# FUNGAL CELLULASES

# XVII.\* THE BEHAVIOUR OF t-BUTYL ALCOHOL, PINACOL, AND METHANOL AS ACCEPTORS FOR THE $\beta$ -GLUCOSIDASE OF STACHYBOTRYS ATRA

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#### Summary

The affinity of  $\beta$ -glucosidase for t-butyl alcohol is similar to that for methanol but the rate of decomposition of the enzyme-two-substrate complex is much decreased for t-butyl alcohol. Pinacol is very similar to t-butyl alcohol in its behaviour as an acceptor.

## I. INTRODUCTION

The behaviour of t-butyl alcohol as an acceptor presents almost an exact opposite to that of hexane-1,6-diol (Jermyn 1966b), since it is only effective as an acceptor at relatively high concentrations and is a less effective acceptor than water. Nevertheless, it will be shown that many of its characteristics can be elucidated in exactly the same way as for hexanediol. Since the concentrations of the acceptor, considered solely as a solute, are sufficient to modify considerably the properties of the solution, it will be obvious that the numerical values deduced refer to fictional aqueous environments, and are to be regarded only as parameters of the enzyme reaction and not as referring to the real behaviour of the enzyme in a real environment.

Some data are also presented for methanol to illustrate a case intermediate between those of t-butyl alcohol and hexanediol. The data for pinacol illustrate an acceptor even more inefficient than t-butyl alcohol.

# II. MATERIALS AND METHODS

The methods were as set out in Parts XV and XVI of this series (Jermyn 1966*a*, 1966*b*). t-Butyl alcohol was fractionally distilled just before use and the bottle kept tightly stoppered to allow minimum access of moisture. Methanol (A.R.) and pinacol hexahydrate (m.p.  $46^{\circ}$ C) were used as received.

### III. Results

# (a) General Characteristics of t-Butyl Alcohol as an Acceptor

For hexanediol, the effect of pH on transfer fraction and  $T_{50}$  were sufficiently minor to make the one pH illustrated (Jermyn 1966b) a reasonable exemplar for all. For t-butyl alcohol this is not true. Figure 1 for four selected cases illustrates the profound effect of pH on transfer fractions and hence of the derived values of  $T_{50}$ . It is apparent on mere inspection, without proceeding to calculations, that the relative affinity of the enzyme for water and t-butyl alcohol is very different at different pH values.

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Lineweaver-Burk plots of the effect of increasing t-butyl alcohol concentration on the enzyme reaction are similar to those for hexanediol, although the effect of



Fig. 1.—Effect of pH (McIlvaine buffers) on the transfer of the glucosyl residue from phenyl  $\beta$ -D-glucopyranoside to t-butyl alcohol at 28°C by the  $\beta$ -glucosidase of *S. atra.* + pH 3.5;  $\times$  pH 5.0;  $\bigcirc$  pH 6.5;  $\bullet$  pH 8.0.

added acceptor is opposite in sense (Fig. 2). The steps outlined for hexanediol (Jermyn 1966b) can be applied to the t-butyl alcohol data to give plots of the type



Fig. 2.—Effect of increasing t-butyl alcohol concentration on Lineweaver–Burk plots of the relation between phenyl  $\beta$ -D-glucopyranoside concentration and the rate of breakdown of the glucoside by the  $\beta$ -glucosidase of *S. atra* at 28°C and pH 5.

of Figure 3. For all pH values studied, as for pH 5 in the figure,  $V_{\rm B}$  is much less than  $V_{\rm W}$ . On the other hand the extrapolation to the  $V_{\rm B}$  of t-butyl alcohol is much further than to the  $V_{\rm H}$  of hexanediol, and the value is relatively less certain.

One interesting consequence follows from the linearity of the points in plots such as in Figure 3 and the corresponding plots for hexanediol. Such a linearity



Fig. 3.—Relative rates of breakdown of the t-butyl alcohol and water complexes during the action of the  $\beta$ -glucosidase of *S. atra* on phenyl  $\beta$ -D-glucopyranoside at 28°C and pH 5.

precludes any general activation or depression of the enzyme by the acceptor interacting with the enzyme otherwise than as substrate, except in the unlikely

PYRANOSIDE TO C-BUTYL ALCOHOL BY THE P-GLOCOSIDINOL OF XTHE INC.									
pH	Rate of Decomposition of Enzyme– Glucoside– Alcohol relative to Enzyme– Glucoside– Water	Т <sub>50</sub> (м)	% of Acceptor Sites Occupied by Alcohol at $T_{50}$	% Transfer to Alcohol when Acceptor Sites Equally Occupied	Molarity at which Acceptor Sites Equally Occupied (calc. from Fig. 1)	Affinity of Acceptor Site for Alcohol relative to that for Water			
$3 \cdot 5$	0.622	$1 \cdot 7$	$61 \cdot 5$	$38 \cdot 5$	$1 \cdot 2$	40			
$5 \cdot 0$	0.417	19	70.5	$29 \cdot 5$	$5 \cdot 3$	10			
$6 \cdot 5$	0.456	$5 \cdot 5$	68.7	$31 \cdot 3$	2.8	20			
8.0	0.385	$1 \cdot 6$	72.2	27.8	0.42	130			

Table 1 summarized data on the transfer of glucosyl residue at 28°C from phenyl  $\beta$ -d-glucopyranoside to t-butyl alcohol by the  $\beta$ -glucosidase of S. ATRA for four distinct pH values

case where the two interactions remain directly proportional to each other over the entire concentration range.

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The most fundamental information that can be derived from data such as that of Table 1 is set out in Figures 4 and 5. The figures for the relative rate of decomposition of the two complexes may be combined with the pH-activity curve for enzymic activity with water as acceptor to give Figure 4. Here are set out both a direct comparison of the rate of decomposition of the two complexes over a pH range and of the shape of the two pH-activity curves.



Fig. 4.—Effect of pH on the rate of breakdown of the water and t-butyl alcohol complexes during the action of the  $\beta$ -glucosidase of *S. atra* at 28°C. (a) Rate in  $2 \times 10^{-9}$ M phenyl  $\beta$ -D-glucopyranoside at pH 5 taken as unity. (b) Rate at respective pH maxima taken as unity.

Figure 5 sets out the relative affinity of the acceptor site for t-butyl alcohol and water over the pH range. The values could as logically have been inverted, and a plot constructed of (affinity for water/affinity for t-butyl alcohol) against pH. The plot would then have had the same fundamental meaning, though not the same derivation, as a plot of Michaelis constant against pH where, also, the highest value indicates the lowest affinity. If it is objected that the view of the Michaelis constant as a measure of affinity is an unduly simple one, especially with a complex twosubstrate-enzyme mechanism, then inspection of the equations set out elsewhere (Jermyn 1962) will demonstrate that this is equally true of the acceptor "affinity".

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# (b) Pinacol as an Acceptor

The results of Part XV (Jermyn 1966a) indicate that primary alcohols with an unbranched carbon chain form the most efficient acceptors. Inspection of the formulae of t-butyl alcohol,  $(CH_3)_3COH$ , and pinacol,  $(CH_3)_2C(OH)C(OH)(CH_3)_2$ ,



Fig. 5.—Effect of pH on the relative affinity of enzyme-phenyl  $\beta$ -glucopyranoside complex for t-butyl alcohol and water at 28°C.

suggests that all the disadvantages of the former should be multiplied in the latter, and indeed pinacol was the most inefficient acceptor that could be shown positively to be an acceptor.



Fig. 6.—Effect of increasing pinacol concentration on Lineweaver– Burk plots of the relation between phenyl  $\beta$ -D-glucopyranoside concentration and the rate of breakdown of the glucoside by the  $\beta$ -glucosidase of *S. atra* at 28°C and pH 5.

The study of pinacol as an acceptor is limited by its limited solubility in water ( $\simeq 0.3$  m at 28°C) and the data cannot be analysed as far as with t-butyl alcohol since transfer and other effects are small and extrapolations so influenced by small experimental errors as to be quite unreliable.

The data for pH 5.0 only will be presented here; Figure 6 demonstrates the closeness of the series of Lineweaver–Burk plots to those expected for an "acceptor analogue", the molecules of which merely occupy the acceptor centre



Fig. 7.—Relative rates of breakdown of the pinacol and water complexes during the action of the  $\beta$ -glucosidase of *S. atra* on phenyl  $\beta$ -D-glucopyranoside at 28°C and pH 5.

without permitting transfer; the observed transfer to 0.3M pinacol at pH 5 is only 13%. Figure 7 indicates the length and uncertainty of the extrapolation involved in evaluating  $V_{\rm P}$  as against  $V_{\rm W}$ . If  $V_{\rm P}$  is taken as 0.4 (and it almost certainly lies

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Acceptor	Т <sub>50</sub> (м)	Rate of Decomposition of Acceptor Complex relative to that of Water Complex	Molarity at which Acceptor Sites Equally Occupied by Acceptor and Water	Relative Affinity of Enzyme for Acceptor as against Water				
Methanol	$1 \cdot 1$	$4 \cdot 3$	$4 \cdot 5$	11				
Pinacol	160	$0 \cdot 4$	10	4 - 5				
t-Butyl alcohol	19	$0 \cdot 42$	$5 \cdot 3$	10				
Hexane-1,6-diol	0.011	4.8	0.09	600				

TABLE 2 COMPARISON OF THE PARAMETERS OF ACCEPTOR ACTION FOR ENZYME-PHENYL β-D-GLUCOPYRANO-SIDE AND t-BUTYL ALCOHOL, PINACOL, METHANOL, AND HEXANE-1.6-DIOL AT DH 5:0

in the range 0.2-0.6), then the two molecules occupy the acceptor sites in equal numbers when the transfer ratio  $\simeq 29\%$ , i.e. at "10m" according to the extrapolated plot of log [t/(1-t)] against pH. The value for the relative affinity of the enzyme for pinacol and water is 4.5.

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Taken overall, these results suggest that pinacol at pH 5 is only a marginally worse acceptor than t-butyl alcohol. Quite small shifts in the constants can produce the observed difference in the values of  $T_{50}$  (160m as against 19m). Essentially similar results were obtained at other pH values.

# (c) Methanol as an Acceptor

Methanol was studied as an acceptor only at pH 5, since no more than comparative results with other acceptors were required. The detailed working up of the data was exactly as for hexanediol and t-butyl alcohol. Table 2 summarizes the results with t-butyl alcohol, pinacol, and methanol at pH  $5 \cdot 0$  and compares them with those for hexanediol at the same pH.

#### IV. DISCUSSION

The conclusions to be drawn from Table 2 seem inescapable, even if considerable latitude is allowed for error in the values quoted. The tables of Part XV (Jermyn 1966a) show the acceptor behaviour of  $HO(CH_2)_nOH$  to be very similar to that of  $CH_3(CH_2)_nOH$  and for our purposes hexane-1,6-diol may be taken as the equivalent of "soluble" heptan-1-ol, i.e. methanol with a lengthened carbon chain. Table 2 then shows that chain-lengthening increases the affinity of the enzyme-glucoside for the acceptor without much affecting the rate of decomposition of the complex once formed. The change from methanol to t-butyl alcohol has little effect on the affinity but slows down the decomposition of the complex. It is as yet far too early to offer any explanation of these results in terms of molecular forces.

#### V. Acknowledgment

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#### VI. References

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