Enzymic Glucosylation of Phenols

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1. A transglucosylase fraction has been obtained from the mycelium of *Aspergillus niger*. 2. The preparation will transfer α -D-glucopyranosyl residues from maltose and other α -D-glucopyranosides to phenolic and alcoholic hydroxyl groups and to carboxylic acid groups. 3. α -Isomaltosides and α -maltosides are formed when resorcinol and catechol are used as acceptors. 4. pH precipitation and DEAE-cellulose chromatography were used to resolve the activity into two fractions. The properties, in particular polyol inhibition, of one of these fractions have been examined in detail.

Nucleoside derivatives serve as D-glucose donors for the formation of phenolic β -D-glucosides in plants (reviewed by Pridham, 1965*a,b*) and insects (Dutton & Duncan, 1960; Trivelloni, Recondo & Cardini, 1962) and these transfer reactions can readily be demonstrated *in vitro*. 'Low-energy' β -D-glucosides such as cellobiose and methyl β -D-glucoside do not appear to act as donors to phenolic hydroxyl groups in the presence of higher-plant enzyme systems (Pridham, 1965*a,b*). *p*-Hydroxyphenyl β -D-fructofuranoside has, however, been prepared from sucrose and quinol with enzyme preparations from wheat germ and yeast (Nakamura & Miwa, 1964) and *Escherichia coli* β -D-galactoside galactohydrolase will transfer β -D-galactosyl and β -D-fucosyl units to phenolic hydroxyl groups (Pridham & Wallenfels, 1964). Pridham (1961) reported the α -D-glucosylation of phenols by an *Aspergillus niger* enzyme preparation, and the present paper describes further studies of this activity.

MATERIALS AND METHODS

General methods. The u.v. spectra were measured with a Unicam SP.500 spectrophotometer (1 cm. cell). Infrared measurements were made with a Perkin-Elmer 137 spectrophotometer with Nujol mulls.

***Aspergillus niger* culture.** The fungus *A. niger* (Birmingham strain 152) was stored at 5° on potato-agar slopes. Cultures for enzyme production were prepared by spore inoculation of Currie's (1917) medium and leaving at 25° for 6–9 days. The harvested mycelium was stored at 5°.

Preparation of crude enzyme. The mycelium (15 g.) was macerated with distilled water (100 ml.). The pH of the resulting slurry was approx. 5, and it was centrifuged to remove cell debris (2000g for 30 min.). The supernatant was dialysed against distilled water overnight. The

temperature was not allowed to rise above 5° during these operations.

Preparation of aryl glucosides. The crude aqueous enzyme preparation (400 ml.) was added to 0.1 M-sodium acetate buffer, pH 5.1 (400 ml.), 0.60 M-maltose (140 ml.), 4.3 M-resorcinol (or 2.2 M-catechol) (40 ml.) and 1.2 M-L-cysteine (20 ml.). The digest was incubated at 25° for 24 hr. and the reaction then stopped with 1.2 N-trichloroacetic acid (40 ml.). After concentration to a syrup at 40° (reduced pressure) the aryl glucoside components were fractionated on paper chromatograms (solvent A).

Hydrolysis of glucosides. Complete hydrolysis was effected with N-HCl at 100° for 1 hr. HCl was removed under reduced pressure and the hydrolysates were then examined on paper chromatograms. Partial acid hydrolysis was carried out by heating at 100° for approx. 30 sec. and alkaline hydrolysis of glucose ester derivatives with N-NaOH at room temperature (1 hr.).

Methylation of resorcinol glucosides. This was achieved by methylating twice with the Kuhn reagent (Kuhn, Trischmann & Löw, 1955). The products were methanolysed by refluxing with methanolic 3% (w/v) HCl and then examined by gas-liquid chromatography as described by Aspinall (1963).

Determination of sugar/phenol ratios. The 0.05 M-resorcinol glucoside (or diglucoside) solution (4 ml.) was incubated with 0.1 M-sodium acetate buffer, pH 6.5 (4 ml.), and maltase (α -D-glucoside glucosylhydrolase) solution (25 mg./ml.; 2 ml.) (L. Light and Co., Colnbrook, Bucks.) at 30° until hydrolysis was complete. The reactions were stopped by addition of ice-cold 0.3 N-HClO₄ solution (10 ml.). Portions of the digests were extracted exhaustively with diethyl ether to remove resorcinol and the remaining glucose was then determined with glucose oxidase (β -D-glucose-oxygen oxidoreductase) (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) (Huggett & Nixon, 1957). The resorcinol was determined by the vanillin method (see below).

Oxidation of catechol glucosides. Approx. 10 mg. of each diglucoside was dissolved separately in water (0.5 ml.). Freshly prepared FeCl₃ solution (2%, w/v; 1 ml.) was then

added and the mixture incubated at 35° for 10 min. After deionization with Bio-Deminrolit the oligosaccharides were adsorbed on a small column of activated charcoal. This was eluted successively with water (5 ml.), aq. 5% (v/v) ethanol (10 ml.) and aq. 10% (v/v) ethanol (10 ml.). Each fraction was concentrated under reduced pressure and examined on paper chromatograms (solvent A). The remainder of each fraction was reduced to the corresponding alcohol derivative with NaBH₄ and then subjected to paper electrophoresis (see below).

Paper chromatography. The ascending technique was used with Whatman papers: no. 1 for sugars and no. 3 for phenols and phenolic glucosides. The following solvent systems were employed, A and B for phenols and C for sugars: A, ethyl acetate-acetic acid-water (9:2:2, by vol.); B, butan-1-ol-ethanol-water (40:11:19, by vol.); C, ethyl acetate-pyridine-water (10:4:3, by vol.). Phenols were detected with diazotized *p*-nitroaniline (Swain, 1953) and reducing sugars with aniline oxalate (Hough, Jones & Wadman, 1950) and AgNO₃-NaOH (Trevelyan, Procter & Harrison, 1950).

Thin-layer chromatography. Methylated sugars were compared with authentic derivatives on kieselguhr plates with toluene-ether (1:2, v/v) solvent. Detection was effected by spraying with aq. 5% (v/v) H₂SO₄ followed by heating (Gee, 1963). Resorcinol monomethyl ether was chromatographed on an alumina thin layer with benzene-acetone (1:1, v/v) solvent.

Paper electrophoresis. The methods described by Pridham (1959) were used to examine phenols and phenolic glucosides. The procedure for the electrophoresis of sugar alcohols with a molybdate electrolyte was that used by Bourne, Hutson, & Weigel (1959).

Enzyme assay. Standard reaction mixtures (total vol. 5 ml.) contained enzyme solution (2.0 ml.), 0.1 M-sodium acetate buffer, pH 5.1 (2.0 ml.), 0.60 M-maltose (0.7 ml.; final concn. 0.083 M) 4.3 M-resorcinol (0.2 ml.; final concn. 0.17 M) and 1.2 M-L-cysteine (0.1 ml.). The digests were incubated at 24° for 60 min. Under these conditions a zero-order reaction was obtained, the rate depending only on enzyme concentration. The reaction was stopped by the addition of 1.2 N-trichloroacetic acid (0.2 ml.) and the resorcinol present was then removed by extracting each digest 20 times with diethyl ether (15 ml.). The amount of *m*-hydroxyphenyl α -D-glucopyranoside formed (only traces of the diglucosides were present under these conditions) was determined by adding vanillin reagent [30 ml.; 1% (w/v) vanillin in aq. 70% (v/v) H₂SO₄ (Swain & Hillis, 1959)] to 3 ml. samples of the extracted digests. After 18 min. the red colour formed was measured at 500 m μ . The method was standardized with a purified preparation of *m*-hydroxyphenyl α -D-glucoside. Protein determinations were made by the method of Lowry, Rosebrough, Farr & Randall (1951) with human serum albumin as standard.

Purification of the enzymes. Crude enzyme solution (21.) at 0° was acidified to pH 3.9 by careful addition, with stirring, of 5 N-acetic acid. The protein precipitate that resulted after 30 min. standing was centrifuged off and discarded. The pH of the centrifugate was then lowered to 2.9, and the precipitated protein was again collected by centrifugation, taken up in water (60 ml.) and dialysed against water for 20 hr. (5°). This solution was applied to the top of a DEAE-cellulose (Whatman DE50) column (25 g.; 20 cm. $\times$ 5 cm.) that had been converted into the OH⁻ form and then washed with M-H₃PO₄ and finally equilibrated with 0.02 M-

sodium phosphate buffer, pH 6.8. The column was eluted by the linear ionic gradient method with 0.02 M-sodium phosphate buffer, pH 6.8 (600 ml.), into which a 0.07 M-sodium phosphate buffer (pH 6.8)-0.4 M-KCl mixture (600 ml.) was gradually introduced. The fractions (14 ml.) collected were assayed quantitatively for protein (Goodwin & Morton, 1964). Samples from these fractions were also incubated with maltose and resorcinol under the conditions described for the enzyme assay. The appearance of *m*-hydroxyphenyl α -D-glucoside was then checked qualitatively by paper chromatography with solvent A. (The complexity of the quantitative method for this enzyme did not permit its application to all the collected fractions.) Two regions of activity were detected (X and Y, see Fig. 1); the appropriate fractions were combined separately and each was dialysed against distilled water (5°) for 20 hr.

Properties of the enzymes. The standard conditions used for assay of enzymic activity were employed to study the properties of the enzyme. Temperature optima and thermal stabilities for fractions X and Y were both measured at pH 5.7. pH optima were obtained by varying the pH of the acetate buffer between 3.8 and 5.6. Donor specificity was investigated with resorcinol as the acceptor (concn. of donor 1-2%, w/v) and maltose was used as the glucosyl donor to examine acceptor specificity (concn. of acceptor 1-2%, w/v); these experiments were qualitative and digests were examined on paper chromatograms.

For the study with inhibitors and activators the L-cysteine solution in the standard digest was replaced by a solution of the appropriate compound. The effect of preincubation with some of the thiol reagents for 40 min. at room temperature before assay was also studied. Reversal of mercaptide formation was examined by adding excess of cysteine 30 min. after preincubation with *p*-chloromercuribenzoate and then assaying by the standard procedure. Photo-oxidation of the enzyme in the presence of methylene blue was carried out at room temperature by the method of Jørgensen (1964).

RESULTS AND DISCUSSION

Incubation of resorcinol and maltose with the crude *A. niger* preparation produced three glucosidic derivatives (I, II and III) that all gave pink azo dyes when allowed to react with diazotized *p*-nitroaniline-sodium hydroxide. This suggested the presence of free phenolic hydroxyl groups. The chromatographic properties of the glucosides are summarized in Table 1. The glucose/resorcinol ratio was determined after hydrolysis with yeast α -D-glucoside glucosylhydrolase and found to be 2:1 for compounds I and II and 1:1 for compound III. None of the compounds was hydrolysed by almond β -D-glucoside glucosylhydrolase. This established that compounds I and II were *O*- α -D-glucosyl α -D-glucosides and that compound III was an α -D-glucoside (*m*-hydroxyphenyl α -D-glucoside). Compound I was cleaved by pullulanase, which is specific for α -(1 $\rightarrow$ 6)-D-glucosidic linkages, yielding compound III and glucose. The products of partial acid hydrolysis of compounds I and II are given in Table 1, and this clearly indicates that compound I was the

Table 1. *Chromatographic properties and oxidation and partial-acid-hydrolysis products of phenolic glucosides*

Compound	Glucoside	R_F		Oxidation or partial-acid-hydrolysis products*
		Solvent A	Solvent B	
I	<i>m</i> -Hydroxyphenyl α -isomaltoside	0.34	0.28	Resorcinol, glucose, isomaltose
II	<i>m</i> -Hydroxyphenyl α -maltoside	0.45	0.36	Resorcinol, compound III, glucose, maltose
III	<i>m</i> -Hydroxyphenyl α -D-glucopyranoside	0.62	0.54	—
IV	<i>o</i> -Hydroxyphenyl α -isomaltoside	0.31	0.40	Isomaltose
V	<i>o</i> -Hydroxyphenyl α -maltoside	0.41	0.48	Maltose
VI	<i>o</i> -Hydroxyphenyl α -D-glucopyranoside	0.61	0.67	Glucose

* Oxidation products from *o*-hydroxyphenyl α -D-glucosides and partial-acid-hydrolysis products from *m*-hydroxyphenyl α -D-glucosides.

Table 2. *Relative retention times on gas-liquid chromatography of methylated and methanolysed derivatives from resorcinol glucosides*

A butane-1,4-diol succinate polyester column was used under the conditions described by Aspinall (1963).

Resorcinol glucoside	Relative retention time		Identity of components
	Unknown	Standard	
I	1.00	1.00	β -Anomer
	1.23	1.24	α -Anomer
	1.37	1.36	β -Anomer
	1.85	1.88	α -Anomer
	0.78	0.79	Resorcinol monomethyl ether
II	1.02	1.00	β -Anomer
	1.42	1.36	α -Anomer
	1.78	1.76	β -Anomer
	2.40	2.37	α -Anomer
	0.76	0.78	Resorcinol monomethyl ether
III	1.00	1.00	β -Anomer
	1.35	1.36	α -Anomer
	0.75	0.78	Resorcinol monomethyl ether

α -isomaltoside and compound II the α -maltoside of resorcinol. Final proof of the structures of the three glucosides was obtained by methylation followed by methanolysis and gas-liquid (Aspinall, 1963) and thin-layer (Gee, 1963) chromatographic analysis (Table 2).

Structurally related D-glucosides (IV, V and VI; Table 1) were also synthesized when resorcinol was replaced by catechol in the crude enzyme digest. These were characterized by a less rigorous treatment. Compound VI was assumed to be *o*-hydroxyphenyl α -D-glucopyranoside, as on acid hydrolysis it yielded glucose and catechol and on paper chromatograms its behaviour was very similar to that of authentic *o*-hydroxyphenyl β -D-glucopyranoside (Pridham & Saltmarsh, 1963). Treatment of compounds IV and V with dilute ferric chloride solution at 35° oxidized the catechol residues and liberated isomaltose and maltose respectively. The disaccharides were further

characterized by reduction with sodium borohydride followed by paper electrophoretic examination of the resulting alcohols with a molybdate electrolyte (Bourne *et al.* 1959). These results, considered together with the structures for compounds I and II, leave little doubt that compound IV is *o*-hydroxyphenyl α -isomaltoside and that compound V is the corresponding α -maltoside.

Replacement of maltose in the crude enzyme digests by isomaltose also resulted in the formation of resorcinol glucosides, but D-glucose, α -D-glucose 1-phosphate, α -methyl D-glucopyranoside, β -methyl D-glucopyranoside, cellobiose and sucrose did not serve as glucosyl donors in this system. The crude enzyme also exhibited a low specificity with regard to phenolic acceptors. The phenols that were tested and the resulting products, which were presumably all α -D-glucopyranosyl derivatives, are listed in Table 3. Tentative identification of structures is based on the formation of glucose on

Table 3. *Phenol glucosylation products obtained with crude enzyme*

Substrate	Probable structure of aglycone residue	R_F values in solvent B	Electrophoretic mobilities relative to salicylic acid (see Pridham, 1959)			Colour with diazotized <i>p</i> -nitroaniline-NaOH
			E1*	E2*	E3*	
Quinol	<i>p</i> -Hydroxyphenyl	0.54				Blue
Pyrogallol	2,3-Dihydroxyphenyl (?)	0.44				Prussian blue
Phloroglucinol	3,5-Dihydroxyphenyl	0.39				Olive
1,2,4-Trihydroxybenzene	2,4-Dihydroxyphenyl	0.49				Blue-green
<i>p</i> -Nitrophenol	<i>p</i> -Nitrophenyl	0.68				No colour; detected with Ehrlich's reagent (Maddy, 1959)
<i>o</i> -Hydroxybenzyl alcohol	<i>o</i> -Hydroxybenzyl	0.64				Crimson
<i>m</i> -Hydroxybenzyl alcohol	<i>m</i> -Hydroxybenzyl	0.59				Crimson
<i>p</i> -Hydroxybenzyl alcohol	<i>p</i> -Hydroxybenzyl	0.54				Purple-blue
<i>p</i> -Coumaric acid	<i>p</i> -Coumaroyl	0.64	0			Pink
Caffeic acid	Caffeoyl	0.45 (0.30†)	0	0.78	0.46	Olive
Ferulic acid	Feruloyl	0.49	0			Purple
Sinapic acid	Sinapoyl	0.46	0	0.67		Blue

* Electrolytes: E1, acetate, pH 5.2; E2, borate, pH 10.0; E3, molybdate, pH 5.2.

† R_F value on molybdate-treated paper.Table 4. *Purification of enzymes*

A unit of maltose-resorcinol glucosyltransferase activity is defined as the amount of enzyme producing 1 μ mole of *m*-hydroxyphenyl α -D-glucoside in 1 min. under the standard conditions.

Fraction	Vol. (ml.)	Total activity (units)	Total protein (mg.)	10 ² $\times$ Sp. activity (units/mg. of protein)
Crude	1600	63.4	1860	3.4
Precipitated at pH 3.9-2.9	62	1.3	22	6.0
DEAE-cellulose { X	90	0.7	3.3	22.0
{ Y*	92	0.6	2.0	28.0

* With enzyme Y the standard period of incubation for 60 min. gives only an approximate indication of the level of activity (see Fig. 3).

acid hydrolysis (and alkaline hydrolysis for the glucose esters) and paper chromatographic comparison with the corresponding authentic β -D-glucosides (Pridham & Saltmarsh, 1963) and glucose esters (Harborne & Corner, 1961) coupled with colour reactions with diazotized *p*-nitroaniline-sodium hydroxide. For caffeic acid, the ester character of the glucosyl derivative was further confirmed by paper electrophoresis in acetate and molybdate electrolytes, both at pH 5.2. In the former the derivative did not migrate, indicating the absence of a free carboxyl group; in the latter it moved as brown molybdate complex (with an electrophoretic mobility, relative to salicylic acid, of 0.46, which showed that it possessed an *o*-dihydroxy grouping (Pridham, 1959). The *p*-coumaroyl and feruloyl derivatives of glucose were also immobile when subjected to electro-

phoresis in weakly acidic or neutral electrolyte solutions.

The crude preparation of maltose-dihydroxybenzene glucosyltransferase showed no decrease in activity when centrifuged at 98 000*g* for 1 hr. Limited attempts to obtain a highly purified enzyme were only partially successful. Heat denaturation of the crude *A. niger* preparation at 70° resulted in a sixfold decrease in activity, and attempted ammonium sulphate and acetone precipitations also gave poor results. Some purification was eventually obtained by a two-step process (Table 4) involving fractional precipitation by change of pH followed by ion-exchange chromatography. The former procedure gave fractions between pH 3.9 and 2.9 with an approximately two- to three-fold increase in specific activity. These fractions were then chromatographed on DEAE-

Fig. 1. Fractionation of acid-precipitated protein on DEAE-cellulose. A linear gradient was produced by the gradual addition of 600ml. of 0.07M-sodium phosphate buffer, pH 6.8, containing KCl (0.4M) to 600ml. of 0.02M-sodium phosphate buffer, pH 6.8.

Fig. 2. Rate-time curve for *m*-hydroxyphenyl α -D-glucoside (MHPG) formation obtained with transferase X. Conditions are given in the Materials and Methods section.

Fig. 4. Optimum pH values for *m*-hydroxyphenyl α -D-glucoside (MHPG) formation obtained with transferase X (a) and transferase Y (b). Sodium acetate buffers (0.1M) were used.

Fig. 3. Rate-time curve for *m*-hydroxyphenyl α -D-glucoside (MHPG) formation obtained with transferase Y. Conditions are given in the Materials and Methods section.

cellulose with chloride gradient elution. A typical protein concentration profile from an ion-exchange column is shown (Fig. 1) and the positions of two distinct bands (X and Y) of transferase activity

that were found. The final purification factors for fractions X and Y were seven- and eight-fold respectively, although only a small fraction of the total activity in the crude enzyme preparation was recovered. This loss occurred almost entirely at the pH precipitation stage.

An investigation of the properties of the transferase fractions X and Y showed that the former gave a normal rate-time curve for *m*-hydroxyphenyl α -D-glucoside formation (Fig. 2). With fraction Y, however, anomalous curves (Fig. 3) were obtained. It is possible that *m*-hydroxyphenyl α -D-glucoside was formed initially by one enzyme and was then lost as glucose, which was transferred to another unknown acceptor. The later rise could conceivably be due to the presence of a second transferase. The pH optima for fractions X and Y differed in acetate buffer, being 4.5 and 5.1 respectively (Fig. 4). A further difference was the temperature optimum: fraction X exhibited maximum activity at 38° and fraction Y at 24° (Fig. 5). The enzymes could also be distinguished by their thermal stabilities at 50°. Fraction Y was stable for at least 1 hr. at this temperature, whereas fraction X suffered a 50% decrease in activity during this time (Fig. 6).

Further examination of fraction X by using various inhibitors and activators suggested that

Fig. 5. Optimum temperatures for *m*-hydroxyphenyl α -D-glucoside (MHPG) formation obtained with transferase X (a) and transferase Y (b), in acetate buffer, pH 5.7 (0.1 M).

Fig. 6. Heat stabilities (50°C) of transferases X and Y. Enzymes were incubated in acetate buffer, pH 5.7 (0.1 M). Δ , Transferase X; $\circ$, transferase Y.

thiol groups were required for activity. Thus cysteine, GSH and 2,3-dimercaptopropan-1-ol in concentrations of about 1 mM enhanced the activity (Table 5). GSSG unexpectedly activated the enzyme, but this was probably due to the fact that high concentrations of resorcinol in the digests were capable of reducing disulphide groups to thiol groups. It must of course be assumed that direct reduction of protein disulphide groups by resorcinol is impossible for steric reasons. Iodoacetate had no effect on the activity of fraction X; even after preincubation with the alkylating reagent the in-

Table 5. Inhibition and activation of transferase X

Reagent	Concn. (mM or mg.ions/l.)		Activity (% of control)
	During pre-incubation	In digest	
L-Cysteine	0	2.5	143
2,3-Dimercaptopropan-1-ol	0	1.0	160
Glutathione (reduced)	0	1.0	277
Glutathione (oxidized)	0	1.0	206
Iodoacetate	0	1.0	100
	1.0	4.0	77
Phenylmercuric acetate	0	1.0	58
	1.0	0.4	53
<i>p</i> -Chloromercuribenzoate	0	0.66	47
	0.66	0.26	26
<i>p</i> -Chloromercuribenzoate + L-cysteine	0.066	0.026	140
	1.7	0.7	
Hg ²⁺	0	1.0	26
Ag ⁺	0	1.0	46
Pb ²⁺	0	1.0	50
Cu ²⁺	0	1.0	53
Fe ³⁺	0	1.0	70
D-Glucose	0	150	66
α -Glucono-(1 $\rightarrow$ 5)-lactone	0	150	58
Methylene blue (photo-oxidation)	0	0	102*
	0	0.16	102*
Be ²⁺	0	1.0	101

* Illumination time of 60 min.

hibition was comparatively small. Phenylmercuric acetate behaved similarly. Preincubation with 0.66 mM *p*-chloromercuribenzoate gave 74% inhibition. This reaction was reversed by the addition of cysteine. The necessity to preincubate the enzyme with the thiol reagents suggests that these groups occupy protected positions in the protein (cf. Boyer, 1960). Heavy-metal ions that may react with thiol groups also inhibited activity, Hg²⁺ being most effective (Table 5). A small inhibition (12%) produced by EDTA was not reversed by addition of 1 mg.ion/l. concentrations of Na⁺, K⁺, Ca²⁺, Mg²⁺, Zn²⁺, Co²⁺ or Mn²⁺.

L-Histidine residues appear to participate in the hydrolysis of glycosides by various glycosidases (e.g. Wallenfels, 1960; Jørgensen, 1964; Halvorson & Ellias, 1958). Attempts to inhibit fraction X by reaction with Be²⁺ (Wallenfels, 1960) or photo-oxidation in the presence of methylene blue (Wallenfels, 1960; Jørgensen, 1964) failed, however. This may mean either that histidine is not involved in the catalytic reaction or possibly that the residues are again in protected positions. Kelemen & Whelan (1965, 1966a,b) have investigated the competitive inhibition of glycosidases by polyols and have attempted to correlate structure with degree of inhibition. The effect of polyols (0.5 M) on

Fig. 7. Polyol (0.5M) inhibition of transferases X and Y. 1, Ethanediol; 2, propane-1,2-diol; 3, propane-1,3-diol; 4, butane-1,3-diol; 5, butane-1,4-diol; 6, butane-2,3-diol; 7, 2,2-dimethylpropane-1,3-diol; 8, pentane-1,5-diol; 9, 2,5-dimethylhexane-2,5-diol; 10, glycerol; 11, erythritol; 12, pentaerythritol; 13, D-mannitol; 14, D-glucitol. ●, Transferase X; ▲, transferase Y.

the transfer of glucose to resorcinol by the *A. niger* transferases X and Y is illustrated in Fig. 7. In general there appears to be a gradual decrease in inhibition with increasing numbers of hydroxyl groups in the polyol. The kinetics of this inhibition have not been investigated, but it is assumed to be competitive in view of the high concentrations of the polyols that were required to effect significant inhibition.

No detailed and adequate explanation of these results can be attempted at present as it is obvious that many factors may play important roles in this inhibition. By analogy with studies on *E. coli* β -galactosidase (β -D-galactoside galactohydrolase) (Wallenfels, Lehmann & Malhotra, 1960) and almond β -D-glucosidase (Baumann & Pigman, 1957) it may be perhaps assumed that, with the *A. niger* enzymes, the hydroxyl groups on C₍₂₎, C₍₃₎, C₍₄₎ and C₍₆₎ of the non-reducing glucosyl residue of maltose are all involved in enzyme-substrate complex-formation. Thus polyol inhibition can be explained in part on the basis of a similarity between the structure of the polyol and the donor glucosyl moiety. Thus β -diols could bond across enzyme sites normally reserved for C₍₂₎ and C₍₄₎ or C₍₄₎ and C₍₆₎

hydroxyl groups of the donor. The former bonding site would require virtually no conformational change from the normal staggered form of the β -diol. δ -Diols, on the other hand, which are less effective inhibitors of the *A. niger* enzymes, would be required to undergo extensive conformational changes in order to fit the C₍₂₎-C₍₆₎ enzyme site. This theory is based on the assumption that the non-reducing glucosyl residue of maltose is in the C-1 conformation (Rao & Foster, 1963). Other factors that must contribute to the observed order of diol inhibition (i.e. $\beta > \alpha \geq \gamma > \delta$) and the indirect relationship between the number of hydroxyl groups and the percentage inhibition include the relative importance of the individual hydroxyl-binding sites. With almond β -D-glucoside glucohydrolase the primary hydroxyl group on the substrate is apparently not so important for binding as the secondary hydroxyl groups on C₍₂₎, C₍₃₎ and C₍₄₎ (Baumann & Pigman, 1957). The conformational state of the polyol in solution is also largely unknown. With the higher-molecular-weight compounds there are presumably many favoured forms in addition to the conformers with groupings that are exactly staggered. Finally, anti-competitive inhibition may, particularly with the diols, play an important part. β -Diols, for example, could readily occupy sites on the enzyme that are normally reserved for the hydroxyl groups of resorcinol. Two minor anomalies exist in Fig. 7. First, 2,2-dimethylpropane-1,3-diol produces a relatively low inhibition compared with other β -diols. This may be explained by steric hindrance caused by the *gem*-dimethyl group. Secondly, with both enzymes X and Y, D-glucitol produces a somewhat greater inhibition than mannitol. This is probably due to the configurational similarity between D-glucitol and the non-reducing glucosyl residue of the donor.

The phenol-glucosylating ability of the protein fractions from *A. niger* is no doubt due to transglucosylase activity by α -D-glucoside glucohydrolase(s). It is not known whether phenol glucosylation is a fungal detoxification mechanism, as has been postulated for higher plants (see Pridham, 1965a,b).

The formation of disaccharide glucosides probably occurs by a stepwise mechanism (cf. Anderson, Hough & Pridham, 1960), i.e. glucosylation of the phenol followed by further glucosylation of the resulting phenolic glucoside. Visual examination of chromatograms of reaction mixtures of the transferase with maltose and resorcinol shows clearly that the α -isomaltoside is always produced first and that the α -maltoside is always the minor product. It might be expected from steric considerations that an α -(1 $\rightarrow$ 6)-linkage would be synthesized more readily than an α -(1 $\rightarrow$ 4)-linkage and this has been observed with other transglucosylase

systems (Peat, Hinson & Whelan, 1952; Barker, Bourne, Hewitt & Stacey, 1955). The enzymic transfer of a β -D-glucosyl unit from a donor, other than a nucleoside diphosphate glucose derivative, to a phenolic hydroxyl group has yet to be demonstrated. From the evidence obtained so far, however, there appears to be no fundamental reason why this should not be achieved.

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