D-Ribulose Production by a Thiamine-requiring *Corynebacterium* Species†

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The effect of thiamine on the D-ribulose production from gluconate by a thiamine-requiring *Corynebacterium* species was investigated. The D-ribulose production by the cells previously grown in a thiamine-deficient medium was higher than that by the cells grown in a thiamine-rich medium and supplementation of the thiamine-deficient cells with thiamine resulted in a significant depression of the D-ribulose production. Gluconokinase and NADP-linked phosphogluconate dehydrogenase were detected in the cell-free extract of this organism. Oxidation and anaerobic dissimilation of D-ribose 5-phosphate by the cell-free extract of the thiamine-deficient cells are reduced and the addition of thiamine pyrophosphate to the extract enhanced the catabolic activities for D-ribose 5-phosphate. These results suggest that the accumulation of D-ribulose by the thiamine-deficient cells is a consequence of a reduction of transketolase activity.

In the previous paper,¹⁾ it was shown that a thiamine-requiring species of *Corynebacterium* was able to accumulate D-ribulose extracellularly when grown on gluconate as a sole carbon source. The accumulation of this ketopentose was depended on the thiamine concentration in the growth medium and the maximum accumulation was observed at the restricted concentration of thiamine for growth.

Accordingly, thiamine appeared to play a significant role in the accumulation of D-ribulose.

The experiments described in this paper were undertaken to investigate the effect of a deficiency of thiamine upon the accumulation of D-ribulose.

MATERIALS AND METHODS

Organism. *Corynebacterium* sp. No. 208, which was isolated by the authors, was employed for this study. This organism strictly requires thiamine for growth and is characterized by preferential utilization of gluconate as compared to other carbon sources.²⁾

† Gluconate Metabolism by a Thiamine-requiring Species of *Corynebacterium*. Part V. For Part IV, see Reference 1). This work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan in Nagoya, April 2, 1968.

Cultural conditions. The organism was grown on a bouillon agar slant for 48 hr at 30°C and a loopful of cells was inoculated to the growth medium. The growth medium consists of potassium gluconate, 6.0%; (NH₄)₂SO₄, 0.4%; KH₂PO₄ 0.1%; MgSO₄·7H₂O, 0.05%; and thiamine hydrochloride (pH 6.5). For the production of thiamine-rich and thiamine-deficient cells, thiamine hydrochloride was added to the medium at 100 µg and 10 µg per liter, respectively. Cultivation was carried out at 30°C on a reciprocal shaker operating at 210 rpm.

Preparation of washed cell suspension. After growth for 48 hr in the media described above, the cells were harvested by centrifugation in the cold, washed 3 times with distilled water, and suspended in 0.05 M phosphate buffer (pH 7.0).

Preparation of cell-free extracts. The washed cells were resuspended in 0.05 M phosphate buffer (pH 7.0) or 0.05 M tris-(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.5) and disrupted by sonic oscillation for 60 min in a 200 watt, 10 kc sonic generator. The disrupted cells suspension was centrifuged at 19,000 × g for 40 min to remove cell debris. The supernatant fluid was then treated with charcoal and dialyzed against 0.05 M phosphate buffer or 0.05 M Tris buffer. The dialyzed solution was used as a cell-free extract of the organism throughout the experiments.

Enzyme assays. Phosphogluconate dehydrogenase activity was assayed spectrophotometrically by following the reduction of NADP at 340 mµ with 6-phos-

phogluconate as the substrate at 30°C. The reaction mixture contained 0.5 μ mole of NADP, 2 μ moles of 6-phosphogluconate, 0.5 μ mole of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 M Tris buffer (pH 7.5), and the cell-free extract (1.25 mg of protein) in a total volume of 3.0 ml. Gluconokinase activity was measured by coupling the formation of 6-phosphogluconate to phosphogluconate dehydrogenase present in the extract and following reduction of NADP at 340 m μ . The assay was carried out under the same conditions as for phosphogluconate dehydrogenase activity except that 1.0 μ mole of ATP and 2.5 μ moles of gluconate were substituted for 6-phosphogluconate. Transketolase activity was assayed according to the method of Brin³⁾ except that the temperature of incubation was 30°C. This method is based on the conversion of D-ribose 5-phosphate to hexose phosphate in the presence of thiamine pyrophosphate.

Analytical methods. D-Ribulose was determined in the cysteine-carbazole reaction as described previously.¹⁾ The concentration of protein in the cell-free extracts was determined by the method of Lowry *et al.*⁴⁾ Oxygen uptake was determined by conventional Warburg manometric techniques at 30°C.

Chemicals. ATP, NAD, NADP, 6-phosphogluconate, D-ribose 5-phosphate and glyceraldehyde-3-phosphate were products of Sigma Chemical Company. Thiamine hydrochloride and thiamine pyrophosphate were purchased from Tokyo Kasei Company.

RESULTS

Production of D-ribulose from gluconate by washed cell suspensions

As shown in Fig. 1, the maximum production of D-ribulose from gluconate under growing conditions occurred at a thiamine hydrochloride concentration of 10 μ g per liter and further increase in the thiamine concentration caused a marked decrease in the production though an abundant cell growth was observed.

Therefore, the cells previously grown in media containing thiamine hydrochloride at 10 μ g and 100 μ g per liter were employed for the investigation of the effect of thiamine on the D-ribulose production. The cells thus obtained were referred to herein as thiamine-deficient and thiamine-rich cells, respectively.

The D-ribulose production from gluconate by the thiamine-deficient cells was higher than

FIG. 1. Effect of Thiamine Concentration in the Growth Medium on D-Ribulose Accumulation and Cell Growth.

The basal growth medium was as described in Materials and Methods.

Cultivation was carried out at 30°C for 96 hr on a reciprocal shaker operating at 210 rpm.

○—○, D-ribulose; ●—●, dry cell weight.

that by the thiamine-rich cells as indicated in Table I. Moreover, supplementation of the

TABLE I. D-RIBULOSE PRODUCTION FROM GLUCONATE BY WASHED CELL SUSPENSION

The incubation mixture consisted of 1 mmole of potassium gluconate, 25 μ moles of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 ml of cell suspension (80 mg of dry cell), and 4 ml of 1/30 M phosphate buffer at pH 7.0 in a total volume of 10 ml.

Incubation was carried out at 30°C for 18 hr on a reciprocal shaker operating at 210 rpm.

Addition to incubation mixture	D-Ribulose produced (mmoles)	
	Thiamine-deficient cells	Thiamine-rich cells
None	0.16	0.08
Thiamine hydrochloride, 10 μ g	0.03	0.05
Oxythiamine, 100 μ g	0.15	0.07

thiamine-deficient cells with thiamine resulted in a significant depression of the production of D-ribulose.

These data suggest that the D-ribulose accumulation in this organism was introduced

by the reduction of thiamine in the cells. Oxythiamine had no effect on the D-ribulose production by the thiamine-rich cells.

Demonstration of gluconokinase and phosphogluconate dehydrogenase activities in cell-free extracts

Since D-ribulose 5-phosphate is an intermediate in the hexose monophosphate pathway, the presence of gluconokinase and phosphogluconate dehydrogenase in the cell-free extract prepared from the thiamine-deficient cells was examined. Figures 2 and 3 show that

FIG. 2. Gluconokinase Activity in a Cell-free Extract.

Reaction mixture was as described in Materials and Methods.

○—○, ATP was added; ●—●, no addition.

gluconokinase and NADP-dependent phosphogluconate dehydrogenase are present in the extract. As shown in Table II, gluconate oxidation by this extract occurred in the presence of ATP. In addition, gluconate oxidation and pyruvate production by the washed cells suspension were found to be inhibited by the presence of 0.001 M monoiodoacetate. In *Corynebacterium* species, the hexose monophosphate pathway is believed to be the major route for the assimilation of gluconate.⁵⁾

It appears from these results that in this organism gluconate is metabolized *via* the hexose monophosphate pathway after con-

FIG. 3. Phosphogluconate Dehydrogenase Activity in a Cell-free Extract.

Reaction mixture was as described in Materials and Methods.

○—○, NADP; ●—●, NAD.

TABLE II. EFFECT OF ATP ON GLUCONATE OXIDATION BY A CELL-FREE EXTRACT

The Warburg vessel contained 10 μ moles of potassium gluconate, 10 μ moles of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 ml of cell-free extract (12.5 mg of protein), and 0.5 ml of 1/10 M phosphate buffer at pH 7.0. The central well contained 0.2 ml of 20% KOH. All vessels contained a total volume of 3.0 ml. Endogenous oxygen uptake was subtracted.

Addition to reaction mixture	Oxygen uptake (μ l of O_2 /mg of protein/hr)
None	0
NAD ^{a)} + NADP ^{a)}	0
NAD + NADP + ATP ^{b)}	7.7

^{a)} 1 mole.

^{b)} 5 moles.

verting to 6-phosphogluconate.

Oxidation of D-ribose 5-phosphate by cell-free extracts

It has previously been shown that pyruvate was oxidized at a decreased rate by the suspension of the thiamine-deficient cells.⁶⁾

The oxidation of several substrates was examined using cell-free extracts prepared from the thiamine-deficient and thiamine-rich cells. As shown in Table III, the extract of the thiamine-deficient cells demonstrated poor oxidative ability for D-ribose 5-phosphate and pyruvate.

TABLE III. OXIDATION OF VARIOUS SUBSTRATES BY CELL-FREE EXTRACTS

The Warburg vessel contained 10 μ moles of substrate, 10 μ moles of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 μ moles of ATP, 1 μ mole of NAD, 1 μ mole of NADP, 1.0 ml of cell-free extract (12.5 mg of protein), and 0.5 ml of 1/10 M phosphate buffer at pH 7.0. The center well contained 0.2 ml of 20% KOH. All vessels contained a total volume of 3.0 ml. Endogenous oxygen uptake was subtracted.

Substrate	Oxygen uptake (μ l of O_2 /mg of protein/hr)	
	Thiamine-deficient cells	Thiamine-rich cells
Gluconate	6.6	7.5
6-Phospho-D-gluconate	3.2	3.5
D-Ribose 5-phosphate	2.5	6.5
Glyceraldehyde 3-phosphate	3.4	3.3
Pyruvate	0.1	1.6

Since the low rate of D-ribose 5-phosphate oxidation was presumed to be due to a decreased amount of thiamine in the extract, thiamine pyrophosphate was added to the extract to determine the effect of thiamine pyrophosphate on the D-ribose 5-phosphate oxidation. The data are presented in Fig. 4. The addition of thiamine pyrophosphate had some stimulating effect on the rate of D-ribose 5-phosphate oxidation in the thiamine-rich cells but a much greater effect was observed in the thiamine-deficient cells.

Dissimilation of D-ribose 5-phosphate by cell-free extracts

Further investigation was undertaken to determine the effect of thiamine on the dissimilation by the extracts according to the method of Brin.³⁾ This method has been widely used to determine the transketolase activity in the erythrocytes and the various tissues of animals. In this method, transketolase activity is obtained by the measurements of the amount of pentose which is consumed and the amount of hexose which is formed during the incubation. Moreover, the levels of restoration of the enzyme activity by the addition of thiamine pyrophosphate,

FIG. 4. Effect of Thiamine Pyrophosphate on D-Ribose 5-Phosphate Oxidation by Cell-free Extracts. The experimental conditions were the same as those in Table III. Endogenous oxygen uptake was subtracted.

○—○, thiamine-deficient cells; ●—●, thiamine-rich cells.

+TPP, thiamine pyrophosphate was added to a final concentration of 3×10^{-6} M.

which is called "TPP effect," can also be estimated. Therefore, this method appeared to be suitable to evaluate the effect of thiamine pyrophosphate on the dissimilation of D-ribose 5-phosphate by the extract of thiamine-deficient cells. The results are given in Table IV. In the extract of the thiamine-deficient cells, both the consumption of pentose and the formation of hexose were significantly reduced. In addition, the remarkable restoration of the dissimilation of D-ribose 5-phosphate by the addition of thiamine pyrophosphate was observed in the extract of the thiamine-deficient cells.

DISCUSSION

Previous papers⁶⁾ in this series have shown that a thiamine-requiring strain of *Corynebacterium* accumulated pyruvic acid extracellularly when grown under the restricted condition for thiamine and the accumulation of the keto acid was introduced by the depression

TABLE IV. DISSIMILATION OF D-RIBOSE 5-PHOSPHATE BY CELL-FREE EXTRACTS

Assay procedure: The assay system in a total volume of 1.15 ml consisted of 0.2 ml of D-ribose 5-phosphate (7.0 mg of ribose per ml), 0.45 ml of Brin buffer (pH 7.4), and 0.5 ml of cell-free extract (12.5 mg of protein per ml) with and without the addition of 0.45 ml of thiamine pyrophosphate (0.11 mg of thiamine pyrophosphate per ml). Incubation was carried out at 30°C for 60 min.

Determination of pentose and hexose: After 60 min 6.0 ml of 7.5% trichloroacetic acid was added to the reaction mixture. The protein free filtrate was used for the determination of the amount of the pentose consumed by the orcinol method with a 20-min heating time at 670 m μ and of the hexose formed by the anthrone method with a 10-min heating time at 620 m μ .

Cell-free extract	Pentose consumption (μ g/mg of protein/hr)			Hexose formation (μ g/mg of protein/hr)		
	No addition	+TPP ^{a)}	TPP effect ^{b)} (%)	No addition	+TPP	TPP effect (%)
Thiamine-deficient cells	90.4	246.0	172.1	40.0	168.2	302.5
Thiamine-rich cells	270.0	287.1	6.3	181.9	205.9	13.2

a) Thiamine pyrophosphate was added to the reaction mixture.

b) Values presented are percentage changed due to the presence of added thiamine pyrophosphate.

of the activity of pyruvate oxidation due to the decreasing the thiamine content of the cells.

As shown in Table I, the accumulation of D-ribulose by this organism was also found to be the result of the thiamine deficiency of the cells.

It is probable that the accumulation of D-ribulose by the thiamine-deficient cells, like that of pyruvic acid, is due to the decreased activity of thiamine-requiring enzymes involved in the catabolism of gluconate.

In the thiamine-deficient cells as shown in Fig. 4, D-ribose 5-phosphate was oxidized at a decreased rate and a marked increase in the oxidation rate was caused by the addition of thiamine pyrophosphate. Similarly, anaerobic dissimilation of D-ribose 5-phosphate in the thiamine-deficient cells was significantly enhanced by the addition of thiamine pyrophosphate (Table IV).

Thiamine pyrophosphate is known to function as a coenzyme for the enzymes involving decarboxylation and transketolation. It has been reported that the activity of transketolase in microorganisms depended on the thiamine content of the medium.^{7,8)} Sasajima and Yoneda⁹⁾ reported that transketolase-lacking mutants of a *Bacillus* species accumulated D-ribose and D-ribulose 5-phosphate 3-epimerase-lacking mutants accumulated both D-ribose and D-ribulose.

Although further work is required to investigate the participation of some enzymes responsible for the catabolism of pentose phosphate to the accumulation of D-ribulose, it may be concluded from the present studies that the accumulation of D-ribulose by the thiamine-deficient cells is probably a consequence of a reduction of transketolase activity and that the reduced transketolase activity is not the result of a depletion of apoenzyme but a deficiency of coenzyme.

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