Pyridoxol Biosynthesis by *Saccharomyces fragilis*[1] (37024)

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In the 35 yr that have elapsed since the isolation of pyridoxine, comparatively little has been learned about the mechanism of its biosynthesis by autotrophic organisms. Some early work indicated that a mixture of glycine and hydroxyacetaldehyde could partly replace pyridoxal in the growth of pyridoxal-requiring strains of *E. coli* (1). Dempsey (2) has implicated C_3 of serine as a direct precursor of one of the hydroxymethyl groups of pyridoxol in some *E. coli* mutants, and most recently Hill, *et al.* (3) have concluded that glycerol and pyruvate contribute to the synthesis of pyridoxol by *E. coli*, the presumed intermediate being 5-deoxyxylulose (4).

The yeast *Saccharomyces fragilis* produces 250–300 μg of pyridoxol/liter when grown on a minimal medium containing glucose as the sole carbon source. This observation offered an opportunity to identify possible B_6 precursors by determining the extent to which various compounds, added to a culture of *S. fragilis* growing on ^{14}C-glucose, spared the incorporation of glucose carbons into pyridoxol. In addition, the extent to which several ^{14}C-labeled compounds other than glucose (acetate, aspartic acid, formate, methionine, and serine) contribute to the formation of labeled pyridoxol by *S. fragilis* has been examined.

Methods. S. fragilis C-351[3] was grown in a medium containing 83 m*M* glucose, 28 m*M* $(NH_4)_2SO_4$, 6 m*M* KCl, 4 m*M* sodium citrate, 1.1 m*M* $CaCl_2$, traces of Fe^{3+} and Mn^{2+}, and a mixture of inositol, thiamine, nicotinamide, calcium pantothenate and biotin; it was buffered (pH 5.6) with 80 m*M* phosphate. The yeast was grown at 30° with slow aeration.

Pyridoxol was isolated from the cell-free medium by two successive Dowex-50 chromatograms. The first column was prepared as described by Storvick (5) and was equilibrated with 0.01 *M* KOAc, pH 4.5. After applying the sample, previously adjusted to pH 3.9, pyridoxol and its analogues were eluted by a KOAc gradient (0.02 *M*, pH 5.5 → 0.10 *M*). The column was monitored by measuring the optical density of the effluent at 255 and 320 nm. Pyridoxol was the only compound absorbing at the latter wavelength which was eluted by this gradient and a reliable estimate of the total amount of pyridoxol produced could be obtained by measuring the absorbance at 320 nm of the first peak to emerge after beginning the gradient. When labeled pyridoxol was to be isolated, a known amount (usually 1 mg) of carrier pyridoxol was added to the solution before beginning its fractionation. Pyridoxol isolated from the first Dowex column was rechromatographed on a second Dowex column, this time at pH 6.05, and eluted with 0.05 → 0.15 *M* KOAc. This column efficiently separated pyridoxol from its chief radioactive contaminant, adenine. After a third chromatogram to replace acetate by chloride, additional carrier pyridoxol (15 mg) was added, and pyridoxol was recrystallized to constant specific activity (and E_{320}/E_{255} ratio) from isopropanol. The total radioactivity incorporated into pyridoxol was then calculated from the specific activity of the recrystallized material, after correcting for all losses.

To determine the effects of a particular

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[3] Culture generously supplied by Dr. Beverly Guirard, University of California.

substrate upon pyridoxol labeling by ^{14}C-glucose, uniformly labeled glucose was added to a 60- or 72-hr culture of *S. fragilis*. After 144 hr, labeled pyridoxol was isolated, recrystallized to constant specific activity, and the total amount of glucose carbon (cpm of ^{14}C-glucose) converted to pyridoxol was calculated. This value provides a measure of the carbon flow from glucose to pyridoxol. The experiment was then repeated, this time adding an (unlabeled) potential precursor along with labeled glucose. If the compound added is a direct precursor of pyridoxol, the radioactivity of the pyridoxol formed in the second experiment would be expected to be reduced compared to the first, the difference in activities reflecting the proximity of the compound to pyridoxol as well as the fraction of the carbon atoms in pyridoxol which it provides.

Information concerning the location of radioactive carbon in radioactive pyridoxol was obtained by chromic acid oxidation. This converts both C'_2 and C_2 (the angular methyl group and its adjacent ring carbon) into acetic acid (6), which was separated from the reaction mixture by distillation. In model experiments, 77% of the theoretical amount of acetic acid was routinely recovered in the distillate. Radioactivity was measured in a Packard tri-carb scintillation spectrometer, with Bray's mixture (7) as the counting fluid.

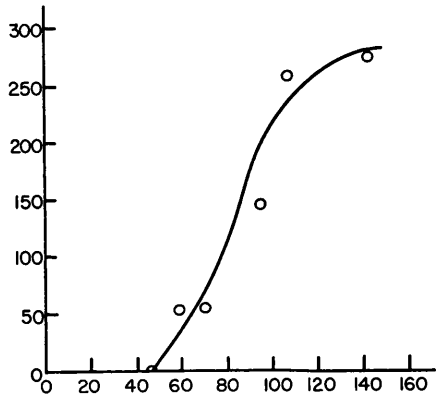

FIG. 1. Pyridoxol production by *S. fragilis*. The pyridoxol content of the cell-free medium was estimated chromatographically. Abscissa: incubation time in hours; ordinate: micrograms of pyridoxol per liter of medium. Other details in text.

TABLE I. Pyridoxol Labeling by Glucose-^{14}C.[a]

Glucose added (mCi)	Time of addition (hr)	Recovered (cpm) as pyridoxol
0.10	0	2900
0.05	60	9500
0.05	60	10,500
0.10	72	11,200
0.10	72	10,000

[a] ^{14}C-Glucose (UL) was added aspetically to cultures of *S. fragilis* C-351 at the times indicated. The cultures were harvested at 144 hrs and the pyridoxol was isolated after the addition of carrier. The values in the last column represent the counts in pyridoxol after correction for all losses.

Efficiency for ^{14}C was about 60%.

Results. Figure 1 shows the rate of pyridoxol production by an aerated culture of *S. fragilis* at 30°. Total cell population, as indicated by turbidity measurements, does not change significantly after 48 hr. On the other hand, no pyridoxol appears in the medium for the first 2 days of growth. After 48 hr, the concentration of pyridoxol increases abruptly, reaching a plateau (265 μg/liter) after about 96 hr. Nearly the same amount of pyridoxol (220 μg/liter) may be recovered if the cells were collected after 49 hr incubation, resuspended in fresh medium containing 0.5–1.5% glucose, and incubated for another 60–70 hr. That the precipitous rise in B_6 concentration does not simply represent release of preformed pyridoxol was indicated by the fact that labeled pyridoxol can still be isolated if ^{14}C-glucose was added as late as 72 hr after inoculation (Table I). These data suggest, in fact, that most of the pyridoxol produced by *S. fragilis* is formed between 60 and 72 hr after inoculation. In most of the experiments described, therefore, labeled glucose and possible precursors were added after 60 or 72 hr.

A total of 14 compounds were examined for their effects upon pyridoxol labeling by ^{14}C-glucose. The data are summarized in Table II. Sodium acetate, alanine, asparagine, glutamic acid, serine, and tryptophan had minimal (less than 20%)[4] effect upon pyri-

[4] As indicated in Table II, experiments with labeled glucose alone were reproducible to ± 10%.

TABLE II. Effects of Supplements upon the Formation of ^{14}C-Pyridoxol from ^{14}C-Glucose.[a]

Compound added	Amount added (mmoles)	Counts recovered as ^{14}C-pyridoxol (cpm)	Percentage change
Expt I			
None	—	10,600 (10,000; 11,200)	—
L-Aspartic acid	*3.75*	*5850 (4950; 6750)*	*—45*
Glycine + hydroxyacetaldhyde	*3.33* / *4.15*	*7050*	*—34*
Pyridoxol	*0.01*	*4150*	*—61*
L-Serine	4.75	10,100	— 5
Expt II			
None	—	10,000 (9500; 10,500)	—
L-Alanine	5.60	8150	—18
L-Asparagine	3.75	10,850	+ 8
L-Glutamic acid	3.40	11,850	+18
Glycine	*6.67*	*17,700*	*+77*
Hydroxyacetaldehyde	*8.30*	*6200 (6100; 6300)*	*—38*
Nicotinic acid	*4.05*	*12,450*	*+25*
Sodium acetate	5.00	11,700	+17
Sodium oxaloacetate	*3.75*	*17,000*	*+70*
Sodium pyruvate	*4.50*	*12,550*	*+25*
D-Ribose	*3.10*	*15,050*	*+50*
L-Tryptophan	2.45	11,600	+16

[a] In Expt I, 0.10 mCi of uniformly labeled glucose (and the unlabeled compound) were added to a 72-hr culture of *S. fragilis;* in Expt II, 0.05 mCi glucose and the supplement were added to a 60-hr culture. In both experiments, labeled pyridoxol was isolated from the medium after 144 hr. The italicized data are those which show a significant (more than 20%) effect, compared to the control experiment.

doxol labeling. Five compounds—glycine, nicotinic acid, oxaloacetic acid, pyruvic acid and ribose—increased pyridoxol labeling by 25–70% when added 60 hr after inoculation. Aside from pyridoxol itself, only two compounds, aspartic acid and hydroxyacetaldehyde, appreciably reduced labeling of pyridoxol by glucose.

Table III includes the results of experiments in which radioactive compounds other than glucose were studied as precursors of pyridoxol by *S. fragilis*. In these experiments, serine-3-^{14}C was added at the time of incubation; the other compounds were added to 60-hr cultures. As before, radioactive pyridoxol was isolated after 144 hr.

Discussion. The data of Table II indicate that the pyridine ring of vitamin B_6 does not arise from tryptophan; nor, seemingly, is nicotinic acid used in its biosynthesis. On the other hand, the fact that the addition of aspartic acid reduced glucose labeling of pyridoxol by nearly 50% encouraged the speculation that a direct pathway from aspartate to B_6, analogous to that from aspartate to nicotinic acid in *C. butylicum* (8) might be operative in *S. fragilis*. Experiments with labeled aspartic acid (Table III) at first supported this hypothesis; however, degradation of pyridoxol derived from both aspartate-1-^{14}C and aspartate-3-^{14}C indicated essentially random labeling by both precursors.[5] It was subsequently discovered that the addition of aspartic acid to a 60-hr culture of *S. fragilis* reduced the pyridoxol content of the medium from 270 to 140 μg/liter; the data of Table II can thus be accounted for by a reduced quantity of glucose carbons being used for B_6 synthesis. In contrast, the addition of hydroxyacetaldehyde (glycolaldehyde) had no effect upon the total amount of pyridoxol formed. This compound remains, therefore, a likely candidate as a direct precursor of pyridoxol; its involvement would be consistent

[5] Pyridoxol contains 8 carbon atoms. If the distribution of labeled carbon is completely random, the acetic acid obtained upon chromic acid oxidation should contain 25% of the total radioactivity.

TABLE III. Incorporation of Labeled Substrates into Pyridoxol.[a]

Substrate	Amounted added (mCi)	(mmoles)	Recovered (cpm) as pyridoxol	Counts recovered as acetic acid (%)
Aspartate-1-^{14}C	0.023	0.375	3500	19.7
Aspartate-3-^{14}C	0.023	0.375	6250	19.6
Aspartate-4-^{14}C	0.023	0.375	500	—
Acetate-1-^{14}C	0.10	1.0	2600	—
Acetate-2-^{14}C	0.10	1.0	2550	—
Serine-3-^{14}C	0.10	5.0	7400	23.8
Formate-^{14}C	0.10	1.0	Trace	—
Methionine-$^{14}CH_3$	0.10	0.34	Trace	—

[a] *S. fragilis* was grown in a minimal medium (see text) at 30°. Radioactive substrates were added aseptically, serine at the time of inoculation, aspartate at 60 hr, and the others at 72 hr. The cultures were harvested at 144 hr and pyridoxol was isolated and counted. The last column refers to the fraction of pyridoxol counts recovered as acetic acid after chromic acid oxidation.

with a proposed pathway for pyridoxol formation via 5-deoxyxylulose (4). Hough and Jones (9) have reported that hydroxyacetaldehyde, like acetaldehyde, can engage in an aldolase-catalyzed reaction with triose phosphate to form xylulose; further reaction with glyceraldehyde phosphate and ammonia would lead ultimately to 3-hydroxy-2,4,5-trihydroxymethyl pyridine; Argoudelis (10) has shown that the latter compound can be transformed into pyridoxol by *S. carlsbergensis*. The unavailability of labeled hydroxyacetaldehyde has, however, thus far prevented a direct test of this hypothesis.[6]

The failure of pyruvate to spare the incorporation of glucose carbons into pyridoxol (indeed, as Table II shows, incorporation slightly *increased*) suggests that pyruvate may not play as critical a role in vitamin B_6 biosynthesis in *S. fragilis* as it has been postulated to do in *E. coli* (3).

Two pathways have been suggested for the incorporation of carbon 3 of serine into pyridoxol in *E. coli* mutants. In one, the hydroxymethyl group is transferred intact (2); in the other, 90% of the labeled carbon appears in C_2 or C'_2 of pyridoxol (3). From the data of Table III, neither of these mechanisms appear to be operative in *S. fragilis*; although pyridoxol was efficiently labeled by serine-3-^{14}C, the distribution of label in the final product appeared to be essentially random. The data of Table III also indicate that neither formate, acetate, nor methionine (methyl) supplies carbon atoms for direct incorporation into pyridoxol.

Several compounds, including glycine, oxaloacetic acid, and ribose, added 60 hr after inoculation, greatly *increased* pyridoxol labeling by glucose (Table II). These results could not be explained by an increase in pyridoxol yield; glycine and ribose did not affect the final concentration of pyridoxol, and the addition of 3.75 mmoles of oxaloacetic acid actually reduced pyridoxol formation (to 105 and 95 μg in duplicate experiments).

Summary. During 6 days of growth in a medium containing glucose as the major carbon source, *Saccharomyces fragilis* C-351 secretes about 270 μg of pyridoxol/liter of culture fluid. Most of this is produced between 60 and 108 hr after inoculation.

Studies of the effects of a number of compounds upon the conversion of labeled glucose to pyridoxol indicates that hydroxyacetaldehyde (glycolaldehyde) is a probable direct precursor of pyridoxol in *S. fragilis*.

[6] Very recently Tani and Dempsey (Abstr. 228, 164th ACS meeting, Sept., 1972, New York) have presented evidence for the direct incorporation of labeled glycolaldehyde into B_6 by *E. coli*.

1. Morris, J. G., J. Gen. Microbiol. **20**, 597 (1959).

2. Dempsey, W. B., Biochim. Biophys. Acta **222**, 686 (1970).

3. Hill, R. E., Rowell, F. J., Gupta, R. N., and Spenser, I. D., J. Biol. Chem. **247**, 1869 (1972).

4. Leete, E., *in* "Biogenesis of Natural Compounds" (P. Bernfeld, ed.), 1st ed., p. 748. Pergamon, Elmsford, NY (1963).

5. Storvick, C. A., Benson, E. M., Edwards, M. A., and Woodring, M. J., *in* "Methods of Biochemi-

cal Analysis" (D. Glick, ed.), Vol. 12, p. 255. Wiley (Interscience), New York (1964).

6. Kuhn, R., and Wendt, G., Chem. Ber. **72**, 305 (1939).

7. Bray, G. A., Anal. Biochem. **1**, 279 (1960).

8. Scott, T. A., and Mattey, M., Biochem. J. **107**, 606 (1968).

9. Hough, L., and Jones, J. K. N., J. Chem. Soc. 4047 (1952).

10. Argoudelis, C. J., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **27**, 1895 (1968).

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