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Kinetic Modeling of the Hydrolysis of Sucrose by Invertase

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Summary

The kinetics of the enzymatic hydrolysis of sucrose by invertase have been examined, with particular emphasis on high substrate concentration. Initial rates of reaction were determined by following the production of glucose directly as a function of time over a wide range of substrate concentrations (0.04M to 2.06M). The resulting data reveal a reaction rate that increases gradually until the sucrose concentration reaches about 0.29M, after which the reaction velocity decreases with increasing sucrose concentration. Previous workers (e.g., Nelson and Schubert[1]) have reported a peak reaction velocity as determined by indirect polarimetric measurements of glucose, at a sucrose concentration of about 0.17M. These measurements, however, neglect the intermediate oligosaccharides formed by the transferase action of invertase,[8–10] and assume equal amounts of glucose and fructose. According to Anderson *et al.*,[10] these oligosaccharides interfere by producing an erroneously low reaction rate. Experimental results of this work confirm Anderson's observations, and show a further reaction rate increase of nearly 20% between sucrose concentrations of 0.177M and 0.285M under the same conditions of temperature, pH, and enzyme concentration.

Effects of substrate diffusion, solution viscosity, water concentration, and substrate inhibition were experimentally studied and the results incorporated into a kinetic model that has proven satisfactory in modeling the experimental results. This model takes into account inhibition by primary substrate, with concentration of the secondary substrate water, as a rate limiting factor at sucrose concentrations greater than 0.285M.

The effects of mixing, in terms of volumetric power input, on the reaction rate have been tested. Approximately 40-fold increase in volumetric power input caused no increase in the reaction rate. These experiments have shown that bulk mass transfer is not a rate limiting factor under the experimental conditions.

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INTRODUCTION

A necessary first step toward the goal of good utilization of enzyme in the chemical process industries is the correlation of enzyme function with enzyme structure. Elucidation of enzyme function begins with the formulation of a kinetic model which is capable of describing the observed kinetic data. Such a model will provide an understanding of the catalytic mechanism of the particular enzyme, and will also serve as the starting point in the design of enzyme reactors.

Various kinetic models have been proposed to describe the hydrolysis of sucrose in the presence of yeast invertase (β-fructofuranosidase).[1–4] All of the proposed models are derived from the Michaelis–Menten model, which is then modified by incorporating the individual effects of water concentration, viscosity of the medium, or substrate inhibition. Previous modeling, based on the effect of the individual factor considered, did not result in a satisfactory kinetic model.

The purpose of this investigation is threefold: 1) to obtain experimental data by directly following the formation of glucose by using an enzymatic glucose assay method, 2) to observe how well the various theoretical models will predict the actual experimental data, and 3) to investigate the possibility of bulk mass transfer as a rate limiting factor for the reaction under consideration.

MATERIALS AND METHODS

Enzyme

100 mg invertase (Schwarz/Mann Labs) was dissolved in 20 ml of buffer solution for each run. The specific activity of the invertase preparation was 108 U/mg (μmole sucrose/min/mg) at 25°C, pH 5.0, and an initial sucrose concentration of 0.285M.

Buffer

Phosphate buffer of 0.162M KH_2PO_4 and 0.001M $Na_2HPO_4 \cdot 7H_2O$ (pH 4.5) represented about 5% of the total reaction volume.

Substrate

Reagent grade sucrose (Allied Chemical Corp.) was used throughout. Initial sucrose concentrations were determined by a polarimetric technique.

Glucose

Glucose was determined by the glucose oxidase method[5] (Glucostat Special, Worthington Biochemical Corp.). This enzyme is specially purified glucose oxidase which contains no traces of invertase or maltase.

Apparatus and Reaction Conditions for Kinetic Studies

All enzymatic hydrolysis reactions were carried out in a 2 liter fermentor (Fermentation Design). This unit provides both temperature and agitation control. The pH inside the reactor was continuously monitored and controlled with the aid of an Ingold glass electrode. All reactions were performed at 25 ± 0.5°C, pH 5.0 ± 0.1, agitation of 300 rpm (flat blade turbine impeller), and a total reaction volume (including enzyme) of 1050 ml.

The glucose oxidase reaction was run at pH 7.0 and room temperature (≈23°C) inside a Beckman DB-G Grating spectrophotometer (440 mμ).

Apparatus and Reaction Conditions for the Mass Transfer Studies

Reactions were carried out in a 1 liter morton flask immersed in a constant temperature bath (25°C). Agitation control was provided by an Eberbach high speed stirrer equipped with a paddle-type blade (5.2 cm in diameter and 0.9 cm in width), capable of speeds up to 1300 rpm. The reaction conditions were pH 5.0, total reaction volume (including enzyme) of 1 liter, and a sucrose concentration of 0.585*M*.

PROCEDURE

Kinetic Studies

One ml samples of the reaction medium were removed at one minute intervals from the reaction vessel and placed into a test tube immersed in a 110–115°C bath. Invertase denaturation was complete and irreversible[6] after one minute. This procedure was followed over a ten minute period for each run. The samples were then refrigerated until assayed for glucose. The reaction rate thus determined varies less than ±7.0% at the 95% confidence level.

Mass Transfer Studies

The reaction was followed by recirculating the sucrose invertase solution through a polarimeter and then back into the reaction vessel.[7] Readings were recorded each minute for a 12 min period. The mean holding time inside the polarimeter and tubing is negligibly small.

RESULTS AND DISCUSSION

Experimental Data

The reaction rate data presented in Table I and plotted in Figure 1 demonstrate more truly the kinetics of the hydrolysis of sucrose to glucose and fructose. The most notable feature of these results is the shift in peak activity from the commonly accepted value of about 0.17M sucrose to that of about 0.29M sucrose.

TABLE I

Rate of Hydrolysis of Sucrose by Invertase as Determined from Direct Glucose Measurements[a]

Sucrose concentration		Reaction velocity,
(g/100 cc)	(moles/liter)	moles glucose/liter-min
1.35	0.040	.00279
2.26	0.066	.00472
3.31	0.097	.00652
4.32	0.126	.00719
6.05	0.177	.00862
9.77	0.285	.01030
19.29	0.564	.00929
36.09	1.055	.00717
48.95	1.431	.00663
60.30	1.763	.00503
70.56	2.060	.00404

[a] pH 5.0 ± 0.7, 25.0°C ± 0.5°C.

This shift in peak activity is not entirely unexpected. Albon *et al.*,[8] Gross *et al.*,[9] and Anderson *et al.*[10,11] have shown that the intermediate oligosaccharides formed by the transferase action of invertase,

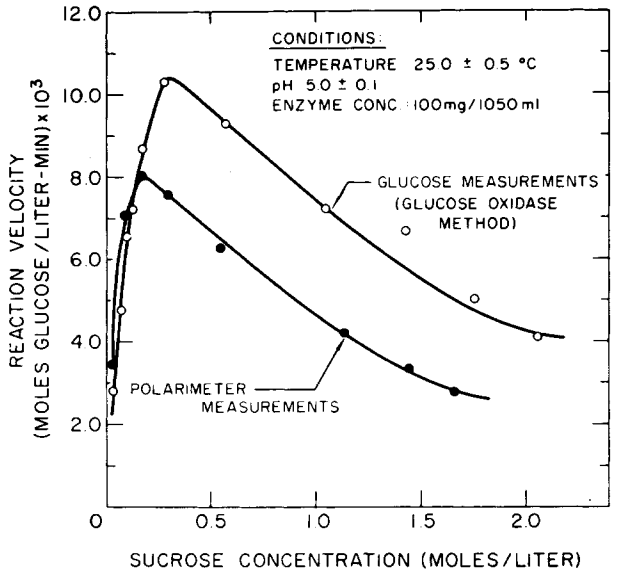


Fig. 1. Rate of hydrolysis of sucrose by invertase vs. sucrose concentration as determined from both polarimeter and enzymatic glucose assay techniques.

will introduce a significant error in polarimetric measurements during the time interval commonly used in determining initial reaction rates. According to Anderson *et al.*[10] these di-, tri-, and tetra-saccharides interfere by producing an erroneously low reaction rate. A comparison of kinetic data taken under similar reaction conditions, as determined by indirect polarimetric measurements of glucose and by an enzymatic glucose assay method, is shown in Figure 1. These results confirm Anderson's observations.

Michaelis–Menton Model

Michaelis–Menten theory[12] predicts the following two step mechanism for enzyme catalysis:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_2} E + P \qquad (1)$$

where E is the enzyme; S the substrate; $[ES]$ the enzyme–substrate complex; and P the product.

The rate expression can be written as

$$R_1 = \frac{V_m \cdot [S]}{K_m + [S]} \tag{2}$$

where R_1 is the initial reaction velocity in moles/liter-min; $[S]$, the substrate concentration in moles/liter; K_m, the Michaelis constant is a combination of rate constants equal to $[(k_{-1} + k_2)/k_1]$; V_m is the maximum reaction velocity, which is equal to the product of k_2 and the total enzyme concentration $[E_0]$.

The constants K_m and V_m have been determined by plotting the experimental data in the form of $1/R_1$ vs. $1/[S]$. This plot is linear at sucrose concentrations below $0.29M$, and shows K_m equal to $0.160M$ and V_m equal to 0.0164 moles/liter-min. The Michaelis–Menten model is shown in Figure 2. The zero order prediction at high substrate concentrations does not fit the experimental data.

Viscosity Model

Various attempts have been made to correlate the experimental data for the sucrose–invertase reaction with a kinetic model that incorporates the change in solution viscosity at sucrose concentrations above $0.17M$. The concept is that the deviation from Michaelis–Menten behavior may be due to a diffusional limitation of sucrose to the active site of the enzyme. According to the Stokes–Einstein equation for the diffusion of solutes in liquids

$$D_L/T = f(1/\eta) \tag{3}$$

where η is the solution viscosity, T the temperature, and D_L the diffusion constant.

McLaren[3] proposed that the initial reaction rate will be proportional to the relative fluidity of the reaction medium. Thus the empirical model advanced by McLaren, and the equivalent one tested in this report is

$$R_2 = \frac{1.2\ V_m[S]}{K_m + [S]}\ (\eta_0/\eta) \tag{4}$$

where η_0 is the viscosity of water at 25°C, η the viscosity of the sucrose solution, and 1.2 the associated constant used to correct the Michaelis–Menten model to the experimental data.

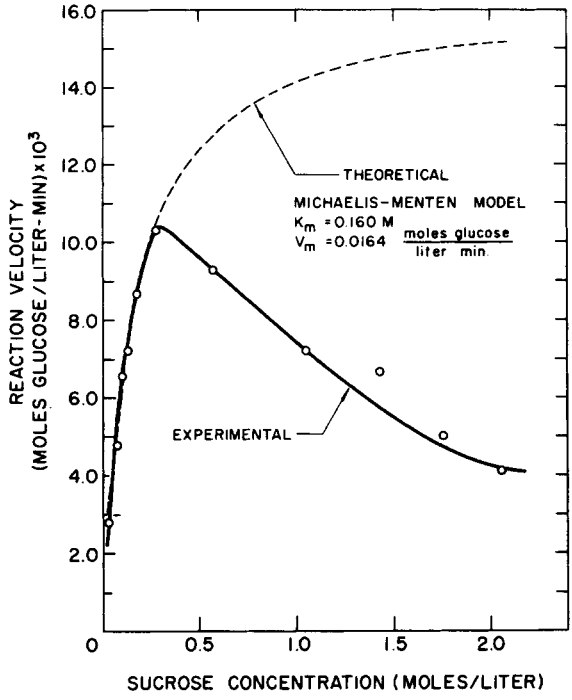


Fig. 2. Theoretical Michaelis–Menten model plotted for comparison to the experimental data.

The viscosity data is listed in Table II. Equation (4) is plotted in Figure 3 for comparison with the experimental data. Clearly the term (η_0/η) is inadequate in explaining the observed experimental data.

TABLE II

Viscosity of Sucrose Solutions (Refs. 13, 14)[a]

Weight % sucrose	η(cp) at 25°C
10	1.17
20	1.71
30	2.75
40	5.21
50	11.49
60	44.02

[a] η_0 at 25°C = 0.8937 cp.

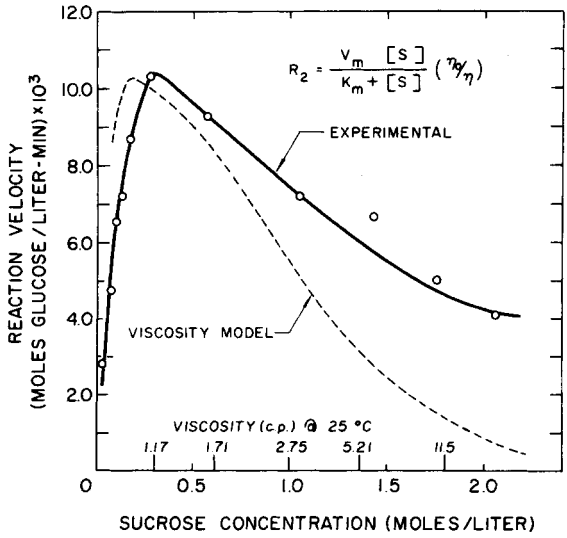


Fig. 3. Empirical test of the effect of viscosity on the rate of hydrolysis.

Effect of Mixing on Reaction Rate

Experimental results indicate that a 40-fold increase in volumetric power input (0.023 watts/liter to 1.01 watts/liter) did not cause an increase in the reaction rate. If bulk mass transfer had been a rate limiting factor, a 2.5-fold increase in reaction rate should have been observed. This is according to the relationship between the mass transfer coefficient (k_L) and the volumetric power input (P/v), which can be expressed for sucrose solutions as

$$k_L = f[(P/v)^{1/4}(\mu/\rho^2)^{1/4}(1/N_{sc})^{2/3}] \quad (5)$$

according to Oyama.[15] The viscosity (μ), density (ρ), the Schmidt number (N_{sc}) remain constant at each point.

Effect of Water Concentration on Reaction Rate

Nelson and Schubert[1] observed that as the concentration of sucrose increases, the water concentration decreases. If the reaction velocity is plotted against the water concentration a curve is obtained which is similar to the velocity vs. sucrose concentration curve.

Further experimentation by Nelson and Schubert led to their conclusion "that the concentration of water is a factor in determining the

magnitude of the velocity of hydrolysis of sucrose by invertase." Kertesz[16] also found the reaction velocity dependent on the concentration of water.

The following reaction mechanism was formulated as a test for the limitation of water:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \tag{6}$$

$$ES + W \xrightarrow{k_2} E + P \tag{7}$$

where W represents water.

The kinetic model can be written as

$$R_3 = \frac{V_m[S]}{K_m + [S]}[W] \tag{8}$$

where $[W]$ is the total water concentration; i.e., the sum of the concentrations of free water and water hydrogen bonded to sucrose (see Table III). In terms of the relative change in free plus bound water, this model can be written as

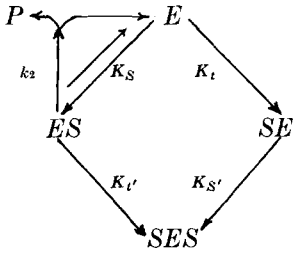
$$R_4 = \frac{1.04\ V_m[S]}{K_m + [S]}\left(\frac{[W]}{[W_0]}\right) \tag{9}$$

where $[W_0]$ is equal to 55.33 moles H_2O/liter at 25°C, and 1.04 is the associated constant used to correct the Michaelis–Menten model to the experimental data.

The plot in Figure 4 shows that the effect of decreasing water concentration reduces the velocity predicted by the Michaelis–Menten model at high sucrose concentrations, but not enough to fit the experimental data.

Substrate Inhibition Model

A kinetic model for substrate inhibition, as described by Laidler,[17] can be formulated from the following network of reactions:



(10)

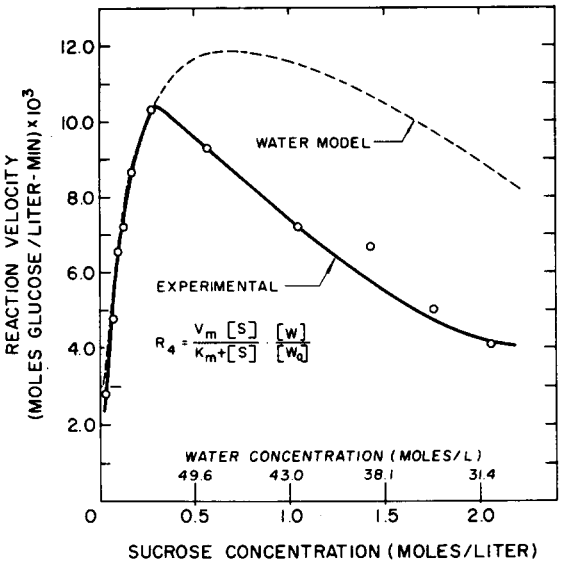


Fig. 4. Effect of water concentration on the rate of hydrolysis.

where *ES* represents an active complex in which substrate is correctly attached to the active sites, while *SE* and *SES* are postulated inactive complexes due to the incorrect binding of substrate. This type of mechanism has been used to explain the kinetics of other enzymes. For example, Alberty *et al.*[18] on fumarase, Wilson and Bergman[19] on acetylcholinesterase, Laidler and Hoare[20] on urease, and Lumry *et al.*[21] on carboxypeptidase have interpreted their results in terms of substrate inhibition.

The kinetic model for substrate inhibition can be written for our case as

$$R_5 = \frac{k_2 K_S [E_0][S]}{1 + (K_S + K_t)[S] + K_S K_t'[S]^2} \tag{11}$$

The three groups of constants in this equation have been determined by linear regression analysis of the experimental data (see Laidler[17]), they are

$$k_2 K_S [E_0] = 0.0103 \text{ mole Glucose/moles Sucrose} \cdot \text{min} \tag{12}$$

$$K_S + K_t = 6.27 \text{ liter/mole Sucrose} \tag{13}$$

$$K_S K_t' = 7.26 \text{ liter}^2/(\text{mole Sucrose})^2 \tag{14}$$

Constants (12) and (13) were obtained from the experimental data in the region of low substrate concentration (below 0.29*M*), while constant (14) from the region of high substrate concentration (above 0.29*M*).

Equation (11) multiplied by the associated constant of 1.19 to once again correct the Michaelis–Menten model, is plotted in Figure 5. The experimental data falls below the hypothetical curve when the sucrose concentration is greater than 0.29*M*, although a fairly good fitting can be seen for sucrose concentrations lower than 0.29*M*.

Nature of the Active Enzyme–Substrate Complex

There are two basic assumptions inherent with the substrate inhibition model that we have used: 1) the enzyme has two active sites, and 2) a substrate molecule must have access to both of these sites for reaction to take place. On this basis it is possible to arrive at a model for the active enzyme–substrate complex. The mechanism of reaction, initially proposed by Laidler,[17] and illustrated in Figure 6, accounts for complexing at both the acidic and basic groups of the invertase active sites.

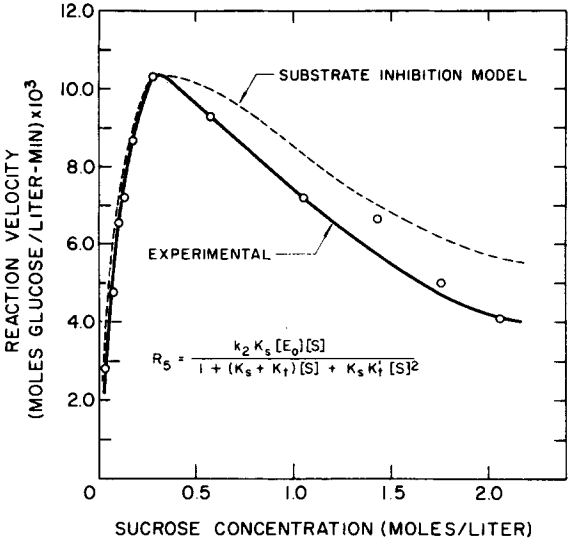


Fig. 5. Inhibition of sucrose hydrolysis by substrate.

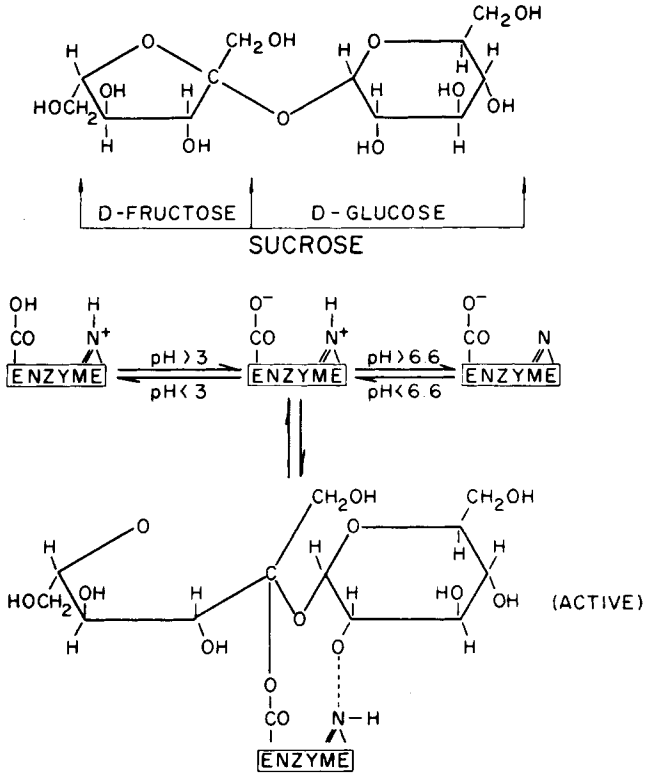


Fig. 6. Suggested sites of binding for the active invertase–sucrose complex.

Combined Effect of Substrate Inhibition and Water Concentration

Although the substrate inhibition model permits a closer fit to the experimental data than the other models, it must be further modified to account for the observed remaining deviation. McLaren,[3] who first suggested a substrate inhibition model, implied that viscosity limitation of diffusion may account for the disagreement between the model and experimental data. If viscosity is incorporated in eq. (11) by the term (η_0/η) it is obvious that the end result of the combined effects will once again be a curve similar to the viscosity model in Figure 3, and show greater deviation from the experimental values.

Equation (11) can also be modified by incorporating the decreasing water concentrations. The term that is sought is the decrease in the

concentration of free water $[W_F]$ relative to the concentration of free plus bound water $[W]$. The concentration of free plus bound water can be determined from the density of sucrose solutions.[4] The free water concentration $[W_F]$ varies with sucrose concentration. If the change in free water concentration is responsible for the deviation between the substrate inhibition model and the observed data, then $[W_F]$ will vary in such a way as to produce the observed lack of fit of the unmodified substrate inhibition model. On this basis we have incorporated the change in free water concentration at sucrose concentrations greater than $0.29M$ into the substrate inhibition model:

$$R_6 = \frac{k_2 K_S[E_0][S]}{1 + (K_S + K_t)[S] + K_S K_t'[S]^2} \left(\frac{[W_F]}{[W]}\right) \tag{15}$$

The ratio $[W_F]/[W]$ is plotted as a function of sucrose concentration in Figure 7. The substrate inhibition model incorporating the term $[W_F]/[W]$ is shown in Figure 8. From these results one can conclude that eq. (15) successfully predicts the observed data for the enzymatic hydrolysis of sucrose by invertase.

From Table III, one can observe an interesting phenomenon. The theoretical values of free water concentration, calculated on the basis of 7 moles of water hydrogen bound to each mole of sucrose (Einstein[22]), are lower than the experimentally determined values of free water concentration. What this could mean is that at high sucrose

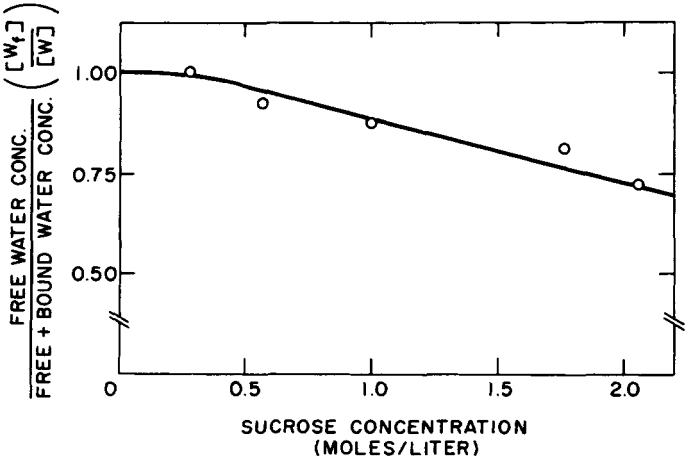


Fig. 7. Distribution of free and bound water as a function of sucrose concentration.

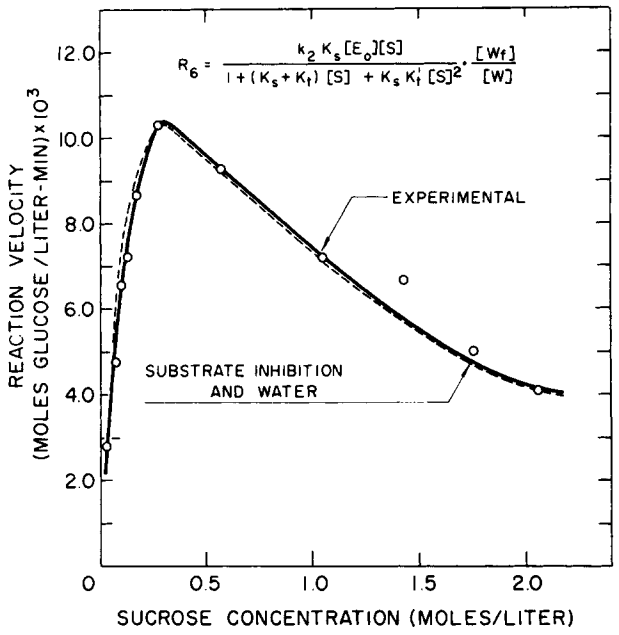


Fig. 8. Combined effect of substrate inhibition and water concentration on the rate of hydrolysis of sucrose by invertase.

concentrations a sucrose molecule may be incompletely hydrated by water. This suggests a possibility of sucrose molecules hydrogen bonding to each other, so as to form clusters that are inaccessible to the active site of the enzyme.

TABLE III

Concentration of Water in Sucrose Solutions

Sucrose, g/100 cc	Concentration, moles/liter	Free + bound water,[4] $[W]$ moles/liter	Experimental free water, $[W_F]$ moles/liter	Theoretical free water, moles/liter
9.77	0.285	52.0	52.0	—
19.29	0.564	48.7	45.0	44.7
36.09	1.055	42.9	37.5	35.5
48.95	1.431	38.3	36.1	28.3
60.30	1.763	34.5	27.9	22.2
70.56	2.060	30.8	22.2	16.4

Tying together all these concepts, Figure 9 is a schematic representation of the various processes capable of explaining the kinetics of the hydrolysis of sucrose by invertase. Although this diagram displays a valid picture for this process of catalysis, the next step must involve the empricial confirmation of the inactive complexes formed due to substrate inhibition.

Kinetic Modeling of Polarimeter Data

Initial modeling of the kinetics of this reaction was performed with polarimeter data. Final results, obtained by this laboratory, showed that a substrate inhibition model (eq. 11) fit the data up to 1.4M sucrose, with decreasing water concentration as a rate limiting factor above this point. A much improved model (eq. 12) was derived to fit kinetic data as determined from an enzymatic glucose assay method. This method allows for the interference effect of oligosaccharide formation, and also provides a more precise technique for determining reaction rates in the region between 0.03M and 0.3M sucrose.

CONCLUSION

From the results presented in this paper, the enzymatic hydrolysis of sucrose by invertase can be satisfactorily explained by a substrate inhibition model (eq. 12) modified to incorporate the effects of water binding, and substrate aggregation as additional rate limiting factors at sucrose concentrations greater than 0.29M.

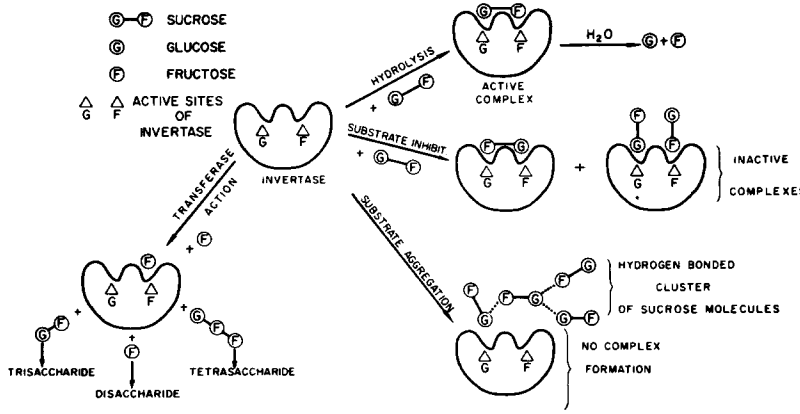


Fig. 9. Suggested enzymatic pathway for the hydrolysis of sucrose by invertase.

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