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Pyridoxamine (Pyridoxine) 5′-Phosphate Oxidase Activity in Rat Tissues during Development of Riboflavin or Pyridoxine Deficiency (40589)[1]

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The use of pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5) activity as an index of riboflavin status potentially offers a major advantage over current methods. This flavin mononucleotide (FMN)-dependent enzyme catalyzes the oxidation of pyridoxamine 5′-phosphate (PMP) and pyridoxine 5′-phosphate (PNP), two of the vitaminic forms of B_6, to the active coenzymic form, pyridoxal 5′-phosphate (PLP) (1). Thus, in contrast to erythrocyte glutathione reductase (EGR), a flavin adenine dinucleotide (FAD)-dependent enzyme widely employed for assessment of riboflavin status, PMP oxidase activity should reflect the availability of the more limiting of the two riboflavin coenzymes.

An experiment was designed to determine if PMP oxidase activity could be used as a sensitive and specific measure of riboflavin nutriture. This study compared changes in PMP oxidase and EGR activities during riboflavin depletion in rats. To establish if these changes were unique to riboflavin status and not the result of concomitant pyridoxine deficiency, liver PLP concentration was measured. In addition, the effect of pyridoxine depletion on PMP oxidase activity was determined.

Materials and methods. Male, Sprague–Dawley rats were obtained from a commercial supplier (ARS/Sprague–Dawley, Madison, Wis.) and were housed individually in stainless-steel cages with wire-mesh bottoms. During a 5-day acclimation period, all animals were fed a purified diet (prepared by ICN Pharmaceuticals, Inc., Cleveland, Ohio), which contained 18% vitamin-free casein, 66% sucrose, 10% vegetable oil, 4% salt mix (2), and 2% vitamin mix (Vitamin Diet Fortification Mixture, ICN). Three rats then were killed (Week 0) as described below, and the remaining rats were randomly assigned to control (CD), riboflavin-deficient ($-B_2$), or pyridoxine-deficient ($-B_6$) groups. The mean body weight of rats in each group was 74 g. Rats in the CD group continued to be fed the diet used during the acclimination period; those in the $-B_2$ and $-B_6$ groups were fed a purified diet of the same composition prepared by omitting the named vitamin from the vitamin mix.

Weekly for 6 weeks, three rats from each diet group were anesthetized with ether and bled from the heart until dead. Washed red blood cells were prepared and stored at −20° until assayed for EGR activity (3). The liver, kidneys, and brain of each rat were rapidly excised, blotted, weighed, quick-frozen in liquid nitrogen, and stored at −20° overnight.

Tissues were homogenized for 10 (liver and brain) or 15 sec (kidneys) in 4 vol of cold 0.02 *M* potassium phosphate buffer, pH 7.0, using a Polytron (Brinkmann Instruments, Westbury, N.Y.). Cellular debris was precipitated by centrifugation at 27,000*g* for 20 min at 4°. The protein concentration of the supernatant was determined by the biuret method (4) using bovine serum albumin as the standard. Liver PLP content was determined by the tyrosine apodecarboxylase method (5) following trichloroacetic acid (TCA) precipitation and ether extraction of the supernatants (6).

A modification of the method of Wada (7) was used for determination of PMP oxidase activity. Under subdued light, a 3.5-ml reaction mixture containing 0.17 *M* Tris–HCl buffer, pH 8.0, 0.08 *M* potassium phosphate buffer, pH 8.0, 2.9×10^{-6} *M* FMN, 2.9×10^{-4} *M* PMP, and 0.3 ml of the tissue sample

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was incubated with shaking for 30 min at 37°. The reaction was stopped by the addition of 0.2 ml of 100% TCA. After addition of 0.3 ml of 4-nitrophenylhydrazine color reagent (3.5×10^{-3} *M* in 30% ethanol:concentrated sulfuric acid (18:7, v/v) diluted 1:5 with water and filtered before use), samples were allowed to stand for 10 min at room temperature. Protein was precipitated by centrifugation at 27,000*g* for 20 min at 4°, and the absorbance of the 4-nitrophenylhydrazone of PLP in the supernatant was read at 435 nm (8).

Statistical comparisons were made by Student's *t* test (9); $P < 0.05$ was accepted as significant.

Results. The body weights of rats fed $-B_2$ or $-B_6$ were significantly lower than those fed CD after 2 weeks; the gap between the control and the two vitamin-deficient groups widened as the experiment progressed. The same pattern was observed for liver and kidney weights (significant by Week 3). Differences between the groups were smaller for brain weights than for the other organs. There was, however, a significant difference in brain weight between rats fed CD and those fed $-B_2$ from Week 3 until the end of the study.

The effect of riboflavin or pyridoxine deficiency on PMP oxidase activity in liver, kidney, and brain (expressed as nmole PLP formed/g/hr) is illustrated in Fig. 1. In liver, PMP oxidase values decreased in all diet groups during the 6-week experimental period. The decrease was most pronounced in the rats fed $-B_2$; over the first 4 weeks, PMP oxidase activity decreased rapidly to 27% of initial values and remained at that low level. The difference in PMP oxidase activity between the riboflavin-deficient and control rats was statistically significant after only 1 week and grew larger as the study progressed. Rats fed $-B_6$ generally had lower liver PMP oxidase activities than those fed CD; this difference did not reach statistical significance.

Kidney PMP oxidase activities were variable during the experimental period (Fig. 1). At Week 2 and thereafter, values in rats fed $-B_2$ were significantly lower than in those fed CD.

During the study, brain PMP oxidase activity decreased by 50% in all groups (Fig. 1).

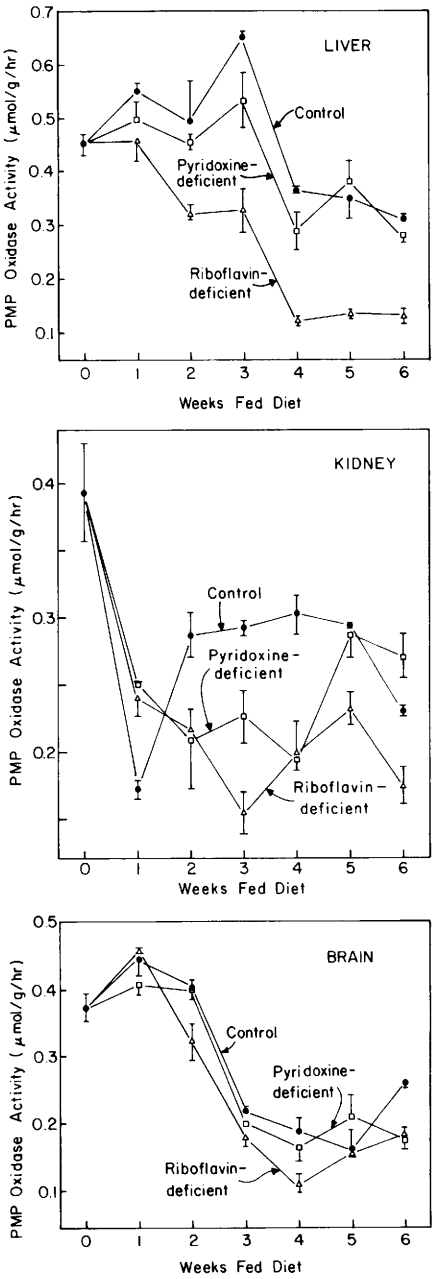

FIG. 1. Effect of riboflavin or pyridoxine deficiency on PMP oxidase activity in rat liver, kidney, and brain during the 6-week experimental period. Mean ± SEM is depicted; $n = 3$ for each point (except $n = 2$ for the control group at Week 5).

Except at Week 6, values in rats fed $-B_6$ were not different from those fed CD. In contrast, values in rats fed $-B_2$ were significantly lower

than in those fed CD except at Weeks 1 and 5.

EGR activity coefficients increased rapidly during the first 2 weeks in the rats fed $-B_2$, remaining at a high level for the duration of the experiment (Fig. 2). In this group, EGR activity coefficients showed a significant negative correlation ($r = 0.469$) with PMP oxidase activities. Rats fed CD and $-B_6$ maintained normal EGR activity coefficients throughout the 6-week period.

Liver PLP concentration remained essentially the same throughout the experiment in rats fed $-B_6$ (Fig. 3). However, in rats fed CD or $-B_2$, liver PLP concentration increased steadily to values approximately double initial determinations.

Discussion. Developmental changes in PMP oxidase activity have been studied only in rat liver. Nakahara *et al.* (10) found that values increased steadily during the suckling period, were higher in nonpregnant adults, and were highest (~200 nmole/g/hr) in pregnant animals. All of the values shown in Fig. 1 for rats fed CD exceed those of Nakahara *et al.* (10).

Pogell's paper (11) describing the first partial purification of PMP oxidase compared enzyme activities in a variety of rat tissues. He found that brain had about one-third the activity of kidney, which, in turn, had about one-third the activity of liver. The same activity ranking was observed in the present study, but values in the three tissues, in contrast to Pogell's finding, were quite similar.

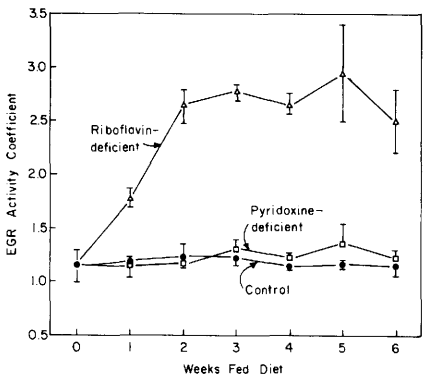

FIG. 2. Effect of riboflavin or pyridoxine deficiency on EGR activity coefficient during the 6-week experimental period. Mean ± SEM is depicted; $n = 3$ for each point (except $n = 2$ for the control group at Week 5).

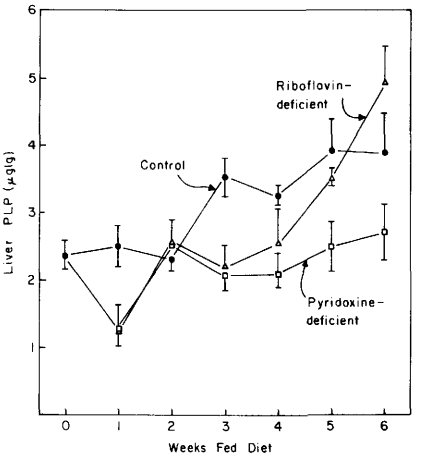

FIG. 3. Effect of riboflavin or pyridoxine deficiency on PLP concentration in rat liver during the 6-week experimental period. Mean ± SEM is depicted; $n = 3$ for each point (except $n = 2$ for the control group at Week 5).

Lakshmi and Bamji (12) found that liver PMP oxidase values in weanling (46 g) rats fed a riboflavin-deficient diet for 50 days were only 15% of values in controls fed *ad libitum*. The present study confirms those findings. PMP oxidase activity in rats fed $-B_2$ was significantly less than in those fed CD during most of the weeks in all tissues. Although PMP oxidase activity in rats fed $-B_6$ was frequently less than in those fed CD, the difference rarely reached statistical significance in any of the tissues examined. PMP oxidase activity thus appears to reflect riboflavin nutriture specifically.

The EGR activity coefficients in control and riboflavin-deficient rats were similar to those obtained by Tillotson and Sauberlich (5). Values were maximal after only 2 weeks of deficient feeding in both their study and in the present experiment. The EGR activity coefficient is, therefore, an early indicator of riboflavin deficiency but is not sensitive to further depletion. Liver PMP oxidase activity also provided an early index of riboflavin deficiency; values in rats fed $-B_2$ were significantly less than values in rats fed CD after only 1 week. Unlike the EGR activity coefficient, however, PMP oxidase activity continued to change as the deficiency progressed, reaching minimal values only after 4 weeks of deficient feeding. PMP oxidase activity

thus represents a more-sensitive index of riboflavin status than the EGR activity coefficient.

Literature reports of liver PLP concentration vary considerably. The results shown in Fig. 3 for control rats at the end of the study are similar to those Lakshmi and Bamji (12) and Nakahara *et al.* (10) obtained using the apotryptophanase method. Other investigators, using either the apotryptophanase method (13) or the tyrosine apodecarboxylase method (6) employed here, reported values two- to threefold greater. The liver PLP concentration of rats fed $-B_6$ was, as expected, significantly less than in those fed CD. The pyridoxine status of rats fed $-B_2$ appeared to be compromised early in the experiment but later was not different from that of rats fed CD.

Summary. Compared with animals fed the control diet, PMP oxidase activity decreased significantly in the livers, kidneys, and brains of young rats fed a riboflavin-deficient purified diet for 6 weeks. PMP oxidase activity in tissues from rats fed a pyridoxine-deficient diet was not different from controls. PMP oxidase activity was more sensitive than the EGR activity coefficient to the degree of riboflavin depletion. It was concluded that PMP oxidase activity was a sensitive and specific index of riboflavin status.

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