Studies on Glucose Isomerase of Bacteria

Part I. Formation of Glucose Isomerase by *Aerobacter aerogenes*, Strain HN-56, and its Relationship to Xylose Isomerase*

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A bacterial strain, HN-56, having an activity of D-glucose isomerization was isolated from soil, and was identified to be similar to *Aerobacter aerogenes* (Kruse) Beijerinck. D-Glucose-isomerizing activity was induced when HN-56 was precultured in the media containing D-xylose, D-mannose, lactate, especially D-mannitol. Paper chromatography showed that the ketose formed in reaction system containing D-glucose was D-fructose alone. The optimum pH for the reaction was 6.5~7.0. Sulfhydryl reagents inhibit the reaction, but metal inhibitors affect little if any. With the washed living cells as enzyme source, only arsenate could accumulate D-fructose. In addition, the cells grown with D-mannitol and D-mannose showed no activity of D-xylose isomerase.

Marshall et al. and Tsumura et al. found that *Pseudomonas hydrophila*¹⁾ and *Aerobacter cloacae*^{2,3)} grown in the medium containing D-xylose as sole source of carbon could isomerise both D-glucose and D-xylose to D-fructose and D-xylulose but those grown in D-glucose or D-fructose could isomerize neither D-glucose nor D-xylose. And in consistence with the view of Simpson et al.⁴⁾, Tsumura et al.³⁾ stated that an identical configuration on carbons No. 1 to No. 4 was observed between the sugar as carbon source of cell growth and the sugar as substrate of isomerization, i.e., D-xylose and D-glucose, L-arabinose and D-galactose. Further, Palleroni and Doudoroff⁵⁾ have shown that manose isomerase

catalyzing interconversion of D-mannose to D-fructose is produced by a mutant strain of *Pseudomonas saccharophila* when the organism was grown on the medium containing D-fructose, and Bertfay⁶⁾ has suggested the presence of glucose isomerase in barley malt. These information led us to a possible speculation that under proper nutritional conditions, some bacteria might produce an enzyme which would convert free D-glucose to D-fructose without phosphorylation by cooperation of hexokinase.

With a hope to find out the bacteria capable of converting D-glucose to D-fructose, and to elucidate further the nature in detail, several strains fitted for the aim were isolated from soil. Of these, one strain, HN-56 which was identified to be *Aerobacter aerogenes*, produced the glucose isomerase when the bacterium was grown in medium containing

* This paper was presented at the Meeting of Kansai Division of Agricultural Chemical Society of Japan, Kyoto, October 28, 1961, and at the Annual Meeting of this Society, Sendai, April 28, 1962.

1) R.O. Marshall and E.R. Kooi, *Science*, **125**, 648 (1957).

2) N. Tsumura and T. Sato, *This Journal*, **25**, 616 (1961).

3) N. Tsumura and T. Sato, *ibid.*, **25**, 620 (1961).

4) F.J. Simpson, M.J. Wolin and W.A. Wood, *J. Biol. Chem.*, **230**, 457 (1958).

5) N.J. Palleroni and M. Doudoroff, *ibid.*, **218**, 535 (1956).

6) J. Bartfay, *Nature*, **185**, 924 (1960).

either D-mannitol, D-mannose or D-xylose as a carbon source. In contrast with the view of Marshall¹⁾ that enzyme preparations having D-xylose isomerase activity could convert D-xylose and D-glucose to corresponding ketoses; the present organism showed no activity of D-xylose isomerase under both D-mannitol and D-mannose nutrition.

The present work deals with the descriptions of the chosen bacterium, the conditions necessary for the production of the isomerase and some characters of the enzyme.

EXPERIMENTAL

Isolation of the Strain.

The technique employed was essentially the same with that described by N. Tsumura et al.²⁾ The collected soil samples were dispersed once in sterile water and one drop of the supernatants was inoculated into 5 ml portions of liquid medium containing 2% D-xylose, 0.6% $(\text{NH}_4)_2\text{HPO}_4$ and 0.025% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The inoculants were maintained at 28°C for 2 days and then cultivated on the plate with 2% agar medium having the same composition as was employed in the enrichment cultures. After incubation at 28°C for 2 days, the colonies formed were transferred on slant agar medium. Each strain of the slant cultures was then subjected to grow in 50 ml-aliqouts of the above xylose medium at 28°C for 24 hours with reciprocal agitation, and then cell suspension of each strain was prepared and tested for their activities on glucose isomerization by the methods mentioned in the following items.

Preparation of Cell Suspension.

The cells were harvested by centrifugation at 8,000 r.p.m. for 10 minutes, washed once each with 0.75% saline and deionized water, and then suspended in water. The cell suspensions thus obtained were employed immediately either as an enzyme solution or to the preparation of acetone dried cells.

Preparation of Acetone Dried Cells.

The technique employed was the same as has been described by Tsumura et al.²⁾: an aqueous cell suspension was added with vigorous stirring to about 20-fold volume of acetone previously cooled to $-8 \sim -10^\circ\text{C}$. After brief standing, the supernatant is decanted off, and the residue was collected on a Buchner's funnel,

and washed several times with chilled acetone. The cake of cells was transferred to a desiccator containing calcium chloride, and then dried overnight in vacuo.

Analytical Methods.

1) Estimation of Isomerization Activity.

The reaction mixture for estimation of isomerization activity is shown in Table I. The reaction was allowed to carry on at 40°C for 24 or 48 hours and stopped by adding 4.0 ml of 0.5N perchloric acid to each reaction mixture. After centrifugation and suitable dilution, ketose accumulated in the system was estimated by a slight modification in color reaction of cysteine-carbazole method⁷⁾. The color reaction was developed at 40°C for 30 minutes and intensity of color was read with a "Hitachi FPW-4" photoelectric photometer at 570 m μ .

TABLE I. COMPOSITION OF REACTION MIXTURE FOR ISOMERIZATION

D-Glucose	180 mg
0.2 M $\text{Na}_2\text{HAsO}_4\text{-HCl}$ solution (pH 7.0) ...	0.20 ml
Enzyme source:	
Washed cell suspension.....	0.5 ml
or acetone dried cell.....	30~50 mg
Water, to make up total volume to 1.0 ml	

2) Identification of Ketoses.

The identification of ketoses formed in the reaction mixture was achieved by the ascending paper chromatography. The solvent system used for present purpose was a mixture of *n*-butanol-acetic acid-water (4:1:1). Spots on chromatogram were developed by spraying ammoniacal silver nitrate solution⁸⁾ or resorcinol reagent²⁾.

RESULTS AND DISCUSSION

Identification of the Strain.

- 1) Morphological: Rod cells, 0.5~0.8 μ by 1.0~2.2 μ , occurring singly. Motile with peritrichous flagella and Gram-negative.
- 2) Cultural:

Bouillon-agar colonies: Circular, smooth, entire, convex, translucent and milk-white.

Bouillon-agar slant: Abundant, spreading growth, glistening and milk-white.

7) Z. Dishe and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

8) Partridge, *Biochem. J.*, **42**, 238 (1948).

Potato: Thick, yellowish white and spreading growth.

Gelatin stab: No liquefaction.

Nutrient broth: Turbid and slightly sedimented.

3) Physiological:

Litmus milk: Acid curd. Reduction of litmus begins in 2 days.

Nitrates reduced to nitrites.

Indole is not produced.

Hydrogen sulfide is produced in broth culture.

Methyl red test: Negative.

Voges-Proskauer test: Positive.

Growth in sodium citrate medium: Present.

Growth in uric acid medium: Present.

Catalase produced.

Acid and gas from D-glucose, D-fructose, D-galactose, D-xylose, L-arabinose, L-rhamnose, D-lactose, D-maltose, dextrin, D-mannitol, D-sorbitol and D-inositol. Glycerol is fermented with the production of acid and gas. Sucrose is not fermented.

Relation to temperature: Good growth at 27~35°C; annihilation by heating at 65°C in 10 minutes.

Relation to free oxygen: Facultative anaerobic.

4) Source: Isolated from cultivated soil in Sasayama, Hyogo Prefecture.

According to Bergey's Manual⁹⁾, present strain, HN-56, seems to be similar to *Aerobacter aerogenes* (Kruse) Beijerinck.

Effect of Carbon Source on Glucose-isomerizing Activity.

By using various sugars, alcohols and salts of organic acids listed in Table II, the effects of carbon sources in the growth media on the glucose-isomerizing activity were compared. The results obtained are shown in Table II. In consistence with Marshall and

Kooi¹⁾, and Tsumura and Sato³⁾, the cells grown in the medium containing D-glucose, D-fructose or D-galactose could not induce isomerization of D-glucose to D-fructose. However, the present strain showed ketose producing activity when sodium lactate, D-mannose, and especially D-mannitol as well as D-xylose was used as sole carbon source.

TABLE II. EFFECTS OF CARBON SOURCES IN CULTURAL MEDIUM ON GLUCOSE ISOMERIZING ACTIVITY

Carbon source	Ketose formed, mg	Carbon source	Ketose formed, mg
D-Xylose	20.0	Sucrose	0
D-Glucose	0	Glycerol	9.0
D-Fructose	0	Sorbitol	0
D-Galactose	0	D-Mannitol	38.0
D-Mannose	15.0	Ethanol	0
L-Arabinose	0	K citrate	8.0
L-Rhamnose	0	Na succinate	0
Lactose	0	Na acetate	0
Maltose	0	Na lactate	20.0

Composition of growth medium: 2% of carbon source listed was added to the basal solution same as employed in the isolation experiment of active strains.

Condition of reaction: 0.5 ml of the washed cell suspension (ca. 50 mg dry cell) was incubated in the reaction mixture (pH 7.0) at 40°C for 48 hours.

Identification of D-Fructose.

The ketose formed by the cells grown in synthetic medium containing D-xylose, D-mannose or D-mannitol was identified by the paper chromatographic method as mentioned above

FIG. 1. Paper Chromatogram of the Ketose Formed by the Cells Grown in Synthetic Medium Containing Xylose, Mannose, and Mannitol.

The spots were obtained by spraying with the resorcinol reagent.

⁹⁾ R.S. Breed et al., "Bergey's Manual of Determinative Bacteriology", The Williams and Wilkins, Co., 1957, p. 342.

FIG. 2. Another paper Chromatogram obtained in the experiment of FIG. 1.

The spots were obtained by spraying with ammoniacal silver nitrate reagent.

(Figs. 1 and 2). Fig. 1 shows spots obtained by spraying with the resorcinol reagent²⁾ which reacts only with ketoses and their derivatives; Fig. 2 is the map of both aldose and ketose obtained by spraying with the ammoniacal silver nitrate reagent⁷⁾. Figs. 1 and 2 undoubtedly indicate that the ketose converted from D-glucose was D-fructose alone.

The effects of pH of the reaction mixture and the various metabolic inhibitors on the glucose-isomerization activities of the cells grown in D-mannitol synthetic medium were investigated in two further experiments, on the basis of new findings that not only the present strain had an activity under D-mannitol nutrition, but also in this experiment the activity was the highest of all carbon sources tested (Table II).

Effect of pH on Isomerization.

The optimum pH for isomerization by the acetone dried cells, grown under D-mannitol nutrition, was proved to be between 6.5~7.0, when the reaction was carried out in 0.04 M arsenate-HCl buffer (Fig. 3).

Effect of Metabolic Inhibitor on D-Fructose Accumulation and D-Glucose Isomerization.

By use of the washed living cells and the acetone dried cells, each of which was grown in D-mannitol synthetic medium, effects of several inhibitors listed in Table III on ac-

FIG. 3. Effect of pH of Reaction Mixture.

Condition of reaction: 40 mg of the acetone dried cells grown in mannitol synthetic medium was incubated at 40°C for 40 hours. pH of the reaction system was controlled with 0.04 M arsenate-HCl buffer.

cumulation of D-fructose and on isomerization of D-glucose were investigated respectively.

The left side of Table III illustrates the results with the living cells in the absence of the buffer, revealing that only arsenate permitted the accumulation of D-fructose. Since arsenate inhibits competitively by arsenolysis the formation of organic phosphate bond with ΔF (hydrolysis) more than 4,000 cal¹⁰⁾, this observation suggests that the process of glucose isomerization may proceed without passing through the phosphorylation, but the D-fructose, once formed, may be metabolized immediately after phosphorylation.

The effects of inhibitors on the glucose-isomerizing activities were examined by incubating the acetone dried cells together with inhibitors in the ordinary reaction system containing arsenate-HCl buffer (Table I). The results obtained are summarized in the right side of Table III. Metal chelating agents, for example sodium azide, potassium

10) J.B. Neilands et al., "Outlines of Enzyme Chemistry", John Wiley and Sons, Inc., 1955, p. 91.

TABLE III. EFFECTS OF VARIOUS INHIBITORS ON ISOMERIZING ACTIVITY

Buffer	None*		0.04 M arsenate buffer**		
	Final conc. of inhibitor, M	Fructose formed, mg	Final conc. of inhibitor, M	Fructose formed, mg	Relative isomerizing activity
Control	—	—	—	19.6	100
NaN ₃	4×10^{-2}	—	1.8×10^{-2}	23.6	120
KCN	4×10^{-2}	—	1.8×10^{-2}	17.7	90
NaF	4×10^{-2}	—	1.8×10^{-2}	18.0	92
EDTA***	—	—	1.8×10^{-2}	20.6	105
MIA***	4×10^{-2}	—	3.0×10^{-3}	15.4	79
pCMB***	—	—	3.0×10^{-5}	10.5	54
Ferricyanide	—	—	1.8×10^{-2}	4.9	25
Arsenite	4×10^{-2}	—	1.8×10^{-2}	25.5	130
2,4-DNP***	4×10^{-2}	—	3.0×10^{-4}	14.6	74
Arsenate	4×10^{-2}	15.0	—	19.6	100

* 30 mg (as dry matter) of the washed living cells was incubated at 40°C for 24 hours in 1 ml of the reaction mixture containing 180 mg of glucose in the absence of buffer.

** 40 mg of the acetone dried cells was incubated at 40°C for 24 hours in 1 ml of the ordinary reaction mixture containing 180 mg of glucose with 0.04 M arsenate-HCl buffer at pH 7.0.

*** The following abbreviations were used: EDTA, disodium salt of ethylenediaminetetraacetate; MIA, monoiodoacetic acid; pCMB, *p*-chloromercuribenzoic acid and 2,4-DNP, 2,4-dinitrophenol.

cyanide, sodium fluoride and disodium ethylenediaminetetraacetate did not show any appreciable effects, while free sulfhydryl reagents, i.e., monoiodoacetic acid, *p*-chloromercuribenzoic acid, especially ferricyanide apparently inhibited the isomerization activity. The isomerizing activity was stimulated by 30 per cents of the original activity by the addition of potassium arsenite, but 2,4-dinitrophenol inhibited by 25 per cents of the original activity. Reasons for such effects of arsenite and 2,4-dinitrophenol are obscure. Nevertheless, from these data, it appears that sulfhydryl group of glucose-isomerizing enzyme from the present strain, HN-56, is involved in the enzymatic activity and metals have little effects.

Presence of Glucose Isomerase and its Relationship to Xylose- and Mannose- Isomerase.

As mentioned above, Marshall et al.¹⁾ and Tsumura et al.^{2,3)} observed that only xylose among several sugars employed as sole carbon source of synthetic medium could give rise of both glucose- and xylose-isomerizing activities to *P. hydrophila*¹⁾ and *A. cloacae*^{2,3)},

and Marshall et al.¹⁾ stated that enzyme preparations including D-xylose isomerase will convert D-glucose to D-fructose, and also Tsumura et al.³⁾ found the identity of configurations on C₁~C₄ between the sugar as carbon source and the sugar as substrate of conversion. However, because it has been found that mannose isomerase is produced in *P. saccharophila* grown on D-fructose synthetic medium⁵⁾ and glucose isomerase is present in barley malt⁶⁾, an alternative possibility that some bacteria produce D-glucose isomerase under proper nutritional conditions may come up. Therefore, it is very interesting to investigate whether cells of the present strain grown under D-mannitol- or D-mannose nutrition have the D-xylose isomerase activity as well as those grown under xylose nutrition. In order to obtain a definite information in this respect, effects of D-mannitol, D-mannose and D-xylose as carbon source on various aldose-isomerization activities were investigated with the present strain, HN-56 of *A. aerogenes*, and a strain, HN-70, belonging to *Escherichia freundii*, isolated from soil in our

TABLE IV. INFLUENCE OF XYLOSE- MANNOSE- OR MANNITOL- NUTRITION ON THE ISOMERIZING ACTIVITIES ON SOME ALDOSES

Carbon source in growth medium	D-Xylose			D-Mannitol			D-Mannose		
	Mannose	Glucose	Xylose	Mannose	Glucose	Xylose	Mannose	Glucose	Xylose
<i>Aerobacter aerogenes</i> , HN-56.	0	16.7	7.0	5.9	36.5	0	0	13.0	0
<i>Escherichia freundii</i> , HN-70.	15.0	55.1	10.7	0	38.5	0	3.7	33.3	0

Condition of reaction: 36 mg (*A. aerogenes*) or 36 mg (*E. freundii*), as dry matter, of the washed living cells was incubated in 1 ml of the reaction mixture (pH 7.0) at 40°C for 48 hours.
 Figures in table show the ketose formed in mg.

laboratory. The results are illustrated in Table IV. The cells of two strains grown under D-mannitol and D-mannose nutrition had glucose-isomerizing activity though they had no activity of D-xylose isomerase, whereas those grown in D-xylose synthetic medium showed activities of both D-glucose and D-xylose isomerization. On the other hand, little or no D-mannose isomerase activity was found in all of the enzyme preparations, except for *E. freundii*, Strain HN-70 which was grown under D-xylose nutrition (Table IV). The results in Table IV decidedly demonstrate that glucose-isomerizing activity, at least under conditions of D-mannitol and D-mannose nutrition, had no direct relation to both xylose- and mannose-isomerase activities. It may be concluded that the D-glucose isomerase which converts free D-glucose to D-fructose without undergoing the phosphorylation process is produced under proper nutritional conditions, here under D-mannitol, D-mannose, and possibly D-xylose nutrition, and then D-glucose-isomerizing activity of the present strain is due to the activity of the D-glucose isomerase itself.

Further investigations on the D-glucose-isomerizing activity in other species of microorganisms, and on the specificity of carbon source in the enzyme formation would be described in subsequent report.

SUMMARY

A bacterial strain, HN-56, having D-glucose-isomerizing activity under D-xylose nutrition was isolated from soil, and from the results of investigations on its characters, it was concluded that the strain was similar to *Aerobacter aerogenes* (Kruse) Beijerinck. The strain also had the activity of D-glucose isomerization under D-mannose, sodium lactate, especially D-mannitol as well as under D-xylose nutrition. The ketose formed in the reaction system containing D-glucose was identified to be D-fructose, according to paper chromatographic method. The optimum pH for the reaction with acetone dried cells grown in D-mannitol synthetic medium was 6.5~7.0, when the 0.04 M arsenate-HCl buffer was used. Among the inhibitors tested, only arsenate could accumulate the D-fructose in the reaction system without any buffer, and it was found in the ordinary reaction system containing arsenate-HCl buffer that sodium azide, potassium cyanide, sodium fluoride and disodium ethylenediaminetetraacetate did not show appreciable effects, whereas monoiodoacetic acid, *p*-chloromercuribenzoic acid and ferricyanide apparently inhibited the D-glucose-isomerizing activity. These results suggest that the process of glucose isomerization may proceed without going through phosphorylation, but the D-fructose formed may be metabolized immediately after phosphoryla-

tion, and that sulfhydryl group of the glucose-isomerizing enzyme from the present strain is involved in the enzymatic activity, but metals have little effects. The cells grown under D-mannitol and D-mannose nutrition showed D-glucose-isomerizing activity though they had no activity of D-xylose isomerase, whereas those grown in xylose synthetic medium had both glucose- and xylose-isomerizing activities. Practically no D-mannose isomerase activity was found in all of the enzyme preparations, except for *E. freundii*, Strain HN-

70, grown under D-xylose nutrition. From these results, it may be concluded that some D-glucose isomerase bearing no relation to both D-xylose- and D-mannose isomerases, is present in *A. aerogenes*, Strain HN-56 and *E. freundii*, Strain HN-70, isolated from soil in our laboratory.

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