THE BIOLOGICAL ACTIVITY OF VITAMIN B₆ ANALOGS IN THE RAT

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Summary The effects of three vitamin B₆ analogs were studied in young adult and weanling rats. The 2-ethyl analog was the most active. It produced higher growth rates, an average feed efficiency equal to the control and elevated liver glycogen levels. Xanthurenic acid excretion remained low with the 2-ethyl analog and pyridoxine in the diet and plasma amino acid concentration was low. Response to the 2-n-propyl analog was similar to that of a B₆-deficient diet, with a large increase in xanthurenic acid excretion. Rats receiving the 2-isopropyl analog were intermediate between the ethyl group and B₆-deficient animals in all parameters measured.

These in vivo results parallel those reported in the in vitro yeast pyridoxine dehydrogenase enzyme system. The ethyl pyridoxine analog was the most active of all the analogs in the yeast dehydrogenase system, in supporting growth and in maintaining normal xanthurenic acid excretion in the rat.

Keywords vitamin B₆, pyridoxine analogs, xanthurenic acid, B₆ deficiency, B₆ effect on glycogen

Three vitamin B₆(pyridoxine hydrochloride)analogs,¹ modified in the 2 position of the pyridine ring by replacing the methyl group with an n-propyl(I), isopropyl(II), or ethyl(III) group were synthesized by modified procedures previously reported by Melius and Marshall (1) and Melius and Greene (2). These investigators found that the ethyl analog has essentially the same substrate activity in yeast pyridoxine dehydrogenase as the naturally occurring vitamin compound, that the isopropyl analog was approximately one-fourth as active as the ethyl analog and that the n-propyl analog was almost inactive. The ethyl pyridoxal phosphate analog has been shown to be active in the apotyrosine decarboxylase system by Morgan (12), while the n-propyl pyridoxal phosphate analog only

¹ The n-propyl analog is 3-hydroxy-4,5-bis(hydroxymethyl)-2-n-propylpyridine, the isopropyl analog is 3-hydroxy-4,5-bis(hydroxymethyl)-2-isopropylpyridine and the ethyl analog is 3-hydroxy-4,5-bis(hydroxymethyl)-2-ethylpyridine. All were synthesized in the Auburn University Department of Chemistry by P. E. Morgan, II.
slightly activated the apotyrosine decarboxylase. In the apotryptophanase system
the ethyl pyridoxal phosphate analog could replace pyridoxal-5'-phosphate, but the
n-propyl- and the 5-[(2-hydroxyethylthio)methyl]pyridoxal phosphate analogs
failed to activate the enzyme at 100 times the concentration of the pyridoxal-5'-
phosphate.

Sandman and Snell (3), reported that ω-methyl-pyridoxine (ethyl analog)
supported growth in rats but not quite as effectively as pyridoxine. They also found
that ω-methylpyridoxic acid was excreted in the urine and that glutamate aspartate
transaminase activity in plasma was only 11% of the normal value. Thus we know
that analog III can be oxidized to the aldehyde form enzymatically; can support
growth in the rat; and acts as a coenzyme (when oxidized and phosphorylated) for
Glu-Asp transaminase although much less effectively than pyridoxal phosphate.
The present growth studies were carried out to investigate further the biological
activities of the above B₆ analogs in an in vivo system, i.e., to assess their ability to
sustain metabolic activity in the whole animal. An evaluation of various
biochemical parameters such as liver glycogen, plasma amino acids, and
xanthurenic acid excretion may aid in the identification of critical enzyme systems
for further in vitro studies.

METHODS

Experiment 1. A semi-purified basal diet was formulated as shown in Table
1. This diet was essentially devoid of B₆, containing only the amount present in
vitamin-free casein (0.63 μg/g of casein). The basal diet was supplemented with the
recommended level (4) of vitamin B₆ (7.8 mg/kg of diet), or an identical amount of

Table 1. Composition of vitamin B₆-deficient basal diet.

| Component                   | g/100 g |
|-----------------------------|---------|
| Casein, vitamin-free        | 20.0    |
| Corn oil                    | 10.0    |
| Cornstarch                  | 30.0    |
| Sucrose                     | 30.0    |
| Vitamin premix¹             | 2.0     |
| Salt mix²                   | 4.0     |
| Alphacel²                   | 3.1     |
| DL-Methionine               | 0.3     |
| Choline chloride            | 0.6     |

¹ Supplied the following amounts of vitamins (mg/kg of diet): menadione, 50;
riboflavin, 20; thiamine-HCl, 20; CA pantothenate, 50; niacin, 100; inositol, 200; d-α-
tocopherol acetate, 55; vitamin A palmitate, 100,000 USP units; folacin, 2; biotin, 0.5;
vitamin D₃, 0.04; and vitamin B₁₂, 0.05. ² Jones and Foster salt mix (7) and alphacel,
non-nutritive bulk, purchase from Nutritional Biochemicals Corp., Cleveland, Ohio.
one of the three analogs, producing four dietary treatments. The unsupplemented basal diet served as a negative control. Male Sprague-Dawley rats (140–187 g) were randomly assigned to five groups of six rats each and housed individually in suspended wire-bottomed galvanized cages. Each group was fed ad libitum on a different experimental diet. Body weights were recorded twice weekly.

At the end of 9 weeks, all animals were anesthetized with sodium pentobarbital and terminal blood samples were obtained by cardiac puncture using vacutainer tubes containing EDTA. Plasma was separated and frozen for subsequent amino acid analyses by the Beckman 121M automatic amino acid analyzer. Livers were removed and frozen prior to glycogen determinations by the direct anthrone method of Seifter et al. (5). Data were subjected to one-way analysis of variance, and treatment means were separated by Duncan’s new multiple range test (6).

Experiment 2. Thirty-six male weanling rats (31–51 g) were fed on the B₆-deficient basal diet for 14 days in order to deplete tissue stores of the vitamin. They were then placed in six groups of six rats each. Five of the groups were each fed ad libitum on a different diet, each dietary treatment being the same as those in Experiment 1. The sixth group was fed ad libitum on a diet formulated by supplementing the basal diet with pyridoxine at one-half the NRC recommended level. Feed intake was recorded daily for the first 4 days of the experiment and body weights were recorded at irregular intervals during the 3-week experimental period. At the end of 3 weeks, termination procedures were carried out as described in the first experiment.

Experiment 3. Thirty weanling Sprague-Dawley male rats were fed on the basal diet ad libitum for 2 weeks and were then divided into five groups containing six rats each. Each group was fed for 8 days on one of the experimental diets used in experiment 1. Feed intake and body weights were recorded every other day. On day 6 the rats were placed in metabolism cages for the collection of 48-hr urine specimens, which were frozen until analyzed for creatinine by the method of Jones and Foster (7) and xanthurenic acid by the method of Lyon and Porter (8). Urinary xanthurenic acid was expressed in mg xanthurenic acid/g creatinine in order to eliminate the variance in xanthurenic acid synthesis due to differences in metabolic body size and consequent dietary tryptophan intake. The rats were then sacrificed by cervical dislocation and the livers were removed and frozen in liquid air prior to glycogen determination.

RESULTS AND DISCUSSION

Experiment 1

Figure 1 shows that the growth curve for rats given analog I was almost identical to that resulting from consumption of the basal diet. Analog II initially depressed growth slightly below the basal level, but the trend reversed during week 8. Analog III administration exhibited growth activity over that of the basal diet.
Fig. 1. Growth curves of young adult male rats fed on a vitamin B₆-deficient basal diet and the same diet supplemented with 7.8 mg B₆/kg as control, the 2-n-propyl analog (I), the 2-isopropyl analog (II), or the 2-ethyl analog (III). Animals were fed on the diets ad libitum for 9 weeks and had free access to water.

Table 2. Effect of feeding vitamin B₆ analogs for 9 weeks on growth, liver glycogen and plasma amino acids of young adult male rats¹ (Experiment 1).

| Dietary treatment² | Body wt. change (%) | Liver glycogen (mg/g) | Total plasma amino acids (mm) |
|--------------------|---------------------|-----------------------|-------------------------------|
| Control            | 137 ± 15ᵃ           | 50 ± 6ᵈ               | 5.4 ± 0.5ᵃ (5)                |
| Basal (B₆-deficient) | 68 ± 11ᶜ            | 74 ± 10ᵃ              | 5.5 ± 1.5ᵃ (3)                |
| n-Propyl analog (I) | 75 ± 15ᶜ            | 68 ± 11ᵃᵇ             | 6.1 ± 1.3ᵃ (3)                |
| Isopropyl analog (II) | 87 ± 14ᶜ           | 56 ± 8ᶜᵈ              | 5.3 ± 1.1ᵃ (3)                |
| Ethyl analog (III)  | 107 ± 20ᵇ           | 62 ± 4ᵇᶜ              | 4.4 ± 0.6ᵃ (3)                |

¹ Values are means ± SEM of 6 rats or the number given in parentheses. Means within a column without a common superscript are significantly different (p < 0.05) by Duncan’s new multiple range test. ² Control and analog diets contained 7.8 mg of B₆ or the appropriate analog per kg of diet.

but was not equivalent in magnitude to the control. Average gain of the control group for the 9-week period was 203 g compared to 168 and 105 g for analog III and the basal groups, respectively. Table 2 shows that the average body weight change of animals receiving analogs I and II was 75 and 87%, respectively, but was not significantly different from the basal value of 68% (Table 2). Analog III produced a mean body weight gain of 107%. This was significantly (p < 0.05)
higher than that of the basal groups but lower than the control gain of 137%.

The highest liver glycogen concentrations were found in the basal (74 mg/g) and analog I (68 mg/g) groups. The lowest glycogen concentration, 50 mg/g, was found in the control. One possible explanation of these data is depressed activity of phosphorylase a, which requires B6 as coenzyme. Large individual variations were observed in total plasma amino acid concentrations, with mean values ranging from 4.4 mM in the analog III group to 6.1 mM in the analog I group, the differences not being significant.

Experiment 2

The basal and analog I growth curves are almost superimposed (Fig. 2), and are very similar to growth curves observed in Experiment 1. As in the previous experiment, analog II initially depressed growth below the basal level. This trend reversed with a sharp increase in growth rate between days 3 and 7, resulting in a total body weight gain very similar to that with analog III. This similarity of growth response suggests that some type of metabolic adaptation may be a prerequisite to utilization of the compound for growth stimulation above the basal level. Analog III consumption produced a growth response slightly lower than that of the two control groups. Body weight changes averaged a little more than 20% and were not significantly different at weeks 1 or 3. Consumption of analogs II and III resulted in respective increases of 57 and 56% in mean body weights at the end of week 1. These values were significantly lower than the low control mean of 69%. By week 3, the growth rate of all groups except the basal had decreased, with body weight changes for analogs II and III being 34 and 40%, respectively, and not statistically different from the low control.

Melius and Marshall (1) found that the ethyl analog of pyridoxine was converted to the ethyl analog of pyridoxal just as actively as pyridoxine was oxidized by yeast pyridoxine dehydrogenase. Present data are comparable in that
growth of both adult and weanling rats given the analog III-supplemented diet was similar to that of those given pyridoxine. Analog I produced a growth rate similar to that with the basal or B₆-deficient diet, which correlates with the fact that the n-propyl analog was only weakly oxidized in the pyridoxine dehydrogenase system and thus would produce only small amounts of pyridoxal phosphate analog for pyridoxal phosphate requiring enzyme systems. The isopropyl analog is intermediate in activity in both the enzyme system and in its ability to support growth in the rat. Although growth in the rat involves a very complex set of conditions, the evidence presented here indicates that the ethyl and isopropyl analogs form pyridoxal phosphate derivatives capable of activating many pyridoxal phosphate-requiring enzyme systems.

Mean feed efficiency values (g gain/g feed) were 0.62, 0.37, and 0.21 for analogs III, II, and I, respectively; all were significantly ($p<0.05$) different from each other. The analog III value was not significantly different from the control or the low control and the analog I value was not significantly different from the basal. These data, calculated from the initial 4 days consumption, provided an early and surprisingly sensitive differentiation of analog activity which paralleled that observed in the yeast dehydrogenase system (1).

Rats fed analog III had the highest concentration of liver glycogen, the mean in the weanling rat being 71 mg/g (Table 3). All other groups had lower concentrations that were significantly different ($p<0.05$) from analog III but not from each other. The total plasma free amino acids levels ranged from a maximum of 5.2 mM in the control group to 3.2 mM in the analog III group. It is not clear why

Table 3. Effect of vitamin B₆ analogs on 3 weeks growth, feed efficiency, liver glycogen, and plasma amino acids of weanling rats¹
(Experiment 2).

| Dietary treatment² | Body wt. change (%) | Feed efficiency³ | Liver glycogen (mg/g) | Total plasma amino acids (mm) |
|-------------------|---------------------|------------------|----------------------|-----------------------------|
|                   | Wk. 1   | Wk. 3   | (g gain/g feed)     |                      |                             |
| Control (NRC)     | 75 ± 5⁵ | 51 ± 7⁵ | 0.63 ± 0.05⁴       | 39 ± 9b             | 5.2 ± 0.5b (4)             |
| Low control (50% NRC) | 69 ± 4b | 41 ± 6b | 0.63 ± 0.08a       | 33 ± 6b             | 4.0 ± 0.6b (3)             |
| Basal (B₆-deficient) | 19 ± 3d | 26 ± 6c | 0.24 ± 0.07e       | 43 ± 12b            | 4.2 ± 0.7b (3)             |
| n-Propyl analog (I) | 22 ± 2d | 23 ± 10d| 0.21 ± 0.07d       | 44 ± 3b             | 3.8 ± 0.4b (3)             |
| Isopropyl analog (II) | 57 ± 3c | 34 ± 2b c| 0.37 ± 0.06b       | 26 ± 10b            | 2.5 ± 0.7b (3)             |
| Ethyl analog (III) | 56 ± 4c | 40 ± 7b | 0.62 ± 0.07a       | 71 ± 11a            | 3.2 ± 1.0b (3)             |

¹ Values are means±SEM of 6 rats or the number of animals given in parentheses. Means within a column not having a common superscript letter are significantly different ($p<0.05$) by Duncan's multiple range test. ² Control and analog diets contained 7.8 mg of B₆ or the appropriate analog per kg of diet. Low control contained 3.9 mg B₆/kg diet. ³ Calculations based on initial 4-day feed intake.
these glycogen and amino acid data do not substantiate those of Experiment 1. Possible factors responsible are experimental differences such as length of experimental period (9 weeks vs. 3 weeks), initial age (young adult vs. weanlings previously depleted of B<sub>6</sub>), and final body weight (180 g vs. 300 g). It is also possible that analog III is converted to the pyridoxal phosphate analog, which competitively inhibits the action of phosphorylase a in the weanling more effectively than in the adult animal.

**Experiment 3**

Table 4 shows that the growth rate and feed intake were similar in the control and analog III groups. These parameters were significantly lower and also similar in the basal, analog I and analog II groups. Liver glycogen in the control group was significantly lower than in rats fed on the basal and analog I diets. The significance of these glycogen data is not clear. Lyon and Porter (8) found that liver glycogen increased in severely pyridoxine-deprived mice. Liver glycogen was diminished in vitamin B<sub>6</sub>-deficient rats studied by Beaton (9), however, Guggenheim and Diamant (10) found liver glycogen concentrations similar in control and vitamin B<sub>6</sub>-deprived rats. Muscle glycogen apparently does not change during vitamin B<sub>6</sub> deficiency (10), therefore, our results in this experiment especially agree with Lyon and Porter’s (8). In our experiment, analog III appears to be substituting for pyridoxine (at least partially), as the liver glycogen level is quite close to that of the control group.

Xanthurenic acid (XA) excretion is increased in rats during vitamin B<sub>6</sub> deficiency (11). There was a very large increase of XA excretion in the B<sub>6</sub>-depleted animals (basal diet) as well as in the animals fed analogs I and II (isopropyl and n-

| Dietary treatment<sup>3</sup> | Body wt. change<sup>3</sup> (%) | Feed intake<sup>3</sup> (g) | Liver glycogen (mg/g) | Xanthurenic acid excretion<sup>4</sup> (mg/g creatinine) |
|-----------------------------|-------------------------------|-----------------------------|-----------------------|----------------------------------------------------------|
| Control                     | 42 ± 2<sup>a</sup>             | 96 ± 6<sup>a</sup>          | 55 ± 3<sup>c</sup>    | 26 ± 4<sup>b</sup> (5)                                    |
| Basal (B<sub>6</sub>-deficient) | 6 ± 2<sup>b</sup>         | 65 ± 5<sup>b</sup>         | 74 ± 7<sup>ab</sup>   | 114 ± 27<sup>a</sup> (5)                                  |
| n-Propyl analog (I)         | 11 ± 1<sup>b</sup>           | 67 ± 4<sup>b</sup>         | 81 ± 8<sup>a</sup>    | 117 ± 41<sup>ab</sup> (4)                                 |
| Isopropyl analog (II)       | 8 ± 1<sup>b</sup>            | 59 ± 3<sup>b</sup>         | 67 ± 5<sup>abc</sup>  | 175 ± 44<sup>a</sup> (5)                                  |
| Ethyl analog (III)          | 40 ± 3<sup>a</sup>           | 89 ± 5<sup>a</sup>         | 60 ± 2<sup>b</sup>    | 30 ± 17<sup>b</sup> (5)                                    |

<sup>1</sup> Values are means ± SEM of 6 rats or the number of animals given in parentheses. Means within a column not having a common superscript letter are significantly different (p<0.05) by Duncan’s multiple range test. <sup>2</sup> Control and analog diets contained 7.8 mg of B<sub>6</sub> or the appropriate analog per kg of diet. <sup>3</sup> Totals for 6-day period. <sup>4</sup> Based on analysis of 48-hr urine samples collected after consumption of experimental diets for 6 days.
propyl B₆ analogs). Xanthurenic acid excretion was extremely low and similar in magnitude for the control and analog III groups, indicating the ethyl analog is capable of replacing pyridoxine in tryptophan catabolism. The ethyl analog apparently substitutes extremely well for the pyridoxine in the enzyme systems involved in tryptophan metabolism. The weight gain and low XA excretion of the animals fed the analog III suggests that it is adequately replacing the natural vitamin in the metabolic requirements of the rat. It is interesting that analog II, which was the least effective for weight gain of the animals, was responsible for a sevenfold increase of XA excretion over the controls. The basal diet resulted in more than a fourfold increase of the XA excretion, and analog I quadrupled XA excretion. Analog II may have inhibited some of the enzyme systems involved in XA catabolism.

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REFERENCES

1) Melius, P., and Marshall, D. L. (1967): Synthesis and enzymological activity of some pyridoxine analogs. J. Med. Chem., 10, 1157–1158.
2) Melius, P., and Greene, J. L., Jr. (1972): Synthesis and enzymological activity of 3-hydroxy-2-n-propyl-4,5-pyridinedimethanol. J. Med. Chem., 15, 206.
3) Sandman, R., and Snell, E. E. (1955): Some effects of ω-methyl analogues of vitamin B₆ in rats. Proc. Soc. Expl. Biol. Med., 90, 63–67.
4) National Research Council (1972): Nutrient Requirements of Laboratory Animals, National Academy of Sciences, Washington, D.C., p. 64.
5) Seifter, S., Dayton, S., Novic, B., and Mutwyler, E. (1950): The estimation of glycogen with the anthrone reagent. Arch. Biochem. Biophys., 25, 191–200.
6) Steel, R. G. D., and Torrie, J. H. (1960): Principles and Procedures of Statistics, McGraw-Hill Book Co., New York, pp. 107–109.
7) Jones, J. H., and Foster, C. (1942): A salt mixture for use with basal diets either low or high in phosphorus. J. Nutr., 24, 245–256.
8) Lyon, J. B., and Porter, J. (1962): The effect of pyridoxine deficiency on muscle and liver phosphorylase of two inbred strains of mice. Biochim. Biophys. Acta, 58, 248–254.
9) Beaton, J. R., (1955): Further studies on carbohydrate metabolism in the vitamin B₆ deprived rat. Can. J. Biochem. Physiol., 33, 161–166.
10) Guggenheim, K., and Diamant, E. J. (1954): Carbohydrate metabolism in pyridoxine-deficient rats. J. Biol. Chem., 224, 861–869.
11) Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J. (1943): Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine-deficient rats. J. Biol. Chem., 149, 195–201.
12) Morgan, P. E. (1978): Synthesis and enzymatic activity of pyridoxine and pyridoxal-5'-phosphate analogs. Ph.D. Dissertation, Auburn University.