Precursor of Carbon Atom Five and Hydroxymethyl Carbon Atom of the Pyrimidine Moiety of Thiamin in *Escherichia coli*

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Summary The pyrimidine moiety of thiamin can be synthesized in bacteria from 5-aminoimidazole ribotide (AIR), an intermediate in purine biosynthesis. To transform the imidazole ring of AIR to the pyrimidine, the bond between C-4 and C-5 of the imidazole ring should be ruptured and a two-carbon unit should be inserted.

We investigated a precursor of the two-carbon unit which should be inserted to form the pyrimidine. To examine the possibility of the two-carbon compound as a precursor which is formed from a three-carbon intermediate in the glycolytic pathway and its three-carbon derivative by decarboxylation in cells, we studied the incorporation of [2-14C]glycerol, [2-14C]pyruvate, [U-14C]alanine and [3-14C]serine into the pyrimidine by *Escherichia coli*. Glycerol, pyruvate and alanine were not incorporated into the pyrimidine. Radioactive carbon of [3-14C]serine was incorporated into C-2 of the pyrimidine via one-carbon units pool. Then, we investigated the possibility of a ribose moiety of AIR being a precursor of the two-carbon unit. As ribose has low permeability into *E. coli* cells, the ribose moiety of AIR was labeled with [14C]glucose which was added to the growth medium. The results show that two radioactive carbons of [U-14C]glucose were incorporated into the pyrimidine and radioactive carbon of [6-14C]glucose was incorporated into hydroxymethyl carbon attached to C-5 of the pyrimidine. The dilution rates of the specific radioactivity of the pyrimidine from [U-14C]glucose and [6-14C]glucose well coincide with those of the ribose moiety of the nucleotide (AMP). Radioactive carbon of [1-14C]glucose was not incorporated into the pyrimidine and nucleotide. It is concluded that the two-carbon fragment derived from C-4’ and C-5’ of the ribose moiety of AIR can be incorporated into the pyrimidine moiety of thiamin as the precursor of C-5 and hydroxymethyl carbon.

Key Words biosynthesis of thiamin, pyrimidine moiety of thiamin, 5-aminoimidazole ribotide, *Escherichia coli*
Newell and Tucker (1) proposed that in *Salmonella typhimurium*, the pyrimidine moiety of thiamin is synthesized from AIR, an intermediate in purine biosynthesis. The transformation of the imidazole ring of AIR into the pyrimidine should be accomplished by ring expansion reaction. Basing on the incorporation of radioactive carbons of [1-14C]glycine and [2-14C]glycine into C-4 and C-6 of the pyrimidine in *S. typhimurium*, Estramareix (2) concluded that the imidazole ring of AIR ruptures at the bond between C-4 and C-5 atoms and a two-carbon unit can be inserted to form the pyrimidine. He suggested that the two-carbon unit to be inserted could be glycolaldehyde (2). However, the origin of the two-carbon unit has not been established.

To examine a precursor of the two-carbon unit to be inserted into the imidazole ring, we studied the incorporation of 14C-labeled compounds into the pyrimidine by *E. coli*.

We now present evidence that the two-carbon fragment derived from C-4' and C-5' of the ribose moiety of AIR can be incorporated into C-5 and hydroxymethyl carbon of pyrimidine moiety of thiamin.

A preliminary communication of this paper has been presented (3).

**MATERIALS AND METHODS**

**Materials.** Radioactive compounds were purchased from Radiochemical Centre, Amersham, England. Taka-diastase from Sankyo Co., Ltd., Tokyo. All other chemicals used were of analytical grade.

**Production and determination of thiamin.** *Escherichia coli* B (IFO 13168) was grown as follows. One liter of the synthetic medium described by Davis and Mingioli (4), supplemented with [2-14C]glycerol, [2-14C]pyruvate and L-[U-14C]alanine was inoculated with *E. coli*. Using DL-[3-14C]serine, [U-14C]glucose, [1-14C]glucose and [6-14C]glucose as tracer, *E. coli* was grown in the glycerol-inorganic medium described by us (5). After incubation for 6 h at 37°C with vigorous shaking, the cells were harvested by centrifugation and washed twice with water. The cell pellet was suspended in 50 ml of water and 0.5 ml of 12 N HCl was added. The suspension was heated in boiling water bath for 10 min to extract thiamin from cells. The heated mixture was adjusted to pH 4.5 with 1 N NaOH and then centrifuged to remove the cell debris. The supernatant was incubated with 100 mg of Taka-diastase at 47°C for 2 h to convert phosphorylated thiamin into thiamin. Determination of thiamin was carried out by the fluorometric method as described previously (5).

**Purification of thiamin.** The extract was added with 10 μmol of carrier thiamin. The solution was adjusted to pH 5.9 with 1 N NaOH and applied to a column (2.5 × 6.5 cm) of Amberlite CG-50 (H⁺). The column was washed with water by the disappearance of UV absorption at 255 nm and then 1 N HCl was passed through the resin to elute thiamin. The thiamin-containing fractions were located by measuring optical densities at 255 nm.
Isolation of 4-amino-2-methylpyrimidyl-5-methane sulfonic acid (pyrimidine sulfonic acid) and 4-hydroxy-2-methylpyrimidine-5-methylthioacetic acid (pyrimidine thioacetic acid). Productions of pyrimidine sulfonic acid and pyrimidine thioacetic acid from thiamin were carried out by the method described by Kumaoka and Brown (6).

Degradation of pyrimidine sulfonic acid by Kuhn-Roth oxidation. The radioactive pyrimidine sulfonic acid was added with 0.5 mmol of nonradioactive pyrimidine sulfonic acid to act as carrier. The mixture was recrystallized and this purified pyrimidine sulfonic acid was degraded to acetate and carbon dioxide by the Kuhn-Roth oxidation procedure as described by Kumaoka and Brown (6). Acetate was recovered as sodium salt and carbon dioxide, also a byproduct of oxidation, was recovered as barium carbonate. Where necessary, acetate was further decomposed to methylamine and carbon dioxide by the Schmidt reaction as described by Phares (7). Methylamine was recovered as hydrochloride. Carbon dioxide was recovered as barium carbonate.

Degradation of pyrimidine thioacetic acid. To the radioactive pyrimidine thioacetic acid was added nonradioactive pyrimidine thioacetic acid as carrier and the mixture was recrystallized. The product was degraded to 1,3-diamino-2-methylpropane by the method described by David et al. (8). Where necessary, 1,3-diamino-2-methylpropane was decomposed by Kuhn-Roth oxidation to acetate and carbon dioxide and acetate was further degraded to methylamine and carbon dioxide.

Separation of amino acid and ribose phosphate from E. coli cells. Amino acids were isolated from cell protein hydrolyzate of E. coli by amino acid autoanalyzer. The content of amino acid was determined by the ninhydrin reaction. AMP was isolated from cell RNA and purified by the procedure described previously (9). AMP was hydrolyzed by heating in 1 N HCl to adenine and ribose phosphate. Adenine and ribose phosphate were purified by paper chromatography with developing solvent consisting of methanol/12 N HCl/water (70:20:10, v/v). Each compound eluted from developed chromatogram was further purified by paper chromatography with the developing solvent of methanol/ethanol/12 N HCl/water (50:25:6:19, v/v). The content of AMP and adenine were determined by measuring optical density at 260 nm.

Determination of radioactivity. Radioactivities were determined with a Nuclear Chicago gas-flow counter. Where necessary, corrections were made for self-absorption.

RESULTS AND DISCUSSION

Investigation of incorporation of glycerol, pyruvate, alanine and serine into the pyrimidine moiety of thiamin

As shown in Table 1, radioactive carbon of [2-14C]glycerol, [2-14C]pyruvate and [U-14C]alanine was incorporated into the pyrimidine with significantly high
Table 1. Incorporation of [2-14C]glycerol, [2-14C]pyruvate, L-[U-14C]alanine and DL-[3-14C]serine into the pyrimidine moiety of thiamin.

| Labeled compound | Specific activity of labeled compound (cpm/mmol) | Thiamin produced (nmol) | Pyrimidine sulfonic acid | Amino acid in protein |
|------------------|-----------------------------------------------|-------------------------|-------------------------|----------------------|
|                  | Amount (μmol) | Count (cpm) | Specific activity (cpm/mmol) | Dilution (times) | Specific activity (cpm/mmol) | Dilution (times) |
| [2-14C]Glycerol  | $9.77 \times 10^8$ | 21.5 | 6.36 | $1.45 \times 10^2$ | $2.28 \times 10^6$ | $1.06 \times 10^7$ | 92.2 | Glycine | $1.12 \times 10^7$ | 87.0 |
|                  |                |         |     |                     |                      |                     |                 | Alanine | $2.41 \times 10^7$ | 40.5 |
| [2-14C]Pyruvate  | $5.90 \times 10^8$ | 23.6 | 4.28 | 27.1 | $6.33 \times 10^3$ | $2.69 \times 10^6$ | 219.0 | Glycine | $1.66 \times 10^6$ | 355.0 |
|                  |                |         |     |                     |                      |                     |                 | Alanine | $1.91 \times 10^7$ | 30.9 |
| L-[U-14C]Alanine | $9.46 \times 10^8$ | 48.4 | 5.34 | 96.1 | $1.80 \times 10^4$ | $3.74 \times 10^6$ | $253$ (three C) | $168$ (two C) | $84$ (one C) | Glycine | $1.78 \times 10^8$ | 5.3 |
|                  | ($3.15 \times 10^8$ cpm/C atom) | | | | | | | | | Alanine | | | |
| DL-[3-14C]Serine | $9.23 \times 10^8$ | 120.8 | 7.74 | $2.14 \times 10^3$ | $2.76 \times 10^5$ | $2.31 \times 10^7$ | 39.9 | Serine | $2.04 \times 10^7$ | 45.2 |
|                  |                |         |     |                     |                      |                     |                 | Methionine | $1.69 \times 10^7$ | 54.6 |

1) 14C-Labeled glycerol, pyruvate and serine were added to 0.5 liter of the growth medium at a concentration of 1.0 mM. [14C]Alanine was added to 1 liter of the growth medium at a concentration of 1.0 mM. 2) The specific activities (*) of the pyrimidine sulfonic acid were corrected to the value of the pyrimidine portion of thiamin produced by the cells on the basis of dilution by carrier thiamin. 3) Dilution is the ratio of specific activities of the 14C-labeled compounds added to the medium and the pyrimidine portion of thiamin and amino acids produced by the cells.
dilution of specific radioactivities. These facts show that none of these compounds are a precursor of the two-carbon unit to be inserted into the imidazole ring of AIR for the formation of the pyrimidine ring of thiamin. Radioactivity of DL-[3-14C]-serine was incorporated into the pyrimidine with 39.9-fold dilution of the specific radioactivity. The dilution rate coincides well with those (45.2- and 54.6-fold) of the specific radioactivities of serine and methionine from E. coli cell protein.

It has been reported by Tani and Dempsy (10) that glycolaldehyde from serine is incorporated into C-5 and hydroxymethyl group attached to C-5 of pyridoxal phosphate in E. coli cells. Estramareix (2) had suggested that the two-carbon compound to be inserted into the imidazole ring of AIR to give the pyrimidine ring may be glycolaldehyde. If radioactive carbon of [3-14C]serine was incorporated into the pyrimidine via glycolaldehyde the radioactive carbon should be located in hydroxymethyl carbon attached to C-5 of the pyrimidine. On the other hand, hydroxymethyl carbon of serine is well known to be converted into one-carbon units. If radioactive carbon of [3-14C]serine was incorporated into the pyrimidine via one-carbon units, the radioactivity should be located in C-2, at which formate is incorporated.

In order to decide which carbon atom of the pyrimidine is labeled with [3-14C]serine, the pyrimidine sulfonic acid was degraded by Kuhn-Roth oxidation to yield acetate and carbon dioxide. The acetate was further decomposed to methylamine and carbon dioxide by the Schmidt reaction. Table 2 shows that of the radioactivity present in the pyrimidine sulfonic acid, the majority was recovered in

Table 2. Results of degradation of the pyrimidine moiety of thiamin labeled with DL-[3-14C]serine.

| Compound                        | Total count (cpm) | Amount (mmol) | Yield (%) | Specific activity (cpm/mmol) |
|---------------------------------|------------------|---------------|-----------|-----------------------------|
| 4-Amino-2-methylpyrimidyl-5-methane sulfonic acid (C-2, 4, 5, 6, 7, 8) | 532              | 0.336         |           | 1.59 x 10^3                 |
| Acetic acid (C-2, 8)            | 300              | 0.182         | 54.2      | 1.65 x 10^3                 |
| Carbon dioxide (C-4, 5, 6, 7)   | negligible       | 0.523         | 38.9      |                             |
| Acetic acid (C-2, 8)            | 300              | 0.182         |           | 1.65 x 10^3                 |
| Carbon dioxide (C-2)            | 219              | 0.163         | 89.6      | 1.34 x 10^3                 |
| Methylamine (C-8)               | 39.9             | 0.145         | 79.7      | 27.4                        |

Pyrimidine sulfonate (0.336 mmol) was degraded by Kuhn-Roth oxidation to yield acetic acid and carbon dioxide as products. The evolved carbon dioxide was trapped as barium carbonate. The acetic acid was recovered as sodium acetate. The acetate was further decomposed to methylamine and carbon dioxide by the Schmidt reaction. Methylamine was recovered as hydrochloride.

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carbon dioxide derived from C-2 of the pyrimidine. It is concluded that radioactive carbon of [3-\textsuperscript{14}C]serine was incorporated into C-2, and serine was not a precursor of the two-carbon unit. Three-carbon intermediates in glycolytic pathway and their derivatives are not a precursor of C-5 and hydroxymethyl carbon attached to C-5 of the pyrimidine.

Investigation on the incorporation of a two-carbon fragment derived from the ribose moiety of AIR into the pyrimidine by using [\textsuperscript{14}C]glucose

The ribose moiety of GTP has been found to participate in the constitution of vitamins such as folic acid (11) and riboflavin (12). It has been considered that the pyrimidine is synthesized from AIR, but the ribose phosphate is liberated from the imidazole ring and does not participate in the formation of the pyrimidine from AIR (1, 2).

We consider that on the transformation of AIR into the pyrimidine, the ribose moiety participates in the constitution of the pyrimidine ring as a precursor of a two-carbon unit to be inserted into the imidazole ring. We investigated the incorporation of radioactive ribose moiety of AIR into the pyrimidine.

Since ribose has low permeability into E. coli cells (13), [\textsuperscript{14}C]glucose was added to the growth medium containing glycerol as carbon source. [\textsuperscript{14}C]Glucose is transformed into the ribose moiety of AIR via a pentose phosphate shunt in E. coli cells (14).

Table 3 shows that [U-\textsuperscript{14}C]glucose was incorporated into the pyrimidine moiety of thiamin with 14.3-fold dilution of the specific activity (as for the incorporation of two carbon atoms from glucose). The dilution rate coincides well with that (13.5-fold) of the specific activity of the ribose moiety of AMP. The fact suggests strongly that two carbon atoms from the ribose moiety of a nucleotide (AIR) are incorporated into the pyrimidine. Table 3 also shows that with [1-\textsuperscript{14}C]-glucose, radioactive carbon was incorporated into the pyrimidine with a significantly high dilution of specific activity and it was not incorporated into the ribose moiety of AMP. On the other hand, radioactive carbon of [6-\textsuperscript{14}C]glucose was well incorporated into the pyrimidine at the same dilution rate (14.9-fold) of specific activity as that (14.1-fold) of the ribose moiety of AMP.

By the addition of nonradioactive glycine and formate into the medium along with radioactive glucose, the incorporation of radioactive carbon of glucose into glycine was reduced to a vanishingly small level (1,405.1-fold with [1-\textsuperscript{14}C]glucose and 694.6-fold with [6-\textsuperscript{14}C]glucose). Radioactive carbon of [1-\textsuperscript{14}C]glucose and [6-\textsuperscript{14}C]glucose was incorporated into alanine at 58.4- and 58.2-fold dilution, respectively. These results suggest that the incorporation of radioactive carbon of [U-\textsuperscript{14}C]glucose into the pyrimidine is not via glycine, formate and other degradative compounds derived from radioactive glucose and show strongly the possibility of the incorporation of the two-carbon fragment derived from the ribose moiety of AIR into the pyrimidine.

To decide which carbon atom of the pyrimidine is labeled with [6-\textsuperscript{14}C]glucose,
Table 3. Incorporation of radioactive glucose into the pyrimidine moiety of thiamin.

| Glucose supplied | Thiamin produced (mmol) | Pyrimidine sulfonic acid | Amino acid and ribose in cells |
|------------------|-------------------------|-------------------------|-----------------------------|
|                  | Specific activity (cpm/mmol) | Specific activity (cpm/mmol) | Count (cpm) | Amount (µmol) | Dilution (times) |
| [U-¹⁴C]Glucose   | 7.20 × 10⁸               | 1.20 × 10⁸               | 175         | 3.16         | 4.89 × 10⁷  |
|                   | (1.20 × 10⁸ cpm/C atom)  |                        |            | 9.14 × 10³  | 1.68 × 10³  |
| [¹⁴C]Glucose     | 9.92 × 10⁸               | 5.58 × 10⁸              | 55           | 3.40         | 2.29 × 10³  |
| [¹⁴C]Glucose     | 8.96 × 10⁸               | 6.75 × 10⁸              | 67           | 4.13         | 1.60 × 10³  |

1) Radioactive glucose was added to the glycerol-inorganic medium at a concentration of 1.0 mM. 2) With [¹⁴C]glucose, E. coli was grown in 1 liter of the growth medium. 3) The specific activities of the pyrimidine sulfonic acid were corrected to the value of the pyrimidine portion of thiamin produced by the cells on the basis of dilution by carrier thiamin. 4) Dilution is the ratio of specific activities of glucose added to the medium and the products produced by the cells (the pyrimidine portion of thiamin, ribose, amino acid, and AMP).
Table 4. Localization of radioactivity in the pyrimidine moiety derived from thiamin labeled with [6-14C]glucose.

| Compound                        | Amount (μmol) | Yield (%)a | Radioactivity present (cpm) | Specific activity (cpm/mmol) |
|--------------------------------|---------------|------------|-----------------------------|-----------------------------|
| 4-Hydroxy-2-methylpyrimidine-5-methylthioacetic acid | 1,101.6       | 2,678      | 2.43 × 10³                  |                             |
| 1,3-Diamino-2-methylpropane (C-4, 5, 6, 7) | 421.0         | 38.2       | 1,023                       | 2.43 × 10³                  |
| Carbon dioxide (C-4, C-6)       | 334.2         | 79.4       | negligible                  |                             |
| Acetic acid (C-5, 7)            | 193.8         | 46.0       | 473.5                       | 2.44 × 10³                  |
|                                 | + 200.0b      |            |                             | 1.20 × 10³                  |
| Carbon dioxide (C-5)            | 279.6         | 71.0       | 22.8                        | 8.16 × 10                  |
| Methylamine (C-7)               | 330.9         | 84.0       | 303.4                       | 9.17 × 10²                  |

1) Pyrimidine thioacetic acid (1.1016 mmol) was degraded into 1,3-diaminopropane. 1,3-Diaminopropane was degraded by Kuhn-Roth oxidation to yield acetate and carbon dioxide. Acetate was further decomposed to methylamine and carbon dioxide. 2) The figures in yield (%) represent the percentage of theoretical yield from the preceding compound. 3) Cold compound (200.0 μmol b) was added for next degradation.

Fig. 1. Proposed biosynthetic route to OMP phosphate from AIR.

The pyrimidine thioacetic acid, derived from thiamin labeled with [6-14C]glucose, was degraded into 1,3-diamino-2-methylpropane, which was further decomposed to carbon dioxide and acetate by Kuhn-Roth oxidation. The acetic acid was further decomposed into methylamine and carbon dioxide. Table 4 shows that almost all of the radioactivity present in the pyrimidine thioacetic acid was recovered in methylamine derived from hydroxymethyl carbon in the pyrimidine. It shows the incorporation of radioactive carbon of [6-14C]glucose into the hydroxymethyl carbon of the pyrimidine.

From these results, we deduced that the two-carbon fragment derived from C-4’ and C-5’ of the ribose moiety of AIR is a precursor of the C-5 and hydroxymethyl group of the pyrimidine moiety of thiamin. We propose that the pyrimidine moiety of thiamin is synthesized from AIR by the pathway shown in Fig. 1. The proposed biosynthetic route satisfies the results described above. The first step of the
transformation of AIR into the pyrimidine is cleavage of the bond between C-3' and C-4' of the ribose moiety and the second step is addition of the two carbon fragment derived from C-4' and C-5' of the ribose moiety to C-4 and C-5 of the imidazole ring to form a 3-membered ring, which then opens to form the pyrimidine ring of thiamin.

The pyrimidine moiety biosynthesized prior to the condensation into thiamin has been thought to be 4-amino-2-methyl-5-hydroxymethylpyrimidine (OMP). Our proposed biosynthetic pathway suggests that the pyrimidine moiety derived from AIR in cells is OMP phosphate. If OMP is synthesized from the purine biosynthetic route, it should be transformed from 5-aminoimidazole riboside, not from AIR. It remains unsolved to decide whether the pyrimidine transformed from the purine biosynthetic intermediate is OMP or OMP phosphate.

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