Some Effects of ω-Methyl Analogues of Vitamin B_6 in Rats. (21938)

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ω-Methylpyridoxine (2-ethyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine), in which the methyl group of pyridoxine has been replaced by an ethyl group(1), was one of the first analogues of vit. B_6 to be synthesized and tested in living organisms. Robbins found the compound replaced pyridoxine in promoting growth of excised tomato roots(2), but was toxic for *Ceratostomella ulmi*(3). Corresponding analogues of pyridoxal and pyridoxamine also have been synthesized(4). For lactic acid bacteria such as *Streptococcus faecalis*, these latter compounds replace pyridoxal as growth factors under some conditions(4-6), but may inhibit growth under slightly different conditions, depending upon the vit. B_6-dependent process which limits growth(5,6). Like ω-methylpyridoxine(7), they inhibit growth of yeast (*Saccharomyces carlsbergensis*); this inhibitory effect is overcome completely by pyridoxine, pyridoxamine, or pyridoxal.

A single dose of ω-methylpyridoxine was less than 2% as effective as pyridoxine in alleviating symptoms of vit. B_6 deficiency in rats(1). No other reports of the physiological effects of these compounds in animals have appeared; results of such an investigation are presented below.

Materials and methods. Synthesis of ω-methylpyridoxal and ω-methylpyridoxamine has been described earlier(4). ω-Methylpyridoxine(1) and an authentic sample of the lactone of 4-pyridoxic acid(8) were gifts from Dr. Karl Folkers. Weanling albino rats were caged individually, and fed vit. B_6-deficient ration of Sarma *et al.*(9) modified by substituting vitamin-free casein (Labco) for blood fibrin. Supplements were added to the diet or given separately by stomach tube. In the latter event a tuberculin syringe and a dulled no. 15 needle, bent and cut off at the proper length, were used. Food and water were given *ad libitum*.

Results. a. Activity of ω-methylanalogues of vit. B_6 during preventive assay. Weanling rats (Sprague-Dawley) were fed the deficient diet supplemented wtih pyridoxal, one of the analogues, or both pyridoxal and analogue, as indicated in Table I. Over a 4-week period, the ω-methyl analogues possess considerable growth-promoting activity. However, the higher levels of analogues were no more effective than the lower levels, and no amount of analogue permitted a maximum growth rate. The compounds were not irreversibly toxic, for when fed together with pyridoxal, a high rate of growth was achieved.

When supplementation was continued for a longer time, it became evident that the high level of all 3 ω-methyl analogues permitted even less growth than the lower level. Such results are shown for ω-methylpyridoxal in Fig. 1. Eventually the rate of growth of supplemented animals slowed to that of the deficient controls. Thus, while the analogues permit growth for a limited length of time in animals containing initially adequate supplies of vit. B_6, they become unable to support growth once bodily supplies of vit. B_6 reach a sufficiently low level.

More striking evidence than growth rates, of the inability of these analogues fully to replace vit. B_6 was provided by the observed symptomatology. Beginning about 2 weeks after supplementation with the analogues, practically all animals of Groups 5 through 10, Table I, underwent frequent epileptiform fits, especially during weighing periods. Occurrence of such convulsions has been noted frequently in animals fed vit. B_6-deficient diets alone (*e.g.*10), but is not a prominent feature of vit. B_6 deficiency on this basal ration, and was not noted in any of the deficient animals of Group 1 (Table I) until fully 4 weeks *after* their occurrence had become common in the analogue-fed animals. Even then, the incidence of the attacks in deficient Group 1 was not more than 25% of that in analogue-fed animals. The 3 analogues, therefore, pro-

TABLE I. Comparative Effects of Pyridoxal and ω-Methyl Analogues of Vitamin B_6 on Growth of Rats. Each group contained 6 animals.

Group	Supplement	Amt of supplement/day, γ	Wt gain in 4 wk, g	Apparent molar activity (pyridoxal = 100)
1	0	—	46	
2	Pyridoxal	1.6	71	
3	"	4.1	94	
4	"	8.2	114	
5	ω-Methylpyridoxal	16.6	100	32
6	"	83	100	6.5
7	ω-Methylpyridoxamine	17.8	92	23
8	"	88	101	6.4
9	ω-Methylpyridoxine	16.6	100	32
10	"	83	93	5.1
11	Pyridoxal + ω-methylpyridoxal	82 83	140	
12	Pyridoxal + ω-methylpyridoxamine	82 88	134	
13	Pyridoxal + ω-methylpyridoxine	82 83	126	

duce a deficiency of a type in which these fits are an unusually common feature—an observation of interest in view of the observed occurrence of convulsions in infants receiving suboptimal amounts of vit. B_6(11). No such attacks occurred in animals of groups 11-13, which received pyridoxal together with the high level of analogue.

b. *Activity of ω-methyl analogues of vit. B_6 during curative assay.* The apparent ability of the analogues to support growth only during the first few weeks (Fig. 1) suggests that as depletion of vit. B_6 proceeds, growth responses to the analogue would become less marked. This was checked by holding weanling animals (derived from the Wistar strain) on the deficient ration for 5 weeks, then supplementing the diet with high levels of ω-methylpyridoxine. A pronounced stimulation of growth occurred in the analogue-fed group during the first week, which decreased greatly in the course of the second and third weeks to a value below that of the deficient control group (Table II). Four of the 5 animals of the analogue-supplemented group died between the 34th and 41st days of supplementation, and the experiment discontinued. None of the deficient control animals had died during this period. This high level of ω-methylpyridoxine was non-toxic when fed together with pyridoxine. Thus the unusual result is observed that a compound that apparently corrects early deficiency symptoms eventually makes these symptoms more pronounced, and changes the intensity of individual symptoms relative to one another.

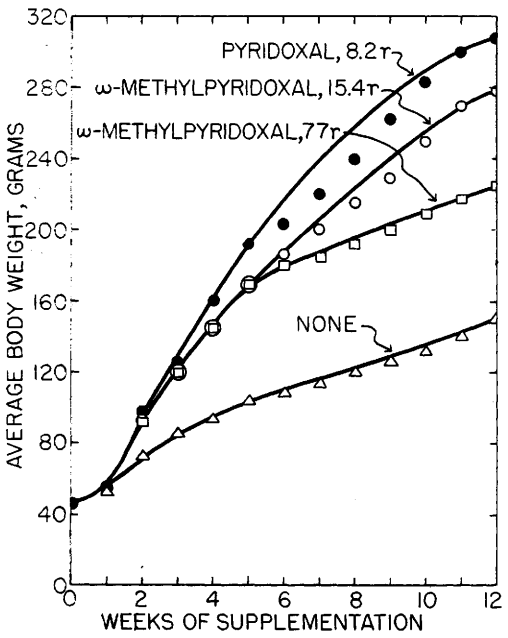

FIG. 1. Effect of time on comparative growth-promoting activities of pyridoxal and ω-methylpyridoxal for rats. Supplements indicated were administered daily.

c. *Metabolism of ω-Methylpyridoxine in Rats.* Application of the method of Heyl(8) for synthesis of 4-pyridoxic acid to a small sample of ω-methylpyridoxine yielded a product with an ultra-violet absorption spectrum essentially identical with that of 4-pyridoxic

TABLE II. Effect of ω-Methylpyridoxine on Growth and Survival of Vit. B_6 Deficient Rats. Each group contained 5 animals.

Supplement	Amt fed per day, γ	Avg wt gain/wk during 1st	2nd	3rd	4th	5th wk	Survivors at 41 days
0		3.2	1.3	.9	3.3	.5	5
Pyridoxine	8.2	27	16	15	16	8.3	5
ω-Methylpyridoxine	820	28	7.9	1.0	.1	−10.5	1

acid. After treatment in such a way as to form the lactone(12) the absorption spectrum proved identical with that of 4-pyridoxic acid lactone. On paper chromatograms the product migrated somewhat faster than 4-pyridoxic acid in all solvents, as might be expected from its slightly less polar character. The method of synthesis, the spectrum of the free acid, and the spectrum of the lactone, all demonstrate that the product is ω-methyl-4-pyridoxic acid. Different animals excrete variable proportions of administered pyridoxine as 4-pyridoxic acid(13); pyridoxal and pyridoxamine are also oxidized to this same product(14). To determine whether ω-methylpyridoxine was similarly metabolized, rats were fed high levels of ω-methylpyridoxine (4600 mμ moles/day) of pyridoxine (4900 mμ moles/day) for 24 hours. Chromatographic comparisons (Table III) showed that administration of ω-methylpyridoxine gave rise to ω-methylpyridoxic acid in the urine. Quantitative determination by the method of Møller(12) showed, in agreement with results of Huff and Perlzweig(13), that only about 0.5% of administered pyridoxine was recovered as 4-pyridoxic acid; similarly, 0.6% of the administered ω-methylpyridoxine was recovered in the urine as ω-methyl-4-pyridoxic acid. In neither case are the other products of metabolism known.

TABLE III. Excretion of ω-Methyl-4-Pyridoxic Acid by Rats Fed ω-Methylpyridoxine. Approximately 30 mμmoles (.01 ml) of acids or lactones and 0.05 ml of urine samples were applied to 18¼ by 22½ inch sheets of Whatman No. 1 filter paper, and developed with butanol-ethanol-water (4:1:1) in an ammoniacal atmosphere. Zones of these fluorescent substances were detected by observation under ultra-violet light.

Substance chromatographed	R_f values Untreated	Treated for lactone formation
4-Pyridoxic acid	.68	.40
ω-Methyl-4-pyridoxic acid	.77	.53
Urine of pyridoxine-fed rats	.66	.44
Urine of ω-methylpyridoxine-fed rats	.77	.46

d. *Some Effects of ω-Methylpyridoxine Administration on Enzyme Levels in Tissues.* D-Amino acid oxidase, the glutamic-aspartic transaminase, and thionase (cysteine desulfhydrase) were selected as representative of one vit. B_6-independent and 2 vit. B_6-dependent enzymes and their levels in appropriate tissues from normal (Group 13, Table I), deficient (Group 1, Table I), and analogue-fed (Group 10, Table I) animals compared. Animals were killed by decapitation, the tissues excised and homogenized. Results (Table IV) show no diminution in activity of the vit. B_6-independent kidney D-amino acid oxidase, but demonstrate a moderate reduction in activity of liver transaminase and a severe reduction in liver thionase in both vit. B_6-deficient and analogue-fed animals. The differences in symptomatology observable in simple vit. B_6-deficiency and in analogue-fed animals thus cannot be explained in terms of the effects on these 2 enzymes, but must result from differential effects upon one or more of the multitude of other vit. B_6-dependent enzymes of tissues. *Rates* of diminution in enzyme activity, an effect not considered in the above comparisons, probably are also of importance here.

The result shows that the ω-methyl analogues are not the functional equivalent of vit. B_6 in activation of these enzymes, and if they are converted to ω-methylpyridoxal phosphate by animal tissues (as they are by bacteria (5,6)) the latter must be *comparatively* ineffective as an activator of these two enzymes. This was checked by comparing the effectiveness of ω-methylpyridoxal phosphate(4) and pyridoxal phosphate as activators of enzyme preparations obtained from rats fed the vit. B_6-deficient ration for 37 days. The analogue coenzyme activated liver glutamic-aspartic

TABLE IV. Comparison of Kidney D-Amino Acid Oxidase, Heart Glutamic-Aspartic Transaminase and Hepatic Thionase between Normal, Vitamin B_6-Deficient, and ω-Methylpyridoxine-Fed Rats. Each determination was carried out on pooled tissues of 3 animals. Normal and analogue-fed rats received vit. B_6-free diet and supplements for 86 days from weaning. Vit. B_6-deficient rats received the vit. B_6-free diet only for 49 days.

Treatment of animal	D-Amino acid oxidase,* μl O_2/hr/mg wet wt	Enzyme systems — Transaminase,† μl CO_2/10 min./mg wet wt	Thionase,‡ μg H_2S/2 hr/g wet wt
Normal	1.4	6.2	166
Analogue-fed	1.8	4.5	45
Vit. B_6-deficient	1.3	4.4	25

* By method of Armstrong, *et al.*(15).
† " " " Ames and Elvehjem(16).
‡ " " " Thompson and Guerrant(17).

transaminase, but was only 11% as active on the weight basis as pyridoxal phosphate. Liver thionase was not fully reactivated by any concentration of the analogue coenzyme; the partial reactivation achieved required over 50 times the concentration that sufficed when pyridoxal phosphate was the coenzyme. The behavior of these enzymes of mammalian origin to the analogue coenzyme resembles closely that of the corresponding bacterial enzymes(6).

Discussion. Under appropriate conditions in certain bacteria, ω-methylpyridoxal and ω-methylpyridoxamine support continued growth because they are converted to ω-methylpyridoxal phosphate, which activates the essential vit. B_6-dependent enzyme in place of pyridoxal phosphate(5,6). Under closely related conditions, when another set of vit. B_6-dependent enzymes is made limiting, the same vitamin analogues inhibit growth, presumably because these vit. B_6-dependent enzymes are not activated by the analogue coenzyme, which consequently behaves as a structural antagonist. That similar relationships hold in animal systems seems probable from the observations that ω-methylpyridoxine and pyridoxine are metabolized similarly, and that ω-methylpyridoxal phosphate varies in its ability to activate various vit. B_6-dependent enzymes of the rat. The observed facts that these analogues first promote, then inhibit, growth on vit. B_6-free rations might be readily interpreted in terms of these effects. It may be supposed that as the stores of vit. B_6 within the body become depleted, various vit. B_6-dependent enzymes become hypofunctional in turn, depending, upon their affinities for pyridoxal phosphate (*cf.* 18). If the first of these to become hypofunctional were also activated by ω-methylpyridoxal phosphate, this analogue would be expected to promote growth. When, however, additional essential vit. B_6-dependent enzymes that are not activated by the analogue coenzyme become hypofunctional, then the analogues no longer promote growth. Since the analogues act also as displacing agents, pyridoxal phosphate displaced by the analogue coenzyme from one apoenzyme may become available for activation of a second apoenzyme having different relative affinities for the coenzyme and analogue coenzyme.* This displaced pyridoxal phosphate may initially contribute to the growth effects of the analogue. At the same time, however, if the enzyme from which this pyridoxal phosphate has been displaced itself catalyzes reactions essential for growth, and is not activated (or is activated to an insufficient extent) by available concentrations of the analogue coenzyme, such displacement would explain the active toxicity exhibited by the analogues after more prolonged periods of administration. Since the enzymes from which pyridoxal phosphate is displaced are not necessarily the same as those that would lose this coenzyme first in simple vitamin deficiency,† administration of the analogue might be expected to produce a pattern of deficiency symptoms differing in some respects from those produced by simple deficiency, as is actually observed. Finally, a part of the vit. B_6 ingested by the animal normally is made unavailable for growth processes through oxidation to 4-pyridoxic acid (and unidentified products) and excretion. The participation of ω-methylpyridoxine as a competitive substrate in these latter reactions would decrease the amount of vit. B_6 destroyed in this way, and thus spare additional amounts for essential metabolic uses.

The similarity of these observations to

* That different enzymes of a single organism may show such varying affinities has been demonstrated in *Streptococcus faecalis*(5,6).

those made with "diethylriboflavin"(20) and with lyxoflavin(21) suggests a fairly widespread occurrence of similar phenomena.

Summary. ω-Methylpyridoxal, ω-methylpyridoxamine and ω-methylpyridoxine support growth of rats for several weeks following deletion of vit. B_6 from the ration. Eventually, however, growth rate in the presence of these compounds slows. and becomes equal to or less than that of vit. B_6-deficient control animals. The period of growth promotion is decreased greatly when the compounds are administered to depleted rats. Following this period of growth-promotion, growth with high concentrations of ω-methylpyridoxine is distinctly less than that in control animals; and death results well before any deaths are noted in the control group. In uncomplicated vit. B_6 deficiency on this ration convulsive seizures are infrequent and were observed only following approximately 6 weeks on the deficient diet. In contrast, in the animals fed the ω-methyl analogues of vit. B_6 convulsions occurred frequently. in all animals, starting about 2 weeks after supplementation while substantial weight gains were still occurring. All of these symptoms were prevented by feeding pyridoxal together with the analogues. Following administration of ω-methylpyridoxine to animals. ω-methyl-4-pyridoxic acid appears in the urine in amounts similar to those found for 4-pyridoxic acid following pyridoxine administration. Glutamic-aspartic transaminase and thionase are reduced in ω-methylpyridoxine-fed animals to about the levels found during uncomplicated vit. B_6 deficiency. Correspondingly, ω-methylpyridoxal phosphate was only about 11% as active as pyridoxal phosphate in activating the glutamic-aspartic transaminase, and did not fully activate liver thionase at any concentration. The facts that feeding these analogues first promotes, but eventually inhibits growth, and produces a deficiency of a different type than that found in uncomplicated vit. B_6-deficiency, may be interpreted in terms of (a) the dual character of ω-methylpyridoxal phosphate as an activator for some vit. B_6-dependent enzymes, but not for others, (b) its role as a structural antagonist for vit. B_6 in those enzymes that it does not activate, and (c) the role of these analogues as competitive substrates for vit. B_6 in reactions leading to destruction of the vitamin.

† Dietrich and Shapiro(19) have observed that the enzymatic disabilities produced by feeding 4-desoxypyridoxine to rats do not coincide exactly with those produced by simple vitamin deficiency. This result should be expected, since the degree to which an analogue displaces pyridoxal phosphate depends not only upon the dissociation constant of the various proteins for pyridoxal phosphate, but also upon their affinity for the analogue coenzyme and upon the rate at which the enzyme is inactivated and reformed within the tissues.

1. Harris, S. A., and Wilson, A. N., *J. Am. Chem. Soc.*, 1941, v63, 2526.

2. Robbins, W. J., *Am. J. Bot.*, 1942, v29, 241.

3. Robbins, W. J., and Ma, R., *Bull. Torrey Bot. Club,* 1942, v69, 342.

4. Ikawa, M., and Snell, E. E., *J. Am. Chem. Soc.*, 1954, v76, 637.

5. Olivard, J., and Snell, E. E., *J. Biol. Chem.*, 1955, v213, 203.

6. ———, *ibid.*, 1955, v213, 203.

7. Rabinowitz, J. C., and Snell, E. E., *Arch. Biochem. and Biophys.*, 1952, v43, 408.

8. Heyl, D., *J. Am. Chem. Soc.,* 1948, v70, 3434.

9. Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v165, 55.

10. Chick, H., El Sadr, M. M., and Worden, A. V., *Biochem. J.*, 1940, v34, 595.

11. Maloney, C. J., and Parmelee, A. H., *J. Am. Med. Assn.*, 1954, v154, 405.

12. Møller, P., *Acta Chem. Scand.*, 1951, v5, 1418

13. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, 1944, v155, 345.

14. Rabinowitz, J. C., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 235.

15. Armstrong, K. F., Feldott, G., and Lardy, H. A., *ibid.*, 1950, v73, 159.

16. Ames, S. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v166, 81.

17. Thompson, R. Q., and Guerrant, N. B., *J. Nutrition,* 1953, v50, 161.

18. Snell, E. E., *Physiol. Rev.*, 1953, v33, 509.

19. Dietrich, L. S., and Shapiro, D. M., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 555.

20. Lambooy, J. P., and Aposhian, H. V., *J. Nutrition,* 1952, v47, 539.

21. Snell, E. E., Klatt, O. A., Bruins, H. W., and Cravens, W. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 583.

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