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Biosynthesis of Vitamin B_6: Incorporation of Three-Carbon Units

Abstract. *Pyridoxol, one of the forms of vitamin B_6, is derived from three glycerol units. One of these is incorporated by way of pyruvate as a two-carbon fragment at the oxidation level of acetaldehyde. The other two glycerol units are incorporated intact, possibly by way of triose phosphate.*

Although it is well recognized that vitamin B_6 is biosynthesized by plants, algae, and most microorganisms, little is known of the pathways involved (*1*). We now offer definite evidence concerning the primary precursors of pyridoxine (that is, pyridoxol), one of the forms of the vitamin.

The major obstacle in the investigation of the biosynthesis of vitamin B_6 is the minute concentration (40 to 200 ng per milligram of dry cell material) (*2*) at which it is present in systems that produce it. Success in biosynthetic tracer studies of the origin of B_6 is thus contingent on a high radiochemical yield in the incorporation of radioactive tracer, such that the specific activity of the product, even after manyfold dilution with inactive carrier, is still high enough for degradation studies. The incorporation of activity from ^{14}C-labeled substrates into pyridoxamine in the yeasts *Candida utilis* (*3, 4*) and *C. albicans* (*5*) was insufficient for this purpose. Nonrandom incorporation of activity from [2-^{14}C]glycerol (*6*) and from DL-[2-^{14}C]aspartate (*7*) into pyridoxamine was demonstrated in a bacterial strain isolated from soil. Partial degradation indicated that little of the activity was present at the aminomethyl and the hydroxymethyl carbon atoms (C-4′ and C-5′). The sites of labeling were not further investigated, however.

We have examined the biosynthesis of pyridoxol in a mutant of *Escherichia coli* (*8*), which is blocked between pyridoxol and pyridoxal (*9*). When deprived of exogenous pyridoxal, this mutant generates pyridoxol and excretes it into the culture medium. Cultures of the mutant were grown to exponential phase in a minimal medium (*9*) supplemented with pyridoxal. The cells were then harvested, washed free of pyridoxal, and resuspended in pyridoxal-free minimal medium to which labeled substrate had been added. Incubation was resumed and continued for 5 to 6 hours, at which time labeled pyridoxol hydrochloride was isolated from the medium by ion exchange (Dowex 50 × 8) and thin-layer (silica gel G) chromatography, after addition of inactive carrier (Table 1).

The labeled samples of pyridoxol hydrochloride were purified to constant radioactivity by repeated crystallization and vacuum sublimation. Kuhn-Roth oxidation then yielded acetic acid, isolated as the α-naphthylamide (*10*), from C-2 and the adjacent C-methyl group (C-2′). Degradation of the acetic acid by the Schmidt reaction in turn gave the C-methyl group as methylamine, isolated as the *N*-dinitrophenyl derivative. The specific activities of the samples of pyridoxol and the corresponding degradation products are presented in Table 2.

Pyridoxol, obtained from the cultures incubated with [1-^{14}C]glycerol (experiments 1 and 2), contained approximately one-fifth of its label in the two-carbon unit (C-2′, C-2) (acetic acid), irrespective of whether glycerol or glucose had served as the general carbon source. Since virtually all the activity of this acetic acid was located at the C-methyl group (C-2′) (methylamine), distribution of label from [1-^{14}C]glycerol was nonrandom (*11*) (experiment 2). It may be inferred from this result that five of the eight carbon atoms of pyridoxine might be ultimately derived from the terminal carbon atoms of glycerol.

In an attempt to define more precisely the relation to pyridoxol of three-carbon compounds related to glycerol, incorporation of label from pyruvic acid was investigated. All activity of pyridoxol from [2-^{14}C]pyruvate (experiment 5) and from [3-^{14}C]pyruvate (experiment 3) was found in the Kuhn-Roth acetate (C-2′, C-2), whose C-methyl carbon (C-2′) is supplied entirely by the C-methyl group of pyruvate (experiment 4). The two-carbon unit (C-2′, C-2) of pyridoxol is thus derived specifically from the methyl and the carbonyl carbon atoms of pyruvate.

To test whether the carboxyl carbon of pyruvate serves as the source of C-3 of pyridoxol, that is, whether an intact pyruvate moiety yields the three-

Table 1. Incorporation of three-carbon units into pyridoxol. The general carbon source in experiment 1 was glucose (0.2 percent, weight to volume). In all other experiments the general carbon source was glycerol (0.2 percent, weight to volume). The culture volume in experiment 6 was 2 liters. In all other experiments it was 1 liter.

Expt. No.	Substrate*	Nominal		Carrier added (g)	Pyridoxol hydrochloride specific activity (10^4 count min^{-1} $mmole^{-1}$)
		Total activity (mc)	Specific activity (mc/mmole)		
1	[1-^{14}C]Glycerol	0.1	15.4	0.04	1.78 ± .05
2	[1-^{14}C]Glycerol	0.5	0.023†	0.12	2.38 ± .06
3	[3-^{14}C]Pyruvic acid	0.1	35.6	0.04	1.20 ± .03
4	[3-^{14}C]Pyruvic acid	0.1	35.6	0.09	0.54 ± .01
5	[2-^{14}C]Pyruvic acid	0.1	31.7	0.04	0.72 ± .02
6	[1,3-$^{14}C_2$]Pyruvic acid‡	0.3	30.8	0.08	1.05 ± .02
7	[2-^{14}C]Acetic acid	0.1	54.7	0.04	0.36 ± .01

* Labeled compounds were obtained from Amersham/Searle (experiments 1, 3–7) and from Commissariat à l'Energie Atomique (experiment 2). † Obtained by mixing [1-^{14}C]glycerol, nominal specific activity 10 mc/mmole (4.6 mg), with inactive glycerol (2 g). ‡ Obtained by mixing [1-^{14}C]-pyruvic acid (0.15 mc, 27.2 mc/mmole) and [3-^{14}C]pyruvic acid (0.15 mc, 35.6 mc/mmole). For determination of distribution of activity, see Table 2, last note (§).

Table 2. Specific activity of pyridoxol and its degradation products. SA, specific activity (10^4 count min^{-1} $mmole^{-1}$); RSA, relative specific activity (percent) (intact pyridoxol = 100).

Expt. No.	Substrate	Pyridoxol SA	Acetic acid* (C-2′, C-2)		Methylamine† (C-2′)	
			SA	RSA	SA	RSA
1	[1-^{14}C]Glycerol	1.78 ± 0.05	0.39 ± 0.01	22 ± 1		
2	[1-^{14}C]Glycerol	2.38 ± 0.06 1.12 ± 0.03‡	0.51 ± 0.01	21 ± 1	0.20 ± 0.01	18 ± 1
3	[3-^{14}C]Pyruvic acid	1.20 ± 0.03	1.15 ± 0.03	96 ± 3		
4	[3-^{14}C]Pyruvic acid	0.54 ± 0.01			0.47 ± 0.01	88 ± 2
5	[2-^{14}C]Pyruvic acid	0.72 ± 0.02	0.68 ± 0.02	94 ± 3		
6	[1,3-$^{14}C_2$]Pyruvic acid§	1.05 ± 0.02	0.94 ± 0.02	90 ± 2	0.87 ± 0.02	84 ± 2
7	[2-^{14}C]Acetic acid	0.36 ± 0.01	0.21 ± 0.01	58 ± 2		

* Isolated as acetyl α-naphthylamide. † Isolated as *N*-methyl-2-4-dinitroaniline. ‡ Obtained from the pyridoxol, specific activity (2.38 ± 0.06) × 10^4 count min^{-1} $mmole^{-1}$, by further dilution with inactive pyridoxol. § To confirm the distribution of label in this substrate, a small sample (~2 μc) was diluted with inactive pyruvic acid and converted to the phenylhydrazone [SA = (3.47 ± 0.02) × 10^4 count min^{-1} $mmole^{-1}$] and degraded by Kuhn-Roth oxidation to yield acetic acid* [SA = (1.65 ± 0.01) × 10^4 count min^{-1} $mmole^{-1}$] (RSA = 48 ± 1 percent).

carbon fragment, C-2′, C-2, C-3, of pyridoxol, an attractive possibility on structural grounds, an experiment with [1,3-$^{14}C_2$]pyruvate (experiment 6) was carried out. Intact incorporation of [1, 3-$^{14}C_2$]pyruvate [52 ± 1 percent of the total activity of this pyruvate was located at the carboxyl carbon, 48 ± 1 percent of the total activity at the methyl carbon, Table 2, last note (§)] would have led to pyridoxol which on degradation should have yielded acetate (C-2′, C-2) and methylamine (C-2′) containing 48 percent of the activity of the vitamin.

In fact the acetate contained 90 percent of the activity (experiment 6), most of which was located at C-2′ of pyridoxol and was therefore derived (experiments 3 and 4) from the C-methyl group of pyruvate. It follows that the carboxyl group of pyruvate had not been incorporated into pyridoxol. Thus, it is a two-carbon unit, corresponding to the methyl and carbonyl group of pyruvate, which supplies the two-carbon unit, C-2′, C-2, of pyridoxol. Since incorporation of acetate into this two-carbon unit is not quantitative (experiment 7) and takes place in low radiochemical yield, it is likely that the pyruvate-derived two-carbon unit enters at the oxidation level of acetaldehyde.

The structural correspondence of glycerol, pyruvate, and pyridoxol (Fig. 1, box), and the steps of a plausible reaction sequence (*12*) leading from the precursors to the product, are shown in Fig. 1. This sequence is consistent with all the available evidence: Of eight carbon atoms of pyridoxol, five (starred) are derivable from the primary carbons (starred) of glycerol (experiments 1, 2), and three (dotted) [which do not include C-4′ and C-5′ (*6*)] from the secondary carbon atom (dotted). Since glycerol enters the glycolytic pathway, by way of glycerol phosphate and glyceraldehyde-3-phosphate, and is hence convertible into py-

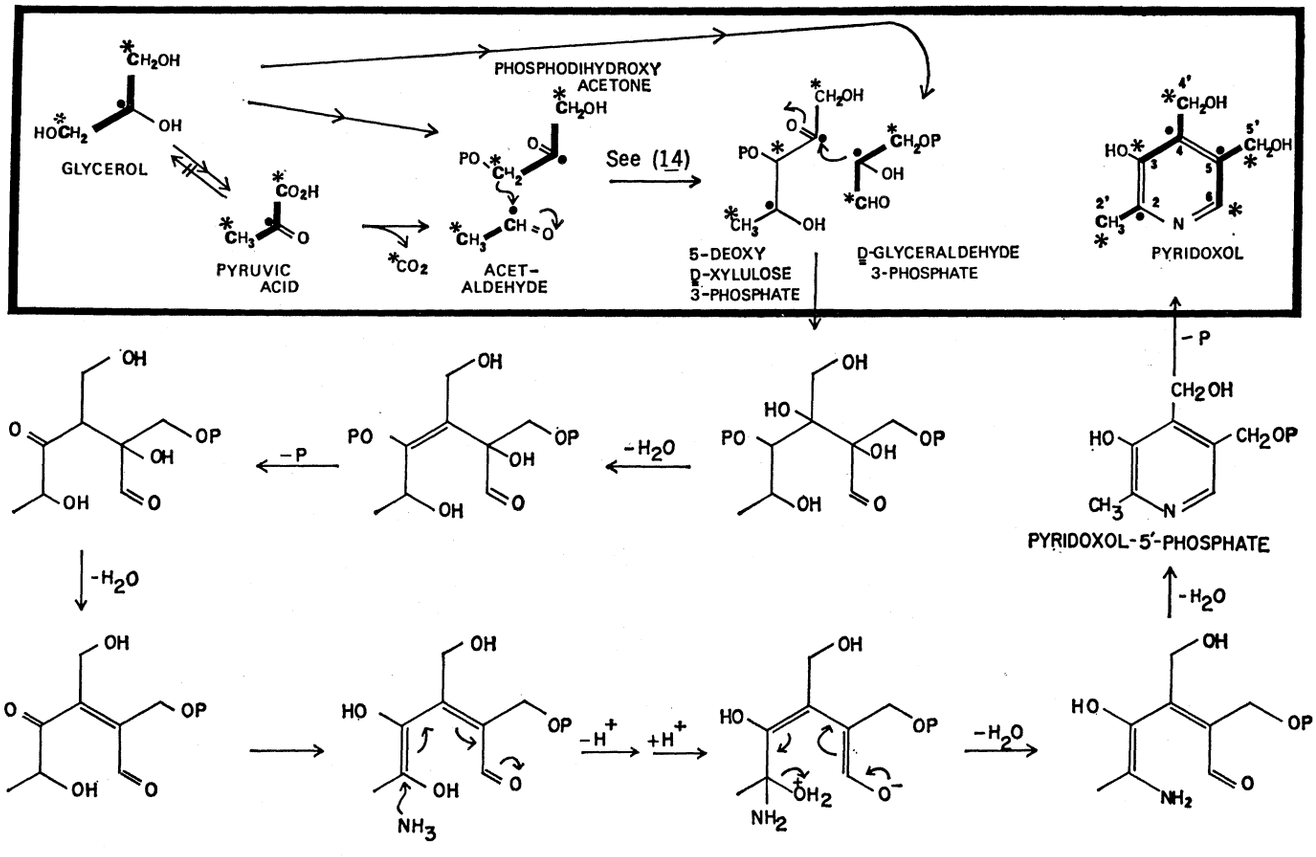

Fig. 1. Biosynthesis of pyridoxol. Structural correspondence of glycerol, pyruvate, and pyridoxol (box) and hypothetical biosynthetic sequence.

ruvate, glycerol can serve as a source of the pyruvate-derived carbon atoms of pyridoxol. Since the final step on this route from glycerol to pyruvate, the dephosphorylation of phosphoenolpyruvate, is essentially irreversible and since reconversion of pyruvate into triose phosphate by alternative pathways is, in any case, unlikely to be important under the chosen experimental conditions, pyruvate cannot replace glycerol as a complete source of the carbon skeleton of pyridoxol. It has yet to be established whether phosphoserine, whose implication in pyridoxol biosynthesis has been inferred on the basis of growth studies with blocked mutants (*13*), enters by way of pyruvate, by way of triose phosphate, or directly.

ROBERT E. HILL
IAN D. SPENSER

Department of Chemistry,
McMaster University,
Hamilton, Ontario, Canada

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11. Random incorporation of label would have supplied one-eighth of the total activity to each of the eight carbon atoms of pyridoxol and would have resulted in the recovery of one-quarter of the total activity in the Kuhn-Roth acetate and one-eighth in the methylamine obtained by degradation.
12. A speculative biogenetic hypothesis, embodying the major features of this scheme, has been put forward on purely structural grounds [E. Leete, in *Biogenesis of Natural Compounds*, P. Bernfeld, Ed. (Pergamon Press, New York, ed. 1, 1963), p. 748].
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Mercury Poisoning: Prevention by Spironolactone

Abstract. *The renal tubular necrosis and calcification as well as the mortality induced by mercuric chloride in the rat are readily prevented by prior treatment with well-tolerated amounts of spironolactone.*

Spironolactone can protect the rat against intoxication with digitoxin (*1*), various anesthetics (*2*), dimethylbenzanthracene (*3*), indomethacin (*4*), and numerous other agents (*5*). Such protective effect is shared by many other "catatoxic steroids" which are known to act through the induction of hepatic drug-metabolizing enzymes; hence the question arose whether spironolactone could also protect against the poisoning with a toxic heavy metal salt, which could not be disposed of by enzymatic degradation.

Female Sprague-Dawley rats (30) with a mean body weight of 100 g (range 90 to 110 g) were divided into two equal groups. Group 1 was not treated; group 2 received 10 mg of spironolactone (Searle) in 1 ml of water by stomach tube, twice daily, beginning on the first day and continuing until termination of the experiment. On the fourth day the rats of both groups were given a single dose consisting of 400 μg of $HgCl_2$ in 1 ml of water intravenously.

The control group began to show signs of prostration within 48 hours, and all of them died within 3 days after the injection of $HgCl_2$. By contrast, the animals that were treated with spironolactone before being given the $HgCl_2$ remained in excellent condition and were killed with chloroform when mortality reached 100 percent in the controls.

At autopsy the kidneys of each of the control rats showed heavy cortical calcification with severe perirenal edema. In the spironolactone-treated animals the kidneys and their surroundings were normal. Upon histologic examination of alcohol-formol fixed specimens stained with the von Kóssa or the celestin blue techniques, calcification and necrosis were found to be limited to the proximal convoluted tubules. The rest of the renal tissue, particularly the medulla and the vascular system, remained unaffected. The histologic appearance of the kidneys of the spironolactone-treated animals was essentially normal; only in two rats did we find two to three calcified tubules per cross section (Fig. 1).

These findings are reminiscent of earlier observations on the protection by testosterone (*6*) and cortisol (*7*) against $HgCl_2$-induced renal damage in the mouse and rat; but spironolactone, though devoid of classic hormone actions, is much more potent in this respect.

It would be premature to speculate about the mechanisms through which steroids exert their prophylactic effect. Spironolactone, which possesses a thioacetate group, may be particularly efficacious in introducing sulfur into the organism for the detoxication of a heavy metal such as mercury. The less potent steroids might promote the synthesis of SH-containing compounds, enhance the elimination of mercury, protect the kidney directly through a renotrophic effect (for example, testosterone), or reduce the mercuric salt to the less toxic mercurous form. The latter possibility is suggested by the observation that ascorbic acid apparently protects

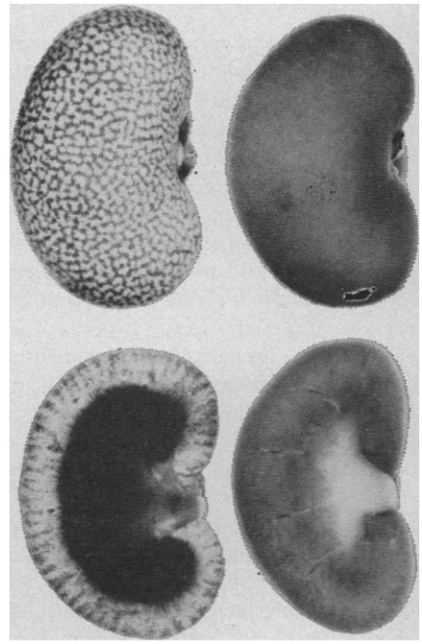

Fig. 1. Protection of the kidney by spironolactone against mercury-induced damage. Fresh specimens. (Left) Heavy calcification of the renal cortex in a rat of group 1 treated with $HgCl_2$. (Right) Complete prevention of calcification by prior treatment with spironolactone. (Top: external surface; bottom: cut surface.)