TRANSFERASE ACTIVITY OF THE β-GLUCOSIDASES OF *ASPERGILLUS ORYZAE*

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Summary

The *p*-nitrophenyl-β-glucosidase of *Aspergillus oryzae* is able to transfer a glucose residue to methanol, ethanol, and *n*-butanol. The enzymes hydrolysing salicin and cellobiose carry out this transfer to a much less degree if at all. There is evidence that these enzymes can transfer glucose residues to other sugar molecules to build up more complex saccharides.

I. INTRODUCTION

Takano and Miwa (1950) have shown that there exist in the apricot (*Prunus armenica*), *Aspergillus niger,* and *Penicillium chrysogenum* enzymes capable of transferring the β-glucosyl residue of *p*-nitrophenyl-β-glucoside to methanol and butanol to give the corresponding glucosides. They concluded from observation of the parallel changes in the two activities during purification that the glucotransferase and β-glucosidase were identical in all three cases.

Pazur and French (1951) found that culture filtrates of *A. oryzae* contained a transglucosidase catalysing such reactions as:

$$\underset{\text{(maltose)}}{4(\alpha\text{-D-glucopyranosyl})\text{-D-glucose}} + \underset{\text{(isomaltose)}}{6(\alpha\text{-D-glucopyranosyl})\text{-D-glucose}} \rightarrow \text{glucose} + \underset{\text{(dextrantriose)}}{6(\alpha\text{-isomaltosyl})\text{-D-glucose}}$$

In this case an enzyme with α-glucosidase activity acts as a transferase to build up a dextran chain by transglucosidation. Indeed it seems that none of the carbohydrases act only as simple hydrolases. Wallenfels (1951) observed formation of galactosidolactose from lactose in the presence of *A. oryzae* extracts. Bacon and Edelman (1950) have shown that even in the classic case of invertase, hydrolysis is accompanied by transglycosidation and the synthesis of oligosaccharides.

The present study arose from observation of the non-equivalence of the glucose and *p*-nitrophenol produced during the hydrolysis of *p*-nitrophenyl-β-glucoside by enzymes of *A. oryzae* (Jermyn 1952*a*). All fissions of β-glucosidic linkages by these enzymes have kinetics too complex to be those of simple hydrolyses.

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II. Experimental

Enzymic hydrolysis of *p*-nitrophenyl-β-glucoside was followed by determining the glucose and *p*-nitrophenol produced at 37°C. in a mixture of 50 ml. of 10^{-3}M glucoside, 20 ml. of pH 5.0 McIlvaine citrate-phosphate buffer, enzyme solution, alcohol, and water to give a total volume of 100 ml. Samples were heated in boiling water for 5 min., glucose determined by the Somogyi-Nelson micromethod (Somogyi 1945) after correction for reduction by the enzyme itself, and *p*-nitrophenol by direct colorimetry. Neither *p*-nitrophenol nor its glucoside had a reducing effect on the Somogyi-Nelson reagents, nor did they affect the reduction of the reagent by glucose.

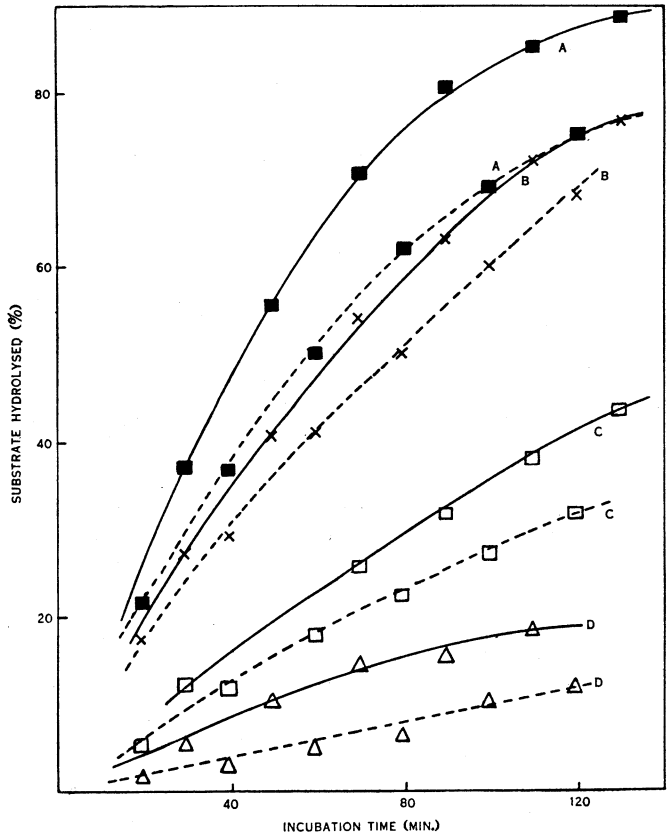

Fig. 1.—Hydrolysis of *p*-nitrophenyl-β-glucoside by various concentrations of crude *A. oryzae* enzyme. *A*, 450 μg./ml.; *B*, 300 μg./ml.; *C*, 120 μg./ml.; *D*, 50 μg./ml. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

The decomposition of salicin was followed at the same initial substrate concentration (5×10^{-4}M), determining liberated glucose in the same way and saligenin by the method of Folin and Ciocalteau (1927).

When it was desired to make a chromatographic examination of the products of enzymic action, the enzyme was first inhibited by boiling and the

cooled solutions passed through a column of "Bio-Deminrolit" to remove buffer salts, *p*-nitrophenol, and enzyme material. The deionized material was then concentrated by distillation under reduced pressure, transferred to a small crucible, and evaporated at 30°C. in a current of air to a small volume (0.1-0.2 ml. from 100 ml.). The total time occupied in this process was 1-2 hr. so that there could have been no appreciable microbiological action.

The paper chromatography of sugars was carried out according to the methods of Jermyn and Isherwood (1949) using ethyl acetate + pyridine + water as the solvent.

III. Results

(*a*) *Enzymic Hydrolysis of* p-*Nitrophenyl-β-glucoside*

Figure 1 shows the course of the hydrolysis of 5×10^{-4}M *p*-nitrophenyl-β-glucoside by various concentrations of crude *A. oryzae* enzyme. Figure 2 shows how this hydrolysis is modified by the presence of methanol and *n*-butanol.

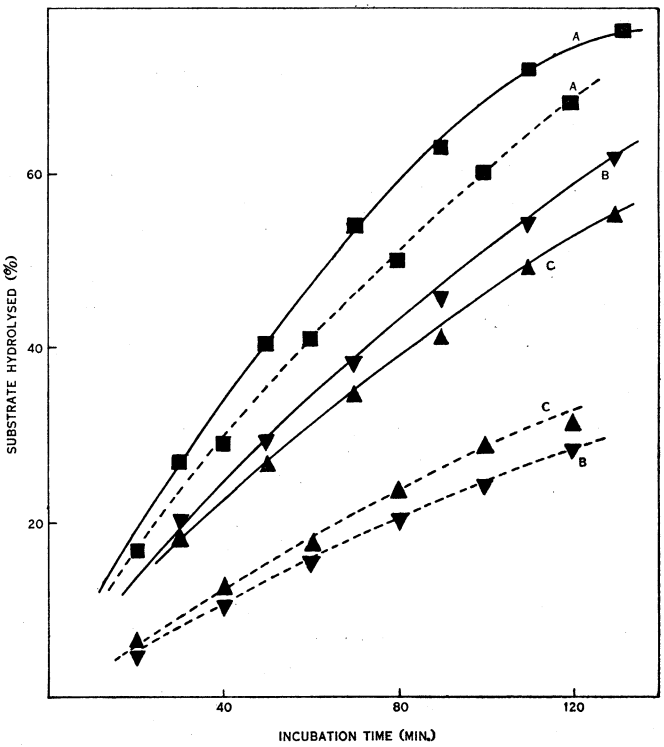

Fig. 2.—Hydrolysis of *p*-nitrophenyl-β-glucoside at a concentration of 300 μg./ml. of crude *A. oryzae* enzyme. *A*, no addition; *B*, 3M MeOH; *C*, 0.5M *n*-BuOH. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

The data of Figure 2 and other data not there inserted for the sake of clarity can be recalculated to give the curves of Figure 3 for the effect of various additions on the ratio of glucose liberated to *p*-nitrophenol liberated.

(*b*) *Enzymic Hydrolysis of Salicin*

Figure 4 shows the effect of methanol on the hydrolysis of salicin by the crude *A. oryzae* enzyme. Figure 5 is a recalculation of Figure 4 in terms of the glucose/*p*-nitrophenol ratio.

(*c*) *Other Observations*

When 5×10^{-4}M glucose was incubated at pH 5.0 and 37°C. with 300 μg./ml. of crude enzyme, there was no change in reducing values whether in the presence or absence of 3M methanol. Cellobiose (5×10^{-4}M) was hydrolysed rapidly by the enzyme under the same conditions and this hydrolysis was inhibited by methanol to about the same degree as that of salicin. The rate of hydrolysis of 5×10^{-4}M β-methyl glucoside (*c.* 1 per cent./hr.) was too low to have any effect on the course of experiments in the presence of methanol.

Hydrolysis of 5×10^{-4} *p*-nitrophenyl-β-glucoside by boiling 0.05N HCl showed exactly equivalent amounts of glucose and *p*-nitrophenol at all stages.

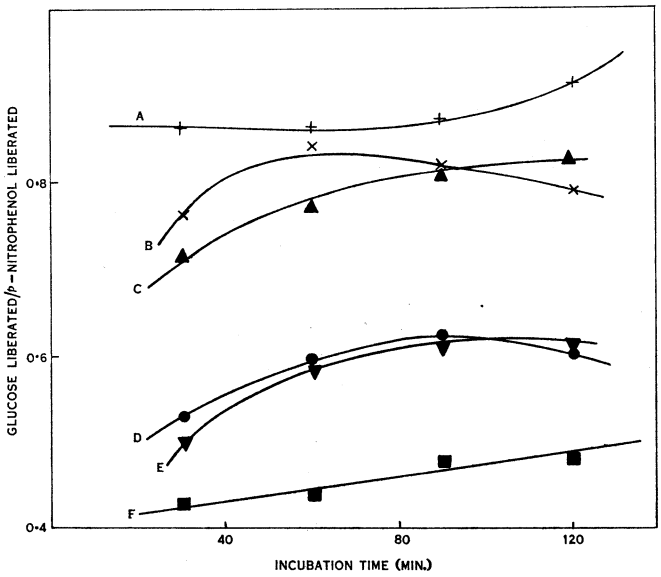

Fig. 3.—Variation in the ratio of glucose liberated to *p*-nitrophenol liberated on addition of various alcohols to the incubation mixture. *A*, no addition; *B*, 1M EtOH; *C*, 1M MeOH; *D*, 2M MeOH; *E*, 0.5M *n*-BuOH; *F*, 3M MeOH.

(*d*) *Chromatographic Experiments*

Salicin, cellobiose, and *p*-nitrophenyl-β-glucoside were incubated with the crude enzyme in the presence and absence of 3M methanol. The enzyme was inactivated when hydrolysis of the glucoside was half completed, as measured by the amount of aglycone liberated and the solution was examined by paper chromatography. The chromatographic observations are set out in Table 1. In addition to the substances listed, traces of sugars identified as galactose and

mannose were invariably present, even when the enzyme was incubated alone. They apparently arise from breakdown of the polysaccharide material contain-

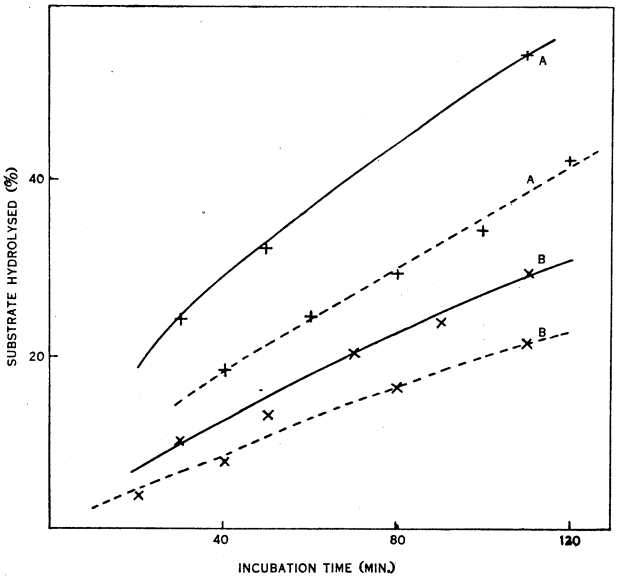

Fig. 4.—Hydrolysis of salicin at a concentration of 300 µg./ml. of crude enzyme. *A*, no addition; *B*, 3M MeOH. Solid line, liberated *p*-nitrophenol; broken line, liberated glucose.

ing galactose and mannose which Jermyn (1952*a*) showed to be present in the crude mould enzyme. Other carbohydrate material was not detected on chromatograms from enzyme solutions incubated without substrate.

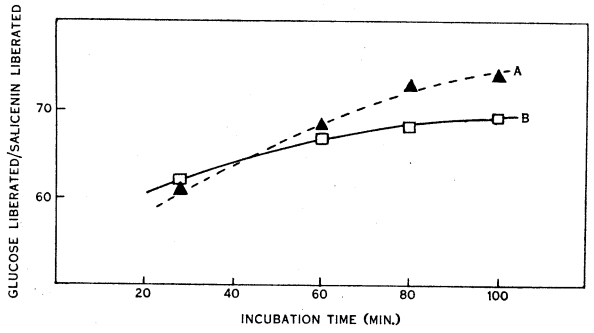

Fig. 5.—Ratio of glucose liberated to saligenin liberated in the experiments of Figure 4. *A*, no addition; *B*, 3M MeOH.

The substrate labelled "methyl glucoside" in Table 1 was identical with β-methyl glucoside in its chromatographic properties under all conditions tried. However, the difference between α- and β-methyl glucosides is so slight that a positive identification could not be made. The substances labelled "polysac-

charide" were tested for by the use of numerous spraying reagents since it cannot be predicted *a priori* which reagents will react with the free reducing groups in such saccharides; ammoniacal silver nitrate and aniline phosphate were the most useful of these reagents. The non-formation of substances of the type glucosyl-glucosyl-aglucone could be demonstrated by spraying the developed paper with a solution of the enzyme and incubating. The liberation of *p*-nitrophenol and saligenin could be detected at a single locus — that of the corresponding simple glucosides.

When *p*-nitrophenyl-β-glucoside was incubated with the crude enzyme in the presence of 1.0M ethanol or 0.5M *n*-butanol, the formation of substances chromatographically identical with β-ethyl glucoside and β-(*n*-butyl)-glucoside respectively could be demonstrated by the technique used with the methyl glucoside. In view of the extreme difficulty of detecting these glucosides on paper, only the methanol system was further investigated.

TABLE 1

SUMMARY OF THE PAPER CHROMATOGRAPHIC ANALYSES OF CONCENTRATED ENZYME DIGESTS

+, Presence indicated; —, not detected

Substrate	Addition	Methyl Glucoside	Glucose	Cellobiose	Oligosaccharides* (mobile)	Polysaccharide* (immobile)
p-Nitrophenyl-β-glucoside	None	—	+	—	+	+
	3M MeOH	+	+	±	—	+
Salicin ..	None	—	+	—	—	+
	3M MeOH	—	+	—	—	+
Cellobiose ..	None	—	+	+	+	+
	3M MeOH	—	+	+	—	+

* The use of these terms is purely a matter of convenience and implies no final conclusions on the structure of the substances so labelled.

IV. DISCUSSION

The transfer of sugar residues by enzymes has been demonstrated in many systems and has been attributed (Wallenfels 1951) to a reaction of the type:

$$\text{Enzyme} - \text{OH} + \text{ROGl} \longrightarrow \text{Enzyme} - \text{OGl} + \text{ROH} \quad (1)$$

$$\text{Enzyme} - \text{OGl} + \text{R'OH} \longrightarrow \text{Enzyme} - \text{OH} + \text{R'OGl}. \quad (2)$$

Where the enzyme glycoside complex reacts with water (R' = H) the result is hydrolysis. With an alcohol a new glycosidic linkage is formed. The enzymes of *A. oryzae* that produce methyl glucoside in solutions containing *p*-nitrophenyl-β-glucoside and methanol do not bring about a similar transfer of a glucose residue from salicin or cellobiose, a fact best explained by the theory of the presence of a number of β-glucosidases having different specificities (Jermyn 1952*b*). The order of effectiveness of different alcohols as competitors with water in reaction (2) (*n*-butanol > methanol > ethanol) differs from that

found by Takano and Miwa (1950) for *A. niger* (methanol > butanol) but is the same as that of the enzymes of *P. chrysogenum*.

Takano and Miwa also demonstrated the transfer of a glucoside residue from *o*-cresyl-β-glucoside, a substrate closely related to salicin. Enzymes from different sources must be supposed to have widely different specificities for both substrate and acceptor. In *A. oryzae*, transfer of the glucosyl residue of *p*-nitrophenyl-β-glucoside to water and other acceptors is carried out by only one of eight enzymes present. This enzyme also acts upon salicin and cellobiose, but even if it catalyses some transfer to alcohols from these substrates its effect will be negligible compared with the total hydrolysis due to the non-transferring enzymes.

The presence of the alcohols appears to exert an inhibiting effect on the hydrolysis of both *p*-nitrophenyl-β-glucoside and salicin, with a more marked effect on the latter reaction. The inhibition is not very specific since methanol, ethanol, and *n*-butanol are about equally effective, and is not increased by the addition of more than a certain minimum amount of the inhibitor, methanol giving much the same inhibition at 1M, 2M, and 3M. The inhibition does not appear to have any direct significance for the transferase activity.

The β-glucosidases of *A. oryzae* can now be added to the list of enzymes that catalyse the synthesis of polysaccharide material by transglycosidase activity. Under the experimental conditions used the intermediate oligosaccharides formed in this synthesis appear to be present in very small concentrations. Since the products are those of a number of enzymes, it is unwise to attempt any theoretical interpretation of this observation. The upward trend of the curves of Figures 3 and 5 presumably means that the polysaccharides built up by transglycosidation are degraded by subsequent hydrolysis. This cannot be taken as a proof of the β configuration of the synthesized linkages; the crude *A. oryzae* material contains enzymes capable of hydrolysing all types of linkage tested. Even from cellobiose the new linkages synthesized would not necessarily be the β 1:4 of the cellulose chain and the formation of new linkages of the original type is in fact far from general in transglycosidation (Hehre 1951). Pazur and French (1951) have shown that the α-glucosidase of *A. oryzae* transforms 1:4 to 1:6 linkages.

V. Acknowledgment

The authors are deeply indebted to Mrs. M. C. Wilkinson for technical assistance.

VI. References

Bacon, J. D., and Edelman, J. (1950).—*Arch. Biochem.* **28**: 467.
Folin, O., and Ciocalteau, V. (1927).—*J. Biol. Chem.* **73**: 627.
Hehre, E. J. (1951).—*Advanc. Enzymol.* **11**: 297.
Jermyn, M. A. (1952*a*).—*Aust. J. Sci. Res.* B **5**: 409.
Jermyn, M. A. (1952*b*).—*Aust. J. Sci. Res.* B **5**: 433.
Jermyn, M. A., and Isherwood, F. A. (1949).—*Biochem. J.* **44**: 402.
Pazur, J. H., and French, D. (1951).—*J. Amer. Chem. Soc.* **73**: 3536.
Somogyi, M. (1945).—*J. Biol. Chem.* **160**: 61.
Takano, K., and Miwa, T. (1950).—*J. Biochem., Tokyo* **37**: 435.
Wallenfels, K. (1951).—*Naturwissenschaften* **38**: 306.