

ABSTRACT

ABSTRACT: The synthesis of phenolic glycosides by the action of a specific enzyme has been studied. The enzyme was purified from a source and its activity was determined. The reaction was studied under various conditions and the effect of different substrates was investigated. The results are discussed in the following sections.

ENZYMIC SYNTHESIS  
of  
PHENOLIC GLYCOSIDES

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by

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Doctor of Philosophy.

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ABSTRACT

An enzyme from the mould Aspergillus niger with maltose: dihydroxybenzene glucosyltransferase activity has been studied in vitro.

The products from a crude enzyme digest with maltose as the glucosyl-donor and resorcinol as the acceptor were isolated and identified. These were m-hydroxyphenyl  $\alpha$ -D-glucoside (MHPG) and the corresponding  $\alpha$ -isomaltoside, (I), and  $\alpha$ -maltoside, (II). Analogous o-hydroxyphenyl compounds were isolated from digests using catechol as the acceptor.

An assay procedure for the enzyme is described, using a vanillin reagent to determine the concentration of the MHPG formed.

Preliminary fractionation procedures, examined in an attempt to purify the enzyme, included heat denaturation,  $(\text{NH}_4)_2\text{SO}_4$ , acetone and pH precipitation. The latter proved to be the most efficient. As a second step, gel filtration on Sephadex proved less effective than ion exchange chromatography on DEAE-cellulose, using  $\text{Cl}^-$  gradient elution. This method resolved the maltose: dihydroxybenzene glucosyltransferase activity into two components, X and Y.

The pH values for optimum activity of X and Y were 4.5 and 5.1, respectively. At pH 5.1, the respective temperature optima were  $38^\circ$  and  $24^\circ\text{C}$ . X was thermolabile at  $50^\circ\text{C}$ , whereas Y was stable

at this temperature.

In time-course studies on enzymic formation of MHPG, X exhibited Michaelis-type kinetics, whereas Y did not.

Donor and acceptor specificities for X and Y proved to be very similar, that for the acceptor being relatively low.

Preliminary kinetic evidence suggested that X, and possibly Y also, has an acceptor site as well as a donor site. Inhibitor studies provided some evidence as to the possible nature of these active sites.

O- $\alpha$ -D-glucosyl- $\alpha$ -D-glucose derivatives (I) and (II) were formed enzymically by stepwise glucosylation of resorcinol with X. Y appeared to form (I), but not (II).

ACKNOWLEDGEMENTS

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## INTRODUCTION

The existence of phenolic glycosides in plants has been known for more than a century. In 1845, Piria, (1) isolated salicin from *Populus* and *Salix* species and showed that it was a compound of glucose (2-hydroxybenzyl

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and other sugars, such as the  $\alpha$ -D-galactosidase ( $\alpha$ -D-galactoside galactohydrolase) and the  $\alpha$ -D-glucosidase ( $\alpha$ -D-glucoside glucosylhydrolase) of yeast, he was able to synthesize monoglycosides of a variety of mono- and di-hydric alcohols. (7 - 12)

The phenolic glycosides occur to a greater or lesser extent in most parts of the plant, (5,15) and the majority of naturally occurring glycosides are phenolic derivatives.

Conversely, most phenols in plants occur in combination with sugars. (5,14 - 15) In a recent, extensive review of phenolic

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See also for abbreviations and nomenclature for ring systems.

INTRODUCTION \*

The existence of phenolic glycosides in plants has been known for more than a century. In 1845, Piria, <sup>(1)</sup> isolated salicin from Populus and Spiraea species and showed that it was a compound of glucose and saligenin (o-hydroxybenzyl alcohol). Several hundreds of naturally occurring glycosides have since been identified, and their distribution is widespread. <sup>(2 - 5)</sup> Bourquelot <sup>(6)</sup> found glycosides in 205 of the 281 species of phanerogams that he examined. He was the first worker to carry out systematic biochemical studies in vitro on the structure of plant glycosides, using the glycoside-cleaving enzymes of sweet almond emulsin. With these, and other enzymes, such as the  $\alpha$ -galactosidase ( $\alpha$ -D-galactoside galactohydrolase) and the  $\alpha$ -glucosidase ( $\alpha$ -D-glucoside glucohydrolase) of yeast, he was able to synthesize monoglycosides of a variety of mono- and di-hydric alcohols. <sup>(7 - 12)</sup>

The phenolic glycosides occur to a greater or lesser extent in most parts of the plant, <sup>(5,13)</sup> and the majority of naturally occurring glycosides are phenolic derivatives. Conversely, most phenols in plants occur in combination with sugars. <sup>(5,14 - 19)</sup> In a recent, extensive review of phenolic

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\*See p. 145 for abbreviations and nomenclature for ring systems.

glycosides in plants, Harborne (5) states that the following parts of the plant contain these substances: flowers, leaves, fruits, seeds, pollens, roots, tubers and woody tissues. The pattern of glycosides varies from tissue to tissue. In flower petals, anthocyanins ( see p.145) are the main scarlet, red, mauve and blue pigments and yellow colouring may be due to the glycosides of aurones, chalcones or flavonols (see p.145), although much yellow is due to carotenoids. Flavone glycosides are present in many white or ivory-coloured flowers. Phenolic glycosides in fruit are generally simpler in structure than the flower constituents. In leaves, anthocyanin pigmentation is comparatively rare, but flavonol glycosides are widespread. Some seeds contain free phenols, but there is a tendency for these organs to produce complex glycosides. Wheat bran, for example, contains a substance, niyacin, which yields nicotinic acid, o-aminophenol, D-glucose, D-xylose, L-arabinose, ferulic acid and sinapic acid on hydrolysis. (20) Woody tissues contain mainly free phenols, although a few flavanone glycosides are also present.

Williams, (21) in his study of the phenolics of fruit trees, showed that the glucoside phloridzin, (2-hydroxyhydrocinnamyl-3,5-dihydroxyphenyl  $\beta$ -D-glucopyranoside; See p. 18), the main phenolic



of apple trees, is present in the leaf and bark, and, to some extent, in the seeds. The concentration is highest in the root-bark and negligible in the fruit flesh. All parts of the tree contain glycosides of quercetin (5,7,3',4'-tetrahydroxyflavonol) and kaempferol (5,7,4'-trihydroxyflavonol), in varying relative amounts. Quercetin  $\alpha$ -L-arabinoside predominates in the bark (22) and the  $\beta$ -D-galactoside in the leaf and fruit. (23) A phloretin arabinoglucoside occurs in the bark only. Pear trees differ considerably from apple trees in their glycoside components. Phloridzin is entirely replaced in pear by arbutin (p-hydroxyphenyl  $\beta$ -D-glucoside), which, like phloridzin, is present in leaf, bark and seeds, but not in fruit flesh.

The arbutin content of the root-bark, however, is low. Other differences include the structures of the flavonol glycoside components. In pear, kaempferol glycosides predominate and neither quercetin nor kaempferol occurs as the L-rhamnoside. Hybrids of apple and pear contain superimposed glycoside patterns. (24)

The universal occurrence of arbutin in Pyrus species and the widespread existence of phloridzin in Malus is taxonomically significant.

Phenolic glycosides are optically-active compounds which are readily hydrolysed by dilute mineral acids, dilute alkali or

a suitable enzyme. The naturally occurring compounds have the general structure shown in Fig. 1. (25-31)

The glycosidic linkage is almost invariably  $\beta$ - with a D-sugar and  $\alpha$ - with an L-sugar.

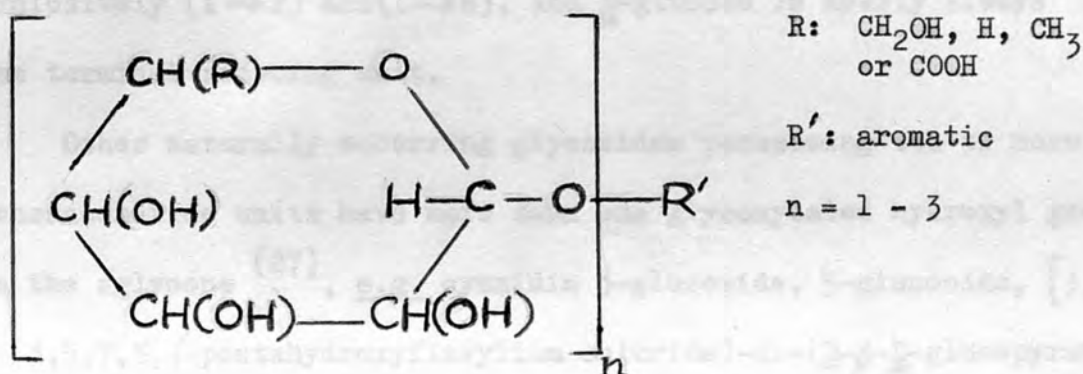


Fig. 1.

The glycone moiety of the phenolic glycoside can be D-glucose, D-galactose, D-fructose, L-rhamnose, D-xylose, L-arabinose or D-glucuronic acid. (25) D-glucose is most common in the natural glycosides, while D-fructose and D-glucuronic acid are rare.

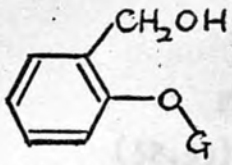
Disaccharide glycosides of phenols are quite common e.g. rutin [3-(5,7,3',4'-tetrahydroxyflavonol)-O- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside] and primeverin [methyl-4-methoxysalicylate O- $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside.] Ten disaccharides combined as phenolic glycosides are known. (5) Of these, sophorose (2-O- $\beta$ -D-glucopyranosyl- $\beta$ -D-glucopyranose), rutinose (6-O- $\alpha$ -L-rhamnopyranosyl- $\beta$ -D-glucopyranose) and sambubiose (2-O- $\beta$ -D-

xylopyranosyl- $\beta$ -D-glucopyranose) are common. Six trisaccharides have been found in phenolic glycosides. (5) All are uncommon. Two rare branched trisaccharides are also known (5,26). Many of the oligosaccharides mentioned above occur only in combination with phenols. The glycosidic linkages in these sugars are almost exclusively (1 $\rightarrow$ 2) and (1 $\rightarrow$ 6), and D-glucose is nearly always the terminal reducing unit.

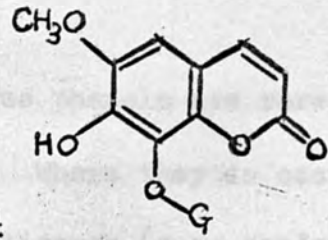
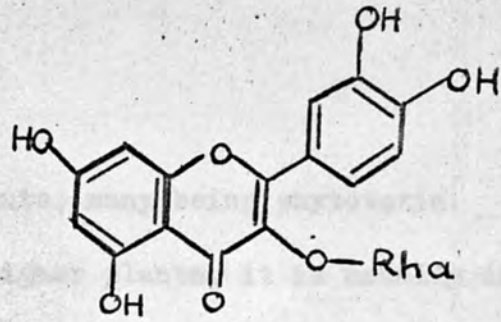
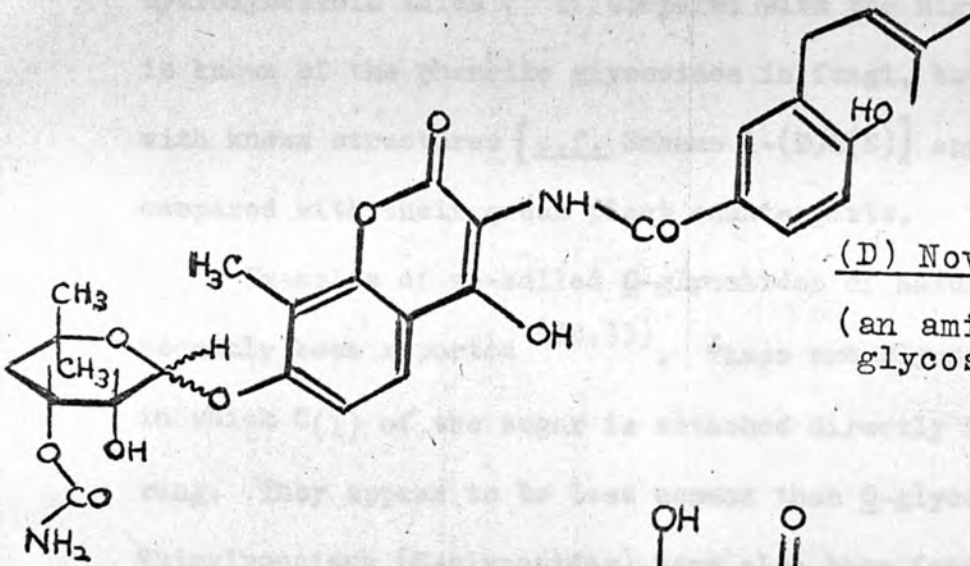
Other naturally occurring glycosides possessing two or more monosaccharide units have more than one glycosylated hydroxyl group in the aglycone (27), e.g. cyanidin 3-glucoside, 5-glucoside, [3, 5-(3,5,7,3',4'-pentahydroxyflavylium chloride)-di-(O- $\beta$ -D-glucopyranoside)]. In some antibiotics of fungal origin, more complex sugars occur e.g. the nitrogenous, methylated deoxy-sugars, novobiiose, (28) in novobiocin, and rhodosamine, in pyrromycin (29). (Scheme 1.).

In the great majority of phenolic monoglycosides, the D-monosaccharide residue is in the pyranoid form and has a  $\beta$ -D-configuration at C(1). The only two  $\alpha$ -D-glucosides known to occur naturally are 3-(3,5,7,3',4'-pentahydroxyflavylium chloride)-O- $\alpha$ -D-arabinoside (5) and 3-(5,7,3',4'-tetrahydroxyflavonol)-O- $\alpha$ -D-arabinoside (5) (avicularin), which both occur in several species.

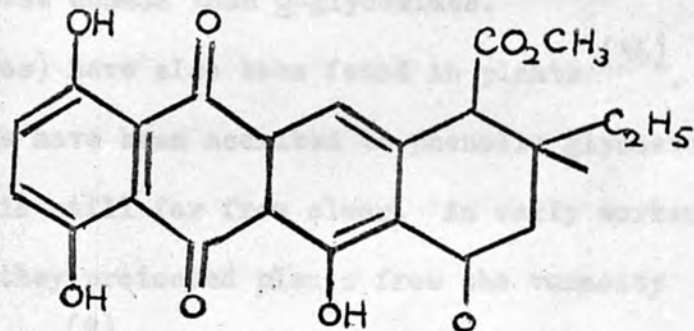
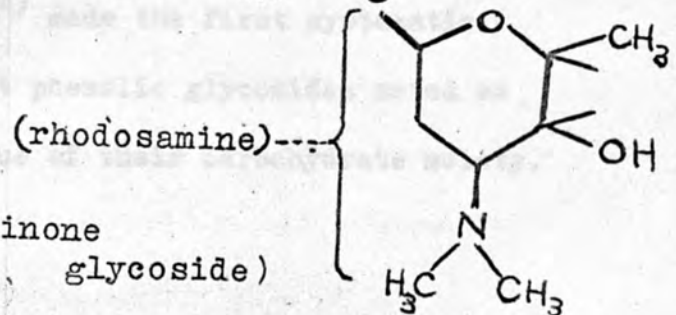
The aglycones of the natural phenolic glycosides may be simple phenols e.g. saligenin [Scheme 1(A)] or they may contain condensed ring systems (e.g. Schemel; c.f. Appendix p.145).

(A) Salicin

(G = glucosyl)

(B) Fraxin(a coumarin  
glucoside)(C) Quercitrin(Rha = rhamnosyl;  
a flavonol glycoside)(D) Novobiocin(an aminocoumarin  
glycoside)

(novobiiose)

Scheme 1(E) Pyrromycin (a rutilantinone  
glycoside)

Free phenols are rare in plants, many being phytotoxic (32,33). Where they do occur in higher plants, it is usually in storage tissues (e.g. seeds and berries) and in dead tissue (e.g. heartwood) (5). All the groups of phenols which occur in higher plants form glycosides, but lower plants, particularly fungi, contain free anthraquinones, hydroxycinnamic acids and hydroxybenzoic acids (5). Compared with the higher plants, little is known of the phenolic glycosides in fungi, but those compounds with known structures [c.f. Scheme 1-(D)&(E)] appear to be complex compared with their green plant counterparts.

Examples of so-called C-glycosides of natural products have recently been reported (34,35). These are glycosyl derivatives in which C(1) of the sugar is attached directly to an aromatic ring. They appear to be less common than O-glycosides. Thioglycosides (S-glycosides) have also been found in plants (36).

A number of functions have been ascribed to phenolic glycosides in plants, but their role is still far from clear. An early worker, Errera (37), claimed that they protected plants from the voracity of animals! In 1897, Pfeffer (2) made the first systematic investigation and concluded that phenolic glycosides acted as secondary food reserves by virtue of their carbohydrate moiety.

He noted that their production was a periodic phenomenon and refuted the suggestion that these substances were merely waste-products.

By the 1920's, workers in the field were strongly divided in their opinion on the physiological role of glycosides. Pfeffer's hypothesis was supported by the work of Bourquelot (38) on oleuropin in olives and by that of Vintesco (38) on syringin in Syringa and Ligustrum species, which showed that glycosides accumulated in winter and disappeared during vegetative growth. Weevers (39) detected seasonal variations in the salicin content of Salix and in the amounts of other glycosides in Pyrus, Populus and Vaccinium species. Wasicky (40), however, attributed such variations to osmoregulation. Hérissé (41) extensively reviewed the evidence at this period and decided in favour of Pfeffer's hypothesis. Goris (3) was the author of an equally comprehensive review supporting the theory that phenolic glycosides are waste-products of plant metabolism. He claimed that glycosides were present to a greater extent in the external parts of the plant and supposed this to be evidence of their end-product nature. This view was supported by Straub (42), who observed that glycosides in Digitalis seeds underwent no change after formation.

In 1926, Bridel (4) attempted to resolve the dilemma by

showing that the two theories were compatible. He demonstrated that perennials, such as Menyanthes, showed no ability to utilise glycosides as food reserves, whereas annuals, such as Rhinanthus, used up some of their glycoside reserve before the onset of photosynthesis. Guignard <sup>(43)</sup> found that Phaseolus lunatus used about a third of its cyanohydrin glucoside when grown in the dark for a month. Bridel <sup>(4)</sup> considered that the two theories differed only in the relative importance attached to the different reactions. On the one hand, the important part of the molecule was the aglycone, and the sugar entered the nutrition cycle as a secondary phenomenon; on the other hand, the aglycone was of lesser importance, and the glycoside was cleaved in order to utilise the glycone. He concluded that the food reserve function was of greater consequence.

The waste product theory has never been proved and would seem unlikely considering the wide variety of naturally occurring glycosidic residues.

The food reserve role of glycosides cannot yet be stated with certainty. It is possible that they are used up under starvation conditions, and Hutchinson <sup>(44)</sup> et al. showed that phlorin synthesis decreased and the phloroglucinol content increased in Pelargonium and Gerbera species under starvation.

The glycosides may be important sources of less common sugars, such as L-arabinose and L-rhamnose, at all growth stages.

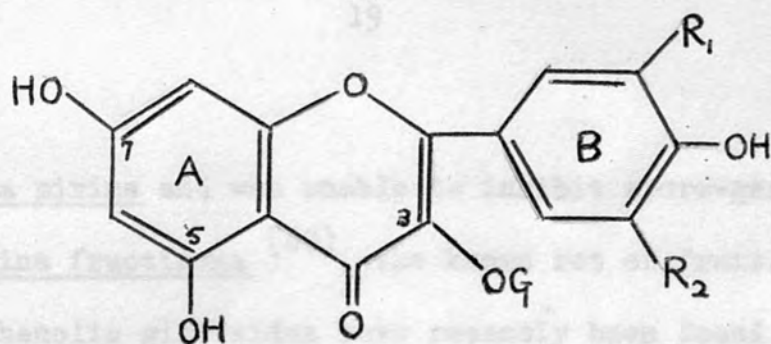
Reviews by Armstrong (13) and Frey-Wyssling (45) suggested that no single function might be assigned to the glycosides. They perform a variety of functions resulting from the diversity of their glycones and aglycones. Frey-Wyssling believed that glycoside formation was a means of solubilising the phenols in the cell sap. However, some glycosides e.g. hesperidin, are insoluble in aqueous systems and others are less soluble than the corresponding aglycones, for example salicin.

The only well-established function of phenol glycosylation is the detoxification of phytotoxic aglycones. Several workers have shown that foreign phenols are rapidly converted to mono- $\beta$ -D-glucosides, when fed to leaves and seedlings of higher plants (44,46 - 53). Kosuge and Conn (50) showed that o-coumaric acid fed to sweet clover shoots was rapidly and almost entirely converted to the phenolic  $\beta$ -D-glucoside. By contrast, Harborne and Corner (52) found that other cinnamic acid derivatives gave rise mainly to D-glucose esters, when fed to a variety of plants. Only caffeic acid gave some of the phenolic D-glucoside (3-caffeic acid  $O$ - $\beta$ -D-glucoside). Harborne suggested that esterification rather than phenol glucosylation occurred in vivo

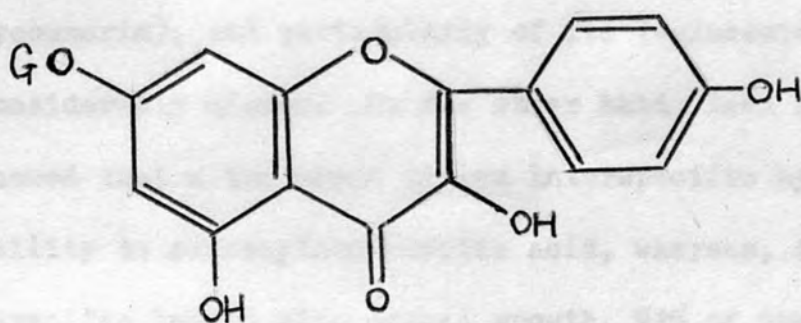


with this group of compounds in order to neutralize the acid groups and enhance sap-solubility. There may be a connection between these facts and the recently-discovered (54,55) special role of phenol glucosylation in the biosynthesis of coumarins and 7-hydroxycoumarins from hydroxycinnamic acid derivatives. This is one of the rare cases so far known, where glucosides, rather than the free aglycones, are essential intermediates in biosynthesis. It is interesting to note that 3-caffeic acid O-β-D-glucoside has been found in wild, tuberous Solanum species, but is absent from the non-tuberous, cultivated species. The latter, however, contain considerable amounts of a glucose ester, 1-p-coumaroyl-D-glucose, which is absent from the wild species. Pridham (56) showed that the ability to glucosylate foreign phenols is widespread in the plant kingdom. It was prominent among gymnosperms and angiosperms, with the exception of a few water plants. The various ferns and the single liverwort tested all formed glucosides. Many of the mosses that were investigated gave traces of glucoside, but this ability was entirely absent from the algae and fungi that were examined. The conversion of phenols to their glucosides in the plant kingdom is analogous to the formation of phenolic glucuronides as a mechanism for phenol detoxification in animals.

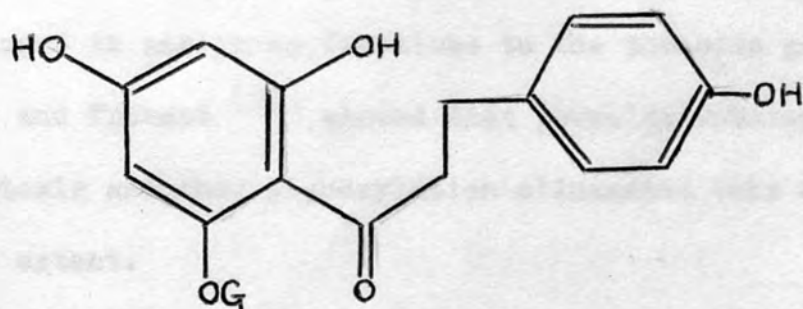
A further function of phenol glycosylation in plants may be the protection of the phenol from enzymic oxidation. In studies on potato phenolics, by Baruah and Swain <sup>(18)</sup>, and on tea phenolics, by Roberts and Wood <sup>(57)</sup>, the actions of the accompanying polyphenol oxidase (polyphenol: O<sub>2</sub> oxidoreductase) on the glycosides phloridzin, aesculin, chaerophyllin, quercitrin, rutin and myricitrin, and on their free aglycones were compared. The latter were all substrates for the enzyme, whereas all glycosides except phloridzin and chaerophyllin were total inhibitors of enzyme action. All the non-substrates were glycosylated at C<sub>(3)</sub> and Roberts <sup>(58)</sup> pointed out that such compounds are non-planar, due to steric effects between the C<sub>(3)</sub>- glycosyl group and the aryl group on C<sub>(2)</sub>. Chaerophyllin, which is glycosylated at C<sub>(7)</sub>, and phloridzin, which is glycosylated at C<sub>(5)</sub>, are not, however, sterically hindered in this way (see Scheme 2). Hence the shape of the glycoside molecule determines its inhibitor action with polyphenol oxidase. Similar results were obtained by Siegelman <sup>(23)</sup> using quercetin glycosides with apple-skin polyphenol oxidase.



(A) 3-Flavonol glycoside  
(G = glycosyl)



(B) 7-Flavonol glycoside



(C) Phloridzin

Scheme 2

It has frequently been claimed that phenolics inhibit growth and sporulation of fungi which attack fruit. Thus, Kirkham and Flood (59) showed that arbutin inhibited the growth of Venturia, an infection of apple and pear, whereas quinol stimulated it. On the other hand, phloridzin stimulated the growth of

Venturia pirina and was unable to inhibit spore-germination of Sclerotinia fructigena (60), the brown rot of fruit.

Phenolic glycosides have recently been found in tumourous tissue of interspecific Nicotiana hybrids (61). Compared with the parent species, the levels of scopoletin (6-methoxy-7-hydroxycoumarin), and particularly of its 7-glucoside, scopolin, were considerably higher. On the other hand, Asen and Emsweller (62) showed that a tumourous Lilium interspecific hybrid had poor ability to glucosylate ferulic acid, whereas, in an intra-specific hybrid with normal growth, 94% of the ferulic acid occurred as the glucose ester. These results may be significant in assigning functions to the phenolic glycosides. Deysson and Truhaut (63) showed that phenolic substances interfered with mitosis and that glycosylation eliminated this effect to a large extent.

It has been suggested that the flavonol glycosides almost universally present in plant leaves may act as growth regulators (5). In general, glycosides of the kaempferol type, with a single hydroxyl group at the 4'- position of the B - ring [Scheme 2(A):  $R_1=R_2=H$ ] were inhibitors of indoleacetic acid oxidase (N-acetylxindoxyl: $O_2$  oxidoreductase). Glycosides of

quercetin, with two hydroxyl groups in the B-ring, at the 3'- and 4'-positions, [e.g. Scheme 2(A) :  $R_1=OH$ ;  $R_2=H$ ] acted as co-factors for the enzyme.

The range of sugar components in phenolic glycosides is very striking and, although its significance is not yet known, future work may reveal relationships between the repeating units of polysaccharides and the glycones present in the plant (5).

The function of oligoglycosides is not known, but Pridham (27) tentatively suggested that they might be used as starting-materials for biosynthesis of complex heteroglycans, where they might be pre-formed as phenol oligoglycosides and then transferred in toto to the heteroglycan by transglycosylation. In this connection, disaccharides with transferase activity, which hydrolyse primeverosides and rutinosides to the aglycone and disaccharide, have been found in Rhamnus species (64,65).

The biosynthesis of the aglycone and glycone components of phenolic glycosides has been partially understood for some time. The carbohydrates (66) arise from chlorophyllic assimilation of carbon dioxide in sunlight, and the phenols are derived either from shikimic acid or from condensations of acetyl coenzyme A units (67,68). Until recently, the mechanism for the formation in vivo of phenolic glycosides from these

components was unknown. It was generally supposed that the sugar moiety was attached at a late stage in the process, after the formation of the phenol.

Ciamician and Ravenna (47,69,70) were the first workers to demonstrate the biosynthesis of phenolic glucosides from phenols, by feeding saligenin, catechol and quinol (47) to Zea mays and other plants. Pridham and Saltmarsh (51), however, obtained mainly the alcoholic glucoside (o hydroxybenzyl  $\beta$ -D-glucoside), when feeding saligenin to Zea mays and Salix daphnoides, and Bourquelot<sup>(6)</sup> found the same product when D - glucose and saligenin were incubated with almond emulsin. Recent work with Vicia faba suggests that both the alcoholic and phenolic glucosides are formed in significant amounts in vivo from saligenin, but that the former predominates (71). Miwa (48) et al. found that the formation of phenolic glucosides from several simple phenols and D-glucose infused into leaf discs of various plants, required oxygen, and occurred more rapidly in the presence of  $\alpha$ -D-glucose -1-phosphate. Hutchinson (44) and co-workers demonstrated that glucosides of simple phenols could be formed in the presence of D-glucose with infused Malus and Gerbera leaf discs, but that no glycosylation occurred with D-fructose, L-rhamnose, D-xylose and L-arabinose, under these conditions. In contrast to the

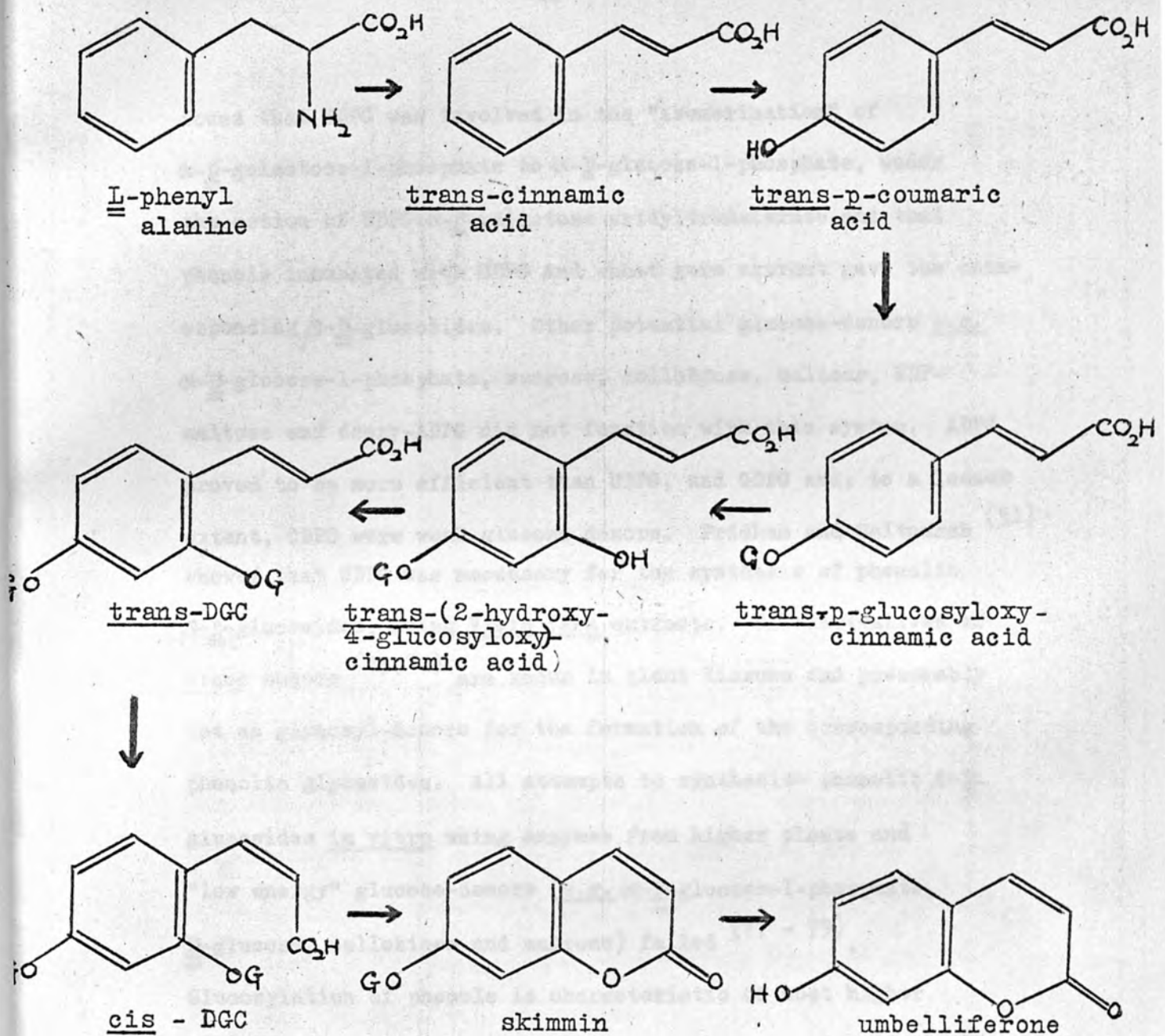
observations of Harborne and Corner <sup>(52)</sup>, (see p.15 ), that many species convert cinnamic acid derivatives almost entirely to the D-glucose esters, Runeckles and Woolrich <sup>(53)</sup> found that tobacco leaf discs, infused with cinnamic acids, formed appreciable amounts of both the phenolic glucosides and the glucose esters. The former predominated in the ratio of 2:1 and of 4:1 for p-coumaric and ferulic acids, respectively. Pridham and Saltmarsh <sup>(51)</sup> showed that the major products of mono-, di- and tri-hydric phenols, fed to Vicia faba seeds, were the mono- $\beta$ -D-glucosides. Phenol itself proved to be highly toxic to the plant, but Nystrom <sup>(72)</sup> obtained phenyl  $\beta$ -D-glucoside by feeding traces of phenol to barley and wheat leaves.

The rule of the attachment of the sugar moiety to the phenol after the formation of the latter has one notable exception i.e. in the biosynthesis of coumarins <sup>(54,55)</sup>. (Scheme 3).

The phenolic glycosides formed in the biosyntheses mentioned are all  $\beta$ -D-anomers and the D-glucose-donor in vivo is believed to be the "high energy" nucleotide sugar, UDPG, which occurs very widely in plant tissues <sup>(73)</sup> and was first isolated by Cardini and Leloir <sup>(74 - 76)</sup>, in 1949. They

coumarin biosynthesis

[ $\beta$ -D-glucosyl ; UDPG - 2,4-di-( $\beta$ -D-glucosyloxy)-cinnamic acid]



Scheme 3 : Role of glucosides in coumarin biosynthesis

[G = D-glucosyl ; DGC = 2,4-di-( $\beta$ -D-glucosyloxy)-cinnamic acid]



found that UDPG was involved in the "isomerisation" of  $\alpha$ -D-galactose-1-phosphate to  $\alpha$ -D-glucose-1-phosphate, under the action of UDPG: $\alpha$ -D-galactose uridyltransferase and that phenols incubated with UDPG and wheat germ extract gave the corresponding  $\beta$ -D-glucosides. Other potential glucose-donors e.g.  $\alpha$ -D-glucose-1-phosphate, sucrose, cellobiose, maltose, UDP-maltose and deoxy-ADPG did not function with this system. ADPG proved to be more efficient than UDPG, and GDPG and, to a lesser extent, CDPG were weak glucose donors. Pridham and Saltmarsh (51) showed that UDPG was necessary for the synthesis of phenolic  $\beta$ -D-glucosides, using Vicia faba extracts. UDP-derivatives of other sugars (73,77) are known in plant tissues and presumably act as glycosyl-donors for the formation of the corresponding phenolic glycosides. All attempts to synthesize phenolic  $\beta$ -D-glucosides in vitro using enzymes from higher plants and "low energy" glucose-donors (e.g.  $\alpha$ -D-glucose-1-phosphate, D-glucose, cellobiose and maltose) failed (77 - 79).

Glucosylation of phenols is characteristic of most higher plants, but, as previously mentioned, occurs slowly, if at all with Bryophytes and Thallophytes. Since UDPG is ubiquitous in plant tissues, inability to glucosylate phenols appears to be due to a lack of the glucosyltransferase (80).

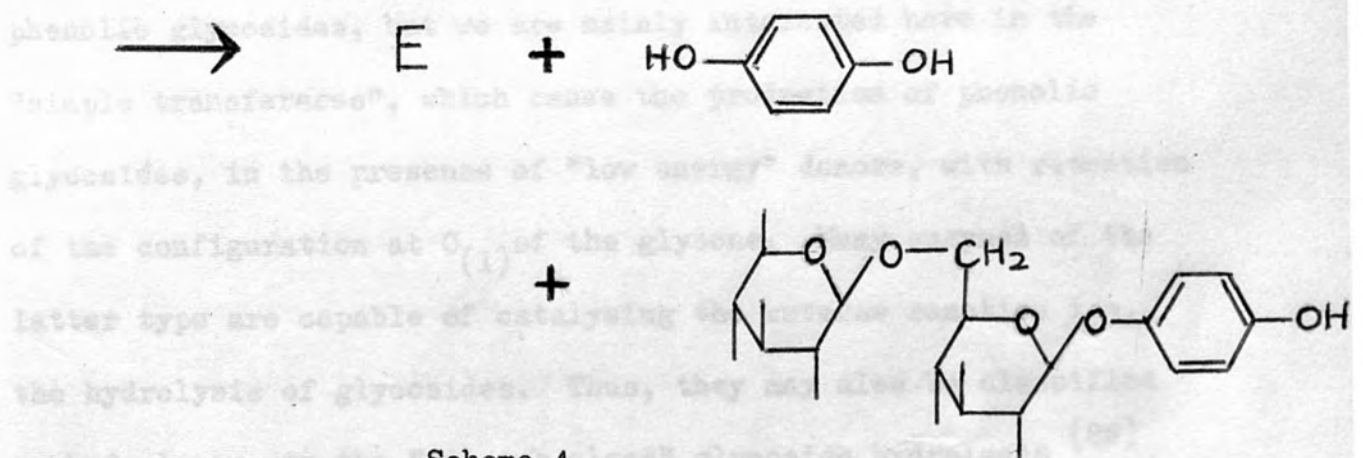
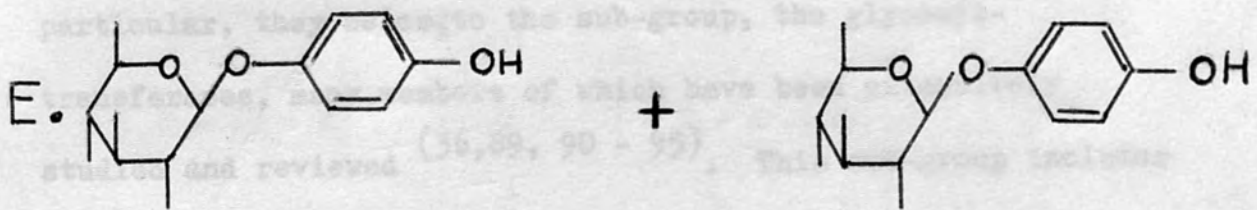
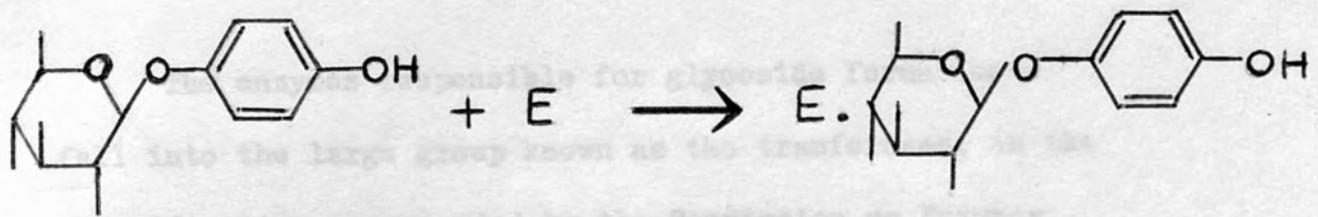
The first synthesis in vitro of a phenolic glucoside from a "low energy" glucosyl-donor was reported by Pridham <sup>(81)</sup>, in 1961. This was achieved with an Aspergillus niger extract using maltose or isomaltose as the donor and various simple phenols as acceptors. The corresponding  $\alpha$ -D-glucosides were formed. Glucose-donors which were not substrates in this reaction were  $\alpha$ -D-glucose-1-phosphate, sucrose, methyl  $\alpha$ -D-glucoside and cellobiose.

Two other instances of the conversion of phenols to their glycosides in the presence of a "low energy" donor and a lower plant or bacterial enzyme have recently been reported. Miwa <sup>(82)</sup> transferred D-fructose from sucrose to quinol, to give the  $\beta$ -D-fructoside, using yeast invertase ( $\beta$ -D-fructoside fructohydrolase) and Pridham and Wallenfels <sup>(83)</sup>, obtained phenolic  $\beta$ -D-fucosides using o-nitrophenyl  $\beta$ -D-fucopyranoside as a donor with E. coli  $\beta$ -galactosidase ( $\beta$ -D-galactoside galactohydrolase). With lactose,  $\beta$ -D-galactosides were formed, together with two possible O-digalactosyl derivatives.

Miller <sup>(84)</sup> was the first worker to report biosynthesis of a phenolic diglucoside in feeding experiments. He obtained o-chlorophenol  $\beta$ -gentiobioside [o-chlorophenyl-O- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 6)- $\beta$ -D-glucopyranoside] by the treatment of

gladioli corms with o-chlorophenol. Pridham (85) observed the production of p-hydroxyphenol  $\beta$ -gentiobioside with arbutin (p-hydroxyphenyl  $\beta$ -D-glucoside) and a Populus grandidentata extract. Almond emulsin and a Vicia faba enzyme preparation gave similar results with arbutin (79). Cardini and Yamaha (77,86) found two enzymes in wheat germ, one of which converted quinol to p-hydroxyphenyl  $\beta$ -gentiobioside, and the other of which converted the monoglucoside, arbutin, to the gentiobioside. Both required UDPG. In the presence of the Vicia faba enzyme of Pridham, quinol and D-glucose or quinol and gentiobiose produced neither arbutin nor the gentiobioside. The  $\beta$ -D-glucosidase and synthetic activities appeared to be due to one enzyme, and it was suggested that arbutin molecules act as both acceptors and donors in the gentiobioside formation. UDPG is not involved in this scheme (Scheme 4) (27).

Scheme 4  
 [5- $\beta$ -D-glucoside glucosyltransferase]  
 Barber (87) converted quercetin to quercetin 5- $\beta$ -D-glucoside using UDPG or TDPG, and thence to the diglycoside rutin, using TDP-L-rhamnose, in the presence of a Fusarium leaf preparation.



Scheme 4

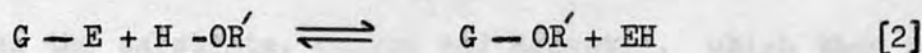
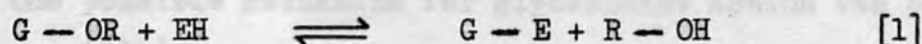
[E =  $\beta$ -D-glucoside glucohydrolase]

Barber <sup>(87)</sup> converted quercetin to quercetin 3- $\beta$ -D-glucoside using UDPG or TDPG, and thence to the diglycoside rutin, using TDP-L-rhamnose, in the presence of a Phaseolus aureus leaf preparation.

The enzymes responsible for glycoside formation fall into the large group known as the transferases, in the classification recommended by the Commission on Enzymes of the International Union of Biochemistry, 1961 (88). In particular, they belong to the sub-group, the glycosyltransferases, many members of which have been extensively studied and reviewed (36, 89, 90 - 95). This sub-group includes enzymes which use "high energy" nucleotide sugars as donors, as well as those which use oligosaccharides or glycosides. Members of both types of glycosyltransferase are capable of forming phenolic glycosides, but we are mainly interested here in the "simple transferases", which cause the production of phenolic glycosides, in the presence of "low energy" donors, with retention of the configuration at C<sub>(1)</sub> of the glycone. Many enzymes of the latter type are capable of catalysing the reverse reaction i.e. the hydrolysis of glycosides. Thus, they may also be classified as hydrolases, in the "sub-sub-class" glycoside hydrolases (88), the members of which are often known by the trivial name "glycosidases". They include those enzymes which hydrolyse low molecular weight glycosides (i.e. alkyl and aryl glycosides and transferases known which do not possess hydrolase activity and many hydrolases are also transferases. One transferase without hydrolase activity is the D-enzyme of potato (maltodextrin).

oligosaccharides) and also those which are responsible for the build-up and break-down of polysaccharides, in the absence of "high energy" glycosyl-donors.

The general transglycosylation reaction may be represented by the following two-step scheme (91,92,96):



#### Scheme 5

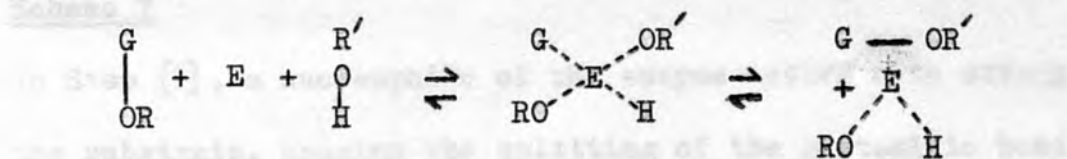
[E = glycosyl-transferase, G = glycosyl group.]

Step [1] is the formation of a complex or complexes between the glycosyl-donor and the active-site of the enzyme.

R may be alkyl, aryl or glycosyl. Step [2] is rate-determining (93) and involves an attack on the complex by the acceptor molecule, R'O-H. It may be a transfer reaction, if R' is aryl, glycosyl or alkyl (primary or secondary alcoholic, but usually not tertiary), or a hydrolysis, if R' = H. Hence hydrolysis is really only a special case of transfer and the two activities usually run parallel during enzyme purifications, indicating that a single enzyme is responsible for both (97,98). There are few transferases known which do not possess hydrolase activity and many hydrolases are also transferases. One transferase without hydrolase activity is the D-enzyme of potato (maltodextrin:

D-glucose 4-maltodextrinyltransferase), which transfers polyglucosyl chains from starch and maltodextrins to D-glucose, with no trace of hydrolytic products (99,100). It is possible, therefore, that some enzymes of this group operate by markedly different mechanisms from the one described in Scheme 5.

One possible mechanism for glycosidase action was suggested by Jermyn (101). This involves the formation of a ternary complex between the substrate, enzyme and acceptor, which then breaks down to give the products (Scheme 6).



Scheme 6

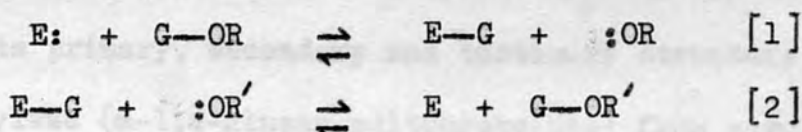
In this model, the displacement is a frontal one, which accounts for the retention of the anomeric configuration of the glycone. The scheme does not, however, explain the high specificity towards the glycone, as compared with the aglycone.

The ternary complex hypothesis was supported by kinetic studies on Stachybotrys atra  $\beta$ -glucosidase (102) ( $\beta$ -D-glucoside glucohydrolase) and Stringer (103) and Neely (104)

showed that it is probably the mechanism for dextransucrase ( $\alpha$ -1,6 -glucan:D-fructose 2-glucosyltransferase), which transfers  $\alpha$ -D-glucosyl residues from sucrose to suitable carbohydrate acceptors. This latter enzyme is unusual, however,

since it is not a hydrolase. There is no evidence to suggest that Jermyn's hypothesis fits glycosidase action generally.

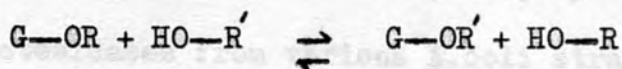
Koshland <sup>(105)</sup> proposed another possible mechanism for glycosidase action, which gives overall retention of the anomeric configuration by double displacement. This is illustrated in Scheme 7.



#### Scheme 7

In Step [1], a nucleophile of the enzyme active site attacks the substrate, causing the splitting of the glycosidic bond and formation of a covalent bond between the glycosyl residue and the enzyme surface.

Step [2] is the nucleophilic attack on this species by the group  $\text{-OR}'$  from the acceptor. In both steps, the nucleophilic attack is on the side opposite to the outgoing group, giving overall retention of the anomeric configuration. An instance of this mechanism is known on the exchange of phosphate by sucrose phosphorylase <sup>(106)</sup> (disaccharide glucosyltransferase), a non-specific enzyme, which catalyses the general reaction:



in which  $\text{R-OH}$  and  $\text{R}'\text{-OH}$  may be various ketoses, L-arabinose,



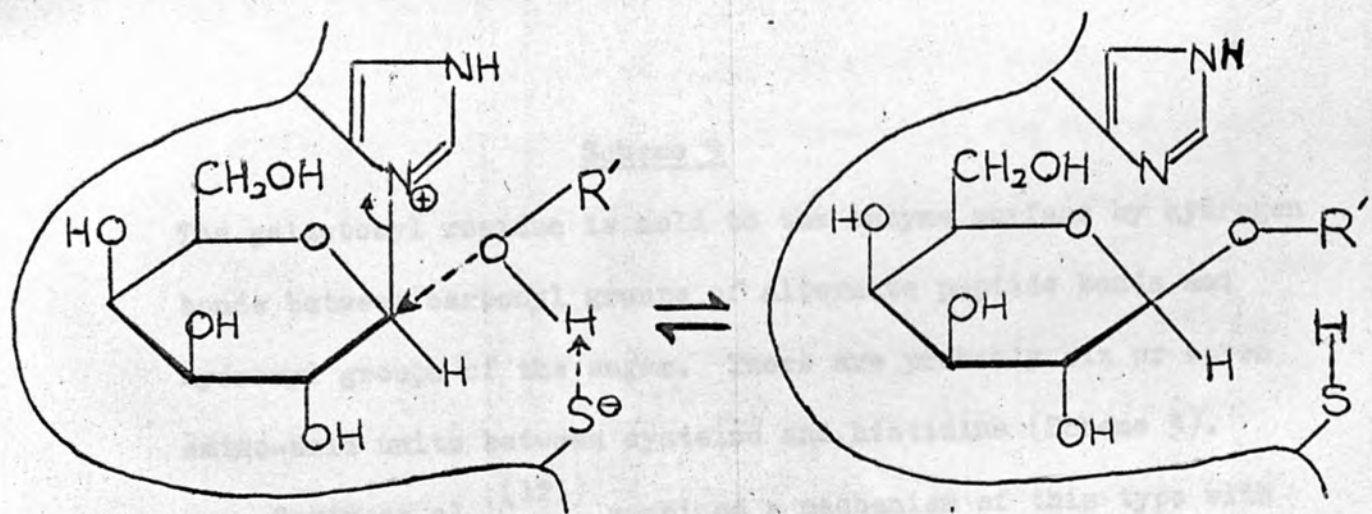
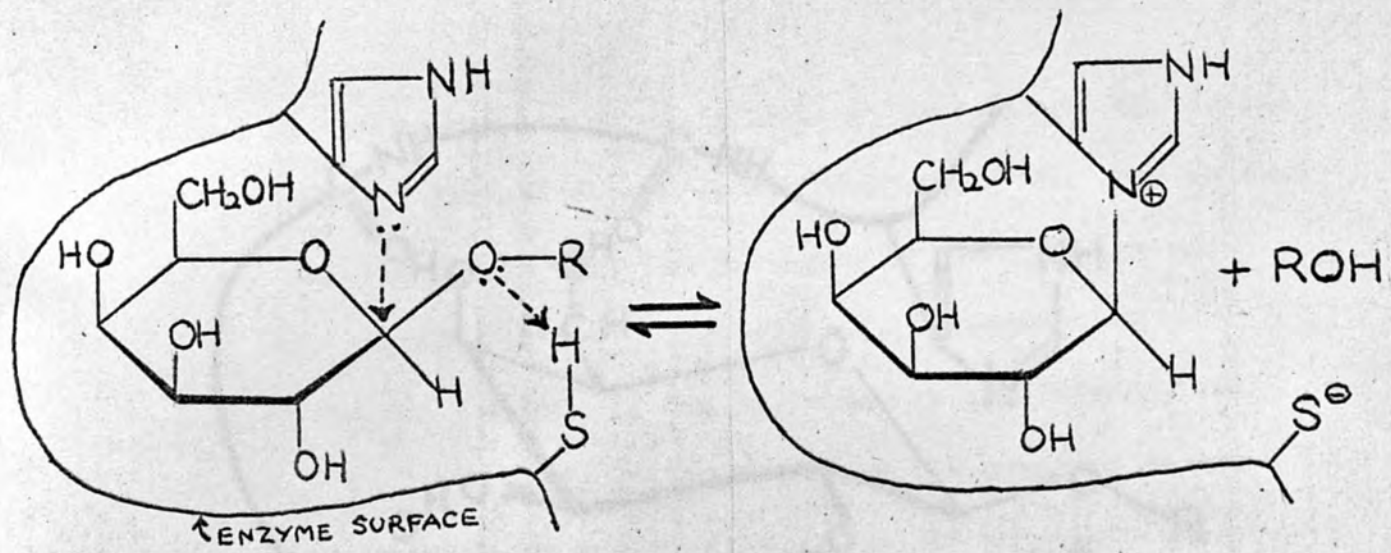
phosphate or arsenate.

One enzyme of the glucosidase group that deserves special mention is the E.coli  $\beta$ -galactosidase, because it is the only glycosyltransferase to be completely purified and crystallised and to be extensively studied by chemical and physical methods [although the glycosidase lysozyme (N-acetylmuramide glycanohydrolase) has been crystallised and much of its primary, secondary and tertiary structure elucidated (107).  $\beta$ -Amylase ( $\alpha$ -1,4-glucan maltohydrolase) from soya bean was also recently obtained in a high state of purity (108).]

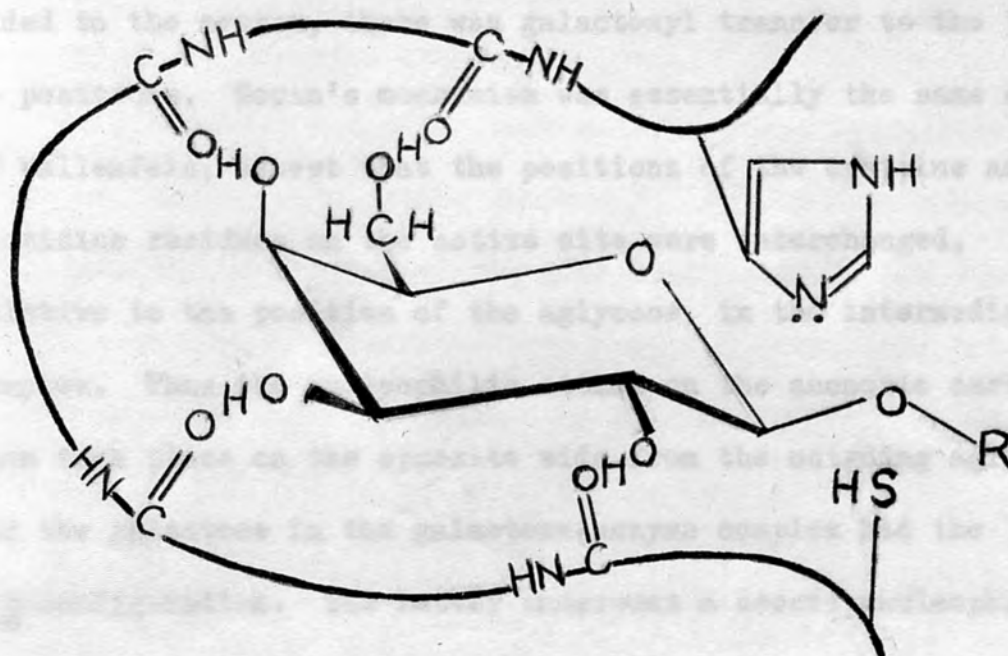
Samples of E.coli  $\beta$ -galactosidase were prepared from strain ML 309 by Wallenfels (109 - 111), strain ML 308 by Hu (112) and strain ML 35 by Sund (113). All appeared to have very similar chemical properties. Evidence from several sources (93,111,113 - 117) suggests that the molecule normally exists as a tetramer, of molecular weight  $5.2 \times 10^5$ . Each sub-unit has a C-terminal lysine unit, and a probable hexamer (93), of molecular weight  $7.5 \times 10^5$ , had four N-terminal threonine residues and two N-terminal glutamic acid units. Anfinsen (118) suggested that the sub-units of the molecule were chemically different. Appel (116) formed by induction several higher polymers from single, tetrameric  $\beta$ -galactosidases from various E.coli strains and hybrids.

Wallenfels (93,119) extensively studied the chemical properties of E.coli  $\beta$ -galactosidase. It had both hydrolase and transferase activities, which were enhanced by  $\text{Na}^+$  and  $\text{K}^+$  ions (120). Sugars, alcohols, phenols (83) and water all functioned as acceptors and transfer products could be detected after less than one minute reaction time. The glycone specificity was comparatively high, being restricted to  $\beta$ -D-galactopyranosides with unsubstituted hydroxyl groups on  $\text{C}_{(2)}$ ,  $\text{C}_{(3)}$ ,  $\text{C}_{(4)}$ , and  $\text{C}_{(6)}$ . Replacement of the primary alcohol group by  $\text{-H}$  or  $\text{-CH}_3$  (giving  $\alpha$ -L-arabinosides or  $\beta$ -D-fucosides, respectively) resulted in a decreased rate of hydrolysis. By contrast, the aglycone specificity was wide, embracing glycosyl, alkyl and aryl groups. The hydrolysis rate, however, depended upon the aglycone. Inhibitor studies indicated the presence of L-cysteine and L-histidine in the enzyme active site.

These observations led Wallenfels (93) to postulate a mechanism of the type shown in Scheme 5. This more detailed scheme is illustrated in Scheme 8.



Scheme 8



#### Scheme 9

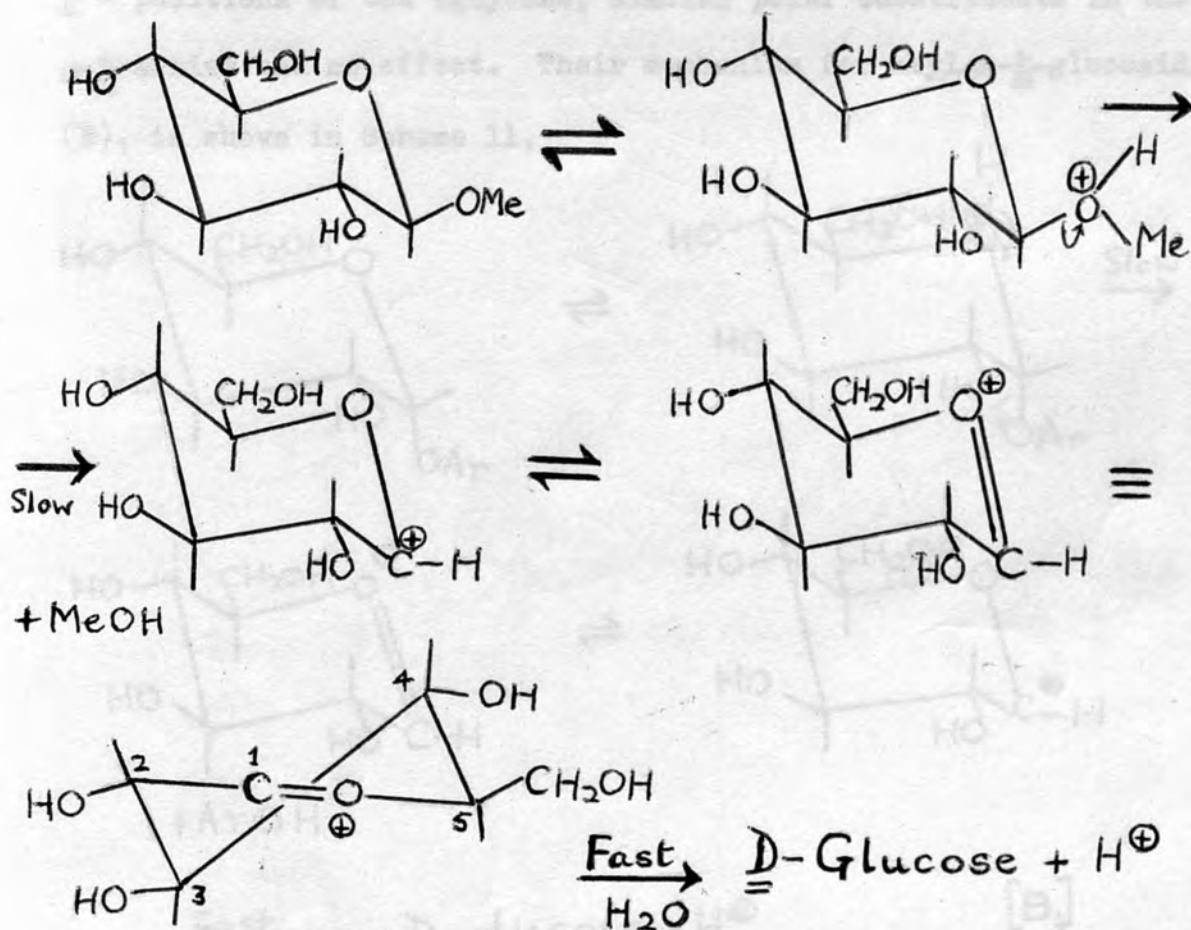
The galactosyl residue is held to the enzyme surface by hydrogen bonds between carbonyl groups of alternate peptide bonds and hydroxyl groups of the sugar. There are probably six or seven amino-acid units between cysteine and histidine (Scheme 9).

Gorin et al. <sup>(121)</sup> combined a mechanism of this type with Koshland's <sup>(105)</sup> double displacement theory to explain the action of Sporobolomyces singularis  $\beta$ -galactosidase (lactose: lactose 4-galactosyltransferase) which synthesized digalactosyl- and tri-galactosyl- glucosides from lactose in vivo. If D-glucose was

added to the system, there was galactosyl transfer to the 3- and 6- positions. Gorin's mechanism was essentially the same as that of Wallenfels, except that the positions of the cysteine and histidine residues on the active site were interchanged, relative to the position of the aglycone, in the intermediate complex. Thus the nucleophilic attack on the anomeric carbon atom took place on the opposite side from the outgoing aglycone, and the galactose in the galactose-enzyme complex had the  $\alpha$ -D-configuration. The latter underwent a second nucleophilic attack to revert to the  $\beta$ -D-configuration. This, Gorin considered, was more in accord with the energy requirements for the reaction at the anomeric carbon atom (i.e. the reaction was probably an  $S_N2$  type, where the nucleophile attacks on the opposite side from the outgoing hydroxyl group, while the other substituents on  $C_{(1)}$  lie approximately in a plane. This arrangement minimises the exchange energy between bonds which are breaking and those which are forming, and gives rise to inversion<sup>(122)</sup>.) Gorin noted that, unlike many enzymes of this type, which glycosylate the  $C_{(6)}$  primary alcohol group most readily, the S.singularis enzyme in vivo preferentially glycosylated secondary hydroxyl groups of the acceptor, which had a vicinal hydroxyl group. This explained the comparative lack of hydrolysis in the system and

suggested that the acceptor might also be bound to the enzyme surface by its hydroxyl groups.

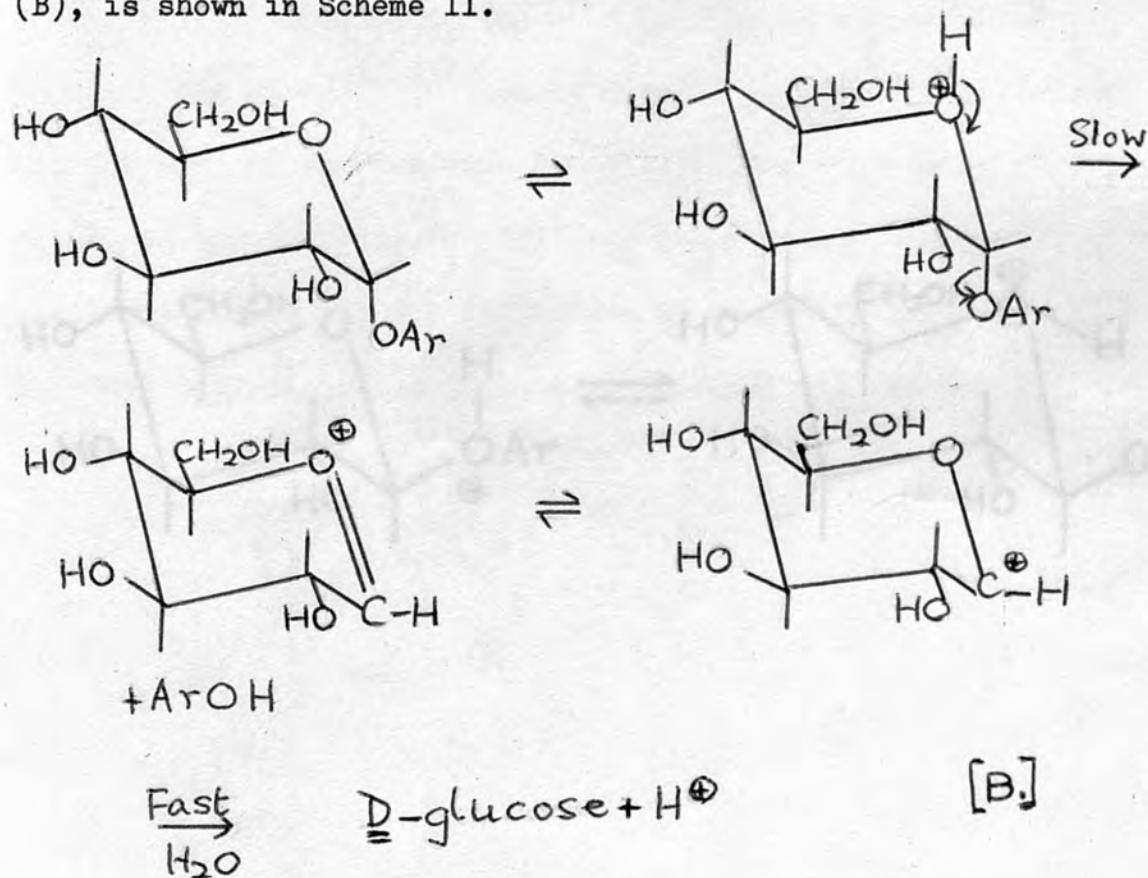
A similarity has often been noted between hydrolyses of glycosides by acid and by enzymes <sup>(123)</sup>, which both occur by aldose-oxygen fission of the glycosidic bond. This does not accord with all the observed facts, however. The acid hydrolysis mechanism, (A), well-established by Vernon <sup>(124 - 126)</sup> and Overend, <sup>(129 - 130)</sup> is shown in Scheme 10.



Scheme 10

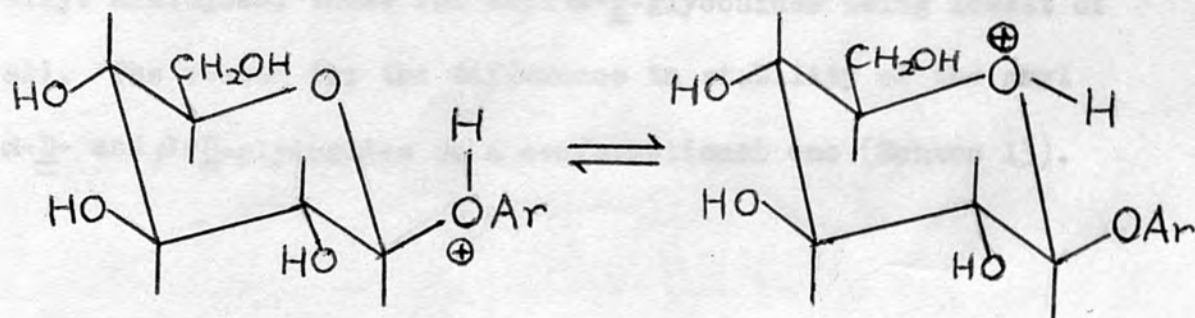
[A]

This mechanism appears to be valid for all glycosides, except tert-alkyl compounds, which do not undergo glycone-oxygen fission, because of a strong tendency to form stable carbonium ions by alkyl-oxygen fission. Nath and Rydon <sup>(131)</sup>, however, postulated another mechanism for acid hydrolysis of aryl  $\alpha$ -D-glycosides, based on their observation that whereas, in the  $\beta$ -D-series, there was increased reactivity due to electron-repelling groups and decreased reactivity due to electron-attracting groups substituted in o - and p - positions of the aglycone, similar polar substituents in the  $\alpha$ -D-series had no effect. Their mechanism for aryl  $\alpha$ -D-glucosides, (B), is shown in Scheme 11.



Scheme 11

In this mechanism protonation is axial, (due to shielding of the equatorial position by the aryloxy group) and decomposition of the conjugate acid by trans-elimination of two axial groups is controlled by steric, rather than electronic, effects. With aryl glycosides, the ring oxygen atom is more basic than the glycosidic oxygen atom (due to the electron-withdrawing effect of the aryl group) and the apparent anomaly of protonation of the glycosidic oxygen in the aryl  $\beta$ -D-series was explained by invoking mesomerisation of the conjugate acid in this case, caused by the proximity of the two oxygen atoms, (Scheme 12).



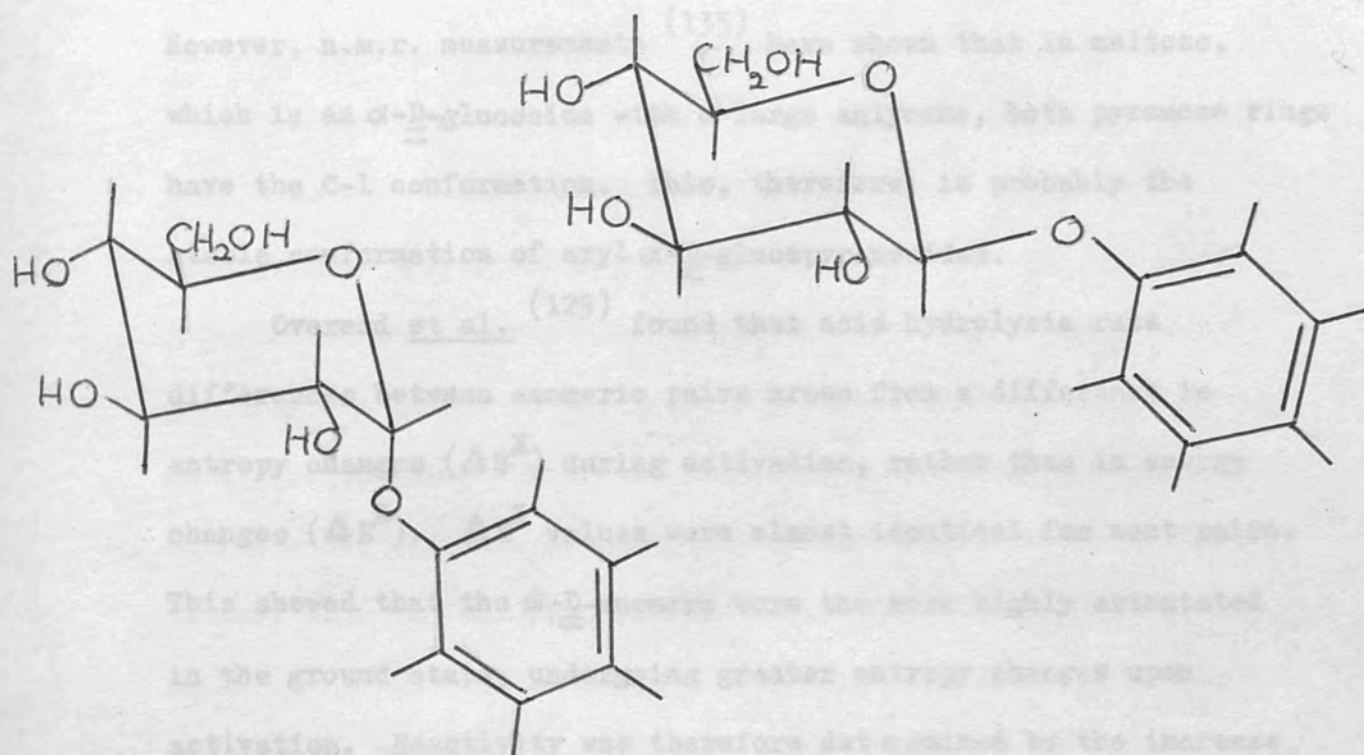
Scheme 12



Timell, (132) however, believed that the apparent lack of effect of polar groups in the  $\alpha$ -D-case was due to the coincidental cancelling of the opposite effects which such groups have on the protonation of the glycosidic oxygen, on the one hand, and on the heterolysis of the conjugate acid, on the other.

In the  $\beta$ -D-case, these effects happen to only partially cancel, but their difference is small.

The early data of Moelwyn-Hughes (133) on activation energies of glycosides during acid hydrolysis, was expanded by Heidt and Purves (134) and by Overend et al. (129). Values for the aryl glycosides were generally lower than their alkyl analogues, those for aryl  $\alpha$ -D-glycosides being lowest of all. The reason for the difference in stability of the aryl  $\alpha$ -D- and  $\beta$ -D-glycosides is a conformational one (Scheme 13).



Aryl  $\alpha$ -D-glucoside

Aryl  $\beta$ -D-glucoside

Scheme 13

In the C-1 conformation the  $\alpha$ -D-anomer has an ( $Ar_{1a}$ ) group, which is subjected to more repulsion than the ( $Ar_{1e}$ ) group of the  $\beta$ -D-anomer. It is questionable whether the aryl  $\alpha$ -D-glucopyranosides exist in the C-1 conformation, since it is conceivable that the combined ( $H_{3a}$ )-(Ar<sub>1a</sub>) and ( $H_{5a}$ )-(Ar<sub>1a</sub>) repulsions might be greater than ( $OH_a$ ) interactions of some other conformation, having an ( $Ar_{1e}$ ) group (e.g. the 1-C conformation).

However, n.m.r. measurements <sup>(135)</sup> have shown that in maltose, which is an  $\alpha$ -D-glucoside with a large aglycone, both pyranose rings have the C-1 conformation. This, therefore, is probably the stable conformation of aryl  $\alpha$ -D-glucopyranosides.

Overend et al. <sup>(129)</sup> found that acid hydrolysis rate differences between anomeric pairs arose from a difference in entropy changes ( $\Delta S^{\ddagger}$ ) during activation, rather than in energy changes ( $\Delta E^{\ddagger}$ ).  $\Delta E^{\ddagger}$  values were almost identical for most pairs. This showed that the  $\alpha$ -D-anomers were the more highly orientated in the ground state, undergoing greater entropy changes upon activation. Reactivity was therefore determined by the increase in entropy due to restriction of rotation of the aglycone by ( $H_{3a}$ ) and ( $H_{5a}$ ), rather than by the increase in potential energy of the molecule, due to repulsion between these substituents and the aglycone (see Scheme 13).

Similar results were found by Timell <sup>(132)</sup> for the anomeric pair maltose and cellobiose. This supports the claim that aryl  $\alpha$ -D-glucosides are conformational analogues of maltose. It is interesting to note that Semke et al. <sup>(136)</sup>, studying the effect of polar substituents in glycone and aglycone upon the hydrolysis rates of two series of aryl  $\beta$ -D-glucosides, found that changes in the aglycone were not reflected in  $\Delta S^{\ddagger}$  or  $\Delta E^{\ddagger}$  values, while

the introduction of a carboxyl group at C<sub>(6)</sub> of the glycone (glucuronides) increased the magnitude of  $\Delta E^X$ , but not that of  $\Delta S^X$ . This suggested that aryl  $\beta$ -D-glucuronides undergo acid hydrolysis by the same mechanism as aryl  $\beta$ -D-glucosides, and that the increased stability of the  $\beta$ -D-glucuronides is due to the inductive effect of the carboxyl group on C<sub>(5)</sub>. If this effect were transmitted as far as the glycosidic oxygen atom, it would decrease the partial negative charge on the latter, thus diminishing the concentration of the conjugate acid. It would also oppose the heterolysis rate. Both of these effects would lower the hydrolysis rate. Even if the inductive effect of the carboxyl group is only transmitted as far as the ring oxygen atom, the tendency of the latter to stabilise the carbonium ion by an electron density drift to C<sub>(1)</sub> (giving partial oxonium ion character) would be diminished and a lower hydrolysis rate would result <sup>(136)</sup>. In some cases, (e.g. *n*-alkyl carboxylic acids), the inductive effect of a carboxyl group is transmitted to some extent through the solvent molecules.

It therefore seems likely that the lower hydrolysis rate of phenyl  $\beta$ -D-glucuronide, compared with phenyl  $\beta$ -D-glucoside, is due to the stabilising effect of the C<sub>(5)</sub>-carboxyl group. In the case of methyl  $\alpha$ -D-glucuronide and methyl  $\alpha$ -D-galacturonide,

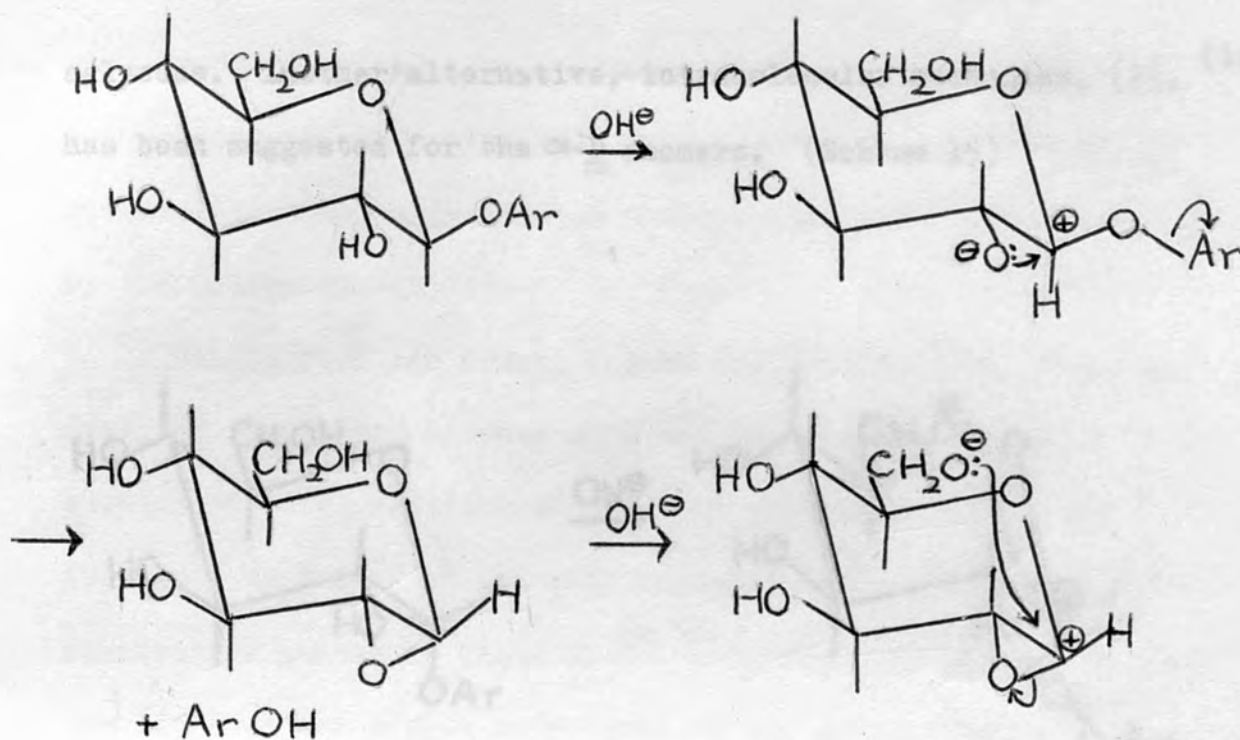
however, the  $\Delta S^\ddagger$  values are significantly different from those of the corresponding methyl  $\alpha$ -D-glycosides (137,138). In this case, the mechanism of acid hydrolysis of the glycosides probably differs from that of the glucuronides, although the latter has not so far been elucidated.

Some of the data of Overend, (129) Timell (132) and Semke (136) are summarized in Tables 1 and 2. (p. 50)

Nath and Rydon (131,139,140) produced the first data which give a clue to the probable nature of enzymic hydrolysis and synthesis of glycosides, although their interpretation was too narrow. (Tables 3 & 4; p. 52) These workers compared the action of acid and of alkali upon a comprehensive group of aryl  $\alpha$ - and  $\beta$ -D-glucosides with the enzymic hydrolysis, using brewer's yeast  $\alpha$ -glucosidase ( $\alpha$ -D-glucoside glucohydrolase) and almond emulsin ( $\beta$ -D-glucoside glucohydrolase), respectively. They noted a resemblance between alkaline and enzymic hydrolysis of the two series of glucosides, both being facilitated by electron-attracting substituents in the aglycone. Inspection of their data, however, shows that nearly all the electron-repelling substituents tested also increased the enzymic hydrolysis rate, which is a feature of acid hydrolysis and a fact that Nath and Rydon overlooked. Another

important fact emerges from the values for the break-down rate constant of the enzyme-substrate complex. In all groups of compounds tested (i.e., Cl, CH<sub>3</sub>O, CN and NO<sub>2</sub>), with one minor exception, the rates for the three positional isomers were o- > m- > p- (although data for the substituted α-D-glucosides are restricted mainly to m- and p- examples). Such an order of rates can only be attributed to steric factors, which must, therefore, be relatively more important in enzymic hydrolysis. It is interesting to note that Wallenfels<sup>(141)</sup> found with E.coli β-galactosidase that the order of reactivity of a series of thiogalactosides is 2:4:6-trinitrophenyl S-β-D-thiogalactoside >> o-nitrophenyl S-β-D-thiogalactoside > phenyl S-β-D-thiogalactoside. This might be due to electronic effects, but could also be due to a steric selectivity of the enzyme for o-substituted aryl aglycones.

Alkaline hydrolysis of phenyl β-D-glucosides probably occurs by the mechanism (C), shown schematically in Scheme 14, in which the glycosidic bond splits by aglycone-oxygen fission<sup>(142)</sup>.



→ 1,6 - anhydro - D - glucose

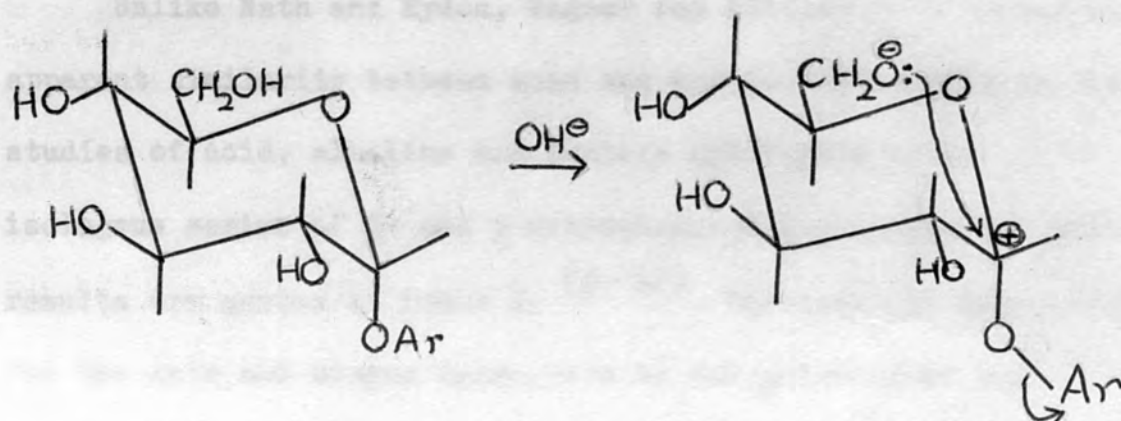
→ 1,6 - anhydro - D - glucose + [C]

Scheme 14

The nucleophilic attack on the anomeric carbon atom is enhanced by electron-attracting substituents in the aglycone, which make the anomeric carbon atom more acidic.

Phenyl  $\alpha$ -D-glucosides react only very slowly with alkali. If mechanism (C) is correct, this slowness would be due to the difficulty of forming an epoxide ring where there is a cis, rather than a trans-, arrangement of the C<sub>(2)</sub>-hydroxyl group and the

aglycone. Another alternative, intramolecular mechanism, (D), (142) has been suggested for the  $\alpha$ -D-anomers. (Scheme 15)



→ 1,6-anhydro-D-glucose + ArOH

[D]

Scheme 15

Overend et al. (143) showed that aryl 2-deoxy- $\alpha$ -D-glucopyranosides were also cleaved by alkali, which seems to preclude mechanism (C) for aryl  $\alpha$ -D-anomers, since 2-deoxy-sugars cannot form 1,2-epoxides. He also found that 1,6-anhydrides were



only produced in relatively strong alkali. At lower hydroxyl ion concentrations, solvolysis predominated to give 2-deoxy-D-glucose (together with its 3,6 - anhydride, which arose from it by the action of alkali).

Unlike Nath and Rydon, Wagner and Metzner <sup>(123)</sup> found an apparent similarity between acid and enzymic hydrolysis in their studies of acid, alkaline and emulsin hydrolysis of an isologous series of o- and p-nitrophenyl  $\beta$ -D-glucosides. Their results are quoted in Table 5. (p. 54) The order of reactivity for the acid and enzyme hydrolysis of the p-isologues was  $\underline{O} \gg \underline{Se} > \underline{S}$ . This was also the order for acid in the o-series, while that for the enzyme in this series was  $\underline{O} \gg \underline{S} \gg \underline{Se}$ , an order which Wagner attributed to steric repulsion between the nitro-group and the Se atom. The latter order, however, is that which would be expected from the decreasing electronegativity of oxygen, sulphur and selenium. The order  $\underline{O} \gg \underline{Se} > \underline{S}$  is not readily explicable. The order for alkali in all cases was quite different *i.e.*  $\underline{Se} > \underline{O} > \underline{S}$ .

Complete analogies cannot therefore be drawn between enzymic and acid hydrolyses, on the one hand, or enzymic and alkaline hydrolyses, on the other. The duality which exists in the present reassessment of Nath and Rydon's results strongly

suggests that glycosidase action is of a general acid-base catalysis type, as proposed by Wallenfels<sup>(141)</sup> and Eigen<sup>(144)</sup>, inter alia. Inspection of the postulated mechanism of Wallenfels (Scheme 8), or the similar one due to Gorin, shows this acid-base nature. There is both protonation of the glycosidic oxygen atom by cysteine and nucleophilic attack on C<sub>(1)</sub> of the glycone by the basic nitrogen atom of histidine. Glycosides resemble esters in their ability to be cleaved by both acids and bases, and hydrolase action in general (including esterases) has been attributed to acid-base catalysis<sup>(145)</sup>.

Nath and Rydon suggested that, in addition to the hydrogen-bonding of the donor molecule to the active site of the enzyme, the acceptor was also bonded, but by weaker forces. This they attributed to van der Waals attraction<sup>(131)</sup>.

Gorin's<sup>(121)</sup> work suggests that hydroxyl groups in the acceptor, as well as those in the donor, may form hydrogen-bonds with the enzyme. In the case which he studied, one hydroxyl group in the acceptor appeared to be sufficient, whereas the donor was much more rigidly bound, by up to four hydroxyl groups hydrogen-bonded to the enzyme. This would explain both the weaker bonding of the acceptor as compared with the donor and the relatively low specificity of the enzyme towards the acceptor.

Table 1: Thermodynamic data for the acid hydrolysis of  
 (129,132)  
 glycosides

Glycoside	$\Delta E^*$ (Kcal.mole.) <sup>-1</sup>	$\Delta S^*$ (cal.deg.mole.) <sup>-1</sup>
Phenyl $\alpha$ - <u>D</u> -glucopyranoside	31.1	+13.3
Phenyl $\beta$ - <u>D</u> -glucopyranoside	31.0	+10.8
p-Nitrophenyl $\alpha$ - <u>D</u> -glucopyranoside	30.3	+10.5
p-Nitrophenyl $\beta$ - <u>D</u> -glucopyranoside	30.3	+ 6.4
Phenyl $\alpha$ - <u>D</u> -galactopyranoside	30.2	+13.5
Phenyl $\beta$ - <u>D</u> -galactopyranoside	28.1	+ 4.1
Methyl $\alpha$ - <u>D</u> -glucopyranoside	34.1	+14.8
Methyl $\beta$ - <u>D</u> -glucopyranoside	34.3	+16.5
Maltose [ <u>O</u> - $\alpha$ - <u>D</u> -glucopyranosyl (1 $\rightarrow$ 4)- <u>D</u> -glucopyranose]	32.7	+14.0
Cellobiose [ <u>O</u> - $\beta$ - <u>D</u> -glucopyranosyl (1 $\rightarrow$ 4)- <u>D</u> -glucopyranose]	31.5	+ 9.0

Table 1: Thermodynamic data for acid, alkali and enzyme hydrolysis for substituted aryl- $\beta$ -D-glucosides (131,139)

Table 2: Thermodynamic data for the acid hydrolysis of substituted aryl- $\beta$ -D-glucosides (136)

Glycoside	$\Delta E$ (Kcal.mole. <sup>-1</sup> )	$\Delta S$ (Cal.deg. <sup>-1</sup> mole. <sup>-1</sup> )
Phenyl $\beta$ -D-glucopyranoside	29.9	+10 <sup>+1</sup>
p - Cresyl $\beta$ -D-glucopyranoside	30.3	+11 <sup>+1</sup>
p - Chlorophenyl $\beta$ -D-glucopyranoside	30.1	+9 <sup>+2</sup>
Phenyl $\beta$ -D-glucuronopyranoside	32.2	+10 <sup>+1</sup>
p - Cresyl $\beta$ -D-glucuronopyranoside	32.3	+10 <sup>+1</sup>
p - Chlorophenyl $\beta$ -D-glucuronopyranoside	34.4	+11 <sup>+2</sup>

- $K_{eq}$  = equilibrium constant for  $\alpha$ -glucosidase hydrolysis (under given conditions)
- $k_{+2}$  = first order velocity constant for break-down of enzyme-substrate complex
- $k_{acid}$  = first order velocity constant for hydrolysis in 0.1N-HCl
- $k_{alk}$  = first order velocity constant for hydrolysis in 3.9N-NaOH

Table 3: Thermodynamic data for acid, alkali and enzymic hydrolysis for substituted phenyl- $\alpha$ -D-glucosides (131,139)

Substituent in aglycone	$\bar{X}$ $K_a$	$\bar{X}$ $k_{+2}$	$\bar{X}$ $10^4 \times k_{acid}$ ( $\text{min}^{-1}$ )	$\bar{X}$ $10^4 k_{alk}$ ( $\text{min}^{-1}$ )
H	1270	7.55	11.7	0.06
<u>o</u> - Me	3968	9.51	7.7	0.02
<u>m</u> - Me	1798	10.27	11.5	0.02
<u>p</u> - Me	1496	6.21	9.2	0.03
<u>m</u> - MeO	2095	10.40	12.2	0.07
<u>p</u> - MeO	1820	5.02	10.7	-
<u>m</u> - Cl	3885	13.12	12.0	0.09
<u>p</u> - Cl	2229	8.40	9.7	0.20
<u>m</u> - NO <sub>2</sub>	3097	11.89	9.1	10.7
<u>p</u> - NO <sub>2</sub>	6272	7.48	10.0	18,000

$\bar{X}$

$K_a$  = equilibrium constant for  $\alpha$ -glucosidase hydrolysis (under given conditions)

$k_{+2}$  = first order velocity constant for break-down of enzyme-substrate complex

$k_{acid}$  = first order velocity constant for hydrolysis in 0.1N-HCl

$k_{alk}$  = first order velocity constant for hydrolysis in 3.9N-NaOH

Table 4: Thermodynamic data for acid, alkaline and enzyme (140)  
hydrolysis of substituted phenyl  $\beta$ -D-glucosides

Substituent	$\bar{x}K_a$ (l.mole. <sup>-1</sup> )	$\bar{x}10^8 \times K'_3$ (min. <sup>-1</sup> )	$\bar{x}10^4 \times k_{acid}$ (min. <sup>-1</sup> )	$\bar{x}10^4 \times k_{alk}$ (min. <sup>-1</sup> )
H	36.75	22.5	1.15	2.54
<u>o</u> -Me	43.4	904	1.81	1.16
<u>m</u> -Me	52.95	56.75	1.405	1.92
<u>p</u> -Me	76.9	17.1	2.32	0.960
<u>o</u> -Pri <sup>i</sup>	345	56.05	-	0.512
<u>p</u> -Pri <sup>i</sup>	238	9.11	-	0.750
<u>o</u> -Bu <sup>t</sup>	132	6.675	-	0.137
<u>p</u> -Bu <sup>t</sup>	157	3.62	-	0.672
2:4-Me <sub>2</sub>	171	24.9	-	0.554
2:6-Me <sub>2</sub>	28.8	13.3	-	1.152
<u>o</u> -MeO	58.1	965.5	8.96	7.62
<u>m</u> -MeO	38.5	132	0.770	6.28
<u>p</u> -MeO	53.45	58.8	2.407	0.944
<u>o</u> -Cl	222	832	1.73	230.3
<u>m</u> -Cl	102.5	228	0.760	19.7
<u>p</u> -Cl	119.5	44.0	0.830	8.96
<u>m</u> -CN	182	690.5	0.509	-
<u>p</u> -CN	419.5	286	0.366	-
<u>o</u> -NO <sub>2</sub>	161.5	3515	1.66	>50,000
<u>m</u> -NO <sub>2</sub>	222.5	460	0.404	147.4
<u>p</u> -NO <sub>2</sub>	500	385	0.330	~5,000

 $\bar{x}$ 

$K_a$  = equilibrium constant for emulsin hydrolysis (under given conditions).

$K'_3$  = first order velocity constant for decomposition of enzyme-substrate complex.

$k_{acid}$  = first order velocity constant for hydrolysis by 0.1N-HCl.

$k_{alk}$  = first order velocity constant for hydrolysis by 4 N-NaOH.

Table 5: Thermodynamic data for the acid, enzyme and alkali hydrolysis for isologous series of o- and p-nitrophenyl  $\beta$ -D-glucosides (123)

Position of $\text{-NO}_2$	Glycosidic bridging atom	$x_{k_{\text{enz}}}$ ( $\text{hr.}^{-1}$ )	$x_{k_{\text{acid}}}$	$x_{k_{\text{alk}}}$
p -	<u>O</u>	56.65	2.303	9.00
p -	<u>S</u>	0.823	0.047	1.482
p -	<u>Se</u>	3.442	0.084	~ 60
o -	<u>O</u>	61.04	5.187	15.04
o -	<u>S</u>	0.32	0.035	0.748
o -	<u>Se</u>	0.00	0.049	~ 50

$x_k$  = rate constant for decomposition of the glycoside

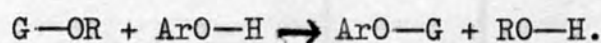
MAIN SECTION

The work with Aspergillus niger maltose-  
 dihydroxybenzene glucosyltransferase can be divided into  
 four main sections, namely, identification of the reaction  
 products, development of a routine assay method, purification  
 of the enzyme and experiments with the purified enzyme.

I : Identification of the Reaction Products

The enzyme from the mould A.niger which catalyses the  
 formation of phenolic  $\alpha$ -D-glucosides from suitable glucosyl-  
 donors and a variety of phenolic acceptors was first reported  
 by Pridham (81) and some preliminary investigations were  
 carried out by Pridham and Saltmarsh (146).

The general reaction catalysed by the enzyme may be  
 summarised by the following equation:



R is an alkyl or glucosyl group, G is an  $\alpha$ -D-glucopyranosyl  
 residue and Ar is an aryl group.

Donor and acceptor substrates for the enzyme will be  
 described in detail in part IV.

Incubation of resorcinol, maltose and the A.niger enzyme,  
 at pH 5.1, for 24 hr, produced three monosubstituted  
 resorcinol derivatives [(I),(II) and (III), numbered in order of



increasing  $R_f$  values; see Table 6], each giving a pink spot with the diazotised *p*-nitroaniline/NaOH reagent (Spray 1).

Table 6 :  $R_f$ -values [Solvents (A) & (B)] for *o* and *m*-hydroxyphenyl  $\alpha$ -glucosides.

Glucoside	Colour of Spot (Spray 1)	$R_f$		Identity
		A	B	
(I)	Pink	0.34	0.28	<u><i>m</i></u> -hydroxyphenyl $\alpha$ -iso/maltoside
(II)	Pink	0.45	0.36	<u><i>m</i></u> -hydroxyphenyl $\alpha$ -maltoside
(III)	Pink	0.62	0.54	<u><i>m</i></u> -hydroxyphenyl $\alpha$ - <u>D</u> -glucopyranoside
(IV)	Crimson	0.31	0.40	<u><i>o</i></u> -hydroxyphenyl $\alpha$ -iso/maltoside
(V)	Crimson	0.41	0.48	<u><i>o</i></u> -hydroxyphenyl $\alpha$ -maltoside
(VI)	Crimson	0.61	0.67	<u><i>o</i></u> -hydroxyphenyl $\alpha$ - <u>D</u> -glucopyranoside

Samples of glucosides were isolated from a large-scale enzyme digest by means of preparative paper chromatography. They were stored in the freeze-dried state. The ratio of carbohydrate to phenol in each glucoside was found by hydrolysis with yeast

maltase ( $\alpha$ -D-glucoside glucohydrolase) <sup>(147)</sup>, followed by determination of the liberated resorcinol with vanillin reagent and the glucose with glucose oxidase ( $\beta$ -D-glucose oxidoreductase) reagent <sup>(148)</sup>. The course of the hydrolysis by maltase is shown for each glucoside in Fig. 2, (p. 58).

Complete hydrolysis required about 200, 30 and 10 min.

for (I), (II) and (III), respectively.

The glucose-resorcinol ratios are given in Table 7.

Table 7 : Glucose-resorcinol ratios for the m-hydroxyphenyl  $\alpha$ -D-glucosides

Glucoside	Final glucose concentration in Hydrolysate ( $\mu\text{mole.ml}^{-1}$ )	Final resorcinol concentration in hydrolysate ( $\mu\text{mole.ml}^{-1}$ )	Glucose-resorcinol ratio
(I)	0.10	0.049	2.0
(II)	0.042	0.021	2.0
(III)	1.10	1.08	1.0

This established that (I) and (II) were O- $\alpha$ -D-glucosyl- $\alpha$ -D-glucosides and that (III) was the  $\alpha$ -D-glucoside. The nature of the linkages at the various anomeric carbon atoms was verified by a series of experiments using glycosidases, i.e. emulsin <sup>(149)</sup>

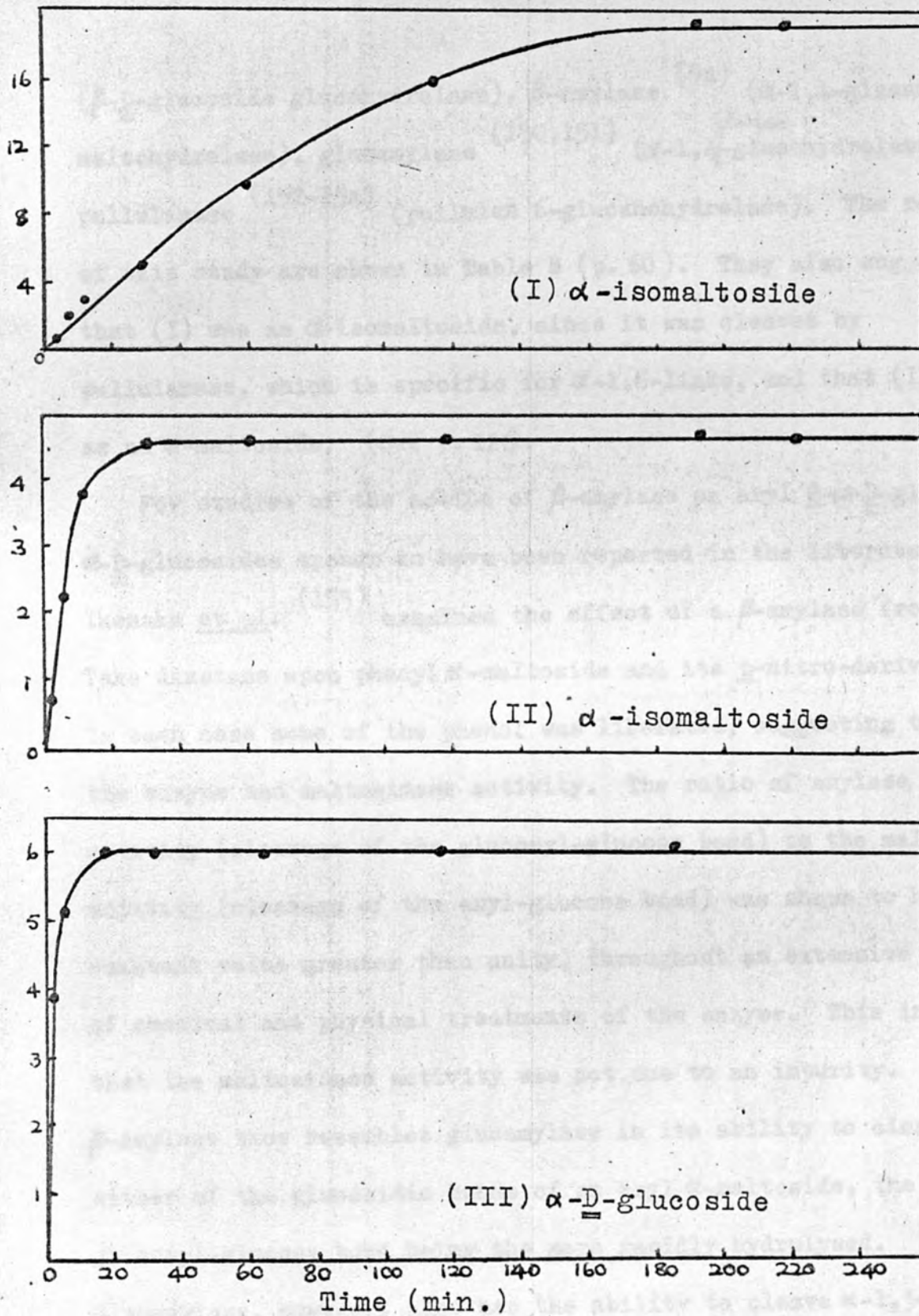


Fig.2 : Maltase hydrolysis of m-hydroxy-phenyl  
 $\alpha$ -D-glucosides

( $\beta$ -D-glucoside glucohydrolase),  $\beta$ -amylase<sup>(94)</sup> ( $\alpha$ -1,4-glucan maltohydrolase), glucamylase<sup>(150,151)</sup> ( $\alpha$ -1,4<sup>glucan</sup>-glucohydrolase), pullulanase<sup>(152-154)</sup> (pullulan 6-glucanohydrolase). The results of this study are shown in Table 8 (p. 60). They also suggested that (I) was an  $\alpha$ -isomaltoside, since it was cleaved by pullulanase, which is specific for  $\alpha$ -1,6-links, and that (II) as an  $\alpha$ -maltoside. (See p. 126).

Few studies of the action of  $\beta$ -amylase on aryl O- $\alpha$ -D-glucosyl- $\alpha$ -D-glucosides appear to have been reported in the literature. Ikenaka et al.<sup>(155)</sup> examined the effect of a  $\beta$ -amylase from Taka diastase upon phenyl  $\alpha$ -maltoside and its p-nitro-derivative. In each case some of the phenol was liberated, suggesting that the enzyme had maltosidase activity. The ratio of amylase activity (cleavage of the glucosyl-glucose bond) to the maltosidase activity (cleavage of the aryl-glucose bond) was shown to have a constant value greater than unity, throughout an extensive range of chemical and physical treatments of the enzyme. This indicated that the maltosidase activity was not due to an impurity.  $\beta$ -Amylase thus resembles glucamylase in its ability to cleave either of the glucosidic bonds of an aryl  $\alpha$ -maltoside, the glucosyl-glucose bond being the more rapidly hydrolysed. Glucamylase, however, also has the ability to cleave  $\alpha$ -1,3- and

**Table 8 :** Enzymic and acid hydrolysis of m-hydroxyphenyl  $\alpha$ -D-glucosides (I), (II) and (III).

Substrate	Emulsin	$\beta$ -Amylase	Maltase	Glucamylase	Pullulanase	Acid
(I)	—	—	R,G	(R),G,III	G,III	R,G, I <sub>m</sub>
(II)	—	(R),(M) (G),(III)	R,G	(R),G,(M),	—	R,G, M,(III)
(III)	—	—	R,G	(R),(G)	—	R,G, (III)
Methyl $\beta$ -D-glucoside	G		—	—		
Phenyl $\beta$ -D-glucoside	G		—	—		
p-Hydroxyphenyl $\beta$ -D-glucoside	G		—	—		
Maltose				G		
Amylose		M		G		
Glucamylase					(152) G,M, M <sub>3</sub>	
Limit dextrin					(152) M, M <sub>3</sub>	
$\beta$ -Limit dextrin						

**Key**

R = resorcinol

G = glucose

M = maltose

I<sub>m</sub> = isomaltoseM<sub>3</sub> = maltotriose

— = no hydrolysis

( ) = trace

(See also Expt. 6, p.126)

$\alpha$ -1,6-glucosidic bonds and aryl monoglucosides. (The relative glucamylase hydrolysis rates found by Pazur & Kleppe <sup>(151)</sup> for maltose, nigerose, isomaltose and phenyl  $\alpha$ -D-glucoside were 100, 6.6, 3.6 and 0.87, respectively).

The products of the action of these two enzymes on the m-hydroxyphenyl glucosides (I), (II) and (III) of this present study (Table 8, p. 60) are in accord with observations of Ikenaka and of Pazur and Kleppe.

Partial acid hydrolysis (see Table 8, p. 60) confirmed the identity of the  $\alpha$ -isomaltoside and also further suggested that (II) was an  $\alpha$ -maltoside.

Further proof of these structures was obtained by methylation of the glucosides (Kuhn's method <sup>(156)</sup>), followed by methanolysis. The resulting products were identified by thin layer <sup>(157)</sup> (Table 9, p 62) and vapour phase chromatography <sup>(158)</sup> (Table 10, p 63).

The I.R. spectrum of (III) was also obtained and the peaks in the region  $700-950\text{cm.}^{-1}$ , were examined and compared with those found by Barker and Bourne <sup>(159)</sup> for  $\alpha$ - and  $\beta$ -D-glucosidic links. These are shown in Table 11. The peaks at  $920$  and  $770\text{cm.}^{-1}$ , could have been due to either  $\alpha$ - or  $\beta$ -D-glucosidic links, but the presence of a peak at  $843\text{cm.}^{-1}$ , and the absence of a peak in the  $890\text{cm.}^{-1}$  region showed that an  $\alpha$ -link was present.

Table 9 : R<sub>F</sub>-Values [solvent (D)] of the permethylated/  
methanolised m-hydroxyphenyl α-D-glucosides \*(157)

† Standard			Permethylated/methanolysed glucosides		
(A <sub>1</sub> )	(A <sub>2</sub> )	(A)	(I)	(II)	(III)
0.02 0.06 0.13			0.03 0.06 0.14		
	0.03 0.11			0.03 0.11	
0.08 0.20 0.34	0.07 0.19 0.34	0.08 0.20 0.33	0.08 0.21 0.33	0.07 0.21 0.34	0.21 0.35

† Standards :

(A): Methyl 2,3,4,6-tetra-O-methyl α-and β-D-glucosides (from methylated D-glucose).

(A<sub>1</sub>): Mixture of (A) and 2,3,4-tri-O-methyl α-and β-D-glucosides (from methylated isomaltose and isomaltotriose).

(A<sub>2</sub>): Mixture of (A) and 2,3,6-tri-O-methyl α-and β-D-glucosides (from (methylated maltose)).

\* Reaction mixtures contain both α-and β-D-anomers and pyranoid and furanoid isomers, some or all of which may be separated by thin-layer chromatography; hence, the multiplicity of spots, many of which cannot yet be individually identified (157).

Table 10 : Relative retention times, (T), of the permethylated /methanolysed m-hydroxyphenyl  $\alpha$ -D-glucosides and standards by vapour phase chromatography

<u>Glucoside</u>		<u>Standard</u>		<u>Identity of Components</u> <sup>(158)</sup>
<u>Identity</u>	<u>T</u>	<u>Identity</u>	<u>T</u>	
(I)	1.00	(A)	( 1.00 )	methyl 2,3,4,6,-tetra- <u>O</u> -methyl- <u>D</u> -glucosides
	1.23		( 1.24 )	
	1.37		( 1.36 )	methyl 2,3,4-tri- <u>O</u> -methyl- <u>D</u> -glucosides
	1.85		( 1.88 )	
	0.78	(B)	0.79	resorcinol monomethyl ether
(II)	1.02	(A <sub>2</sub> )	( 1.00 )	methyl 2,3,4,6-tetra- <u>O</u> -methyl- <u>D</u> -glucosides
	1.42		( 1.36 )	
	1.78		( 1.76 )	methyl 2,3,6-tri- <u>O</u> -methyl- <u>D</u> -glucosides
	2.40		( 2.37 )	
	0.76	(B)	0.78	resorcinol monomethyl ether
(III)	1.00	(A)	( 1.00 )	methyl 2,3,4,6-tetra- <u>O</u> -methyl- <u>D</u> -glucosides
	1.35		( 1.36 )	
	0.75	(B)	0.78	resorcinol monomethyl ether

No data is at present available to distinguish between pairs of peaks due to anomers and/or alternative ring forms (except in the case of 2,3,4,6-tetra-O-methyl  $\alpha$ - and  $\beta$ -D-glucopyranosides).

Standards :

(A), (A<sub>1</sub>) & (A<sub>2</sub>): see Table 9, p. 62 .

(B) Authentic resorcinol monomethyl ether.



Table 11 : I.R. absorption spectrum peaks for glucoside (III) in the region 700-950cm.<sup>-1</sup>

Values given by Barker & Bourne (159)		Values observed with glucoside (III) (cm. <sup>-1</sup> )
$\alpha$ -link (cm. <sup>-1</sup> )	$\beta$ -link (cm. <sup>-1</sup> )	
917 $\pm$ 13	920 $\pm$ 5	920
844 $\pm$ 8	891 $\pm$ 7	843
766 $\pm$ 10	774 $\pm$ 9	772

It was not possible to obtain I.R. spectra of (I) and (II), since they deliquesced rapidly on mulling and water interferes in the region 700-950cm.<sup>-1</sup>

In conclusion, the overall results indicated clearly that (I), (II) and (III) were m-hydroxyphenyl  $\alpha$ -isomaltoside, m-hydroxyphenyl  $\alpha$ -maltoside and m-hydroxyphenyl  $\alpha$ -D-glucopyranoside, respectively.

Related D-glucosides were also formed when resorcinol was replaced by catechol in the enzyme digest. Thus, three o-hydroxyphenyl-glucosides (IV), (V) and (VI) were produced (Table 6, p. 56) with glycone groups corresponding to (I), (II)

and (III). The structures of (IV) and (V) were determined by treatment with ferric chloride solution at 35°C, which produced oxidative cleavage and yielded the intact glucone and *o*-benzoquinone in each case. The glucones produced from (IV) and (V) were separated from the ferric chloride oxidation mixture, after deionisation, by absorption on charcoal, followed by stepwise elution with aqueous ethanol. The glucose from (IV) co-chromatographed with authentic isomaltose and that from (V) with authentic maltose (Table 12).

Table 12 :  $R_G$ -Values [Solvent (C)] of the free glucones produced by oxidation of *o*-hydroxyphenyl glucosides

Standard	$R_G$ values	Glucone (IV)	Glucone (V)
Isomaltose	0.41	0.40	
Maltose	0.45		0.44
Glucose	1.00	1.0 (trace)	1.0 (trace)

The disaccharides liberated from (IV) and (V) were also reduced to the corresponding sugar-alcohols with sodium borohydride (160). These were examined on paper electrophoreto-

grams, using molybdate buffer. The reduced disaccharide from (IV) migrated at the same rate as authentic isomaltitol ( $M_s = 0.6$ ; i.e. characteristic of a 6-substituted derivative of D-glucitol) and the reduced glucone (V) co-electrophoresed with authentic maltitol ( $M_s = 0.3$ ; i.e. characteristic of a 4-substituted D-glucitol). These results strongly suggested that (IV) and (V) were the isomaltoside and maltoside respectively of catechol and, by analogy with the resorcinol derivatives, it is reasonable to assume that both glycosidic residues were  $\alpha$ -linked to the phenol.

The structures of the  $\alpha$ -D-glucosides (I) to (V) are summarized in Fig.3.

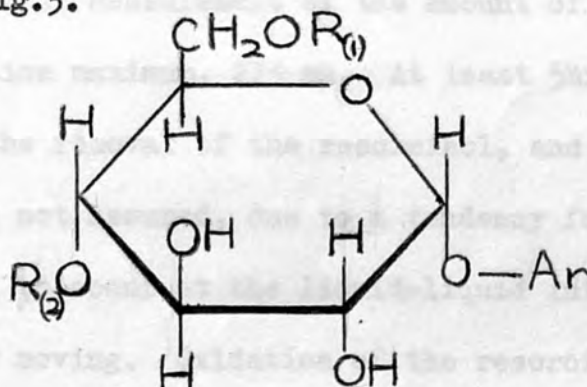


Fig.3 : Structures of the aryl  $\alpha$ -D-glucosides.

(I)-(III) : Ar = m-hydroxyphenyl  
 (IV)&(V) : Ar = o-hydroxyphenyl

(I)&(IV) :  $R_1 = \alpha$ -D-glucopyranosyl;  $R_2 = H$   
 (II)&(V) :  $R_1 = H$ ;  $R_2 = \alpha$ -D-glucopyranosyl  
 (III) :  $R_1 = R_2 = H$ .

II : Development of an assay method for the enzyme

The main product of the standard enzyme digests, with maltose and resorcinol, detected by chromatography, was m-hydroxyphenol  $\alpha$ -D-glucopyranoside (MHPG). [Glucoside (III); see part I.] MHPG was detectable after a few minutes of reaction, while glucosides (II) and (III) were not detected in "short-term" digests (1 hr.). It was accordingly decided to determine the amount of MHPG formed as a measure of enzyme activity.

The first method which was attempted consisted of the removal of residual resorcinol from the reaction mixture with ether in liquid-liquid extractors, followed by direct U.V. spectrophotometric measurement of the amount of MHPG formed, at the absorption maximum, 273 m $\mu$ . At least 5hr. were required for the removal of the resorcinol, and complete extraction was not assured, due to a tendency for emulsification to occur at the liquid-liquid interface, which was constantly moving. Oxidation of the resorcinol also took place in the crude enzyme digest, when using this method, and the oxidation products absorbed strongly in the same region as MHPG.

A second method which was tried consisted of forming a red azo-dye from the MHPG present in the ether-extracted

digest, using diazotised p-nitroaniline, followed by alkali. The compound exhibited a maximum absorption at 510  $\mu$ . This failed as a quantitative means of determining the glucoside, since the intensity of the red colour produced varied with the speed at which the alkali was added, a factor which could not readily be controlled in routine analysis.

The third method met with some success and was used in early determinations. This was based on quantitative chromatographic separation of the components of the digest. MHPG was then located by means of marker spots and quantitatively eluted. It was determined spectrophotometrically at 273 $\mu$ . This method gave reproducible results but suffered from two defects, namely that it was slow (7 to 9 hr.) and the molar extinction coefficient of the MHPG was too low for accurate work. However, it was used initially to estimate the optimum conditions of time, temperature and pH for the maximum enzyme activity, in order to define the conditions for a standard digest.

The plot of MHPG concentration against reaction time for the crude enzyme (c.f. Fig. 6, p. 83), the other parameters being fixed, showed that the rate curve was linear between 20 and 80min., and that, after this time, the reaction rate gradually fell to zero at 120min. Accordingly, 60min. was chosen as the reaction

time for standard digests. Some apparently anomalous concentration values for MHPG were found between 0 and 20min., using the crude enzyme. These were explicable, however, in terms of the properties of the purified enzyme. [See part (IV)].

Since the quantitative chromatographic method for estimation of the enzyme activity was too slow, and rather inaccurate for routine assay during the purification of the enzyme, another method was sought. The use of liquid-liquid extractors for removing residual resorcinol was replaced by manual extraction, using separating funnels. A preliminary experiment showed that about fifteen extractions with 3 volumes of ether to 1 volume of reaction mixture removed all the resorcinol, providing that 10 to 15min. was allowed for the liquid-liquid interface to settle completely. A series of digests could be extracted simultaneously and the total assay time was reduced to about 6hr. (including reaction time and the determination of the MHPG.) Chloroform was also tested as an extraction solvent, but this proved to be less efficient. The vanillin reagent of Swain and Hillis (161, 162), which had been used for the determination of resorcinol and its derivatives, was found to give a red colour with MHPG. This was accordingly used to estimate the MHPG concentrations in aliquots of ether-

extracted digests.

It was found necessary to prepare the reagent freshly each day and to standardise it on each occasion. This method was used to determine the enzyme activity, which was defined as follows: (88)

One unit (U) of enzyme activity is the amount which produces 1  $\mu$ mole of MHPG in 1min., under standard conditions.

The standard digest (5.0ml.) was:

- 2.0ml. enzyme solution
- 2.0ml. 0.1M-acetate buffer (pH 5.1)
- 0.7ml. 0.60M-maltose solution
- 0.2ml. 4.3M-resorcinol solution
- 0.1ml. 1.2M-L-cysteine solution

Temperature : 24°C      Time : 60min.

Protein determinations were made using the Folin-Ciocalteu-Lowry method (163,164).

The specific activity of the enzyme was defined as the number of units of enzyme per mg. of protein.

Specific activities and total activities were calculated as follows:

If x = concentration of MHPG ( $\mu$ mole.ml.<sup>-1</sup>) in standard solution

y = absorbance of colour produced by standard with vanillin reagent

a = absorbance of colour produced with unknown MHPG with vanillin reagent,

### III : Purification of the Enzyme

then concentration of MHPG in the unknown is given by  $\left(\frac{ax}{y}\right) \mu\text{mole.ml.}^{-1}$

A 5ml. standard digest contains 2ml. enzyme solution

i.e., under standard conditions, 2ml. enzyme solution produces

$\left(\frac{5ax}{y}\right) \mu\text{mole MHPG per hr.}$

In 1min., 1ml. enzyme solution produces  $\left(\frac{5ax}{y} \times \frac{1}{120}\right) \mu\text{mole.MHPG}$

i.e., activity of enzyme solution =  $\left(\frac{ax}{24y}\right) \text{Units}$

If v ml = total volume of enzyme solution

total activity =  $\left(\frac{vax}{24y}\right) \text{Units}$

If b mg.ml.<sup>-1</sup> = protein concentration,

total protein = (vb)mg.

Specific activity =  $\left(\frac{ax}{24by}\right) \text{Units.mg.protein}^{-1}$

Inspection of the data in Table I shows that salting-out

produces a sevenfold increase in the most active fraction (No.5),

but that this fraction contains only about 2% of the total number

of enzyme units present in the crude extract. It is interesting

to note that, in the purification of *E. coli*  $\beta$ -galactosidase

(118) found that out of a total of about 75 proteins

in the crude extract, only five were completely removed by



### III : Purification of the Enzyme

High-speed centrifugation of the crude enzyme extract did not decrease the activity. This showed that the enzyme is soluble and not associated with a particulate fraction.

Several methods of protein fractionation were tested to find a suitable initial purification step for the crude enzyme. The procedures tried were fractionation with ammonium sulphate, acetone precipitation, fractionation by variation of pH and heat denaturation<sup>(165)</sup>. The latter method gave a sixfold decrease in the specific activity of the enzyme and was thus unsuitable as a means of purification. The specific activities of typical fractions produced by the ammonium sulphate, acetone and pH-change methods are shown in Tables 13, 14 and 15, respectively.

Inspection of the data in Table 13 shows that salting-out produces a sevenfold increase in the most active fraction (No.5), but that this fraction contains only about 2% of the total number of enzyme units present in the crude enzyme. It is interesting to note that, in the purification of E.coli  $\beta$ -galactosidase Anfinsen et al<sup>(118)</sup> found that out of a total of about 23 proteins in the crude extract, only five were completely removed by ammonium sulphate fractionation (up to 40% saturation), the

Table 13 :  $(\text{NH}_4)_2\text{SO}_4$ -Fractionation of the crude enzyme

Fraction	Volume (ml.)	% $(\text{NH}_4)_2\text{SO}_4$ saturation	Total activity (U)	Total protein (mg.)	Specific activity (U.mg <sup>-1</sup> )
Crude	1,600	0	232	1610	1.4
(1)	100	20	3.5	60	0.6
(2)	70	40	3.3	4	8.9
(3)	70	60	2.4	2	10.9
(4)	50	80	1.8	4	4.2
(5)	60	100	4.5	4	12.5
<b>* % Recovery :</b>			6.6	4.4	

\* Excluding supernatant fraction at 100%  $(\text{NH}_4)_2\text{SO}_4$  saturation.

Table 14 : Acetone fractionation of the crude enzyme

Fraction	Volume (ml.)	% Acetone concentration	Total activity (U)	Total protein (mg.)	Specific activity (U.mg <sup>-1</sup> )
Crude	40	0	1.8	14	0.13
(1)	10	20	0.28	2.4	0.12
(2)	10	40	0.24	1.2	0.20
(3)	11	60	0.29	1.2	0.24
(4)	10	80	0.32	1.4	0.23
(5)	94	✕	0.65	7.2	0.14
% Recovery :			100	95	

✕ Supernatant at 80% acetone concentration.

Table 15 : Fractionation of the crude enzyme by change of pH

Fraction	Volume (ml.)	pH	Total activity (U)	Total protein (mg.)	Specific activity (U.mg. <sup>-1</sup> )
Crude	50	5.2	8.2	93	0.09
(1)	20	4.4	1.2	7.6	0.15
(2)	10	3.9	0.5	2.8	0.18
(3)	10	3.5	0.6	7.6	0.21
(4)	20	2.9	0.8	3.0	0.27
(5)	49	✕	4.3	71	0.07
% Recovery :			90	94	

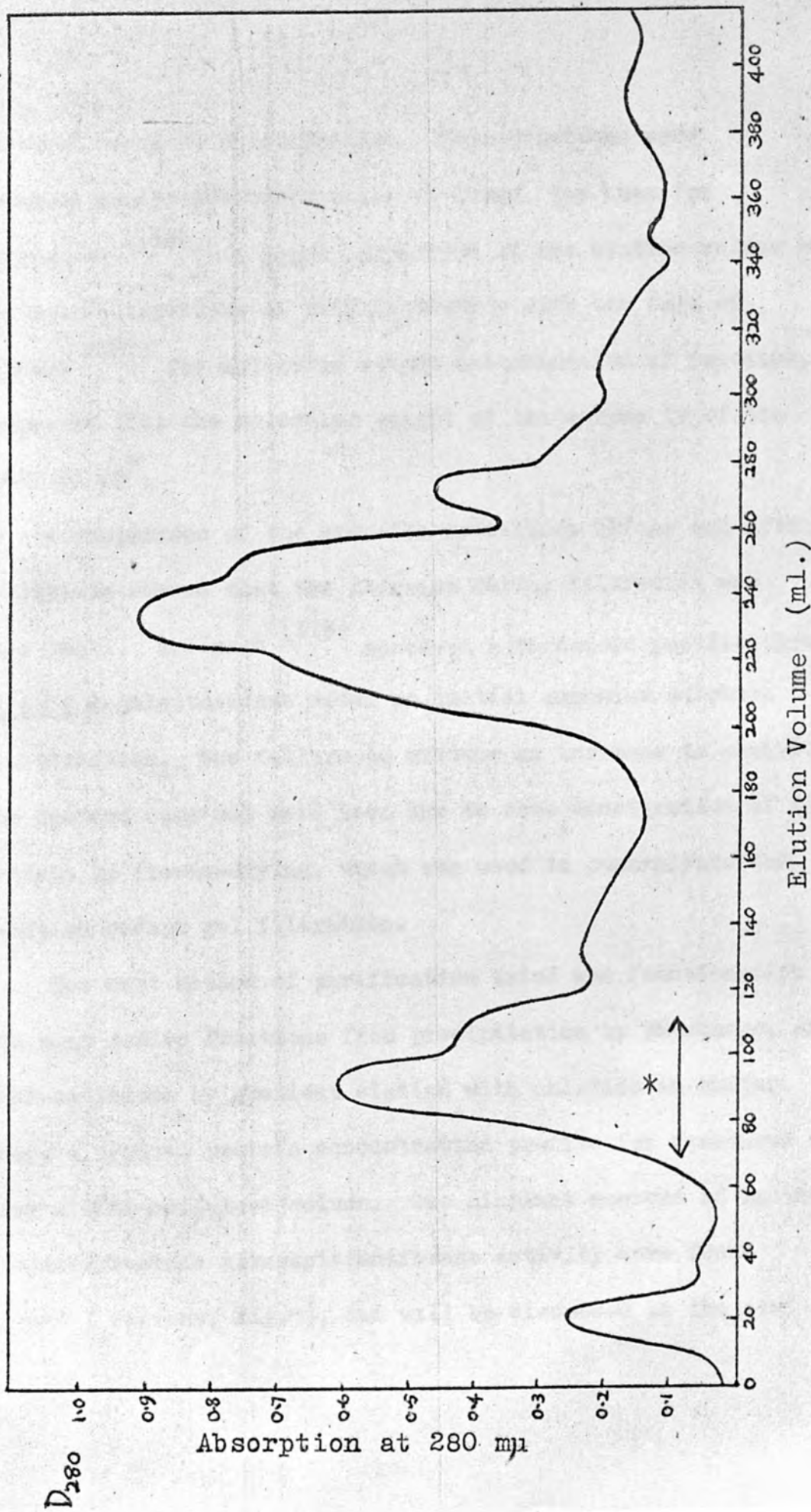
✕ Supernatant at pH 2.9.

concomitant specific activity increase being threefold.

Table 14 shows that some purification was achieved with acetone, but that there was a spread of activities over the various fractions and no single fraction had a markedly higher activity. A similar spread was observed by Askonas <sup>(166)</sup> with several enzymes, using this method, although separation was generally sharper with acetone than with other organic solvents.

The method which gave the best results was fractionation by change of pH <sup>(165)</sup>. This procedure also has the advantage of being more rapid than salt or organic solvent precipitation. Examination of Table 15 showed that fractions (3) and (4) had the highest specific activities. Although the purification factor ( $\sim 3$ ) was lower than with ammonium sulphate, the combined fractions (3) and (4) contained about 17% of the initial total activity and it was decided to use the fraction precipitated between pH 3.9 and 2.9 for the next purification step. (The percentage recovery of activity at this step varied from one extract to another, being as low as 2% in some instances c.f. Table 16.).

While the above investigations were in progress, gel filtration of some of the fractions of higher specific activity on Sephadex G-100 was attempted <sup>(167)</sup>. Fig.4 shows the protein concentration profile obtained with the combined fractions (2)-(5) from an



Elution Volume (ml.)

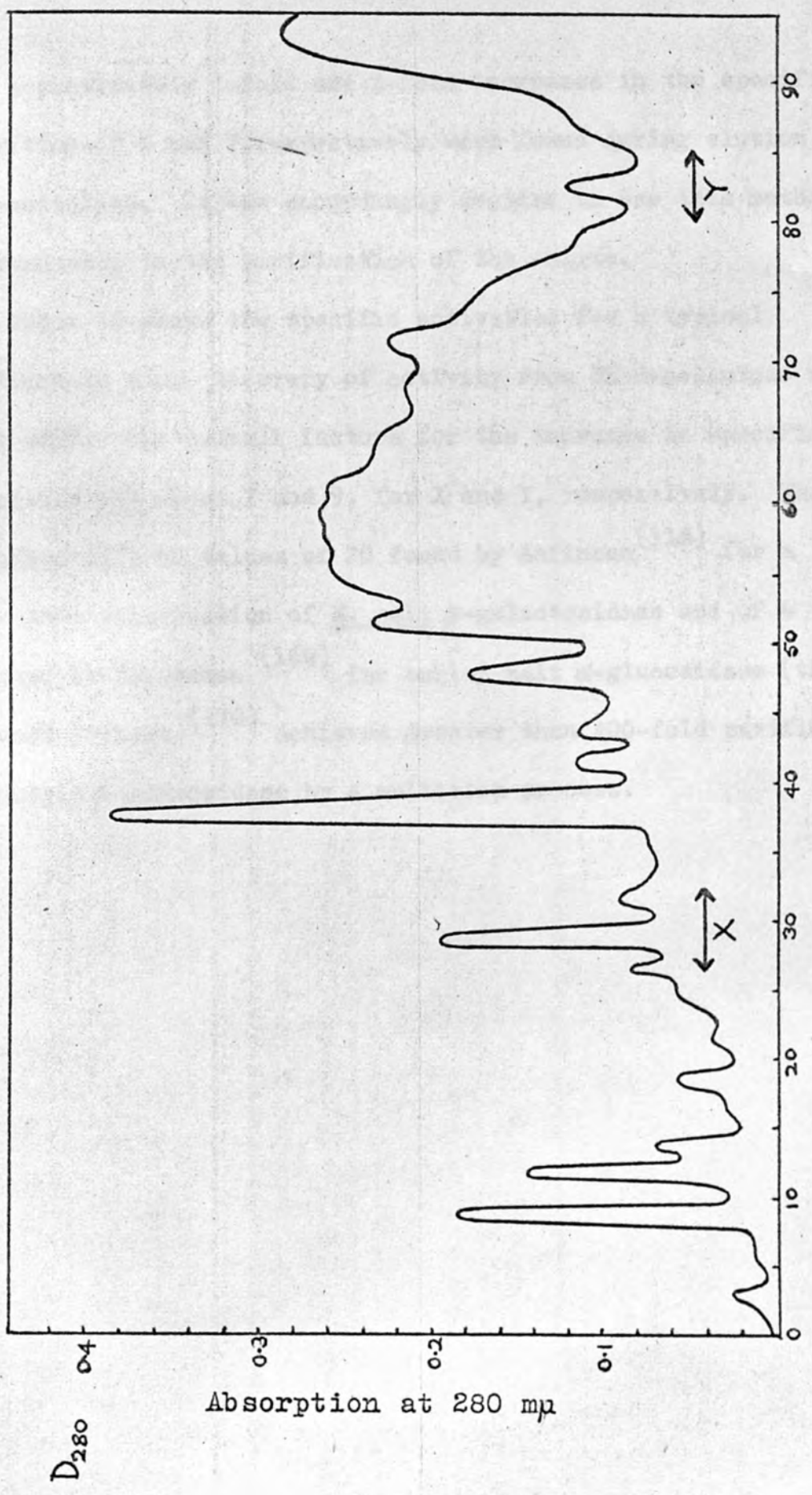
(\*denotes region of enzyme activity)

Figure 4: Fractionation of  $(\text{NH}_4)_2\text{SO}_4$ -precipitated protein on Sephadex G-100

ammonium sulphate fractionation. Concentrations were measured spectrophotometrically at 280m $\mu$ , the  $\lambda_{\text{max}}$  for tryptophan <sup>(168)</sup>. A rough comparison of the elution volume of the active fractions of this filtration with the data of Andrews <sup>(167)</sup> for molecular weight determination of proteins, suggested that the molecular weight of the enzyme is of the order of 10<sup>5</sup>.

A comparison of the specific activities before and after gel filtration showed that the increase during filtration was very small. Anfinsen <sup>(118)</sup> achieved a threefold purification of E. coli  $\beta$ -galactosidase after an initial ammonium sulphate fractionation. The failure to produce an increase in activity in the present case may have been due to some denaturation of the protein by freeze-drying, which was used to concentrate the solution before gel filtration.

The next method of purification tried was fractionation of the most active fractions from precipitation by pH-change, on DEAE-cellulose by gradient elution with chloride in buffer. Fig.5 shows a typical protein concentration profile for fractions eluted from a DEAE-cellulose column. Two distinct regions of maltose: dihydroxybenzene glucosyltransferase activity were found (X and Y regions, Fig.5), and will be discussed in the next section.



Fractionation of protein precipitated in the pH-range 3.9 - 2.9 on DEAE-cellulose.

Figure 5: Fractionation of protein precipitated in the pH-range 3.9 - 2.9 on DEAE-cellulose.



Approximately 4-fold and 5-fold increases in the specific activities of X and Y, respectively, were found during elution from DEAE-cellulose. It was accordingly decided to use this method as a second step in the purification of the enzyme.

Table 16 shows the specific activities for a typical purification run. Recovery of activity from DEAE-cellulose was about 100%. The overall factors for the increase in specific activities are about 7 and 9, for X and Y, respectively. These are comparable to values of 20 found by Anfinsen<sup>(118)</sup> for a three-step purification of E. coli  $\beta$ -galactosidase and of 6 obtained by Jørgensen<sup>(169)</sup> for barley malt  $\alpha$ -glucosidase (three-step), although Joubert<sup>(170)</sup> achieved greater than 400-fold purification of emulsin  $\beta$ -glucosidase by a multistep process.

Protein			
	1600	62	90
Crude			
		3.9-2.9	
Precipitated pH			
			92
Eluted from			
DEAE-cellulose			
			X
			Y

Table 16 : Two-step purification of the crude enzyme

Fraction	Volume (ml.)	Total activity (U)	Total protein (mg.)	Specific activity (U.mg. <sup>-1</sup> )
Crude	1600	63.4	1860	0.034
Precipitated pH 3.9-2.9	62	1.3	22	0.060
Eluted from DEAE-Cellulose	90 92	0.74 0.58	3.3 2.0	0.22 0.28

#### IV : Properties of the Purified Enzyme

Early experiments with the crude enzyme, performed to find the optimum conditions for assay purposes, showed that the enzyme had a maximum activity at pH 5.1 and 24°C. An investigation of the time-course of the reaction revealed that the velocity was constant between 20 and 80min. and fell to zero between 80 and 120min. (using the standard conditions described in Expt.14). Anomalous "humps" were found in the time course curve for the period 0-20min. with the crude enzyme.

The time-courses for the DEAE-cellulose column eluates, X and Y, were investigated and are shown in Fig.6. X gave a normal curve for a straightforward enzyme reaction <sup>(171,172)</sup> i.e. the rate of MHPG formation was initially constant (0-60min.) and then fell to zero as equilibrium was attained (100min.). The time-course for Y was unusual (Fig.6). There was a rapid build-up and breakdown of MHPG during the first 20min., after which time re-formation of MHPG proceeded normally, the rate falling to zero at between 50 and 80min., depending upon the enzyme concentration, [E]. The final MHPG concentration was only slightly less than the initial (12min.) maximum. The initial part of the curve for Y resembled that found by Pridham and Wallenfels <sup>(83,173)</sup> for the formation of m-hydroxyphenyl  $\beta$ -D-fucoside and  $\beta$ -D-galactoside with

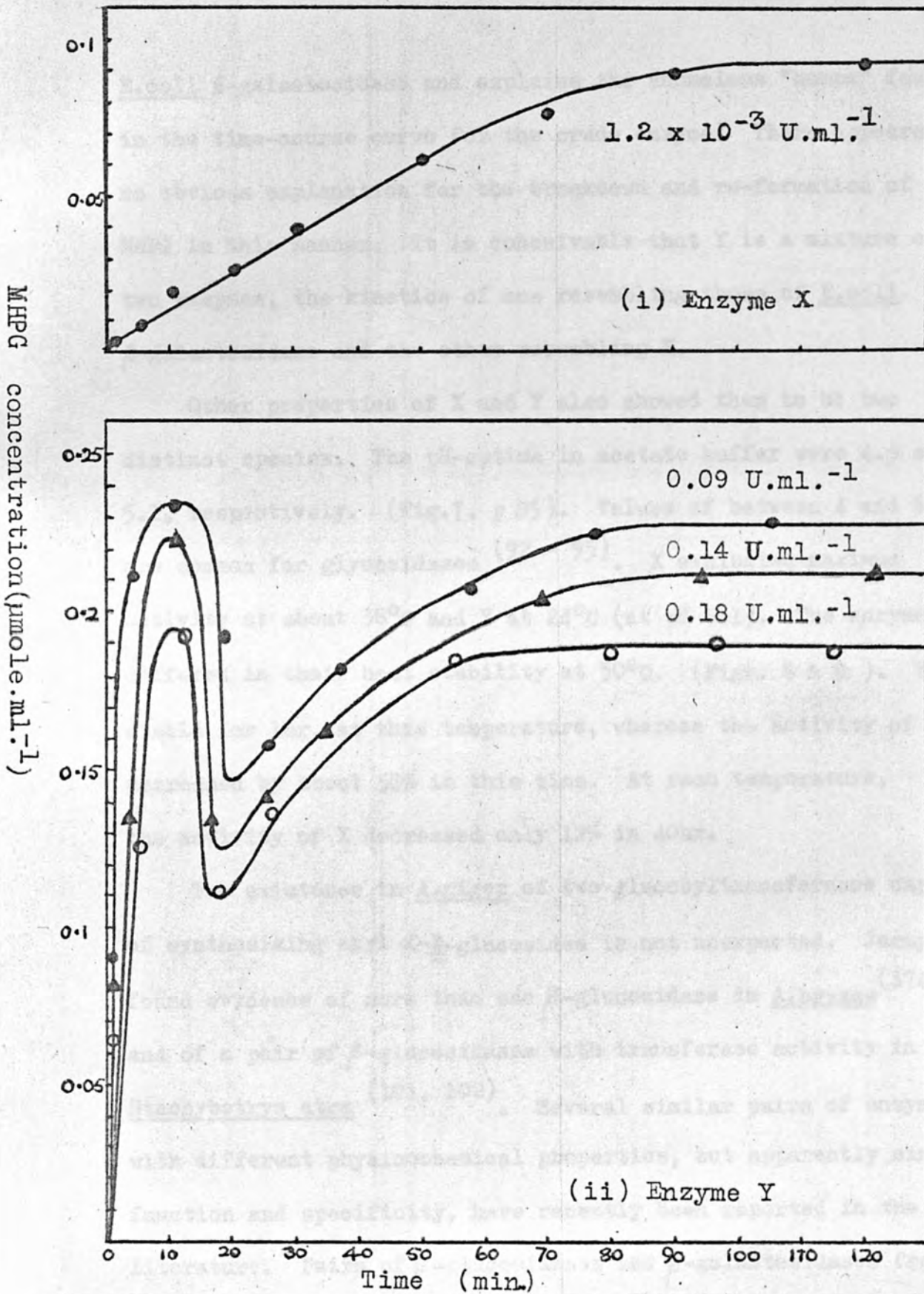


Fig. 6 : Time-course of enzymic formation of MHPG

E.coli  $\beta$ -galactosidase and explains the anomalous "humps" found in the time-course curve for the crude enzyme. There appears to be no obvious explanation for the breakdown and re-formation of MHPG in this manner. It is conceivable that Y is a mixture of two enzymes, the kinetics of one resembling those of E.coli  $\beta$ -galactosidase and the other resembling X.

Other properties of X and Y also showed them to be two distinct species. The pH-optima in acetate buffer were 4.5 and 5.1, respectively. (Fig.7, p 85). Values of between 4 and 6 are common for glycosidases (92 - 95). X exhibited maximum activity at about 38°C and Y at 24°C (at pH 5.1). The enzymes also differed in their heat stability at 50°C. (Figs. 8 & 9 ). Y was stable for 1hr. at this temperature, whereas the activity of X decreased by about 50% in this time. At room temperature, the activity of X decreased only 12% in 40hr.

The existence in A.niger of two glucosyltransferases capable of synthesizing aryl  $\alpha$ -D-glucosides is not unexpected. Jermyn found evidence of more than one  $\beta$ -glucosidase in A.oryzae (174) and of a pair of  $\beta$ -glucosidases with transferase activity in Stachybotrys atra (101, 102). Several similar pairs of enzymes with different physicochemical properties, but apparently similar function and specificity, have recently been reported in the literature. Pairs of  $\beta$ -glucosidases and  $\beta$ -galactosidases from various strains of the fungus Neurospora crassa have been

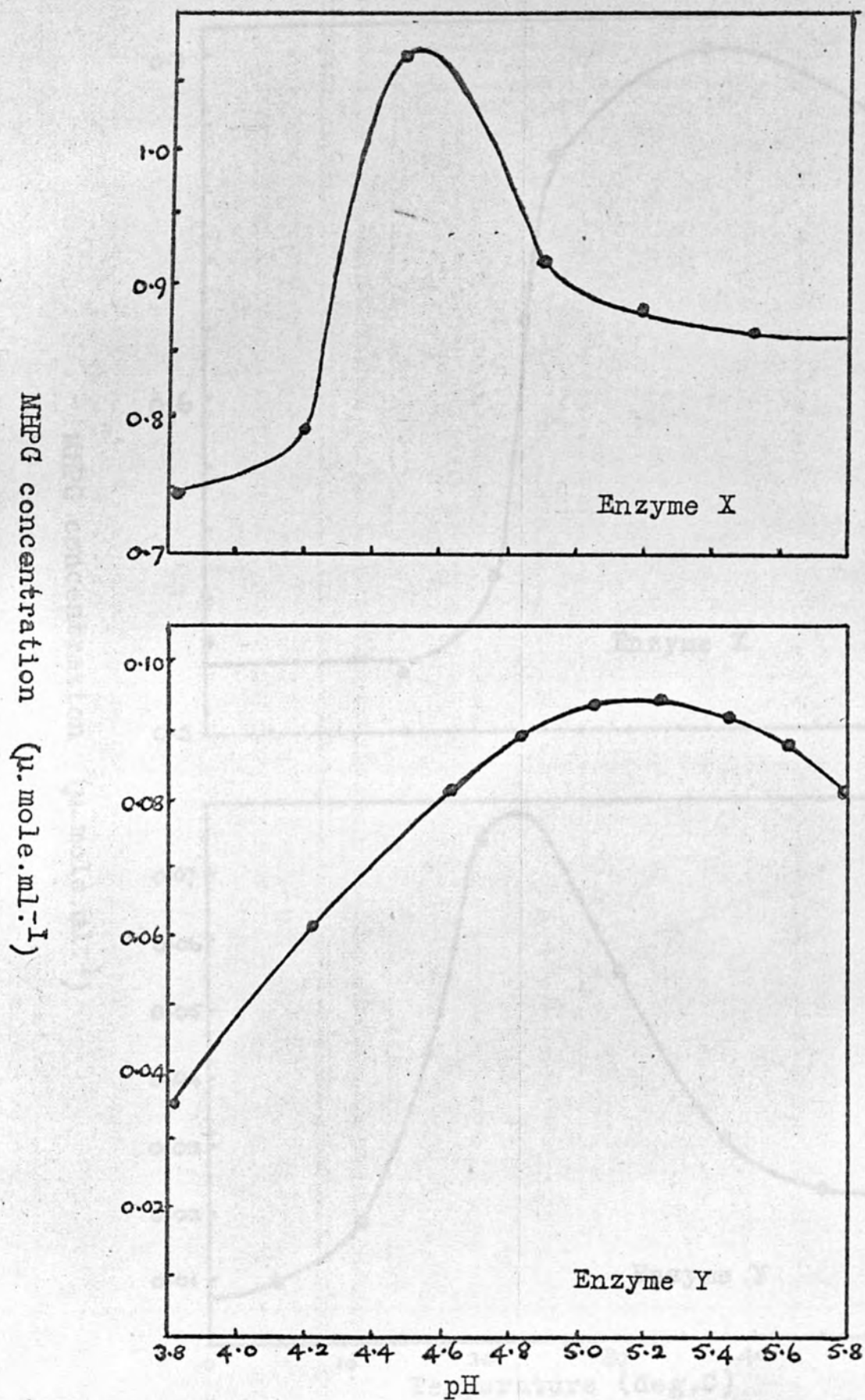


Fig. 7 : pH-dependence of enzymic formation of MHPG.

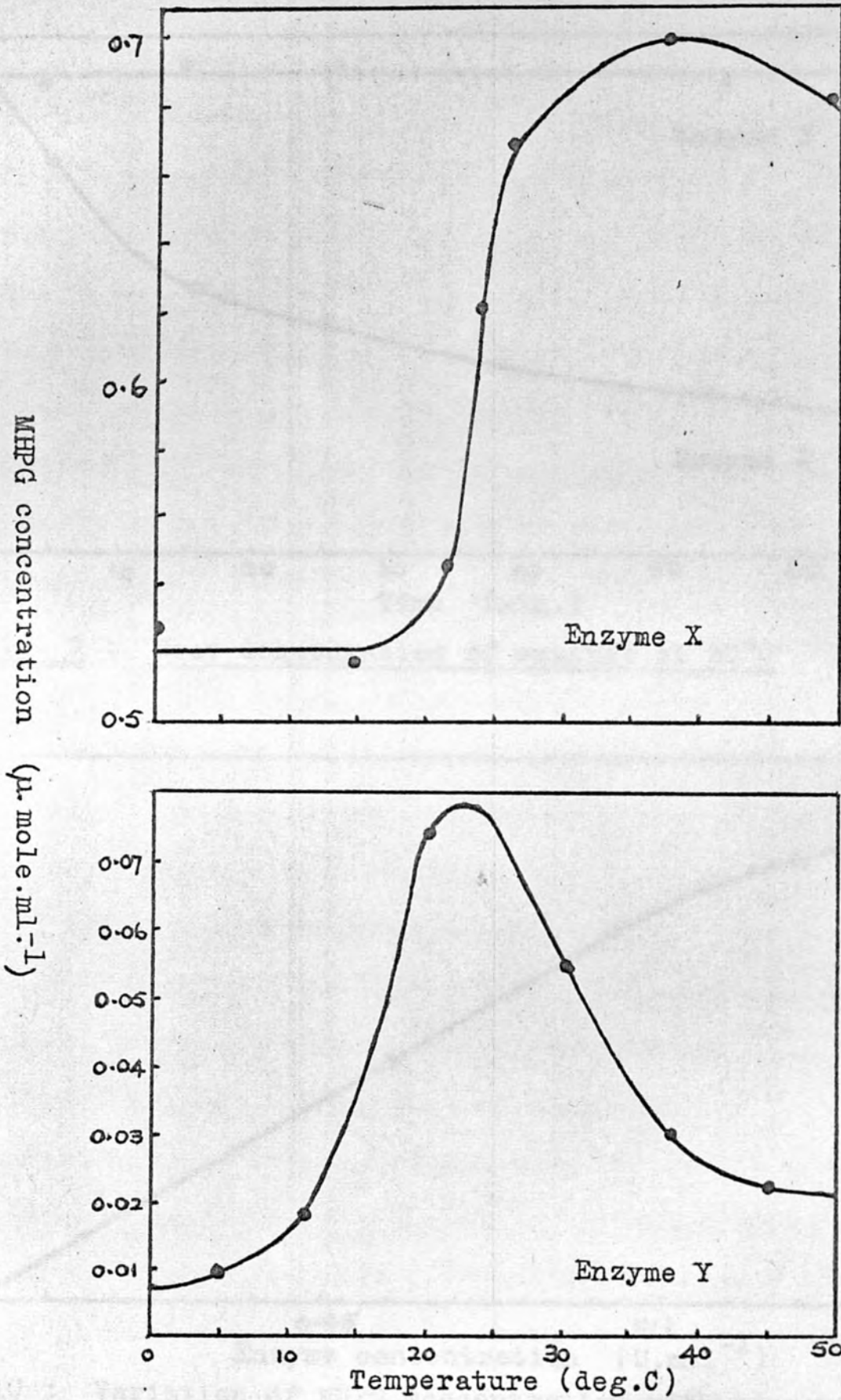


Fig. 8 : Temperature-dependence of enzymic formation of MHPG

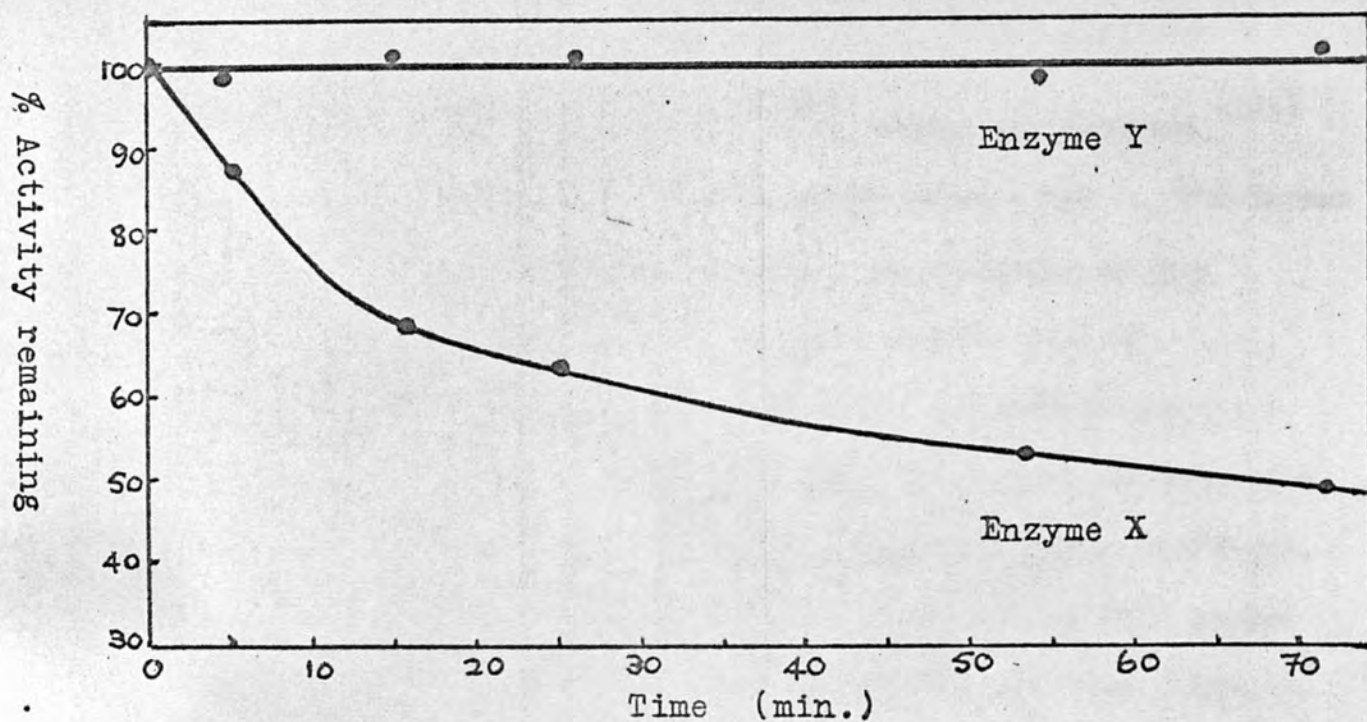


Fig. 9 : Heat denaturation of enzymes at 50°C

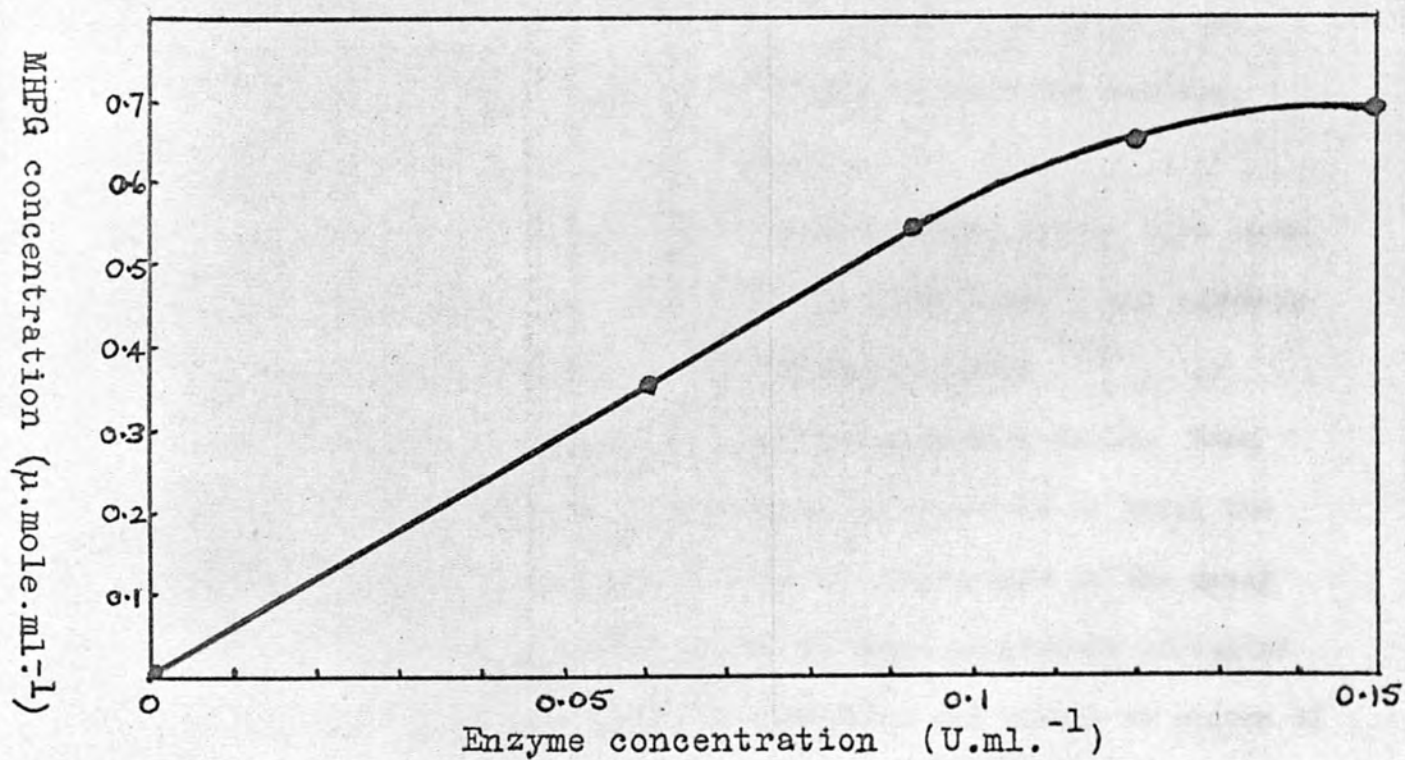


Fig.10 : Variation of MHPG concentration with concentration of enzyme X



studied by several workers (175 - 178). Bates and Woodward (175) found two  $\beta$ -galactosidases with pH-maxima about 4 and 7. The former was thermolabile at 50°C and the latter thermostable at this temperature. Eberhart (176,177) found a similar pair of  $\beta$ -glucosidases in N.crassa: a thermolabile cellobiase and a thermostable aryl  $\beta$ -glucosidase. Lester (178) found evidence of a possible difference in function for the N.crassa  $\beta$ -galactosidases, viz. the pH 4 enzyme may be primarily a hydrolase and pH 7 enzyme a transferase. Other pairs of disaccharidases have been found in N.crassa, e.g. trehalases (179) [ $\alpha,\alpha$ -trehalose glucohydrolases] and invertases (180) [ $\beta$ -D-fructofuranoside fructohydrolases]. Sussman (181) found that the relative trehalase activities and invertase activities varied significantly between the conidia, ascospores and mycelia of the fungus.

A plot of MHPG concentration against [E] for enzyme X is shown in Fig. 10 (p. 87). For [E] values  $\leq 0.09 \text{ U.mg.}^{-1}$ , the curve is linear, as normally observed with enzyme reactions (171). At higher [E] values, however, the gradient gradually falls. Such non-linear curves have been attributed to reactions in which the substrate becomes exhausted (171) or to limitations in the assay method. It is unlikely that either of these conditions prevailed here, since the concentration of substrates was vastly in excess of that of the product, (the extent of the forward reaction  $\nabla$  1%), and aliquots of the digest were diluted to within the limit of MHPG concentration which may be assayed with vanillin reagent,

( $\leq 0.7 \mu\text{mole.ml}^{-1}$ ). A possible explanation for the shape of this curve is that the enzyme has two sites, one for the donor, (c.f. Schemes 8 and 9, pp. 34 and 35), and a second for the acceptor, (c.f. (101, 102)). At high  $[E]$  values, the probability of any one enzyme molecule having both an acceptor and a donor molecule bound to it is lower than at smaller  $[E]$  values, giving an  $[MHPG] - [E]$  curve with a maximum point (102), which represents the optimum  $[E]$  value for MHPG formation. The values plotted in Fig.10 could represent the portion of such a curve for which  $\frac{d}{d[E]} [MHPG] \geq 0$ .

The non-Michaelis kinetics of enzyme Y (Fig.6, p.83) made it difficult to study the variation of  $[MHPG]$  with  $[E]$  in this case. However, some indication of the nature of this variation was obtained from the set of time-course curves at three different  $[E]$  values, (Fig.6, p.83). This shows that the  $[MHPG]$  value decreases as  $[E]$  increases. The  $[E]$  values used for Y were somewhat higher than for X, and the  $[MHPG]$  values found for Y would fit a curve of the type due to dual active sites, as suggested for X. In the case of Y, they could be to the right of the maximum point, where  $\frac{d}{d[E]} [MHPG] < 0$ . Since X and Y have very similar specificities both towards donors and towards acceptors (c.f. p.90), it is feasible that their mechanism of action is similar.

In preliminary investigations with the crude enzyme, using resorcinol as the acceptor, Pridham (81) found that the following

substances were not glycosyl-donors: D-glucose,  $\alpha$ -D-glucose-1-phosphate, methyl  $\alpha$ -D-glucoside, methyl  $\beta$ -D-glucoside, cellobiose, sucrose and UDPG. Both maltose and isomaltose were substrates for the crude enzyme.

A selection of potential donors was investigated in the present study with the purified enzymes (24hr. incubation). Maltose and UDPG (1%) were substrates for both X and Y, giving MHPG and D-glucose. The failure to detect MHPG formation from UDPG with the crude enzyme may have been due to too low a UDPG concentration. Neither D-glucose, nor methyl  $\alpha$ -D-glucoside were substrates for X or Y. An isomaltose/isomaltotriose mixture gave MHPG with X only. Amylose was also tested as a substrate. It had no effect with Y and gave only traces of MHPG and D-glucose in 24hr. with X. Maltose also underwent rapid hydrolysis to D-glucose in the absence of an acceptor with both X and Y.

Saltmarsh <sup>(146)</sup> investigated a number of phenols, hydroxycinnamic acids, phenolic alcohols and coumarins as potential acceptors with the crude enzyme. Substances which were not glucosylated were p-hydroxybenzoic acid, cinnamic acid, shikimic acid, quercetin (5,7,3',4'-tetrahydroxyflavonol) and umbelliferone (7-hydroxycoumarin). A variety of phenolic substances acted as acceptors with the crude enzyme. All gave mono- $\alpha$ -D-glucosides,

which were tentatively characterised by Saltmarsh <sup>(146)</sup> by chromatographic comparison with authentic  $\beta$ -D-glucosides of these compounds.

Most of these acceptors were reinvestigated in the present study with the purified enzymes and the products were detected chromatographically [solvent (B), spray (1)]. The results agreed with Saltmarsh's <sup>(146)</sup> observations on the crude enzyme, except that no evidence of formation of 2,6-dimethoxyphenyl  $\alpha$ -D-glucoside from 2,6-dimethoxyphenol was found with the purified enzymes, either from its U.V. absorption or its colour reaction with spray (1) in the absence of alkali [c.f. <sup>(146)</sup>].

The products of the reactions with various acceptors are shown in Table 17 (p. 92). They were identified tentatively from chromatographic data of Saltmarsh. With o- and m-hydroxyphenols, the alcoholic group was glucosylated and hydroxycinnamic acid derivatives showed evidence of  $\alpha$ -D-glucose ester formation. The latter compounds were characterised by Saltmarsh from chromatographic and electrophoretic comparisons with authentic  $\beta$ -D-glucose esters of these acids. Molybdate chromatography and electrophoresis <sup>(182)</sup> showed the presence of a free o-dihydroxy grouping in the caffeic acid derivative. There was no evidence of formation of phenolic  $\alpha$ -D-glucosides with the hydroxycinnamic acids. In this present

Table 17 : Products from glucosylation of phenolic acceptors with enzymes X &amp; Y

Phenol	R <sub>f</sub> values (solvent B)				Fluorescence		Colour of product with spray (1)	* Probable identity of aglucone of product
	Substrate	* Cr.		U.V.	U.V.+ NH <sub>3</sub>			
		X	Y					
Catechol	0.92	0.63	0.63	0.61			Crimson	<u>o</u> -hydroxy- $\Phi$
Resorcinol	0.85	0.55	0.57	0.56			Pink	<u>m</u> -hydroxy- $\Phi$
Quinol	0.87	0.54	0.59	-			Blue	<u>p</u> -hydroxy- $\Phi$
Pyrogallol	0.74	0.44	0.45	0.48			Blue	2,3-dihydroxy- $\Phi$
1,2,4-trihydroxy- benzene	0.75	0.49	0.52	0.50			Blue-green	2,4-dihydroxy- $\Phi$
Phloroglucinol	0.73	0.39	0.36	0.38			Olive	3,5-dihydroxy- $\Phi$
Saligenin	0.89	0.64	0.60	0.56			Crimson	<u>o</u> -hydroxy-Bz
<u>m</u> -Hydroxybenzyl alcohol	0.84	0.59	0.55	0.55			Crimson	<u>m</u> -hydroxy-Bz
<u>p</u> -Coumaric acid	0.84	0.64	0.62	0.60	Ab.purple	-	Pink	1- <u>p</u> -coumaroyl †
Caffeic acid	0.73	0.45	0.47	0.49	Pale blue	Blue	Olive	1-caffeoyl †
Ferulic acid	0.73	-	0.50	0.49	Ab.purple	-	Purple	1-feruloyl †
Sinapic acid	0.70	0.45	0.46	0.46	Pale blue	Green	Blue	1-sinapoyl †

Ab. = Absorbs; Cr. = crude enzyme;  $\Phi$  = phenyl; Bz = benzyl

\* Data of Saltmarsh (146)

All products except those marked † are  $\alpha$ -D-glucosides (only aglycone moiety is given).†  $\alpha$ -D-glucose esters of carboxylic acids.

study, phosphate electrophoresis <sup>(182)</sup> and chromatography using bromocresol green as spray, confirmed that the -COOH group was the one glucosylated in these compounds. In addition, slower-moving compounds giving intense spots of the same colour as the  $\alpha$ -D-glucose esters were seen on chromatograms, using spray (1). The reaction with bromocresol green suggested that these latter compounds did not contain free-COOH groups. All had  $R_f$  values of  $< 0.1$ , too low for the phenolic  $\alpha$ -D-glucosides [e.g. Harborne <sup>(52)</sup> found  $R_f$  values of 0.55 and 0.46 in the same solvent for caffeic acid 3- $\beta$ -D- and 4- $\beta$ -D- glucosides, respectively.] It is possible that these products were the O - $\alpha$ -D-glucosyl- $\alpha$ -D-glucose esters of the hydroxycinnamic acids.

The standard maltose and resorcinol incubation was carried out with X at various maltose concentrations, [s], in an attempt to determine the Michaelis constant,  $K_m$ , for maltose. [ $K_m$  has no meaning for Y, which exhibits non-Michaelis kinetics]. Fig.11 shows the Lineweaver-Burk <sup>(183)</sup> plot for X. Contrary to a straightforward Michaelis reaction, involving a single active site and the absence of inhibition, the curve obtained (Fig.11) suggested that inhibition by the substrate was taking place <sup>(171)</sup>. This occurs at high [s] values if two active sites for the donor are involved. At high [s] values, each of the sites may

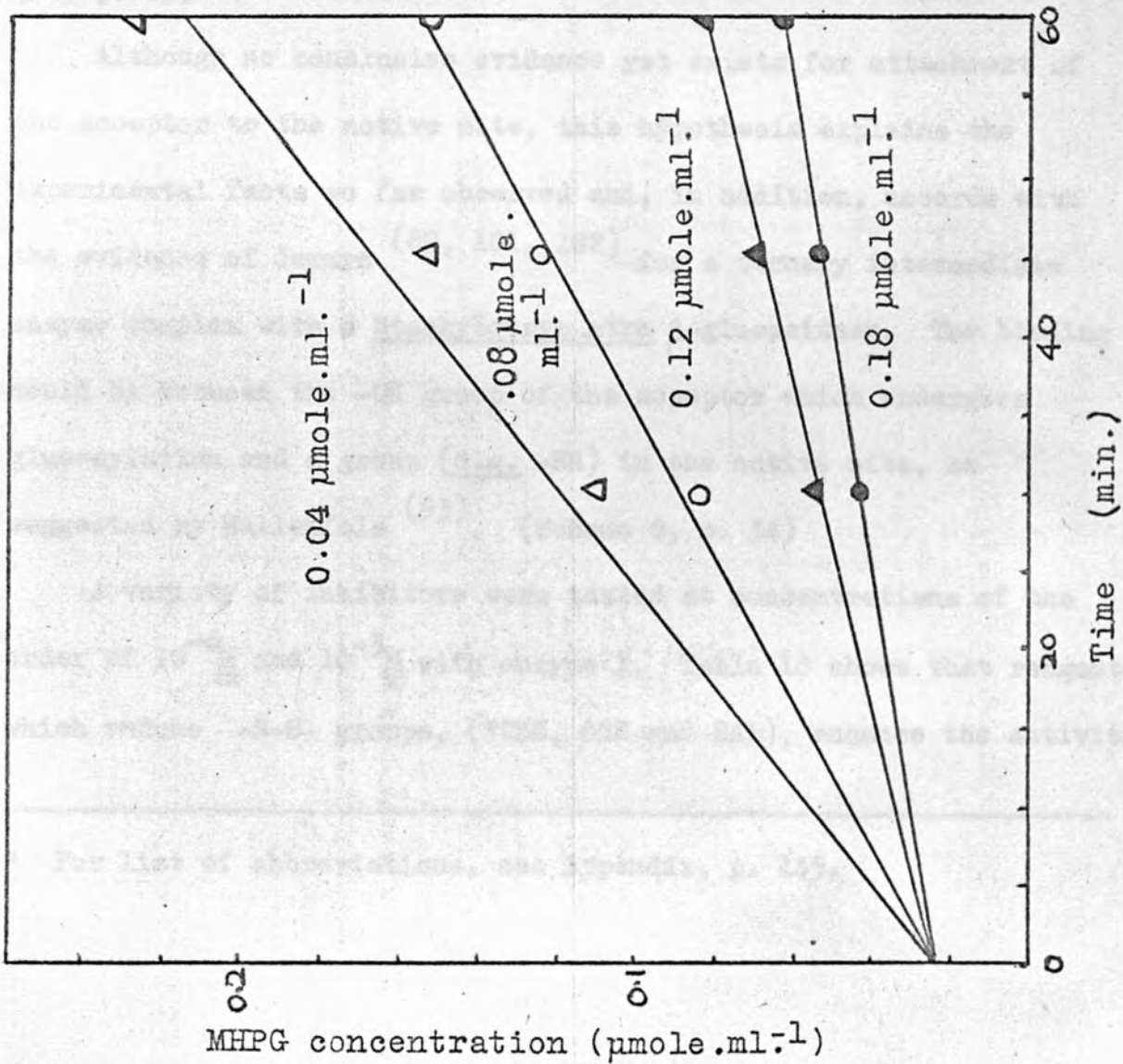
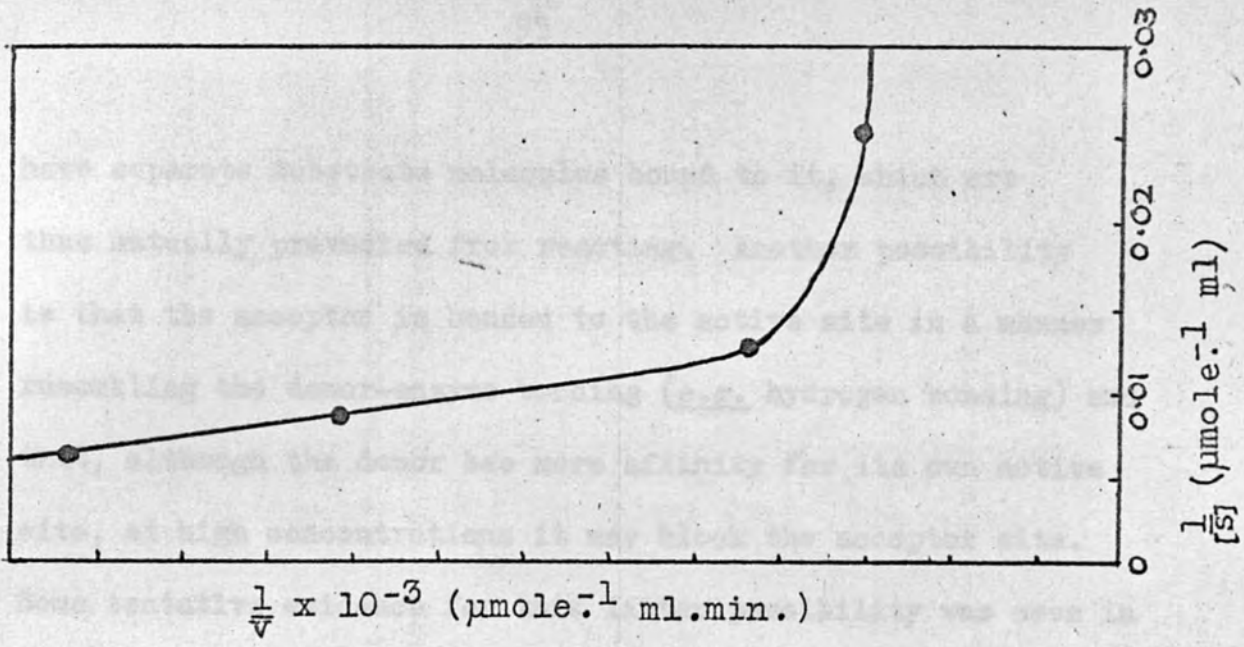


Fig. 11: Variation of rate of MHPG formation (v) with substrate concentration (s) for enzyme X

have separate substrate molecules bound to it, which are thus mutually prevented from reacting. Another possibility is that the acceptor is bonded to the active site in a manner resembling the donor-enzyme bonding (e.g. hydrogen bonding) and that, although the donor has more affinity for its own active site, at high concentrations it may block the acceptor site. Some tentative evidence for this latter possibility was seen in the effect of increasing  $[E]$  for enzyme X, (and possibly Y, also). (See p. 88)

Although no conclusive evidence yet exists for attachment of the acceptor to the active site, this hypothesis explains the experimental facts so far observed and, in addition, accords with the evidence of Jermyn (89, 101, 102) for a ternary intermediate enzyme complex with a Stachybotrys atra  $\beta$ -glucosidase. The binding could be between the -OH group of the acceptor which undergoes glucosylation and a group (e.g. -SH) in the active site, as suggested by Wallenfels (93). (Scheme 8, p. 34)

A variety of inhibitors were tested at concentrations of the order of  $10^{-4}$  M and  $10^{-3}$  M with enzyme X. Table 18 shows that reagents which reduce -S-S- groups, (\*CSH, GSH and BAL), enhance the activity

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\* For list of abbreviations, see Appendix, p. 145.



**Table 18** : **Effect of sulphhydryl reagents upon enzyme X**

Activity of untreated enzyme = 100%

Reagent*	Type of reaction	Concentration in digest ( <u>M</u> )	% Activity remaining
CSH	Reduction of -S-S- groups	$2.5 \times 10^{-3}$	143
		$2.5 \times 10^{-2}$	177
		$7.5 \times 10^{-2}$	149
BAL	"	$1 \times 10^{-4}$	115
		$1 \times 10^{-3}$	160
GSH	"	$1 \times 10^{-4}$	242
		$1 \times 10^{-3}$	277
GSSG	Oxidation of - SH groups	$1 \times 10^{-4}$	138
		$1 \times 10^{-3}$	206
DTNB	"	$2 \times 10^{-5}$	160

(continued overleaf)

\* For list of abbreviations see Appendix, p.145.

Table 18 : (Continued)

Reagent*	Type of reaction	Preincubation concentration M (M)	Concentration in digest	% Activity remaining
Iodoacetic acid	Alkylation of -SH groups	---	$1 \times 10^{-4}$	100
		---	$1 \times 10^{-3}$	100
		$1 \times 10^{-4}$	$4 \times 10^{-5}$	90
		$1 \times 10^{-3}$	$4 \times 10^{-4}$	77
Phenyl mercuri-acetate	Mercaptide (-S-Hg-) formation	---	$1 \times 10^{-4}$	88
		---	$1 \times 10^{-3}$	58
		$1 \times 10^{-4}$	$4 \times 10^{-5}$	66
		$1 \times 10^{-3}$	$4 \times 10^{-4}$	53
PCMB	"	---	$6.6 \times 10^{-5}$	70
		---	$6.6 \times 10^{-4}$	47
		$6.6 \times 10^{-5}$	$2.6 \times 10^{-5}$	32
		$6.6 \times 10^{-4}$	$2.6 \times 10^{-4}$	26
PCMB+ excess CSH <sup>†</sup>	Reversal of mercaptide formation	$(6.6 \times 10^{-5})_+$ $(1.7 \times 10^{-3})$	$(2.6 \times 10^{-5})_+$ $(7 \times 10^{-4})$	140
		$(6.6 \times 10^{-4})_+$ $(1.7 \times 10^{-3})$	$(2.6 \times 10^{-4})_+$ $(7 \times 10^{-4})$	127

\* For list of abbreviations see Appendix, p.145.

--- 40min. preincubation of enzyme with inhibitor

† CSH added during preincubation, 30min. after PCMB

of the enzyme. In the case of CSH, a high concentration ( $7.5 \times 10^{-2} \underline{\underline{M}}$ ) was less effective than an intermediate one ( $2.5 \times 10^{-2} \underline{\underline{M}}$ ). The increase in activity of the enzyme was as high as 2.8-fold with  $10^{-3} \underline{\underline{M}}$ -GSH. GSSG at the same concentration was also found to activate the enzyme, although this reagent normally oxidises -SH groups and has the reverse effect to GSH. However, in the system used, the excess resorcinol may have appreciably reduced GSSG to GSH. Another dithio-compound, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), also behaved as an activator and a similar explanation may apply.

The -SH group alkylating agent, iodoacetic acid, only decreased the activity of X when preincubated with the enzyme for 40min., and then only by 10% and 23% at  $10^{-4} \underline{\underline{M}}$  and  $10^{-3} \underline{\underline{M}}$ , respectively. This reagent has been found to give zero or low inhibition with other glycosyltransferases. Jørgensen <sup>(184)</sup> found it to be ineffective with barley malt  $\alpha$ -glucosidase and Igaue <sup>(185)</sup> observed low inhibition (5-10%) at  $10^{-3} \underline{\underline{M}}$  with a rice Q-enzyme, ( $\alpha$ -1,4-glucan:  $\alpha$ -1,4-glucan 6-glucosyltransferase).

Phenyl mercuriacetate and PCMB were also more effective as inhibitors when preincubated with the enzyme than when used

directly in incubation. Addition of excess CSH after PCMB, during preincubation, completely reversed the inhibitory effect of PCMB and caused activation of X (as found with CSH alone).

Heavy metal ions inhibited the activity of X, (Table 19), the order of effectiveness at  $10^{-3}$  g.ion.l.<sup>-1</sup> being  $\text{Hg}^{2+} \gg \text{Ag}^+ > \text{Pb}^{2+} > \text{Cu}^{2+} > \text{Fe}^{3+}$ .  $\text{Fe}^{3+}$  ion was only a feeble inhibitor. The exact action of heavy metals in inhibition is unknown (186), but there is evidence that they react, at least in part, with -SH groups.

The above evidence suggests that for enzyme X, -SH groups are involved either in the active site of the enzyme or in maintaining the enzyme in an active configuration. The pH-maximum of the enzyme also suggests that L-cysteine may be involved in its activity (93).

The failure to produce complete inhibition with -SH group reagents was probably due to some of these groups occupying a protected position in the protein. A number of instances of this are known (187). The highest degree of inhibition produced, (with  $6.6 \times 10^{-4}$  M-PCMB and  $10^{-3}$  M- $\text{Hg}^{2+}$ ), is 74%, in both cases. This suggests that about a quarter of the -SH groups involved in the enzyme activity may be protected in some way under the conditions employed.

EDTA inhibited X to a low degree (12% at both  $10^{-4}$  M and  $10^{-3}$  M). A number of metal ions were added to EDTA-treated enzyme samples to

**Table 19 : Effect of heavy metal ions upon enzyme X**

Activity of untreated enzyme = 100%

Ion	Concentration in digest (g.ion.l. <sup>-1</sup> )	% Activity remaining
Hg <sup>2+</sup>	1 x 10 <sup>-4</sup>	77
	1 x 10 <sup>-3</sup>	26
Ag <sup>+</sup>	1 x 10 <sup>-4</sup>	72
	1 x 10 <sup>-3</sup>	46
Pb <sup>2+</sup>	1 x 10 <sup>-4</sup>	69
	1 x 10 <sup>-3</sup>	50
Cu <sup>2+</sup>	1 x 10 <sup>-4</sup>	86
	1 x 10 <sup>-3</sup>	53
Fe <sup>3+</sup>	1 x 10 <sup>-4</sup>	96
	1 x 10 <sup>-3</sup>	70

ascertain whether the inhibitory effect of EDTA was due to the removal of metal ions (Table 20). None of the metal ions tested restored the activity, and  $K^+$  and  $Ca^{2+}$ , and possibly  $Mg^{2+}$ , further inhibited the enzyme slightly. (The assay method has a maximum error of 2%).

Wallenfels (93), Jørgensen (184) and Halvorson (188) found evidence of participation of L-histidine in the hydrolysis of glycosides by E.coli  $\beta$ -galactosidase, barley malt  $\alpha$ -glucosidase and Saccharomyces italicus  $\alpha$ -glucosidase, respectively (c.f. Wallenfels' mechanism of glycosidase action, Scheme 8, p.34).

However, two imidazole reagents,  $Be^{2+}$  (93) and illuminated methylene blue (93, 184) (Table 21), failed to decrease the activity of enzyme X. It is not possible to say whether or not L-histidine exists in the active site of X. It is possible that it is present in a protected position and is thus unreactive (c.f. the -SH groups discussed above).

D-glucose and 1,5-D-gluconolactone, in concentrations of about  $2x[s]$ , inhibited enzyme X appreciably. These probably behave as competitive inhibitors, as their structures are closely related to the non-reducing D-glucosyl moiety of the substrate.

Inhibition of glycosidases by polyols has recently been found by Kelemen and Whelan (189). Jermyn found with Stachybotrys atra  $\beta$ -glucosidase that lower polyols were both competitive and anticompetitive inhibitors (i.e. they blocked

Table 20 : Effect of metal ions upon EDTA-treated enzyme X

Activity of untreated enzyme = 100%

" " EDTA-treated " = 88%

Metal ion ( $10^{-3}$ g.ion.l. $^{-1}$ )	% Activity (of untreated enzyme) remaining
Na <sup>+</sup>	86
K <sup>+</sup>	84
Ca <sup>2+</sup>	84
Mg <sup>2+</sup>	85
Zn <sup>2+</sup>	89
Co <sup>2+</sup>	89
Mn <sup>2+</sup>	89

Table 21 : Effect of imidazole reagents upon enzyme X

Activity of untreated enzyme = 100%

Reagent	Concentration ( <u>M</u> )	Time of illumination (min.)	% Activity remaining
Methylene <sup>‡</sup> blue (in photo- oxidation)	0	60	102
	$1.6 \times 10^{-4}$	0	100
	$1.6 \times 10^{-4}$	30	101
	$1.6 \times 10^{-4}$	60	102
Be <sup>2+</sup>	$1 \times 10^{-4}$	--	99
	$1 \times 10^{-3}$	--	101

‡ Enzyme preincubated with inhibitor and illuminated by 500W lamp; inhibitor removed by dialysis.



**Table 22 : Effect on enzyme X of compounds resembling D-glucose**

Activity of untreated enzyme = 100%

Compound	Concentration in digest ( <u>M</u> )	% Activity remaining
<u>D</u> -glucose	$1.5 \times 10^{-1}$	66
1,5- <u>D</u> -glucono-lactone	$1.5 \times 10^{-1}$	58
Erythritol <sup>⊠</sup>	$5 \times 10^{-1}$	73
<u>D</u> -mannitol <sup>⊠</sup>	$5 \times 10^{-1}$	82
<u>D</u> -glucitol <sup>⊠</sup>	$5 \times 10^{-1}$	71

<sup>⊠</sup> Data from Table 23, included for comparison.

both donor and acceptor sites), whereas higher polyols were almost entirely competitive inhibitors. The degree of inhibition in the latter case appeared to depend upon the extent to which the substance resembled that non-reducing D-glucosyl moiety of the substrate. Kelemen (190) found that monohydric alcohols gave total inhibition of enzymes of this type. Although the possibility of denaturation of the enzyme in the presence of monohydric substances cannot be excluded, the amount of denaturation at the concentrations of alcohols used ( $\leq 0.5M$ ) is unlikely to be great, and therefore monohydric compounds probably have a strong tendency to hydrogen-bond with one or both of the active sites.

In this present study, a series of polyols (2 to 6 -OH groups) was tested with enzyme X. The results are shown in Table 23. Fig.12 (data of Table 23) shows the variation of inhibition produced with the number of -OH groups present in the polyol. Since comparatively high concentrations of polyols were required, the inhibition was probably competitive. D-glucitol (see Fig.12) gave a higher inhibition than predicted by the curve, presumably due to its considerable resemblance to D-glucose. It was, however, less effective as an inhibitor than compounds resembling the substrate and possessing a pyranoid structure (See Table 22).

Inhibition with diols was high in the present case,

Table 23 : Effect of polyols<sup>\*</sup> upon enzyme X

Activity of untreated enzyme = 100%

* Ref.no.	Polyol	No.of-OH groups	Distribution of -OH groups in diols	%Inhibition
1	Ethandiol	2	$\alpha$	67
2	Propan-1,2-diol	2	$\alpha$	72
3	Propan-1,3-diol	2	$\beta$	77
4	Butan-1,3-diol	2	$\beta$	74
5	Butan-1,4-diol	2	$\gamma$	65
6	Butan-2,3-diol	2	$\alpha$	65
7	2,2-Dimethyl propan-1,3-diol )	2	$\beta$	65
8	Pentan-1,5-diol	2	$\delta$	56
9	2,5 Dimethyl hexan-2,5-diol )	2	$\gamma$	61
10	Glycerol	3	--	30
11	Erythritol	4	--	27
12	Pentaerythritol	4	--	26
13	<u>D</u> -mannitol	6	--	18
14	<u>D</u> -glucitol	6	--	29

\* See Fig:12, p107.

Fig. 12: Inhibition by polyols of MHPG formation

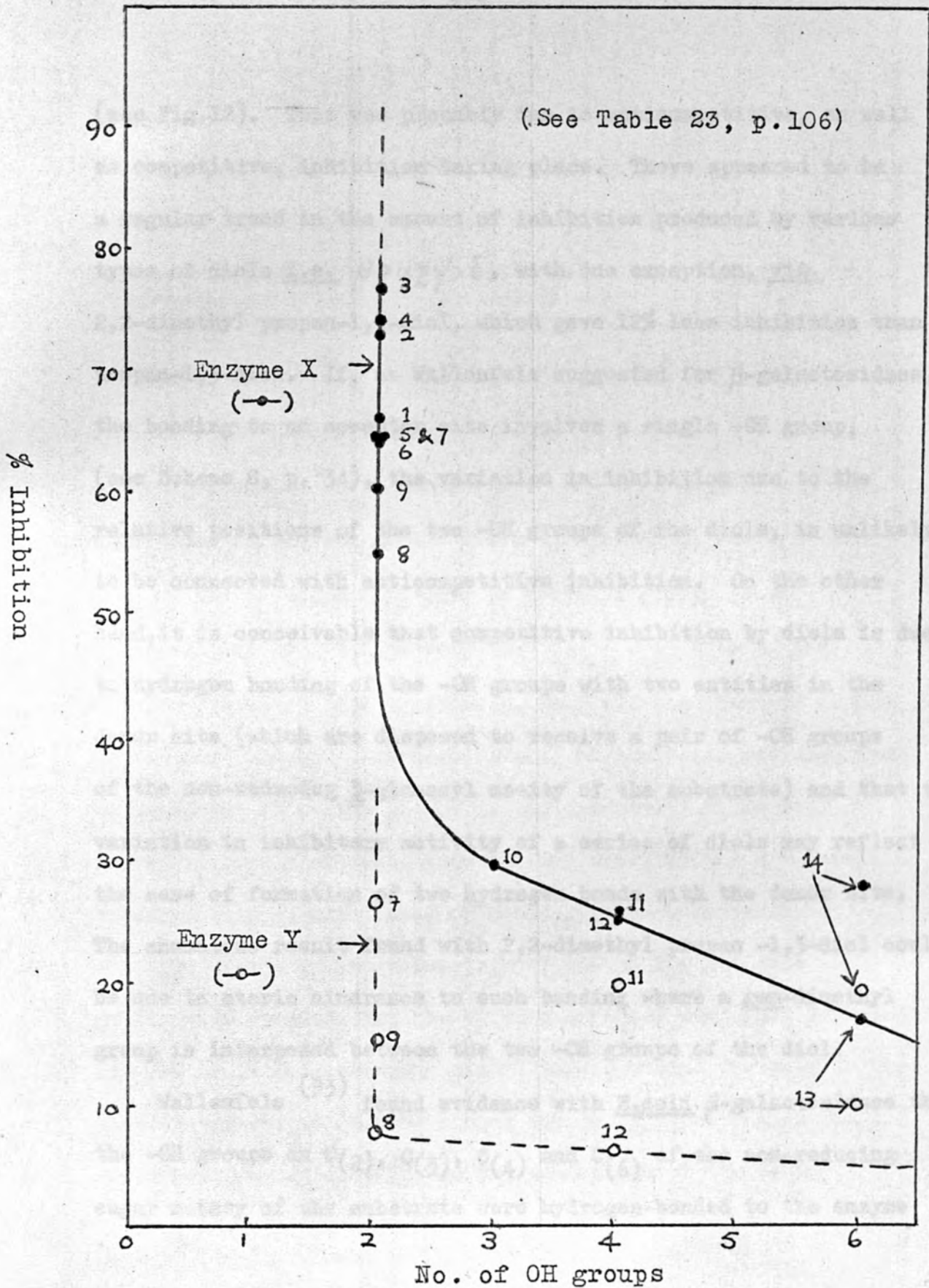


Fig. 12: Inhibition by polyols of MHPG formation

(see Fig.12). This was probably due to anticompetitive, as well as competitive, inhibition taking place. There appeared to be a regular trend in the amount of inhibition produced by various types of diols i.e.  $\beta > \alpha > \gamma > \delta$ , with one exception, viz. 2,2-dimethyl-propan-1,3-diol, which gave 12% less inhibition than propan-1,3-diol. If, as Wallenfels suggested for  $\beta$ -galactosidase, the bonding to an acceptor site involves a single -OH group, (see Scheme 8, p. 34), the variation in inhibition due to the relative positions of the two -OH groups of the diols, is unlikely to be connected with anticompetitive inhibition. On the other hand, it is conceivable that competitive inhibition by diols is due to hydrogen bonding of the -OH groups with two entities in the donor site (which are disposed to receive a pair of -OH groups of the non-reducing D-glucosyl moiety of the substrate) and that the variation in inhibitory activity of a series of diols may reflect the ease of formation of two hydrogen bonds with the donor site. The anomalous result found with 2,2-dimethyl propan -1,3-diol could be due to steric hindrance to such bonding where a gem-dimethyl group is interposed between the two -OH groups of the diol.

Wallenfels <sup>(93)</sup> found evidence with E.coli  $\beta$ -galactosidase that the -OH groups on C<sub>(2)</sub>, C<sub>(3)</sub>, C<sub>(4)</sub> and C<sub>(6)</sub> of the non-reducing sugar moiety of the substrate were hydrogen-bonded to the enzyme

in the enzyme-substrate complex (see Scheme 9, p. 35) and the inhibition by various types of diol in the present case could be explained on the theory that all the free -OH groups of the D-glucosyl moiety of the donor are involved in bonding to the donor site. Thus a  $\delta$ -diol could hydrogen bond across groups on the enzyme disposed to receive the C<sub>(2)</sub> and C<sub>(6)</sub> hydroxyl groups of the D-glucosyl moiety of the substrate. A  $\gamma$ -diol could bond across the analogous C<sub>(3)</sub> and C<sub>(6)</sub> sites. A  $\beta$ -diol might bond across the normal sites of bonding of the C<sub>(2)</sub> and C<sub>(4)</sub> or the C<sub>(4)</sub> and C<sub>(6)</sub> pair of hydroxyl groups in the substrate and an  $\alpha$ -diol across the C<sub>(2)</sub> - C<sub>(3)</sub> site or the C<sub>(3)</sub> - C<sub>(4)</sub> site. The hypothesis, that all the free -OH groups of the non-reducing D-glucosyl moiety of the donor are involved in anchoring it to the enzyme during the reaction, is only tentative, and must await experimental observations with modified substrates (e.g. methylated or deoxy-D-glucose derivatives.)

The effect of inhibitors upon Y has so far not been extensively studied, but the effects of a few are shown in Table 24. There is some evidence with Y that cysteine residues in the protein participate in the enzyme action (c.f. effect of GSH and phenyl mercuriacetate and the optimum pH of the enzyme).

Table 24 : Effect of various reagents upon enzyme Y

Activity of untreated enzyme = 100%

Compound <sup>‡</sup>	Concentration ( <u>M</u> )	% Activity remaining
GSH	$1 \times 10^{-3}$	170
GSSG	$1 \times 10^{-3}$	100
Iodoacetic acid	† $1 \times 10^{-3}$	100
Phenyl mercuriacetate	† $1 \times 10^{-3}$	60
Methylene blue <sup>‡‡</sup>	$1.6 \times 10^{-4}$	93
Be <sup>2+</sup>	$2 \times 10^{-3}$	94
1,5- <u>D</u> -gluconolactone	$1.5 \times 10^{-1}$	89
2,2-Dimethyl propan-1,3-diol )	$5 \times 10^{-1}$	73
Pentan-1,5-diol	$5 \times 10^{-1}$	92
2,5-Dimethyl hexan-2,5-diol )	$5 \times 10^{-1}$	84
Erythritol	$5 \times 10^{-1}$	79
Pentaerythritol	$5 \times 10^{-1}$	92
<u>D</u> -mannitol	$5 \times 10^{-1}$	88
<u>D</u> -glucitol	$5 \times 10^{-1}$	79

‡ For abbreviations used see Appendix, p.145.

† Preincubation concentration (otherwise incubation)

‡‡ Photo-oxidation (500W lamp; 60min. preincubation).

The participation of histidine (c.f. effects of  $\text{Be}^{2+}$  and photo-oxidation with methylene blue) is doubtful, however. The anomalous effects of GSSG and iodoacetic acid found with this enzyme were discussed in conjunction with X. 1,5-D-gluconolactone acts as an inhibitor, probably competitively. A limited selection of polyols was tested with Y. Only three diols were examined, but the variation of the amount of inhibition according to the positions of the -OH groups was the same as for X, i.e.  $\beta > \gamma > \delta$ . A possible curve (with limited data) of the amount of inhibition produced, against the number of -OH groups in the polyol, is included in Fig.12 (p.107), for comparison with X. Again, with Y, D-glucitol inhibition was substantially higher than predicted. The values with D-mannitol and erythritol were also high with Y. D-mannitol differs from the non-reducing glucose moiety of maltose only on  $\text{C}_{(1)}$  and  $\text{C}_{(2)}$  and erythritol resembles the D-glucosyl group on  $\text{C}_{(3)}$ ,  $\text{C}_{(4)}$  and  $\text{C}_{(6)}$ .

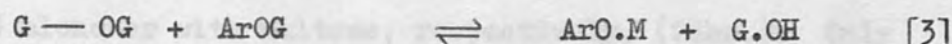
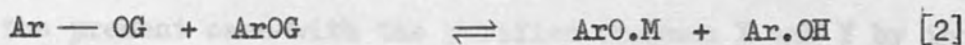
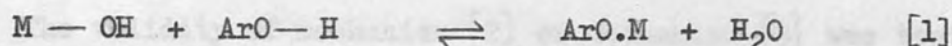
To summarize, the enzymes X and Y are two distinct species varying somewhat in physicochemical properties, but very little in substrate specificities. The substrate specificity towards the donor is comparatively high and could involve hydrogen-bonding between the -OH groups on  $\text{C}_{(2)}$ ,  $\text{C}_{(3)}$ ,  $\text{C}_{(4)}$  and  $\text{C}_{(6)}$  of the non-reducing D-glucose moiety of the donor of a type first



postulated by Wallenfels <sup>(93)</sup>, (c.f. <sup>(121)</sup>). The acceptor specificity is fairly low. Acceptors may be bonded to the active site (101, 102, 121), e.g. by a hydrogen bond. Cysteine residues probably participate in the action of both enzymes. No evidence for the presence of histidine in the active site of X was obtained, and its presence was doubtful in the case of Y.

The reaction between maltose and resorcinol produced O-α-D-glucosyl-α-D-glucosides in addition to a monoglucoside (see part I of this section). A qualitative examination of the formation of the diglucosides using the crude enzyme over a period of 24hr. indicated that traces of m-hydroxyphenyl α-D-isomaltoside were only present after 1.5-2hr., the concentration gradually increasing until about 8hr., when it remained steady. The α-maltoside was first detected after 10hr., and reached a constant concentration in 18hr. From the intensity of the chromatographic spots, the amount of α-maltoside formed was less than the amount of α-isomaltoside, after 24hr., both being considerably less than the amount of MHPG. Both X and Y gave the α-isomaltoside, but only X appeared to give the α-maltoside in 24hr.

There are three possible mechanisms for the enzymic formation of O-α-D-glucosyl-α-D-glucosides from resorcinol and maltose. These are illustrated in Scheme 16, for the -maltoside.



M = maltosyl; G = D-glucosyl;

Ar = m-hydroxyphenyl

#### Scheme 16

Mechanism [1] would require prior isomerisation of maltose to isomaltose in the case of the  $\alpha$ -isomaltoside formation. In mechanisms [2] and [3], the D-glucosyl residue could be transferred to the -OH group on either C<sub>(4)</sub> or C<sub>(6)</sub> of the acceptor glucone, giving the  $\alpha$ -maltoside or the  $\alpha$ -isomaltoside, respectively. Cases of all three mechanisms are known, although not necessarily with aryl acceptors. Whelan *et. al.* (191) found an enzyme in sweet corn which will repeatedly transfer intact maltosyl residues with <sup>6</sup> $\alpha$ -maltosylmaltotriose as both the donor and the initial acceptor (*c.f.* Mechanism [1]). An instance of mechanism [2] where arbutin molecules act both as donors and as acceptors, was discussed in the Introduction (Scheme 4, p.27). Stepwise glycosylation of an acceptor (mechanism [3]) is common among enzymes responsible for oligo- and polysaccharide formation (192, 193).

The validity of mechanism [2] or mechanism [3] was tested in the present case with the purified enzymes X and Y by incubating MHPG alone or with maltose, respectively, (24hr.). Only MHPG in the presence of maltose gave the diglucosides, indicating that glucosylation was stepwise (mechanism [3]). Replacement of maltose by UDPG or an isomaltose/isomaltotriose mixture as donor gave the same products with X and Y. Replacement of MHPG by its o-isomer, OHPG, gave analogous diglucosyl compounds with X and Y. (See Table 25) Traces of two other compounds with  $R_f$  values [solvent (B)] intermediate between those of the isomaltoside and maltoside and between the maltoside and D-glucoside were also found. They may have been the  $\alpha$ -kojibioside and the  $\alpha$ -nigeroside, respectively.

- isomaltose
- Is<sub>3</sub> - isomaltotriose
- (I) - o-hydroxyphenyl  $\alpha$ -isomaltoside
- (II) - o-hydroxyphenyl  $\alpha$ -maltoside
- (IV) - o-hydroxyphenyl  $\alpha$ -isomaltoside
- (V) - o-hydroxyphenyl  $\alpha$ -maltoside

See part I, p. 55 for identification of o-o-o-glucosyl-o-o-glucosides.

Table 25 : O- $\alpha$ -D-glucosyl- $\alpha$ -D-glucosides formed from MHPG or OHPG and various donors with enzymes X and Y

Donor	Acceptor	Products <sup>*</sup>	
		Enzyme X	Enzyme Y
Maltose	MHPG	(I),(II)	(I)
Im/Im <sub>3</sub>	MHPG	(I),(II)	(I)
UDPG	MHPG	(I),(II)	(I)
Maltose	OHPG	(IV),(V)	(IV)
Im/Im <sub>3</sub>	OHPG	(IV),(V)	(IV)
UDPG	OHPG	(IV),(V)	(IV)

Key : Im = isomaltose  
 Im<sub>3</sub> = isomaltotriose  
 (I) = m-hydroxyphenyl  $\alpha$ -isomaltoside  
 (II) = m-hydroxyphenyl  $\alpha$ -maltoside  
 (IV) = o-hydroxyphenyl  $\alpha$ -isomaltoside  
 (V) = o-hydroxyphenyl  $\alpha$ -maltoside

<sup>\*</sup> See part I, p. 55 for identification of O- $\alpha$ -D-glucosyl- $\alpha$ -D-glucosides.

GENERAL METHODSPaper Chromatography

The descending technique was used with Whatman papers (No. 1 for sugars and No. 3 for phenols) and the following solvents

EXPERIMENTAL SECTION

- (1) Ethyl acetate, acetic acid, water (9:2:1). (194)
- (2) n-Butanol, ethanol, water (20:11:1). (195)
- (3) Ethyl acetate, pyridine, water (10:1:1). (196)

Mobilities of phenolic compounds are expressed as  $R_f$  values and mobilities of sugars are given as  $R_D$  values, where

$$R_D = \frac{\text{distance travelled by a substance}}{\text{distance travelled by D-glucose standard}}$$

Thin Layer Chromatography

The mobile phases were:

- (1) Toluene, ether (1:2). (157)
- (2) Benzene, acetone (1:1).

Sugar Reagents

- (1) Disodium p-nitroaniline / NaOH, (197) for phenols.
- (2) p-anilidine hydrochloride, (198) for reducing sugars.
- (3) Aniline oxalate, (199) for reducing sugars.
- (4) Silver nitrate / NaOH, (199) for sugars.
- (5) Sulphuric acid (1:1), (200) for sugars.

GENERAL METHODSPaper Chromatography

The descending technique was used with Whatman papers (No.1 for sugars and No.3 for phenols) and the following solvent systems (proportions by volume):

- (A) Ethyl acetate, acetic acid, water (9:2:2).<sup>(194)</sup>  
 (B) n-Butanol, ethanol, water (40:11:19).<sup>(195)</sup>  
 (C) Ethyl acetate, pyridine, water (10:4:3).<sup>(196)</sup>

Mobilities of phenolic compounds are expressed as  $R_f$  values and mobilities of sugars are given as  $R_G$  values, where

$$R_G = \frac{\text{distance travelled by a substance}}{\text{distance travelled by } \underline{\underline{D}}\text{-glucose standard}}$$

Thin Layer Chromatography

The mobile phases were:

- (D) Toluene, ether (1:2).<sup>(157)</sup>  
 (E) Benzene, acetone (1:1).

Spray Reagents

- (1) Diazotised p-nitroaniline /NaOH,<sup>(197)</sup> for phenols.  
 (2) p-Anisidine hydrochloride,<sup>(198)</sup> for reducing sugars.  
 (3) Aniline oxalate,<sup>(198)</sup> for reducing sugars.  
 (4) Silver nitrate / NaOH,<sup>(199)</sup> for sugars.  
 (5) Sulphuric acid (5% aq.),<sup>(157)</sup> for sugars.

### Vapour Phase Chromatography

For sugar derivatives, the relative retention time,  $T$ , is given by:

$$T = \frac{\text{Distance of peak due to unknown from base-line}}{\text{(Distance of peak due to methyl 2,3,4, 6-tetra-O-methyl } \beta\text{-D-glucopyranoside from base line)}}$$

### Paper Electrophoresis

This was carried out for sugar alcohols using Whatman No.1 paper and aqueous sodium molybdate (pH 5.0; 1.5% w/v), at 2000v. (200)

$M_S$  values are given by:

$$M_S = \frac{\text{distance travelled by a substance}}{\text{distance travelled by D-glucitol standard}}$$

Electrophoresis of phenolic carboxylic acids was carried out using No.1 paper and phosphate buffer (pH 7.2, 0.1M) at 2,000v. (182)

$M_{SA}$  values are given by:

$$M_{SA} = \frac{\text{distance travelled by a substance}}{\text{distance travelled by salicylic acid standard}}$$

### I.R. Spectrophotometry

Compounds were examined as Nujol mulls on NaCl plates in a Perkin-Elmer 137 Spectrophotometer.

### U.V. and Visible Region Spectrophotometry

Absorbances of aqueous solutions were determined in 1cm. silica cells in a Unicam SP500 Spectrophotometer. Unless otherwise stated, water was used as the reference.

Experiment 1: Cultivation of Aspergillus niger mycelium.Culture Slopes

The spores of the fungus, Aspergillus niger, Birmingham Strain 152, were stored on potato-agar slopes, at 0-5°C.

These were prepared from a solution of agar (Davis, New Zealand; 30g.) in potato extract (1 l.), obtained by boiling sliced potatoes (200g.) for 1hr., followed by straining.

After autoclaving (15lb.in.-2; 20min.), the medium was tubed and allowed to set in an inclined position.

Culture Medium : Currie's synthetic medium (201)

The sucrose medium for mycelial growth contained the following : sucrose (600g.),  $\text{NH}_4\text{NO}_3$  (10g.),  $\text{KH}_2\text{PO}_4$  (4g.),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (4g.),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2g.),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2g.) and water (4 l.). The initial pH was adjusted to 6.7 and the solution divided between 16 penicillin pans. After autoclaving (15lb.in.-2; 20min.), the medium was inoculated with A.niger spores and incubated at 25°C until a firm mat of unspored mycelium had formed (6 to 9 days). After harvesting, the mycelium was washed with distilled water and partially dried between sheets of filter-paper. It was stored at 0-5°C.



Experiment 2 : Preparation of a crude A.niger enzyme extract.

A.niger mycelium, dried partially with filter-paper, was macerated in distilled water (15g.in 100ml.) in a blender (M.S.E. Homogenizer). This gave an aqueous solution of pH about 5. The cell debris was centrifuged off at 0-5°C (M.S.E. Centrifuge; 2,000xg;30min.) and the clear supernatant, dialysed overnight against distilled water (0-5°C), comprised the crude enzyme preparation.

Experiment 3 : Preparation of aryl glucosides (I), (II) & (III)

A large-scale digest containing crude aqueous enzyme solution (400ml.), 0.1M-sodium acetate buffer (400ml; pH 5.1), 0.60M-maltose solution (140ml.), 4.3M-resorcinol solution (40ml.) and 1.2M-L-cysteine solution (20 ml.) was incubated for 24hr. The reaction was stopped with 1.2M-TCA solution (40ml.) and the digest concentrated to a thick syrup, at 40°C, under vacuum. The syrup was then dissolved in methanol (100ml.) and the components of the

buffer/enzyme solution with vigorous stirring

and the whole made up to 600 ml.

It was stored at 4°C in a dark bottle

and remained active for several months.

methanolic solution, which included three resorcinol glucosides, were fractionated on Whatman No.3 paper. (solvent (A); 5-6hr. development). The glucosides were eluted separately from the papers with water, and the solution concentrated to dryness, under vacuum.

Experiment 4 : Determination of the sugar-phenol ratio of the aryl glucosides (I), (II) & (III)

(a) Estimation of glucose by the glucose-oxidase method

This method is applicable to solutions of D-glucose of concentrations up to  $3.5\text{mg}\cdot\text{ml}^{-1}$  and required the following:

Solutions (A) Buffer/enzyme solution (500ml.)

0.12M phosphate buffer, (pH 7)

40  $\mu\text{g}\cdot\text{ml}^{-1}$  horseradish peroxidase

250  $\mu\text{g}\cdot\text{ml}^{-1}$  D-glucose-oxidase.

(B) Chromogen (4ml.)

6.6  $\text{mg}\cdot\text{ml}^{-1}$  o-anisidine hydrochloride.

(C) Glucose reagent (600 ml.)

The chromogen solution was added to the

buffer/enzyme solution with vigorous stirring and the whole made up to 600 ml.

It was stored at  $4^{\circ}\text{C}$  in a dark bottle

and remained active for several months.

- Solutions
- (D) D-Glucose standard solution (20ml.)  
 91µg.ml.<sup>-1</sup> D-glucose.
- (E) Perchloric acid (for deproteinisation)  
 Approximately 3.3% (w/v) i.e. perchloric acid  
 (A.R.; 2.85ml; 70% aq.) diluted to 100ml.  
 with water.

The concentration of D-glucose in the unknowns was found using the following:

- Standard 0.2ml. D-glucose solution (D)  
 5.0ml. glucose reagent (C)
- Blank 0.2ml. water  
 5.0ml. glucose reagent (C)
- Sample 0.2ml. solution containing glucose (unknown)  
 5.0ml. glucose reagent (C)

The samples were allowed to react at room temperature and the absorbance was determined at 436 mµ, after 35min.

(b) Hydrolysis of the glucosides using maltase

A digest (10.0ml.) was prepared with each glucoside as follows:

Glucoside solution

2.0ml. glucoside (I), (II) or (III) solution approximate  
 respective concentrations: 0.1 µmole.ml.<sup>-1</sup>, 0.05 µmole.ml.<sup>-1</sup>  
 & 0.25 µmole.ml.<sup>-1</sup>  
 2.0ml. water

4.0ml.0.1M-sodium acetate buffer (pH 6.5).

#### Maltase solution

25 mg.ml.<sup>-1</sup> maltase (ex yeast, Light & Co.) in water.

#### Blank

The glucoside solution (0.8ml.) was run into a tube standing in ice, containing perchloric acid solution (E) (10.0ml.).

The maltase solution (0.2ml.) was then added.

(Preliminary experiments with methyl  $\beta$ -D-glucoside, phenyl  $\beta$ -D-glucoside and p-hydroxyphenyl  $\beta$ -D-glucoside showed that the maltase was devoid of  $\beta$ -D-glucosidase activity.)

#### Samples

At zero time, maltase solution (1.8ml.) was added to the glucoside solution (7.2ml.). The digest was incubated at 30°C and 8 samples (1.0ml.) were removed at time intervals up to 4hr. These were run immediately into ice-cold perchloric acid solution (10.0ml.) to stop the reaction, and kept at 0°C until the reagent (C) was added.

A preliminary determination of D-glucose in the presence of an equimolar quantity of resorcinol showed that the latter lowered the absorbance readings at 436 m $\mu$  and hence all the resorcinol was extracted from the digests with ether before proceeding to

the glucose-oxidase determination.

The perchloric acid-treated samples were allowed to warm up to room temperature and aliquots (0.2ml.) were treated with reagent (C) (5.0ml.). The absorbance at 436 m $\mu$  was determined after 35min. A plot of the amount of D-glucose liberated with time is shown for each glucoside in Fig. 2. (p. 58)

The D-Glucose concentration after complete hydrolysis was found from the graph.

The aliquot of the digest (1.0ml.) remaining after 4hr.-incubation was used for the determination of the amount of resorcinol in the original glucoside sample. An aliquot (0.150ml.) was removed with a microsyringe and diluted tenfold. The resorcinol was determined with vanillin reagent (Expt.19)

(c) Calculation of sugar-phenol ratios

Glucoside (I)

By comparison with standard D-glucose curve:

final concentration of D-glucose in the hydrolysate

$$= 18.6\mu\text{g.ml.}$$

$$= 0.10\mu\text{mole.ml}^{-1}$$

By comparison with standard curve for resorcinol:

final concentration of resorcinol in the hydrolysate

$$= 5.38\mu\text{g.ml.}$$

$$= 0.05\mu\text{mole.ml}^{-1}$$

$$\text{Ratio of sugar to phenol in (I)} = 0.10:0.05$$

$$= \underline{2:1}$$

Glucoside (II)

Final concentration of D-glucose in the hydrolysate

$$= 0.042 \mu\text{mole.ml}^{-1}$$

Final concentration of resorcinol =  $0.020 \mu\text{mole.ml}^{-1}$

$$\underline{\text{Ratio of sugar to phenol in (II) = 2:1}}$$

Glucoside (III)

Final concentration of D-glucose in hydrolysate

$$= 1.10 \mu\text{mole.ml}^{-1}$$

Final concentration of resorcinol in hydrolysate

$$= 1.08 \mu\text{mole.ml}^{-1}$$

$$\underline{\text{Ratio of sugar to phenol in (III) = 1:1}}$$

Experiment 5 : Partial acid hydrolysis of glycosides (I), (II)  
& (III)

This was carried out by adding IV-hydrochloric acid (2.ml.) to an aqueous solution of the glycoside (1ml;  $5\text{mg.ml}^{-1}$ ) and boiling the solution for about 30sec. The components of the hydrolysate were detected chromatographically. Reducing sugars were detected on Whatman No.1 paper (solvent(A) ) with spray (2), (3) or (4). Resorcinol and its monoglucosides were detected on Whatman No.3 paper (solvent (A) ), on No.1 paper (solvent (B) ) and on thin layer plates (alumina solid phase; solvent (A) ). Spray (1) was used for all phenolic compounds.

Enzyme	Emulsin (ex almond)	$\beta$ -Amylase	Maltase (ex yeast)	Glucamylase (ex <u>A.niger</u> )	Pullulanase
Buffer	0.1M- acetate	0.1M- acetate	0.1M- phosphate	0.1M- acetate	0.1M- acetate
pH	5.5	5.0	6.5	5.1	5.0
Temperature (°C)	37	40	30	50	35
Incubation time (hr.)	18	18	12	18	12
Specificity	$\beta$ -1,4-D- glucose links.	Stepwise degradat- ion from non-reduc- ing end of $\alpha$ -1,4-D- glucose polymers, giving maltose.	$\alpha$ -1,4-D- glucose links. Some $\alpha$ - 1,6-D- activity	$\alpha$ -1,4-links in maltodex- trins. Also cleaves $\alpha$ -1,6- D-glucose links and aryl-glucose -links slowly.	$\alpha$ -1,6-links in D-glucose polymers.
References	(149)	(94)	(147)	(150,151)	(152)

Experiment 6 : Enzymic hydrolysis conditions  
used with glycosides (I), (II) & (III)

Experiment 7 : Permethylation of glucosides (I), (II) & (III)  
(Kuhn's Method) (156)

The glucoside samples (50-100mg.) were dissolved in freshly distilled dimethylformamide dried over anhydrous  $\text{Na}_2\text{SO}_4$  (1-2ml.). and redistilled methyl iodide (dried over  $\text{CaCl}_2$ ) was added at  $0^\circ\text{C}$ . The solution was shaken for 5min. and then dry silver oxide (1g.) was added and the mixture shaken at  $0^\circ\text{C}$ , in the dark, for 1hr. It was then shaken for a further 20hr. at room temperature. The solid formed was dissolved in dry chloroform (10ml.). The solution was filtered and the residue washed with chloroform (3 x 10 ml.). The chloroform extracts were combined and the bulk of the solvent removed under vacuum at  $40^\circ\text{C}$ . The remainder was removed by freeze-drying. The solid or syrup remaining was dried over phosphorus pentoxide. I.R. measurements were made to determine the degree of methylation, (for a fully methylated compound, the -OH band at about  $3,500\text{ cm}^{-1}$  should be smaller by a factor of about ten than the CH band at about  $2,900\text{ cm}^{-1}$ ).

It was necessary to repeat the methylation procedure in order to effect complete substitution of the hydroxyl groups.

Experiment 8 : Methanolysis of permethylated glucosides

Dry methanol was prepared by treating methanol with magnesium ribbon and distilling the resultant solution. Methanolic hydrogen chloride (approx. 3%) was made by the addition of acetyl chloride



(6ml.) to the dry methanol (95ml.). This was prepared fresh a few hours before use.

The permethylated glucosides from Expt. 7 were refluxed for 3-4hr. with methanolic HCl (5ml.) and a small amount of Amberlite 1R-120 resin (H<sup>+</sup>form). The solution was then neutralised with dry silver carbonate and filtered. The filtrate used in Expts. 9 , 10 & 11.

Experiment 9 : Thin layer chromatography of methylated methyl glucosides. (157)

Authentic samples of maltose and a mixture of isomaltose and isomaltotriose (pure isomaltose not being available) were methylated twice by the Kuhn method (Expt. 7) and then methanolised, to serve as standards (Expt. 8).

The standards and sugar components of the m-hydroxyphenyl glucosides were compared by thin layer chromatography on kieselguhr plates, using solvent (D) and spray (5).

Experiment 10 : Thin layer chromatography of resorcinol monomethyl ether.

Authentic resorcinol monomethyl ether was used as standard and alumina as the thin layer solid phase. Chromatograms were developed with solvent (E) and the phenolic ether located with spray (1). Resorcinol monomethyl ether has an R<sub>F</sub> value of about 0.5 under these conditions.

Experiment 11 : Vapour phase chromatography of methylated methyl glucosides and resorcinol monomethyl ether (158)

Samples (2-10mg.) from Expts. 7 and 8 were examined with a Pye Argon Chromatograph, at 175°C. Polyphenyl ether [m-bis-(m-phenoxyphenoxy)-benzene] was used as the stationary phase, with a celite support treated with dimethyl dichlorosilane. The methylated methyl glucosides and resorcinol monomethyl ether were detected on a single column, by comparison with standards.

Experiment 12 : Isolation and mild oxidation of catechol glucosides (IV) & (V)

Samples of catechol (o-hydroxyphenyl) glucosides (IV) and (V) were isolated from a large-scale digest containing crude aqueous enzyme extract (200ml.), 0.1M-acetate buffer (200ml.), 0.6M-maltose solution (70ml.), 2.2M-catechol solution (20ml.) and 1.2M-L-cysteine solution (10ml.).

Approximately 10mg. of each glucoside was dissolved in water (0.5ml.). Fresh ferric chloride solution (2%; 1ml.) was added to the glucoside solution and the mixture incubated at 35°C for about 10 min. It was then run down a small mixed-bed ion exchange column (Biodemineralit; H<sup>+</sup>/HCO<sub>3</sub><sup>-</sup>form), until the eluate gave negative

tests for iron and chloride, and then down a small animal charcoal column (3cm. diameter; 2cm. length). Three fractions were successively eluted with water (5ml.), 5% and 10% aqueous ethanol (10ml.). Each fraction was concentrated (0.5ml.) at 40°C under vacuum and examined on Whatman No.1 paper with solvent (A) and spray (3).

Experiment 13 : Borohydride reduction of disaccharides from the catechol glucosides (160)

The disaccharide solutions from Expt. 12 were treated with sodium borohydride solution (2ml;  $0.03\text{g.ml.}^{-1}$ ) and allowed to stand at room temperature for 3hr. The excess borohydride was destroyed by the addition of HCl. The solutions were concentrated (0.5ml.) and examined electrophoretically, using molybdate buffer (1.5%; pH 5.0; 2,000v; 1hr.). Authentic maltitol ( $M_s = 0.3$ ) and isomaltitol ( $M_s = 0.6$ ) were used as standards.

Experiment 14 : Standard enzyme digests

Maltose was chosen as the glucose-donor and resorcinol as the acceptor, since it is the least oxidisable of the dihydroxybenzenes.

Standard digests (5.0ml.) contained the following: aqueous enzyme solution (2.0ml.),  $0.1M$  - sodium acetate buffer (2.0ml;

pH 5.1), 0.60M-maltose solution (0.7ml; 0.083-M final concentration), 4.3M-resorcinol solution (0.2ml; 0.17M final concentration) and 1.2-M-L-cysteine solution (0.1ml.).

The digests were incubated for 60min. at 24°C (see Expts.16, 17 & 18 .)

The reaction was stopped by the addition of 1.2-M-trichloroacetic acid solution (TCA; 0.2ml.).

Experiment 15 : Quantitative chromatographic method for determination of enzyme activity

Aliquots (0.100ml.) of the TCA-treated digests were spotted on thick paper (No.3) using an 'Agla' micrometer syringe. Solutions of appropriate marker compounds were spotted at the edges of the paper.

The chromatograms were developed for 3.5hr. with solvent (A) and the marker spots were detected with spray (1). The areas of paper containing the glucosides were cut out and eluted with water (5.0ml.). The solutions were centrifuged and the absorbance at 273 m $\mu$  determined (1cm. cell; water reference; Unicam SP500 Spectrophotometer).

Experiment 16 : Time-course of the enzymic reaction with maltose and resorcinol

A number of standard digests were allowed to react for intervals from 1min. to 2hr. Initially crude enzyme preparations were used and the formation of MHPG was followed using the quantitative chromatographic method (Expt.15). The experiment was later repeated using the purified enzymes and the vanillin method (Expt.19), for determination of MHPG. (See Fig. 6, p.83)

Experiment 17 : Determination of the pH-optimum for enzyme activity

Standard conditions (Expt.14) were used, but the pH of the buffer was varied between 3.8 and 5.8. The crude enzyme activity, determined by the chromatographic method (Expt.15), showed a maximum at pH 5.1-5.2.

The experiment was repeated with the purified enzymes using the vanillin method for MHPG determination (Expt.19; Fig. 7, p. 85)

Experiment 18 : Determination of the temperature-optimum for enzyme activity

Standard conditions (Expt. 14) were used, but the temperature was varied between 0°C and 50°C. Using the chromatographic method (Expt. 15), the crude enzyme was shown to have a temperature-optimum of 24°C. The experiment was later repeated with the purified enzymes, the MHPG concentration being determined by the vanillin method. (Expt.19). (Fig.8, p.86 )

Experiment 19 : Ether extraction of digests and vanillin method for determination of activity

Residual resorcinol was removed from each digest by shaking with ether (15-20 x 15ml.) in a separating funnel. The components of spray (1) were used to test for the absence of resorcinol in spot tests on a depression-tile. Resorcinol gives a transient purple colour, whereas MHPG gives a red tint, which is relatively stable.

The vanillin reagent was prepared fresh for each determination by dissolving vanillin (1g.) in sulphuric acid (100ml; 70% aq.). This reagent keeps for a few hours only, and must be standardised before use.

The concentration of MHPG in the extracted digests was found by comparison with the standard, using the following solutions.

<u>Standard</u>	1.5ml. standard MHPG solution 3.0ml. vanillin reagent.
<u>Blank</u>	1.5ml. water 3.0ml. vanillin reagent.
<u>Sample</u>	1.5ml. ether-extracted digest 3.0ml. vanillin reagent.

The vanillin reagent was added rapidly with thorough stirring and the absorbance of the red solution formed was measured at 500 m $\mu$ , after 18min.

Experiment 20 : Direct U.V. spectrophotometric determination of proteins in solution (168)

The concentration of protein in column eluates was determined at 280 m $\mu$ , the  $\lambda$  max. for tryptophan.

Experiment 21 : Folin - Ciocalteu - Lowry Method of protein determination (163,164)

This method is applicable to protein solutions of concentration 25 to 500  $\mu\text{g}.\text{ml}^{-1}$

- Reagents
- (A): 2% sodium carbonate in 0.1N-NaOH
  - (B): 0.5% cupric sulphate in 1% sodium tartrate solution
  - (C): reagent (A)(50ml.) + reagent (B)(1ml.), freshly prepared each day
  - (D): Folin - Ciocalteu phenol reagent, diluted to exactly 1N in acid

Reagent (D), containing phosphotungstic and phosphomolybdic acids, was standardised by titration against 1N-NaOH, to a phenolphthalein end-point. It was stored at 4°C and diluted only a few minutes before use.

The concentration of protein in unknowns was found using the following solutions.

Standard      A standard curve was obtained with a number of serum albumin solutions of known concentration, using:

1.0ml. human serum albumin solution  
5.0ml. solution (C)  
0.5ml. solution (D), added 10.0min. after (C).

Blank            1.0ml. water  
5.0ml. solution (C)  
0.5ml. solution (D) added 10.0min. after (C)

Sample            1.0ml. solution containing protein (unknown)  
5.0ml. solution (C)  
0.5ml. solution (D), added 10.0min. after (C).

Reagents (C) and (D) were added rapidly, with thorough stirring, and the solutions were allowed to react at room temperature in stoppered tubes. The absorbance of the blue solution formed was found at 750 m $\mu$ , 40min. after the addition of (C).

Experiment 22      :      High-speed centrifugation of the crude enzyme

A sample of crude enzyme extract (5ml.) was centrifuged in an ultracentrifuge. [M.S.E. Superspeed 40; 3x5ml.head; 5°C; 1hr; 55,000—98,000xg (top-bottom of tube)].

The specific activity of the supernatant was compared with that of an uncentrifuged control.

Experiment 23      :      Ammonium sulphate fractionation of the crude enzyme <sup>(165)</sup>

Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (A.R., 12g; amounts calculated from data of Dixon & Webb <sup>(165)</sup>) was added to the crude enzyme solution (100ml.)



with stirring to give a 20%  $(\text{NH}_4)_2\text{SO}_4$  saturation. The solution was allowed to stand for 20-30min. and was then centrifuged (2,000xg; 30min; 0-5°C). The supernatant was poured off and the protein precipitate dissolved in a minimal volume of water (5-10ml.).

The procedure was repeated with the supernatant at 40,60,80 and 100%  $(\text{NH}_4)_2\text{SO}_4$  saturations. The 5 fractions were dialysed overnight (water; 0-5°C), to remove traces of  $(\text{NH}_4)_2\text{SO}_4$ . The specific activities of the fractions were determined by the usual method (Expts. 14,19 & 21). (See Table 13, p. 73).

Experiment 24 : Fractionation of the crude enzyme  
by change of pH<sup>(165)</sup>

The initial pH of the crude extract was about 5. Acetic acid (5N) was added with thorough stirring to the enzyme solution (50ml; 5°C) until the pH was lowered by about 0.5. The solution was allowed to stand (20-30min.) and the protein precipitate was then collected by centrifugation (2,000xg; 30min; 0-5°C).

The procedure was repeated several times with the supernatant, to a final pH of about 3. Each of the protein fractions, dissolved in a minimum of water (~5ml.), and an aliquot of the supernatant at pH3, were dialysed against water overnight. The specific activity of each fraction was then determined, (Expts. 14,19 & 21). (See Table 14, p. 74).

This method was selected for the initial purification of the enzyme on a large scale (1-2 l.).

Experiment 25 : Acetone fractionation of the crude enzyme (165,166)

Cold acetone ( $-20^{\circ}\text{C}$ ) was added to the crude enzyme solution (40ml.;  $5^{\circ}\text{C}$ ) to give a concentration of 20%. The solution was allowed to stand (20min.) before centrifugation (2,000xg; 30min;  $0-5^{\circ}\text{C}$ ).

The precipitate was dissolved in a little water ( $\sim 5\text{ml.}$ ) and the procedure repeated at 40,60 and 80% acetone concentration. The specific activities of the 4 fractions and that of the supernatant at 80% concentration were determined, (Expts. 14,19 & 21). (See Table 15, p. 75).

Experiment 26 : Fractionation of the crude enzyme  
by heat denaturation (165)

A sample of crude enzyme solution (10ml.) was heated in a water-bath with stirring ( $70^{\circ}\text{C}$ ; 10min.). The precipitate was removed by centrifugation (2,000xg; 12min;  $0-5^{\circ}\text{C}$ ) and the specific activity of the supernatant was compared with that of an unheated control sample (10ml.), which had been similarly centrifuged.

Experiment 27 : Fractionation of the partially-purified enzyme on Sephadex G-100<sup>(167)</sup>

Sephadex particles (G-100; 25g.) were allowed to swell in water (500ml.) for 3 days. The supernatant liquid was poured off and replaced by fresh water. The gel was then stirred up well to give a slurry which was poured into a column (2.4cm. diameter) to a height of 50cm. The column was washed well with sodium phosphate buffer (0.02M; pH6.8) containing KCl (0.16M). The protein solution was concentrated by freeze-drying in buffer (20ml.). After filtration, it was applied to the column (0-5°C). Phosphate buffer/KCl solution was used to elute the proteins from the column and fractions (10ml.) were collected on an automatic fraction collector. A plot of protein concentration, (Expt.20), against elution volume showed several distinct peaks (Fig.4, p.77). Each peak was tested for maltose: dihydroxybenzene glucosyltransferase activity and the specific activity of the combined active fractions was determined.

Experiment 28 : Fractionation of the partially-purified enzyme on DEAE-cellulose (202, 203)

This method was employed as the second step in the purification of the enzyme (c.f. Expt. 24).

DEAE-cellulose (Whatman DE50; 25g.) was washed by suspension in NaOH (0.1N; 750ml.) followed by neutralisation to pH6.8 with  $H_3PO_4$  (1M). The DEAE-cellulose was collected by centrifugation and

repeatedly washed in sodium phosphate buffer ( $0.02\text{M}$ ; pH6.8; 5x750ml.). The slurry was stirred well and poured into a column (diameter 5.0cm), which had a perforated porcelain disc covered with cotton wool at the base, to a height of 15-20cm.

The DEAE-cellulose was allowed to settle and most of the supernatant buffer was run off. The column was left overnight with a small head of liquid and washed with more buffer before use ( $0.02\text{M}$ ; 300ml.).

A filter paper was placed on the surface of the DEAE-cellulose.

The protein precipitate between pH3.9 and 2.9 from the crude enzyme (1-2 l.) was dissolved in water (30-60ml.) and applied carefully to the column. The column was drained until a small head of liquid remained (< 1cm.). Gradient elution was then commenced using sodium phosphate buffer ( $0.02\text{M}$ ; pH6.8; 600ml.) and a phosphate buffer/KCl mixture ( $0.07\text{M}$ -phosphate, pH6.8;  $0.4\text{M}$ -KCl). Fractions (14ml.) were collected on an automatic collector ( $0-5^{\circ}\text{C}$ .). The protein concentration of each fraction was determined (Expt.20) and plotted against fraction number. The fractions of highest protein concentration were tested for maltose : dihydroxybenzene glucosyltransferase activity. Two distinct peaks of activity were found and designated X and Y (See Fig.5, p.79 ). The fractions of an active peak were combined (80-120ml.) and the solutions dialysed overnight, (water;  $0-5^{\circ}\text{C}$ ). The specific activities were then determined

(Expts. 14,19 & 21) and the solutions used for investigations on the purified enzymes (Expts. 16 - 18 & 29 - 33).

Experiment 29 : Investigation of heat denaturation of enzymes X and Y

Samples of X and Y (3ml.) were heated in a thermostat at 50°C for various time intervals from 0 to 70min. Aliquots of the heat-treated samples were used in standard digests (Expt.14) to determine the fall in percentage activity of the enzyme at this temperature (See Fig. 9, p. 87).

Experiment 30 : Investigation of MHPG formation at various enzyme concentrations

The amount of MHPG formed with enzyme X was investigated using standard digests (Expt.14) and various  $[E]$  values between 0.06 and 0.15 U.ml.<sup>-1</sup> (Fig.10, p. 87).

The non-Michaelis kinetics of enzyme Y made an investigation of MHPG formation with change of  $[E]$  difficult. However, some indication of the mode of variation was found by investigating the complete time-course for Y (0-2hr.; Expt.16) at three different  $[E]$  values (0.09-0.18 U.ml.<sup>-1</sup>) [Fig. 6(ii), p. 83]

Experiment 31 : Investigation of donor and acceptor specificity of enzymes

Donors (1-2% in standard digests) were investigated with resorcinol as the acceptor, and acceptors (1-2%) with maltose as the donor. Products were examined chromatographically [No.3 paper, solvent (B) and spray (1)] and identified tentatively from the chromatographic data of Saltmarsh <sup>(146)</sup> for products with the crude enzyme, (obtained by comparison with authentic  $\beta$ -D-glucose derivatives of these compounds).

The following methods were employed in particular cases.

(1) Preparation of 1,2,4-trihydroxybenzene <sup>(204)</sup>

This compound is rapidly oxidised and a sample was freshly prepared by refluxing the tri-acetate (5g.) with methanol (10ml.) and concentrated HCl (1ml.) for 1hr. The dark green solution was distilled under reduced pressure, below 30°C, until it turned brown. On cooling, grey crystals of 1,2,4-trihydroxybenzene were formed.

(2) Investigation of site of glucosylation of hydroxycinnamic acids

Saltmarsh <sup>(146)</sup> concluded that the -COOH group was glucosylated ( $\alpha$ -D-glucose ester) from the following comparison with authentic 1-caffeoyl and 1-sinapoyl  $\beta$ -D-glucose esters supplied by Harborne <sup>(52)</sup>.

- (i)  $R_f$  values in solvents (A) and (B) were similar, the small differences being attributed to the difference in anomeric configuration.
- (ii) Electrophoretic mobilities of the caffeic acid derivative and 1-caffeoyl  $\beta$ -D-glucose ester in molybdate ( $2\%w/v$ ; pH5<sup>(182)</sup>) were very similar and indicated a free o-dihydroxy grouping, as did the brown colour of the molybdate complex formed.
- (iii) All the unknowns were hydrolysed readily by dilute alkali, a property of glucose esters, but not of glucosides.
- (iv) The sinapoyl derivative had a U.V. spectrum which agreed with the data of Harborne<sup>(52)</sup> for 1-sinapoyl  $\alpha$ -D-glucose ester.
- (v) In sodium acetate buffer ( $0.2M$ , pH5<sup>(182)</sup>), the glucose derivatives had low mobility ( $M_{SA} < 0.1$ ), comparable with the  $\beta$ -D-glucose esters, but considerably lower than the free acid.

In addition, in the present study, two further methods of investigation were used with the hydroxycinnamic acid derivatives (p-coumaric, caffeic, ferulic and sinapic compounds).

- (a) Electrophoresis in sodium phosphate buffer ( $0.1M$ , pH7.2<sup>(182)</sup>) showed that the  $M_{SA}$  values of the derivatives ( $\sim 0.1$ ) were considerably lower than those of the free acid, suggesting that an ester had been formed.

(b) Chromatography in solvent (B), using faintly alkaline bromocresol green as spray (0.1% in 95% aq. ethanol) showed that the glucose derivatives were neutral to this indicator, showing that free - COOH groups were absent.

It was concluded that the D-glucose derivatives were the l-hydroxycinnamoyl  $\alpha$ -D-glucose esters.

Experiment 32 : Investigation of MHPG formation at various maltose concentrations (Lineweaver-Burk plot) <sup>(183)</sup>

Standard digests (Expt.14) were used but the concentration of maltose in the solution was varied.

MHPG was determined for 30, 45 and 60min. reactions, (Expt.19). Plots of [MHPG] against time at each maltose concentration, [s], gave the reaction velocity, v, and a Lineweaver-Burk plot was obtained (Fig.11, p. 94).

The experiment was not attempted with enzyme Y, which does not exhibit Michaelis kinetics.

Experiment 33 : Effect of inhibitors and activators upon enzymes <sup>(93, 184, 186)</sup>

L-cysteine (CSH) in the standard digests (Expt.14), was replaced by inhibitor or activator solution of a suitable strength, (for actual concentrations used see Tables 18 - 24, p. 96 et.seq.).



Preincubations were carried out by treating the enzyme with the inhibitor for 40min. at room temperature, before incubation.

The following particular methods were used:

(1) Photo-oxidation with methylene blue (184)

Enzyme samples (5ml.) were treated with methylene blue (0.16M) in a glass-fronted thermostat for 30 or 60min. The reaction tubes were unstoppered and illuminated by a 500W lamp (20cm. distant). The samples were then exhaustively dialysed (6x4l.water; 36hr; 4<sup>o</sup>C), until only a faint blue colour remained.

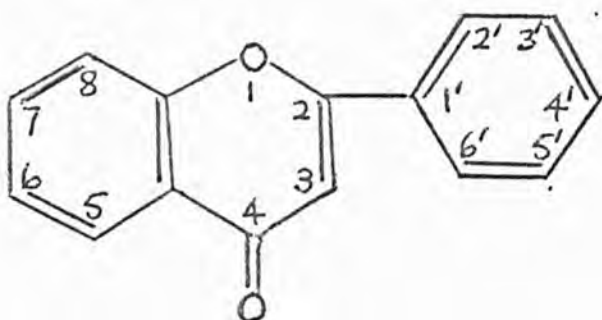
(2) Reversal of PCMB effect with CSH (186)

CSH solution (0.1ml.) was added to the enzyme solution (1.9ml.) 30min. after the addition of PCMB, during preincubation. The mixture was allowed to stand for 10min. before incubation (Expt.14). (For concentrations used, see Table 18, p. 97)

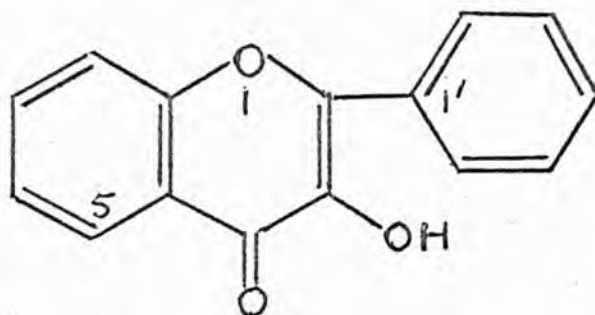
APPENDIX

The following abbreviations are used in the text:

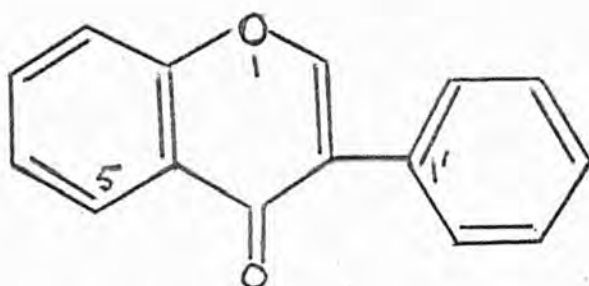
- UDPG : uridine diphosphate glucose [uridine 5' -(D-glucosyl-pyrophosphate)]
- ADPG : adenosine diphosphate glucose [adenosine 5' -(D-glucosyl-pyrophosphate)]
- CDPG : cytidine diphosphate glucose [cytidine 5' -(D-glucosyl-pyrophosphate)]
- GDPG : guanosine diphosphate glucose [guanosine 5' -(D-glucosyl-pyrophosphate)]
- OHPG : o-hydroxyphenyl  $\alpha$ -D-glucoside
- MHPG : m-hydroxyphenyl  $\alpha$ -D-glucoside
- CSH : L-cysteine
- GSH : glutathione, reduced form
- GSSG : glutathione, oxidised form
- BAL : British antilewisite (2,3-dimercapto-propan-1-ol)
- DTNB : 5,5' -dithio-bis-(2-nitrobenzoic acid)
- PCMB : p-chloromercuribenzoic acid
- EDTA : ethylenediamine tetracetic acid
- DEAE : diethylaminoethyl

Ring-systems found in naturally occurring phenolic compounds

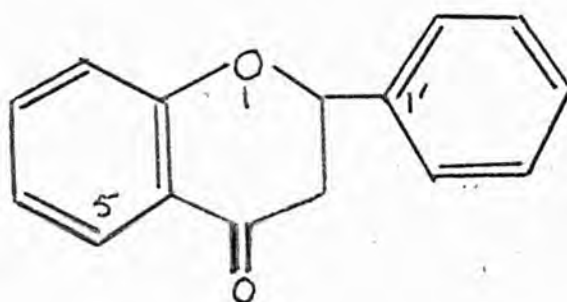
Flavone  
(2-Phenyl benzopyrone)



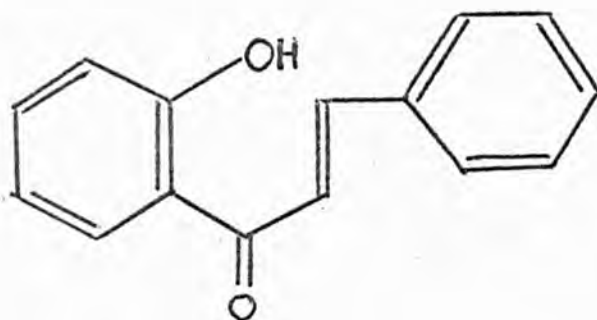
Flavonol  
(2-Phenyl-3-hydroxy-benzopyrone).



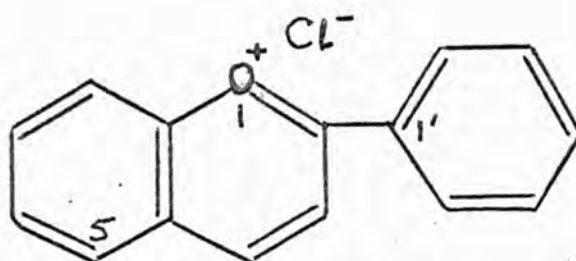
Iso-Flavone  
(3-Phenyl benzopyrone)



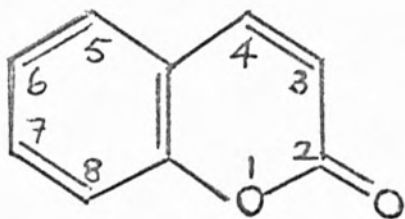
Flavanone  
(2-Phenyl-2,3-dihydro-benzopyrone)



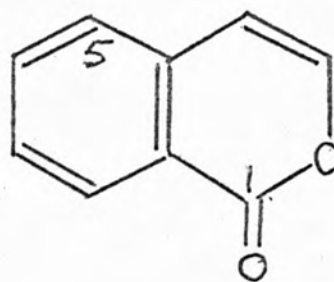
2-Hydroxychalcone



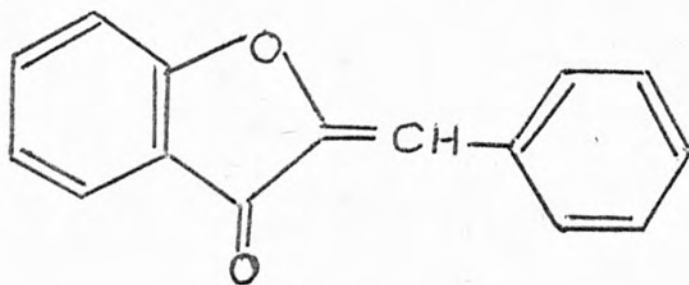
Flavylium chloride  
(Anthocyanidin)



Coumarin



iso-Coumarin



Aurone

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