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INHIBITION OF DEXTRAN SYNTHESIS BY GLUCOAMYLASE AND ENDODEXTRANASE*

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ABSTRACT

In a model experiment, glucoamylase was shown to inhibit α-D-glucan synthesis as catalyzed by potato phosphorylase. Both glucoamylase and endodextranase inhibited dextran synthesis with dextransucrases of *Leuconostoc mesenteroides*. The inhibition could be ascribed to competition between glucoamylase and dextransucrase for the glucosyl groups at the non-reducing end of dextran. The inhibition caused by endodextranase may result from rapid and random hydrolysis of acceptor dextrans. Moreover, significantly low units of glucoamylase, as compared with endodextranase, effectively inhibited dextran synthesis. These results thus present evidence that biosynthesis of dextran occurs by the addition of glucosyl groups at the non-reducing end of the growing dextran. The measurement of initial velocity suggested that the ping-pong Bi-Bi mechanism proposed for the levansucrase of *Bacillus subtilis* is also applicable to dextransucrase.

INTRODUCTION

The biosynthesis of dextran is believed to be catalyzed by dextransucrase (EC 2.4.1.5), which transfers α-D-glucopyranosyl residues from sucrose to the growing dextran chain. Although there are many reports on dextransucrases isolated from strains of *Leuconostoc mesenteroides* and *Streptococcus*[1], several problems relating to the reaction mechanism of dextran biosynthesis remain to be clarified. For example, two possible mechanisms have been reported for the direction of dextran synthesis, as summarized by Sidebotham[1]. Hehre[2] and Brock Neely[3] have claimed that biosynthesis of dextran occurs by the addition of glucosyl groups to the non-reducing ends of the growing dextran. In contrast, Ebert and Schenk[4] have proposed that chain elongation of dextran occurs at the reducing ends by an insertion mechanism. In a more-recent study, Walker[5] has reported that the non-reducing ends of isomaltose-oligosaccharides can be labelled with D-[^{14}C]glucose and that dextran might, in consequence, be synthesized from the non-reducing ends of the chains. However,

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

Robyt *et al.*[6] have shown from pulse-labelling of dextran that the incorporated label is located in the vicinity of the reducing ends, thus supporting the hypothesis of growth from the reducing end.

In the present paper, we substantiate the former hypothesis of the direction of chain elongation by using glucoamylase as an exo-dextranase. In addition, initial-velocity measurements have been performed to elucidate the mechanism of dextran synthesis.

MATERIALS AND METHODS

Substrates. — Native dextran from *Leuconostoc mesenteroides* strain NRRL B-512(F) was prepared by the method of Jeanes[7]. Preparation of dextran from *L. mesenteroides* NRRL B-1299 has been described previously[8].

Materials. — All of the reagents used were of analytical grade. The extracellular dextransucrase of *L. mesenteroides* NRRL B-512(F) was purified by fractionation with ammonium sulfate and by adsorption on hydroxylapatite. The washed cells of *L. mesenteroides* NRRL B-1299 were used as a source of B-1299 dextransucrase[9]. These two enzyme preparations contained 52 and 17% of carbohydrate (calculated as glucose), respectively. Crystalline glucoamylase of *Rhizopus niveus* was purchased from Seikagaku Kogyo Co., Tokyo, Japan. Endo-dextranase of *Chaetomium gracile* was kindly provided by Sankyo Co., Japan[10]. Glucoamylase and endo-dextranase were free from invertase activity. Potato phosphorylase was prepared by the method of Kamogawa *et al.*[11].

Analytical methods. — Total carbohydrate was determined by the phenol–sulfuric acid method[12]. Reducing sugars were determined by the Nelson–Somogyi method[13,14]. The amount of inorganic phosphate (Pi) liberated by phosphorylase was determined by the modified Allen method[15]. D-Glucose was determined by the Glucostat reagent as described previously[16]. Protein was determined by the method of Lowry *et al.*[17].

Enzyme assays. — An incubation temperature of 30° was used throughout this study. Inhibition studies of phosphorylase action with glucoamylase were performed as follows: soluble starch (1.18 mg/ml), D-glucosyl phosphate (10mM), sodium citrate buffer (0.1M, pH 6.0), glucoamylase (0.0014–0.0224 units; determined with soluble starch as a substrate; specific activity, 23.5 units/mg), and potato phosphorylase (0.32 units, 72.5 μg) were incubated for 20 min. The enzymes were inactivated by the addition of M sodium hydroxide. D-Glucose and Pi were determined by the Nelson–Somogyi[13,14] and modified Allen methods[15], respectively.

In standard assays, inhibition studies of dextransucrase action with glucoamylase or endo-dextranase were performed as follows: sucrose (73.1mM), acetate buffer (40mM, pH 5.2), dextransucrase (B-512(F); 2.4 units/55 μg or B-1299; 2.1 units/100 μg cells) and various amounts of glucoamylase (4×10^{-6} to 2.56×10^{-3} dextranase units; determined with clinical dextran as a substrate, specific activity; 0.18 units/mg) or endo-dextranase (0.15–2.4 units, specific activity; 6.0 units/mg) were incubated

for 24 h. The enzymes were inactivated by heating in a boiling-water bath for 10 min. Dextransucrase activity was expressed as the amount of fructose liberated, which was calculated from the difference of the analytical values of reducing sugar determined by the Nelson–Somogyi[13,14] and Glucostat[16] methods.

For measurement of the initial velocity, dextransucrase activity was assayed by incubating the B-512(F) enzyme for 15 min in a mixture containing sucrose (4.57–73.1mM), acetate buffer (40mM, pH 5.2), and B-512(F) native dextran (8.9–142.4 mg/ml). Effects of the addition of glucoamylase or endo-dextranase were also examined.

Analysis of the product dextran. — The amount of dextran produced by the inhibition studies was determined by the phenol–sulfuric acid method[12] after the inactivated digests had been dialyzed exhaustively against distilled water. The molecular-weight distribution of the product dextran was examined by gel filtration on Sepharose 6B, as described previously[18].

RESULTS AND DISCUSSION

Working hypothesis. — Although two distinct mechanisms for the direction of skeletal chain-propagation of dextran have been presented[2–6], a definite conclusion must be drawn from more-extensive studies. As shown in Fig. 1, we propose a working hypothesis to determine the direction of dextran chain-synthesis. When exo-dextranase is added to the reaction mixture for dextran synthesis, this enzyme will exert some inhibitory effect on the chain-propagation if the biosynthesis of dextran occurs by the addition of glucosyl groups to the non-reducing ends of the polymer molecule (Fig. 1a). Therefore, the competition between the two enzymes, exo-dextranase and dextransucrase, for the glucosyl groups of the non-reducing end, should decrease the reaction rate of the glucosyl-transferring step, and consequently decrease the overall reaction rate. In contrast, exo-dextranase will give no significant effect if the skeletal chain grows by insertion at the reducing end, because no competition will occur between the two enzymes (Fig. 1b). Although endo-dextranase does not compete with dextransucrase at both chain-ends of dextran, the hydrolyzing activity on dextran by endo-dextranase will exert some effect on dextran synthesis. Thus, we examined the effect of glucoamylase in comparison with endo-dextranase on dextran synthesis.

As a preliminary study to confirm the working hypothesis, a model experiment was constructed by using a potato α-D-glucan phosphorylase system. It has been well established that phosphorylase transfers the glucosyl group from D-glucosyl phosphate to the non-reducing end of the acceptor (glycogen or starch[19]), and glucoamylase successively hydrolyzes the polysaccharides from the non-reducing end[20,21]. Table I shows that, at low concentrations, glucoamylase inhibits glucan synthesis in the potato phosphorylase system. In the control experiments, theoretical amounts of released Pi, which decreased because of the fall in the acceptor concentration caused by glucoamylase action, were calculated for corrections. Although the amount of soluble starch lost through glucoamylase action could be controlled, that produced

by phosphorylase was difficult to control accurately in the present experiments. The amount of α-D-glucan (320 μg) synthesized by the incubation without glucoamylase was smaller than that added (472 μg) to the incubation mixture as an acceptor. However, the apparent inhibition would be much stronger than the values shown in

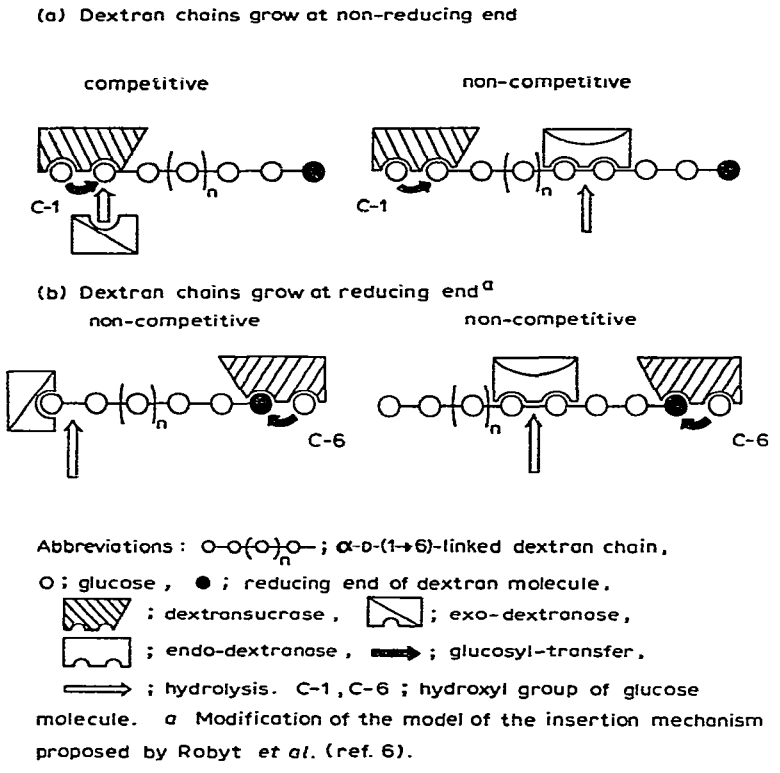

Fig. 1. Inhibition model for dextran synthesis by two types of glucanases.

Table I were the amount of α-D-glucan synthesized by phosphorylase reaction to be included in the total amount of acceptor that contributes to raise the calculated values of the control (%): B. Moreover, a diminution in quality of the soluble starch remaining to serve as an acceptor after the glucoamylase action was examined. When the soluble starch preparation, previously digested (degree of hydrolysis, d.h., 17.4%) by glucoamylase under the same conditions as shown in Table I, was used as the acceptor, almost the same extent of inhibition was observed, and the initial velocity of hydrolysis with glucoamylase was not changed (data not shown). From these results, significant inhibition of the (1→4)-α-D-glucan synthesis by the exo-hydrolyzing enzyme, glucoamylase, was demonstrated.

TABLE I

INHIBITION OF α-D-GLUCAN SYNTHESIS WITH PHOSPHORYLASE BY THE ADDITION OF GLUCOAMYLASE[a]

Glucoamylase (units/ml) added	*Hydrolysis (%) of soluble starch*	*α-D-Glucan synthesized (%)*		*Apparent inhibition (%): 100(1 − A/B)*
		In the presence of glucoamylase[b]: A	*Control[c]: B*	
0.0014	4.4	109.7	96.3	—
0.0028	9.6	105.3	93.1	—
0.0056	16.8	24.8	87.4	71.6
0.0112	20.0	13.8	84.9	83.7
0.0224	25.4	11.9	80.9	85.3

[a]The mixture (0.4 ml) contained soluble starch (472 μg), D-glucosyl phosphate (10mM), sodium citrate buffer (0.1M, pH 6.0), potato phosphorylase (0.32 units, 72.5 μg), and glucoamylase (0.0014–0.0224 units, 0.06–0.95 μg). After incubation at 30° for 20 min, the enzymes were inactivated by the addition of M sodium hydroxide (0.2 ml). Control experiments by using heat-inactivated phosphorylase or glucoamylase were also performed. [b]Amounts of Pi liberated by incubation without glucoamylase are taken as 100%. [c]Values calculated for convenience from the concentration of the acceptor starch, decreased by the action of glucoamylase.

TABLE II

EFFECT OF ADDED DEXTRAN ON THE INHIBITORY ACTION OF GLUCOAMYLASE[a]

Dextran[b] (mg)	*Relative activity of dextran synthesis (%)*	
	Exp. 1 (0 min)[c]	*Exp. 2 (90 min)[d]*
0[e]	100	100
0	38.1	42.3
1.5	42.3	43.9
3.0	47.0	45.6
6.0	52.6	49.2
9.0	65.2	50.0
12.0	74.1	56.4

[a]The mixture (0.8 ml) contained the B-512(F) dextransucrase (0.6 units, 13.8 μg), glucoamylase (6.4×10^{-4} dextranase units, 3.5 μg), sucrose (73.1 mM), acetate buffer (pH 5.2, 40mM), and various amounts of dextran. The rate-enhancing effect of the dextran added to the dextransucrase–sucrose mixture was controlled for the dextransucrase–glucoamylase–sucrose system. [b]Native dextran of B-512(F) was used in this experiment. [c]Exp. 1: Dextran was added to the mixture at zero time. [d]Exp. 2: Dextran was added after 90 min of pre-incubation, with further incubation for 90 min. [e]Incubation was conducted by using heat-inactivated glucoamylase solution (3.5 μg/0.1 ml).

Effects of glucoamylase on dextran synthesis. — As shown in Fig. 2, dextransucrase activities of *L. mesenteroides* NRRL B-1299 and B-512(F) were inhibited by the addition of glucoamylase. Dextransucrase of strain B-512(F) was almost com-

pletely inhibited by 2.56×10^{-3} dextranase units of glucoamylase, whereas the B-1299 enzyme retained 62% of its activity at this concentration. Both enzymes were also inhibited by the addition of glucoamylase during the course of incubation. As shown in Fig. 3, the inhibitory effect of glucoamylase on dextran synthesis was lessened proportionally to the pre-incubation periods, and the degrees of inhibition caused

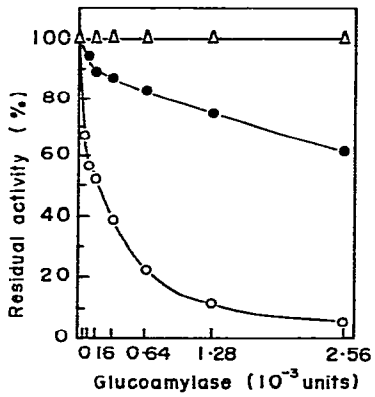

Fig. 2. Effect of glucoamylase on dextran synthesis as catalyzed by dextransucrases from the strains B-512(F) (○) and B-1299 (●). (△). Control (without glucoamylase).

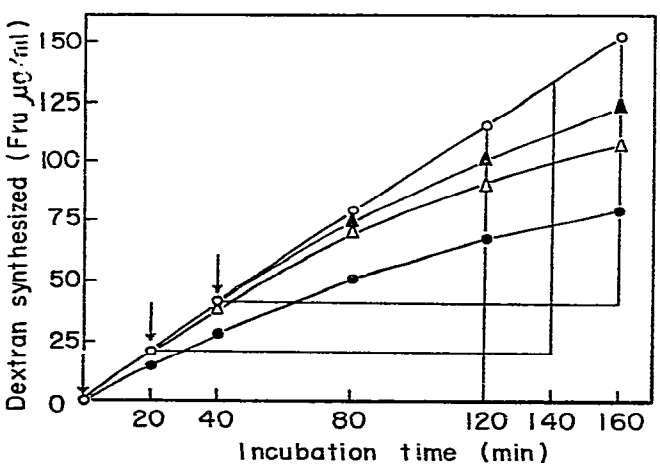

Fig. 3. Inhibition of dextran synthesis by the addition of glucoamylase during the course of reaction. Glucoamylase (6.4×10^{-4} dextranase units/10 μl) was added to the mixture (1.0 ml), containing B-512(F) dextransucrase (2.4 units), sucrose (73.1mM), and acetate buffer (40mM, pH 5.2), at times indicated by arrows; 0 (●), 20 (△), and 40 min (▲). (○), without glucoamylase. A control experiment was also performed with heat-inactivated glucoamylase.

after 0, 20, and 40 min of pre-incubation were 43.5, 32.7, and 24.7%, respectively, with 120 min of incubation. Thus, no direct action of the glucoamylase preparation on dextransucrase could be attributed to the decrease of dextran synthesis. Table II shows that the inhibition of dextransucrase action by glucoamylase is relieved by the addition of dextran to the system. The relative activity of dextran synthesis was restored to the level of 74.1% by addition of native dextran to the mixture prior to incubation. When the dextran was added to the system after 90 min of pre-incubation, the activity of dextransucrase was also restored, although the levels of restoration were lower than that in the former case. Moreover, the degree of inhibition was inversely proportional to the activity of dextransucrase when the concentration of glucoamylase was fixed and that of dextransucrase was varied.

In our previous paper[16], we definitely confirmed the exo-type of action of glucoamylase on various dextrans. After 24 h of incubation, the degrees of hydrolysis (d.h.) of clinical dextran and B-1299 dextran were 16.3 and 0.05%, respectively (Table III). The highly branched structure of B-1299 dextran[22,23] seems to restrict the glucoamylase action. Therefore, inhibition of dextran synthesis by glucoamylase was correlated to the structure of the product dextran, which determined the susceptibility to the hydrolyzing enzymes.

The molecular-weight distribution in the product dextran was examined by gel filtration on Sepharose 6B. Dextrans synthesized in the presence and absence of glucoamylase were both excluded at the void volume. However, the yield of the former dextran was much lower than that of the latter.

It has been suggested that the reducing end of the dextran molecule is likely to be "capped" by a β-D-fructofuranosyl group[24]. However, the glucoamylase could not attack this reducing end preferentially, as the enzyme preparation has been shown to

TABLE III

CHARACTERISTICS OF GLUCOAMYLASE AND ENDO-DEXTRANASE USED IN THE INHIBITION EXPERIMENTS

	Glucoamylase	*Endo-dextranase*
Enzyme activity[a] (Glc μg/min/ml)	$0.45–2.56 \times 10^{-3}$ units	432–2.4 units
Degree of hydrolysis with		
clinical dextran	16.3%	67.7%
B-1299 dextran	0.05%	13.5%
Inhibition by 50% of B-512(F)		
dextran synthesis	1.6×10^{-4} units[b]	1.2 units
Inhibition by 25% of B-1299		
dextran synthesis	1.28×10^{-3} units[b]	<2.4 units

[a]The incubation mixture contained 1.0% of clinical dextran, 40mM acetate buffer (pH 5.2) and each hydrolase. [b]Values are expressed as dextranase units.

be of the exo-type[20,21] and, in addition, it contains no sucrase activity. If elongation of the dextran chain occurred at the reducing end, no competition between dextransucrase and glucoamylase for the D-glucosyl residue at the nonreducing end would be observed. Therefore, the inhibitory effect of glucoamylase on dextran synthesis with dextransucrase is clearly demonstrated from these results.

Effects of endo-dextranase on dextran synthesis. — The dextranase of *Chaetomium gracile* has been shown to hydrolyze dextran T-2000 (Pharmacia Co.) by the endo-type of action[10]. Therefore, the effects of this dextranase on dextran synthesis were examined to compare the inhibition pattern with that of glucoamylase.

As shown in Fig. 4, the endo-dextranase also inhibited dextran synthesis with both B-512(F) and B-1299 dextransucrases. Dextran synthesis with B-512(F) enzyme was inhibited more strongly than synthesis with the B-1299 enzyme. Furthermore, dextran of high molecular weight could not be synthesized in the presence of endo-dextranase because of the random and rapid hydrolysis of the synthesized product by endo-dextranase, and only a trace of dextran was detected as non-dialyzable polysaccharide.

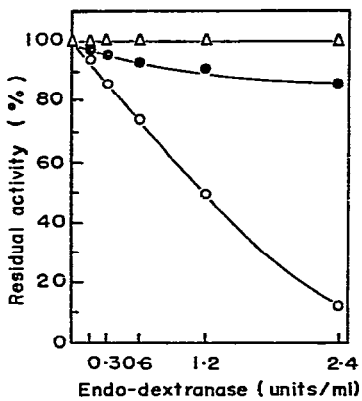

Fig. 4. Effect of endo-dextranase on dextran synthesis catalyzed by dextransucrases from strains B-512(F) (○) and B-1299 (●). (△), Control (without endo-dextranase).

Inhibition of dextran synthesis by hydrolases. — From the foregoing results, two types of dextran-hydrolyzing enzymes, glucoamylase and endo-dextranase, have proved to inhibit dextran synthesis. We have already confirmed that the attack of glucoamylase on dextran is of the exo-type and releases only glucose, and the extents of hydrolysis of highly branched dextrans produced by such strains as B-1299 and B-1307 are very low as compared with those of dextran T-40, T-110, and clinical dextran[16]. Table III shows the characteristics of the two hydrolases. Both enzymes degrade clinical dextran better than B-1299 dextran [which contains high proportions of non-(1→6)-α-D-glucosidic linkages[22,23]]. The initial velocity of hydrolysis of

clinical dextran with endo-dextranase was about one thousand times greater than that with glucoamylase. However, 50% inhibition of dextran synthesis by B-512(F) dextransucrase was attained with 1.6×10^{-4} dextranase units of glucoamylase activity, which releases 0.028 μg of glucose/min (Fig. 5). On the other hand, 50% inhibition by endo-dextranase was attained with 1.2 units of dextranase, releasing 216 μg of glucose/min. The degree of hydrolysis of clinical dextran increased linearly to 16.3% upon incubation with glucoamylase for 24 h, whereas the action of endo-dextranase reached a maximum within 6–8 h incubation and gave a d.h. value of 67.7%. Furthermore, inhibition of the B-1299 dextransucrase by these hydrolases was much smaller than that of the B-512(F) dextransucrase. In each case, the inhibition caused by glucoamylase was far stronger than that caused by endo-dextranase (Fig. 5).

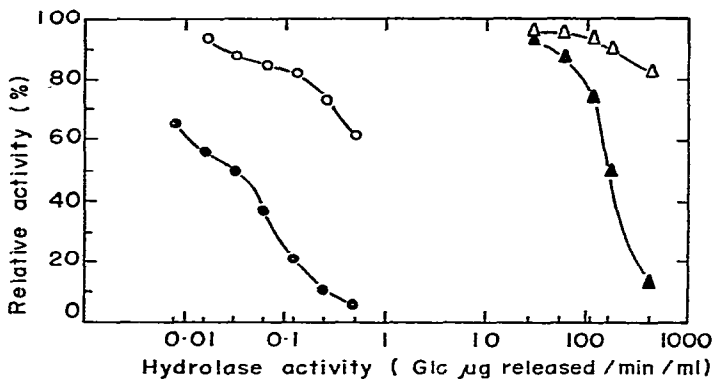

Fig. 5. Degree of inhibition of dextran synthesis correlating with the hydrolyzing activity of glucoamylase and endo-dextranase. Amount of glucose (μg/min/ml) released from the digest is indicated in terms of the potency of each hydrolase added to the mixture as an inhibitor. (○), B-1299 dextransucrase and glucoamylase; (●), B-512(F) dextransucrase and glucoamylase; (△), B-1299 dextransucrase and endo-dextranase; and (▲), B-512(F) dextransucrase and endo-dextranase.

Therefore, such an exo-dextranase as glucoamylase effectively inhibits dextran synthesis catalyzed by dextransucrases of both *L. mesenteroides* NRRL B-512(F) and B-1299 strains. In these instances, the dextransucrases should be in competition with glucoamylase for the glucosyl group at the non-reducing end. As the hydrolytic action of glucoamylase depends upon the degree of branching of the dextrans (Table III) and the degree of inhibition of dextran synthesis corresponds to the susceptibility of each dextran to the glucoamylase (Fig. 2), it is proposed that elongation of the growing dextran chain occurs at the non-reducing ends of the skeletal chains of dextran molecules, as shown in Fig. 1. In contrast, the inhibition by endo-dextranase seemed not to be caused by the competitive effect, but probably by the rapid and extensive hydrolysis of the growing dextran molecules.

Initial-velocity measurements of dextran synthesis. — A series of excellent studies by Chambert *et al.*[25] has shown that levansucrase from *Bacillus subtilis* synthesizes levan from sucrose by the ping-pong Bi-Bi mechanism. Kinetic equations for this enzyme were derived after assuming that the fructose-polymer, levan, has a dual role in levan synthesis: acting both as a substrate and product molecule. Therefore, unlike the common ping-pong Bi-Bi mechanism[26], plots of $1/v = f(1/[\text{sucrose}])$ and $1/v = f(1/[\text{levan}])$ gave linear converging lines, respectively. Moreover, the rapid-equilibrium, random mechanism for the reaction of phosphorylases from several sources[27–29] has been demonstrated by considering the dual role of the polysaccharide.

Therefore, the initial velocity of action of the B-512(F) dextransucrase was examined. In the earlier studies, it was reported that the addition of dextran increased the rate of glucosyl transfer as catalyzed by the B-512(F) dextransucrase[30,31]. This activation was also observed for the B-1299 (ref. 32), *Streptococcus sanguis*[33] and *S. mutans* dextransucrases[34]. As shown in Fig. 6, the double-reciprocal plot

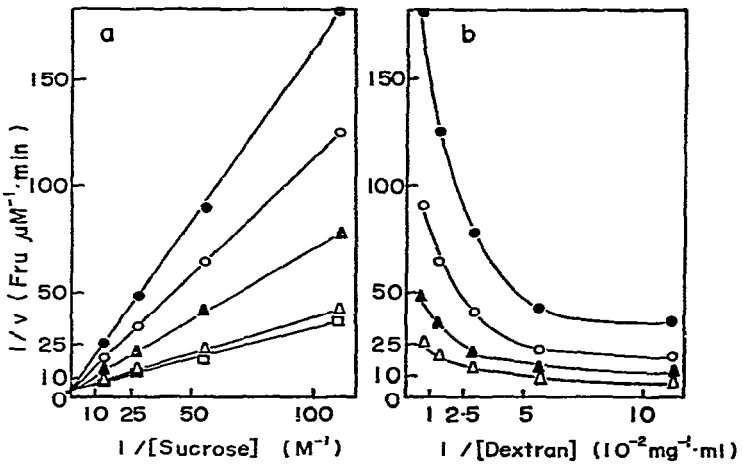

Fig. 6. Double-reciprocal plots of initial rate of dextran synthesis by the B-512(F) dextransucrase. (a) $1/v = f(1/[\text{sucrose}])$, dextran concentrations (mg/ml) were kept constant at 8.9 (●), 17.8 (○), 35.6 (▲), 71.2 (△), and 142.4 (□). (b) $1/v = f(1/[\text{dextran}])$, sucrose concentrations (mM) were kept constant at 9.14 (●), 18.28 (○), 36.55 (▲), and 73.1 (△).

of $1/v = f(1/[\text{sucrose}])$ and $1/v = f(1/[\text{dextran}])$ gave linear-converging and non-linear plots, respectively. Among the ten possible mechanisms of action for dextransucrase, only five types gave a combination of linear-converging and non-linear plots[25]. Furthermore, as in the case of the common two-substrate system, the ping-pong mechanism may readily be distinguished from the other types by secondary plots of the slopes of $1/v = f(1/[\text{sucrose}])$ against [dextran] and its intercept with the ordinate axis against 1/[dextran], both of which give linear plots (data not shown).

As the two secondary plots for the B-512(F) dextransucrase system gave linear plots, a ping-pong mechanism seems to be applicable not only to the levansucrase, but also to the dextransucrase system. However, a more-definite conclusion must be drawn from other experiments, such as isotopic exchange-reactions and inhibition studies.

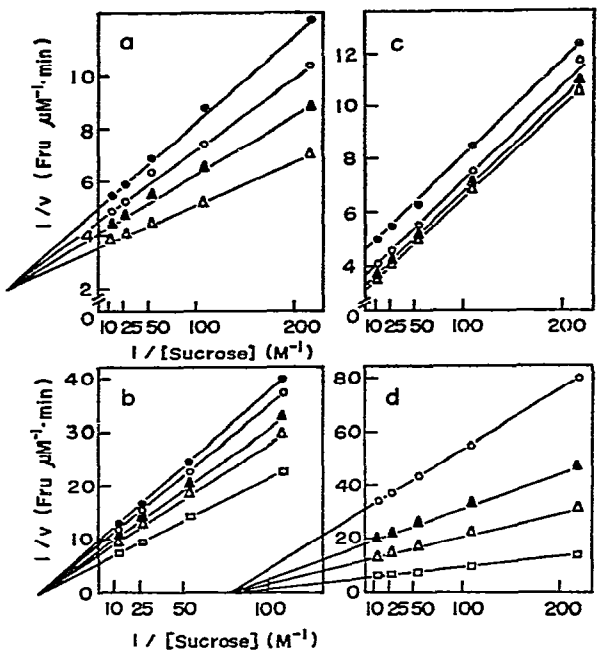

Fig. 7. Double-reciprocal plots of dextran synthesis by the B-512(F) dextransucrase in the presence of glucoamylase and endo-dextranase instead of the acceptor dextran. Plots of $1/v = f(1/[\text{sucrose}])$ were the results from the following incubations: (a), glucoamylase for 15 min; (b), endo-dextranase for 15 min; (c), glucoamylase for 24 h; and (d), endo-dextranase for 24 h. Glucoamylase concentrations ($\times 10^{-3}$ dextranase units) were kept constant at 1.28 (●), 0.64 (○), 0.32 (▲), and 0.16 (△) in (a) and (c). Endo-dextranase concentrations (units) used were 2.4 (●), 1.2 (○), 0.6 (▲), 0.3 (△), and 0.15 (□) in (b) and (d).

Effects of hydrolases on the initial velocity of dextran synthesis. — The double-reciprocal plots of initial velocity of the B-512(F) dextransucrase, measured in the presence of glucoamylase or endo-dextranase added in place of the acceptor dextran, are shown in Figs. 7a and b. Although both plots of $1/v = f(1/[\text{sucrose}])$ gave linear, converging lines, those of $1/v = f(1/[\text{hydrolase}])$ gave non-linear plots (data not shown). After 24 h of incubation under the same assay conditions, the characteristic pattern of converging lines with the endo-dextranase system was unchanged (Fig. 7d), but the plot with the glucoamylase system gave parallel lines (Fig. 7c) as in the case of the common mechanism of the ping-pong Bi-Bi type. However, the plot without hydrolases gave the same patterns, as shown in Fig. 6, even after 24 h of incubation. Interestingly, the patterns of the double-reciprocal plots were changed according to

the incubation periods, and this transformation was observed only when glucoamylase was added to the dextran-synthesizing system. Differences in the patterns of double-reciprocal plot with regard to the foregoing two hydrolase systems may give a clue to the nature of the mechanism whereby hydrolases inhibit dextran synthesis, although further studies will be necessary in order to elecudate the exact mechanism.

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