FUNGAL CELLULASES

XVI.* ALKANE-1,ω-DIOLS AS ACCEPTORS FOR THE β-GLUCOSIDASE OF *STACHYBOTRYS ATRA*

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Summary

Using hexane-1,6-diol as an exemplar, methods are developed for estimating the kinetics of the reaction of enzyme and phenyl β-D-glucopyranoside with an added acceptor. The effects of pH on the rate of decomposition of the enzyme–glucoside–hexanediol complex and on the Michaelis constant of the partial reaction of enzyme–hexanediol with glucoside are considerably different from those for the water complex. Some less certain deductions are also made for the reaction of enzyme with butane-1,4-diol and *p*-nitrophenyl β-D-glucopyranoside.

I. INTRODUCTION

Of the various acceptors selected for further study in Part XV of this series (Jermyn 1966*a*), hexane-1,6-diol, an efficient "activating" acceptor at relatively low concentrations, provides the fewest problems in interpretation. For this reason it will be studied first, and the results used as a background against which other observations can be discussed. Certain relevant observations with butane-1,4-diol will also be described.

II. MATERIALS AND METHODS

Hexane-1,6-diol (Fluka AG) was used as received; its melting point (43°C) agreed with literature and its solutions showed no trace of reaction with the Somogyi–Nelson or Folin–Ciocalteau reagents. Butane-1,4-diol (British Drug Houses) was redistilled before use.

The methods used are described in the previous part (Jermyn 1966*a*). Their generalization in various ways to investigate various problems about acceptor action is described fairly fully in the next section of the paper. The results to be described with other acceptors in succeeding papers make use of these procedures and will not be given in such detail, and the section is in part intended as a general descriptive reference.

III. RESULTS

(*a*) *General Characteristics of Acceptor Behaviour with Hexane-1,6-diol*

It will be shown that the kinetics of the enzyme–donor–acceptor reaction are profoundly affected by such parameters as pH. However, it is impracticable to investigate all possible combinations in detail, and there is no evidence that the

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qualitative nature of the reaction changes. Hence, the transfer reaction has only been investigated in detail under a few conditions and, of them, the reaction at pH 6·5 (McIlvaine buffer) will be set out as an exemplar. Although the conclusions stated remain valid with, say, changing pH, the numerical values of the parameters are widely different.

Figure 1(*a*) shows some of the basic information on which the superstructure is built. A series of Lineweaver–Burk plots are drawn in which the relationship between phenyl-β-D-glucopyranoside concentration and reaction velocity is determined at various hexane-1,6-diol concentrations. Velocity at infinite donor concentration,

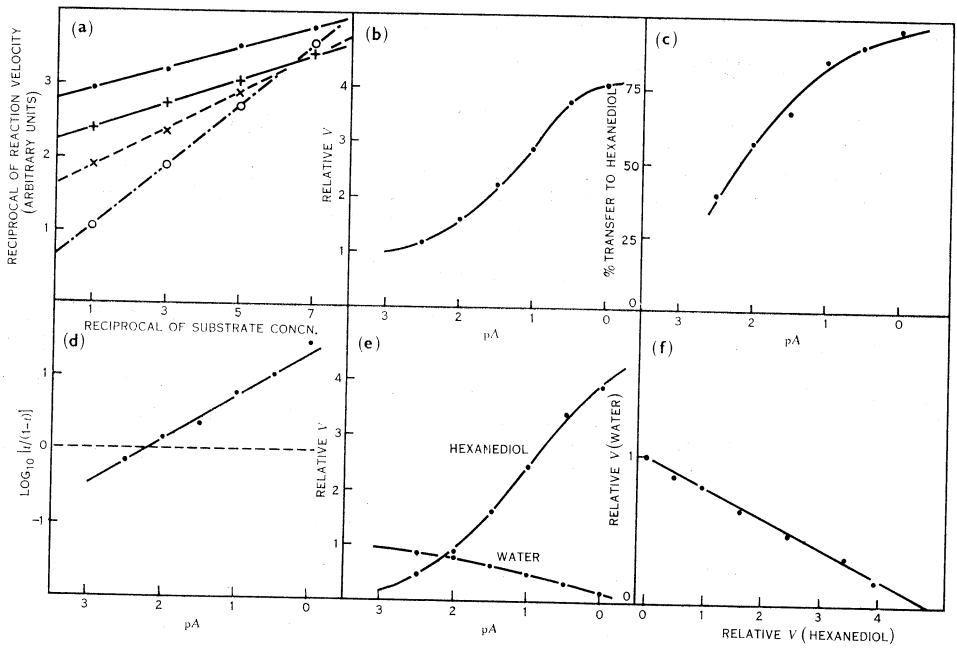

Fig. 1.—(*a*) Lineweaver–Burk plots of the dependence of phenyl β-D-glucopyranoside breakdown by *S. atra* β-glucosidase at 28°C and pH 6·5 on phenyl β-glucoside concentration at hexane-1,6-diol concentrations of 0 (●), 0·003M (+), 0·03M (×), and 1·0M. Unit substrate concentration = 2×10^{-3}M. (*b*) Values of relative V (V with hexanediol/V without hexanediol) plotted against pA, the negative logarithm of the acceptor (hexanediol) concentration. Taken from the data of Figure 1(*a*). (*c*) Percentage of glycosyl residue transferred to hexanediol (pH 6·5, 28°C) from phenyl β-glucoside by *S. atra* β-glucosidase plotted against pA. (*d*) $\mathrm{Log}_{10}\ [t/(1-t)]$, where t = transfer fraction from the data of Figure 1(*c*), plotted against pA. (*e*) Partial relative V for hexanediol and water [derived for the data of Fig. 1(*b*) and corrected transfer fractions from Fig. 1(*d*)] plotted against pA. (*f*) Partial relative V for hexanediol plotted against partial relative V for water [from the data of Fig. 1(*e*)].

V, is determined by extrapolation. If all the experiments are carried at known enzyme concentrations, and V without added hexanediol is set at unity, then it is possible to plot relative values of V at increasing hexanediol concentrations as in Figure 1(*b*).

But the reaction is the sum of two partial reactions, the maximum velocities of which may be written V_H (for hexanediol) and V_W (for water). By means of concurrent experiments in which the dependence of transfer fraction on hexanediol concentration is determined [Fig. 1(*c*)], relative V may be partitioned into relative V_H and relative V_W. Thus if maximum velocity has been doubled at a certain hexanediol concentration, and the transfer fraction is 0·75, then relative V_H will be 1·50 and relative V_W 0·50. It must be emphasized that the validity of this procedure depends entirely on the demonstration that partition fractions are independent of donor concentration (see Part XV, Jermyn 1966*a*).

The actual results of the partition are shown in Figure 1(*e*). In Figure 1(*f*), relative V_H is plotted against relative V_W and the line extrapolated to relative $V_W = 0$. Since the observations have been doubly extrapolated to infinite, i.e. saturating, concentrations of both substances, the resulting numerical value (4·63) is the ratio of the rate of decomposition of the complex (enzyme–hexanediol–phenyl β-glucoside) to that of the complex (enzyme–water–phenyl β-glucoside). This value might conceivably have been arrived at by extrapolation in Figure 2 if the concentration of hexanediol had been raised high enough but the procedure, uncertain enough for hexanediol, would be impossibly uncertain for less efficient acceptors.

This conclusion may be reversed by imagining a hypothetical situation in which hexanediol is the normal solvent and the inefficient acceptor water is added. When large amounts of water are added, the velocity of enzyme action will begin to fall towards a value of 1/4·63 (= 0·22) of the "normal" value. This case is formally identical with the addition of t-butyl alcohol to water next to be considered (Part XVII, Jermyn 1966*b*), except that from T_{50} data the ratio of the affinities of the enzyme for t-butyl alcohol and water is $\simeq 2$ and for water and hexanediol it is $\simeq 10^{-4}$. It is doubtful if added water would have sufficient effect on the observed velocity to make meaningful observations possible.

It may be noted [Fig. 1(*e*)], that the absolute amount of transfer to water decreases monotonically as hexanediol concentration rises. In the absence of a theoretical curve relating these two quantities, the absence of any general activation of the enzyme by hexanediol cannot be disproved (the values on the experimental monotonically decreasing curve may well lie consistently above those calculated from a theoretical curve of almost any shape). On the other hand, the certainty of such an effect that may be deduced from the increase in the absolute amount of transfer to water with increasing acceptor concentration that has been observed in other cases is correspondingly absent. The information that can be gained from plots of the type of Figure 1(*f*) in such cases will be discussed in later papers.

(*b*) *Significance of* T_{50} *for Hexanediol*

By replotting the data of Figure 1(*c*) (as in Fig. 1 of Jermyn 1966*a*) to give Figure 1(*d*) the value of T_{50} for hexanediol may be calculated as $8{\cdot}7 \times 10^{-3}$M. If the complex enzyme–hexanediol–glucoside decomposes to products 4·63 times as fast as the complex enzyme–water–glucoside, then at T_{50} 83% of the enzyme is present as the water complex and 17% as the hexanediol complex, and relative V is 1·66 times that in the absence of hexanediol. Alternatively the two complexes are present in equal amount when transfer to hexanediol is 83%, and relative V is 2·82.

Reference to Figure 1(*d*) gives $7 \cdot 5 \times 10^{-2}$M hexanediol as the concentration necessary to attain this transfer fraction. The true value of the relative affinity of the enzyme for hexanediol and water in the presence of a saturating quantity of phenyl β-glucoside is thus 740 instead of 6400 as estimated from T_{50} data alone.

(*c*) *pH–Activity Curve for Hexanediol*

Hexanediol is a fairly effective acceptor, yet at 0·08M, at which concentration the reaction is proceeding 2·8 times as fast as in the absence of acceptor, 50% of the enzyme is still present as the water complex. This means that, for instance, pH–activity curves for the enzyme are only legitimate for relative activities produced by double extrapolation to infinite concentration of both substrates. Merely "high" concentrations of acceptor and infinite donor concentration will not do, at least not without information showing that but a negligibly small portion of the enzyme is present as the water complex. Similar remarks apply to the temperature–activity relationship.

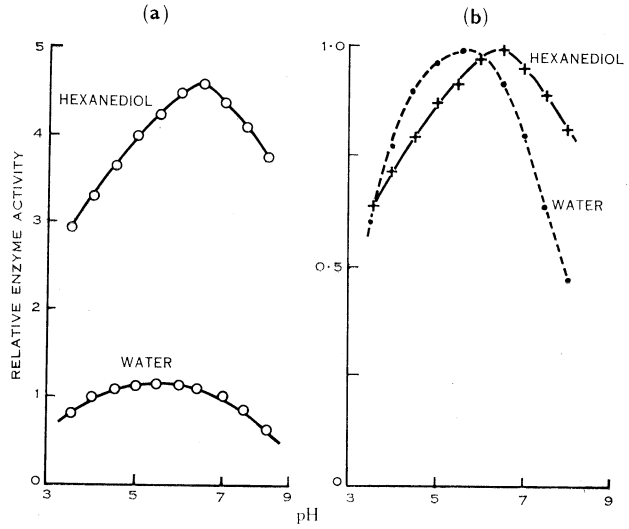

Fig. 2.—Rates of decomposition of the complexes enzyme–phenyl β-glucoside–water and enzyme–phenyl β-glucoside–hexanediol plotted against pH: (*a*) relative to rate of decomposition of water complex at pH 5 = unity; (*b*) relative to maxima of each of the previous curves = unity.

In Figures 2(*a*) and 2(*b*), pH–activity curves are plotted for McIlvaine buffers that compare the relative rates of breakdown of the two complexes (enzyme–glucoside–water and enzyme–glucoside–hexanediol). The displacement of the curve about one pH unit in the alkaline direction for the hexanediol complex as against the water complex seems to be a genuine effect. The differences in the shapes of the curves may or may not be real; too much smoothing of extrapolated data has been involved for useful comment.

(*d*) *Michaelis Constants for Hexanediol*

It is in theory possible to extrapolate the data set out in plots such as Figure 1(*a*) to give an enzyme activity for a given phenyl glucoside concentration and infinite

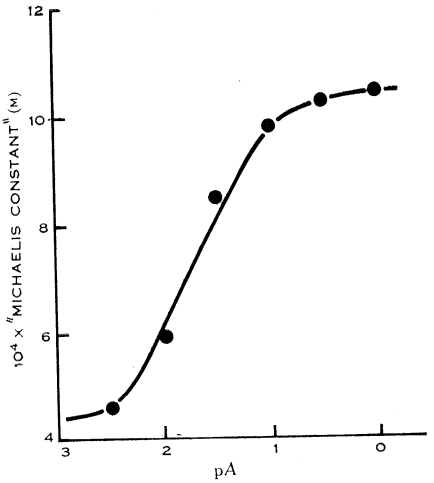

Fig. 3.—Apparent Michaelis constants for the decomposition of phenyl β-D-glucopyranoside by *S. atra* β-glucosidase (pH 6·5, 28°C) plotted against p*A*.

hexanediol concentration. These extrapolated values might then be combined in a Lineweaver–Burk plot to give a Michaelis constant for the enzyme–glucoside–

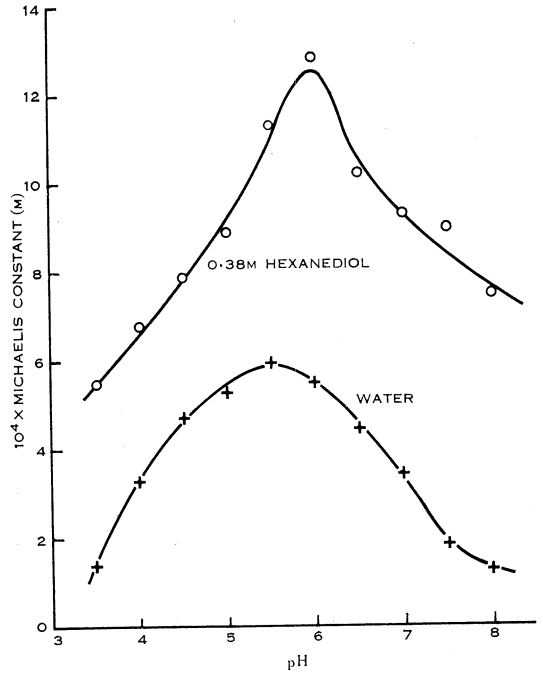

Fig. 4.—Effect of pH (McIlvaine citrate–phosphate buffer) on the Michaelis constant for the breakdown of phenyl β-D-glucopyranoside by *S. atra* β-glucosidase at 28°C in the presence and absence of 0·38M hexanediol.

hexanediol reaction. In practice the points on such plots are too scattered to give useful information.

However, if "Michaelis constants" for increasing concentrations of hexanediol are plotted against hexanediol concentration, the values tend towards a maximum (Fig. 3) which will be the true Michaelis constant of the reaction with hexanediol alone. These "Michaelis constants" for any hexanediol concentration are derived by making a Lineweaver–Burk plot of reaction velocity against phenyl glucoside concentration as if only a single enzyme reaction were taking place. In practice 0·3M hexanediol, at which concentration over 90% of the reaction was proceeding

TABLE 1

EFFECT OF VARIOUS CONCENTRATIONS OF BUTANE-1,4-DIOL ON THE BREAKDOWN OF *p*-NITROPHENYL β-D-GLUCOPYRANOSIDE BY THE β-GLUCOSIDASE OF *S. ATRA*

pH 5·0, 28°C

Butanediol Concn. (M)	Relative V	$10^5 \times$ "Michaelis Constant" (M)	Transfer Fraction (%) if $V_B = 4 \cdot 60$
	(*a*) *First Experiment*		
1·0	4·28	57	97
0·4	4·06	30	96
0·16	2·53	11·8	77
0·064	1·74	7·2	54
0·0256	1·43	6·9	38
0·01024	1·16	5·1	18
None	1·00	5·3	—
	(*b*) *Second Experiment*		
0·2	3·34	20	89
0·1	2·34	11·1	73
0·03	1·52	7·5	44
0·02	1·36	6·0	34

via hexanediol as acceptor, was taken as a convenient concentration for estimating "Michaelis constants" that are nearly Michaelis constants. Figure 4 shows the effect of pH on such constants. The relative, although not the absolute, effect of pH is obviously much less than on the constants of the water reaction.

(*e*) *Butane-1,4-diol and* p-*Nitrophenyl* β-D-*Glucopyranoside as Substrates*

Certain information on these substrates is presented to illustrate a method by which information can be elicited when transfer fractions are not available. As explained in Part XV (Jermyn 1966*a*), repeatable determinations of glucose and hence of the transfer fraction are impossible in the presence of nitrophenol. The basic information which was available is set out in the first two columns of Table 1(*a*).

To deal with this information, the assumption is made that the data fall on a true sigmoid curve. Then if V_B = relative V at saturating butanediol concentration and V_x = relative V at butanediol concentration, C_x, then the plot of $\log[(V_B - V_x)/(V_x - 1)]$ against $\log C_x$ should be linear. Since a straight line of sorts could be drawn through the points whatever value of V_B is assumed, the problem resolves itself into assuming values for V_B, calculating the least-squares line through the resulting points, and calculating the variance of the points about this line. The value of V_B which gives the minimum value for this variance is taken as the value of best fit.

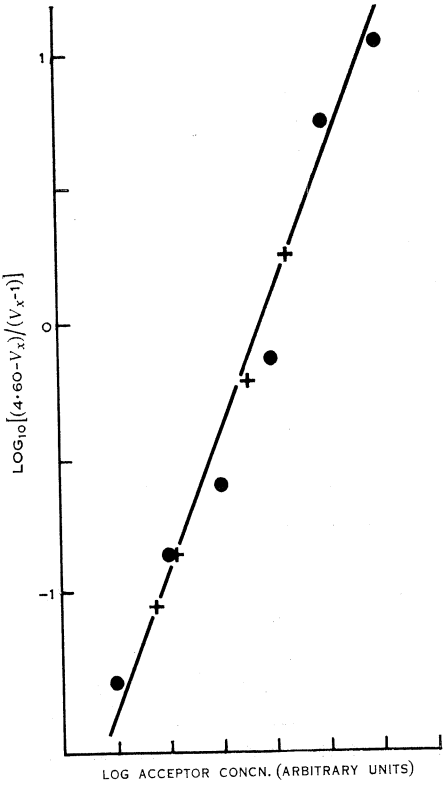

Fig. 5.—Data of Table 1(*a*) plotted on the assumption of minimum variance, leading to $V_B = 4{\cdot}60$ (●), with least-squares line drawn amongst the points. The data of Table 1(*b*) were then calculated on the same basis and plotted (+) on the same graph.

The final value adopted for V_B was 4·60. Figure 5 shows the plot of $\log[(4{\cdot}60 - V_x)/(V_x - 1)]$ against $\log C_x$ for the observations of Table 1(*a*). To check the calculations, a second entirely independent experiment was carried out [Table 1(*b*)]. These observations, when reduced to the same form as those of Table 1(*b*), fit the calculated line rather better than the original ones.

If $V_B = 4{\cdot}60$, then for every value of V_x there is a unique value of the transfer ratio. Thus, when enzyme–glucoside complex is distributed 50/50 between water and butanediol, relative $V = 0{\cdot}50 + (0{\cdot}50 \times 4{\cdot}60) = 2{\cdot}80$ and the transfer ratio equals $2{\cdot}30/2{\cdot}80 = 82\%$. We can now plot $\log[t/(1-t)]$ against butanediol concentration (Fig. 6). The derived "best" values are for T_{50}, 0·44M and for

concentration of equal distribution between complexes, 0·15M. It follows that the affinity of enzyme–*p*-nitrophenyl glucoside for butane-1,4-diol is $\simeq$ 370 times that for water.

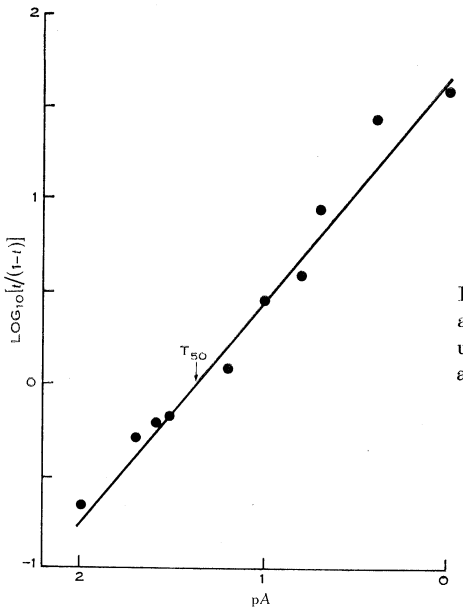

Fig. 6.—Values of transfer ratio (t) derived from the assumption that $V_B = 4 \cdot 60$ for the data of Table 1, used to calculate $\log_{10} [t/(1-t)]$ which is plotted against pA.

IV. Discussion

The behaviour of hexanediol, an efficient acceptor with a high affinity for the enzyme, presents no surprises. It is an excellent starting point for the consideration of other and more complex cases.

The ratio of the rates of breakdown of the two complexes, enzyme–water–glucoside and enzyme–acceptor–glucoside, is nearly the same ($\simeq 4 \cdot 6$) in both cases studied. Inspection of Figure 4 and Table 1 shows that the Michaelis constants for the formation of the two complexes differ in one case by a factor < 2, and in the other case by a factor > 10. The implication is that each case has still to be treated as a separate entity and no generalized correlations or predictions are yet possible.

V. Acknowledgment

I wish to acknowledge the technical assistance of Miss Carol May.

VI. References

Jermyn, M. A. (1966*a*).—*Aust. J. Biol. Sci.* **19**: 903.
Jermyn, M. A. (1966*b*).—*Aust. J. Biol. Sci.* **19**: 927.