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Studies on a Honey Bee Sucrase Exhibiting Unusual Kinetics and Transglucolytic Activity†

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ABSTRACT: Honey bee sucrase (invertase) with transglucolytic activity was found to have unusual kinetics. Fructose or *p*-nitrophenol release from the substrates sucrose and *p*-nitrophenyl α-D-glucoside (pNphGlc), respectively, exhibited nonlinear Hofstee plots and the rates became almost first order at high substrate concentrations while glucose production resulted in conventional linear Hofstee plots. The V_m for release of transglucolytic (transferase) products was first order at all substrate concentrations and a vertical Hofstee plot resulted at higher substrate concentrations. When glucose, Tris (tris(hydroxymethyl)aminomethane), methyl α-D-glucoside, fructose, and other compounds were added initially, differing effects on the rates of production of fructose (or *p*-nitrophenol), glucose and transferase products were noted. The results are accounted for by a mechanism which proposes the formation of an enzyme–substrate intermediate which reacts with water for hydrolysis or with acceptor substrates for transglucolysis. The proposed mechanism predicts the absence of a kinetically relevant binding site for the acceptor substrate. In addition to reacting with acceptor substrates such as glucose, methyl α-D-glucoside, fructose, or Tris the acceptor substrate may be the same as the initial substrate. Thus sucrose and pNphGlc can be both initial and acceptor substrates. The nonlinear and first-order kinetics for the production of fructose (or *p*-nitrophenol) and transferase products are ascribed to the effect of this on the V_m values. The identity and possible route of synthesis of the three main transferase compounds were also investigated.

A report on the properties of sucrase (invertase) from insect sources was first published in 1924 (Nelson and Cohn) on the enzyme from honey. More recently some of the properties of sucrase from *Drosophila* (Marzluff, 1969; Huber and Lefebvre, 1971) and other insects (Kawabata *et al.*, 1973) have been reported. The enzyme is also widely distributed outside of the insect kingdom and most forms of the enzyme are known to be transglycolytic (Stanek *et al.*, 1965). The honey enzyme was shown to have transglucolytic activity and analyses of the transferase products have been previously reported (White and Maher, 1953a,b; Gray and Fraenkel, 1953, 1954; Wolf and Ewart, 1955a,b). One form of sucrase which is present in very large amounts in honeybees has been studied in detail and the enzymatic, physical, and chemical properties of this purified enzyme are being reported elsewhere (Huber, 1973). It was found that the kinetics of this highly purified sucrase from honeybees were very unconventional in that nonlinear and first-order Hofstee plots were obtained for the production of some of the products. The unusual kinetics were found to be the result of the combination of hydrolytic and transglucolytic activities of the enzyme. Since sucrases (invertases) from most other sources are also known to possess both hydrolytic and transglycolytic activity (Stanek *et al.*, 1965), it was felt that a study of the rates of formation of the products of the enzyme reaction of the honey bee enzyme should be of considerable interest and of value for application to other systems especially since the kinetic nature of these reactions has not previously been reported. A model for the action of this enzyme is proposed on the basis of the results which were found.

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Experimental Section

Materials. Enzyme grade sucrose and Tris (tris(hydroxymethyl)aminomethane) were obtained from Schwarz/Mann

while *p*-nitrophenyl α-D-glucoside,[1] methyl α-D-glucoside, trehalose, isomaltose, melezitose, maltotriose, and glucose were from Sigma (methyl α-D-glucoside was recrystallized three times from 95% ethanol to remove glucose). Glucose-free maltose was obtained from Calbiochem and Glucostat reagent was from Worthington. Radiochemicals were from New England Nuclear and ICN. Kieselguhr G was purchased from E. Merck A. G., Darmstadt, while *p*-hydroxybenzoic acid hydrazide was from Aldrich. All other chemicals were Reagent Grade Fisher.

Enzyme. Sucrase was isolated from whole honey bees (*Apis mellifera*) to homogeneity as determined by disc gel electrophoresis (pH 9.1) and isoelectric focusing (Huber, 1973). In one experiment partially heat inactivated and partially urea inactivated enzyme were used. The enzyme was heat inactivated by incubation at 50° until only 40–50% of its activity remained and was then rapidly cooled in an ice bath. For urea inactivation the enzyme was incubated until it had lost 50–60% of its activity whereupon the solution was diluted 10-fold and dialyzed.

Assays for Glucose, Fructose, and p-Nitrophenol. The products of the hydrolysis of sucrose and of pNphGlc were quantitatively analyzed as follows.

(a) Glucose. Enzyme was incubated with sucrose or pNphGlc in 0.03 M sodium citrate buffer (pH 6.0) for 10 min at 30°, and then the reaction was stopped by placing the tube into boiling water for 2 min. After cooling, 1 ml of Glucostat reagent buffered in sodium phosphate (pH 7.0) was added and the color was allowed to develop for 30 min at 30°. The Glucostat reaction was stopped by the addition of 200 μl of 3 N HCl and the absorbance at 420 nm was compared to a glucose standard.

(b) Fructose. Enzyme was incubated as above but the reaction was stopped by adding 1.5 ml of 1% (w/v) of *p*-hydroxybenzoic acid hydrazide in 0.5 M NaOH and boiling for 10 min (Lever, 1972). After cooling the absorbance was read at 410 nm and compared to standard fructose or glucose values (fructose and glucose had identical standard curves) which yielded a value of the total amount of fructose and glucose released. To determine the amount of fructose produced the glucose was determined as above and subtracted. This assay is somewhat tedious and does not work at all in the presence of high amounts of glucose or maltose. Thus, in most cases the effects of various additions on the rate of production of the non-glucose hydrolytic moiety (fructose or *p*-nitrophenol) were studied by following the rate of *p*-nitrophenol production. In those cases where fructose production was compared to *p*-nitrophenol production the overall effect was similar.

(c) *p*-Nitrophenol. Enzyme was placed into 2 ml of pNphGlc solution buffered in 0.03 M sodium citrate (pH 6.0) and the increase in absorbance at 420 nm was measured by means of a recording spectrophotometer.

Kinetic Analyses. The assay results at various substrate concentrations were plotted for analyses on Hofstee plots (Hofstee, 1959) as recommended by Dowd and Riggs (1965). In the case of this study the Hofstee plot is more descriptive of what is occurring than other linear transformations since the values on the ordinate are read directly as rates of product formation, whereas values on the abscissa relate the initial velocity of reaction directly to the substrate concentration. For first order the $v_0/(S_0)$ values would be constant for a series of concentrations and a vertical line will be seen.

In some cases the "V_m," which is derived algebraically, is dependent in a first-order fashion on the substrate concentration and is not, therefore, a constant. These will, however, be referred to as V_m values since they are derived as such.

Analyses by Thin-Layer Chromatography (tlc). The products of the reaction of sucrase with substrates were analyzed by tlc on kieselguhr G. The kieselguhr G, after being thoroughly washed with hot 95% ethanol and dried, was suspended in 0.1 M NaH_2PO_4 and spread in a 0.25-mm thick layer on 5 × 20 cm glass plates. The plates were dried for 1 hr at 110° before spotting. The development systems used were acetone–H_2O (92:8, v/v) and 1-propanol–H_2O (85:15, v/v). Bands were visualized by concentrated sulfuric acid plus heat. The radioactive hydrolytic and transferase compounds produced after incubation in various concentrations of sucrose-*U*-^{14}C, sucrose (fructose-*1*-*t*), glucose-*U*-^{14}C or fructose-*U*-^{14}C were followed by scanning the plates with a Packard radiochromatogram scanner Model 385 (1030 V, gas flow 33 cm^3/min). Relative amounts of products were determined by measuring areas under peaks and by scraping spots into scintillation vials (70 g/l. of naphthalene, 7 g/l. of 2,5-diphenyloxazole, and 0.05 g/l. of 1,4-bis[2-(5-phenyloxazolyl)]benzene in dioxane). Kinetics of the formation of hydrolytic products and transferase compounds from radioactive sucrose-*U*-^{14}C were determined by relating the relative amounts of hydrolytic products and transferase compounds separated by tlc to the total amount of sucrose-*U*-^{14}C originally in solution.

Analyses of Transferase Products. The mobilities of the three main transferase components obtained after incubation of the enzyme with a mixture of 0.2 M sucrose and 0.2 M glucose were compared to the mobilities of various di- and trisaccharides and are reported as $R_{fructose}$. The sources of the monosaccharide moieties of the bands were determined by finding the amount of radioactivity derived from an assay mixture with both 0.2 M sucrose and 0.2 M glucose with sucrose-*U*-^{14}C being the added label in one case and glucose-*U*-^{14}C in the other.

Results

Kinetic Analyses of Hydrolytic Product Formation. Sucrose and pNphGlc. Figure 1 shows the Hofstee plots of the rates of glucose and fructose production from sucrose and the rates of glucose and *p*-nitrophenol production from pNphGlc. With both sucrose and pNphGlc the rate of glucose production resulted in a linear Hofstee plot. The production of fructose and *p*-nitrophenol, on the other hand, resulted in lines with very distinct curvatures. At very high substrate values the lines become almost vertical, indicating that a process which is nearly first order occurs at high substrate concentrations.

Note that the rates for the two substrates are different. That is, if similar substrate concentrations were used the rate of product formation from pNphGlc was significantly greater than with sucrose. This can be noted by the fact that although the scale on the ordinate is the same for both substrates the abscissa scale is much expanded for sucrose as compared to pNphGlc. Thus the moiety attached to the glucose is important in determining the rate of the reaction.

Effects of inhibitors and activators. Figure 2 shows the effect of Tris and methyl α-D-glucoside on the production of glucose from sucrose. Straight lines were obtained at high substrate concentration and these lines have lower intercepts and steeper slopes than the line in the absence of these compounds. At low substrate concentration in the presence of Tris or methyl α-D-glucoside there is definite tailing of the

[1] Abbreviation used is: pNphGlc, *p*-nitrophenyl α-D-glucoside.

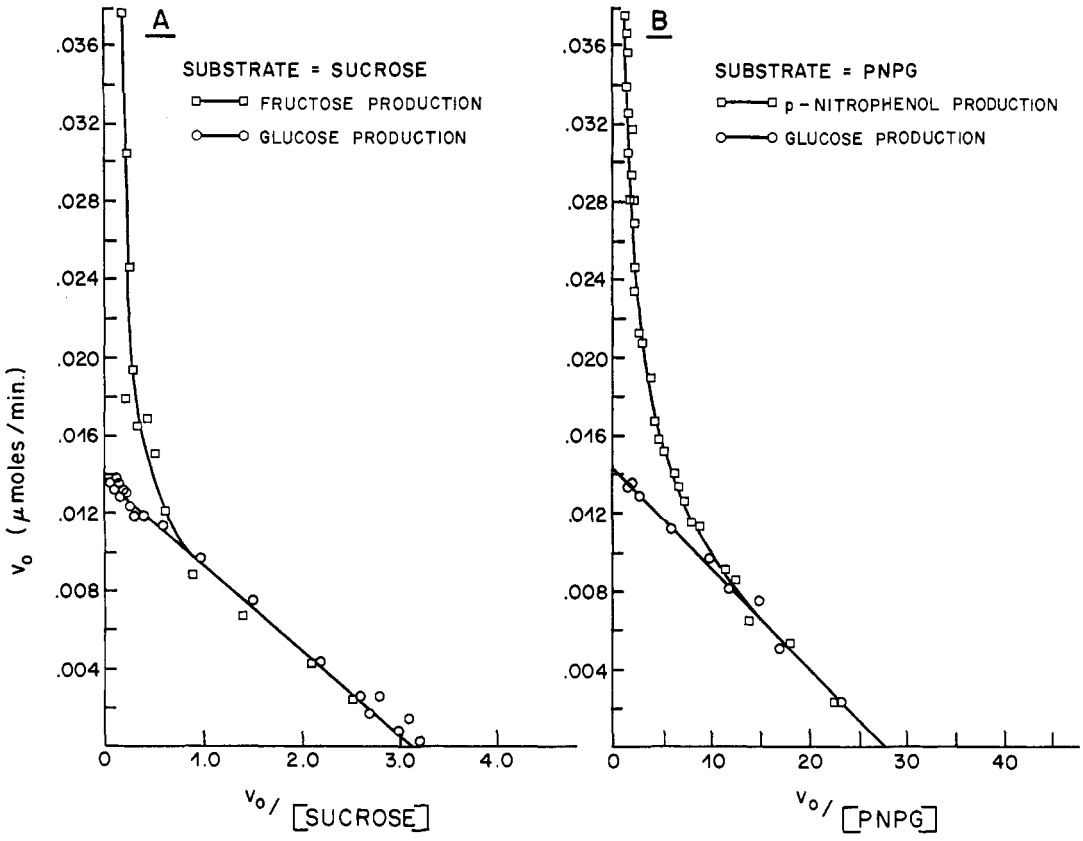

FIGURE 1: Hofstee plots for the hydrolysis products after incubation of the enzyme with (A) sucrose and (B) pNphGlc.

lines. This indicates that relative to the values at high substrate concentrations the v_0 and $v_0/(S_0)$ values at low substrate concentrations are high.

Figure 3A shows the effects of sucrose and Tris on the kinetics of *p*-nitrophenol production from pNphGlc. Two concentrations of sucrose are shown. Sucrose causes definite inhibition of *p*-nitrophenol production and the lines become linear. At high sucrose levels the *p*-nitrophenol production rates are almost first order at all pNphGlc levels since the abscissa values are essentially constant. Tris, which is known to be an inhibitor of sucrases and other disaccharidases (Huber and Lefebvre, 1971), also inhibited *p*-nitrophenol production. Low concentrations of Tris inhibited at low pNphGlc concentration but had little effect on the rate at high pNphGlc concentration, this effect being what is expected for competitive type inhibition. High levels of Tris, however, depressed the rate of *p*-nitrophenol production considerably and gave results which were again almost first order, since the lines are essentially vertical.

Figure 3B illustrates that methyl α-D-glucoside activated the rate of *p*-nitrophenol production by a constant ratio over the entire range. It is interesting that methyl α-D-glucoside, which inhibited glucose production, activated the production of *p*-nitrophenol while Tris, which inhibited glucose production, also inhibited *p*-nitrophenol production.

Figure 3B also shows the effect of glucose on *p*-nitrophenol production. Very dramatic effects are noted. The line became linear and a great deal of activation was observed.

Lactose, glycerol, and amylose were found to have no effect on *p*-nitrophenol production while fructose and galactose acted in a similar fashion to methyl α-D-glucoside. Maltose action on *p*-nitrophenol production was similar to the action of sucrose on *p*-nitrophenol production. Maltose has been found to be a substrate for the enzyme (Huber, 1973) and thus this result is not unexpected.

It is obvious that various compounds have different effects on glucose production than on fructose or *p*-nitrophenol production. If the mechanism were a simple hydrolytic one the production rates for both the glucose moiety and the fructose or *p*-nitrophenol moiety should be similar under all conditions.

Partially Inactivated Enzyme. Although the sucrase studied had only one protein band by both disc gel electrophoresis and isoelectric focusing (Huber, 1973) it was thought desirable to show that partially inactivated enzyme gave the same kinetic results as the fully active enzyme in order to rule out the pos-

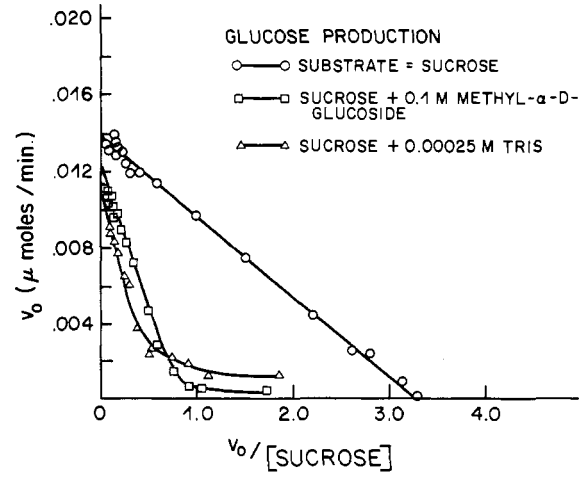

FIGURE 2: Hofstee plots for the production of glucose from sucrose in the presence of Tris and methyl α-D-glucoside.

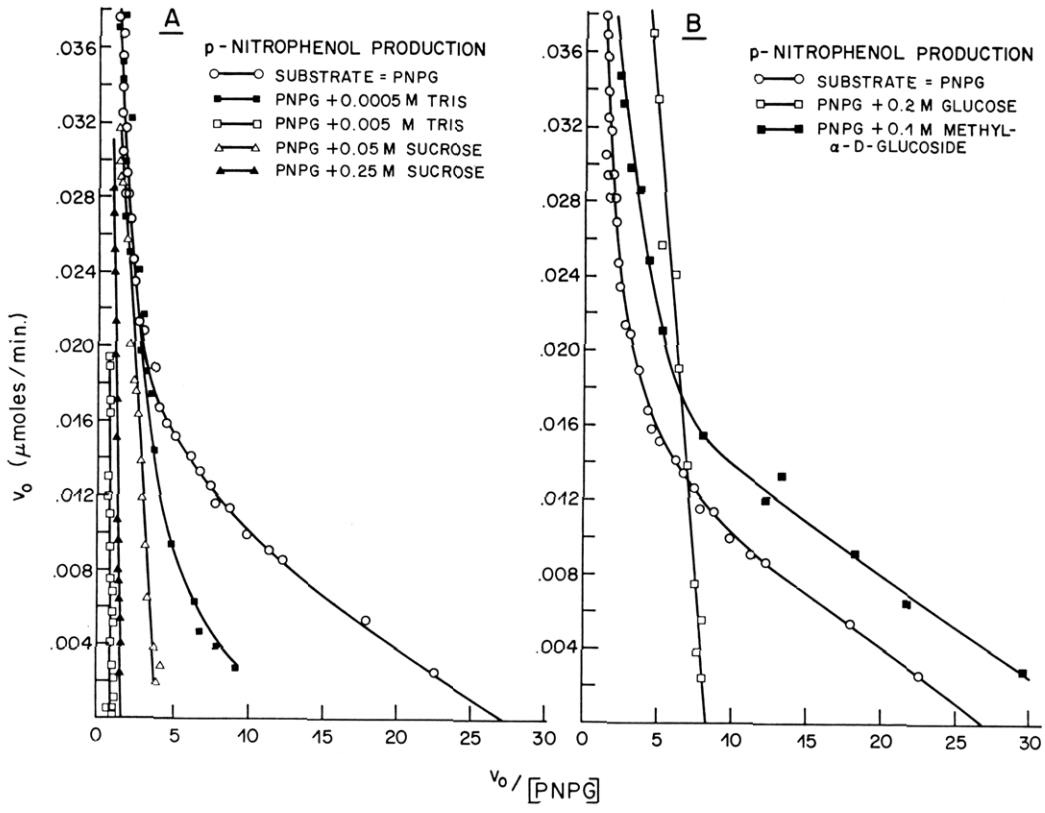

FIGURE 3: Hofstee plots for the production of *p*-nitrophenol from pNphGlc in the presence of (A) sucrose and Tris and (B) methyl α-D-glucoside and glucose.

sibility that the results are due to two different enzymes. With both heat and urea denatured enzymes, kinetics similar to the above were found at all substrate and inhibitor concentrations which were checked. An example of this is shown in Table I where similar ratios of activity are found at all concentrations of pNphGlc when the heat inactivated enzyme was compared to the normal enzyme. Therefore, unless two enzymes had exactly the same denaturation rates with heat and with urea, it is unlikely that the results are caused by the presence of two different enzymes.

TABLE I: Ratio of Activity of Partially Heat-Inactivated Enzyme to Totally Active Enzyme at Different Concentrations of pNphGlc.

pNphGlc Concn (M)	Ratio of Act.
0.0003	0.426
0.0007	0.454
0.001	0.436
0.002	0.431
0.003	0.424
0.004	0.436
0.005	0.415
0.006	0.438
0.007	0.424
0.008	0.438
0.10	0.427
0.013	0.422
0.015	0.401
0.017	0.431
0.025	0.418

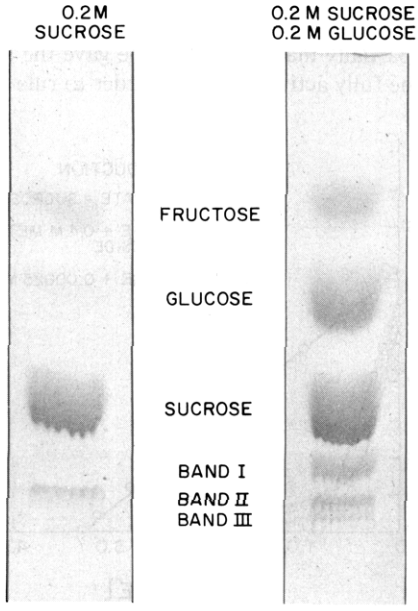

FIGURE 4: Tlc plates showing the products produced from sucrose in the absence and presence of glucose.

Thin-Layer Chromatographic Analyses. SUCROSE. Figure 4 is an indication of the products obtained from sucrose when analyzed by tlc after incubation both in the presence and absence of glucose. Note the presence in both cases of fructose, glucose, and sucrose as well as of additional bands. When only sucrose was present initally one additional band is seen and this band moved more slowly than sucrose, but when sucrose and glucose were initially present three bands (I, II, and III)

TABLE II: Distribution of Radioactivity in the Products of Enzymatic Reaction with Sucrose-*U*-^{14}C.[a]

		%		
		Glucose	Fructose	Transferase Compounds
High sucrose (0.2 M)	Alone	19	30	51
	+0.2 M Glucose	20	30	50
	+0.005 M Tris	15	28	57
	+0.2 M Methyl α-D-glucoside	18	42	40
Low sucrose (0.002 M)	Alone	43	43	14
	+0.2 M Glucose	16	38	46
	+0.005 M Tris	45	29	26
	+0.2 M Methyl β-D-glucoside	26	44	30

[a] In all cases the time of incubation did not result in significant differences in proportion of products. The error in these data is about ±3% in all cases.

moving more slowly than sucrose are seen. One of these (band II) has a mobility similar to the band found when only sucrose was present. (Under certain conditions there was also a band present between band I and band II but this was present in only minor amounts.) Because of the mobility of these bands and because transglycolytic activity has been previously observed with sucrases (Stanek *et al.*, 1965), it was concluded that these bands were transglycolytic products. Evidence below regarding the composition of these bands confirms that this is the case. When the reaction was allowed to proceed for a longer time period before tlc analysis more bands were noted which did not move very far from the origin and which were probably high oligosaccharides.

The results of the analyses of the approximate proportions of hydrolytic and transglycolytic products produced, as determined by radiochromatoscans of the tlc plates after the action of sucrase on sucrose-*U*-^{14}C under a variety of conditions, are shown in Table II. Note that the makeup of the products was highly dependent on the sucrose concentration. In the absence of any additions about one-half of the products at high sucrose concentration were transglycolytic while at low sucrose concentration only about one-eighth of the products were transglycolytic. In addition, the proportions of glucose and fructose produced were different at the different sucrose concentrations. At low sucrose concentrations about equal amounts of glucose and fructose are formed while at high sucrose concentrations much larger proportions of fructose than glucose are formed. These results agree with the above kinetic results (Figure 1). In the presence of glucose the proportions of products at high and low sucrose concentrations were similar and again this is expected from the kinetic results above where a linear Hofstee plot was obtained (Figure 3B). When methyl α-D-glucoside was added the proportion of products produced at low sucrose concentration changed but not nearly to the extent to which they were changed by glucose. In the case of methyl α-D-glucoside the main transferase component which was formed moved ahead of fructose and was probably glucosylmethyl α-D-glucoside. Tris resulted in very little change in the composition of product at high sucrose but at low sucrose there are increases in the apparent glucose produced and in the transferase compounds formed. The inflated glucose value is, however, probably an artifact because a compound with an amino group was found by ninhydrin spray in exactly the same region that glucose moved in both solvent systems. Thus it is thought that at low sucrose concentration in the presence of a high Tris concentration a significant proportion of the product is a transferase component which is formed from glucose and Tris. This component was not chromatographically separable from glucose in the systems used.

pNphGlc. A tlc plate illustrating the products formed at three different concentrations of pNphGlc is shown in Figure 5. At high pNphGlc (0.02 M) mainly pNphGlc and a transferase component moving somewhat more slowly than pNphGlc were evident. At an intermediate concentration (0.005 M) both the transferase component and glucose were present while at low pNphGlc (0.001 M) only glucose is seen. In all cases in this study the reactions were allowed to proceed until similar amounts of *p*-nitrophenol were produced. Thus, as with sucrose, there are changes in proportions of products depending on the substrate concentrations.

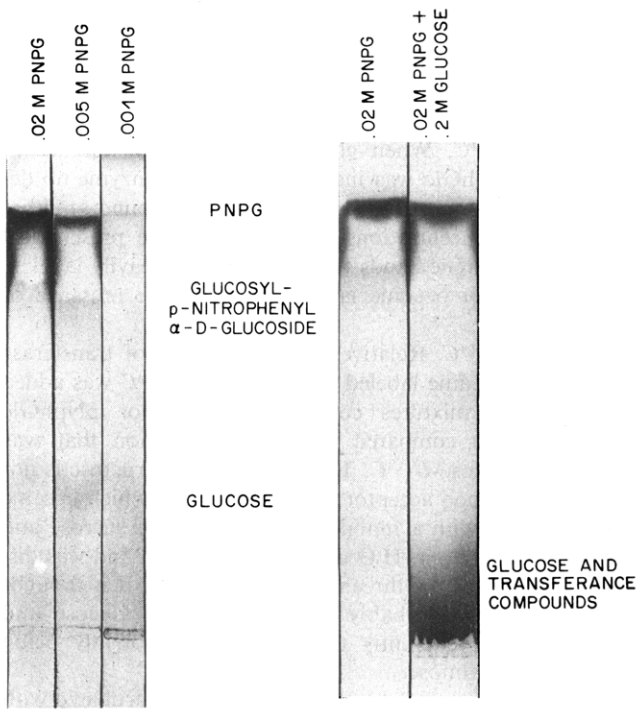

FIGURE 5: Tlc plates showing the products produced from pNphGlc at three different pNphGlc concentrations and in the presence of glucose.

TABLE III: Mobilities of Standards and of the Three Transferase Bands Relative to Fructose in Two Different Solvent Systems on Kieselguhr G.

Sugar	$R_{fructose}$ in Acetone–H_2O (93:7)	$R_{fructose}$ in 1-Propanol–H_2O (85:15)
Fructose	1.00	1.00
Glucose	0.85	0.89
Sucrose	0.50	0.82
Maltotriose	0.040	0.48
Melezitose	0.085	0.62
Raffinose	0.031	0.36
Maltose	0.31	0.69
Isomaltose	0.038	
Cellobiose	0.31	
Patulose	0.37	
Turanose	0.43	
Trehalose	0.14	0.82
Band I	0.28	0.67
Band II	0.075	0.58
Band III	0.039	0.48

Figure 5 also shows how glucose changes the products formed. Essentially no fast moving transferase component was formed in the presence of large amounts of glucose.

When pNphGlc was incubated with the enzyme and high concentrations of sucrose-*U*-^{14}C a small amount of the fast-moving transferase component of Figure 5 became labeled, but the amount was very small compared to the other dispositions of the sucrose-*U*-^{14}C (into fructose, glucose, and other transferase products). The component did not become labeled at all in the presence of high concentrations of glucose-*U*-^{14}C or fructose-*U*-^{14}C. These studies indicate that the main transferase component formed from pNphGlc is derived from pNphGlc itself. In the presence of sucrose a small amount of this compound can come from the sucrose but free glucose or fructose do not become incorporated into this compound. From the position of the migration, the compound is probably glucosyl-*p*-nitrophenyl α-D-glucoside.

GLUCOSE-*U*-^{14}C. When glucose-*U*-^{14}C in the absence of sucrose or pNphGlc was incubated with the enzyme no detectable conversion of the glucose-*U*-^{14}C was found at either high or low concentrations of glucose. In the presence of sucrose or pNphGlc bands I and III became heavily labeled. Band II did not become labeled at all in the presence of glucose-*U*-^{14}C.

FRUCTOSE-*U*-^{14}C. Relatively small amounts of transferase components became labeled when fructose-*U*-^{14}C was added to incubation mixtures containing sucrose or pNphGlc especially when compared to the incorporation that was found with glucose-*U*-^{14}C. This indicates that fructose is not a particularly good acceptor. The largest peak which became labeled moved with a mobility between that of sucrose and band I in the acetone–H_2O development system and was different than any of the three major components. It is thought that this peak is probably a disaccharide of glucose and fructose linked differently than in sucrose, possibly being turanose or palatinose.

SUCROSE (FRUCTOSE-*1*-t_3). When enzyme was incubated with sucrose (fructose-*1*-t_3) only fructose and band II became labeled. This indicates that of the three major bands only band II has detectable amounts of fructose in its structure. The other transferase components must be composed entirely of glucose.

TABLE IV: The Approximate Amounts of Sucrose or Glucose in the Transferase Components in Terms of Moles of Transferase Component Which Derived from These Substrates.[a]

	30 min		60 min		Approx.
	Sucrose-*U*-^{14}C	Glucose-*U*-^{14}C	Sucrose-*U*-^{14}C	Glucose-*U*-^{14}C	S:G Ratio
BI	0.0000400	0.000036	0.0000690	0.0000588	1:1
BII	0.0000092	0.0000009	0.0000130	0.0000016	1:0
BIII	0.0000164	0.0000069	0.0000456	0.0000177	2:1

[a] Enzyme was incubated with a mixture of 0.2 M sucrose and of 0.2 M glucose for 30 and 60 min. In one case sucrose-*U*-^{14}C was added and in the other glucose-*U*-^{14}C.

Analyses of the Identity and Source of the Three Major Transferase Components. The $R_{fructose}$ values of the three major transferase bands found after the enzyme was incubated in the presence of sucrose and glucose and of some standards are shown in Table III for the acetone–H_2O (93:7, v/v) and 1-propanol–H_2O (85:15, v/v) systems. Band I migrates with approximately the mobility of maltose in both systems, band II as melezitose and band III as maltotriose.

Table IV shows how much of sucrose-*U*-^{14}C and how much of glucose-*U*-^{14}C was incorporated into the three major transferase components and the approximate ratio of the amount derived from sucrose as compared to glucose. Band I is approximately 1:1 from sucrose and glucose, band II seems to be entirely derived from sucrose and band III two-thirds from sucrose and one-third from glucose. The values for incorporation from sucrose are somewhat high compared to glucose because glucose which is released can react as free glucose and thus increase the amount of product derived from sucrose. These data suggest that glucose moieties from sucrose are transferred to acceptors. In the case of band I the glucose moiety from sucrose is transferred to free glucose, for band II the glucose moiety from sucrose is transferred to sucrose and for band III the glucose moiety is transferred to the band I compound.

On the basis of the above studies and the fact that band II was the only band containing fructose the bands were identified as follows: Band I = maltose, band II = melezitose, and band III = maltotriose. Bands found after longer periods of time which moved more slowly than band III are probably higher oligosaccharides of glucose since they also were found not to incorporate label from sucrose-*fructose-1-*t_3.

Some of these compounds have been established as being formed by honey sucrase previously (Stanek *et al.*, 1965) but the previous workers reported that maltotriosucrose and maltotetrasucrose were formed while no evidence for these latter two compounds was found in this study. The honey enzyme that was previously studied may have been a different enzyme than the one studied here and the methods used in the previous study were also different than in this study as no radioactive tracer studies were carried out.

Rates of Formation of the Transferase Components. Figure 6 shows the percentage of sucrose-*U*-^{14}C which was incorporated into the three main transferase components as a function of time from an incubation mixture of 0.2 M sucrose and 0.2 M glucose. Note that initially band I increases at a rapid rate, then levels out and eventually falls off. Band III, on the

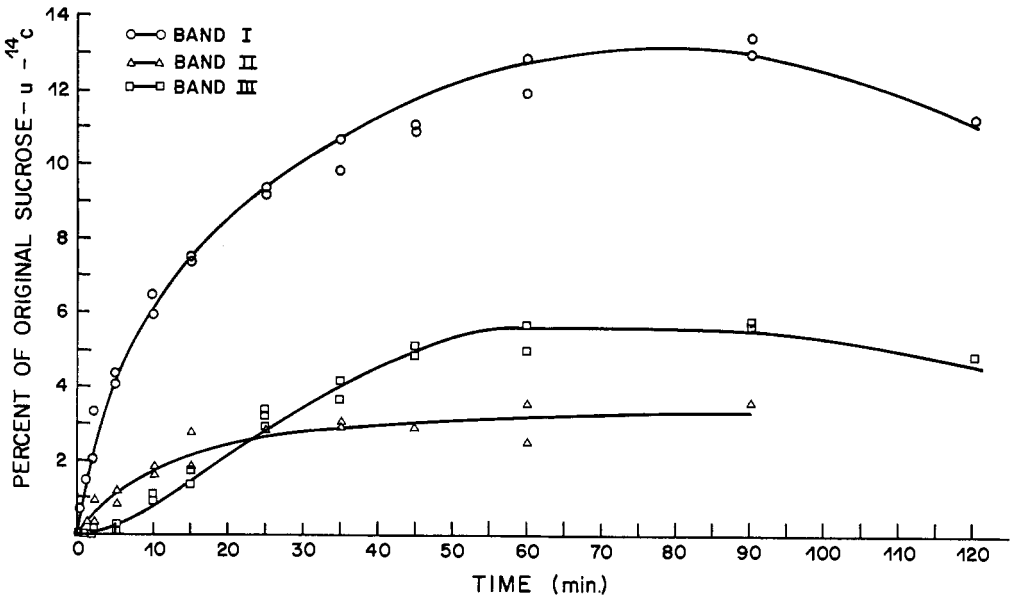

FIGURE 6: The rates of formation of the three main transferase components after incubation of the enzyme in a mixture of 0.2 M sucrose-*U*-^{14}C and 0.2 M glucose (unlabeled).

other hand, has a definite early lag, then increases, levels out and falls off. Since band I is probably maltose and band III maltotriose, this is suggestive evidence that maltose is a precursor to the formation of maltotriose as was suggested above. Band II increased at an early rapid rate but its formation levelled out quite soon.

Hofstee Plots for the Formation of Transferase Compounds. Figure 7A is a Hofstee plot for the production of fructose, glucose and the transferase components as determined by tlc after the incubation of the enzyme in various concentrations of sucrose-*U*-^{14}C in the absence of any other sugars. Note that the production of fructose and glucose is as shown previously. The production of the transferase components (in this case mainly band II) gave a line which is very steep, being essentially first order. At very low substrate values, however, the transferase activity approached zero and the curve bends backward toward the ordinate.

Figure 7B shows the Hofstee plot obtained for the production of fructose, glucose, and transferase products in the presence of sucrose-*U*-^{14}C and 0.2 M unlabeled glucose as well as with 0.5 M glucose. Three linear lines which are essentially parallel were obtained at each glucose concentration for the production of the three products. The parallel lines indicate that all three compounds have the same K_m. The K_m increased with increasing glucose. Both the production of fructose and glucose as well as the production of transferase components is greatly enhanced as compared to the normal case. Although the V_m for production of fructose and transferase compounds increases as glucose is increased, this is not the case for glucose production where the V_m remained constant regardless of the amount of glucose present.

Discussion

The unusual kinetic results obtained can be accounted for by the mechanism summarized in Figure 8A. The proposed mechanism is somewhat unique since sucrose (or pNphGlc) can be the substrate at two points (initial substrate or acceptor substrate) and the enzyme does not have a kinetically relevant binding site for the second substrate (either water or acceptor substrate).

In the presence of only sucrose or pNphGlc (*i.e.*, these compounds are both initial and acceptor substrates) the steady-state V_m values for each of the products for the mechanism proposed in Figure 8A can be derived as

P_1 (fructose or *p*-nitrophenol)

$$V_{mP_1} = \frac{[k_1k'_2 + k_1k_3(S)](E_t)}{k_3 + k_1} \quad (1)$$

P_2 (glucose)

$$V_{mP_2} = \frac{k_1k'_2(E_t)}{k_3 + k_1} \quad (2)$$

P_3 (transferase compounds)

$$V_{mP_3} = \frac{k_1k_3(S)(E_t)}{k_3 + k_1} \quad (3)$$

The K_m for all three products would be

$$K_m = \frac{k'_2 + k_{-1}}{k_3 + k_1} \quad (4)$$

Consideration of Figures 1A,B and 7A indicate that these equations are in agreement with the results. At low substrate concentrations the V_m for both P_1 and P_2 production should be independent of the substrate concentrations and should be essentially the same for both products since the component in eq 1 which includes the substrate concentration would be negligible. The value of V_{mP_2} is independent of substrate concentrations at all substrate values while V_{mP_1} becomes dependent in a first-order fashion on substrate concentration at high (S_0) values, since in this case the constant term in eq 1 becomes negligible. The V_{mP_3} should be dependent on substrate concentration throughout the entire range. The K_m should be the same for all three products and should be independent of the substrate concentration. All of these conditions are seen to be the case in Figures 1A,B and 7A. The fact that both the K_m and V_m for glucose (P_2) production are independent of substrate concentration is important. If a

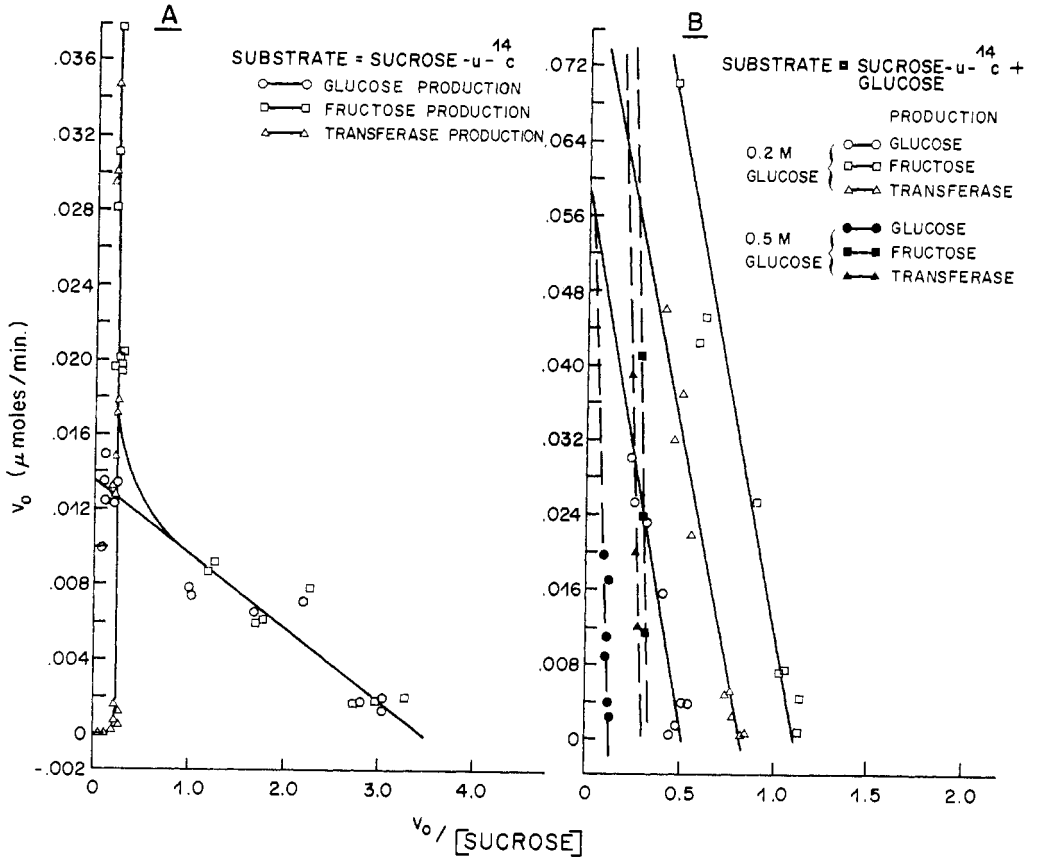

FIGURE 7: Hofstee plots for the production of hydrolysis and transferase products from sucrose-U-^{14}C: (A) only sucrose present; (B) sucrose and 0.2 M glucose, sucrose, and 0.5 M glucose.

kinetically relevant binding site for the acceptor substrate (in this case sucrose or pNphGlc) were postulated, then the V_m obtained for large substrate concentrations would decrease since the V_{mP_2} derived for a mechanism of this type would be inversely dependent on substrate concentration. There is no evidence for this as the lines for glucose production do not bend downward. It should be noted that a mechanism with a kinetically relevant binding site for H_2O would give similar overall relationships as this mechanism does and a mechanism with a kinetically relevant site for water is therefore a possibility.

If the acceptor substrate is a molecule such as glucose, which when present in high concentrations reacts at such a rapid rate that the reaction of initial substrate as an acceptor is negligible, the steady-state V_m values for each of the products would be

P_1 (fructose or *p*-nitrophenol)

$$V_{mP_1} = [k'_2 + k_3(N)](E_t) \quad (5)$$

P_2 (glucose)

$$V_{mP_2} = k'_2(E_t) \quad (6)$$

P_3 (transferase component)

$$V_{mP_3} = k_3(N)(E_t) \quad (7)$$

The K_m for all three products would be

$$K_m = \frac{k_{-1} + k'_2 + k_3(N)}{k_1} \quad (8)$$

Hinberg and Laidler (1972) discussed a complete series of mechanisms for systems in which acceptor molecules other than initial substrates were reactants. The kinetic constants

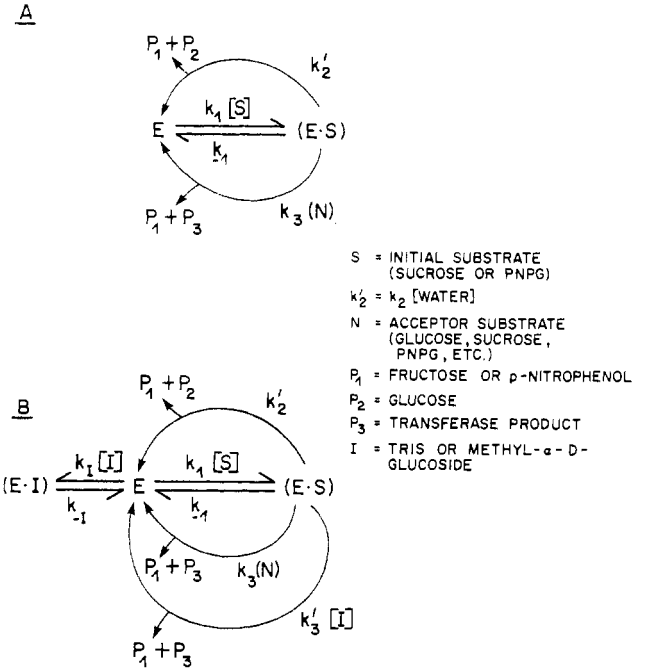

FIGURE 8: Proposed mechanisms: (A) basic mechanism; (B) mechanism in the presence of compounds which are both competitive inhibitors and acceptor substrates.

derived in this paper have the same values as those calculated by Hinberg and Laidler in their mechanism II.

The above equations predict that in the presence of an acceptor for which the magnitude of k_3 is large one would obtain linear Hofstee plots for the mechanism of Figure 8A due to the fact that the K_m and all of the V_m's are calculated to be independent of initial substrate concentration. Consideration of Figures 2A and 7B indicate that indeed linear plots are obtained in the presence of high glucose. The K_m's should be dependent on the concentration of glucose and should be the same for all three substrates at each glucose concentration. Figure 7B shows this to be the case since the lines are parallel and become steeper as glucose concentration is increased. The V_m values should be dependent on glucose concentration for the production of fructose and transferase compounds but not for glucose production, and again this is the case (Figure 7B).

Tris and methyl α-glucoside have more complex effects and these can be best visualized by the scheme in Figure 8B where these compounds (designated as I) react reversibly with free enzyme as dead end inhibitors and also act as acceptor compounds. The kinetic constants are as follows

P_1 (fructose or *p*-nitrophenol)

$$V_{mP_1} = \frac{k_1k_{-I}[k'_2 + k'_3(I) + k_3(S)](E_t)}{[k_{-I}k_3 + k_Ik_1 + k_Ik_3(I)]} \quad (9)$$

P_2 (glucose)

$$V_{mP_2} = \frac{k_1k_{-I}k'_2(E_t)}{[k_{-I}k_3 + k_{-I}k_1 + k_Ik_3(I)]} \quad (10)$$

P_3 (transferase compounds)

$$V_{mP_3} = \frac{k_1k_{-I}[k'_3(I) + k_2(S)](E_t)}{[k_{-I}k_3 + k_{-I}k_1 + k_Ik_3(I)]} \quad (11)$$

K_m for all three products

$$K_m = \frac{(k_{-I}k_{-1} + k_{-I}k'_2) + (k_{-I}k'_3 + k_Ik_{-1} + k_Ik'_2)(I) + k_Ik'_2(I)^2}{[k_{-I}k_3 + k_{-I}k_1 + k_Ik_3(I)]} \quad (12)$$

The effect of I on V_{mP_1} will depend on the relative values of $k_1k_{-I}k'_3$ and k_Ik_3. If $k_1k_{-I}k'_3(I) \gg k_Ik_3(I)$, V_{mP_1} will increase with increasing I, whereas, if $k_Ik_3(I) \gg k_1k_{-I}k'_3(I)$, V_{mP_1} will decrease with increasing I. In the case of Tris and methyl α-D-glucoside two opposite effects are seen. Tris seems to act more readily as a dead end inhibitor of the free enzyme than as an alternate acceptor of the glucose moiety and thus for Tris, V_{mP_1} values are lower than they are in the absence of Tris (Figure 3A). At high Tris concentrations (0.005 M), the V_m is considerably suppressed and increases in rate are seen only at high (S_0) values and then in a first-order fashion giving almost vertical Hofstee plots. In contrast, methyl α-D-glucoside seems to be a better alternate acceptor than a dead end inhibitor and V_{mP_1} is increased rather than decreased (Figure 3B). In the case of methyl α-D-glucoside the Hofstee plot is parallel to the curve in the absence of additions and thus while there is a dependence of V_{mP_1} on substrate concentration, the V_m is increased by a constant amount as predicted by eq 9.

The V_{mP_2} value should decrease as I is increased for both Tris and methyl α-D-glucoside and this is clearly seen by the results of Figure 2 (the intercept is lowered). An interesting feature of glucose production in the presence of Tris or methyl α-D-glucoside is the unusual tailing at low sucrose levels, where both v_0 and $v_0/(S_0)$ values are higher than expected. This can be explained by the fact that at low sucrose (or pNphGlc) levels the reaction of initial substrates as acceptors of glucose becomes insignificant and only the reaction with Tris or methyl α-D-glucoside as acceptor is important. The V_m for glucose production then becomes: $V_{mP_2} = k'_2(Et)$, which is the same as it was when large amounts of glucose were added (eq 6). This value will be higher than the value for V_{mP_2} in eq 10 above and thus at very low sucrose the v_0 and thus the $v_0/(S_0)$ values will be higher than expected.

The effect of I on K_m (eq 12) would depend on the values of the various rate constants concerned but in most cases it would increase the value of K_m and thus cause the slope of the Hofstee plot to become steeper. The effect of I on K_m is best seen in Figure 2 for the production of glucose where one sees a steeper slope produced in the presence of Tris or methyl α-D-glucoside than in their absence.

The effect of sucrose on the release of *p*-nitrophenol from pNphGlc would be similar to the effect of Tris except for the fact that sucrose is a substrate of the enzyme and is hydrolyzed or converted to transferase product on its own. One, however, expects results similar to those of Tris on the rate of *p*-nitrophenol (P_1) production, and this is found (Figure 3A).

The overall kinetic relationships found would hold for a mechanism in which (E·S) was converted to (E–G·X) before reaction with the acceptor substrate but would not hold for a system in which X (fructose or *p*-nitrophenol) is released before water or the acceptor had acted. In the case of mechanisms of this type, V_{mP_2} (for glucose production) would be an inverse function of substrate concentration and thus the Hofstee plot for glucose production would curve downwards at high (S_0) values. In addition V_{mP_1} for fructose (or *p*-nitrophenol) release as well as transferase product release would not be first-order functions of substrate concentration but rather more complicated hyperbolic functions. Since this is not the case in this system, the possibility of release of P_1 before reaction with acceptor can be discounted.

Further evidence that the proposed mechanism is correct is that the pNphGlc and sucrose reactions with enzyme occur at different rates. Hinberg and Laidler (1972) summarized a very comprehensive series of mechanisms which have been proposed by several workers to account for reactions of systems producing transferase products and only two of the mechanisms proposed would allow for the fact that different rates are found for different substrates. One of these mechanisms is that proposed in this present study, while the other is similar but proposes a kinetically relevant binding site for water. A site for binding of water is definitely a possibility and the kinetic data obtained do not eliminate the possibility that it exists.

Therefore, of all of the mechanisms which were considered the proposed one seems to fit the data best although a mechanism with a binding site for water is also feasible. Not only is the reaction in the presence of the two substrates explained but activation and inhibition effects by various additional compounds are also accounted for. Thus, the enzyme seems to be a hydrolytic enzyme which possesses transglucosidic action forming various oligosaccharides by transferring glucose moieties from the substrate itself. The kinetics are explained on the basis of the proposed mechanism.

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