-1,5-lactone (lactobiono-δ-lactone)			87
Others			
p-Nitrophenyl-β-D-xyloside			3·7
Menthyl-β-D-glucuronide			1,200
Glycerol			*c.* 20,000
Ascorbic acid			140

(protons free or bound to the enzyme) to the *R*-O bond, and on the fit of the substrate to the enzyme surface. It may be predicted qualitatively that *Q* groups capable of acting as electron sinks will increase the reaction rate and, in agreement with this, k_3 for phenyl-β-glucoside is about 10 times greater than for *cyclo*hexyl-β-glucoside (and other secondary alkyl-β-glucosides).

This prediction cannot be applied in detail, however, and if k_3 values for *para*-substituted phenyl-β-glucosides are compared, we find the order

$$C_6H_5 > Cl > NO_2 > H > OCH_3 > CH_3 > I,$$

which does not coincide with any series of electronic effects. It is apparent that a large number of factors are involved in the stability of the transition complex. It seems unnecessary, however, to postulate with Pigman (1944) that there are any specific forces of attraction between the aglycone and the enzyme; indeed with hydrocarbon radicals this seems most unlikely (cf. Gottschalk 1950). Where the aglycone is another sugar radical which would be expected to have some affinity for the enzyme, the reaction rate is zero for the *S. atra* enzyme.

The *S. atra* β-glucosidase agrees with the fungal β-glucosidases from *Aspergillus oryzae* and *A. niger* studied by Miwa *et al.* (1937) in hydrolysing *ortho*-substituted phenyl-β-glucosides much more slowly than the parent β-glucoside. This effect extends to 1-naphthyl-, 8-quinolinyl-, and substituted 2-naphthyl-β-glucosides. V_{max} is diminished and K_m is increased, and since the effects do not always run in parallel, it is apparent that k_1, k_2, and k_3 are probably all affected by the *ortho*-substituent. It has already been shown (Fig. 11) that

the preponderating effect must be on k_1, the rate of attachment of substrate to enzyme to form the active complex. One way in which this effect could arise is by restricting free rotation about the C-O-C bonds to allow the required fit of the glucoside to the enzyme surface and experiments with models show that rotation in 2,6-dimethylphenyl-β-glucoside is almost completely hindered. The order for the K_m of mono-*ortho*-substituted phenyl-β-glucosides ($-OCH_3 < H < Cl$, $CH_2OH < CH_3 < I < CH(CH_3)_2 < NO_2$) contains deviations from the accepted list of radical sizes and specific effects must be invoked here also.

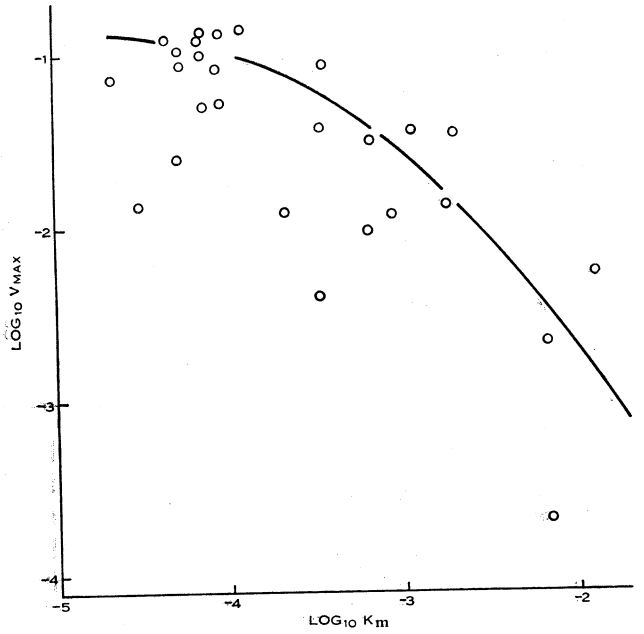


Fig. 11.—Relationship between the Michaelis constant (K_m) and relative V_{max} (proportional to k_3) for substrates of *S. atra* β-glucosidase. The expected relationship from the equation $K_m \simeq k_3/k_1$ is that V_{max} should increase as K_m increases, i.e. the trend line should lie from lower left to upper right. Each point gives the values (taken from Table 6) of K_m and relative V_{max} for a single substrate.

The effect of 2,6-dimethyl and 2-iodo-6-methyl substitution can scarcely be due to other than steric causes, and this behaviour has been generally, but not so strongly, observed by other workers (Helferich and Scheiber 1934; Veibel and Shin-Lin Yang 1952). The *ortho* effect is also visible in the much smaller efficiency of *N-o*-tolyl-β-D-glucosylamine as a competitive inhibitor than the *p*-tolyl derivative.

In agreement with the conclusions set out by Veibel (1950) for a number of β-glucosidases the *S. atra* β-glucosidase hydrolyses *sec.*-alkyl-β-glucosides more rapidly than *prim.*- and *tert.*-alkyl-β-glucosides. This behaviour is shown not

only in a lower K_m but apparently in a higher V_{max}. The *prim.*- and *tert.*-alkyl-β-glucosides are in fact unsatisfactory substrates for study with the *S. atra* β-glucosidase since secondary effects interfere with any attempt to determine the Michaelis constant.

It will be seen that the specificities of the *S. atra* β-glucosidase represent no major departure from those recorded in the literature for related enzymes. For one departure from expected behaviour, however, no explanation can be offered at present. This is the non-equivalence of K_m for most of the phenolic β-glucosides with K_i for their competitive inhibition of the hydrolysis of *p*-nitrophenyl-β-glucoside (Table 6). These glucosides apparently fulfil the criteria for competitive inhibition (linear Lineweaver-Burk plots tending to a constant value of $1/V_{max}$, constant K_i with changes in $[I]$, and give linear Lineweaver-Burk plots when their K_m is determined directly. It is apparent that one or both of K_m and K_i are not true dissociation constants.

One conclusion that can be drawn from the observations made on inhibitors of the *S. atra* β-glucosidase is that decisions about enzyme specificity made after the study of competitive inhibition by the techniques reviewed by Levvy and Marsh (1954) need to be treated with some caution. Thus no substrate of the enzyme failed to act as a competitive inhibitor, but competitive inhibitors are not necessarily substrates (e.g. β-xylosides). Glucose is a competitive inhibitor and β-glucosides are substrates of the enzyme but, although mannose is an almost equally effective competitive inhibitor, neither α- nor β-mannosides are substrates.

IX. Acknowledgments

The author is indebted to Misses S. Plaisted and M. Dunne for technical assistance.

X. References

Conchie, J. (1954).—*Biochem. J.* **58**: 552.
Crane, R. K., and Sols, A. (1954).—*J. Biol. Chem.* **210**: 596.
Ezaki, S. (1940).—*J. Biochem., Tokyo* **32**: 91.
Gottschalk, A. (1950).—*Advanc. Carbohyd. Chem.* **5**: 49.
de Grandchamp-Chaudun, A., and Moreau, M. T. (1953).—*Bull. Soc. Chim. Biol., Paris.* **35**: 743.
Gross, M. (1954).—*Arch. Biochem.* **53**: 119.
Hattori, S., and Kurihara, Y. (1950).—*Science (Japan)* **20**: 571.
Helferich, B., and Scheiber, H. E. (1934).—*Hoppe-Seyl. Z.* **226**: 272.
Hestrin, S., and Lindegren, C. C. (1950).—*Nature* **165**: 158.
Hodgson, R., Rijker, A. J., and Peterson, W. H. (1945).—*J. Biol. Chem.* **158**: 89.
Horikoshi, K. (1942).—*J. Biochem., Tokyo* **35**: 39.
Jermyn, M. A. (1952).—*Aust. J. Sci. Res.* B **5**: 433.
Jermyn, M. A. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 541.
Jermyn, M. A. (1955*b*).—*Aust. J. Biol. Sci.* **8**: 563.
Jermyn, M. A. (1955*c*).—*Aust. J. Chem.* **8**: 403.
Koshland, D. E. (1953).—*Biol. Rev.* **28**: 416.
Koshland, D. E. (1954).—"Symposium on the Mechanism of Enzyme Action." p. 608. (Ed. W. D. McElroy and H. B. Glass.) (Johns Hopkins Press: Baltimore.)
Koshland, D. E., and Stein, S. S. (1954).—*J. Biol. Chem.* **208**: 139.

LEDERBERG, J. (1950).—*J. Bact.* **60**: 381.
LEVVY, G. A., and MARSH, C. A. (1952).—*Biochem. J.* **52**: 690.
LEVVY, G. A., and MARSH, C. A. (1954).—*Science* **119**: 337.
LINEWEAVER, H., and BURK, D. (1934).—*J. Amer. Chem. Soc.* **56**: 658.
MASSART, L. (1950).—"The Enzymes, Chemistry and Mechanism of Action." Vol. I, Part I. Ch. 7. (Ed. J. B. Sumner and K. Myrbäck.) (Academic Press Inc.: New York.)
MIWA, T. (1932).—*Acta Phytochim., Tokyo* **6**: 154.
MIWA, T., and TANAKA, K. (1949).—*Symp. Enzyme Chem., (Japan)* **2**: 19.
MIWA, T., CHENG, C., FUJISAKI, M., and TOISHI, A. (1937).—*Acta Phytochim., Tokyo* **10**: 155.
MOORE, S., and LINK, K. P. (1940).—*J. Biol. Chem.* **133**: 293.
MORITA, Y. (1952).—*J. Japan Biochem. Soc.* **24**: 189.
NELSON, N. (1944).—*J. Biol. Chem.* **153**: 375.
NISHIZAWA, K. (1942).—*Sci. Rep. Tokyo Bunrika Daig.* **6**: 43.
NISHIZAWA, K. (1951).—*J. Fac. Text. Sericult. Shinshu Univ.* **1**: 213.
NISHIZAWA, K., and WAKABAYASHI, K. (1951).—*Symp. Enzyme Chem. (Japan)* **6**: 26.
NIWA, K. (1951).—*J. Biochem., Tokyo* **38**: 109.
PIGMAN, W. W. (1944).—*Advances in Enzymology* **4**: 41.
SOLS, A., and CRANE, R. K. (1954).—*J. Biol. Chem.* **210**: 581.
TSOU, K. C., and SELIGMAN, A. M. (1952).—*J. Amer. Chem. Soc.* **74**: 5605.
VEIBEL, S. (1950).—"The Enzymes, Chemistry and Mechanism of Action." Vol. I, Part I, Ch. 16, 17. (Ed. J. B. Sumner and K. Myrbäck.) (Academic Press Inc.: New York.)
VEIBEL, S., and ERIKSEN, E. (1937).—*K. Danske Vidensk. Selsk., Mat.-fys. Medd.* **14**, No. 15.
VEIBEL, S., and LILLELUND, H. (1940).—*K. Danske Vidensk. Selsk., Mat.-fys. Medd.* **17**, No. 6.
VEIBEL, S., and SHIH-LIN YANG (1952).—*Acta Chim. Scand.* **6**: 1366.
WILLSTATTER, R., and KUHN, R. (1924).—*Hoppe-Seyl. Z.* **129**: 33.