Biosynthesis of Vitamin B₆

I. Incorporation of ¹⁴C-Glycerol, Aspartic Acid and Leucine into Vitamin B₆

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1. Two bacteria stems producing vitamin B₆ were obtained. One is Klebsiella producing 2 mg of pyridoxamine per liter in 60 hours and the other is Flavobacterium producing 4 mg of pyridoxal per liter in 60 hours.

2. Resting cell suspension of Flavobacterium was able to synthesize pyridoxal. Maximal synthesis was achieved in the presence of glycerol and an amino acid (aspartic acid or leucine).

3. The carbon chains of both ¹⁴C-labeled glycerol and ¹⁴C-labeled leucine were efficiently incorporated into newly synthesized pyridoxal by the resting cell system. ¹⁴C-pyridoxal produced by cells incubated with 2-¹⁴C-aspartic acid was too low to permit a definitive conclusion.

Regardless of long history of research on vitamin B₆ biosynthesis, very little information is available about the precursors and the pathway of the biosynthesis of vitamin B₆. In 1942, Snell considered for some time that D-alanine, which played the role of pyridoxine in the nutritional requirement of Lactobacillus casei and Streptococcus faecalis, might be a precursor of this vitamin. But it was later revealed that pyridoxine was concerned with the synthesis of D-alanine which was an essential component of the cell wall of many bacteria (1-3).

Amano investigated the biosynthesis of vitamin B₆ by two strains of pyridoxine requiring mutants obtained from Escherichia coli. He described that DL-alanine, DL-glutamic acid, L-aspartic acid, DL-leucine and L-cystine promoted the growth of these bacteria (4).

In 1959, Woods and Morris used E. coli and studied the biosynthesis of vitamin B₆. By testing the growth effects of varying ingredients of the media on which a series of vitamin B₆-requiring mutants of E. coli were grown, Woods and Morris came to the conclusion that serine, glycine and glycolaldehyde are possible precursors in the biosynthesis of vitamin B₆ in E. coli. However, no isotopic experiments have yet been performed to confirm the hypothesis that any or all of these compounds are true precursors of vitamin B₆ (5, 6).

Recently, Lunan and West isolated the ¹⁴C-labeled pyridoxamine from Candida utilis. Serine-3-¹⁴C, acetate-1-¹⁴C, alanine-1-¹⁴C and acetate-2-¹⁴C gave rise to ¹⁴C-labeled pyridoxamine. But the observed extents of incorporation were too low to permit a definitive interpretation of these results in terms of the biosynthesis of vitamin B₆. The failure of

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these studies were due to the difficulty to obtain the bacteria which produced B₆ in plenty (7).

The authors obtained two strains of B₆-biosynthesizing bacteria. One strain belongs to Klebsiella and the other to Flavobacterium. Both bacteria produce pyridoxamine and pyridoxal abundantly.

In the present paper we report the possible precursors of biosynthesized vitamin B₆ and studies on incorporation of the radioactive compounds into vitamin B₆. These studies have shown that glycerol and leucine are two possible precursors on the biosynthesis of the vitamin.

**EXPERIMENTALS**

1. *Chemicals*

Pyridoxine was obtained from Nakarai Chemical Co., Kyoto. Pyridoxamine was obtained from Sigma Chemicals, and pyridoxal was purchased from Cal-Bio Chem. Co. ¹⁴C-Aspartic acid and ¹⁴C-leucine were purchased from Daiichi Pure Chemicals, and ¹⁴C-glycerol was obtained from the Radio-Chemical Centre, Amersham, England.

2. *Strains of Bacteria*

B₆-synthesizing bacteria grown on the B₆-less culture medium were obtained from soil. Several strains were picked up from the agar plate of B₆-less culture medium and tested the ability of B₆ biosynthesis. On this experiment we could obtain a strain producing about 4 mg of pyridoxamine or pyridoxal per liter. This bacterium was identified as Klebsiella sp. by Haruna (8). Another strain of B₆-biosynthesizing bacterium, Flavobacterium, was generously provided by Professor Koichi Ogata.

3. *Culture Condition*

The medium contained 4 g of casamino acid, 25 µg of inositol, 250 µg of thiamine, 2.5 mg of nicotinic acid, 2.5 mg of calcium pantothenate, 8 µg of biotin, 425 mg of KCl, 50 g of glucose, 12.5 mg of CaCl₂, 125 mg of MgSO₄, 2.5 mg of MnSO₄, 550 mg of KH₂PO₄, 2.5 mg of FeCl₃, 5 g of potassium citrate, 1 g of citric acid and 2 mg of pyridoxine per liter. To the bioassay of B₆, a pyridoxine-omitted medium was used.

4. *Bioassay of Vitamin B₆*

Amount of vitamin B₆ was routinely measured by the growth rate of Saccharomyces carlsbergensis 4228 ATCC 9080 grown in the medium mentioned above (9). Types of vitamin B₆ were detected on paper chromatograms either by absorption of ultraviolet light or by colorization of a dilute 2,6-dichloroquinochloroimide spray.

5. *Growing Cell*

The organism was cultured in the medium used for growing cell experiment at 26°. This medium contained, per liter, 3 g of pepsin, 0.5 g of KH₂PO₄, 1.5 g of K₂HPO₄, 0.5 g of yeast extract and 5.0 g of glucose. Cells were harvested by centrifugation, washed with 0.85% NaCl solution twice and used for the growing cell experiment of bacteria.

6. *Growing Cell Experiment*

Cells from a 24-hour culture of bacteria grown at 27° were suspended to inoculate in 50 ml of growth medium. The growth medium contained, per 50 ml, 30 mg of KH₂PO₄, 6 mg of CaCl₂, 6 mg of MgSO₄, 0.01 mg of thiamine, 0.07 mg of inositol and either 0.1% of amino acid or 1 g of carbohydrate. The culture was incubated at 27° on a shaker for 24 or 48 hours. After each period, two aliquots (5 ml + 5 ml) were withdrawn from each culture medium. One was used for determination of the dry cell weight and the other for determination of the pyridoxal content in the cells or in the medium. To determine the dry weight of the cells, 5 ml of cell suspension were centrifuged at 4,000 rpm for 15 min and the resulted cell pellet was washed once with 5 ml of distilled water. The supernatant solution was discarded and the cells were transferred quantitatively to a previously weighed aluminum planchette. The cells were dried at 100° to a constant weight. A 5 ml aliquot to be used for the determination of B₆ was centrifuged to separate the cells from the medium. The cell pellet was then washed with 5 ml of distilled water and recentrifuged. The supernatant solution from the washings was added to the original supernatant fluid. The supernatant solution and the cell suspension were acidified to the final concentration of 1 N with 10 N H₂SO₄. The supernatant solution and the cell suspension were then autoclaved at 125° for 30 min to hydrolyze the B₆ esters to free vitamin B₆. After hydrolysis, the samples were cooled, neutralized and 1 (from supernatant) or 0.5 ml (from the cell suspension) of aliquots were transferred into 9.0 or 9.5 ml of the bioassay medium to determine the vitamin B₆ with Saccharomyces carlsbergensis.

7. *Resting Cell Experiment*

The cells from a 24-hour culture slant were inoculated in one liter of growth medium. Growth medium contained, per liter, 1.5 g of KH₂PO₄, 0.5 g of K₂HPO₄, 10.0 g of glucose, 2.0 g of peptone and 1.0 g of yeast extract. After inoculation for 24 hours at 26°
TABLE 1

Effects of sugars and amino acids on the production of pyridoxal by growing cells of Flavobacterium

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>Cells produced</th>
<th>Amounts of PAL</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose + NH₄⁺</td>
<td>3.1</td>
<td>0.08</td>
<td>0.74</td>
<td>0.83</td>
<td>265</td>
</tr>
<tr>
<td>Glucose + casamino acid</td>
<td>10.8</td>
<td>0.08</td>
<td>1.08</td>
<td>1.17</td>
<td>108</td>
</tr>
<tr>
<td>Glucose + casamino acid + ribose</td>
<td>20.8</td>
<td>0.08</td>
<td>2.33</td>
<td>2.41</td>
<td>119</td>
</tr>
<tr>
<td>Casamino acid + ribose</td>
<td>23.8</td>
<td>0.08</td>
<td>2.33</td>
<td>2.41</td>
<td>101</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>18.5</td>
<td>0.33</td>
<td>4.35</td>
<td>4.68</td>
<td>253</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.5</td>
<td>0.67</td>
<td>3.75</td>
<td>4.42</td>
<td>385</td>
</tr>
<tr>
<td>Alanine</td>
<td>17.8</td>
<td>0.58</td>
<td>3.25</td>
<td>3.48</td>
<td>219</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid + glucose</td>
<td>52.1</td>
<td>0.07</td>
<td>3.60</td>
<td>3.67</td>
<td>70</td>
</tr>
<tr>
<td>Aspartic acid + glutamic acid</td>
<td>32.0</td>
<td>0.13</td>
<td>4.30</td>
<td>4.47</td>
<td>140</td>
</tr>
<tr>
<td>Aspartic acid + alanine</td>
<td>31.6</td>
<td>0.11</td>
<td>3.06</td>
<td>3.17</td>
<td>100</td>
</tr>
<tr>
<td>Glutamic acid + alanine</td>
<td>30.2</td>
<td>0.45</td>
<td>2.55</td>
<td>3.00</td>
<td>99</td>
</tr>
</tbody>
</table>

The growth medium was "Basal medium" plus the additions shown above. NH₄⁺ was added as NH₄Cl at 1 mg per ml of medium. Casamino acid, glutamic acid, aspartic acid, or L-alanine was added at 1 mg per ml of medium. Glucose was added at 20 mg per ml. The cells were grown in 50 ml of growth medium at pH 6.0 for 24 hr.

Results and Discussion

1. Amount and Sort of B₆ Produced

As shown in Fig. 1, Klebsiella produced...
FIG. 1 Amount and sort of B6 produced

The composition of incubation medium was shown in the text. Amounts of B6 produced were measured by the growth rate of Saccharomyces carlsbergensis. 2 mg of vitamin B6 in 1 liter of the complete culture medium in 40 hours. Flavobacterium also produced 4 mg of vitamin B6 in 1 liter of the complete culture medium in 60 hours. By ascending paper chromatography with the solvent system of n-butanol-acetic acid-water (12 : 3 : 5), it revealed that Klebsiella produced pyridoxamine (Rf = 0.09) mainly, whereas Flavobacterium produced pyridoxal (Rf = 0.33) mainly. On the basis of this result, we used mostly Flavobacterium.

2. Experiments Conducted with Growing Cells

The experiments to be reported below were performed to determine which substances, when added to the basal medium, might stimulate the production of pyridoxal by growing cultures of Flavobacterium. It was hoped that such experiments might provide clues to the identity of the precursors of the vitamin which would direct the course of subsequent work with resting cell preparation. The pyridoxal content of the cells and the growth medium containing various additions is shown in Table 1. If the cells grown in a medium containing glucose as the sole carbon source and NH4Cl as the nitrogen source, it may be seen that replacement of the ammonium salt by casamino acid results in an increase in a cell yield. However, no increase in pyridoxal production per unit weight of cells was observed.

Cells grown in a medium containing glutamic acid, aspartic acid or alanine produced almost six times as much pyridoxal as the cells grown in a glucose and NH4Cl medium. Thus, amino acids appear to stimulate the production of pyridoxal by Flavobacterium. The results of experiment 2, Table 1, show the stimulatory effects of the mixture of amino acids. Maximal production was achieved with the combination of aspartic acid and glutamic acid.

3. Experiments Conducted with Resting Cells

The foregoing experiments suggested that certain amino acids stimulated the biosynthesis of vitamin B6. Experiments were designed to decide whether resting cells of Flavobacterium could synthesize the vitamin, if they were
Table 3

Effect of amino acid on pyridoxal formation by resting cells of Flavobacterium

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>PAL (mg per ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µmoles per ml</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>780</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>740</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>840</td>
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<tr>
<td>L-Valine</td>
<td>1,680</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>2,210</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>1,480</td>
</tr>
<tr>
<td>L-Serine</td>
<td>730</td>
</tr>
<tr>
<td>L-Treonine</td>
<td>910</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>1,820</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>1,690</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>550</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>440</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>680</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1,110</td>
</tr>
<tr>
<td>L-Proline</td>
<td>490</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>630</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>980</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>860</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>530</td>
</tr>
</tbody>
</table>

The incubation mixture contained per 2 ml: 125 µmoles of phosphate buffer at pH 6.0, 75 µmoles of NH₄Cl, 20 µmoles of α-ketoglutaric acid, 40 µmoles of glycerol, 15.0 mg (dry weight) of cells, and additions as shown in 20 µmoles. Incubation was carried out for 22 hr at 26°C.

The data of Table 2 show that resting cells were able to produce small amounts of B₆ in the presence of NH₄Cl and either sugar, carboxylic acid or an amino acid. In this experiment maximal production of the vitamin was achieved when a mixture of aspartic acid, α-ketoglutaric acid and glycerol was supplied. A comparison of the stimulatory effects of the various amino acids when each was tested in the presence of glycerol (Table 3), showed that leucine, aspartic acid and glutamic acid were approximately equally active and that valine was somewhat less active. The stimulatory effects of these compounds were not duplicated by any of the other amino acids. The results summarized in the resting cell experiment, show that maximal synthesis was achieved when glycerol and either leucine or aspartic acid were added to the reaction mixture. By varying the concentration of both glycerol and the amino acid, it was determined that maximal synthesis resulted when each component was used at a concentration of 5 or 10 µmoles per ml respectively. Investigation of pyridoxal synthesis as a function of time and of pH showed that maximal synthesis was achieved in about 10 hours and that pH 6.0 was best.

Table 4

Incorporation of ¹⁴C-compounds into pyridoxal by resting cells

<table>
<thead>
<tr>
<th>¹⁴C-substrate</th>
<th>Specific activity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol-1,3</td>
<td>1,030,000</td>
<td>626,000</td>
</tr>
<tr>
<td>Glycerol-2</td>
<td>130,000</td>
<td>62,500</td>
</tr>
<tr>
<td>L-Leucine-U</td>
<td>214,400</td>
<td>18,500</td>
</tr>
<tr>
<td>DL-Leucine-1</td>
<td>248,400</td>
<td>0</td>
</tr>
</tbody>
</table>

Incubation mixture contained bacteria (240 mg dry weight), 4 mmoles of potassium phosphate buffer at pH 6.0, 2.4 mmoles of NH₄Cl, 16 µmoles of α-ketoglutaric acid and radioactive compound in a final volume of 64 ml. 320 µmoles of 1,3-¹⁴C-glycerol, 320 µmoles of 2-¹⁴C-glycerol, or 40 µmoles of leucine was used as the radioactive compound. Incubation was carried out for 12 hr at 26°C. Detailed methods of separation and purification of radioactive pyridoxal are shown in text.

4. Incorporation of ¹⁴C-Compounds into B₆ by Resting Cells

The results of above experiments suggested that glycerol and a certain amino acid could be used as precursors of pyridoxal. To decide whether these compounds are actually incorporated into pyridoxal, the suspected precursors labeled with ¹⁴C were incubated with resting cell preparations and after hydrolysis and the addition of unlabeled pyridoxal as carriers, the vitamin was isolated in the form of pyridoxine as mentioned in the experimentals. Such pyridoxine obtained was sublimated and recrystallized to a constant specific activity. The results of several such experiments are summarized in Table 4. Carbon from glycerol and leucine, was incorporated into pyridoxal with significant dilution. But even if aspartic acid stimulated the production of pyridoxal, its carbon was incorporated into the vitamin with very high dilution.

The data presented above provide con-
clusive evidence that the carbon chains of glycerol and leucine are utilized in the synthesis of pyridoxine. However, they do not permit any conclusions regarding the location within the pyridoxal molecule of any specific radioactive carbon atom derived from a known radioactive substrate. The results of this analysis for the localization of radioactive carbon atom will be presented in the next paper.

Acknowledgements

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References