FUNGAL CELLULASES

XV.* ACCEPTOR SPECIFICITY OF THE ARYL β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

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[*Manuscript received March* 31, 1966]

Summary

A parameter has been devised that gives a general measure of acceptor efficiency in the reaction of an enzyme with two substrates. The numerical value of this parameter has been determined for the β-glucosidase of *Stachybotrys atra*, phenyl β-D-glucopyranoside, and a large number of hydroxylic acceptors. There are certain correlations between acceptor efficiency and structure.

I. INTRODUCTION

Those glycosidases that transfer the glycosyl residue to an acceptor with retention of the configuration about the anomeric carbon atom may be taken as "typical". Although "atypical" glycosidases which give rise to products that have inverted configurations or are unsaturated may share with the typical enzymes many common elements in mechanism, not enough is yet known about the nature and function of active centres in either class to make this more than a speculation. The observations recorded in this and the following papers are therefore only directly relevant to the problem of the mechanism of typical glycosidases. The latter enzymes and those of the protease–esterase group both catalyse (for hydroxylic acceptors) reactions of the type

$$\mathrm{XOR}+\mathrm{R'OH} \rightleftharpoons \mathrm{XOR'}+\mathrm{ROH}.$$

The crucial difference in mechanism appears to be that the protease–esterase reaction proceeds in two steps,

$$\mathrm{enzymeOH}+\mathrm{XOR} \rightarrow \mathrm{enzymeOX}+\mathrm{ROH}$$
$$\mathrm{enzymeOX}+\mathrm{R'OH} \rightarrow \mathrm{enzymeOH}+\mathrm{XOR'}$$

but that formation of enzymeOGly by glycosidases appears to occur as only a transient, perhaps as only a virtual, stage that is part of a single-step mechanism. This mechanism involves the decomposition of a ternary complex in which GlyOR and R'OH are simultaneously bound to the enzyme. No mechanism has yet been postulated, however, for glycosidase action that does not include such a virtual intermediate, implied as it is by the necessity of a double Walden inversion to restore the configuration about the anomeric carbon.

* Part XIV, *Aust. J. Biol. Sci.*, 1966, **19**, 715–17.

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The best-known and most easily purified proteases have been studied exhaustively. The sequence of residues in their peptide chains and much of the geography and chemistry of their active centres are known. The advantage in clarity that this gives can be seen by comparing such relatively sophisticated expositions of mechanisms as that of Bender and Kezdy (1964) for chymotrypsin and that of Ebert and Stricker (1964) for dextransucrase. In the first case defined bonds can be made to definite residues; in the second case complex thermodynamic data obtained by ingenious experiments must still be interpreted by full or broken lines pointing towards conveniently spaced functional groups on the surface of an undefined enzymic "plum pudding".

For protease–esterases, direct evidence for the postulated mechanism is therefore possible in favourable circumstances, and a number of acyl–enzyme intermediates have in fact been isolated and the nature and position of the acylated group determined. For glycosidases the evidence is all indirect, in the sense that observations have been made that are incompatible with the two-step hypothesis. Thus the nature of the glycoside may determine the point of substitution in a polyhydroxylic acceptor (Miwa *et al.* 1956), or the ratio of the products when two competing acceptors are present (Jermyn 1962*b*). The idea of a ternary complex is itself susceptible to further analysis, and Ebert and Stricker (1964) have applied such an analysis to dextransucrase. Here, the highly anomalous kinetics of a complex situation with several competing processes involving a number of molecular species, each of which can serve as both donor and acceptor, can be simply interpreted in terms of the rates at which various bonds are formed and broken in a ternary complex.

Glycosidases thus seem to be only a special case of the class of "two-substrate" enzymes. The glycoside is only one of the substrates, certainly not *the* substrate; the acceptor is the other. Although much work has been carried out on the specificity of the reaction between enzymes and glycosides, little is known, on the other hand, about the specificity of the reaction between enzymes and acceptors. This little has usually taken the form of measurement of the relative transfer to water and acceptor at one or two concentrations of a few acceptors under fixed conditions. The β-glucosidase of *Stachybotrys atra* is very suitable for a more detailed analysis of acceptor specificity since certain complicating factors such as transfer of glucosyl residue to substrate glucoside, or product glucose and glucoside, or the hydrolysis of the product glucoside are absent or quantitatively negligible.

An additional reason for the study was the suspicion that the hydrolytic action of the enzyme may not be a true indication of the role it plays in the integrated metabolism of the mould. The enzymic synthesis of a disaccharide by the transfer of a glycosyl residue to a glycose and the enzymic hydrolysis of a disaccharide are formally identical. Since the latter process is frequently highly specific for both moieties of the disaccharide, a sugar appeared to be the most likely candidate for the role of "true acceptor" of the β-glucosidase. The site at the active centre that binds the acceptor would then be expected to display a "true" specificity as narrow as that for the donor (Jermyn 1955*b*). The observed functioning of water and alcohols as acceptors would be no more relevant that the ability of all sorts of

hydroxylic molecules at high enough concentrations to bind to the donor site, although this site is actually narrowly specific for aryl β-D-glucopyranosides.

This paper records a qualitative investigation into the specificity of the β-glucosidase of *S. atra* for a wide range of acceptors; the following papers outline details of the investigation of the kinetics of suitable examples from the various classes of acceptors that could be distinguished. Since some of these acceptors appear to show a non-specific effect on the enzyme protein in their character as solutes, the behaviour of a related non-hydroxylic solute was studied. Many of the most effective acceptors are polyhydroxylic and quantitative data on their behaviour can only be assessed after the identification of the site(s) in the molecule to which the glucosyl residue is transferred. Substitution in the glycitols, the mono-β-D-glucopyranosides of which were wanted for another purpose (Jermyn 1965), has therefore been examined. Interpretations previously given of earlier observations in terms of enzymic mechanisms are now seriously out of date; this series of papers attempts to synthesize what is known of the mode of action of the β-glucosidase of *S. atra* into a new and more satisfactory picture.

II. Theoretical Background and Definitions

Any substance that acts as an acceptor for the β-glucosidase does so in the presence of an inevitable second competing acceptor, water. The scheme that covers the various reactions taking place (Jermyn 1962*a*) may be written (PhOGlu = aryl glucoside):

$$
\begin{array}{lllll}
 & & \overset{k_4}{\underset{k_{-4}}{\rightleftharpoons}} & & \\
 & +\mathrm{PhOGlu} & & \mathrm{E.PhOGlu.H_2O} & \\
 & & \overset{k_5}{\underset{k_{-5}}{\rightleftharpoons}} & & \\
 & +\mathrm{H_2O} & & & \\
 & & \overset{k_6}{\underset{k_{-6}}{\rightleftharpoons}} & & \\
 & +\mathrm{ROH} & & & \\
 & & \overset{k_7}{\underset{k_{-7}}{\rightleftharpoons}} & & \\
 & +\mathrm{PhOGlu} & & &
\end{array}
\qquad
\begin{array}{lll}
\overset{k_8}{\underset{k_{-8}}{\rightleftharpoons}} & & \overset{k_{12}}{\underset{k_{-12}}{\rightleftharpoons}} \\
\mathrm{PhOH} & +\mathrm{E.GluOH} & \mathrm{GluOH} \\
\overset{k_9}{\underset{k_{-9}}{\rightleftharpoons}} & & \\
\mathrm{GluOH} & & \\
\overset{k_{10}}{\underset{k_{-10}}{\rightleftharpoons}} & & \\
\mathrm{GluOR} & & \\
\overset{k_{11}}{\underset{k_{-11}}{\rightleftharpoons}} & & \overset{k_{14}}{\underset{k_{-14}}{\rightleftharpoons}} \\
\mathrm{PhOH} & +\mathrm{E\cdot GluOR} & \mathrm{GluOR}
\end{array}
$$

$$
\mathrm{E}+\mathrm{H_2O} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} \mathrm{E.H_2O}
$$

$$
\mathrm{E}+\mathrm{PhOGlu} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \mathrm{E.PhOGlu}
$$

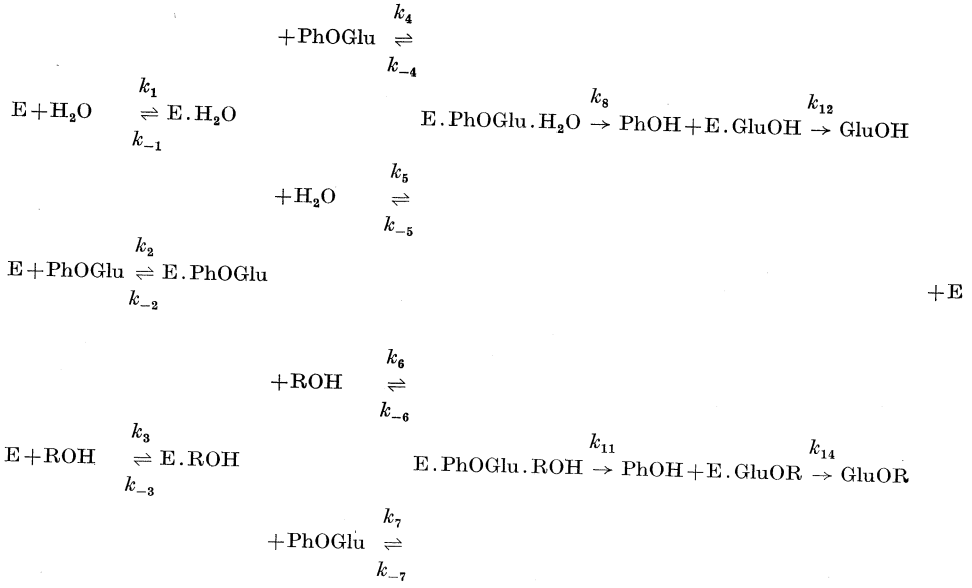
$$
\mathrm{E}+\mathrm{ROH} \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \mathrm{E.ROH}
$$

$$
\mathrm{E.PhOGlu.ROH}
$$

$$
+\mathrm{E.PhOH} \overset{k_{13}}{\underset{k_{-13}}{\rightleftharpoons}} \mathrm{PhOH} + \mathrm{E}
$$

We cannot tell from kinetic data anything about events within the Michaelis complex (Cleland 1963) and hence such transformations as E.PhOGlu.ROH ⇌ E.PhOH.ROGlu are excluded from the scheme. At the concentrations used, [PhOGlu] ≃ 10^{-3}M, and a degree of reaction not exceeding 1–10%, the resulting [GluOR] and [GluOH] ≃ 10^{-5}–10^{-4}M, and the binding of these species to the enzyme

is negligible; in addition, all observations on the enzyme suggest that the affinity for PhOH is zero, so that all paths involving the species E.PhOH may be eliminated from discussion (Jermyn 1962*a*). The assumptions are, of course, far from valid for other glycosidases, but within their ambit the scheme reduces to:



The mathematics of such a scheme have been developed elsewhere (Jermyn 1962*a*), but certain of its consequences may be deduced by qualitative reasoning. Let us define T_{50} as the concentration of added acceptor at which 50% of the glucosyl residue is transferred to this added acceptor and 50% to water. T_{75} and T_{25} etc. follow obviously and the definition can be extended to any transferring enzyme. If t is the fraction of transfer to the added acceptor, then T_{50} is the concentration of added acceptor at which $t/(1-t) = 1$. It is apparent that two separate comparisons are confounded in T_{50}—the partition of E between E.GluOPh.ROH and E.GluOPh.H_2O in the steady state, and the relative rates of decomposition of the two complexes. Nevertheless it gives a measure of the "efficiency" of any given acceptor and no acceptor will be specific for which T_{50} is not very low. It is thus a useful parameter for a general survey of acceptors. In comparing adjacent members of homologous series it will give fairly close estimates of relative affinity; thus it will be justifiable to compare methanol, ethanol, and n-propanol, but not, say, methanol and sucrose.

No useful meaning can be given to T_{50} unless the fraction of transfer, when the concentration of the added acceptor is fixed (that of water being fixed by circumstances) is independent of the concentration of the donor, $[D]$. Otherwise the fraction of transfer will be a constantly changing quantity during an experiment, as donor is used up, and will depend also on the initial concentration of donor. A different value of T_{50} will thus be deduced from the results of different experiments.

The formula that expresses $t/(1-t)$ in terms of $[D]$ that emerges from the mathematical analysis (Jermyn 1962*a*) may be simplified to a power series of the form

$$a_0+a_1[D]^{-1}+a_2[D]^{-2}+\ldots,$$

with the net conclusion that $t/(1-t)$ will become visibly dependent on $[D]$ only at low concentrations of donor, the concentration range involved depending on the value of the constants a_1, a_2, . . . etc. In any range where the value of $t/(1-t)$ for any acceptor concentration is independent of $[D]$, the value of T_{50} will be independent of $[D]$. Qualitatively, dependence of T_{50} on $[D]$ will occur only when [PhOGlu] is so low that the enzyme is essentially present only as $E.H_2O$ or E.ROH and the relative affinity of PhOGlu for the two complexes $[(k_4+k_8)/k_{-4}$ as against $(k_7+k_{11})/k_{-7}]$ controls the kinetics.

Empirically, it has been found that for certain well-studied acceptors (ethanol, t-butyl alcohol, hexane-1,6-diol), the values of T_{50} deduced under standard conditions, with the usual (2×10^{-3}M) initial concentration of donor phenyl β-D-glucopyranoside, are constant within experimental error, although no attempt was made to standardize the amount of phenyl β-glucoside broken down in any experimental series. A deliberate experiment was set up with the acceptor methanol (concn. 1M) and phenyl β-glucoside at initial concentrations over the range 10^{-1}–10^{-4}M. From these mixtures an approximately constant amount of the β-glucoside, equivalent to a concentration of 5×10^{-5}M (0·05–50% of the total glucoside present), was removed enzymically. No significant variation in the value of t could be detected.

The expression for the dependence of $t/(1-t)$ on the concentration of added acceptor is complex and involves a large number of constants (Jermyn 1962*a*, 1962*b*); the problem of determining T_{50} empirically may be simplified by the observation that the relation between t and c (the concentration of added acceptor) takes a generalized sigmoid form. If it were a true sigmoid curve, the equation

$$\log[t/(1-t)] = K_1 \log c+K_2$$

would hold. As Figure 1 shows, the relationship between $\log[t/(1-t)]$ and $\log c$ is near enough to linear for a number of acceptors for a straight line to be drawn through the experimental points and T_{50} determined as the concentration value at which this line cuts $\log[t/(1-t)] = 0$. Provided that $15\% < t < 85\%$, approximately, the linear relationship holds well; beyond these points deviations become marked as the equations in Jermyn (1962*a*) suggest that they will at the ends of the range. Where low solubility or inefficiency of an acceptor has meant that t must be determined at acceptor concentrations well away from T_{50}, the resultant extrapolations are therefore highly uncertain and the derived T_{50} values are no more than an indication of the "true" values. Apart from theoretical considerations, the value of $t/(1-t)$ is obviously greatly affected by small experimental errors.

The effective concentration of water is 55·6M; an acceptor with $T_{50} = 10^{-3}$M is thus $5\cdot6\times10^4$ times as efficient as water, one with $T_{50} = 1$M is 55 times as effective, and one with $T_{50} = 10^3$M is 5×10^{-2} times as effective. The concentration value in the last example, the calculation of which moreover assumes that the enzyme is functioning in an aqueous environment, is obviously fictitious. T_{50} in such cases

can be regarded only as a parameter of the reaction, giving a measure of acceptor efficiency, to which no physical reality is to be ascribed.

The effect of added acceptor on overall enzyme kinetics may also be described in a qualitative fashion. Figure 2 illustrates the expected results in terms of Lineweaver–Burk plots.

Although mathematical investigation shows that Lineweaver–Burk plots are not necessarily linear when both of two competing reactions are taking place to a significant degree, no departures from linearity have been observed in this study; certainly none such that the known degree of experimental error would justify any other procedure than drawing a straight line through the experimental points. The range of glucoside concentrations covered was probably not sufficient to produce noticeable effects.

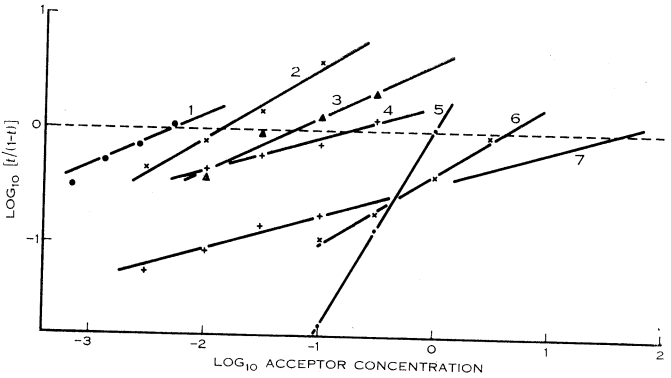


Fig. 1.—Relation between t, the fraction of transfer, and acceptor concentration for a variety of acceptors chosen to cover the range. Donor, phenyl β-D-glucopyranoside, initially at 4×10^{-3}M; pH 5·0 and 28°C. *1*, decane-1,10-diol; *2*, pentan-1-ol; *3*, 2,2-dimethylpropan-1-ol; *4*, 2-methylbutan-2-ol; *5*, methyl β-DL-arabinoside; *6*, DL-butane-2,3-diol; *7*, hexan-2-ol.

III. Materials and Methods

(*a*) *General Considerations*

Reducing sugar was measured by the Somogyi–Nelson method (Nelson 1944), and phenol according to a modified version of the method of Folin and Ciocalteau (1927). For the survey experiment set out in Table 1, commercial samples of the acceptors were used as received, unless the blanks in either the sugar or phenol determinations were above the tolerable limit. In this case liquids were redistilled and solids recrystallized to give an acceptable product.

Butan-2-ol was resolved according to Kantor and Hauser (1953), and pentan-2-ol according to Pickard and Kenyon (1911). Commercial inactive butane-2,3-diol was used to prepare *meso*-butane-2,3-diol and DL-butane-2,3-diol according to Wilson and Lucas (1936), and commercial cyclohexane-1,4-diol to prepare the *cis* and *trans* isomers according to Perrine and White (1947). Many of the sugar derivatives were samples prepared previously in this laboratory for other purposes. All relevant details about the acceptors are noted in Table 1.

Phenyl β-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside were laboratory preparations; salicin was a commercial (Fluka) product. All of them gave no detectable reaction in solution up to concentrations of 10^{-2}M for reducing sugar; phenyl β-D-glucopyranoside and salicin were free of detectable phenols.

The aryl β-glucosidase of *S. atra* liberates equimolecular amounts of phenols and glucose from aryl β-D-glucopyranosides at concentrations of the latter below 0·1M, a level much above the working concentrations ($1–4 \times 10^{-3}$M) used in this study.

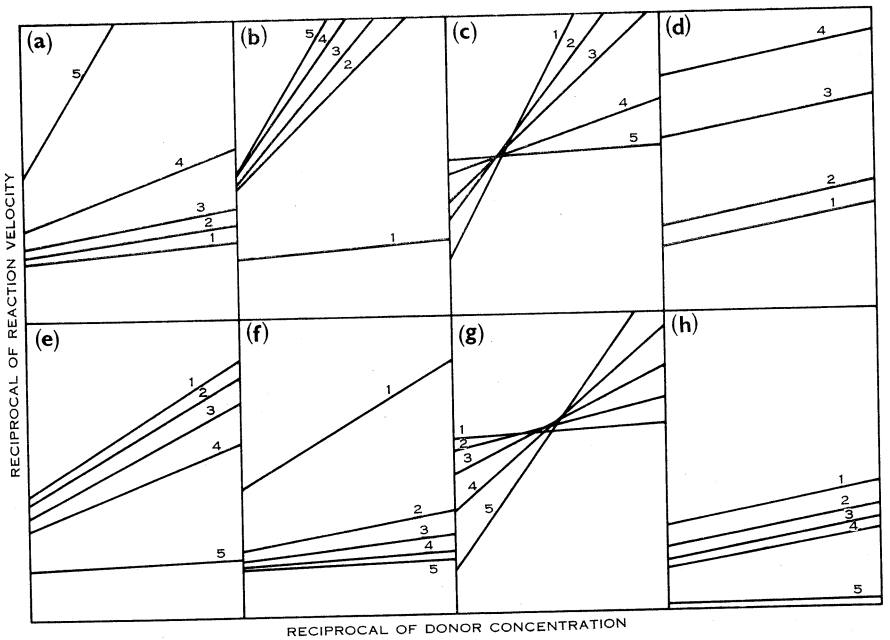


Fig. 2.—How various relations between the kinetic constants will alter the effect of added acceptor on Lineweaver–Burk plots. Throughout, *1* = no added acceptor; *2,3,4* = low, medium, high concentrations of added acceptor; *5* = theoretical reaction with acceptor only. *a,b,c,d*: $k_8 > k_{11}$. *a,b*: $K_m[ROH] > K_m[H_2O]$ with steady-state favouring E.GluOPh.H_2O in *a* and E GluOPh.ROH in *b*. *c*: $K_m[ROH] < K_m[H_2O]$. *d*: $k_{11} = 0$; the series of parallel lines are diagnostic for "anti-competitive" inhibition, i.e. competitive inhibition of that substrate the breakdown of which is *not* being measured. *e,f,g,h*: $k_{11} > k_8$. *e,f*: $K_m[ROH] < K_m[H_2O]$ with steady-state favouring E.GluOPh.H_2O in *e* and E.GluOPh.ROH in *f*. *g*: $K_m[ROH] > K_m[H_2O]$. *h*: $k_{11} \gg k_8$; the series of parallel lines are typical of the situation with low concentrations of very efficient acceptors—"anti-competitive activation".

This point has been repeatedly checked in the past (cf. Jermyn 1962*b*); however, since preparations that have not been stringently purified contain carbohydrates that may be potential acceptors (Jermyn 1962*c*), this point has been rechecked for each new batch of enzyme used in this study. The same finding has been made on each occasion. In view of this result, the source of enzyme that has been used, without further purification, is a dialysed and lyophilized preparation of the medium into which enzyme had been secreted after phenyl β-D-thioglucopyranoside induction of washed mycelium (Jermyn 1965). This material is in any case much freer of extraneous polymers than non-induced enzyme preparations from growing cultures.

TABLE 1

VALUES OF T_{50} FOR A VARIETY OF ACCEPTORS

Donor phenyl β-D-glucopyranoside at pH 5·0 (McIlvaine citrate–phosphate buffer at final dilution of 1 in 50) and 28°C. Acyclic acceptors are listed in order of increasing chain length; acceptors with heteroatoms are assimilated to this arrangement by using the "oxa-" and "thia-" nomenclature. More usual names and any other necessary comments are also given

No.	Name	Synonym or Comments	T_{50} (10^{-3}M = 1)	No.	Name	Synonym or Comments	T_{50} (10^{-3}M = 1)
			Acyclic Compounds				
1	Methanol		1,100	32	DL-3-Methylbutan-2-ol		27
2	Ethane-1,2-diol		240	33	Pentane-1,2,3,4,5-pentol	D-Arabitol	210
3	Ethanol		580	34		L-Arabitol	350
4	Propane-1,2,3-triol	Glycerol	170	35		Xylitol	290
5	Propane-1,3-diol		100	36		Adonitol	100
6	2,2-Dimethylpropane-1,3-diol		31	37	Pentane-1,5-diol		10
7	2,2-Di(hydroxymethyl)propane-1,3-diol	Pentaerythritol	4·3	38	3-Oxapentane-1,5-diol	Diglycol	410
				39	3-Thiapentane-1,5-diol	Thiodiglycol	10
8	2-Hydroxymethyl-2-aminomethylpropane-1,3-diol	Tris, self-buffered at pH 5·4	42	40	Pentan-1-ol		14
				41	3-Oxapentan-1-ol	Ethyl cellosolve	240
9	DL-Propane-1,2-diol		55	42	4-Oxo-3-Oxapentan-1-ol	Ethylene glycol monoacetate	170
10	Propan-1-ol		320				
11	2-Methylpropan-1-ol	Isobutyl alcohol	150	43	DL-Pentan-2-ol		380
12	2,2-Dimethylpropan-1-ol	Neopentyl alcohol	55	44	D-Pentan-2-ol	99% D-isomer	220
13	Propan-2-ol		480	45	"L"-Pentan-2-ol	86% L-isomer	87
14	2-Methylpropan-2-ol	t-Butyl alcohol	19,000	46	Pentan-3-ol		260
15	*meso*-Butane-1,2,3,4-tetrol	Erythritol	140	47	Hexane-1,2,3,4,5,6-hexol	D-Mannitol	3,500
16	DL-Butane-1,2,4-triol		79	48		Dulcitol; D-glucitol	480
17	*meso*-Butane-2,3-diol		110	49		Sorbitol; galactitol	300
18	DL-Butane-2,3-diol		4,000	50	Hexane-1,6-diol		8·7
19	L-Butane-2,3-diol		470	51	Hexan-1-ol		12
20	2,3-Dimethylbutane-2,3-diol	Pinacol	160,000	52	DL-Hexan-2-ol		34
21	Butane-1,4-diol		90	53	"L"-3-Oxo-4-oxahexan-2-ol	Commercial ethyl lactate	58
22	DL-Butane-1,3-diol		110				
23	Butan-1-ol		74	54	Heptane-1,7-diol		12
24	2-Oxo-3-oxabutan-1-ol	Methyl glycollate	300	55	Heptan-1-ol		33
25	3-Oxabutan-1-ol	Methyl cellosolve	1,000	56	3-Oxaheptan-1-ol	Butyl cellosolve	73
26	DL-2-Methylbutan-1-ol		26	57	Octan-1-ol		12
27	L-2-Methylbutan-1-ol		3·4	58	Nonane-1,9-diol		9·1
28	3-Methylbutan-1-ol	Isoamyl alcohol	65	59	Decane-1,10-diol		5·0
29	DL-Butan-2-ol		1,300	60	Carbowax 1500 [$HO(CH_2CH_2O)_nCH_2CH_2OH$]	Polyethyleneglycol, chain length = 100	23
30	D-Butan-2-ol	98% D-isomer	4,400				
31	2-Methylbutan-2-ol	t-Amyl alcohol	170				

TABLE 1 (*Continued*)

No.	Name	Synonym or Comments	T_{50} (10^{-3}M = 1)	No.	Name	Synonym or Comments	T_{50} (10^{-3}M = 1)
			Cyclic Compounds				
61	Benzyl alcohol		46	65	Cyclohexane-1,4-diol	Commercial mixed isomers	140
62	Cyclopentanol		43				
63	Cyclohexanehexol (one isomer)	myoInositol	220	66	*trans*-Cyclohexane-1,4-diol		220
64	*trans*-Cyclohexane-1,2-diol		89	67	*cis*-Cyclohexane-1,4-diol		110
				68	Cyclohexanol		110
			Sugars and Related Compounds				
69	Methyl β-D-arabinopyranoside		42,000	80	Potassium D-galactonate		54
70	Methyl β-DL-arabinopyranoside	Equimolecular mixture of both isomers		81	Dipotassium mucate		830
				82	D-Galactono-γ-lactone		No transfer
			1,300	83	Methyl α-D-galactopyranoside		87
71	Methyl β-L-arabinopyranoside		260	84	*p*-Nitrophenyl β-D-thiogalacto-pyranoside		120
72	Potassium D-arabonate		No transfer				No transfer
73	D-Arabono-γ-lactone		36	85	Methyl α-D-mannopyranoside		No transfer
74	Potassium L-arabonate		No transfer	86	Benzyl β-D-fructopyranoside		56
75	Potassium D-xylonate		83	87	D-*Glycero*-D-*gulo*-heptonolactone		No transfer
76	Potassium D-gluconate		100	88	Trehalose		360
77	Potassium hydrogen D-saccharate		100	89	Potassium lactobionate		130
78	Methyl α-D-glucopyranoside		100	90	Melezitose		
79	Phenyl β-D-thioglucopyranoside		78				

The basic technique in transfer experiments was as follows. Phenyl β-D-glucopyranoside (10 mg) was dissolved in a solution (9 ml) containing water, acceptor, and buffer and equilibrated at the working temperature. At τ_0, temperature-equilibrated enzyme solution (1 ml) was added and the reaction allowed to proceed for a predetermined time that depended on the enzyme preparation used and the effect, if any, of the acceptor on overall enzyme activity, but was calculated to allow about 10% decomposition of the phenyl β-glucoside. At τ_0 and τ_x duplicate samples for each determination were pipetted into either the Folin–Ciocalteau or copper reagents, both of which immediately destroy enzyme activity; the rest of the procedure is described elsewhere (Jermyn 1962*b*). A group of experiments with different concentrations of the same acceptor was usually run simultaneously, together with a control without added acceptor, and a blank with the highest concentration of acceptor to make allowances for any reactive impurities present in the acceptor sample. Occasional small alterations in this procedure were necessary to achieve specific objects, but they were in all cases sufficiently obvious not to need to be set out in detail.

The fraction of transfer is given by

$$1-\frac{\text{moles of glucose liberated}}{\text{moles of phenol liberated}},$$

since glucosyl residues transferred to form alkyl glucoside are not determined as reducing sugar, like those transferred to water. In practice, small variations in standards and reagents displace the control value of the ratio of liberated glucose to liberated phenol a little from unity in any given set of experiments. Provided this ratio was within the limits of $1{\cdot}00\pm0{\cdot}03$, the experiments were not rejected, and the observed control value of the ratio was used as a multiplier to correct the values of the ratio obtained in the presence of acceptor.

p-Nitrophenyl β-D-glucopyranoside, although it is the most convenient substrate for measuring the concentration and general properties of enzyme solutions, cannot be used with any accuracy in experiments where glucose is to be determined by alkaline copper reagents. The glucose determinations become quite erratic, depending strongly on the heating schedule. The reduction of copper and of the aromatic nitro group are both possible reactions for glucose in hot alkaline solutions, and under the conditions of the determination they are apparently competitive. This substrate has therefore only been used in these studies for cases in which a small amount of potential acceptor was available. This was then tested under the normal conditions for estimating the Michaelis constant for *p*-nitrophenyl β-glucosidase activity, and its pattern of reaction with the enzyme interpreted by means of Lineweaver–Burk plots (cf. Fig. 2).

Similar considerations prevent the testing of reducing sugars as acceptors; they have been replaced in this study, not perhaps entirely homologously, by their simple glycosides. Nor can the most efficient acceptor discovered, pentaerythritol, be used in more precise kinetic studies because of its slow reaction with the Folin–Ciocalteau reagent. This behaviour is not a consequence of impurities in the pentaerythritol, but appears to be due to slow reversal of the normal method of synthesis of the molecule, under the conditions of the determination, to give formaldehyde.

A more serious limitation is imposed by the fact that an appreciable fraction of any acceptor with a low enough value of T_{50} will be used up during the experiment if this is carried out using acceptor concentrations comparable with T_{50}. For phenyl β-glucoside the lowest values of T_{50} found ($\simeq 4\times10^{-3}$M) are just within the range where errors are not too great. For salicin, the values marked "undeterminable" in Table 2, represent in fact a T_{50} so low that significant partial transfer to both acceptor and water takes place only at acceptor concentrations ($1–5\times10^{-4}$M) where the acceptor is rapidly used up and apparent transfer fractions are continually altering. Such systems will require another type of experimental approach altogether in which true initial rates can be measured, presumably by continuous spectrophotometry. A search for alkyl glucosides with a markedly different ultraviolet absorption spectrum from the parent alcohols has so far yielded disappointing results.

TABLE 2

COMPARISON OF THE VALUES OF T_{50} FOR THE TWO DONORS, PHENYL β-D-GLUCOPYRANOSIDE AND SALICIN, AT 28°C AND pH 5·0, AND CERTAIN SELECTED ACCEPTORS

Acceptor	T_{50} for Phenyl β-D-Glucopyranoside (10^{-3}M = 1)	T_{50} for Salicin (10^{-3}M = 1)
Methanol	1100	210
Ethanol	580	200
Propan-1-ol	320	63
Butan-1-ol	74	6·3
3-Methylbutan-1-ol	65	4·2
Pentan-1,5-diol	10	Undeterminable
Pentaerythritol	4·3	Undeterminable

IV. Results and Discussion

(a) T_{50} Values

Table 1 presents a comprehensive table of values of T_{50} using phenyl β-D-glucopyranoside as the donor at 28°C and pH 5·0. The principle of arrangement used in Table 1 is not the only one that could have been used, and not all cross-relationships can be covered in a finite series of tables.

The limit to the substances that can be investigated is set by solubility; the data of Butler, Thomson, and Maclennan (1933) and Ekwall, Daniellson, and Henrikson (1952), used to calculate the molarity of octan-1-ol and decane-1,10-diol solutions, can be extrapolated to give approximate solubilities at 20°C of $1·2\times10^{-3}$M for nonan-1-ol and $1·5\times10^{-3}$M for undecane-1,11-diol against limiting solubility and T_{50} of 4·5 and 12×10^{-3}M for octan-1-ol and 5·7 and $5·3\times10^{-3}$M for decane-1,10-diol.

It appears from the data of Table 1 that, upon an overall tendency towards a lower value of T_{50} with increasing chain length for alkan-1-ols and n-alkane-1, ω-diols, is superimposed a much more specific interaction which is maximal at a

chain length of 5–6 carbon atoms. The observed value of T_{50} may be the result of two interactions, a general one with the enzyme as a whole and a secondary one with a specific site. This observation may be joined by the further deductions that a primary hydroxyl group, a compact molecule that does not sterically hinder approach to this hydroxyl group, and the L-configuration favour a low value of T_{50}. The resulting prediction is that minimum values of T_{50} should be found for the L-isomers of such hexanols as 2,3-dimethylbutan-1-ol, 2-methylpentan-1-ol, and 3-methylpentan-1-ol.

There is no evidence from the table that polyhydroxylic compounds, and specifically sugars, are necessarily more efficient acceptors. However, the variation in acceptor efficiency amongst compounds alike structurally but not sterically is so great as to suggest that steric factors may play an overriding part in the availability of these relatively rigid molecules as acceptors; the variation between methyl

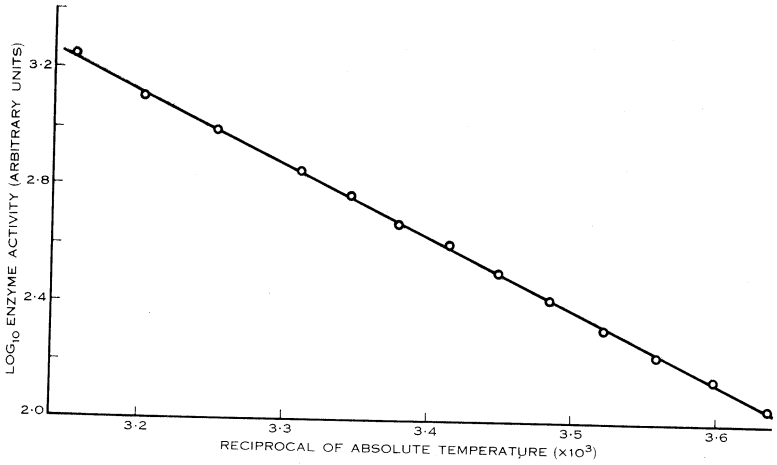


Fig. 3.—Enzymic breakdown of 10^{-3}M *p*-nitrophenyl β-D-glucopyranoside at pH 5·0 in McIlvaine (citric acid–sodium phosphate) buffer at pH 5·0 in the presence of 0·5M pentaerythritol over the range 2–45°C.

D- and L-arabinopyranosides or between methyl D-manno- and D-galactopyranosides is quite striking. If sugars are the "natural" acceptors such differences may be quite enough to regulate the type of syntheses performed by the enzyme without calling for high specificity for the acceptor.

Table 2 illustrates the fact that the values of T_{50} for selected acceptors vary with the nature of the donor. The dependence of degree of transfer on the nature of the donor has already been adequately established (Jermyn 1962*b*). Table 2 re-expresses these observations in terms of a new parameter.

(*b*) *Activation Energy of the Transfer Reaction*

Before any discussion of reaction mechanisms is attempted, it is necessary to be sure that a single mechanism is in fact operating over the temperature range studied. The most sensitive test would appear to be the construction of an Arrhenius plot; and Ebert and Stricker (1964) have shown breaks in the slope of the plot for the dextransucrase reaction corresponding to changes in mechanism. For a test

reaction, the accurately measurable decomposition of *p*-nitrophenyl β-D-glucopyranoside was chosen with pentaerythritol as acceptor, since Table 1 indicated that the latter should almost totally exclude water from the reaction at concentrations that would not modify the liquid environment too drastically. In fact, liberation of free reducing sugar in the presence of 0·5M pentaerythritol was undetectable (below 1%) for phenyl, *p*-nitrophenyl, and *o*-cresyl β-D-glucopyranosides, and for salicin. The results are presented in Figure 3. There are no breaks in the Arrhenius line; the activation energy is 11,250±100 kcal/mole. The enzymic hydrolysis of this glucoside with water as acceptor has the significantly different activation energy of 7800±220 kcal/mole (Jermyn 1955) in the same buffer at the same pH; the activation energy is also outside the range (7800–9300 kcal/mole) found for transfer to water from all β-D-glucopyranosides tested.

It may be concluded that a single rate-determining step for transfer to pentaerythritol exists over the entire temperature range, and that it is different from the rate-determining step for transfer to water. Therefore this step cannot be $E+GluOPhNO_2 \rightarrow EOGlu+NO_2PhOH$; but the data will not allow us to make the positive choice between the reaction of glycosyl enzyme with acceptor and steps involving the formation or decomposition of ternary complexes.

Since the reaction used was not selected on any other grounds than convenience the conclusion reached may, in the absence of strong evidence to the contrary, be taken as typical for the enzyme and all substrate pairs.

(*c*) *Variation of Transfer with Temperature*

Whatever view is taken of the mechanism of transfer to water and the second acceptor, the reactions can be considered quite generally as two processes each with its characteristic rate, at a given temperature, T_1, of $P.\exp(-E_1/\boldsymbol{R}T)$ and $Q.\exp(-E_2/\boldsymbol{R}T)$. Here P and Q are constants, E_1 and E_2 are energies of activation, and $\boldsymbol{R}$ is the gas constant. The ratio of the rates then has the form $Z.\exp[(E_2-E_1)/\boldsymbol{R}T]$. But this ratio is exactly the transfer ratio, $t/(1-t)$, discussed earlier. If

$$t/(1-t) = Z.\exp[(E_2-E_1)/\boldsymbol{R}T],$$

then

$$\log_e[t/(1-t)] = \log_e Z+[(E_2-E_1)/\boldsymbol{R}]\cdot(1/T).$$

Hence if all other variables are fixed then the distribution of transfer between two acceptors should depend on temperature, in such a way that the relation between the logarithm of the transfer ratio and the reciprocal of absolute temperature is linear.

An experiment was set up in which the transfer to ethanol from phenylglucoside was measured at pH 5 in 1M ethanol over a temperature range. The raw data are set out in Table 3; when the logarithm of the transfer ratio was plotted against the reciprocal of absolute temperature, a linear relationship was found. Moreover, the slope of the line can be used to calculate a value for (E_2-E_1); this comes out to be 2840 cal/mole. The transfer of the glucosyl residue from phenyl β-D-glucopyranoside to ethanol thus requires $\simeq$ 3000 cal/mole more than that to water, when 1M ethanol is the solvent.

The use of this difference to calculate a value for the energy of activation of the enzyme–phenyl glucoside–ethanol reaction that will be comparable to that for the enzyme–phenyl glucoside–water reaction depends on the assumption that 1M ethanol as solvent has had no general activating or depressing effect on the enzyme. Later papers in these series will demonstrate the amount of work needed to establish this point. The question can, however, be tackled empirically by observing the dependence of the calculated value of (E_2-E_1) on ethanol concentration.

At 0·3M ethanol the value of (E_2-E_1) came to 3140 cal/mole and at 0·1M to 3020 cal/mole, the same within experimental error as the value with 1M ethanol. There is thus reasonable justification for using the mean value of (E_2-E_1), 3000 cal/mole, and the known value (9300 cal/mole, Jermyn 1955*a*) for the water reaction, to calculate a value of 12,300 cal/mole for the enzyme–phenyl glucoside–ethanol reaction in a purely aqueous solution at pH 5.

TABLE 3

VARIATION IN THE TRANSFER BY THE β-GLUCOSIDASE OF *S. ATRA* OF THE GLUCOSYL RESIDUE TO ETHANOL WITH VARYING TEMPERATURE, USING 2×10^{-3}M PHENYL β-D-GLUCOPYRANOSIDE IN 1M ETHANOL AT pH 5·0 (McILVAINE BUFFER)

Temperature (°C)	Percentage Transfer to Ethanol	Transfer Ratio
42	75	3·00
35	72	2·57
28	67	2·03
21	61	1·57
14	53	1·13
7	49	0·96
1	43	0·75

The rest of the studies set out in these papers have been carried out at the standard temperature of 28°C. It will be obvious that all the numerical values set out are no more than the values of temperature-dependent variables for this particular temperature. There is no evidence, however, that the nature of the effects, as opposed to their magnitude, is temperature-dependent.

(*d*) *Choice of Acceptors for Further Study*

The considerations illustrated graphically in Figure 2 suggest the following acceptors as worth investigating, when they are combined with the empirical observations of the activating or depressing effects of various acceptors which were accumulated during the experiments leading to the T_{50} values of Table 1:

(1) Activating, T_{50} concentration low: i.e. the enzyme binds acceptor much more readily than water and the resulting complex is more active than that involving water. Hexane-1,6-diol was chosen for investigation since it was solid, highly soluble, and inert to the reagents.

(2) Repressing, T_{50} concentration low: i.e. the enzyme binds acceptor much more readily than water and the resulting complex is less active than that involving water. Ethyl lactate was the only simple example found. The extreme case where there was no transfer to the repressor was represented by benzyl β-fructopyranoside.

(3) Activating, T_{50} concentration high: i.e. the binding of water and acceptor is of the same order of magnitude and enzyme–acceptor is the more active of the two complexes. Methanol was chosen as having the highest T_{50} value amongst simple compounds.

(4) Repressing, T_{50} concentration high: i.e. the binding of water and acceptor is of the same order of magnitude and enzyme–acceptor is the less active of the two complexes. t-Butyl alcohol was the simplest representative of the class.

V. Acknowledgment

The author wishes to acknowledge the technical assistance of Miss Carol May.

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