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# PURIFICATION, CRYSTALLIZATION AND PROPERTIES OF THE D-XYLOSE ISOMERASE FROM *LACTOBACILLUS BREVIS*

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SUMMARY

1. D-Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) has been purified and crystallized from the extracts of D-xylose-grown cells of *Lactobacillus brevis*.

2. The crystalline enzyme appears from its sedimentation pattern to be homogeneous. D-Lyxose is inert with respect to this enzyme. In the reverse reaction, xylose is the only product of D-xylulose, but D-lyxose is not detected in the reaction products of D-xylulose.

3. This enzyme catalyzes the isomerization of D-xylose, D-glucose and D-ribose. The optimum pH is identical for these substrates, 6.0–7.0, while the Michaelis constants are very different: $5 \cdot 10^{-3}$ M for D-xylose, 0.92 M for D-glucose and 0.67 M for D-ribose.

4. This enzyme required manganese ions for its activity and the Michaelis constant was found to be $6.1 \cdot 10^{-6}$ M.

## INTRODUCTION

D-Xylose isomerase (D-xylose ketol-isomerase, EC 5.3.1.5) has been found in *Lactobacillus pentosus* (*L. plantarum*) by MITSUHASHI AND LAMPEN[1], in *Pseudomonas hydrophila* by HOCHSTER AND WATSON[2], and in *Pasteurella pestis* by SLEIN[3]. A procedure for the partial purification of this enzyme has been reported[4,5]. The same enzyme has also been found in *Lactobacillus brevis*, *L. pentoaceticus*, *L. fermenti*, *L. gayonii*, *L. xylosus*, and *Leuconostoc mesenteroides*[6].

The enzymatic conversion of D-glucose to D-fructose was found to occur in the D-xylose-grown cells of *Pseudomonas hydrophila* by MARSHALL AND KOOI[7] in 1957. Arsenate was required for this reaction. YAMANAKA[8] had found, however, the direct enzymatic isomerization of D-glucose to D-fructose by the D-xylose isomerase preparation. Arsenate was not required. Furthermore, arsenate inhibited bacterial growth and production of D-xylose isomerase and of D-glucose isomerizing activity in the medium, and also inhibited both activities of isomerization of D-xylose and D-glucose in the case of lactic acid bacteria[9].

In connection with D-glucose isomerizing activity, *Aerobacter cloacae*[10,11], *Aerobacter aerogenes*[12], *Escherichia intermedia*[13], *Bacillus megaterium*[14], *Brevibacterium*

*pento-aminoacidicum*[15], *Paracolobacterum aerogenoides*[16], *Streptomyces phaeochromogenus*[17], *Bacillus coagulans*[18], and Streptomyces sp.[19] were reported to produce this activity with or without arsenate. No attempt, however, was made to investigate the relation between this activity and the D-xylose isomerase activity in these preparations. There was no direct evidence that the same enzyme would catalyze both reactions in these preparations.

In lactic acid bacteria, D-glucose isomerization was always found in the D-xylose isomerase preparations and several heterofermentative lactic acid bacteria, which were known to produce D-xylose isomerase, *Lactobacillus brevis, L. mannitopoeus, L. gayonii, L. pentoaceticus, L. fermenti, L. lycopersici* and *Leuconostoc mesenteroides*, produced the D-glucose isomerizing activity and D-xylose isomerase in a constant ratio[9]. Both activities were eluted in the same fraction by chromatography on DEAE-cellulose or on DEAE-Sephadex[9]. In this connection, there is no positive evidence of the presence of a so-called specific D-glucose isomerase.

This paper reports a procedure for purification of D-xylose isomerase from *L. brevis* which produces large crystals. It also demonstrates that one enzyme, D-xylose isomerase, catalyzes the isomerization of D-xylose, D-glucose, and D-ribose.

## MATERIALS AND METHODS

### *Culture*

*L. brevis* (Institute for Fermentation, Osaka, Japan, 3960) was grown in a medium composed of 1% sodium acetate, 1% peptone, 0.2% yeast extract, 0.05% $MnSO_4 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CoCl_2 \cdot 6H_2O$, 1% D-xylose, and 0.1% D-glucose. Manganese ions enhanced the yield of the D-xylose isomerase by *L. brevis*[20], and D-xylose was required for enzyme production. The sugar and salt solutions were autoclaved separately and added just before inoculation. An inoculum of 8 ml of the above medium was incubated overnight at 32° in a test tube. The whole culture was transferred to 400 ml of the same medium and incubated for 16 to 20 h, and then transferred to 10 l of medium. Cells were harvested by a Sharples centrifuge after 45 h of incubation at 32°.

### *Enzymatic assay*

D-Xylose isomerase activity was determined by measuring the increase of xylulose[21]. Unless otherwise specified, the standard reaction mixture was composed of 30 $\mu$moles maleate buffer (pH 6.0), 0.5 $\mu$mole $MnCl_2$, 0.01 to 0.05 ml of enzyme praparation with adequate dilution, and distilled water to a final volume of 0.95 ml. After equilibration for 5 min at 35°, 0.05 ml of 0.1 M D-xylose was added and incubated at 35° for 10 min. Xylulose was determined by the cysteine–carbazole reaction[22]. 1 unit of enzyme is defined as the amount which produces 1 $\mu$mole of xylulose under these conditions. Specific activity is expressed as unit per mg of protein. Protein was determined from the absorbances at 280 m$\mu$ and 260 m$\mu$ (ref. 23).

For the isomerization of D-glucose, the reaction mixture contained 30 $\mu$moles maleate buffer (pH 6.0), 0.5 $\mu$mole $MnCl_2$, 0.5 $\mu$mole $CoCl_2$, and enzyme preparation. After equilibration at 50°, 0.30 ml of 2 M D-glucose was added and incubated at 50° for 30 min. Fructose was determined by the cysteine–carbazole test. Color was developed at 50° for 30 min and assayed at 560 m$\mu$. For the isomerization of D-ribose,

D-glucose was replaced by D-ribose and reaction was allowed to proceed at 50° for 10 min. Ribulose was determined by the same test after a 20-min incubation at 20°. 1 unit of these enzymatic activities is also expressed as the amount which produces 1 $\mu$mole of fructose or ribulose under these conditions.

*Column chromatography of pentoses*

Dowex 1 resin, borate form, was used for the separation of pentoses[24]. As xylulose, ribose and lyxose can be eluted in the same fraction by 0.02 M borate, modifications were made in the concentration of borate and the pH of the eluting solution which allowed xylulose, ribose and lyxose to be separated. Authentic sugar samples were applied onto a column of Dowex 1 resin, borate form, which was washed with a sufficient volume of a mixture of 0.005 M borate and 0.1 M boric acid. Sugars were eluted by the same borate–boric acid solution. The peaks of the 3 sugars appeared in the following order: D-lyxose, D-xylulose, and D-ribose. Sugar was assayed by the method of phenol–$H_2SO_4$ (ref. 25).

*Chemicals*

D-Xylulose and D- and L-ribuloses were prepared by epimerization of D-xylose and D- and L-arabinoses, respectively, with dry pyridine[26]. The bulk of the remaining aldose was removed by crystallization in a refrigerator. After repeating the crystallization procedure, the mixture was applied onto a column of Amberlite IRA-400 (bisulfite form). Ketopentose was eluted first by 75% *n*-propanol according to the method of ADACHI AND SUGAWARA[27]. The fractions containing ketopentose were pooled, and propanol was removed by distillation under reduced pressure. The condensed solution was passed through a column of ion retardation resin, AG11A8 (Dowex 1), to remove bisulfite ions. The fractions were collected, condensed and passed through a column of Sephadex G-25 with deionized water. The purified preparation was free of any other reducing sugars as judged by paper chromatography. Samples were kept in a deep freeze. Levigated alumina, 300 mesh, chromatographic grade, was purchased from the Wako Co., Osaka, Japan.

## RESULTS

*Purification procedure*

All procedures were performed at 2° unless otherwise stated. The washed cells (wet weight 23.5 g from 10 l of medium) were disrupted by grinding with alumina. D-Xylose isomerase was extracted with 0.02 M Tris–acetate buffer (pH 7.4). Alumina and cell debris were removed by centrifugation (crude extract). To the crude extract was added dropwise 10.5 ml of 1 M $MnCl_2$. pH was maintained at 6.2 to 6.6 by adjusting with 1 M NaOH. After centrifugation, the precipitate was discarded (Mn treatment). Solid ammonium sulfate (51 g) was added to give 0.45% of saturation, and the precipitate was removed by centrifugation. Ammonium sulfate (71 g) was added to the supernatant solution (0.95 saturation). The precipitate was collected, dissolved in 0.01 M Tris–acetate buffer (pH 7.4), and dialyzed overnight against the same buffer containing $5 \cdot 10^{-3}$ M $MnCl_2$ (ammonium sulfate fraction I). $MnCl_2$ (1.5 ml of 1 M solution) was added and the enzyme solution, in an erlenmeyer flask, was immersed with stirring in a water bath at 80°. The flask was transferred to another water bath at

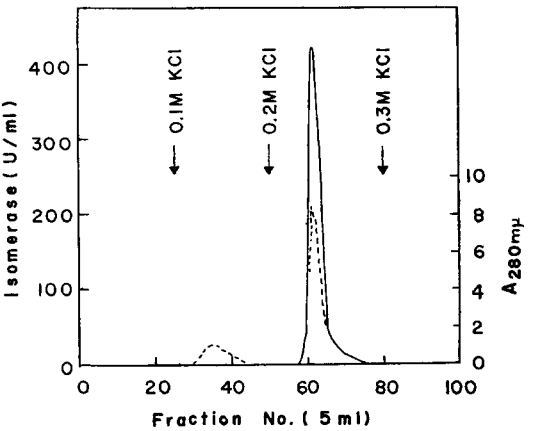



Fig. 1. Chromatography of D-xylose isomerase on DEAE-Sephadex. ———, enzyme activity; - - - - -, protein ($A_{280\ m\mu}$). For conditions see text.

50° when the temperature of the enzyme solution reached 49°. The flask was kept at 50° for 5 min. After cooling, the coagulated protein was discarded by centrifugation (heated fraction). The pH of the heated fraction was adjusted to 5.0 with 0.2 M acetic acid and was chilled to near 0° (27.5 ml). Acetone (7.2 ml), previously chilled to −20°, was added dropwise with stirring (20% acetone). The precipitated protein was removed by centrifugation at −10°. To the supernatant (32.5 ml) was added 11.4 ml of chilled acetone to give 40% acetone. The precipitate was collected and dissolved in 4.0 ml of 0.02 M Tris–acetate buffer (pH 7.4). Insoluble protein was removed by centrifugation (acetone fraction). Enzyme solution was then applied to a column of DEAE-Sephadex A-50 (1.5 cm × 20 cm) which had been equilibrated with 0.01 M Tris–acetate buffer (pH 7.4). The protein was eluted stepwise with 0.02 M Tris–acetate buffer (pH 7.4), increasing the concentration of KCl to 0.3 M (Fig. 1). The fractions were collected, and the enzyme was precipitated with ammonium sulfate between 0.6 and 0.9 saturation (ammonium sulfate fraction II).

TABLE I

PURIFICATION OF ENZYME

| *Fraction* | *Volume (ml)* | *Protein (mg)* | D-*Xylose isomerase* | | |
|---|---|---|---|---|---|
| | | | *Total units* | *Specific activity* | *Recovery (%)* |
| Crude extract | 210 | 2860 | 16 100 | 5.6 | 100 |
| $Mn^{2+}$-treated | 205 | 1390 | 15 000 | 10.8 | 93 |
| Ammonium sulfate I | 29 | 810 | 13 900 | 17.1 | 86.5 |
| Heated | 28 | 495 | 14 500 | 29.4 | 90.3 |
| Acetone | 4.7 | 260 | 9 400 | 40 | 58.4 |
| DEAE-Sephadex | 55 | 200 | 10 500 | 53 | 65 |
| Ammonium sulfate II | 3.7 | 142 | 8 000 | 56 | 50 |
| 1st crystals | — | 95 | 5 800 | 61 | 36 |
| 2nd crystals | — | 55 | 3 500 | 64 | 21.7 |

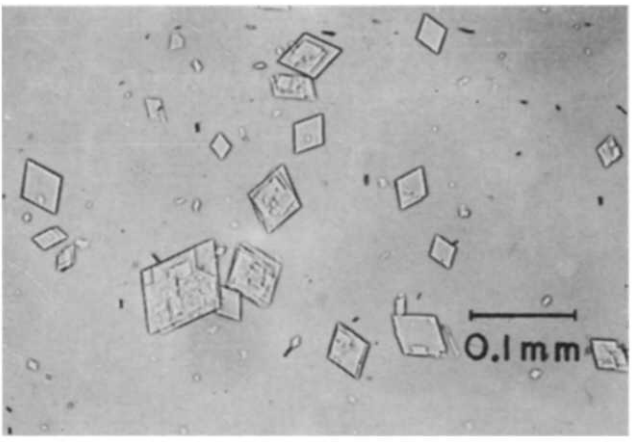



Fig. 2. Crystals of D-xylose isomerase from *L. brevis*. (Second crystals.)

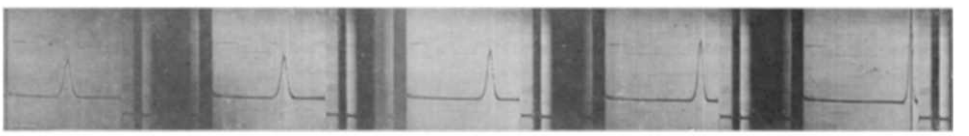


Fig. 3. Sedimentation pattern of D-xylose isomerase. Recrystallized enzyme was used in 0.02 M Tris–acetate buffer (pH 7.4). The photographs were taken at 0, 8, 16, 24, and 32 min after reaching 59 780 rev./min. The sedimentation is from right to left.

*Crystallization*

After dialysis against 0.5 l of 0.02 M Tris–acetate buffer (pH 7.4) overnight, 6.4 ml of saturated ammonium sulfate solution was added to 3.7 ml of the purified enzyme preparation (0.64 saturation). A trace of precipitated protein was removed by centrifugation, and the solution was kept at 5° overnight. The first crystals were collected, dissolved in the same buffer and dialyzed against the same buffer containing $5 \cdot 10^{-3}$ M $MnCl_2$. Recrystallization was carried out by the same procedure at 62%

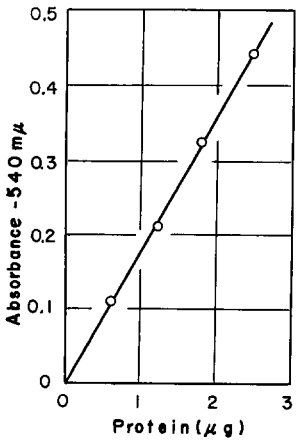



Fig. 4. Linear relationship between activity and enzyme protein.

ammonium sulfate saturation. The purification procedure is summerized in Table I. This procedure yielded 22% of the initial activity as crystals with a purification of slightly over ten-fold. As reported earlier[20,21], D-xylose isomerase of *L. brevis* from $Mn^{2+}$-fortified medium has a specific activity about 10 times higher than that obtained with the basal medium. The crystals were large, regular rhombic plates with the long diagonal dimension of the largest crystals of 0.1 mm (Fig. 2). The second crystals showed a single symmetrical moving peak on ultracentrifuge analysis (Fig. 3).

A linear relationship was obtained between the absorbance at 540 mμ by cysteine–carbazole reaction and the amount of enzyme protein up to 2.5 μg (Fig. 4).

*Properties*

*Substrate specificity*. The enzyme was specific for aldoses of 5 or 6 carbons bearing *cis* hydroxyl groups in positions 2 and 4 (Table II). D-Xylose, D-glucose and D-ribose are isomerized by this single enzyme. This results confirmed the earlier

TABLE II

SUBSTRATE SPECIFICITY OF THE ENZYME

The reaction took place in 0.05 M maleate buffer (pH 6.0) in the presence of $5 \cdot 10^{-3}$ M $MnCl_2$ at 35° for 60 min with 100 μg of crystalline enzyme protein. Ketoses were assayed by the cysteine–carbazole method and expressed as xylulose, ribulose or as fructose.

| *Substrate (2/3 M)* | *Ketose (μmoles)* |
|---|---|
| D-Xylose | 32.0 |
| D-Xylulose* | −59.0 |
| D-Glucose | 1.5 |
| D-Fructose | −3.0 |
| D-Ribose | 0.8 |
| D-Ribulose* | −21.9 |
| D-Arabinose | 0 |
| L-Arabinose | 0 |
| L-Ribulose* | −0.2 |
| L-Xylose | 0 |
| D-Lyxose | 0 |
| D-Mannose | 0 |
| D-Galactose | 0 |

* Concentration was 0.33 M.

observation on glucose isomerization activity of D-xylose isomerase preparation[8]. The ratio of isomerization activities on D-xylose, D-glucose and D-ribose remained constant throughout purification to the second crystals (Table III). After incubating the enzyme solution for 30 min at 20°, 30°, 40°, 50° and 60°, the percents of residual activities in the 3 isomerizations were identical at every temperature (Fig. 5).

*Effect of pH on isomerization reaction*. The enzyme activity varies little between pH 5.7 and 7.0, although it is reduced about 42% and 20% at pH 4.5 and 4.0, respectively. At alkaline pH, activity decreases to about 50% of maximum at pH 7.5 with Tris–maleate buffer. The optimal pH's for the isomerization of D-xylose, D-glucose and D-ribose were the same, 6.0–7.0 (Fig. 6).

TABLE III

RATIO OF ENZYME ACTIVITIES

| *Step* | *Isomerization of** | | |
|---|---|---|---|
| | D-*Xylose* | D-*Glucose* | D-*Ribose* |
| Ammonium sulfate 0.45–0.9 ppt. | 100 | 107 | 16.1 |
| Acetone 20–40% ppt. | 100 | 107 | 17.5 |
| DEAE-Sephadex | 100 | 108 | 16.0 |
| 1st crystals | 100 | 107 | 16.1 |
| 2nd crystals | 100 | 108 | 16.5 |

* Isomerizing activities were calculated as units per ml. Activity of D-xylose isomerase was expressed as 100 in each purification step. The other 2 isomerizing activities were expressed as percent of D-xylose isomerase activity.

*Metal requirement.* In our earlier experiment, the activity of D-xylose isomerase was inhibited by EDTA, and recovered by further addition of manganese ions[28]. The enzyme was dialyzed against 0.01 M Tris–acetate buffer (pH 7.4) containing $5 \cdot 10^{-3}$ M EDTA for 44 h at 2°, then dialyzed against the same buffer without EDTA for 24 h, replacing the buffer every 8 h. This dialyzed enzyme preparation showed no activity with respect to D-xylose without the addition of manganese ions (Table IV). The enzyme activity was recovered by manganese ions specifically. But magnesium ions were completely ineffective in activating this enzyme. Cobalt and zinc ions showed a slight effect on enzyme activation. The Michaelis constant for manganese ions was found to be $6.1 \cdot 10^{-6}$ M (Fig. 7).

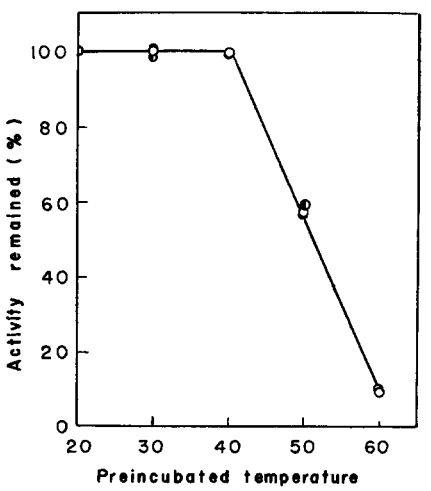



Fig. 5. Effect of temperature on enzyme stability. ○—○, D-xylose isomerase; ●—●, D-glucose isomerizing activity; ◐—◐, D-ribose isomerizing activity. Remaining activity was measured at 40° for 10 min with D-xylose or D-ribose, and for 20 min with D-glucose, and expressed as percent of original activity.

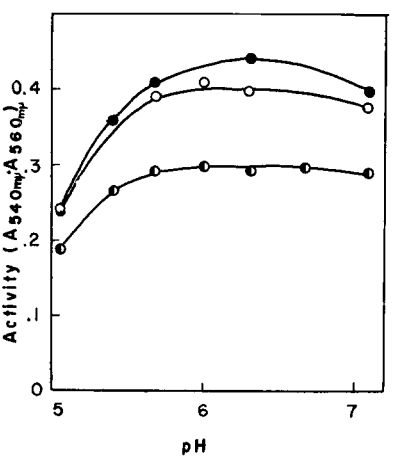



Fig. 6. Effect of pH. ○—○, with D-xylose, enzyme protein (2.5 μg) was incubated at 35° for 10 min; ●—●, with D-glucose, enzyme protein (454 μg) was incubated at 50° for 10 min; ◐—◐, with D-ribose, enzyme protein (45.4 μg) was incubated at 50° for 10 min.

TABLE IV

METAL REQUIREMENT

Dialyzed enzyme was incubated with 5 $\mu$moles of D-xylose and $10^{-6}$ to $10^{-2}$ M of $Mn^{2+}$, $Mg^{2+}$, $Co^{2+}$, or $Zn^{2+}$ ions at pH 6.0 for 10 min at 35°. Activity was expressed as the absorbance at 540 m$\mu$ by cysteine–carbazole test.

| *Addition* | *Activity* ($A_{540\ m\mu} \times 10^3$) | | | | | | |
|---|---|---|---|---|---|---|---|
| *Metal (M)* | 0 | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $5 \cdot 10^{-3}$ | $10^{-2}$ |
| $Mn^{2+}$ | 2 | 3 | 29 | 185 | 204 | 155 | 160 |
| $Mg^{2+}$ | | 2 | −4 | 7 | −1 | 7 | −15 |
| $Co^{2+}$ | | 3 | −14 | 31 | 38 | 49 | 50 |
| $Zn^{2+}$ | | 4 | −8 | 3 | −16 | 45 | 6 |

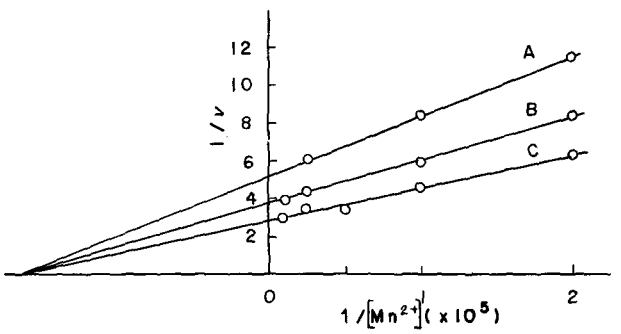



Fig. 7. Effect of concentration of manganese ions on D-xylose isomerase. The reaction took place in 0.025 M maleate buffer (pH 6.0) at 35° for 10 min. The enzyme was the same preparation described in Table IV. EDTA-dialyzed enzyme preparation was used in each run. The concentration of manganese ions varied from $5 \cdot 10^{-6}$ M to $1 \cdot 10^{-4}$ M in the presence of different amounts of D-xylose, 0.0015 M (A), 0.003 M (B) and 0.005 M (C).

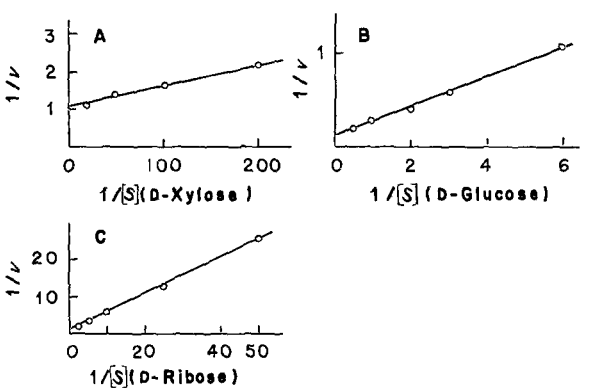



Fig. 8. Effect of substrate concentration. A, D-xylose; B, D-glucose; C, D-ribose.

*Effect of substrate concentration.* The Michaelis constants obtained for the 3 sugars were as follows: $5 \cdot 10^{-3}$ M for D-xylose, 0.92 M for D-glucose and 0.67 M for D-ribose (Fig. 8).

*Stereospecificity of enzyme action.* As shown in Table II, ketose was not detected in L-arabinose or D-lyxose *plus* an excess amount of enzyme. The differences in OH configuration between these sugars and D-xylose are at the C-2 and C-4 positions. The presence of xylulose was not demonstrated in the reaction mixture of D-lyxose by column chromatography. Another experiment seemed to be very significant in the

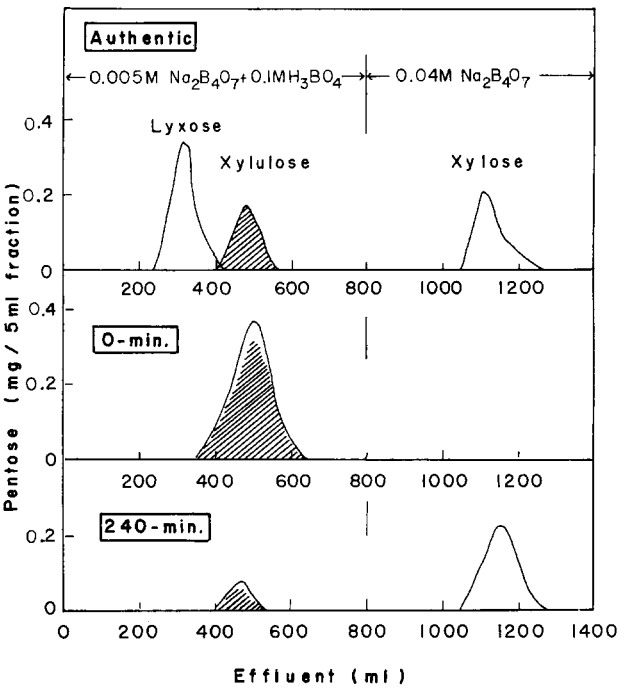



Fig. 9. Chromatography on Dowex 1 of the reaction product from D-xylulose. The reaction mixture was composed of 150 $\mu$moles maleate buffer (pH 6.7), 5 $\mu$moles $MnCl_2$, 200 $\mu$moles D-xylulose and 200 units of enzyme in a total volume of 5.0 ml. The reaction took place at 35° for 240 min. Authentic samples of D-xylose, D-xylulose and D-lyxose were also chromatographed separately and their chromatograms were combined at the upper part. A blank test, run as a reference, is shown in the middle. The lower part was the chromatogram of the reaction mixture. The shaded area represents the fraction which is positive to the cysteine–carbazole test and expressed as xylulose.

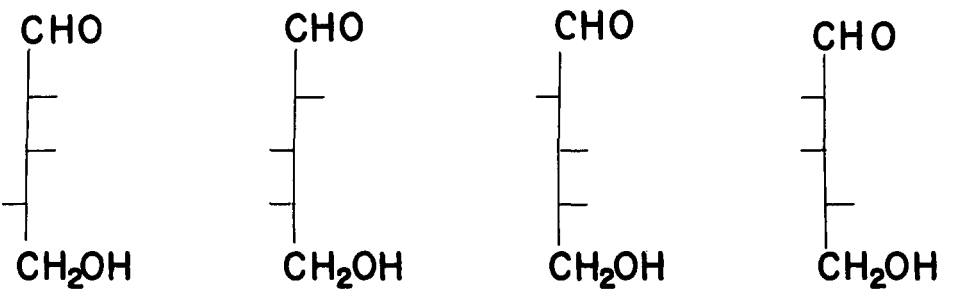
identification of the stereospecificity of the enzyme action. D-Xylulose (30 mg, 200 $\mu$moles) was incubated with 200 units of enzyme at 35° for 240 min. Chromatography of the reaction mixture on a Dowex 1 borate column (1.6 cm × 14 cm) revealed that the newly formed sugar was xylose, and not lyxose. D-Lyxose may possibly be produced from D-xylulose by the reverse reaction. The enzymatic reaction, however, was found to be highly stereospecific and only xylose was recovered from D-xylulose by the action of D-xylose isomerase (Fig. 9).

## DISCUSSION

D-Xylose isomerase was first obtained in the pure state as crystals from *L. brevis*. The crystalline enzyme is fairly specific towards its substrate.

D-Xylose and D-xylulose are the most important substrates, but D-glucose and D-ribose are also significantly isomerized. There is no striking affinity of the enzyme toward other sugars whose structures are similar to that of D-xylose. SIMPSON, WOLIN AND WOOD[29] demonstrated that the crude extract from L-arabinose-grown cells of *Aerobacter aerogenes* showed the isomerization activity not only on L-arabinose but also on D-galactose. Similar results were also obtained for the isomerization of glucose by D-xylose isomerase preparation[8]. Little was known, however, about the correlation between the two activities.

Evidence has been presented here that a single enzyme, D-xylose isomerase, catalyzed the isomerization of D-xylose, D-glucose and D-ribose. These 3 naturally occurring sugars of the D series have in common a *cis* configuration of OH groups at C-2 and C-4. D-Xylose and D-glucose have the same stereochemical configuration at C-2, C-3 and C-4. L-Xylose is inert with respect to this enzyme; L-glucose and L-ribose were not assayed. Pentoses with a *trans* configuration of OH groups at C-2 and C-4 do not act as substrates for this enzyme. L-Arabinose, D-arabinose and D-lyxose are not isomerized by the enzyme, and L-lyxose does not occur naturally.




L-Lyxose L-Arabinose D-Arabinose D-Lyxose

In contrast to the case of D-xylose isomerase, ANDERSON AND WOOD[30] found that L-xylose was isomerized to L-xylulose by crude extracts from the L-xylose-grown cells of *Aerobacter aerogenes*, PRL-R3. L-Xylulose was further isomerized to L-lyxose. It was not known, however, if the isomerization of L-xylose and L-lyxose was catalyzed by the same enzyme or by 2 different enzymes. L-Xylose isomerase seemed to be the same enzyme as D-arabinose isomerase[31], but no details are available concerning this enzyme. Another pentose isomerase, which acts on the unnatural pentose D-lyxose, was found in *Aerobacter aerogenes*, PRL-R3, and named D-lyxose isomerase[32]. The purified enzyme isomerized D-lyxose to D-xylulose and D-mannose to D-fructose. The enzyme is inactive in regard to D- and L-xyloses and D- and L-arabinoses.

D-Xylulose is the product of the action on D-xylose of D-xylose isomerase[1,2,6], and D-xylose is the product of the action of the present isomerase on D-xylulose. The D-xylose isomerase is, therefore, distinct from the D- and L-arabinose isomerases and D-lyxose isomerase. The migration of carbon-bound hydrogen seems to be stereochemically regulated by the enzyme.

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