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The β-glucosidase from *Botryodiplodia theobromae*

Mechanism of enzyme action

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1. In the presence of a high concentration of *p*-nitrophenyl β-D-glucopyranoside (donor) the rates of production of *p*-nitrophenol and a transglucosylation product (1-glyceryl β-D-glucopyranoside) increased, whereas the rate of production of glucose decreased with increasing concentration of glycerol in reactions catalysed by the high-molecular-weight β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) obtained from culture filtrates of *Botryodiplodia theobromae* Pat. 2. When [donor] $> K_m$ the rate of production of *p*-nitrophenol was higher in the presence of glycerol than in its absence, whereas when [donor] $< K_m$ the rate of production of *p*-nitrophenol was lower in the presence of glycerol than in its absence. 3. Glycerol increased both the Michaelis constant (K_m) and maximum velocity ($V_{max.}$), whereas dioxan increased K_m but decreased $V_{max.}$. 4. Up to 1mM-$AgNO_3$ had no effect on enzyme activity. 5. A ^2H-solvent-isotope-effect [$V_{max.}(H_2O)/V_{max.}(^2H_2O)$] value of 1.40 ± 0.05 was found at pH (or p^{2}H) 5.8. 6. α-^2H-kinetic-isotope-effect ($k_H/k_{^2H}$) values of 1.03 ± 0.01 and 1.05 ± 0.01 were found in the absence and presence of glycerol respectively. 7. Although maltose was a non-competitive inhibitor of β-glucosidase activity, the ratio of velocity in the presence of glycerol to that in its absence increased, after an initial decline, with increasing concentration of maltose. 8. These results are discussed in terms of a mechanism involving a solvent-separated glucosyl cation–carboxylate ion-pair, which has greater affinity for alcoholic glucosyl acceptors, and an intimate ion-pair, which has greater affinity for water as a glucosyl acceptor and which could collapse reversibly and rapidly into a preponderance of an unreactive covalent glucosyl-enzyme.

Previous studies (Umezurike, 1977, 1978, 1979) on the high-molecular-weight β-glucosidase from culture filtrates of *Botryodiplodia theobromae* Pat. have shown that the mechanism of action of this enzyme is consistent with the postulation of Wallenfels & Malhotra (1961) that the mechanism of action of glycosidases involves an initial general-acid catalysis by an acidic group on the enzyme to generate a glycosyl cation that is stabilized by an ionized group. In the presence of the dipolar aprotic solvent dioxan, an increase in apparent Michaelis constant (K_m) and a decrease in maximum velocity ($V_{max.}$) were observed (Umezurike, 1977, 1978, 1979). The increase in K_m was attributed to a 'medium effect' caused by changes in the hydration of some groups on the enzyme (Umezurike, 1978), whereas the decrease in $V_{max.}$ was attributed to the collapse of the reactive glucosyl cation–carboxylate ion-pair into an unreactive glucosyl-enzyme as the nucleophilic reactivity of the carboxylate counter-ion would be enhanced in the presence of dioxan. In the present paper, results are presented that appear to provide more information on the nature and reactivity of the glucosyl cation–carboxylate ion-pair generated in β-glucosidase-catalysed reactions.

Materials and methods

Organism

B. theobromae Pat. (I.M.I. 115626; A.T.C.C. 26123) was originally isolated from decaying wood (Umezurike, 1969).

Chemicals

p-Nitrophenyl β-D-glucopyranoside and ^2H_2O were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

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Methods

Enzyme purification. The high-molecular-weight β-glucosidase used in the present work was obtained from culture filtrates of *B. theobromae* and purified as described previously (Umezurike, 1971*a*, 1975*b*).

Enzyme assay. β-Glucosidase activity was determined with *p*-nitrophenyl β-D-glucopyranoside in 0.05 M-sodium acetate/acetic acid buffer (pH 5.0) as described previously (Umezurike, 1971*a*). Michaelis constants (K_m) and maximum velocities ($V_{max.}$) were calculated from plots of v against v/[donor]. Initial-velocity studies in the presence of dioxan were carried out as described previously (Umezurike, 1977, 1978).

Estimation of glucose and transglucosylation product. Glucose estimation was carried out as described previously (Umezurike, 1971*b*). The amount of the transglucosylation product formed in enzyme-catalysed reactions was calculated from the difference between the amount of *p*-nitrophenol and glucose liberated.

Synthesis of ^{2}H-labelled glucoside. p-Nitrophenyl β-D-[1-^{2}H]glucopyranoside was synthesized from [^{2}H]acetobromoglucose (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide) as described by Sinnott & Souchard (1973) for ^{2}H-labelled galactopyranosides. [^{2}H]acetobromoglucose was prepared from the ^{2}H-labelled sugar as described by Lemieux (1963), and [1-^{2}H]glucose was prepared from D-glucono-δ-lactone as described by Sinnott & Souchard (1973) for [^{2}H]galactose.

Concentration of reagents. The reagent concentrations quoted were final concentrations in the reaction mixtures.

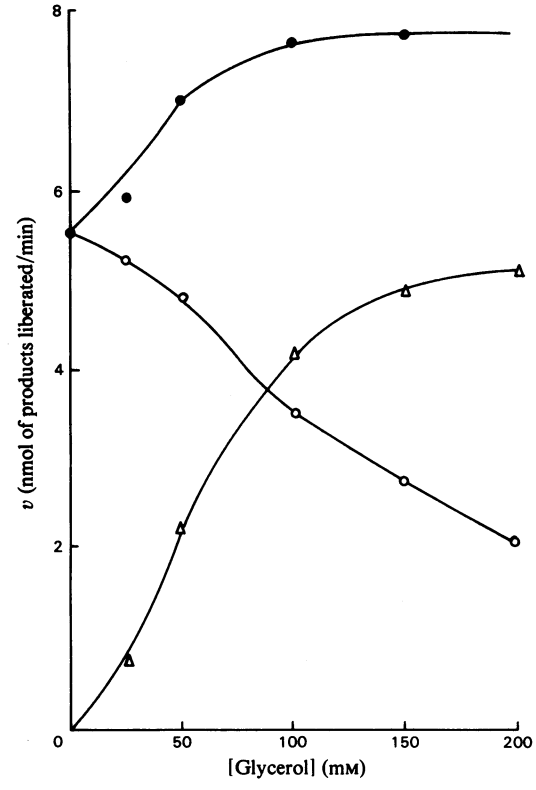


Fig. 1. *Effect of increasing concentration of glycerol on the rate of production of p-nitrophenol* (●), *glucose* (○) *and a transglucosylation product* (△) *by β-glucosidase*
The assay mixture contained 2.0 mM-*p*-nitrophenyl β-D-glucopyranoside. The amount of transglucosylation product formed was calculated from the difference between the amounts of *p*-nitrophenol and glucose liberated.

Results and discussion

Partitioning of intermediate to give two products

Previous studies have indicated that the mechanism of action of β-glucosidase from *B. theobromae* is consistent with acid-catalysed generation of a glucosyl cation stabilized by ion-pairing with an ionized carboxylate group (Umezurike, 1977, 1978, 1979). If the formation of the glucosyl cation is the rate-limiting step, the partitioning of the glucosyl cation–carboxylate ion-pair to products in the presence of two glucosyl acceptors would change in response to changes in the concentration of the acceptors, but the sum of the rates of formation of the two products would be the same and would correspond to the rate of formation of the glucosyl cation–carboxylate ion-pair (cf. Jencks, 1969, p. 53). Fig. 1 shows the results obtained when the rates of formation of *p*-nitrophenol, glucose and a transglucosylation product (previously identified as 1-glyceryl β-D-glucopyranoside; Umezurike, 1975*a*) were measured in the presence of a high donor concentration ([donor] = 7.1 K_m) and increasing concentration of glycerol. There was not much increase in the overall rate of production of the glucosyl cation–carboxylate ion-pair (i.e. rate of *p*-nitrophenol liberation) in the presence of high concentrations of glycerol, but there was a considerable increase in the rate of production of the transglucosylation product and a considerable decrease in the rate of glucose production (hydrolysis). These results indicate that the glucosyl cation–carboxylate ion-pair is a common intermediate for hydrolysis and transglucosylation and that its rate of production appears to be rate-limiting with *p*-nitrophenyl β-D-glucopyranoside as donor. The increase in the rate of production of the glucosyl cation–carboxylate ion-pair with increase in glycerol concentration, particularly in the presence of lower glycerol concentrations, has been ascribed to the

higher nucleophilicity of glycerol than water to the glucosyl cation–carboxylate ion-pair (Umezurike, 1978). About 88 mM-glycerol was reacted to form the transglucosylation product at the same rate as did 55 M-water to produce the same amount of glucose (see Fig. 1), indicating that glycerol is about 6.25×10^2 times better as a glucosyl acceptor than water.

Even though in the presence of high concentrations of donor ([donor] $> K_m$) the rate of production of the glucosyl cation–carboxylate ion-pair (measured by the release of *p*-nitrophenol) was higher in the presence of glycerol than in normal hydrolysis (Fig. 2*a*), when the concentration of donor was low ([donor] $< K_m$) the rate of production of the ion-pair was decreased in the presence of glycerol relative to the rate in normal hydrolysis (Fig. 2*b*). There was about 1.5-fold increase in the ratio of *p*-nitrophenol/glucose produced and about a 5.6-fold increase in the ratio of transglucosylation product/hydrolysis product (glucose) as the donor concentration was increased from 0.1 mM to 2.0 mM in the presence of 100 mM-glycerol. Equimolar amounts of *p*-nitrophenol and glucose were produced in normal hydrolysis at all concentrations of donor (Fig. 2). These results are consistent with glycerol acting as a more efficient nucleophilic solvent than water towards the glucosyl cation–carboxylate ion-pair and with the increase in the rate of transglucosylation at the expense of hydrolysis in the presence of glycerol. The decrease in the overall rate of enzyme activity in the presence of glycerol apparent in Fig. 2(*b*) is due to the 'medium effect' that operates in the presence of alcoholic glucosyl acceptors (Umezurike, 1978, 1979). Glycerol was found to increase both K_m and $V_{max.}$. The quaternary structure of β-glucosidase was not affected by 200 mM-glycerol, as pre-incubation of the enzyme for 30 min at 30°C in its presence before gel filtration on Sephadex G-200 did not lead to dissociation.

Effect of $AgNO_3$

Dey (1969) has shown that Ag^+ is a potent competitive inhibitor (K_i 2.46 μM) of sweet-almond α-galactosidase. The inhibition by Ag^+ was thought to result by salt formation involving Ag^+ and an active-site carboxylate group, stabilized by co-ordination with the tertiary nitrogen atom of the imidazole group at the active site. With β-glucosidase from *B. theobromae*, it was found that up to 1 mM-$AgNO_3$ had no effect on the activity of the enzyme at pH 5.0 in the presence of 0.06–2.0 mM-*p*-nitrophenyl β-D-glucopyranoside. In the presence of 10 mM-$AgNO_3$ the enzyme was precipitated from solution and lost activity completely. This enzyme is therefore similar to the α-galactosidase from *Calvatia cyanthiformis*, which was unaffected by up to 1 mM-Ag^+ (Li & Shetlar, 1964). This result suggests that the glucosyl cation and the carboxylate counter-ion do not exist as dissociated ions in β-glucosidase-catalysed reactions.

Isotope effects

The ^2H solvent isotope effect, $V_{max.}(H_2O)/V_{max.}(^2H_2O)$, on the activity of β-glucosidase from *B. theobromae* at pH (or p^{2}H) 5.8 in the presence of

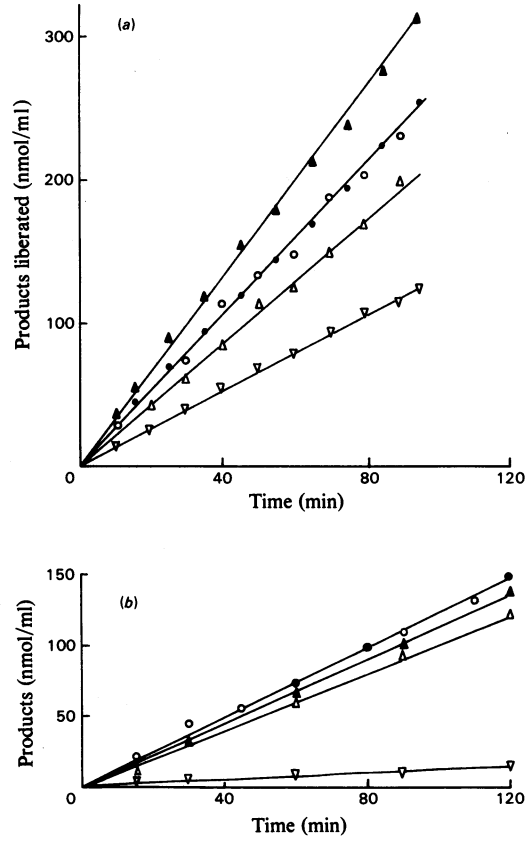


Fig. 2. *Effect of glycerol on the amounts of p-nitrophenol, glucose and transglucosylation product formed by β-glucosidase action*

(*a*) shows plots of the amounts of *p*-nitrophenol (●) and glucose (○) produced in the absence of added glycerol; and the amounts of *p*-nitrophenol (▲), glucose (△) and transglucosylation product (▽) formed in the presence of 100 mM-glycerol when the concentration of *p*-nitrophenyl β-D-glucopyranoside (donor) was 2.0 mM. (*b*) shows plots of the amounts of *p*-nitrophenol (●) and glucose (○) formed in the absence of added glycerol, and the amounts of *p*-nitrophenol (▲), glucose (△) and transglucosylation product (▽) formed in the presence of 100 mM-glycerol when the concentration of donor was 0.1 mM.

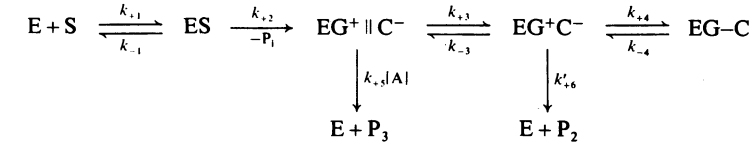
saturating donor concentration ([donor] = $58K_m$) was calculated to be 1.40 ± 0.05. A ^2H solvent isotope effect of 1.25 at pH (or p^{2}H) 7.0 has been found for α- and β-amylase activities, and these are believed to involve an acid-catalysed generation of a glucosyl cation stabilized by a carboxylate counter-ion (cf. Capon, 1969, p. 438). A ^2H solvent isotope effect value of 1.57 has been reported for the intramolecular general acid-catalysed hydrolysis of 2-carboxyphenyl β-D-glucopyranoside (cf. Capon, 1969, p. 438).

However, difficulties in the interpretation of ^2H solvent isotope effects with respect to enzyme-catalysed reactions have been pointed out (cf. Jencks, 1969, p. 277), particularly for enzymes with bell-shaped pH profiles, because of the isotope effects on the pK_a values of ionizing groups.

The α-^2H kinetic isotope effect ($k_H/k_{^2H}$) on the activity of β-glucosidase from *B. theobromae* was determined with *p*-nitrophenyl β-D-[1-^{2}H]glucopyranoside and the non-^2H-labelled compound as substrates. A $k_H/k_{^2H}$ value of 1.03 ± 0.01 was found in the absence of glycerol. When 100mM-glycerol was present in the assay mixture a $k_H/k_{^2H}$ value of 1.05 ± 0.01 was obtained. Dahlquist *et al.* (1969) have reported a $k_H/k_{^2H}$ value of 1.01 ± 0.01 for the hydrolysis of phenyl β-D-glucopyranoside by the β-glucosidase from sweet almond. The α-^2H kinetic isotope effect observed with β-glucosidase appears to be too small for a simple S_N1 departure of the leaving group (*p*-nitrophenolate) and too big for direct intramolecular displacement of the leaving group by the carboxylate counter-ion. It appears that the displacement of the leaving group by the carboxylate counter-ion takes place by an exchange reaction involving an intermediate intimate ion-pair of glucosyl cation and the leaving group, suggesting that the carboxylate counter-ion provides some assistance to acid-catalysed aglycone departure. However, bond-breaking is ahead of bond-forming process with respect to time, and the anomeric carbon atom of the glucosyl moiety will thus have a carbonium ion character. This interpretation is justified by the observation that aryl β-D-glucopyranosides undergo acid-catalysed hydrolysis more slowly than their α-anomers, indicating that the carbonium ion character is less highly developed in the transition states for acid-catalysed hydrolysis of aryl β-D-glucopyranosides than for aryl α-D-glucopyranosides (Hall *et al.*, 1961; Capon, 1969, p. 417).

Kinetics

The increase in $V_{max.}$ (or $k_{cat.}$) with increase in the concentration of added alcoholic glucosyl acceptor (Umezurike, 1978) can be explained by assuming that alcoholic glucosyl acceptors react more readily with a solvent-separated glucosyl cation–carboxylate ion-pair than with an intimate ion-pair, and water reacts more readily with the intimate ion-pair than with the solvent-separated ion-pair (cf. Scheme 1). The equilibrium between these intermediates is believed to lie in favour of a preponderance of the covalent α-D-glucosyl-enzyme. The result is that in the presence of high concentrations of an alcoholic glucosyl acceptor the rate of transglucosylation would increase as that for hydrolysis decreases. It has been proposed that for some solvolyses (e.g. the acetolysis of some alkyl arenesulphonates) both a solvent-separated and an intimate ion-pair exist as intermediates (cf. Alder *et al.*, 1971, p. 88). Alcohols are more reactive than water towards carbonyl-type carbon, and, because a carbonyl-type carbon atom is isoelectronic with a protonated aldehyde, alcohols are believed to be more reactive than water towards the glucosyl cation (cf. Sinnott & Viratelle, 1973; Umezurike, 1979). An alcohol (methanol) has been found to be less nucleophilic than water towards saturated carbon atoms in both S_N1 and S_N2



Scheme 1. *Reaction scheme for β-glucosidase-catalysed hydrolysis and transglucosylation*

E and ES are the free enzyme and the Michaelis enzyme–substrate complex respectively; EG$^+$ ‖ C$^-$, EG$^+$C$^-$ and EG–C are the enzyme-bound solvent-separated glucosyl cation–carboxylate ion-pair, the intimate ion-pair and the unreactive covalent glucosyl-enzyme respectively; and P_1, P_2 and P_3 are *p*-nitrophenol, glucose and the transglucosylation product respectively. It is assumed that the intimate ion-pair is more reactive with water than with an added alcoholic glucosyl acceptor (A), whereas the solvent-separated ion-pair is more reactive with the alcoholic glucosyl acceptor than with water. The rate constants for the forward reactions are denoted as k_{+1}–k_{+6} and those in the reverse direction as k_{-1}–k_{-4}. The rate constant $k'_{+6} = k_{+6}$[water], but as the concentration of water is very high k_{+6}[water] is regarded as a constant (k'_{+6}). Note that the 'medium effect' observed in the presence of alcoholic glucosyl acceptor has been omitted for simplicity.

reactions (Streitweiser, 1962). If, for the purpose of simplification, the well-known 'medium effect' observed in the presence of added alcoholic glucosyl acceptors is ignored, since its effect is mainly on K_m (Umezurike, 1978, 1979), the scheme for β-glucosidase-catalysed reactions in the presence of an added alcoholic glucosyl acceptor can be represented as shown in Scheme 1. The steady-state rate equation for Scheme 1, derived by the method of King & Altman (1956), is of the Michaelis–Menten type (eqn. 1).

Since $k_{cat.}$ (i.e. the rate of liberation of *p*-nitrophenol) is equal to the sum of the rate of transglucosylation ($k_{trans.}$) and that of hydrolysis ($k_{hydr.}$), the equations for $k_{trans.}$, $k_{hydr.}$ and the ratios of $k_{cat.}/k_{hydr.}$ and $k_{trans.}/k_{hydr.}$ are given by eqns. (2)–(5).

Effects of dioxan and maltose

The dipolar aprotic solvent dioxan, which is not a glucosyl acceptor, increased the apparent K_m but decreased $V_{max.}$ in β-glucosidase-catalysed hydrolysis of *p*-nitrophenyl β-D-glucopyranoside just as it did when the donor was o-nitrophenyl β-D-glucopyranoside (Umezurike, 1977, 1978, 1979). It has been argued (Umezurike, 1979) that the decrease in $V_{max.}$ in the presence of dioxan is due to the collapse (internal return) of the glucosyl cation–carboxylate ion-pair into an unreactive covalent glucosyl-enzyme because the nucleophilicity of the carboxylate counter-ion would be enhanced as a result of poor solvation. Thus the ratio of the molar concentration of dioxan to that of water was found to enter into the steady-state rate equation in the presence of dioxan (Umezurike, 1979). The increase in apparent K_m in the presence of dioxan has been attributed to a 'medium effect' (Umezurike, 1979).

D-Maltose is not an effective glucosyl acceptor compared with water and, unlike the efficient alcoholic glucosyl acceptors, it has no effect on apparent K_m but decreases $V_{max.}$ (Umezurike, 1971*b*, 1975*a*, 1978). Fig. 4(*a*) shows the plots of some of the results obtained when β-glucosidase activity was measured in the presence of high donor concentration ([donor] = $7.1K_m$) and in the absence or presence of 100mM-glycerol as a function of maltose concentration. Maltose inhibited β-glucosidase activity in the presence or absence of up to 100mM-glycerol but activity in the presence of glycerol was always higher than activity in its absence. Plots of the data as the ratio of the velocity

$$v/[E]_0 = k_{cat.}/[1 + (K_m/[S])] \quad (1)$$

where

$$K_m = \frac{k_{-1}+k_{+2}}{k_{+1}} \Bigg/ \left(1 + \frac{k_{+2}[k_{+3}(k_{+4}+k_{-4}) + k_{-4}(k_{-3}+k'_{+6})]}{k_{-4}[k_{+5}[A]\,(k_{-3}+k'_{+6}) + k_{+3}\,k'_{+6}]}\right)$$

and

$$k_{cat.} = k_{+2} \Bigg/ \left(1 + \frac{k_{+2}[k_{+3}(k_{+4}+k_{-4}) + k_{-4}(k_{-3}+k'_{+6})]}{k_{-4}[k_{+5}[A]\,(k_{-3}+k'_{+6}) + k_{+3}\,k'_{+6}]}\right)$$

$$k_{trans.} = \frac{k_{+2}\,k_{-4}\,k_{+5}\,[A]\,(k_{-3}+k'_{+6})}{k_{-4}[k_{+5}[A]\,(k_{-3}+k'_{+6}) + k_{+3}\,k'_{+6}] + k_{+2}[k_{+3}(k_{+4}+k_{-4}) + k_{-4}(k_{-3}+k'_{+6})]} \quad (2)$$

$$k_{hydr.} = \frac{k_{+2}\,k_{+3}\,k_{-4}\,k'_{+6}}{k_{-4}[k_{+5}[A]\,(k_{-3}+k'_{+6}) + k_{+3}\,k'_{+6}] + k_{+2}[k_{+3}(k_{+4}+k_{-4}) + k_{-4}(k_{-3}+k'_{+6})]} \quad (3)$$

$$k_{cat.}/k_{hydr.} = 1 + k_{+5}[A]\,(k_{-3}+k'_{+6})/(k_{+3}\,k'_{+6}) \quad (4)$$

$$k_{trans.}/k_{hydr.} = k_{+5}[A](k_{-3}+k'_{+6})/(k_{+3}\,k'_{+6}) \quad (5)$$

Thus eqn. (1) shows that K_m and $k_{cat.}$ would increase with increase in [A] as observed (cf. Umezurike, 1978, 1979). Eqns. (2) and (3) show that $k_{trans.}$ would increase but $k_{hydr.}$ would decrease with increasing concentration of the added acceptor (A) as observed in Fig. 1. A plot of the ratio $k_{cat.}/k_{hydr.}$ against [A] would be a straight line with intercept = 1 when [A] = 0 (cf. eqn. 4). A plot of the ratio $k_{trans.}/k_{hydr.}$ against [A] would also be a straight line and the ratio would have a value of zero when [A] = 0 as shown by eqn. (5). Fig. 3 shows the plots of the ratios of $k_{cat.}/k_{hydr.}$ and $k_{trans.}/k_{hydr.}$, calculated from the data of Fig. 1, against the concentration of glycerol. Both lines have the same slope and agree with the predictions of eqns. (4) and (5).

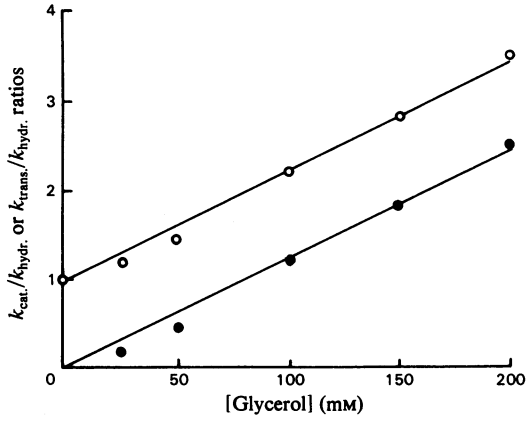


Fig. 3. *Plots of* $k_{cat.}/k_{hydr.}$ (○) *and* $k_{trans.}/k_{hydr.}$ (●) *calculated from the data in Fig. 1 against the concentration of glycerol*

in the presence of glycerol ($v_{glycerol}$) to that in the absence of glycerol (v_0) against the concentration of maltose are shown for three different concentrations of glycerol in Fig. 4(*b*). Even though maltose is a non-competitive inhibitor of β-glucosidase activity (cf. Umezurike, 1971*b*, 1975*a*), the ratio $v_{glycerol}/v_0$ increased, after an initial decline, with increase in maltose concentration. This indicates that there was an increase in the rate of transglucosylation to glycerol at the expense of hydrolysis as the concentration of maltose was increased. These results are consistent with the postulation of two types of ion-pair with different acceptor affinities as shown in Scheme 1. Maltose apparently caused a shift in the equilibrium between the solvent-separated glucosyl cation–carboxylate ion-pair and the intimate ion-pair in favour of the former ion-pair species. This could be an 'ionic strength effect'. The initial decline in the ratio of $v_{glycerol}/v_0$ with increasing concentration of maltose (see Fig. 4*b*) suggests an enhancement of the ionization of the unreactive covalent glucosyl-enzyme to the intimate ion-pair, which in turn is converted into the solvent-separated ion-pair. The transient increase in the concentration of the intimate ion-pair in this sequence of events would increase the rate of hydrolysis until the concentration of the solvent-separated ion-pair is increased. Ultimately, the increase in concentration of the solvent-separated ion-pair, which has less affinity for water, would lead to inhibition of hydrolysis in the absence of an effective alternative alcoholic glucosyl acceptor, and to increasing $v_{glycerol}/v_0$ ratios in the presence of increasing concentration of glycerol.

If Scheme 1 is condensed by eliminating the distinction between the two ion-pair species with respect to their acceptor affinity, it would appear as if only an ion-pair is in equilibrium with the

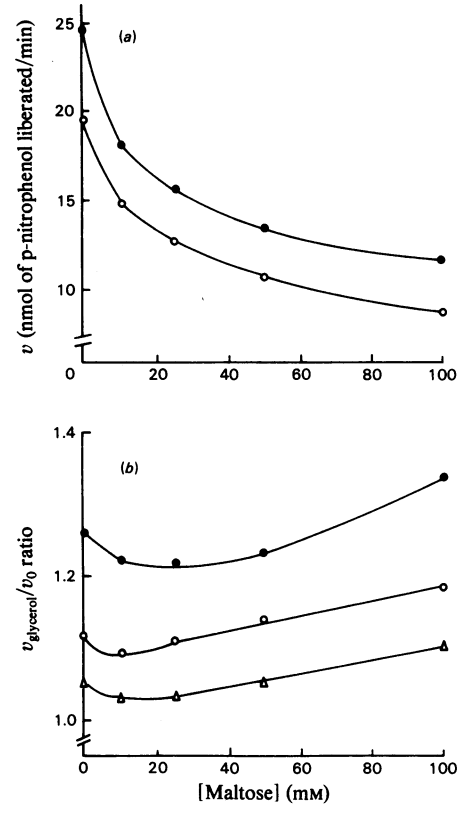


Fig. 4. *Effect of maltose on the activity of β-glucosidase in the absence or presence of glycerol*

(*a*) Plots of enzymic activity in the absence (○) and presence of 100 mM-glycerol (●) in the absence and presence of increasing concentration of maltose, and (*b*) plots of the ratios of enzymic activity in the presence of glycerol to that in its absence as a function of maltose concentration for assays carried out when glycerol concentration was 25 mM (△), 50 mM (○) and 100 mM (●). The final concentration of *p*-nitrophenyl β-D-glucopyranoside in all the assay mixtures was 2 mM.

unreactive covalent glucosyl-enzyme and that water and the alcoholic glucosyl acceptor react with the same ion-pair. The steady-state rate equation for such a scheme is also of the Michaelis–Menten type. The kinetic parameters (K_m, $k_{cat.}$, $k_{trans.}$ and $k_{hydr.}$) would be less complex than the relationships given in eqns. (1)–(5) but the predictions of these equations would still hold. The effect of maltose on enzyme activity and the kinetic behaviour of maltose (cf. Fig. 4) would, however, be difficult to explain. We can, therefore, write the mechanism of β-glucosidase-catalysed hydrolysis of aryl β-D-glucopyranosides as shown in Scheme 1 but without the alcoholic glucosyl acceptor.

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