Properties of 3-Hexulose Phosphate Synthase and Phospho-3-hexuloisomerase of a Methanol-utilizing Bacterium, 77a

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The key enzymes, 3-hexulose phosphate synthase and phospho-3-hexuloisomerase, of the pentose monophosphate pathway for formaldehyde fixation were purified from a methanol-grown bacterium 77a, and their enzymatic properties were investigated. The condensation product between formaldehyde and D-ribulose 5-phosphate by 3-hexulose phosphate synthase was identified as D-arabino-3-hexulose 6-phosphate, on the bases of UV-absorption spectrum, pH stability and color reactions with several reagents on thin-layer chromatography. The apparent K_m value of 3-hexulose phosphate synthase for each substrate was determined: formaldehyde, 0.74; D-ribulose 5-phosphate, 0.081; D-arabino-3-hexulose 6-phosphate, 0.036mM. The apparent K_m values of phospho-3-hexuloisomerase for D-arabino-3-hexulose 6-phosphate and D-fructose 6-phosphate were found to be 0.029 and 0.67 mM, respectively.

No activity of phospho-3-hexuloisomerase was able to detect in the cell-free extract of methanol-utilizing yeasts, *Kloeckera* sp. No. 2201 and *Hansenula polymorpha* DL-1, under the condition tested.

Some bacteria growing on reduced C_1 -compounds incorporate the C_1 unit into cell materials by way of the pentose monophosphate pathway.¹⁾ The pathway involves the condensation of formaldehyde with D-ribulose 5-phosphate to give D-arabino-3-hexulose 6-phosphate which is then isomerized to D-fructose 6-phosphate.^{2~4)} 3-Hexulose phosphate synthase and phospho-3-hexuloisomerase catalyzing the condensation and isomerization, respectively, have been purified from a methane-utilizing bacteria, *Methylococcus capsulatus*, and characterized.⁵⁾

An obligate methylotroph, 77a, has been found to possess the enzyme activity of the condensation of formaldehyde with pentose phosphate.⁶⁾ In this work, the purification and characterization of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from the bacterium 77a were investigated and these properties are compared with those of *M. capsulatus* reported by Ferenci *et al.*⁵⁾

Fujii *et al.*,⁷⁾ reported that formaldehyde

was firstly incorporated to a hexose phosphate by a methanol-utilizing yeast, *Candida* N-16. The activity of pentose phosphate-dependent fixation of formaldehyde has been detected in the cell-free extracts of *Kloeckera* sp. No. 2201,⁸⁾ *Candida boidinii*⁹⁾ and *Candida* N-16.¹⁰⁾ However, this fixation mechanism has not been elucidated in detail. In this paper, it is also described, if the isomerization of D-arabino-3-hexulose 6-phosphate prepared by 3-hexulose phosphate synthase from the bacterium 77a is catalyzed by the cell-free extract of the yeasts.

MATERIALS AND METHODS

Materials. Phosphoriboisomerase and alkaline phosphatase (from calf intestinal mucosa) were purchased from Sigma Chemical Company (St. Louis, U. S. A.). Phosphoglucosomerase, glucose 6-phosphate dehydrogenase and alcohol dehydrogenase were products of Boehringer Mannheim GmbH (Mannheim, West Germany). D-Ribose 5-phosphate sodium salt was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Avicel plate SF® was a product of Asahikasei Co., Ltd. (Tokyo, Japan). All other

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chemicals were obtained from commercial sources and were used without further purification. Formaldehyde was prepared by heating 0.5 g of paraformaldehyde in 5 ml of water at 100°C in a sealed tube for 15 hr.

Organisms and growth. The obligate methylo-troph used, strain 77a, was able to grow on only methanol as carbon source.⁶⁾ It was maintained and grown at 28°C in a medium of composition (per 100 ml): methanol, 2.0 g; NaNO₃, 0.5 g; K₂HPO₄, 0.2 g; NaCl, 0.1 g; MgSO₄·7H₂O, 0.02 g; pH 7.0. The cultivation was carried out in a jar-fermentor (30 liters volume) under the aeration of 1 vvm. After 24~30 hr of growth, cells were harvested by continuous flow centrifugation, washed with 10 mM of potassium phosphate buffer containing 1 mM MgCl₂, pH 7.4, and stored at -18°C until use.

Enzyme assay. *3-Hexulose phosphate synthase* (D-arabino-3-hexulose 6-phosphate formaldehyde lyase). The activity was assayed as a routine by measuring the rate of D-ribulose 5-phosphate-dependent disappearance of formaldehyde according to Ferenci *et al.*⁶⁾ The spectrophotometric assay of 3-hexulose phosphate synthase was done by using the assay mixture of Dahl *et al.*,¹¹⁾ supplemented with 1.3 units of phosphoriboisomerase (EC 5.3.1.6) and 1.0 units of phospho-3-hexuloisomerase purified from the bacterium 77a: in this assay, the addition of phospho-3-hexuloisomerase, phosphoglucosomerase (EC 5.3.1.9) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) links the production of D-arabino-3-hexulose 6-phosphate with NADP⁺ reduction. 3-Hexulose phosphate synthase was assayed in the reverse direction, by measuring the rate of formaldehyde formation from D-arabino-3-hexulose 6-phosphate according to Ferenci *et al.*⁶⁾

Phospho-3-hexuloisomerase. The activity was measured in a system with final volume of 0.5 ml containing 50 mM imidazole-HCl buffer, pH 7.0, 5 mM MgCl₂, 4 mM D-ribose 5-phosphate, 0.9 U phosphoriboisomerase, 2 mM formaldehyde, 1 U 3-hexulose phosphate synthase, 0.7 U phosphoglucosomerase, 0.07 U glucose 6-phosphate dehydrogenase, 0.5 mM NADP and enzyme. After D-arabino-3-hexulose 6-phosphate had been prepared, the sugar phosphate (0.2 mM) was used in the place of the mixture of D-ribose 5-phosphate, phosphoriboisomerase and formaldehyde. These assays were carried out spectrophotometrically at 30°C by following the reduction of NADP⁺ at 340 nm. The enzyme was assayed in the reverse direction by following the formation of formaldehyde from D-fructose 6-phosphate according to Ferenci *et al.*⁶⁾ Phosphoriboisomerase was assayed according to Horecker *et al.*¹²⁾

Analytical method. Inorganic phosphorus was assayed by the method of Takahashi,¹³⁾ formaldehyde by the method of Nash,¹⁴⁾ ribulose 5-phosphate by

cysteine-carbazole method¹⁵⁾ and protein by the method of Lowry *et al.*¹⁶⁾ The formaldehyde solution was standardized with alcohol dehydrogenase (EC 1.1.1.1) according to Bernt and Gutman.¹⁷⁾ D-Fructose 6-phosphate was assayed by the method of Lang and Michal.¹⁸⁾ D-arabino-3-Hexulose 6-phosphate was assayed by isomerization to D-fructose 6-phosphate with 1 unit of phospho-3-hexuloisomerase and the D-fructose 6-phosphate produced was assayed as described above. Nonenzymatic formation of D-fructose 6-phosphate was corrected for by running parallel assays lacking phospho-3-hexuloisomerase.

Partial purification of 3-hexulose phosphate synthase. All operations were performed at 0 to 5°C throughout the purification. All the buffers used were potassium phosphate buffer, pH 7.4, containing 1 mM MgCl₂ and 2-mercaptoethanol, unless otherwise stated.

Step 1. Preparation of cell-free extract. The washed cells (45 g as dry weight) were suspended in 750 ml of 10 mM buffer and treated with a Kaijo Denki ultrasonic oscillator (19 kHz) for 30 min. The cells and debris were removed by centrifugation at 16,000×g for 30 min.

Step 2. Protamine sulfate precipitation. To 850 ml of the supernatant (21.7 g protein) was added 2% protamine sulfate solution, to a final concentration of 140 mg of protamine sulfate to 1 g of protein. After 30 min, the precipitate was discarded by centrifugation at 16,000×g for 30 min. The supernatant was dialyzed against two changes of 10 liters of the buffer described above for 18 hr.

Step 3. Ammonium sulfate precipitation. To 900 ml of dialyzed enzyme was added 504 g of solid ammonium sulfate to 80% saturation. After standing 3 hr, the precipitate was collected by centrifugation at 16,000×g for 20 min and was dissolved in 130 ml of 10 mM buffer. The solution was dialyzed against 10 liters of the same buffer.

Step 4. 1st DEAE-cellulose column chromatography. The adsorbent, equilibrated with 10 mM buffer, was packed into a column (5.5×76 cm). The dialyzed enzyme solution which contained 7 g of protein was placed on the column. Elution was carried out with stepwise increases in the buffer concentration. The enzyme activity was found in the fraction eluted with 50 mM buffer. The active fractions were concentrated by the addition of ammonium sulfate to 70% saturation. The precipitate obtained was dissolved in a small volume of 10 mM of the phosphate buffer, and was dialyzed against the same buffer.

Step 5. 2nd DEAE-cellulose column chromatography. The dialyzed enzyme solution (37 ml) was rechromatographed on a column of DEAE-cellulose (4×39 cm) which had been equilibrated with 20 mM Tris-HCl buffer, pH 8.04. The enzyme activity was found in the fractions eluted with 100 mM Tris-HCl, pH 8.04, con-

taining 100 mM NaCl. The enzyme solution concentrated with the addition of ammonium sulfate (70% saturation) were dialyzed against 10 mM buffer.

Step 6. Hydroxylapatite column chromatography. The dialyzed enzyme solution (10 ml) was applied to a column (2×15 cm) of hydroxylapatite previously equilibrated with 10 mM buffer. The enzyme activity was eluted from the column with the equilibration buffer, close to the void volume of the column. The active fractions (55 ml) were concentrated to 3 ml by the same manner as described above.

Step 7. Sephadex G-200 gel filtration. The enzyme solution was subjected to Sephadex G-200 column chromatography. The gel, which had been equilibrated with 10 mM buffer, was used to pack a column (3×70 cm). The active fractions were pooled to give 40 ml. The enzyme was precipitated by the addition of ammonium sulfate to 70% saturation and stored in a refrigerator at 5 to 7°C.

Partial purification of phospho-3-hexuloisomerase. All operations were performed at 0 to 5°C throughout the purification. The first 3 steps were performed in the same batch as described in the purification of 3-hexulose phosphate synthase.

Step 4. DEAE-cellulose column chromatography. The chromatography of this enzyme was carried out concurrently in the case of 3-hexulose phosphate synthase. Phospho-3-hexuloisomerase activity was found in the fractions eluted with 0.1 M buffer. The active fractions were concentrated by the addition of ammonium sulfate to 80% saturation. The precipitate was dissolved in a small volume of 50 mM buffer and was dialyzed against the same buffer.

Step 5. DEAE-sephadex column chromatography. The dialyzed enzyme solution (146 ml, containing 1.85 g protein) was chromatographed on a column of DEAE-Sephadex (3.8×38 cm) previously equilibrated with 50 mM buffer. The enzyme activity was found in the fractions eluted with 100 mM potassium phosphate buffer. The enzyme solution concentrated (402 mg of protein) by the addition of ammonium sulfate (80% saturation) and dialyzed against 10 mM buffer.

Step 6. Hydroxylapatite column chromatography. A part of the enzyme solution (100 mg protein) was applied to a hydroxylapatite column (2×15 cm) which had been equilibrated with 10 mM buffer. The activity was eluted with the equilibration buffer. The active fraction was concentrated to 2 ml by the addition of ammonium sulfate.

Step 7. Sephadex G-200 gel filtration. This treatment was performed in the same way as described in the case of 3-hexulose phosphate synthase. The resulting enzyme was precipitated by the addition of ammonium sulfate to 80% saturation.

Synthesis of D-arabino-3-hexulose 6-phosphate. D-

arabino-3-Hexulose 6-phosphate was prepared by the method of Strøm *et al.*²¹ The partially purified 3-hexulose phosphate synthase from bacterium 77a was used for the condensation of formaldehyde and D-ribulose 5-phosphate in concentration of 339 U in 100 ml of the reaction mixture. Parallel to decrease of formaldehyde and D-ribulose 5-phosphate, the formation of D-arabino-3-hexulose phosphate was observed. D-arabino-3-hexulose 6-phosphate in eluate was enzymatically assayed as described above. The separation of sugar phosphates was performed according to Khym and Cohn.¹⁹ The elution pattern was closely similar to that reported by Strøm *et al.*²¹ The pH of sugar phosphate solution was adjusted to 3.0. The solution was stored at 5°C.

Dephosphorylation of the sugar phosphate. Sugar phosphate was dephosphorylated by an alkaline phosphatase. The reaction mixtures contained 1 mM sugar phosphate, 20 mM Tris-HCl buffer, pH 8.5, 50 mM MgCl₂, 400 µg alkaline phosphatase in total volume of 16 ml. The reaction was carried out at 37°C for 30 min, and terminated with the addition of 0.1 ml of 4 N HCl.

Preparation of cell-free extract of methanol-utilizing yeasts. The cultures of methanol-utilizing yeasts, *Kloeckera* sp. No. 2201²⁰ and *Hansenula polymorpha* DL-1²¹ were carried out as described previously. The cells in the cultured broth at the middle of exponential growth phase were harvested by centrifugation and washed with 10 mM buffer. The washed cells were disrupted by an ultrasonic oscillator or a Vibrogen Cell Meal.[®] The cells and debris were removed by centrifugation at 16,000×g for 30 min. The supernatant was used as the cell-free extract. The above procedures were carried out below 5°C. The cell-free extracts of yeasts were also prepared by the autolysis with toluene according to Calder *et al.*²²

RESULTS

Properties of D-arabino-3-hexulose phosphate

Some properties of the sugar phosphate which was obtained by the enzyme-catalyzed condensation reaction of formaldehyde with D-ribulose 5-phosphate as described in MATERIALS AND METHODS section were examined. The absorption spectrum of the sugar phosphate at pH 7.0 shows a maximum at 280 nm ($\epsilon=210 \text{ liter} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and a minimum of 250 nm. This is closely similar to the results of D-arabino-3-hexulose phosphate obtained by *Methylococcus capsulatus*²¹ but the molar

extinction coefficient value obtained is relatively higher than that of the latter. The UV-absorption spectrum shows that the sugar phosphate cannot form a ring structure.

The sugar phosphate was more unstable in alkaline solution than in acid. A little non-enzymatic formation of D-fructose phosphate from the original sugar phosphate was observed in slightly alkaline solution (pH 7.5 to 8.5).

The sugar phosphate was identified by dephosphorylation and thin-layer chromatographies using an avicel plate® of the resulting sugars. The dephosphorylated preparation of the sugar phosphate gave a 3-hexulose/inorganic phosphorus ratio of 1:1.19, in which 3-hexulose was determined as the phosphate form. This ratio shows that this sugar phosphate preparation was contaminated with other phosphates. Most of the contaminants was thought to be D-fructose 6-phosphate which was nonenzymatically formed from the original sugar phosphates. The results of color reactions and *R_f* values on the chromatographies are summarized in Table I. The color reactions and *R_f* values of the sugar to be identified were clearly different from those of other sugars tested. On the chromatogram, the sugar gave a pink color with urea-phosphate spray.²³⁾ The sugar was colored yellow-

brown and yellow with diphenylamine-aniline²⁴⁾ and resorcinol-HCl²⁵⁾ reagents, respectively. These color reaction were some different from common hexuloses such as fructose and so on. The properties of the sugar (phosphate) described above are well compatible with those of D-arabino-3-hexulose (6-phosphate) reported by Kemp⁴⁾ and Strøm *et al.*²⁾

Purification and properties of 3-hexulose phosphate synthase

It was reported that the enzyme activity was located in a particulate fraction of a methane-utilizing bacterium, *M. capsulatus*.²⁹⁾ In the bacterium 77a which was able to utilize only methanol as carbon source,⁶⁾ the activity was detected in the supernatant obtained by centrifugation of cell-free extract at 40,000×*g* for 60 min. Through the purification procedures as described in MATERIALS AND METHODS section, the enzyme was purified about 50-fold from the cell-free extract. The specific activity of the partially purified enzyme was 12.6 U/min·mg in the standard conditions.

Since the purified enzyme preparation contained a small amount of phosphoriboisomerase, the preparation catalyzed the condensation of formaldehyde with D-ribose 5-phos-

TABLE I. THIN-LAYER CHROMATOGRAPHIES OF SEVERAL SUGAR AND THE DEPHOSPHORYLATED PRODUCT

	Ribulose	Ribose	Glucose	Fructose	Product
Color reaction					
Spray reagent					
A	brown	NC ^{a)}	NC	gray-green	pink
B	yellow	green	brown	yellow	yellow-brown
C	green	NC	NC	red	yellow
			<i>R_f</i> value		
Solvent system					
a	0.50	0.48	— ^{b)}	0.41	0.34
b	0.48	—	—	0.31	0.36
c	0.64	—	0.56	0.59	0.68

^{a)} no color.

^{b)} did not tested.

Layer: Avicel plate SF®

Spray reagent : (A) urea-phosphate²³⁾ (B) diphenylamine-aniline²⁴⁾ (C) resorcinol-HC^[25]

Solvent system : (a) *n*-butanol: pyridine: H₂O (6: 4: 3 v/v/v)²⁶⁾

(b) acetone: acetic acid: H₂O (20: 6: 5 v/v/v)²⁷⁾

(c) phenol (90% w/v): formic acid: H₂O (500: 13: 167 w/v/v)²⁸⁾

FIG. 1. Effect of pH on the Activities of 3-Hexulose Phosphate Synthase and Phospho-3-hexuloisomerase of the Bacterium 77a.

Both reaction mixtures were the same as described in MATERIALS AND METHODS section except for the uses of different buffers; potassium phosphate buffer, pH 5.5 to 7.5, Tris-HCl buffer pH 8.0 to 9.0, and boric acid-NaOH buffer, pH 10.0.

3-Hexulose phosphate synthase (●—●) and phospho-3-hexuloisomerase (○—○) were used in the concentrations of 0.02 and 0.03 U/ml, respectively.

FIG. 2. Effect of Temperature on the Activities of 3-Hexulose Phosphate Synthase and Phospho-3-hexuloisomerase.

The activity of each enzyme at different temperatures was assayed under the same conditions as described in Fig. 1, with the following enzyme concentrations; 3-hexulose phosphate synthase (○—○), 0.01 U/ml and phospho-3-hexuloisomerase (●—●), 0.013 U/ml.

phate in the standard assay conditions without phosphoriboisomerase. However the synthase activity increase about 3 times by the supplement of the isomerase. Therefore, the true acceptor of formaldehyde in the condensation reaction was thought to be D-ribulose 5-phosphate.

As shown in Fig. 1, the activity of the enzyme was optimum at pH 8.0. The optimum temperature of the enzyme was 40°C (Fig. 2).

The Michaelis constant (K_m) for each of the substrates of the enzyme were determined by the method of Lineweaver and Burk³⁰⁾ (Fig. 3). The apparent K_m for formaldehyde and D-ribulose 5-phosphate were 0.74 mM (determined at 0.4 mM D-ribulose 5-phosphate concentration) and 0.081 mM (determined at

FIG. 3. Determination of Apparent K_m Values of 3-Hexulose Phosphate Synthase for Formaldehyde. (a), D-ribulose 5-phosphate (b) and D-arabino-3-hexulose 6-phosphate (c).

The activities were assayed under the standard conditions as described in MATERIALS AND METHODS section and the text. D-Ribulose 5-phosphate with defined concentration was prepared according to Ferenci *et al.*⁵⁾ Velocity (V) is expressed as μ moles each product formed per min (U/min).

FIG. 4. Determination of Apparent K_m Values of Phospho-3-hexuloisomerase for *D-arabino*-3-Hexulose 6-Phosphate (a) and *D*-Fructose 6-Phosphate (b).

The activities were assayed under the standard conditions as described in MATERIALS AND METHODS section. Velocity (V) is expressed as U/min.

4 mM formaldehyde concentration), respectively. In the reverse direction, the apparent K_m for *D-arabino*-3-hexulose 6-phosphate was 0.036 mM (Fig. 4).

Purification and properties of phospho-3-hexuloisomerase

The isomerase was purified as described in MATERIALS AND METHODS section. The specific activity of the partially purified enzyme was 20 U/min·mg in the standard conditions. The enzyme preparation did not contain the activity of 3-hexulose phosphate synthase.

The enzyme activity was optimum at pH 7.5 (Fig. 1) and at 30°C (Fig. 2). The apparent K_m value for *D-arabino*-3-hexulose 6-phosphate was found to be 0.029 mM. In the reverse reaction, the apparent K_m value for *D*-fructose 6-phosphate was 0.67.

Searchs for 3-hexulose phosphate synthase and phospho-3-hexuloisomerase activities in methanol-utilizing yeasts

The activities of the condensation of formaldehyde with a pentose phosphate in the cell-free extracts of *Kloeckera* sp. No. 2201 and *Hansenula polymorpha* DL-1 was assayed in the standard conditions with or without 5 mM ATP. No activities was detected in any cell-free extract tested. While, formaldehyde not dependent formation of *D*-fructose 6-

phosphate from *D*-ribose 5-phosphate or *D*-ribulose 5-phosphate was obtained. This formation probably occurred by the catalysis of phosphoriboisomerase, ribulose phosphate epimerase, transketolase and transaldolase.

The activity of phospho-3-hexuloisomerase in the cell-free extract of yeasts was assayed using *D-arabino*-3-hexulose phosphate prepared by the bacterial 3-hexulose phosphate synthase as substrate. The reaction mixture was the same as used for the bacterial enzyme except that 0.5 mg of cell-free extract of the yeast was contained. The reaction was carried out at 30°C up to 20 min. An aliquot of the mixture was taken at 5 min intervals, and were added to HClO_4 (final concentration 0.5 M), following neutralization with 2 M K_2CO_3 . *D*-Fructose 6-phosphate formed and *D-arabino*-3-hexulose 6-phosphate disappeared were assayed as described in MATERIALS AND METHODS section. No appreciable disappearance of the substrate nor formation of *D*-fructose 6-phosphate dependent on the cell-free extract used was detected in the standard conditions. No effect of variation of pH (6.0 to 7.5) or addition of ATP (5 mM) and MgCl_2 (1 mM) on the isomerization was observed.

DISCUSSION

The bacterium 77a has been shown to possess the ribulose monophosphate pathway for formaldehyde fixation but not the serine pathway.⁶⁾ In this work, the purifications of the two enzymes, 3-hexulose phosphate synthase and phospho-3-hexuloisomerase, make it possible to confirm, in this bacterium, the enzymic conversion of *D*-ribulose 5-phosphate and formaldehyde into *D*-fructose 6-phosphate via *D-arabino*-3-hexulose 6-phosphate. Some enzymic properties of these two enzymes are summarized in Table II, in which those of both enzymes from *M. capsulatus*⁵⁾ are presented for comparison. A difference in the properties of the two 3-hexulose phosphate synthases is in optimum pH for the activity, which were found to be 8.0 and 7.0 for the enzymes from the bacterium 77a and *M.*

TABLE II. COMPARISON OF THE PROPERTIES OF 3-HEXULOSE PHOSPHATE SYNTHASE AND PHOSPHO-3-HEXULOISOMERASE FROM BACTERIUM 77a AND *M. capsulatus*

Properties	Unit	Value with enzyme from	
		bacterium 77a	<i>M. capsulatus</i> ⁴⁾
3-Hexulose phosphate synthase			
Optimum temperature	°C	40	—
Optimum pH		8.0	7.0
<i>Km</i> for HCHO	mM	0.74	0.49
<i>Km</i> for D-ribulose 5-phosphate	mM	0.081	0.083
<i>Km</i> for D-arabino-3-hexulose 6-phosphate	mM	0.036	0.075
Phospho-3-hexuloisomerase			
Optimum temperature	°C	30	—
Optimum pH		7.5	8.3
<i>Km</i> for D-arabino-3-hexulose 6-phosphate	mM	0.029	0.10
<i>Km</i> for D-fructose 6-phosphate	mM	0.67	1.1

capsulatus, respectively. The affinities for substrates of the enzyme from the bacterium 77a are similar to those from *M. capsulatus*.

There are some differences in the pH optima and the affinities for the substrate of phospho-3-hexuloisomerases from the two organisms. The apparent *Km* value for D-arabino-3-hexulose 6-phosphate of the enzyme from 77a is considerably lower than that of the *M. capsulatus* enzyme.

Evidence has been obtained for occurrence of a C₁-assimilation pathway like the pentose monophosphate pathway in the methanol-utilizing yeasts, on the bases of the isotope studies with intact cells⁷⁾ and cell-free extracts.^{8~10)} In contrast to the bacterial 3-hexulose phosphate synthase, the cell-free extracts of the yeasts have been shown to require ATP for the condensation reaction.¹⁰⁾ Little information on detail of the enzyme reaction or the intermediate has so far been obtained. In present work, no activity of phospho-3-hexuloisomerase was detected in the cell-free extracts of methanol-grown *Kloeckera* sp. No. 2201 and *H. polymorpha* DL-1. Since the reaction conditions for the assay of the yeast enzyme have not completely been investigated, we cannot conclude that phospho-3-hexuloisomerase is absent in the methanol-utilizing yeasts. However, it is possible to say that the yeasts possess no enzyme

activity like that which appeared in the bacteria. If phospho-3-hexuloisomerase is absent in the yeasts, the condensation reaction of C₁- and C₅-compounds is thought to be catalyzed by another type of enzymes than the bacterial ones.

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