column top which upon removal and elution gave crystalline material; yield 100 mg., m.p. 184°, X-ray powder diffraction pattern[21] identical with that of β-sophorose (2-*O*-β-D-glucopyranosyl-β-D-glucose) octaacetate.

(21) A. Thompson, Kimiko Anno, M. L. Wolfrom and M. Inatome, THIS JOURNAL, **76**, 1309 (1954).

Identical partial hydrolysis and analysis[5] of a sample of the unheated amylose (Superlose[14]) yielded only β-D-glucopyranose pentaacetate, β-maltose octaacetate, β-maltotriose octaacetate and no β-isomaltose octaacetate or β-gentiobiose octaacetate.

COLUMBUS 10, OHIO

[CONTRIBUTION FROM THE FERMENTATION SECTION, NORTHERN UTILIZATION RESEARCH AND DEVELOPMENT DIVISION[1]]

# A Kinetic Study of Dextransucrase[2a]

BY C. S. STRINGER AND H. M. TSUCHIYA[2b]

RECEIVED NOVEMBER 11, 1957

Dextran synthesis by cell-free enzyme preparations derived from *Leuconostoc mesenteroides* NRRL B-512F requires both sucrose and a suitable "acceptor" cosubstrate. Molecules of the latter serve as initiators for the formation of new chains. At sucrose concentrations greater than about 0.01 *M*, the initial rate of reaction depends upon the availability of the acceptor, as well as upon sucrose concentration. The effect of a model acceptor substrate, α-methyl glucoside, upon some of the kinetic properties of dextransucrase is reported. The initial rate of reaction has a first order dependence upon α-methyl glucoside concentration in the range 0.15–0.8 *M*; below 0.15 *M* the measured rate is in part due to an unidentified acceptor substrate. Data indicate that this substance is not a contaminant of the enzyme preparation. First-order dependence of the rate upon sucrose concentration is confirmed in the range 0.01–0.11 *M*; the dependence changes through zero order to an inverse dependence (inhibition) at higher sucrose concentrations, as Hehre[3] earlier reported, in the absence of α-methyl glucoside. α-Methyl glucoside relieves the inhibition. Estimates of the Michaelis constant and maximum velocity for either sucrose or α-methyl glucoside depend upon the concentration of the other reactant. An equation is derived which describes the dependence of rate upon the concentrations of sucrose and α-methyl glucoside over the ranges 0.01–0.11 and 0.15–0.80 *M*, respectively. The data are compared with the behavior predicted by certain reaction mechanisms.

Dextransucrase catalyzes a transglucosylation which may be represented empirically as

sucrose + acceptor ⟶ fructose + glucosyl-acceptor

The second product may react repeatedly with sucrose, giving rise to polymeric products. Although the enzyme is, for practical purposes, specific for sucrose as the donor,[3] any of several carbohydrates will serve as acceptor.[4–9] Since the latter serve as chain initiators, some degree of control of the molecular weight distribution has been achieved by making a suitable selection of the acceptor substrate and by the adjustment of the ratio of its concentration to that of sucrose. (Other factors, particularly temperature and enzyme concentration, also affect the molecular weight distribution of the product.[7])

This communication presents a study of the effect of a model acceptor substrate upon some of the kinetic properties of dextransucrase. The acceptor concentration was varied over a range sufficient to shift the average degree of polymerization of the product from the order of several million[10] to the oligosaccharide range.[4,9]

The high molecular weight dextran synthesized by *Leuconostoc mesenteroides* NRRL B-512F is believed to consist of a number of α-1,6-linked main chains, to which short branches (predominately single glucosyl units) are attached by α-1,3-linkages.[11–13] The main chains may be several hundred units in length and are thought to be associated in a "macro-branched" structure.[12] Although it has been shown[14] that the two types of glucosidic linkages are synthesized by separate enzymes, the ratio of α-1,6;α-1,3 linkages in B-512F dextran (95:5)[11] indicates that the predominant activity of the dextransucrase preparations used in this work is the formation of α-1,6 linkages. The oligosaccharides synthesized in the presence of α-methyl glucoside have been shown to be α-methyl isomaltoside and its α-1,6-linked homologs.[9]

Kinetic constants with respect to sucrose have been reported by Hehre,[3] Carlson,[15] Goodman[16] and their co-workers. Earlier studies[3,4] indicated no effect upon the rate of reaction by an added cosubstrate (dextran), possibly because only a very low molar concentration could be attained experimentally. Some saccharides known to be effective acceptors have been shown to have an effect upon the rate of reaction, as well as upon the molecular weight of the product.[4] Bovey has

(1) One of the Divisions of the Agricultural Research Service, U. S. Department of Agriculture. Article not copyrighted.

(2) (a) Presented in part at the 55th General Meeting, Society of American Bacteriologists, New York, N. Y., May 8–12, 1955. Abstracted, Bacteriological Proceedings, 1955, p. 126. (b) Department of Chemical Engineering, Universty of Minnesota, Minneapolis 14, Minn.

(3) E. J. Hehre, *J. Biol. Chem.*, **163**, 221 (1946).

(4) H. J. Koepsell, H. M. Tsuchiya, N. N. Hellman, A. Kazenko, C. A. Hoffman, E. S. Sharpe and R. W. Jackson, *ibid.*, **200**, 793 (1953).

(5) H. M. Tsuchiya, N. N. Hellman and H. J. Koepsell, THIS JOURNAL, **75**, 757 (1953).

(6) E. J. Hehre, *ibid.*, **75**, 4866 (1953).

(7) H. M. Tsuchiya, N. N. Hellman, H. J. Koepsell, J. Corman, C. S. Stringer, S. P. Rogovin, M. O. Bogard, G. Bryant, V. H. Feger, C. A. Hoffman, F. R. Senti and R. W. Jackson, *ibid.*, **77**, 2412 (1955).

(8) M. Killey, R. J. Dimler and J. E. Cluskey, *ibid.*, **77**, 3315 (1955).

(9) R. W. Jones, A. Jeanes, C. S. Stringer and H. M. Tsuchiya, *ibid.*, **78**, 2491 (1956).

(10) F. R. Senti and N. N. Hellman, Abstracts of Papers, Am. Chem. Soc., 121st Meeting 8-O (1952).

(11) J. W. Van Cleve, W. C. Schaefer and C. E. Rist, THIS JOURNAL, **78**, 4435 (1956).

(12) R. W. Jones, R. J. Dimler, A. Jeanes, C. A. Wilham and C. E. Rist, Abstracts of Papers, Am. Chem. Soc., 126th Meeting, 13D (1954).

(13) F. R. Senti, N. N. Hellman, N. H. Ludwig, G. E. Babcock, R. Tobin, C. A. Glass and B. L. Lamberts, *J. Polymer Sci.*, **17**, 527 (1955).

(14) R. W. Bailey, S. A. Barker, E. J. Bourne and M. Stacy, *J. Chem. Soc.*, 3530 (1957).

(15) W. W. Carlson, C. L. Rosano and V. Whiteside-Carlson, *J. Bacteriol.*, **65**, 136 (1953).

(16) (a) A. Goodman, E. Braswell and K. G. Stern, Abstracts of Papers, Am. Chem. Soc., 127th Meeting, 10D (1955); (b) A. Goodman, R. M. Weil, E. Braswell and K. G. Stern, Abstracts of Papers, Am. Chem. Soc. 128th Meeting, 6S (1955).

given a preliminary account of a study of the effect of $\alpha$-methyl glucoside.[17a]

## Methods

**Materials.**—The enzyme preparations employed were derived from cell-free culture filtrates of *Leuconostoc mesenteroides* NRRL B-512F.[18] Except where otherwise described in the text, the preparation used is an alcohol-precipitated concentrate assaying 635 dextransucrase units[19] (D.S.U.) per ml., similar to that used previously.[7]

Stock solutions of $\alpha$-methyl glucoside (twice recrystallized from methanol, m.p. 165°) and sucrose (table quality cane sugar) were prepared on a weight/volume basis, and the concentrations were verified polarimetrically. The low molecular weight dextran used in one experiment was synthesized enzymatically, precipitated three times with methanol and dried *in vacuo* at 40°.

**Procedures.**—All experiments were performed at *p*H 5.1 ± 0.1 and 15°. The synthesis of high molecular weight product is suppressed, although not entirely eliminated, in acceptor-primed reactions at this temperature[7] (in one experiment rates at 30° were also measured). Portions (10 ml.) of sucrose and $\alpha$-methyl glucoside solutions, each at 2.5-fold the desired final concentration, were combined at room temperature. The mixtures were brought to 15° and 5 ml. of stock enzyme solution (also at 15°) were added with rapid mixing. Except where otherwise indicated, the final enzyme concentration was 10 D.S.U. per ml. Aliquots were removed from the reaction mixture at intervals of 6, 12, 18 and 24 minutes after the addition of enzyme and delivered into sufficient dilute NaOH (phenolphthalein endpoint) to inactivate the enzyme. Reducing sugar was determined by the iodometric method of Somogyi[20] and calculated as fructose.

**Calculations.**—Reaction rates were taken from the slope of a graph showing reducing value as a function of sampling time. This is permissible, since no deviation from linearity was detectable within the limits of experimental error. Hehre[3] has established that the rate of formation of reducing sugars is a valid index of the rate of dextran synthesis. Statistical examination of a portion of the data indicates that the standard deviations of the rates are about 4–5%. Reaction rates are expressed as micrograms of fructose per ml. per minute.

Kinetic constants were evaluated by conventional procedures.[21–23] Michaelis constants are expressed as moles per liter.

## Results

**Simultaneous Variation of the Concentrations of $\alpha$-Methyl Glucoside and Sucrose.**—Reaction rates were measured in a series of mixtures containing various concentrations of sucrose and $\alpha$-methyl glucoside. Twelve sucrose concentrations (0.005 to 0.20 $M$) were examined in the presence of each of eight levels of $\alpha$-methyl glucoside (0–0.80 $M$); additional sucrose concentrations to 1.0 $M$ were used at some of the $\alpha$-methyl glucoside levels.

Representative data are given in Fig. 1. The solid line curves represent values calculated by the Michaelis–Menten equation

$$v = (V_s[S])/([S] + K_s) \qquad (1)$$

Appropriate numerical values for $V_s$ and $K_s$ are taken from Table I. Each short dashed line segment at the right side represents a $V_s$ value, the asymptote of the adjacent hyperbola.

For many of the data the rate is simultaneously limited by the concentrations of both sucrose and $\alpha$-methyl glucoside. However, the latter is not required; appreciable rates are measured in its absence (open circles). These represent the synthesis of high molecular weight dextran, which requires no added cosubstrate.[3,7] The data also confirm the observation[3] that the rate of reaction decreases as the sucrose concentration exceeds about 0.2 $M$.

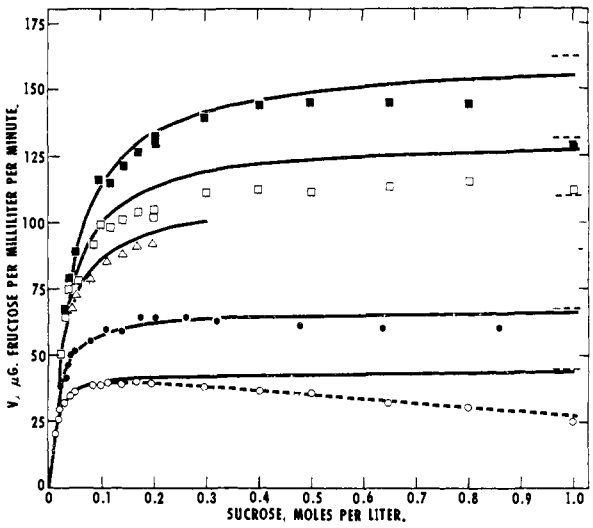


Fig. 1.—Velocity as a function of [sucrose] and [$\alpha$-methyl-glucoside]. Concentrations of the latter are: ○, none; ●, 0.10 M; △, 0.25 $M$; □, 0.40 $M$; ■, 0.80 $M$. All points at the origin and some points in the range below 0.1 $M$ sucrose have been omitted for clarity. Solid lines calculated by eq. 1; dashed curve calculated by eq. 2.

The dashed-line curve of Fig. 1 represents rates calculated by the equation[21]

$$v = (V_s[S])/([S] + K_s + [S]^n/K_2) \qquad (2)$$

Numerical values of the constants $V_s$ and $K_s$ are from Table I; $n$ and $K_2$ were estimated by Lineweaver and Burk's "Case III" procedure. $K_2$, the dissociation constant of the hypothetical inactive enzyme species E-$S_n$, was found to be 1.6. In this case, $n$ was estimated to be 2.3, and is presumed to be a poor approximation of the integer 2. Data obtained at sucrose concentrations up to 1.85 $M$ are satisfactorily described by this equation, confirming the observation[3] that the deviations are the result of an orderly process.

Estimates of $n$ and $K_2$ obtained in the presence of $\alpha$-methyl glucoside are not reported. These vary widely and erratically, probably because experimental error is relatively large as compared with the deviation caused by inhibition. The deviations of the data for 0.4 $M$ $\alpha$-methyl glucoside, for example, do not change appreciably with increasing sucrose concentration, and are probably largely the result of a poor estimate of $K_s$. Nonetheless, the data illustrate that the addition of $\alpha$-methyl glucoside alleviates sucrose inhibition, and shifts the optimum sucrose concentration to a higher value. This is consistent with a theoretical study of substrate inhibition for cofactor-dependent enzymes.[24]

**"Apparent"[25] Kinetic Constants.**—Numerical estimates of the Michaelis constant and maximum velocity were made by the Lineweaver–Burk double reciprocal method.[21] A graph of $1/v$ *vs.* 1/[sucrose] was made for each $\alpha$-methyl glucoside level, and found to be linear over most of the range; points at the higher sucrose levels, where inhibition becomes significant, fell above a line fitting the other points. Similarly, a plot of $1/v$ *vs.* 1/[$\alpha$-methyl glucoside] was made for each sucrose level. At 0.005 and 0.010 $M$ sucrose the points scattered badly. At 0.015 $M$ sucrose and above, each graph was linear over the range 0.15–0.80 $M$ $\alpha$-methyl

(17) (a) F. A. Bovey, Abstracts of Papers, Am. Chem. Soc., 131st Meeting, 9D (1957); (b) *ibid.*, p. 15S.

(18) In 1950, the B-512F substrain supplanted B-512 for all work at the Northern Utilization Research and Development Division. Since that time, this substrain has been designated inexactly as B-512 in numerous publications.

(19) H. J. Koepsell and H. M. Tsuchiya, *J. Bacteriol.*, **63**, 293 (1952).

(20) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

(21) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(22) G. W. Schwert and H. T. Hakala, *Arch. Biochem. and Biophys.*, **38**, 55 (1952).

(23) L. L. Ingraham and B. Makower, *J. Phys. Chem.*, **58**, 266 (1954).

(24) S. Levine, *Enzymol.*, **16**, 265 (1953).

(25) If, in an activator-dependent system, both substrate and activator are simultaneously rate-limiting, the evaluations of the kinetic constants of either or both substrates may depend upon the concentration of the other. Such constants are termed "apparent."[29]

glucoside. Points representing lower levels of α-methyl glucoside fell below a line fitting the other points; the anomalously high velocities represent, in part, the synthesis of high molecular weight dextran. In all cases, 5 or more points were used to establish the line for estimating slope and intercept.

The "apparent" kinetic constants for each of the substrates vary with changing concentration of the other (Tables I and II).

TABLE I

EVALUATION OF THE "APPARENT" KINETIC CONSTANTS OF SUCROSE

| [α-Methyl glucoside], *M* | Maximum velocity | Michaelis constant, *M* |
|---|---|---|
| 0.0 | 44.3[a] | 0.012 |
| .05 | 54.1 | .013 |
| .10 | 67.6 | .017 |
| .15 | 81.5 | .023 |
| .25 | 110 | .027 |
| .40 | 132 | .032 |
| .60 | 143 | .037 |
| .80 | 162 | .042 |

[a] μg. fructose per ml. per minute; enzyme concentration, 10 D.S.U./ml.

TABLE II

EVALUATION OF THE "APPARENT" KINETIC CONSTANTS OF α-METHYL GLUCOSIDE

| [Sucrose], *M* | Maximum velocity | Michaelis constant, *M* |
|---|---|---|
| 0.015 | 47.5[a] | 0.057 |
| .020 | 61.5 | .083 |
| .030 | 81.3 | .108 |
| .040 | 98.0 | .127 |
| .050 | 101 | .125 |
| .080 | 125 | .150 |
| .110 | 135 | .155 |
| .140 | 143 | .154 |
| .170 | 147 | .162 |
| .200 | 152 | .167 |

[a] μg. fructose per ml. per minute; enzyme concentration, 10 D.S.U./ml.

To facilitate initial comparisons with mechanisms described in the literature, it was found expedient to exclude from consideration those data in which the effects of sucrose inhibition or high molecular weight dextran synthesis are apparent. Such data were identified by applying Lineweaver and Burk's "Case II" method[21] (Figs. 2 and 3), using the estimates of $V_s$ and $V_g$ of Tables I and II. First-order dependence of rate upon concentration is found in the ranges 0.005–0.11 *M* sucrose and 0.15–0.6 *M* α-methyl glucoside. (Despite the scatter of points in Fig. 3 at 0.8 *M*, it is assumed that the range can be extended to this concentration.) These ranges establish the limits of validity of the rate equation (eq. 4).

**Kinetic Constants of the Rate Equation.**—It is found that an equation of the form

$$v = \frac{[S][G]V_{SG}}{[S][G] + K_S[G] + K_G[S] + K_{SG}} \quad (3)$$

relates the observed initial rate of reaction, $v$, to the concentrations of the substrates (sucrose, S; α-methyl glucoside, G); $V_{SG}$ is the limiting value of $v$ as the substrate concentrations are simultaneously increased; $K_S$, the Michaelis constant for sucrose, is equal to that concentration of sucrose at which $v = V_{SG}/2$, when α-methyl glucoside is present at sufficiently high concentration that its further increase will not affect the rate; $K_G$, the Michaelis constant for α-methyl glucoside, is similarly defined. These constants are evaluated (Fig. 4).

An estimate of $K_{SG}$ can be obtained from the slopes of the Lineweaver–Burk plots $1/v$ *vs.* $1/[G]$, by the relationship that slope $= (K_G[S] + K_{SG})/[S]V_{SG}$. The dependence of this quantity upon $1/[S]$ (over the range 0.020–0.110 *M* sucrose) was computed by the method of least squares. We find $K_{SG}/V_{SG} = 4.8 (\pm 1.0) \times 10^{-6}$. Taking $V_{SG} = 203$, then $K_{SG} = 9.8 (\pm 2.1) \times 10^{-4}$. The hypothesis that $K_{SG} = 0$ is rejected by the *t*-test at the 99% confidence level.

Similarly, a second estimate, $K_{SG} = 8.8 \times 10^{-4}$, is obtained from the slopes of $1/v$ *vs.* $1/[S]$ for those α-methyl glucoside concentrations of 0.15 *M* and greater. The precision of this estimate ($\pm 5.7 \times 10^{-4}$) is poor; the hypothesis that $K_{SG} = 0$ is not rejected by the *t*-test. We have tentatively accepted the first estimate of $K_{SG}$, rounding the value to 0.0010.

The equation

$$v = \frac{[G][S]203}{[G][S] + 0.0474[G] + 0.221[S] + 0.0010} \quad (4)$$

describes the experimental data within the limits 0.010–0.11 *M* sucrose and 0.15–0.080 *M* α-methyl glucoside for reaction mixtures containing 10 units of dextransucrase per ml. In a series of twenty comparisons covering these ranges of substrate concentrations, the calculated rates deviated from the experimental values by not more than 6%.[26]

**Effect of α-Methyl Glucoside upon the Yield of High Molecular Weight Dextran.**—Reaction mixtures containing 0.30 *M* sucrose, 0.01 *M* acetate buffer (*p*H 5.1 ± 0.1), dextransucrase (40 D.S.U./ml.) and α-methyl glucoside (as indicated in Table III) were incubated for 20 hours at 15°. At the end of this period reducing sugar data indicated complete conversion. An alcohol-precipitation procedure[7] was used to determine the yield of high molecular weight dextran, which is precipitated between the limits 36–40% (v./v.) methanol. The yields between the limits 40–44% are also given; the low yields obtained indicate the sharpness of the precipitation obtained in the 36–40% range.

TABLE III

EFFECT OF α-METHYL GLUCOSIDE UPON YIELD OF HIGH MOLECULAR WEIGHT DEXTRAN

| [α-Methyl glucoside], *M* | Methanol precipitation range 36–40% (v./v.) | 40–44% (v./v.) |
|---|---|---|
| 0.0 | 64.5[a] | 1.5[a] |
| .0006 | 63.0 | 2.0 |
| .01 | 44.0 | 3.0 |
| .15 | 6.5 | 3.0 |
| .30 | 2.5 | 1.0 |
| .60 | 1.0 | 0.0 |

[a] Percentage of the dextran yield theoretically obtainable by complete conversion of the sucrose.

**Effect of Variation of Enzyme Concentration.**—The enzyme preparation was examined for the presence of a diffusible activator or inhibitor[27] by determining the initial velocity at enzyme concentrations ranging from 5 to 80 D.S.U. per ml. Sucrose concentrations of 0.1 and 1.2 *M* were used; sampling times were modified from those ordinarily used as appropriate to the concentration of enzyme. Plots of velocity *vs.* enzyme concentration are linear, as previously shown by Goodman, *et al.*[16a] The absence of a diffusible cofactor is indicated.

**Effect of the Addition of Heat-inactivated Enzyme.**—Reaction mixtures of series A (Table IV) contained: sucrose (0.48 *M*), dextransucrase (unheated, 10 D.S.U./ml.) and α-methyl glucoside as indicated. Reaction mixtures of series B and C contained, in addition, aliquots of a dextransucrase solution inactivated by heating for 10 minutes at 100°. The amounts used were equivalent to 10 and 30 D.S.U./ml., respectively. Preparation D contained only sucrose (0.48 *M*) and heated dextransucrase equivalent to 30 D.S.U./ml. The observed velocities indicate the absence of a heat-stable diffusible activator.

**Comparison of Enzyme Preparations of Differing Degree of Purity.**—Such enzyme preparations would be expected

(26) Extrapolation of the linear ranges of the double-reciprocal plots accomplishes this to the extent that the "apparent" constants for a substrate, A, at any given level of the other, B, are estimated from only those data in which the rate shows a first-order dependence upon [A]. However, the set of constants may reflect a dependence upon [B] of varying order due to side-reactions or inhibition.

(27) J. S. Friedenwald and G. D. Maengwyn-Davies, in "A Symposium on the Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds., Johns Hopkins Press, Baltimore, Md., 1954, pp. 173–176.

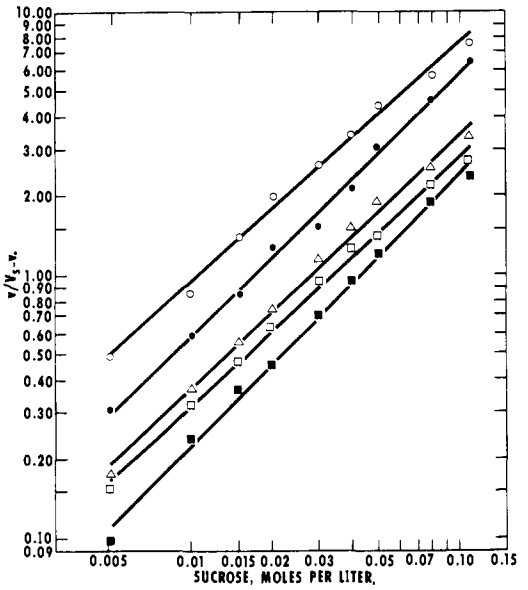


Fig. 2.—Test of reaction order with respect to sucrose. Symbols are the same as in Fig. 1. From top to bottom, the slopes are 0.90 ± 0.03, 0.99 ± 0.03, 0.96 ± 0.03, 0.94 ± 0.05 and 1.03 ± 0.02, calculated from the points lying in the range 0.005–0.11 *M*.

to differ in their content of a diffusible activator, if such exists, and in their response to the addition of α-methyl glucoside. Three enzyme preparations differing at least 30-fold in specific activity were examined: A. A cell-free culture-filtrate of *Leuconostoc mesenteroides* NRRL B-512F, diluted to 50 D.S.U./ml. B. The alcohol-precipitated concentrate described in "**Methods.**" (This preparation

TABLE IV

EFFECT OF HEATED DEXTRANSUCRASE UPON RATE OF REACTION

| [α-Methyl glucoside], *M* | A | B | C |
|---|---|---|---|
| 0 | 37.7[a] | 38.4 | 38.4 |
| 0.05 | 52.6 | 52.3 | 50.7 |
| .10 | 67.3 | 64.3 | 65.1 |
| .15 | 82.6 | 74.4 | 75.6 |
| .20 | 86.6 | 84.4 | 84.6 |
| .25 | 96.0 | 94.8 | 112.0 |
| Preparation D | 0.0 | | |

[a] μg. fructose per ml. per minute.

has an activity of 32 D.S.U./mg. of solids and a total carbohydrate content, by the anthrone procedure, of about 25 μg./D.S.U. Such preparations contain 0.1–0.5 μg. dextran/D.S.U.[28]) The concentrate was diluted to 50 D.S.U./ml. C. A preparation assaying 915 D.S.U./mg., derived by repeated fractionation with $(NH_4)_2SO_4$ from a concentrate similar to B. (This preparation contains approximately 0.005–0.01 μg. dextran/D.S.U.[28]) A solution was prepared in cold 0.02 *M* acetate buffer (*p*H 5.1), assayed and diluted to 50 D.S.U./ml.

Rate measurements at two temperatures were made for each enzyme preparation at levels of 0 and 0.9 *M* α-methyl glucoside. The sucrose concentration was 0.48 *M* throughout. To facilitate comparisons, the ratios of activities observed in the presence ($v_g$) and in the absence of ($v_o$) of α-methyl glucoside are calculated.

The enzyme preparations show (Table V) roughly equal responses to the addition of α-methyl glucoside. The unidentified acceptor which functions in the absence of α-methyl glucoside does not appear to be a contaminant of the enzyme preparations, unless it is one which remains associated with the enzyme throughout the isolation procedure.

(28) E. J. Hehre, personal communication. Estimated from the ability of the enzyme solution to precipitate with dextran-reactive antiserum, as compared with a solution of purified dextran.

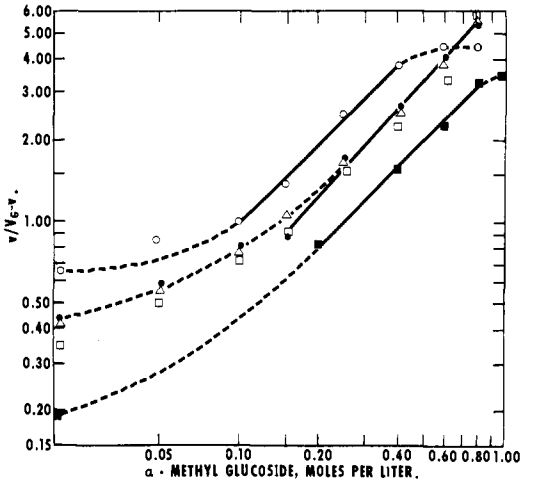


Fig. 3.—Test of reaction order with respect to α-methyl glucoside. Symbols: ○, 0.03 *M* sucrose; ●, 0.08 *M*; ■, 0.80 *M*. Solid lines fitted by method of least squares to these sets of data and have slopes of 0.99 ± 0.03, 1.08 ± 0.03 and 0.99 ± 0.03, respectively. Dashed lines drawn by inspection. Points for 0.11 *M* sucrose, △, and 0.20 *M* sucrose, □, are shown, but the fitted straight lines are omitted for clarity. These lines have slopes 0.99 ± 0.03 and 1.06 ± 0.09, respectively, and are calculated from the points lying in the range 0.15–0.80 *M* sucrose.

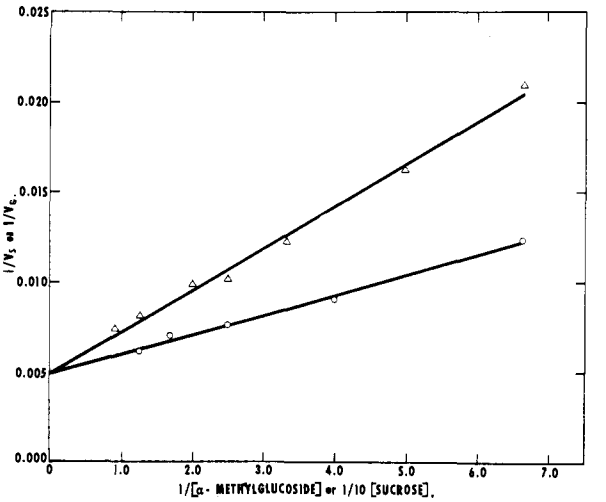


Fig. 4.—Lineweaver-Burk plot of: ○, $V_S^{-1}$ *vs.* $[G]^{-1}$; △, $V_G^{-1}$ *vs.* $(10[S])^{-1}$. The common intercept ($4.93 \times 10^{-3}$) is an estimate of $V_{SG}^{-1}$; the slopes, $1.09 \times 10^{-3}$ and $2.34 \times 10^{-4}$, provide, respectively, estimates of $K_G$ (0.221 *M*) and $K_S$ (0.0474 *M*) when multiplied by $V_{SG}$ ($2.03 \times 10^2$).

**Effect of Low Molecular Weight Dextran upon the Initial Rate of Reaction.**—Reaction mixtures contained 0.48 *M* sucrose and dextran (molecular weight by light scattering, $1.6–1.8 \times 10^4$) at the concentrations 0, 1.26, 2.51 and 5.02 g./100 ml. The reaction rates observed were 38.6, 39.2, 41.3 and 46.2 μg. fructose per ml. per minute, respectively. The change in velocity per unit change in dextran concentration was about 2.7 mg. fructose/ml.-minute/(mole/liter). This is a 9-fold greater effect than is obtained with α-methyl glucoside; it may be related to the superior acceptor power of low molecular weight dextran.[6] Because of the relatively high molecular weight of the latter (as compared with α-methyl glucoside), only low molar concentrations can be attained experimentally. Thus, small increases

TABLE V

| Enzyme preparation | Temp., °C. | $v_s$ | $v_0$ | $v_s/v_0$ |
|---|---|---|---|---|
| A | 15 | 136 | 33.3 | 4.1 |
| B | 15 | 151 | 40.6 | 3.7 |
| C | 15 | 128 | 33.9 | 3.8 |
| A | 30 | 265 | 86 | 3.1 |
| B | 30 | 281 | 84 | 3.3 |
| C | 30 | 231 | 76 | 3.0 |

in rate are observed. The rates found in the absence of added acceptor appear to be much too high to result from contamination of the enzyme preparations with dextran.

## Interpretation of Rate Data

The tests and statements made in this section refer only to the data obtained in the presence of a sufficient concentration of $\alpha$-methyl glucoside to make negligible the competing reaction by which high molecular weight dextran is formed.

**Comparison of Data with Postulated Reaction Mechanisms.**—Rate equations of the form of eq. 3 describe the kinetic behavior of three basically different reaction mechanisms: (a) Formation of a ternary complex by stepwise addition of the substrates in *random* sequence (equilibrium cases of ref. 29, eq. I,1 and ref. 23, mech. II). A simplifying assumption[30] (that adsorption of either substrate does not influence the affinity for the other) does not hold for the data, since the "apparent" Michaelis constant for each substrate varies with concentration of the other.

(b) Formation of a ternary complex by stepwise addition of the substrates in *fixed* sequence (ref. 29, eq. I,2 and I,3: ref. 23, mech. I). Of these, the latter expression is the simplest.

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA}$$

$$\mathrm{EA} + \mathrm{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB} \xrightarrow{k_5} \mathrm{E} + \text{products} \qquad (5)$$

Since the "apparent" Michaelis constants and maximum velocity values are hyperbolic functions of the concentration of the corresponding cosubstrate, the steady state assumption (that $k_5$ is not rate limiting) is required. No identification of the order in which the substrates react with the enzyme can be made.

(c) The Theorell–Chance mechanism (ref. 29, eq. I,4). The third step cannot be much more

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{EA}$$

$$\mathrm{EA} + \mathrm{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EC} + \mathrm{D} \qquad (6)$$

$$\mathrm{EC} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{E} + \mathrm{C}$$

rapid than the second; if $k_5 >> k_3$, the mechanism reduces to a form (ref. 23, mech. III) which would require the common intercept of Fig. 4 to occur at the origin. No identification of the sequence of reaction of the substrates can be made.

Some of these permissible mechanisms may be distinguished by methods[29] based upon the measurement of the equilibrium concentrations of reactants and products; the essential irreversibility of dextran synthesis precludes their use. Other methods have been described.[31]

The data specifically exclude two formulations (ref. 29, eq. I,6 and I,7) of the hypothesis that an intermediate of the form "glycosyl-enzyme" occurs. The usual expression[32]

$$\text{glycosyl–OR} + \text{enzyme–H} \rightleftharpoons \text{glycosyl–enzyme} + \text{H–OR}$$

$$\text{glycosyl–enzyme} + \text{H–OR}' \rightleftharpoons \text{glycosyl–OR}' + \text{enzyme–H} \qquad (7)$$

is rejected on the basis of the same considerations as in the special case ($k_5 >> k_3$) of the Theorell–Chance mechanism. The second formulation invokes the formation of an intermediate ternary complex in each of the step reactions (above); the rate equation, of the form of eq. 3, requires that $K_{SG} = 0$.

An alternative to the glycosyl–enzyme hypothesis, originally suggested[7] to explain the origin of high molecular weight dextran, is rejected for the $\alpha$-methyl glucoside–sucrose system. It may be represented as

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{FA}$$

$$\mathrm{EA} + \mathrm{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAB} \xrightarrow{k_5} \mathrm{EA} + \mathrm{C} \qquad (8)$$

where B and C are sucrose and fructose, respectively. A represents an acceptor substrate molecule or its homolog which has participated one or more times in the transfer reaction. If $k_2 << k_1$, a few acceptor molecules participate repeatedly while the remainder participate only occasionally or not at all. The rate expression[33] for this mechanism is such that the "apparent" maximum velocity with respect to A has a hyperbolic dependence upon [B]. The "apparent" maximum for substrate B, however, is constant and equal to the true maximum velocity at any [A].

## Discussion

**Mechanism.**—Although the data do not distinguish between several permissible mechanisms, it seems clear that the commonly used representation of transglycosylation (eq. 7) cannot be applied to the $\alpha$-methyl glucoside–sucrose system. Rather, all of the permissible mechanisms thus far examined require the reaction of the enzyme with *both* substrates in order to form a product. Those mechanisms which include a ternary complex are consistent with the hypothesis, first made in studies of hydrolases,[34,35] that there is simultaneous

(29) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953).

(30) K. J. Laidler and I. M. Socquet, *J. Phys. Colloid Chem.*, **54**, 530 (1950).

(31) R. A. Alberty, THIS JOURNAL, **80**, 1777 (1958).

(32) M. Doudoroff, H. A. Barker and W. Z. Hassid, *J. Biol. Chem.*, **168**, 725 (1947); E. H. Fischer, L. Kohtes and J. Fellig, *Helv. Chim. Acta*, **34**, 1132 (1951); K. Wallenfels, E. Bernt and G. Limberg, *Ann.*, **584**, 63 (1953).

(33) B. R. Rabin and E. M. Crook, *Biochim. et Biophys. Acta*, **19** 550 (1956).

(34) R. K. Morton, *Discussions Faraday Soc.*, **20**, 149 (1955).

(35) M. A. Jermyn, *Science*, **125**, 12 (1957).

binding of donor and acceptor substrates at adjacent active sites.

Both substrates may be expected to show some affinity for the loci appropriate to the opposite substrate. The binding of $\alpha$-methyl glucoside at the "sucrose" site may, for example, explain the inhibition by $\alpha$-methyl glucoside which is observed at low sucrose concentrations. Similarly, the simultaneous binding of two molecules of sucrose would explain the inhibition observed at high sucrose concentrations, if the subsequent breakdown of the intermediate complex is relatively slow. The decrease in rate at sucrose concentrations greater than the optimum is tentatively interpreted as evidence of a true substrate inhibition, rather than as a result of changes induced by the lowered concentration of water.[3] This is inferred by the fact that $\alpha$-methyl glucoside relieves the inhibition, which may imply a competition between the two substrates for the hypothetical acceptor site.

**Anomalous Rate Data.**—There appears to be a realtionship between the anomalously high velocities observed at the lower concentrations of $\alpha$-methyl glucoside (Fig. 3) and the formation of high molecular weight dextran (Table III). The latter material is believed to be formed by an enzymatic rearrangement of the linear chains having a molecular weight of 3 to 4 million.[17] Presumably the rate values in question represent, in large part, the formation of this linear intermediate. (In the earlier portion of this paper the anomalous rates have been attributed, for convenience of reference, to an "unidentified acceptor.")

The apparent inconsistency implied by the high rate of transfer to a few acceptor molecules becomes plausible when interpreted by the hypothesis[36] that the rate of reaction depends not only upon the concentrations of donor and acceptor substrates, but also upon the degree of polymerization of the acceptor. From this point of view, the linear range of Fig. 3 represents the condition in which acceptor molecules are so numerous that few have a selective advantage due to molecular weight; the rate is governed mainly by the effect of concentration. At the lower concentrations of $\alpha$-methyl glucoside, some of the acceptors are sufficiently large to exert an effect on the rate which is out of proportion to their number.

The hypothesis is consistent with the observation that low molecular weight dextran is a more effective chain initiator than mono- and disaccharides,[6] and that high molecular weight products are formed early in dextran synthesis.[7]

(36) J. Edelman, *Advances in Enzymol.*, **17**, 189, 222 (1956).

**Chain Initiation in the Absence of Added Cosubstrate.**—The fact that dextransucrase is active in the absence of an added cosubstrate suggests that the materials used may be contaminated with an initiator. Kinetic evidence (linearity of rate as a function of enzyme concentration; Tables IV and V) and the low total carbohydrate and dextran content of the enzyme preparations indicate that such a material is not to be found in the enzyme. Since the reaction rates have first-order dependence upon sucrose concentration in the absence of $\alpha$-methyl glucoside, it does not seem likely that the sucrose itself contains a contaminating initiator. One would expect a second-order dependence upon sucrose concentration if the concentration of a rate-limiting acceptor were being varied simultaneously.

Contrary to our earlier view,[2] the suggestion[36] that sucrose may serve both as acceptor and as donor seems likely. This conclusion is based on the following presumptive evidence:

A low molecular weight dextran fraction has been isolated from a reaction mixture which had an initial sucrose content greater than 0.6 *M*, by repeated precipitation between the limits 50-70% (v./v.) ethanol. Treatment of a solution of this dextran with yeast invertase (a transfructosidase) caused an increase in reducing sugar content without change of viscosity; paper chromatography showed the presence of a single sugar, which had the mobility and color reactions of fructose. Treatment of the dextran solution with honey invertase (a transglucosidase) had no effect, demonstrating that the dextran was free of contamination by sucrose.[37]

If the conclusion is correct that this enzymatically synthesized dextran is initiated by the transfer of glucosyl to a second molecule of sucrose, it seems probable that similar molecules of somewhat higher molecular weight are the precursors which are "rearranged"[17] in the formation of high molecular weight dextran.

**Acknowledgment.**—We thank Mrs. Mary Dudas Klein and Miss Sinah E. Kelley for analyses and calculations; Dr. E. J. Hehre of Cornell University Medical College for serological determinations of the dextran content of dextransucrase preparations; Dr. H. J. Koepsell and Mr. E. S. Sharpe for the purified dextransucrase; and Drs. H. L. Tookey, M. E. Slodki and R. G. Benedict for help in the preparation of the manuscript.

PEORIA, ILL.

(37) J. Corman, C. S. Stringer and H. M. Tsuchiya, unpublished data.