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# Biosynthesis of Vitamin $B_6$

## II. Localization of $^{14}C$-Carbons in Vitamin $B_6$

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**$^{14}C$-Labeled pyridoxal was synthesized from 2-$^{14}C$-glycerol and 1,3-$^{14}C$-glycerol. Radioactivity of 2-$^{14}C$-glycerol was incorporated in the pyridine moiety of pyridoxal, whereas that of 1,3-$^{14}C$-glycerol was incorporated in the pyridine moiety of pyridoxal and $CO_2$. From these results we suggested that 5,6-positions of the carbon atoms of pyridoxal and that in hydroxymethyl attached to 5-C of pyridoxal came from glycerol and other carbons from leucine or its related compounds.**

Considerable evidence in the previous paper supports the view that the precursors of vitamin $B_6$ are glycerol and leucine (1). By the results of the radiochemical tracer experiments, using 1,3-$^{14}C$-glycerol, 2-$^{14}C$-glycerol and uniformly labeled $^{14}C$-L-leucine, it was confirmed that they were the precursors of vitamin $B_6$, since they were incorporated into pyridoxal with a very low dilution. In this paper it was aimed to decide the site of $^{14}C$-location and to clarify the part of the mechanism of $B_6$ biosynthesis.

## EXPERIMENTAL

### 1. *Chemicals*

$^{14}C$-U-leucine, 1,3-$^{14}C$-glycerol and 2-$^{14}C$-glycerol were obtained from Radiochemical Centre, Amersham, England. Other chemicals were of the puriest grade available from commercial sources.

### 2. *Preparation of Diazomethane*

Nitrosomethyl urea was prepared from methylamine hydrochloride and potassium cyanate (2). Two grams of nitrosomethyl urea and 5 ml of 40 %KOH aqueous solution was mixed with 20 ml of ether and shaken vigorously for 10 min. The ether layer was dehydrated by the addition of granular potassium hydroxide. The yellow ether layer was diazomethane dissolved in ether.

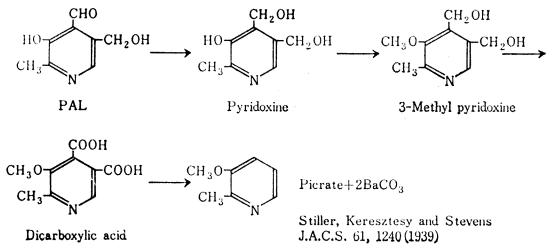


FIG. 1 *Degradation of $^{14}C$-pyridoxal*

### 3. *Degradation of Pyridoxal*

The reduction of pyridoxal to pyridoxine was made according to Matsuo and Greenberg (3). Oxidation of pyridoxine to pyridoxine dicarboxylic acid was carried out by the method of Leete (4), and decarboxylation of pyridoxine dicarboxylic acid by the method of Stiller *et al.* (5).

### 4. *Preparation of Free Base of Pyridoxine*

As described in the previous paper (1), $^{14}C$-pyridoxal was synthesized from $^{14}C$-compounds (1,3-$^{14}C$-glycerol, 2-$^{14}C$-glycerol, or U-$^{14}C$-L-leucine). After

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several recrystallizations, $^{14}$C-pyridoxal was changed to $^{14}$C-pyridoxine by sodium borohydride. It was placed on the column of Dowex-50($H^+$), and eluted with 1 N $NH_4OH$. Pyridoxine fraction was evaporated to dryness and sublimed at 140° ($10^{-4}$ mm Hg), whereby white crystals were obtained.

*5. Methylation of Pyridoxine*

A well-cooled solution of 30 mg of $^{14}$C-pyridoxine in 1 ml of absolute methanol was treated with an excess of diazomethane in 7.5 ml of absolute ether. After standing at room temperature for 16 hours, the solvent and excess diazomethane were removed by distillation. The residual brown oil was taken up in 5 ml of methyl alcohol and treated with 6 volumes of ether. The resultant precipitate was again treated in a similar manner. The combined methyl alcohol-ether solution was applied on paper and developed with the solvent of water-N-propanol-ethyl-acetate (50 : 60 : 80) for 16 hours. Pyridoxine and methyl pyridoxine on the paper chromatogram were detected under the mineral light at the wave length of 350 mμ. $R_F$ of methyl pyridoxine was 0.3–0.4, whereas that of pyridoxine was $R_F$ 0.7–0.8. The zone of methyl pyridoxine was cut and eluted by the water. They were crystallized by chloroform-petroleum ether. The absorption spectrum was very similar to that of pyridoxine, but Gibb's reaction gave a negative result. Yield of methyl pyridoxine was 24.74 mg.

*6. Oxidation of the Methyl Ester of Pyridoxine*

To a solution of 20 mg of pyridoxine methyl ester in 2.0 ml of water, 0.5 ml of 0.1 M barium permanganate was added in a small amount with shaking. It was allowed to stand at room temperature for 16 hours. It was then centrifuged and the washings were combined and condensed. It was dissolved in a small amount of methyl alcohol and subjected to paper chromatography with the same solvent mentioned above. The area of pyridoxine dicarboxylic acid was detected under the mineral light and its $R_F$ value showed 0 25. This zone was eluted and condensed to dryness. Total yield was 10.70 mg.

*7. Decarboxylation of $B_6$ Dicarboxylic acid*

Pyridoxine dicarboxylic acid (3.84 mg) was decarboxylated by refluxing in 30 ml of diphenyl-methane with 10.0 mg of copper chromite catalyst at 280° on the silicon oil bath in a nitrogen atomosphere for 3 hours. The carbon dioxide developed was absorbed in 1 N NaOH. Saturated barium chloride solution was added and resulted $BaCO_3$ was obtained after filtration. 20 mg of picric acid dissolved in 5 ml of ethanol was added from the top of reflux. The combined solution was filtered and excess copper chromite was removed as residue. Excess amount of free picric acid and diphenyl methane were removed from the filtrate with several ether extractions. Water layer was condensed and measured as the picrate of the pyridine part of vitamin $B_6$.

## Results and Discussion

As shown in Table 1, 11,600 cpm of pyridoxine biosynthesized from 2-$^{14}$C-glycerol and 6,620 cpm of pyridoxine biosynthesized from 1,3-$^{14}$C-glycerol were used as the starting materials. After the decarboxylation, the radioactivity of glycerol-2-$^{14}$C was not detected in the $CO_2$. On the otherhand, when pyridoxine originated from glycerol-1,3-$^{14}$C was used as precursor, the ratio of radioactivities, $CO_2$ to pyridine moiety, was almost 1 : 2. By our degradation method, 2 moles of $CO_2$ and one mole of pyridine moiety were produced from one mole of pyridoxal phosphate, *i. e.*, one $^{14}$C of glycerol was incorporated into $CO_2$ and another $^{14}$C of glycerol remained in the pyridine moiety. When 9,250 cpm of $^{14}$C-pyridoxine biosynthesized from 2-$^{14}$C-aspartic acid was tested, $^{14}$C of pyridoxine was distributed equally in the pyridine ring and the side chain. On the other hand, 7,000 cpm of $^{14}$C-pyridoxine biosynthesized from U-$^{14}$C-L-leucine was analyzed, radioactive carbon atoms were distributed almost in the pyridine ring of pyridoxine.

From these results it was suggested that the carbons of glycerol moved to the carbon of hydroxymethyl attached to 5-C of pyridoxal, and the carbons of 5,6-positions of

TABLE 1
*Localization of radioactive $^{14}$C in pyridoxal*

| | 2-$^{14}$C-Glycerol | 1,3-$^{14}$C-Glycerol |
|---|---|---|
| | (*cpm/mmole*) | |
| Pyridoxal | 11,600 | 6,620 |
| $CO_2$ | 160 | 1,710 |
| Pyridine Ring | 8,400 | 3,570 |

Radioactive pyridoxal was biosynthesized by *Flavobacterium* as shown in the previous paper (1). Detailed degradation method of pyridoxine was also described in "Experimental".

the pyridine ring of pyridoxal. Other carbons came from leucine or its related compounds.

In 1960, Ortega and Brown (6) suggested that the pyridine ring of nicotinic acid was derived from glycerol and succinate. Anyway, the fact that both pyridine rings of nicotinic acid and of pyridoxal come from glycerol is of great interest. Further study to determine the localization of $^{14}$C in leucine and the source of nitrogen is now under investigation.

## References


1. Suzue, R. and Haruna, Y., *J. Vitaminol.*, **16**, 154 (1970).
2. Arnold, H. R., *Organic Synthesis*, Coll. Vol. II, p. 442 (1943).
3. Matsuo, Y., and Greenberg, D. M., *J. Biol. Chem.*, **234**, 507 (1959).
4. Leete, E., *J. Am. Chem. Soc.*, **78**, 3520 (1956).
5. Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, **64**, 1240 (1942).
6. Ortega, M. V., and Brown, G. M., *J. Biol. Chem.*, **235**, 2939 (1960).