Vitamin B6 Biosynthesis of a Pdx Mutant, *Escherichia coli* B WG3, in Amino Acid-supplemented Medium[†]

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Alanine, cystine, cysteine, β -hydroxypyruvate, an amino acid mixture and glycolaldehyde, which were found to satisfy the vitamin B6 (B6) requirement of *Escherichia coli* B WG3, a *pdxB* mutant, by paper disc assay method, were examined about the effect on growth of the mutant in liquid cultivation. Alanine and β -hydroxypyruvate did not significantly affected growth under the culture conditions, whereas cysteine and cystine supported growth to some extent. The amount of B6 synthesized by the mutant in the amino acid-supplemented medium was from 0.05 to 0.12 nmol per ml. The amount of B6 synthesized in the medium, which was supplemented with an amino acid mixture composed of L-cysteine, L-threonine and L-methionine, was about 0.15 nmol per ml. This amount of B6 was lower than the amount (0.4 nmol per ml) biosynthesized in glycolaldehyde-supplemented medium. The data suggested that growth of the mutant supplemented with amino acids could not be supported by only the B6 biosynthesized from them, because such amounts of B6 were much smaller than the amount of B6 (0.6 nmol per ml) required for full growth of the mutant in minimal medium.

To find out the biosynthetic intermediate of vitamin B6 (B6), we established a paper disc assay method in a previous paper.¹⁾ As the result, D- and L-alanine, D- and L-cysteine, L-cystine, β -hydroxypyruvate and an amino acid mixture composed of 18 amino acids, besides glycolaldehyde, a precursor of B6,²⁾ were found to be active in the assay system. Although the plate assay method was very convenient to screen such compounds among many samples, detailed examination of these compounds as for the involvement in B6 biosynthesis was difficult by the method.

In the present paper, effect of these compounds on growth and B6-biosynthesis of *Escherichia coli* B WG3, a *pdxB* mutant, was examined in detail by a liquid cultivation. Selection of the essential components in the amino acid mixture to support sufficient growth of the mutant, and B6 biosynthesis in the essential components-supplemented medium were also described.

MATERIALS AND METHODS

Microorganisms. A *pdxB* mutant, *E. coli* B strain WG3,³⁾ which was characterized to lack two isozymes of glycolaldehyde dehydrogenase involved in the first step of B6 biosynthesis,⁴⁾ was used in this study. *Saccharomyces carlsbergensis* (syn., *uvarum*) ATCC 9080 was used for determination of B6.

Chemicals. All chemicals used were usual commercial products and were used without further purification.

Media. Glucose and glycerol media were as described by Dempsey.⁵⁾ Glucose was separately autoclaved to avoid the degradation to glycolaldehyde which could support the growth of *E. coli* WG3. Glycerol was autoclaved together with minimal salts. Cysteine, β -hydroxypyruvate and glycolaldehyde were sterilized by filtration. Other amino acids involving L-cysteine in the amino acid mixture were autoclaved together with minimal salts. When B6 was required, pyridoxine hydrochloride was supplemented into the medium at the final concentration of 0.1 μ g per ml.

Cultivation. A fresh culture of *E. coli* WG3 which was grown with reciprocal shaking at 37°C overnight in a 300-ml Erlenmeyer flask containing 50 ml of pyridoxine-supplemented medium was centrifuged at 6,000 rpm for 10 min and washed twice with each 50 ml of sterilized 0.85% NaCl. The washed cells were suspended into 50 ml of

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sterilized 0.85% NaCl and each 2.0 ml of this suspension was inoculated into each 50 ml of supplemented- or non-supplemented medium. Cultivation was carried out at 37°C with reciprocal shaking in a 500-ml shaking flask containing 50 ml of the medium.

Growth. Growth was followed by measuring optical density at 610 nm with a Hitachi/Model 101 spectrophotometer. All readings were made in test tubes (16.5 by 165 mm) in the range of 0 to 0.5 O.D. units. Dry cell weight was calculated from O.D. units by reference to a calibration curve which was prepared according to the method of Dempsey.⁵⁾ Relative growth was represented as percentage of O.D. units of the culture grown in pyridoxine-supplemented medium.

Determination of B6 amount. The total content of all forms of B6 in culture was assayed by yeast bioassay method after acid-hydrolysis.⁵⁾

RESULTS

Growth of E. coli B WG3 in supplemented liquid medium

Glycolaldehyde, D- and L-alanine, D- and L-cysteine, L-cystine and β -hydroxypyruvate formed growth zone on the assay plate of the paper disc assay method to detect B6 biosynthetic intermediate.¹⁾ However, the growth zone made by these compounds except for glycolaldehyde was thin in the density. To compare the growth in each supplement, *E. coli* WG3 was cultivated in the liquid medium containing each one of these compounds.

Figure 1 shows the growth when glycolaldehyde was supplemented into glucose or glycerol medium at concentration of 25 μ g per ml. The cells which were harvested from pyridoxine-supplemented glucose or glycerol medium were inoculated. Both the glucose- and glycerol-grown cells could grow similarly when they were cultivated in glucose medium. They showed the maxima of growth and B6 biosynthesis after 4-hr cultivation. But the apparent delay of growth of the glucose-grown cells was observed in glycerol medium. The similar behavior of the mutant was also observed when it was cultivated in pyridoxine-supplemented medium. From these results, glucose- and glycerol-grown cells were inoculated into glucose and glycerol media,

FIG. 1. Growth and B6 Biosynthesis of *E. coli* WG3 in Glycolaldehyde-supplemented Media.

The washed cells obtained from pyridoxine-supplemented glucose (○) or glycerol (●) medium, were inoculated into glucose or glycerol medium supplemented glycolaldehyde (25 μ g per ml).

—, growth (mg dry cell weight per ml); ---, B6 amount (nmol per ml).

respectively, in the following experiments.

When any compounds other than glycolaldehyde were supplemented into glucose and glycerol media, no significant growth of the mutant was observed after 6-hr cultivation. In glucose medium, the mutant gradually grew even in non-supplemented medium as the cultivation time was prolonged. As a consequence, no significant difference of growth in non-supplemented and supplemented media was obtained by the prolongation. The growth in non-supplemented glucose medium reached more than 0.5 mg dry cell weight per ml at 22-hr cultivation. The cells obtained from the culture could grow in non-supplemented medium as same rate as in pyridoxine-supplemented medium. This result indicates that reversion of the mutant to wild type can easily occur in glucose medium when the cultivation time become longer. On the other hand, no significant growth was observed in non-supplemented glycerol medium even when the cultivation time was prolonged more

FIG. 2. Growth of *E. coli* WG3 in Glycerol Medium Containing Pyridoxine, Cysteine or Cystine.

The medium used was glycerol medium. The concentrations of supplements were $0.1 \mu\text{g}$ per ml for pyridoxine (PN) and $100 \mu\text{g}$ per ml for D- and L-cysteine and L-cystine, respectively.

than 20 hr. The significant growth in supplemented glycerol medium was observed when the cultivation time was prolonged more than 6 hr.

In Fig. 2, the growth of the mutant in glycerol medium which was supplemented with each $100 \mu\text{g}$ per ml of L-cysteine, D-cysteine or L-cystine is shown in comparison with the growth in non-supplemented and pyridoxine-supplemented media. In cysteine- or cystine-supplemented medium, the mutant began to grow at about 6 hr and reached the full growth at about 12 to 15 hr. Thus, the activity of cysteine and cystine to support the growth of the mutant in the paper disc assay method reappeared in liquid cultivation when glycerol medium is used and the cultivation time is prolonged. Table I shows the growth in glycerol medium supplemented with various concentration of each compound. L- and D-Cysteine and L-cystine showed significant effect on growth of the mutant at the concentration of $100 \mu\text{g}$ per ml, but not at the lower ($10 \mu\text{g}$ per ml) and higher

TABLE I. EFFECT OF THE COMPOUNDS ON GROWTH OF *E. coli* WG3

Each compound was supplemented into glycerol medium.

Cultivated time (hr)	Supplement		Relative growth (%)
		Concentration ($\mu\text{g}/\text{ml}$)	
16	Pyridoxine·HCl	0.1	100
	L-Cysteine	10	13
		100	78
		1000	3
	D-Cysteine·HCl	10	16
		100	67
		1000	2
	L-Cystine	10	19
		100	73
		1000	23
	D-Cystine	10	65
		100	70
		1000	69
	None	0	9
22	Pyridoxine·HCl	0.1	100
	L-Alanine	10	12
		100	11
		1000	10
	D-Alanine	10	12
		100	10
		1000	4
	β -Hydroxypyruvate	10	9
		100	8
		1000	5
	None	0	11

($1000 \mu\text{g}$ per ml) concentrations. But D-cystine, which was not tested in the paper disc assay method, supported significantly the growth even at the lower and higher concentrations. L- and D-Alanine and β -hydroxypyruvate did not show any significant effect on the growth at any concentrations tested, even when the cultivation time was prolonged to 22 hr. These were rather inhibitory in the growth at the higher concentration.

Effect of cysteine and cystine on B6 biosynthesis by E. coli WG3

E. coli WG3 was found to be able to grow in liquid glycerol medium which was supplement-

FIG. 3. Cell Mass and B6 Amount of *E. coli* WG3 Grown in Glycolaldehyde-, Cysteine- or Cystine-supplemented Medium.

E. coli WG3 was cultivated for 6 hr in glycolaldehyde-supplemented glycerol medium and for 16 hr in cysteine- or cystine-supplemented glycerol medium. ●, cell mass (mg dry cell weight per ml); ○ and ●, B6 amount (nmol per ml) and (nmol per mg cell), respectively.

ed with cysteine or cystine and cultivated for more than 6 hr. It was shown that B6-requirement of the mutant was due to lack of two isozymes of glycolaldehyde dehydrogenase which catalyzed oxidation of glycolate to glycolaldehyde as the first step enzyme of B6 biosynthesis.^{4,7)} Consequently, the amount of B6 which was formed during the growth was measured to know whether the activity of cysteine and cystine is due to the replacement of glycolaldehyde as a biosynthetic intermediate or not. Figure 3 shows the cell mass and the B6 amount obtained after 16-hr cultivation with supplement of various concentrations of the amino acids. The values obtained from the cultures after 6-hr cultivation in glycolaldehyde-supplemented medium were also shown for comparison.

With supplement of 100 μ g per ml of D- and L-cysteine which was the best concentration for the growth, the amount of B6 in the culture showed the maximal value, 0.057 and 0.087 nmol per ml with D- and L-cysteine, respectively. The growth and B6 biosynthesis by supplement of D-cystine was not influenced so much by change of the concentration. The highest amount of B6 in the culture was 0.055

TABLE II. EFFECT OF OMISSION OF AN AMINO ACID FROM THE AMINO ACIDS MIXTURE ON GROWTH OF *E. coli* WG3

The medium used was glycerol medium. The growth was measured after 9-hr cultivation. The growth in pyridoxine-supplemented medium was taken as 100%.

Omission	Relative growth (%)
All	11
None	182
L-Cysteine	5
L-Threonine	7
L-Alanine	34
L-Methionine	83
L-Arginine	150
Glycine	157
L-Phenylalanine	160
L-Lysine	166
L-Leucine	167
L-Aspartic acid	169
L-Valine	171
L-Isoleucine	174
L-Proline	174
L-Glutamic acid	177
L-Serine	179
L-Tyrosine	183
L-Histidine	186
L-Tryptophan	189

TABLE III. EFFECT OF AMINO ACIDS COMBINATION ON GROWTH OF *E. coli* WG3

The medium used was glycerol medium. Each amino acid was supplemented at concentration of 100 μ g per ml. The growth was after 9-hr cultivation and shown as same as Table II.

Amino acid mixture	Amino acid composition				Relative growth (%)
	L-Cysteine	L-Threonine	L-Alanine	L-Methionine	
4-A	+	+	+	+	90
3-A	+	+	+	—	75
3-B	+	+	—	+	87
3-C	+	—	+	+	23
3-D	—	+	+	+	22
2-A	+	+	—	—	21
2-B	+	—	—	+	7
2-C	—	+	—	+	3
Cysteine	+	—	—	—	35
Threonine	—	+	—	—	5
Alanine	—	—	+	—	11
Methionine	—	—	—	+	3
None	—	—	—	—	9

nmol per ml. In case of L-cystine, the amino acid concentration higher than 100 μ g per ml was required for the best growth and B₆ biosynthesis. At the concentration of 400 μ g per ml, the highest amount of B₆, 0.119 nmol per ml, was formed, though the maximal growth was obtained at the concentration of 300 μ g per ml. In these cases, the amounts of B₆ per mg cell was almost parallel to the amount of B₆ per ml. On the other hand, in case of glycolaldehyde, the amount of B₆ per mg cell increased with the concentration of supplemented glycolaldehyde. The glycolaldehyde concentrations of 25 μ g and 50 μ g per ml were the best concentration for the growth and B₆ biosynthesis, respectively. At the concentration, 0.403 nmol per ml of B₆ was formed in the culture. Thus, the amount of B₆ formed in cysteine- or cystine-supplemented medium was much less than that in glycolaldehyde-supplemented medium. This result suggests that the amino acids do not function as a biosynthetic intermediate between glycolaldehyde and B₆.

Effect of an amino acid mixture on growth of E.

coli WG3

It was indicated in the previous paper¹⁾ that an amino acid mixture which was composed of 18 natural amino acids formed a growth zone having strong density on the assay plate of *E. coli* WG3 than that formed by each amino acid alone. Effect of the amino acid mixture on growth of the mutant in liquid glycerol medium was examined. The mutant grew very well in the medium containing each 100 μ g per ml of the amino acids. It reached the maximal growth after 6- to 8-hr cultivation and the growth was about 1.5 to 2.0 times higher than that obtained in pyridoxine-supplemented medium. In order to know essential composition of amino acids supporting the growth, the mutant was cultivated in the media in which each one of the amino acids was omitted from the amino acid mixture. Table II shows growth of the mutant after 9-hr cultivation. Omission of L-cysteine, L-threonine, L-alanine or L-methionine reduced the growth very much in comparison with that in 18 amino acids-supplemented medium. Omission of each one of other 14 amino acids did not reduce the growth so much. Then, the

TABLE IV. EFFECT OF GLYCOLALDEHYDE AND AMINO ACID MIXTURE 3-B ON GROWTH AND B6 BIOSYNTHESIS OF *E. coli* WG3

The medium used was glycerol medium. The concentration of the amino acid mixture 3-B is shown as the amount of each amino acid. The growth and the B6 amount were measured after 6-hr or 9-hr cultivation in glycolaldehyde- or amino acid mixture-supplemented medium, respectively.

Supplement	Concentration	Growth	Amount of B6	
	($\mu\text{g/ml}$)	(mg^*/ml)	(nmol/ml)	(nmol/mg cell)
Glycolaldehyde	10	1.235	0.142	0.115
	25	1.290	0.303	0.235
	50	1.080	0.403	0.373
	100	0.800	0.386	0.482
	200	0.314	0.275	0.819
Amino acid mixture 3-B	20	0.940	0.032	0.034
	50	1.260	0.064	0.051
	100	1.230	0.154	0.125
	200	1.175	0.076	0.065

* mg : mg dry cell weight.

mutant was cultivated in the medium containing only these 4 amino acids essential for the growth. It was resulted that the mutant could grow in the 4 amino acids-supplemented medium almost the same level in pyridoxine-supplemented medium. No growth was observed in the medium supplemented with residual 14 amino acids. Subsequently, effect of combination of L-cysteine, L-threonine, L-alanine and L-methionine on growth of the mutant was examined in detail. As shown in Table III, omission of L-threonine or L-cysteine from the amino acid mixture reduced the growth so much. An amino acid mixture, 3-B, supported the growth as same as the complete mixture, 4-A. None of the components of 3-B could be omitted without reducing the growth very much. L-Cysteine itself supported the growth to some extent, whereas L-threonine and L-methionine were rather inhibitory for the growth in themselves or in combination of each with one of 3-B (2-A, -B and -C). These results indicate that an amino acid mixture, 3-B, which is composed of L-cysteine, L-threonine and L-methionine, is the simplest amino acid mixture to support the sufficient growth of the mutant.

Effect of the amino acid mixture 3-B on B6 biosynthesis of E. coli WG3

As mentioned above, a mixture of L-cysteine, L-threonine and L-methionine could replace B6 to support the growth of *E. coli* WG3. Then, the effect of the amino acid mixture 3-B on biosynthesis of B6 by the mutant was studied. The growth and B6 amount in glycerol medium supplemented with various concentrations of the amino acid mixture 3-B were measured to compare with those supplemented with glycolaldehyde. As shown in Table IV, the best growth and the highest B6 amount were obtained at the concentrations of 50 μg and 100 μg per ml of each amino acid, respectively. Growth of the mutant by the amino acid mixture 3-B was good at all concentrations tested but amounts of B6 formed were low in comparison to those formed in glycolaldehyde-supplemented medium. Even the highest amount of B6, 0.154 nmol per ml, in the medium supplemented with the amino acid mixture at concentration of 100 μg per ml was less than half amount of that with glycolaldehyde, 0.403 nmol per ml. The amount was not so much higher than a value of 0.119 nmol per ml, which was formed in the

medium supplemented with L-cystine alone at concentration of 400 μ g per ml.

The results suggest that the amounts of B6 biosynthesized from the amino acids were too small to support sufficient growth of the mutant, because the mutant requires 0.6 nmol per ml of B6 for full growth in minimal medium.

DISCUSSION

In the previous paper,¹⁾ alanine, cysteine, cystine and β -hydroxypyruvate in addition to glycolaldehyde were estimated to function as biosynthetic intermediates of B6 according to a newly developed paper disc assay method. In order to carry out more detailed investigation of the relationship between these compounds and B6 biosynthesis, the effect of these compounds on growth of *E. coli* WG3 in liquid medium was examined. Alanine and β -hydroxypyruvate could not render the mutant to grow in liquid medium under conditions tested. Growth of the mutant in cysteine- or cystine-supplemented glycerol medium was observed when the cultivation time was prolonged.

Morris and Woods indicated first that glycolaldehyde and β -hydroxypyruvate could substitute for pyridoxine in a Pdx mutant of *E. coli* B, strain B166.⁶⁾ They mentioned that β -hydroxypyruvate was active but, since the requiring concentration was about ten times of that of glycolaldehyde, this might have been due to spontaneous or metabolic conversion to glycolaldehyde. Shimizu and Dempsey indicated that β -hydroxypyruvate could entirely substitute for B6 requirement for growth of a *serC* mutant of *E. coli* K12.⁸⁾ They also proved that B6 could be synthesized in the β -hydroxypyruvate-supplemented medium by this group of mutants. This type of mutant (*serC* mutant) of *E. coli* B was isolated and characterized to require B6 and serine for growth.⁹⁾ Investigations concerning the biosynthetic pathway of B6 were carried out by using these mutants,^{10,11)} but no evidence that B6 is synthesized from β -hydroxypyruvate in B

strain have been reported. In the case of *E. coli* B WG3, which is a *pdxB* mutant and differs from *serC* mutant, both glycolaldehyde and β -hydroxypyruvate were effective for growth on the assay plate medium,¹⁾ but only glycolaldehyde but not β -hydroxypyruvate was effective for the growth in liquid medium, as shown in Table I. These results indicate that the conversion of β -hydroxypyruvate to B6 is much more difficult in B strain than in K12 strain when the compound is supplemented exogenously, even if β -hydroxypyruvate is a real biosynthetic intermediate of B6 *in vivo*.

Effect of alanine on growth of some B6-requiring microorganisms has been reported.^{12~16)} In all case, however, role of alanine as a biosynthetic intermediate of B6 was denied. It was also indicated in the present study that alanine could not act as the biosynthetic intermediate in *E. coli* WG3.

Amano indicated that L-cysteine could decrease the amount of B6 which is required for growth of a Pdx mutant of *E. coli*.¹⁷⁾ However, it has not been known that cysteine or cystine has B6-replacing effect for itself on growth of B6-requiring microorganisms. The amount of B6 in the culture of *E. coli* WG3 grown in cysteine- or cystine-supplemented medium was much lower than that in glycolaldehyde-supplemented medium, as shown in Fig. 3. Such effect of an amino acid on growth of a Pdx mutant was reported by Dempsey.¹⁶⁾ In the case, a *pdxK* mutant could grow fully in alanine-supplemented medium, but the total B6 in the culture was as low as about 0.1 nmol per mg cell. Moreover, alanine uniformly labeled with ¹⁴C was not incorporated into pyridoxal phosphate to any significant degree by the mutant.¹⁸⁾ These results indicate that these amino acids affected to allow the mutant to grow only with a low concentration of B6. It means that none of these amino acid act as a biosynthetic intermediate of B6.

Growth of *E. coli* WG3 in the medium supplemented with 18 natural amino acids was 1.5 to 2 times higher than that in pyridoxine-supplemented medium. Such high growth of

the mutant was probably due to the presence of large amount of carbon and nitrogen sources for growth as the result of many amino acids supplementation. As shown in Table III, an amino acid mixture, 3-B, containing L-cysteine, L-methionine and L-threonine at the concentration of each 100 μ g per ml, was the simplest mixture to give nearly the same level of growth as pyridoxine. Amount of B6 synthesized in the amino acid mixture-supplemented medium was 0.125 nmol per mg cell (Table IV), which was scarcely higher than the best amount of B6 synthesized in a single amino acid-supplemented medium (Fig. 3). The result indicated that the reduction of full growth time and some increase of cell mass at full growth time by supplement of amino acids in combination were not caused by the increased amount of B6 biosynthesized. It seemed that some of the essential compounds for growth of the mutant, which are produced by the action of B6, can be produced with use of much smaller amount of B6 in the amino acids-supplemented medium.

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