Studies on the Pentose Isomerases of Lactic Acid Bacteria*

Part IV.* Effects and Requirement of Metal

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D-Xylose isomerase requires manganese ions for its action, but L-arabinose isomerase has a less specific on metal requirement. L-Arabinose isomerase is activated by addition of Mn^{++} or Co^{++} , less effectively by addition of Zn^{++} , Ca^{++} , Mg^{++} , Sr^{++} or Cd^{++} . Moreover, manganese and potassium ions for D-xylose isomerase, and manganese and cobaltous ions for L-arabinose isomerase were also shown to have protective effect on respective enzymes against thermal inactivation.

INTRODUCTION

It has been well known that many enzymes require metallic ions for its action. In some of these enzymes metals are firmly bound with the enzyme proteins, but in others they are combined only loosely with the enzymes¹⁾. Enzymes belonging to the first group do not lose metals during the course of purification, but may be inactivated by a forced removal of metals. In the second group of enzymes, the metals dissociate during the purification, and can be easily removed by dialysis. Many metals were reported to activate one or more enzymes, such as Na^+ , K^+ , Mg^{++} , Mn^{++} , Ca^{++} , Cu^{++} , Co^{++} , Fe^{++} , Mo^{++} , Zn^{++} , Ni^{++} and so on. Concerning these important roles of metals on the activation of enzymes, many investigations were carried out^{1~5)}.

In our previous papers on the pentose iso-

merases^{6~8)}, magnesium chloride was used a priori as an activator in the isomerase reaction, but no attempt was made to clarify the role of metallic ions on the enzyme action. D-Xylose isomerases from *Pseudomonas hydrophila*⁹⁾ and from *Pasteurella pestis*¹⁰⁾ were reported to require manganese or magnesium ions for their activities. Magnesium ions were less effective for both cases.

Pentose isomerases of lactic acid bacteria were not inactivated by dialysis against water, but they were easily inactivated by treatment with EDTA** followed by dialysis against water. The addition of manganese ions restored the pentose isomerase activities of these inactivated enzyme preparations. Cobaltous ions were effective only for L-arabinose isomerase. Further evidences on the effects of metals and of inhibitors are presented in this paper.

* The former serial title is corrected as above. So the reports under "Studies on the Pyruvate and Carbohydrate Metabolisms by Lactic Acid Bacteria" are corrected as follows: Parts IX⁶⁾, X⁷⁾ and XI⁸⁾ under former title are corrected to Parts I, II and III, respectively.

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4) A.L. Lehninger, *Physiol. Rev.*, **30**, 393 (1950).

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6) K. Yamanaka, *Bull. Agr. Chem. Soc. Japan*, **22**, 299 (1958).

7) K. Yamanaka, *ibid.*, **24**, 305 (1960).

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10) M.W. Slein, *J. Am. Chem. Soc.*, **77**, 1663 (1955).

** The following abbreviations were used: Tris, Tris-(hydroxymethyl)-aminomethane; EDTA, disodium salt of ethylenediamine tetraacetate; pCMB, sodium salt of *p*-chloromercuribenzoate and 2, 4-DNP, 2, 4-dinitrophenol.

METHODS

Preparations of Enzymes

L-Arabinose isomerase was partially purified from extracts of L-arabinose-grown cells of *Lactobacillus gayonii*. This bacterium showed strong L-arabinose isomerase activity, while production of D-xylose isomerase was still low in the L-arabinose-medium⁸. For the production of D-xylose isomerase, *Lactobacillus brevis* was used. This bacterium produced a large amount of D-xylose isomerase, but only a small amount of L-arabinose isomerase in the D-xylose-medium⁹. Stock cultures of these organisms were maintained in agar-stabs containing one per cent of L-arabinose or D-xylose, respectively, and stored at 6°C.

The organisms were grown as described below. The medium was composed of 1 per cent peptone, 1 per cent yeast extract, 1 per cent sodium acetate, 0.02 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 per cent $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.001 per cent $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 per cent NaCl, and a sugar as carbon source. Microorganisms were incubated in test tubes with 8 ml each of a liquid-medium containing one per cent of pentose for 20 hours at 37°C. The culture was then transferred to 200 ml of the same pentose-medium and was grown for 20 hours, then it was transferred to 2.5 l of the same medium containing one per cent D-xylose and 0.1 per cent glucose for D-xylose isomerase production, or 0.6 per

cent L-arabinose and 0.5 per cent glucose for L-arabinose isomerase production. Pentoses were effective not only for the production of respective enzymes⁸, but also for promoting better growth of these heterofermenters^{8,11,12}.

After 24 hours culture, cells were harvested by centrifugation and washed twice with cold 0.02 M NaHCO_3 solution. Freshly separated cell pastes were stored at -10°C in frozen state, or were used immediately for preparation of cell-free extracts. About seven to eight grams of wet cells ground with 20 g of levigated alumina for five to eight minutes in the cold, extracted with ten times volume of chilled 0.05 M NaHCO_3 , and centrifuged at $13,000 \times g$ for thirty minutes. The precipitated cells were re-extracted once more with five times volume of 0.05 M NaHCO_3 . Yield of enzyme by second extraction was 10 to 15 per cent of the first extracts. First and second extracts were combined (Crude extracts), and all further procedures were carried out in the ice-cold. To the crude extracts containing about 3.0 to 5.0 mg of protein per ml, 0.05 volume of M MnCl_2 was added, and the resulted precipitate was removed by centrifugation (Mn-treated extracts). Solid ammonium sulfate was added to Mn-treated extracts to 0.60 saturation, and the precipitate was discarded (Fraction A). Further addition of solid ammonium sulfate was made to 0.90 saturation. The precipitate was collected, dissolved in 0.1 M phosphate

TABLE I. PREPARATION OF PENTOSE ISOMERASE

A. D-Xylose isomerase (*L. brevis*)

Fraction	Volume (ml)	Protein (mg)	Xylose isomerase		Arabinose isomerase	
			Specific activity	Total unit	Specific activity	Total unit
Crude extracts	80	386	1.49	574	0.08	30.8
Mn-treated extracts	80	142	5.62	800	0.34	48
Ammonium sulfate (0~0.6 sat.) fraction	13.4	40	0.35	13.7	0.03	1.1
Ammonium sulfate (0.6~0.9 sat.) fraction	13.2	29	12.9	378	0.58	16.9

B. L-Arabinose isomerase (*L. gayonii*)

Fraction	Volume (ml)	Protein (mg)	Arabinose isomerase		Xylose isomerase	
			Specific activity	Total unit	Specific activity	Total unit
Crude extracts	90	477	1.85	882	—	—
Mn-treated extracts	90	205	4.87	999	—	—
Ammonium sulfate (0~0.6 sat.) fraction	16.5	77	0.32	24.7	0	0
Ammonium sulfate (0.6~0.9 sat.) fraction	13.0	40	14.6	590	0.22	8.8

11) M.N. Camien and M.S. Dunn, *Proc. Soc. Exp. Biol. Med.*, **90**, 183 (1955).12) S.A. Koser and J.L. Thomas, *ibid.*, **95**, 89 (1957).

buffer at pH 7.4 and insoluble residues were removed. This solution was dialyzed against 0.02 M phosphate buffer at pH 7.4 and then against distilled water over 40 hours at $6 \pm 1^\circ\text{C}$ (Fraction B). This fraction was mainly used in this experiment. Further fractionation was carried out with acetone for L-arabinose isomerase, and the last acetone fraction (acetone 40~60 per cent) contained 25 per cent of the original activity. This step accomplished 10 fold increase in specific activity. Typical data for preparations of enzymes are shown in Table I. Enzyme preparations were stored at -10°C in frozen state without loss of activity over one month.

Activity Measurement

Enzyme was added to 0.5 ml of tris buffer of pH 7.4 and other ingredients, filled up to 2.8 ml with distilled water, and preincubated at 30°C for ten (or five) minutes, then 20 micromoles of pentose (L-arabinose or D-xylose) were added, and reaction was proceeded at 37°C for ten minutes. Ketopentose was determined by cysteine-carbazole method¹³⁾, and protein by Lowry's method¹⁴⁾.

A unit of enzyme was defined as the quantity which produces one micromole of ketopentose from aldopentose under those conditions, and specific activity was expressed as unit of enzyme per mg of protein.

RESULTS

Effects of Inhibitors

Both on D-xylose and L-arabinose isomerases from lactic acid bacteria, effects of several inhibitors including chelating agents and sulfhydryl or oxidizing reagents were examined. Experiments were performed repeatedly in duplicate or triplicate with enzyme preparations of different batches (25 to 130 μg of protein used). These results were summarized in Table II. Data are shown as averaged value and relative activity is expressed as per cent. With ten minutes' incubation of enzyme with these inhibitors (final concentration at 10^{-3} M), no or little inhibitory effects were observed except EDTA. Chelating agents, such as α, α' -dipyridyl, *o*-phenanthroline, and 8-hydroxyquinoline did not show any appreciable inhibitory effect. Moreover, other metal-

trapping materials such as pyrophosphate, organic acids, cyanide and azide did not inhibit enzyme activity. Only fluoride showed a little inhibitory effect (about ten per cent inhibition). EDTA showed strongly inhibi-

TABLE II. EFFECT OF INHIBITORS

Inhibitor (final, 10^{-3} M)	Relative activity (%)	
	D-Xylose isomerase	L-Arabinose isomerase
A. Chelating agent		
None	100	100
EDTA	97.3	36.7
α, α' -Dipyridyl	100.8	108.4
<i>o</i> -Phenanthroline	95.7	109.2
8-Hydroxyquinoline	91.0	111
Na-Pyrophosphate	100.0	96.4
K ₂ -Oxalate	104.5	88.7
K ₃ -Citrate	98.2	92.0
Na ₂ -Malonate	104	96.2
Chromotropic acid	125.5	95.2
NaF	85.5	87.0
KCN	102.5	90.5
NaN ₃	104	91.2
B. Sulfhydryl reagent		
None	100	100
Na-Moniodoacetate	107.8	87.5
pCMB	86.2	98.5
Cysteine	95.2	109
Cystine	103.5	105.5
C. Oxidizing reagent		
None	100	100
<i>o</i> -Iodosobenzoate	99.0	92.0
K ₃ Fe(CN) ₆	103	101
D. Others		
None	100	100
2,4-DNP	107.5	93.0
Hydroxylamine	105	75.8
Hydrazine	105	71.5
Semicarbazide	104	76.5
<i>m</i> -Phenylenediamine	107	85.2
Uracil	93.2	76.8
Urea	97.4	86.7
Urethane	98.3	94.7
Na-Arsenate	91.4	102
Na-Arsenite	92.2	99.8
Thiourea	102	102.5

After preincubation of enzyme with inhibitor at 30°C for 10 min., reaction was proceeded with addition of pentose at 37°C for 10 min. Addition of metal was omitted.

13) Z. Dische and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

14) O.H. Lowry, N.J. Rosenbrough, A.L. Forr and R.T. Randall, *ibid.*, **193**, 265 (1951).

tory effect on L-arabinose isomerase, and some results will be mentioned later. Sulfhydryl and oxidizing reagents, 2,4-DNP, urea and others were neither inhibitory nor stimulatory. Only slight inhibition was observed with addition of carbonyl reagents on L-arabinose isomerase. Furthermore, cysteine did not show stimulating effect on isomerase activity as shown in Table III.

TABLE III. EFFECT OF L-CYSTEINE ON L-ARABINOSE ISOMERASE ACTIVITY*

L-Cysteine-HCl (final, M)	0	2.5×10^{-3}	1×10^{-3}	5×10^{-4}	1×10^{-4}
Ribulose formed (μ g)	78.8	81.8	85.4	84.3	85.8

* Methods for preincubation and reaction were same as Table II. Addition of metal was omitted. Total protein used was 50 μ g.

Inhibition with EDTA and Reactivation by Metals

L-Arabinose isomerase was strongly inhibited by EDTA at 10^{-4} M in the final concentration (Table IV), while D-xylose isomerase was not inhibited at 10^{-3} M of EDTA. However, addition of more concentrated solution of EDTA and prolonged treatment with EDTA caused considerable inhibition on D-xylose isomerase activity (Tables V and VI). So it may be concluded that pentose isomerases of lactic acid bacteria require metallic ions for their activities. Further studies on reactivation by metallic ions of EDTA-treated enzyme

TABLE IV. INHIBITION BY EDTA ON L-ARABINOSE ISOMERASE*

EDTA (final, M)	0	10^{-3}	5×10^{-4}	10^{-4}	10^{-5}	10^{-6}
Ribulose formed (μ g)	90.5	29.9	29.1	29.9	83.6	90.3
Inhibition (%)	—	66.9	67.0	66.9	7.5	0

* Enzyme was incubated with EDTA for 10 min. at 30°C, total protein used was 50 μ g.

TABLE V. INHIBITION BY EDTA ON D-XYLOSE ISOMERASE*

EDTA (final, M)	0	10^{-2}	5×10^{-3}	10^{-3}
Xylulose formed (μ g)	105	66.6	96.0	104
Inhibition (%)	—	36.5	8.6	0

* Enzyme was incubated with EDTA for 10 min. at 30°C, total protein used was 110 μ g.

TABLE VI. INHIBITION BY EDTA ON D-XYLOSE ISOMERASE*

Time of Preincubation at 30°C (min)	Xylulose formed (μ g)			
	15	30	60	90
Without EDTA	131	136	148	115
With EDTA (final, 10^{-2} M)	102.3	66.5	46.0	35.8

* Enzyme was incubated with and without EDTA at 30°C for a definite time noted in Table, and then D-xylose was added and reaction was proceeded at 37°C for 10 min. Addition of metal was omitted. Protein, 110 μ g.

were made on L-arabinose isomerase. In this experiment, EDTA was added to L-arabinose isomerase and the mixture was preincubated at 30°C for ten minutes, followed by the addition of metallic ions, incubation at 30°C for five minutes, and then the addition of 20 micromoles of L-arabinose and incubation at 37°C for ten minutes for the determination of enzyme activity. As seen in Table VII, among twelve cations, only manganese or

TABLE VII. REVERSAL ACTION BY METAL ON EDTA-INACTIVATED L-ARABINOSE ISOMERASE*

Metal	I**		II***	
	Ribulose formed (μ g)	Relative activity (%)	Ribulose formed (μ g)	Relative activity (%)
Without EDTA-treatment****	73.5	100	58.1	100
+MgSO ₄	41.4	56.4	34.8	60.0
+MnSO ₄	86.3	117	82.7	145.2
+CoCl ₂	66.0	90	66.9	115
+FeSO ₄	46.7	63.5	34.3	59.2
+Fe ₂ (SO ₄) ₃	22.9	31.2	29.0	50.0
+CuSO ₄	21.1	28.8	27.3	47.1
+ZnSO ₄	43.2	58.8	45.4	78.2
+NiSO ₄	35.2	48.0	27.3	47.1
+CdSO ₄	34.3	46.0	37.0	63.8
+CaCl ₂	36.1	49.1	59.9	103
+BaCl ₂	28.3	38.5	27.3	47.1
+SrCl ₂	—	—	29.1	50.0
Without metal	25.5	34.8	21.1	36.4

* Protein used was 50 μ g. Treated enzyme by EDTA for 10 min. at 30°C, then 20 micromoles of L-arabinose added, and reaction was proceeded for 10 min. at 37°C.

** I. EDTA, 5×10^{-4} M, metal, 10^{-3} M; *** II. EDTA, 10^{-4} M, metal, 2×10^{-4} M.

**** Enzyme was preincubated at 30°C for 15 min, at pH 7.4 without addition of EDTA.

cobaltous ions had reactivating effects, and calcium ions were also effective in the case of inhibition by EDTA at 10^{-4} M. Addition of other divalent or trivalent cations had no effect.

Effect of Metallic Ions

An enzyme preparation was further dialyzed for 41 to 45 hours against distilled water at $2\pm 1^\circ\text{C}$, and the effect of added metallic ions on the enzymatic activity was determined. In this experiment, metallic ions were added to the dialyzed enzyme preparation, and the mixture was incubated at 30°C for five minutes at pH 7.4, before enzymatic reaction was initiated. As shown in Table VIII, the addition of manganese ions for D-xylose isomerase, and the addition of manganese, cobaltous and strontium ions for L-arabinose isomerase showed a stimulating effect on the enzyme activities. This effect by these metallic ions was observed at concentrations between 10^{-4} to 10^{-2} M (Table IX). Magnesium, calcium, barium

TABLE IX. STIMULATORY EFFECT BY METAL ON PENTOSE ISOMERASES

Metal (final, M)	Ribulose formed (μg) by arabinose isomerase*		Xylulose formed (μg) by xylose isomerase**	
	MnSO ₄	CoCl ₂	SrCl ₂	MnSO ₄
0	83.6	83.6	91.2	37.1
10^{-2}	109	111.2	127.7	130.8
10^{-3}	99.5	112.5	120.0	129.8
10^{-4}	106.6	104.8	127.7	122.8

* Protein, 62.8 μg ; ** Protein, 110 μg .

and mono-valent cations showed neither stimulatory nor inhibitory effect, but some di- and tri-valent cations, such as Cu^{++} , Zn^{++} , Fe^{+++} , Ni^{++} , Cd^{++} and Hg^{++} were inhibitory. Especially, Cu^{++} and Hg^{++} strongly inhibited the enzymatic activity even at lower concentrations (10^{-4} and 10^{-5} M).

Cofactor Requirement

As noted above, pentose isomerases from lactic acid bacteria were not inactivated by dialysis against water. It was found, however, that when EDTA was added to the enzyme before dialysis against water, isomerase was almost completely inactivated. From the results in Tables VII-IX, manganese or cobaltous ions seemed to be a cofactor for these enzymes. Other chelating agents, such as α, α' -dipyridyl, o-phenanthroline and nitroso-R were less effective (Table X).

In order to demonstrate the metal requirement, the following technique was employed. To an enzyme preparation with suitable dilution, EDTA was added to 10^{-3} M in final concentration, then this mixture was dialyzed against distilled water at 6°C for 42 hours or longer. After dialysis, the enzyme showed only slight activity never exceeding 10 per cent of the original activity. The activity was restored by the addition of manganese ions. With L-arabinose isomerase, the addition of manganese or cobaltous ions restored the isomerase activity, but zinc, cadmium, magnesium, calcium, or strontium was less effective.

TABLE VIII. EFFECT OF METAL

Metal (final, 10^{-3} M)	Relative activity (%)	
	L-Arabinose isomerase*	D-Xylose isomerase**
None	100	100
+MgSO ₄	96.1	102.2
+MnSO ₄	130.5	183.5
+CoCl ₂	133	64.5
+CuSO ₄	6.4	39.3
+ZnSO ₄	51.2	37.3
+FeSO ₄	77	68.8
+Fe ₂ (SO ₄) ₃	14.7	76.7
+BaCl ₂	107.2	89.8
+CaCl ₂	112	61.1
+NiSO ₄	87.8	47.2
+CdSO ₄	79	69.0
+SrCl ₂	140	96.3
+KCl	103	—
+Li ₂ SO ₄	101	—
+HgCl ₂	0	35.6

* L-Arabinose isomerase: acetone fraction, protein 88 μg , dialyzed 41 hrs.

** D-Xylose isomerase: fraction B, protein 110 μg (specific activity, 12.9), dialyzed 45 hrs.

TABLE X. TREATMENT BY CHELATING AGENTS ON DIALYSIS

Treated enzyme with*	Water		Xylulose formed (μ g)		α, α' -Dipyridyl		<i>o</i> -Phenathroline		Nitroso-R	
	—	+	—	+	—	+	—	+	—	+
Reaction with 10^{-3} M MnSO_4										
Dialyzed for (hr.)										
0	94.7									
17	55.0	73.2	14.1	96.7	80.7	74.8	38.4	70.4	55.8	81.2
42	50.0	64.7	5.1	69.2	41.7	57.7	23.0	51.2	38.4	62.7
65	41.7	55.0	6.4	82.6	29.4	64.0	35.2	97.3	33.2	71.0
89	35.2	—	0	66.5	16.0	51.2	19.2	—	22.4	53.7

* D-Xylose isomerase (protein, 1.94 mg/ml 2.0 ml+water or chelating agent (10^{-2} M) 2.0 ml, mixture were dialyzed at $2 \pm 1^\circ\text{C}$. One tenth ml of dialyzed enzyme solution were taken off at every interval for measurement of isomerase activity.

TABLE XI. METAL REQUIREMENT

Metal added (final, M)	D-Xylose isomerase* Xylulose formed (μ g)		L-Arabinose isomerase** Ribulose formed (μ g)
	10^{-3}	10^{-2}	10^{-3}
None	11.5	20.5	4.4
+ MgSO_4	18.5	26.2	14.5
+ MnSO_4	137.0	119	63.4
+ CoCl_2	32.0	33.3	52.4
+ FeSO_4	28.8	—	1.3
+ $\text{Fe}_2(\text{SO}_4)_3$	30.7	—	4.8
+ ZnSO_4	19.2	—	22.4
+ BaCl_2	22.4	—	8.4
+ CaCl_2	25.6	33.3	15.4
+ NiCl_2	7.7	—	9.2
+ CdSO_4	18.5	—	10.1
+ SrCl_2	12.8	12.8	11.0
+ CuSO_4	11.5	—	—
+ KCl	17.3	33.3	5.7
+ NaCl	11.5	23.0	—
+ Li_2SO_4	18.5	19.2	6.6
Before dialysis			
with MnSO_4 (10^{-3} M)	235.5	235.5	103.0

* Dialyzed for 65 hrs., protein used for enzymatic assay, 50 μ g.

** Dialyzed for 42 hrs., protein used for enzymatic assay, 65.4 μ g.

Fig. 1 shows that full activity is attained with about 1×10^{-4} M of Mn^{++} , or with 5×10^{-4} M of Co^{++} . However, the degree of restoration of activity by addition of Co^{++} ions is always lower than by Mn^{++} . For D-xylose isomerase, only manganese ions are able to restore the activity. Cobaltous, magnesium, iron (ferrous, ferric), calcium, barium, zinc, cadmium, or potassium ions show only little effect. Thus

FIG. 1. Requirement of Metal on L-Arabinose Isomerase*

* EDTA-inactivated enzyme (EDTA, final 5×10^{-4} M), protein, 64 μ g/3 ml. Reaction condition was presented in the text.

FIG. 2. Requirement of Mn ion on D-Xylose Isomerase*

* EDTA-inactivated enzyme (EDTA, final 5×10^{-4} M, dialyzed for 65 hrs.) protein 67.5 μ g.

it was concluded that D-xylose isomerase required manganese ions specifically, but L-arabinose isomerase was less specific in the metal requirement.

Effect of Metals on Thermal Inactivation

Pentose isomerases were easily inactivated by heat treatment. As shown in Fig. 3, the incubation of the enzyme at 60°C over two minutes at pH 7.4, destroyed the activity of D-xylose isomerase completely and that of L-arabinose isomerase to about 50 per cent. In this heat treatment of enzyme the addition of specific cofactors (metallic ions) protected the enzyme from thermal inactivation. Besides cofactors, in the case of D-xylose isomerase, potassium chloride, bromide or sulfate was effective in the protection, but potassium carbonate, tartarate or phosphate was completely inactive. Whereas the effect of potassium salts was observed only at very high concentrations, manganese was effective even at low concentrations as shown in Fig. 5. On the other hand, the effect of manganese or cobaltous ions for the protection of L-arabinose isomerase activity from thermal inactivation was observed at concentrations between 1~

FIG. 3. Thermal Inactivation of Pentose Isomerase*

Curve A. L-Arabinose isomerase (protein, 30.9 μ g, specific activity 14.6)

Curve X. D-Xylose isomerase (protein, 95 μ g, specific activity 23.4)

* Experimental conditions for the measurement of thermal inactivation see in Table XII.

TABLE XII. EFFECT OF METAL ON THERMAL INACTIVATION

O.D. 540 $m\mu$ by cysteine-carbazole test

Incubation time at 60°C (min.)	Xylose isomerase*			Arabinose isomerase**	
	10	10	5	10	10
Salt (final, M)	2×10^{-2}	10^{-3}	2×10^{-2}	2×10^{-2}	10^{-3}
KCl	0.428	0.034	0.630	0.327	0.402
KBr	0.373	—	—	0.325	—
K ₂ SO ₄	0.387	—	—	0.288	—
K ₂ HPO ₄ ***	0.063	—	—	0.221	—
K ₂ CO ₃ ***	0.067	—	—	0.238	—
K ₂ -Tartarate	0.128	—	—	0.330	—
NaCl	0.055	0.039	0.132	0.383	0.470
Na ₂ SO ₄	0.018	—	—	0.347	—
Li ₂ SO ₄	0.030	0.033	0.080	0.303	0.469
MgCl ₂	0.004	—	—	—	—
MgSO ₄	0.007	0.070	0.012	0.353	0.372
MnCl ₂	0.023	—	—	0.690	—
MnSO ₄	0.331	0.155	0.704	0.885	0.975
CoCl ₂	0.038	—	0.048	0.534	0.580
BaCl ₂	0.038	—	0.064	—	0.343
CaCl ₂	0.040	—	—	0.385	0.410
SrCl ₂	0.042	0.024	0.068	0.407	0.440
None	0.028	0.005	0.071	0.286	0.348
Without heat treatment	0.691	0.416	0.691	0.741	0.741

* Xylose isomerase (protein 95 μ g, specific activity, 23.4).

** Arabinose isomerase (protein 30.9 μ g, specific activity, 14.6).

*** pH was adjusted with 0.1 N HCl.

After treated enzyme at 60°C for definite time and cooled in ice-bath, enzyme reaction was carried out with added 10^{-3} M of MnSO₄ and 20 micromoles of pentose at 37°C for 10 min.

FIG. 4. Effect of MnSO₄ and of CoCl₂ on Thermal Inactivation of L-Arabinose Isomerase*

* Protein used, 30.9 μ g. After heat-treatment, 10^{-3} M of MnSO₄ and L-arabinose were added.

FIG. 5. Effect of KCl and of MnSO_4 on Thermal Inactivation of D-Xylose Isomerase*

* Protein used, 110 μg . After heat-treatment, 10^{-3} M of MnSO_4 and D-xylose were added.

10×10^{-3} M, and potassium salts were completely inactive.

DISCUSSION

A few reports are available on the inhibition study of pentose isomerase from bacteria. On D-xylose isomerase from *Pasteurella pestis* (Mn-treated, dialyzed preparation)¹⁰, it has been reported that the enzyme was inhibited by pCMB (2.5×10^{-3} M) completely, and its activity was restored by addition of cysteine to 80 per cent of the original activity. The enzyme was stimulated by cysteine and, to a lesser extent, by glutathione in the absence of pCMB. Iodoacetate, iodoacetamide, N-ethylmaleimide and Tris inhibited the activity of this enzyme. With D-xylose isomerase from *Pseudomonas hydrophila* (sonic extracts, acetone powder)⁹, activity was decreased to 20 to 30 per cents of the original activity by addition of 2,4-DNP, or 8-hydroxyquinoline at a final concentration of 10^{-2} M. But the addition of pCMB, iodoacetate, adenine, adenosine, sulfanilamide, fluoroacetate, hydroxylamine, alloxan, citrate, urethane, fluoride and azide showed neither inhibition nor stimulation.

In this paper, D-xylose and L-arabinose isomerases from lactic acid bacteria were not inhibited by pCMB, iodoacetate, 2,4-DNP or

8-hydroxyquinoline at a final concentration of 10^{-3} M, in contrast to the D-xylose isomerase described above. The activity of D-xylose isomerase from *Pasteurella pestis* was stimulated 2 to 3 fold by the presence of cysteine¹⁰, while the pentose isomerases from lactic acid bacteria were not stimulated by cysteine. So, it appears that pentose isomerases from lactic acid bacteria do not require sulfhydryl groups for their action.

Moreover, it has been found that L-arabinose isomerase from lactic acid bacteria is strongly inhibited by EDTA, and that the addition of Mn^{++} , Co^{++} or Ca^{++} ions can restore the full activity of the EDTA-inactivated enzyme.

Further evidences have been presented here for the metal requirements of the enzymes. With dialyzed preparations, L-arabinose isomerase is stimulated by the presence of Mn^{++} , and also by the presence of Co^{++} or Sr^{++} . Moreover, the enzyme was almost completely inactivated by treatment with EDTA followed by dialysis against water. The activity was restored by the addition of Mn^{++} or Co^{++} . Manganese and also magnesium ions were required also for D-xylose isomerases from *Pseud. hydrophila*⁹ and from *Pasteurella pestis*¹⁰. D-Arabinose isomerase from *E. coli* B_{a15} was activated by the addition of manganese ions¹⁵.

SUMMARY

1. Pentose isomerases from lactic acid bacteria were not inhibited by α, α' -dipyridyl, o-phenanthroline, 8-hydroxyquinoline, pyrophosphate, organic acids, cyanide, azide, monoiodoacetate, pCMB, cysteine, cystine, 2,4-DNP, urea, ferricyanide, o-iodosobenzoate, arsenate and arsenite.

2. L-Arabinose isomerase was strongly inhibited by EDTA, but D-xylose isomerase showed a considerable resistance against the EDTA-inhibition. With L-arabinose isomer-

15) D.M. Scott and S.S. Cohen, *Biochem. J.*, **65**, 686 (1957).

ase, enzymatic activity was fully restored by addition of Mn^{++} , Co^{++} or Ca^{++} ions after inactivation by EDTA.

3. Mn^{++} for D-xylose isomerase, and Mn^{++} , Co^{++} , or Sr^{++} for L-arabinose isomerase showed stimulating effect, but Cu^{++} , Zn^{++} and Hg^{++} strongly inhibited both D-xylose- and L-arabinose isomerase activities.

4. Pentose isomerases were inactivated by treatment with EDTA followed by dialysis against water. With addition of Mn^{++} for D-xylose isomerase, and of Mn^{++} or Co^{++} for L-arabinose isomerase, enzymatic activities were restored. Other cations were less effective. It seemed that D-xylose isomerase required Mn^{++} specifically and L-arabinose isomerase Mn^{++} or Co^{++} for their activities.

5. The cofactors were also effective for the protection of the enzymes from the thermal inactivation.

Addendum Dr. Lampen¹⁶⁾ presented in detail his data on the metal requirement of D-xylose and L-arabinose isomerases from *L. pentosus*. Both isomerases were inactivated by dialysis against 0.02 M EDTA over-night. The metal requirement of L-arabinose isomerase appeared to be considerably less specific than that of D-xylose isomerase. Addition of

Mn^{++} , Co^{++} (10^{-4} M) or Ca^{++} (10^{-3} M) gave essentially the same maximal activity, and Mg^{++} , Ni^{++} , or Zn^{++} were less effective on L-arabinose isomerase. D-Xylose isomerase was activated by Mn^{++} (10^{-4} M), and slight activation was observed with Ca^{++} , Co^{++} , and Mg^{++} within about ten per cent of that obtained with Mn^{++} .

We think that our results on metal requirement of pentose isomerases are essentially in agreement with those by Dr. Lampen.

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16) J.O. Lampen, Personal communication (September 15, 1960).