

## Properties of D-Xylose Isomerase from *Streptomyces albus*

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A partially purified D-xylose isomerase has been isolated from cells of *Streptomyces albus* NRRL 5778 and some of its properties have been determined. D-Glucose, D-xylose, D-ribose, L-arabinose, and L-rhamnose served as substrates for the enzyme with respective  $K_m$  values of 86, 93, 350, 153, and 312 mM and  $V_{max}$  values measuring 1.23, 2.9, 2.63, 0.153, and 0.048  $\mu\text{mol}/\text{min}$  per mg of protein. The hexose D-allose was also isomerized. The enzyme was strongly activated by 1.0 mM  $\text{Mg}^{2+}$  but only partially activated by 1.0 mM  $\text{Co}^{2+}$ . The respective  $K_m$  values for  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$  were 0.3 and 0.003 mM.  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$  appear to have separate binding sites on the isomerase. These cations also protect the enzyme from thermal denaturation and from D-sorbitol inhibition. The optimum temperature for ketose formation was 70 to 80 C at pH values ranging from 7 to 9. D-Sorbitol acts as a competitive inhibitor with a  $K_i$  of 5.5 mM against D-glucose, D-xylose, and D-ribose. Induction experiments,  $\text{Mg}^{2+}$  activation, and D-sorbitol inhibition indicated that a single enzyme (D-xylose isomerase) was responsible for the isomerization of the pentoses, methyl pentose, and glucose.

The conversion of D-glucose to D-fructose by microbial enzymes was first described in cell-free extracts of *Pseudomonas hydrophila* by Marshall and Kooi (16). This activity in the genus *Streptomyces* was initially reported by Tsumura and Sato (23). Subsequent studies of the enzyme from this genus, involving purification, crystallization, and kinetic characterization, demonstrated that the inducible enzyme D-xylose keto isomerase (EC 5.3.1.5) converted D-xylose as well as D-glucose to their respective ketoses (21). In addition to these carbohydrates, the isomerases found in *Lactobacillus brevis* (24) and in *Bacillus coagulans* (3) also catalyzed D-ribulose synthesis from D-ribose. The latter activity, however, was not detected in *Streptomyces* (21). This paper characterizes a D-xylose isomerase from *Streptomyces albus* which utilizes, in addition to D-glucose, the following carbohydrates: D-xylose, D-ribose, D-allose, L-arabinose, and L-rhamnose. Furthermore, it describes the functional role of  $\text{Mg}^{2+}$  as an activator of isomerization and discusses the practical importance of the cation.

### MATERIALS AND METHODS

**Organism and cultural conditions.** *Streptomyces albus* (Rossi Doria) Waksman and Henrici NRRL 5778 was isolated by the authors from a local garden

soil by suspending 1 g of soil in 100 ml of sterile distilled water and plating from dilutions of the suspension on an inorganic salts-agar medium (17) containing 0.5% D-xylose as sole carbon source.

All cultures were grown in a medium (RM) containing 0.4% yeast extract, 0.3% malt extract, 0.5% NaCl, and 0.05%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and adjusted to pH 7.3. The cultures were incubated at 29 C on a rotary shaker at 250 rpm. Stock cultures were maintained on RM medium solidified with 1.5% agar.

For mycelium propagation, a 250-ml Erlenmeyer flask with 100 ml of RM was inoculated with spores from 2-day-old slants and incubated for 24 h. The total content of the flask was subsequently added to a 2.8-liter Fernbach flask containing 800 ml of RM supplemented with 0.5% D-xylose. After a 24-h incubation, the mycelium was harvested by filtration through Whatman no. 41 paper, washed twice with distilled water, and resuspended in 0.2 M potassium phosphate buffer (pH 7.2) at room temperature, maintaining a ratio of 2 g of mycelium (wet weight) per 5 ml of buffer.

Cobalt was not included in RM medium since it does not enhance isomerase formation. Cobalt has been reported to stimulate isomerase formation by other strains of *Streptomyces* (10, 21, 22).

**Enzyme preparation.** Enzyme solutions were prepared according to Strandberg and Smiley (20) with the modification that the mycelial suspension was disrupted at 4 C by passage through a French pressure cell at 8,000 lb/in<sup>2</sup>. The final enzyme preparation had a specific activity for D-glucose of 1.085  $\mu\text{mol}/\text{min}$  per mg of protein, corresponding to a two-fold purification over the crude extracts. The enzyme preparation was stored at -5 C.

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**Enzyme assays.** A 1-ml mixture containing 16  $\mu\text{mol}$  of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 100  $\mu\text{mol}$  of potassium phosphate buffer (0.2 M), pH 7.2, and 2 to 4 mg of protein with isomerizing activity was preincubated for 5 min at 70 C. To start the reaction, 160  $\mu\text{mol}$  of each carbohydrate in 1 ml of buffer were added to the preincubated mixture. When required, 0.08  $\mu\text{mol}$  of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  was used. The reaction was incubated at 70 C for 15 min and stopped with 1 ml of 5% trichloroacetic acid. Unless otherwise specified, the enzyme activity was measured under these conditions. Fructose and ketopentoses formed were determined by the cysteine-carbazole method (5), in which color was developed at room temperature for 15 min. The optical density values were read at 540 nm in a Gilford 300-N spectrophotometer. All ketopentoses were determined by comparison with a standard curve prepared with D-ribose-*o*-nitrophenylhydrazine (1) whereas ketohexoses were determined by comparison to a standard curve prepared with D-fructose. Enzyme activities were expressed as micromoles of ketose formed per minute per milligram of protein.

The carbohydrates used as substrates were shown to be at least 99% pure by gas-chromatographic analysis of their peracetylated aldonitrile derivatives (7) and by paper chromatography (9).

Protein was determined by the method of Lowry et al. (14), using bovine serum albumin as standard.

**Chemicals.** All the carbohydrates with the exception of D-allose were purchased from Sigma Chemical Co. D-Allose was prepared at Northern Regional Research Laboratory, Peoria, Ill., by W. Dick essentially as described by Kawana et al. (11).

## RESULTS

The enzymatic conversion of D-glucose to D-fructose, using cell-free extracts of *Streptomyces albus* NRRL 5778, functioned optimally at 70 to 80 C (Fig. 1a) which is in agreement with other *Streptomyces* species (20-22). Enzyme activity was optimal between pH 7 to 9 (Fig. 1b) and is in accord with previously reported values for other *Streptomyces* strains (20, 21) but lower than pH 9.5 reported for *Streptomyces phaeochromogenes* strain SK (22). An Arrhenius plot of the temperature activity values revealed an activation energy of 47,300 J per mol. Since D-fructose can be converted to D-glucose and other sugars through enolization reactions at pH 8 or higher, the present work was done at pH 7.2 and 70 C.

$\text{Mg}^{2+}$  (1.0 mM) either as chloride or as sulfate strongly stimulated D-glucose isomerase activity extracted from the microorganism under study (Table 1);  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  at the same concentrations were weak activators. When the activity was tested in several  $\text{Mg}^{2+}$  or  $\text{Co}^{2+}$  concentrations (Fig. 2), the maximal activity obtained in the presence of  $\text{Co}^{2+}$  was 25% of that with  $\text{Mg}^{2+}$ . Respective affinity constants for

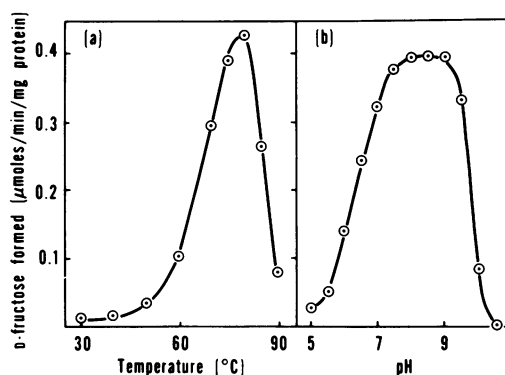


FIG. 1. Effect of temperature (a) and pH (b) on D-glucose isomerase activity. Activity was measured as described in Materials and Methods, except that the temperature and pH values were varied as indicated. Potassium phosphate buffers (0.05 M) were used for pH values of 5.0 to 8.5 and carbonate-bicarbonate buffers (0.05 M) from pH 8.5 to 11.0. All results were corrected for alkaline isomerization.

TABLE 1. Activation of D-glucose isomerase<sup>a</sup> from *S. albus* NRRL 5778 by divalent cations

Metal salt (1 mM)	Relative activity <sup>b</sup> (%)
None	9.85
$\text{FeSO}_4$	0.0
$\text{CuSO}_4$	0.0
$\text{CaCl}_2$	0.0
$\text{NiSO}_4$	5.32
$\text{MnCl}_2$	13.15
$\text{CoCl}_2$	28.5
$\text{MgCl}_2$	100.0
$\text{MgSO}_4$	100.0 <sup>c</sup>
$\text{MgSO}_4$ plus $\text{CoCl}_2$	137.5

<sup>a</sup> The enzyme was dialyzed overnight against demineralized distilled water to remove metallic ions.

<sup>b</sup> The activity was assayed as described in Materials and Methods but omitting the  $\text{MgSO}_4$ .

<sup>c</sup> The activity in the presence of  $\text{MgSO}_4$  was set as 100%.

$\text{Mg}^{2+}$  and  $\text{Co}^{2+}$  of 0.3 and 0.003 mM (Fig. 3) were graphically determined (13).

An additive effect resulted from the presence of both  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$ . To learn more about the additive effect, glucose isomerizing rates were assayed either in the absence or in the presence of a fixed amount of  $\text{Co}^{2+}$  at different  $\text{Mg}^{2+}$  levels. Lineweaver-Burk treatment of the data demonstrated that although  $\text{Co}^{2+}$  does not alter the  $K_m$  for  $\text{Mg}^{2+}$  it increases  $V_{max}$  (Fig. 4). This observation suggests that the cations do not compete for a common binding site on the enzyme molecule.  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$  either singly

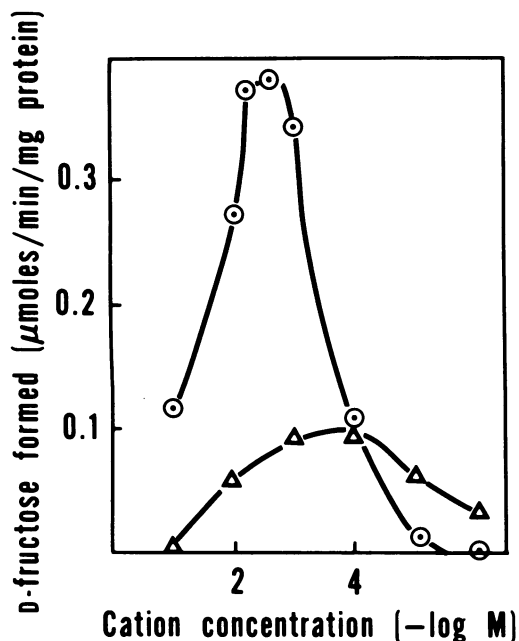


FIG. 2. Effect of  $\text{Co}^{2+}$  ( $\Delta$ ) and  $\text{Mg}^{2+}$  ( $\circ$ ) concentrations on D-glucose isomerase activity. Conditions as described in Materials and Methods but varying  $\text{Mg}^{2+}$  and  $\text{Co}^{2+}$  concentrations.

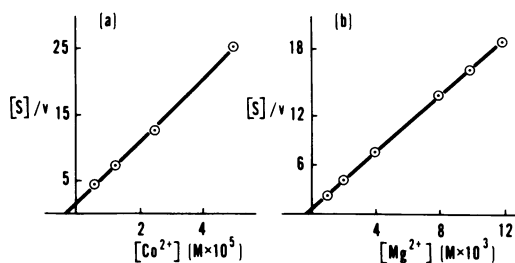


FIG. 3. Plots of the reaction rate of D-glucose isomerase with varying  $\text{Co}^{2+}$  and  $\text{Mg}^{2+}$  concentrations. The reactions were carried out for 10 min under conditions described in Materials and Methods. The initial velocity was expressed as micromoles of D-fructose formed per minute per milligram of protein at 70 C.

or combined inhibited thermal denaturation of the enzyme (Fig. 5a). Consequently, a 24-h preincubation at 70 C did not significantly alter enzyme activity when both cations were present, even though substrate was absent (Fig. 5b).

Surprisingly, the enzyme isomerized an interesting array of substrates. As shown in Table 2, in addition to isomerizing D-glucose and D-xylose, as has been reported for other *Streptomyces* species (20, 21, 23), the enzyme from strain NRRL 5778 also isomerized D-ribose, D-allose, L-arabinose, and L-rhamnose to their

respective ketoses. Because sufficient amounts of D-allose were not available, further work with this sugar could not be done. Gas-chromatographic analyses of the peracetylated aldono-nitrile derivatives (7) and paper chromatography (9) of all the substrates showed them to be >99% pure, thus eliminating contamination of the substrates as the source of ketose formation. Respective  $K_m$  values for D-glucose and D-xylose of 86 and 93 mM and  $V_{max}$  values of 1.23 and 2.9  $\mu\text{mol}/\text{min}$  per mg of protein were graphically determined (13). Additionally, it was found that  $K_m$  values for D-ribose, L-arabinose, and L-rhamnose were 350, 153, and 312 mM, respectively, whereas  $V_{max}$  values were 2.63, 0.153, and 0.048  $\mu\text{mol}/\text{min}$  per mg of protein. Yamanaka and Isumori (25) recently

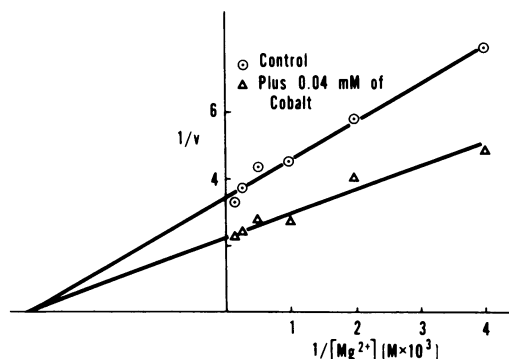


FIG. 4. Double reciprocal plots of the velocity of D-glucose isomerase activity versus varying  $\text{Mg}^{2+}$  concentrations in the absence ( $\circ$ ) or presence ( $\Delta$ ) of a fixed  $\text{Co}^{2+}$  concentration. Conditions as in Fig. 3.

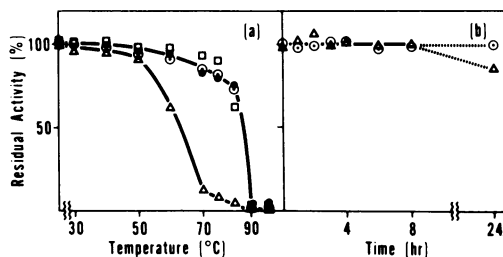


FIG. 5. (a) Thermal tolerance of D-glucose isomerase activity. The enzyme was treated for 10 min in the absence of substrate at the indicated temperatures under the following conditions: no cation ( $\Delta$ );  $4 \times 10^{-5}$  M  $\text{Co}^{2+}$  ( $\bullet$ );  $8 \times 10^{-3}$  M  $\text{Mg}^{2+}$  ( $\circ$ ); and  $\text{Co}^{2+}$  plus  $\text{Mg}^{2+}$  ( $\square$ ). After treatment, the activity was measured as described in Materials and Methods. (b) Heat stability of D-glucose isomerase activity. The enzyme was treated at 70 C during the indicated time in the presence of  $4 \times 10^{-5}$  M  $\text{Co}^{2+}$  plus  $8 \times 10^{-3}$  M  $\text{Mg}^{2+}$  ( $\Delta$ ). Control was at room temperature ( $\circ$ ). After treatment the activity was measured as described in Materials and Methods.

reported on an isomerase from a *Streptomyces* sp. which is active at 35 C and specific only for L-arabinose.

Whether the five activities result from a single isomerase or from five different enzymes could be determined by examining the ability of each substrate utilized to induce isomerizing activity. Only D-xylose effectively induced enzyme synthesis (Table 3). This observation suggests that a single enzyme (D-xylose isomerase) catalyzed the isomerization of the aforementioned carbohydrates. The fact that D-glucose induced no detectable isomerase activity may be due to catabolic repression by D-glucose (15). Table 4 presents further evidence supporting the existence of only one isomerase. Isomerization of the five substrates was markedly activated by Mg<sup>2+</sup> and (with the exception of L-arabinose) partially by Co<sup>2+</sup>. Dixon plots (6) in Fig. 6 show that D-sorbitol competitively inhibited D-glucose, D-xylose, and D-ribose isomerization with identical *K<sub>i</sub>* values of 5.5 mM which also tends to confirm the single-enzyme hypothesis. Sorbitol inhibition of L-arabinose and L-rhamnose isomerization was not tested. Interestingly, D-sorbitol inhibited only when Co<sup>2+</sup> and Mg<sup>2+</sup> were not present in the reaction system (Fig. 7). These cations prevented inhibition of D-xylose isomerization even by high D-sorbitol concentrations. Similar results were obtained in the presence of either Mg<sup>2+</sup> or Co<sup>2+</sup> alone. Sorbitol also failed to inhibit isomerization in the presence of Co<sup>2+</sup> and Mg<sup>2+</sup> when D-xylose was substituted by either D-glucose or D-ribose.

## DISCUSSION

The catalytic conversion of D-glucose to D-fructose was studied using a partially purified enzyme with isomerizing activity obtained from *Streptomyces albus* NRRL 5778. In addition to D-glucose, this enzyme also isomerizes D-xylose, D-ribose, D-allose, L-arabinose, and L-rhamnose. Steric correlations were found in these carbohydrates since, with the exception of L-rhamnose, these sugars have a C1 conformation and the hydroxyl groups on carbon 2 are in equatorial position. If the OH is in an axial position as in D-lyxose or D-mannose, they are not isomerized by the enzyme. When the OH groups of carbon 3 and 4 are equatorial as in D-glucose and D-xylose, maximum isomerization is obtained. However when the OH groups are axial at carbon 3 as in D-ribose and D-allose, the rate of isomerization is diminished. Axial OH at carbon 4 in the pentose L-arabinose further reduces the amount of isomerizations by the enzyme.

TABLE 2. Substrate specificity of *S. albus* NRRL 5778 isomerase

Substrate (80 mM)	Activity <sup>a</sup> (μmol of ketose formed/min per mg of protein)
D-Glucose	1.085
D-Xylose	0.666
D-Ribose	0.488
D-Allose	0.250
L-Rhamnose	0.086
L-Arabinose	0.0369
D-Arabinose	0.0
D-Lyxose	0.0
L-Fucose	0.0
D-Fucose	0.0
D-Mannose	0.0
D-Galactose	0.0

<sup>a</sup> The activity was measured as described in Materials and Methods. Reaction time, 10 min at 70 C.

TABLE 3. Effects of carbon source in growth medium on isomerizing activity of *S. albus* NRRL 5778

Carbon source (1%)	Relative isomerization (%) <sup>a</sup>				
	D-Xylose	L-Arab-inose	D-Ribose	D-Glucose	L-Rham-nose
No addition	14.0	15.45	13.0	10.25	6.55
D-Glucose	0.0	0.0	1.56	0.0	0.0
D-Fructose	5.41	3.8	3.34	1.25	3.12
D-Ribose	7.3	2.67	3.55	2.9	1.11
L-Arabinose	7.6	7.02	4.68	1.93	1.25
L-Rhamnose	4.78	8.3	6.92	2.64	1.49
Glycerol	10.15	10.12	9.6	3.29	3.79
D-Xylose <sup>b</sup>	100.0	100.0	100.0	100.0	100.0

<sup>a</sup> After 24 h of growth in RM medium supplemented with inducer, the isomerization was measured as described in Materials and Methods.

<sup>b</sup> The activity obtained with D-xylose as inducer was set as 100%.

Hexoses or methyl pentoses with axial OH on carbon 4 such as D-galactose or D-fucose are not isomerized. The utilization of L-rhamnose which has a 1C conformation is not understood at present.

It seems probable that a single enzyme (D-xylose isomerase) is responsible for isomerizations of all the aforementioned substrates, since only D-xylose serves as inducer of isomerase biosynthesis and because all five isomerase activities are uniformly stimulated by Mg<sup>2+</sup> and to a lesser extent by Co<sup>2+</sup> cations. D-Sorbitol competitively inhibits isomerization of D-glucose, D-xylose, and D-ribose with identical inhibition constants (*K<sub>i</sub>*) which also suggests that all isomerizations occur on the same active center of a single enzyme.

TABLE 4. Effect of magnesium and cobalt on *S. albus* NRRL 5778 isomerizing activities<sup>a</sup>

Metal addition	Relative activity (%) <sup>b</sup>				
	L-Arabinose	L-Rhamnose	D-Glucose	D-Ribose	D-Xylose
None	0.0	8.87	9.85	6.75	36.5
MgSO <sub>4</sub>	100.0	100.0	100.0	100.0	100.0
CoCl <sub>2</sub>	0.0	11.3	28.5	40.3	41.0

<sup>a</sup>The activities were assayed using the same concentrations of cations and carbohydrates described in Materials and Methods.

<sup>b</sup>The activity obtained in the presence of Mg<sup>2+</sup> was set to 100%.

This enzyme exhibits a fairly high  $K_m$  value for D-xylose (93 mM). In contrast, a relatively high affinity for D-glucose is measured ( $K_m = 86$  mM) when compared to reported values for similar enzymes ( $K_m = 250$  to 920 mM) from several microbial sources including *Streptomyces* (16, 20, 23, 24). It is interesting to note that previously reported  $K_m$  values of this enzyme from either *Streptomyces* (21, 23) or other microbial sources (3, 24) all show higher affinity for D-xylose than D-glucose. The significance of the low affinity for D-xylose is not understood since it alone induces isomerase formation and is therefore presumed to be the natural substrate.

An additional characteristic of the enzyme is that the catalytic function is strongly stimulated by Mg<sup>2+</sup>, but only partially by either Co<sup>2+</sup> or by Mn<sup>2+</sup>. A more stable conformation appears to result from the enzyme-Mg<sup>2+</sup> complex since it has high resistance to thermal degradation. Furthermore, the elimination of D-sorbitol inhibition (competitive) either by Mg<sup>2+</sup> or Co<sup>2+</sup> suggests that the conformational stabilization of the enzyme molecule no longer permits binding of the hexitol; i.e., the cations alter the substrate specificity of the enzyme. Since Co<sup>2+</sup> increases  $V_{max}$  without altering the  $K_m$  for Mg<sup>2+</sup>, we conclude that these cations do not compete for a common binding site on the enzyme molecule. A similar example for isomerizing enzymes was described by Danno for Co<sup>2+</sup> and Mn<sup>2+</sup> in *Bacillus coagulans* (4).

Because the isomerase from *Streptomyces albus* NRRL 5778 is highly stimulated by Mg<sup>2+</sup> and poorly by Co<sup>2+</sup>, it may be of interest for producing corn sugar syrups containing high D-fructose concentrations. D-Glucose isomerase from other microbial sources including *Streptomyces* are preferentially activated by Co<sup>2+</sup> (4, 10, 16). Additionally, in other *Streptomyces* species, even though activation by

Mg<sup>2+</sup> alone has been reported, Co<sup>2+</sup> is apparently required to obtain maximum glucose isomerase formation (20–22). Even though Co<sup>2+</sup> is an essential trace metal for human nutrition (18), it is toxic at higher levels (8, 19). The Co<sup>2+</sup> content of fructose syrups is about 1 mM (2) and this concentration is known to cause toxic effects in rats (12). Consequently fructose syrups must be treated with ion exchanges to reduce the Co<sup>2+</sup> content to acceptable levels. From the standpoint of human health, it would be desirable to eliminate Co<sup>2+</sup>

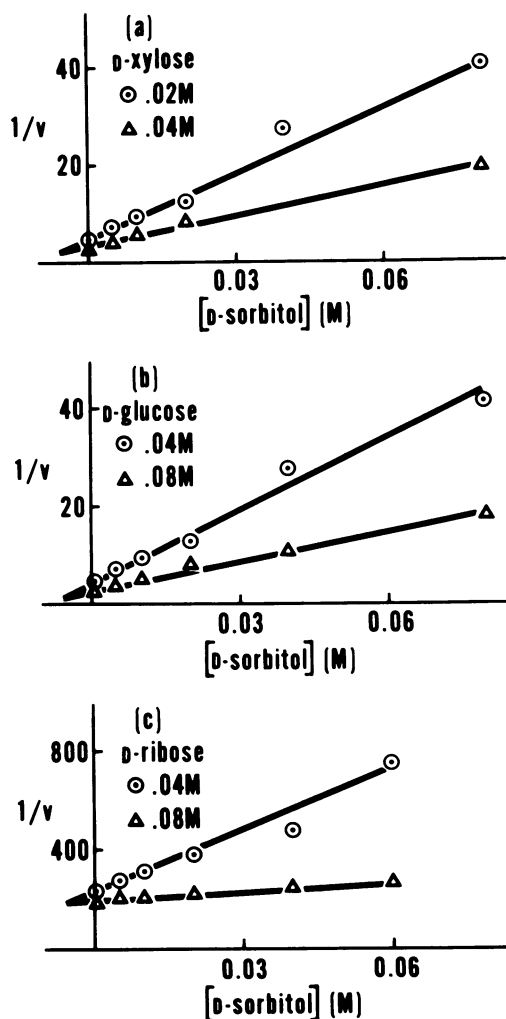


FIG. 6. Dixon's plots for D-sorbitol inhibition of isomerizing activity where D-xylose (a), D-glucose (b), and D-ribose (c) are substrates. The reaction was carried out for 10 min in the absence of cations as described in Materials and Methods. The  $K_i$  value obtained for all three substrates corresponds to 5.5 mM.

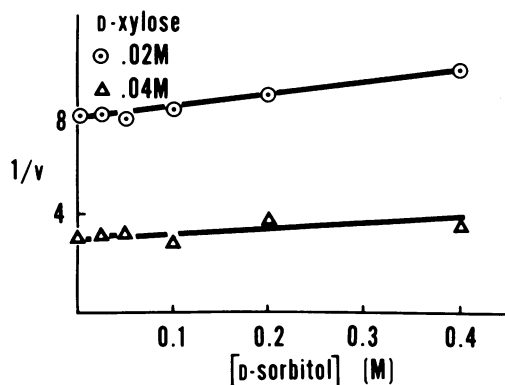


FIG. 7. Prevention of D-sorbitol inhibition of D-xylose isomerization by  $Mg^{2+}$  plus  $Co^{2+}$ . The reaction was measured after 10 min in the presence of  $8 \times 10^{-3}$  M  $Mg^{2+}$  plus  $4 \times 10^{-5}$  M  $Co^{2+}$  by the procedure described in Materials and Methods.

from the process. It seems probable that this could be accomplished by using the isomerase produced by NRRL 5778. Finally since the activity occurs between 70 to 80 C, either the use of mutants with altered D-fructokinase or complicated and expensive enzyme purification methods to prevent further metabolism of D-fructose are avoided. These characteristics, together with good affinity for glucose, make this isomerase a potentially useful industrial enzyme.

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