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Response of Kynurenine Pathway Enzymes to Pregnancy and Dietary Level of Vitamin B-6

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VAN DE KAMP, J. L. AND A. SMOLEN. *Response of kynurenine pathway enzymes to pregnancy and dietary level of vitamin B-6.* PHARMACOL BIOCHEM BEHAV **51**(4) 753–758, 1995.—The kynurenine pathway of tryptophan metabolism produces several neuroactive metabolites including 3-hydroxykynurenine, kynurenic acid, and quinolinic acid. This pathway is sensitive to reductions in vitamin B-6 availability because two key enzymes, kynurenine aminotransferase (KAT) and kynureninase (KYNase), require pyridoxal 5′-phosphate. During pregnancy abnormal concentrations of kynurenine metabolites are also found. We measured the effects of pregnancy and vitamin B-6 availability on KAT and KYNase in liver. DBA/2Ibg and A/Ibg mice were fed diets containing 0.25, 0.5, 2.0, 3.6, or 7.0 mg/kg pyridoxine-HCl (PN-HCl) for 4 weeks. Mitochondrial KAT and cytosolic KYNase were measured in control mice and pregnant mice on gestational days 16–18. The response of the two inbred strains was similar throughout. There were no marked alterations in KAT activity as a function of diet or pregnancy. In contrast, KYNase activities were significantly reduced by dietary restriction of vitamin B-6, and pregnant mice had significantly lower activity than nonpregnant controls for all but the highest dietary level of PN-HCl. These data show that pregnancy has a more pronounced effect on KYNase activity than vitamin B-6 restriction, and that the effects of pregnancy and diet are additive. The alteration in the kynurenine pathway in pregnancy is due to a reduction in KYNase activity, which is resistant to alleviation by vitamin B-6 supplementation.

Kynurenines Kynurenine aminotransferase Kynureninase Vitamin B-6 Pregnancy Mice

DURING pregnancy, most women display some degree of vitamin B-6 deficiency that is of unknown etiology (2,6,16, 28). Although it may represent a normal, relatively benign, physiological change in most pregnant women, it has been reported that the deficit in vitamin B-6 is significantly greater in pregnancies complicated with preeclampsia/eclampsia (toxemia of pregnancy) (4,11). Preeclampsia, which occurs in 4% of all pregnancies (24), is characterized by the development of edema, proteinuria, and hypertension late in the course of an apparently normal pregnancy. Eclampsia, which occurs in 0.3% of all pregnancies (27), is characterized by generalized seizures superimposed on the preeclamptic syndrome. The association of vitamin B-6 deficiency with increased susceptibility to spontaneous seizures in humans (18) and in laboratory animals (25) has long been known.

We previously reported that pregnant mice display depressed plasma vitamin B-6 levels (8) similar to those found in pregnant women. Furthermore, pregnant mice are more susceptible to flurothyl-induced seizures compared to nonpregnant controls (30). This enhanced pregnancy-associated seizure susceptibility is under genetic control because most strains, such as DBA/2Ibg, are more susceptible to seizures when pregnant, whereas others, such as A/Ibg, are not (29). We have suggested that these commonalties make the pregnant mouse a useful model for investigating the potential association of abnormal vitamin B-6 metabolism with eclamptic seizures.

The active coenzyme forms of vitamin B-6, pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) (3), are involved in nearly all amino acid metabolizing enzymes, and deficiency of vitamin B-6 may lead to a host of metabolic abnormalities. Our recent studies have centered on the kynurenine pathway (Fig. 1), which is responsible for 90% of tryptophan metabolism (7). This pathway is capable of producing several neuroactive metabolites, is markedly sensitive to reductions in vitamin B-6 availability, and has been found to be abnormal during pregnancy [reviewed in (5,10,28)].

Tryptophan is metabolized to *N*-formylkynurenine by tryptophan 2,3-dioxygenase [L-tryptophan : oxygen 2,3-oxidoreductase (decylizing), EC 1.13.11.11, also called tryptophan pyrrolase] (5,10). *N*-Formylkynurenine is rapidly converted first to kynurenine (KYN), then to 3-hydroxykynurenine

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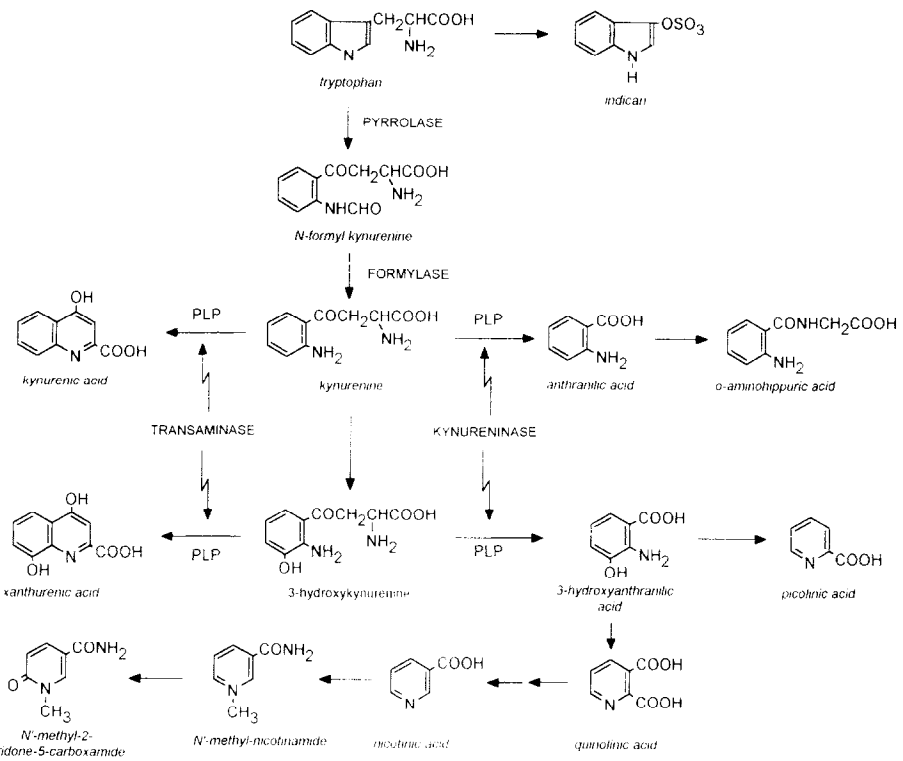

FIG. 1. The kynurenine pathway of tryptophan metabolism. Redrawn from Brown et al. (5), with permission.

(3-HK). Both KYN and 3-HK can be oxidized by the vitamin B-6-requiring enzyme kynureninase (L-kynurenine hydrolase, EC 3.7.1.3, KYNase) to anthranilic acid or 3-hydroxyanthranilic acid (3-HAA), respectively; or they may be transaminated by the vitamin B-6-requiring enzyme kynurenine aminotransferase (L-kynurenine:2-oxoglutarate aminotransferase, EC 2.6.1.7, KAT) to kynurenic acid (KYNA) or xanthurenic acid (XANTH), respectively. 3-Hydroxykynurenine, KYN, KYNA, XANTH, and quinolinic acid (QUIN) are collectively referred to as the kynurenines. They are formed in abnormally high concentrations in pregnancy and during vitamin B-6 deficiency states (14,34). The kynurenines are known to modulate excitatory neurotransmission (12,26), and may be involved in the development of certain neurologic diseases and seizure disorders (22,26). It is thought that the increased production of the neuroactive kynurenines is due a block in the degradation of the kynurenines though the KYNase branch of the pathway.

The present study examines the effect of pregnancy and dietary restriction of vitamin B-6 on the activity of two of the kynurenine degradative enzymes, KAT and KYNase, in the liver of two inbred mouse strains that differ with respect to pregnancy-associated seizures. A preliminary report of this study has been published (32).

METHOD

Materials

Acetonitrile (HPLC grade) was obtained from Baxter (Muskegon, MI). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Experimental Animals

Female A/Ibg and DBA/2Ibg mice were used in this study. Mice were bred at the Institute for Behavioral Genetics. The colony was maintained at 21 ± 2°C under a 12-h photoperiod (lights on 0600–1800 h). Food and water were available ad lib. The mice were fed a semipurified diet (ICN Biomedicals, Cleveland, OH) containing 0.25, 0.5, 2.0, 3.67, or 7.0 mg pyridoxine-HCl/kg diet for at least 2 weeks prior to mating, and then were maintained on the diet until enzyme activities were measured on gestational days 16–18. Nonpregnant control mice were maintained on the diet for the same period of time. Details of the diets and husbandry have been published previously (8,9).

Tissue Preparation

Mice were killed by cervical dislocation. The livers were removed, rinsed in ice-cold saline, blotted dry, weighed, and homogenized in 4 vol. of 0.14 M KCl in 20 mM potassium phosphate, pH 7.0. All subsequent procedures were carried out at 4°C. The homogenate was centrifuged for 10 min at 500 × *g* in a Sorvall RC-2B centrifuge. The supernatant was decanted and centrifuged at 10,000 × *g* for 10 min. Both supernatant (cytosol) and pellet (crude mitochondria) from this centrifugation were used. The supernatant was centrifuged at 100,000 × *g* for 45 min in a Beckman L2-65 ultracentrifuge, and the resulting supernatant was decanted and used for assay of KYNase activity. The crude mitochondrial pellet was washed three times by resuspension in homogenization buffer followed by centrifugation at 10,000 × *g* for 10 min. The final pellet was resuspended in 1.5 ml of 10 mM sodium phos-

phate, pH 7.4, and incubated for 30 min on ice with 40 μl of a 100-mg/ml solution of sodium deoxycholate. The samples were then centrifuged at 14,000 × *g* for 20 min to remove the mitochondrial membranes. The resulting mitochondria supernatant was decanted and used for assay of KAT activity.

Biochemical Assays

Both KAT and KYNase activities were measured in the absence and presence of a saturating concentration of PLP. Holoenzyme activity (−PLP) measures enzyme to which coenzyme is bound and reflects the activity likely to be found in vivo. Total activity (+PLP) includes additional, inactive apoenzyme, and may be used as a rough indicator of enzyme synthesis and stability.

Kynurenine aminotransferase activity was measured by a modification of the method of Takeuchi et al. (31) in which the product of the transamination reaction, XANTH, was quantified by high pressure liquid chromatography (HPLC). The reaction mixture consisted of 200 μl of mitochondrial extract, 2 mM α-ketoglutarate (potassium salt), and 2 mM 3-HK in the absence or presence of 80 μM PLP in a total volume of 250 μl. The reactions were incubated at 37°C for 40 min and terminated by the addition of 1 vol. of 0.55 M perchloric acid (PCA). Blanks had PCA added prior to addition of substrate. The samples were kept at −35°C until HPLC analysis. Samples were stable for at least 1 month under these conditions.

On the day they were assayed, the KAT samples were centrifuged at 12,000 × *g* for 10 min. Then 400 μl of the resulting supernatant was transferred to a 500-μl polypropylene autosampler vial (Sun Brokers, Willmington, NC) containing 20 μl of the internal standard, 3,4-dihydroxyphenylacetic acid (DOPAC, 10 μg/ml). The HPLC consisted of a Beckman (Fullerton, CA) model 110B pump, ISCO (Lincoln, NE) Isis autosampler, BAS (W. Lafayette, IN) phase II ODS 3-μm column, BAS LC-4B electrochemical detector (+0.8 V vs. Ag/AgCl), and linear (Reno, NV) strip chart recorder. A 20-μl aliquot of the sample was injected onto the column, and separation was achieved isocratically with a mobile phase consisting of 130 mM monochloroacetic acid, 0.67 mM EDTA, 0.4 g/l sodium octyl sulfate, and 0.5% acetonitrile at a flow rate of 1.0 ml/min. Quantification of XANTH was accomplished by comparing peak heights with those of standard curves run daily.

Kynureninase activity was measured by a modification of the method of McDermott et al. (17) in which the product, 3-HAA, was quantified by HPLC. The reaction mixture consisted of 300 μl of cytosol and 400 μM 3-HK in the absence or presence of 4 μM PLP in a total volume of 500 μl. The reaction was incubated at 37°C for 10 min and was terminated by the addition of 1 vol. of 0.55 M PCA. Blanks had PCA added prior to addition of substrate. The samples were frozen at −35°C until HPLC analysis. Samples were stable for at least 1 month under these conditions.

On the day of analysis, the KYNase samples were centrifuged at 12,000 × *g* for 10 min. Then 450 μl of the supernatant was transferred to an autosampler vial containing 20 μl of 50 μg/ml DOPAC. A 20-μl aliquot of the sample was analyzed using the HPLC system described above except that the mobile phase consisted of 130 mM monochloroacetic acid, 0.67 mM EDTA, 0.35 g/l sodium octyl sulfate, and 2% acetonitrile. Quantification of 3-HAA was accomplished by comparing peak heights with those of standard curves run daily.

Protein concentrations were measured using the method of Lowry et al. (15) with bovine serum albumin as the standard.

Data Analysis

Data were analyzed by one-, two- or three-way ANOVA as appropriate with a commercial software package (CRUNCH, Oakland, CA) using strain, diet, and pregnancy status (control or pregnant) as independent variables. Following a significant overall main effect, differences in individual group means were determined using the Tukey B post hoc test. An alpha level of <0.05 was considered to represent a significant difference, and is the only level reported.

RESULTS

Kynurenine Aminotransferase

The effects of pregnancy and dietary restriction of vitamin B-6 on mitochondrial KAT activity are shown in Fig. 2. The left panels show holoenzyme activity, which is that measured in the absence of added PLP in vitro. The total enzyme activity (holoenzyme plus additional inactive apoenzyme), measured in the presence of added PLP in vitro, is shown in the right-hand panels. The holoenzyme activity was markedly lower than the total enzyme activity, suggesting that the majority of the enzyme in vivo is in the apoenzyme form. Three-way ANOVA of holoenzyme activity showed significant main effects of diet, $F(4, 155) = 16.6$ (due primarily to the 0.25-mg diet), and pregnancy status, $F(1, 155) = 7.35$ (pregnant groups were generally lower), but not strain. For both strains there was a significant effect of diet [$F(4, 79) = 22.5$, A mice; $F(4, 76) = 3.84$, DBA mice] and a significant diet by pregnancy status interaction [$F(4, 79) = 3.73$, A mice; $F(4, 76) = 4.60$, DBA mice].

Three-way ANOVA of the KAT total enzyme activity showed significant main effects of strain, $F(1, 155) = 5.11$ (DBA mice higher), diet, $F(4, 155) = 7.82$, and pregnancy, $F(1, 155) = 20.35$ (pregnant groups were lower); and for both strains there were significant two-way main effects of diet [$F(4, 79) = 5.48$, A mice; $F(4, 76) = 3.86$, DBA mice] and pregnancy [$F(1, 79) = 15.85$, A mice; $F(1, 76) = 6.98$, DBA mice]. Similar to the results for KAT holoenzyme, the pregnant animals had lower activity than the controls, and the effect of diet, although significant, was not great.

Kynureninase

Figure 3 shows the effects of pregnancy and dietary restriction of vitamin B-6 on cytosolic KYNase activity. In contrast to KAT holoenzyme, KYNase holoenzyme activity was similar in magnitude to the total enzyme activity, suggesting that the majority of the enzyme in vivo was in the holoenzyme form. Three-way ANOVA of the holoenzyme activity showed main effects of diet, $F(4, 154) = 21.9$, and pregnancy status, $F(1, 154) = 58.1$, but not strain. For both strains there were significant two-way main effects of diet [$F(4, 80) = 14.5$, A mice; $F(4, 74) = 9.98$, DBA mice] and pregnancy [$F(1, 80) = 32.8$, A mice; $F(1, 74) = 25.8$, DBA mice], and a significant diet by pregnancy status interaction for the DBA mice, $F(4, 74) = 4.61$. Holoenzyme activity decreased with dietary restriction of PN-HCl, and the activity in the pregnant animals was significantly lower than controls. This indicates that saturation of KYNase with PLP is significantly lower in pregnancy, and that enzyme activity in vivo would be expected to be significantly lower in pregnant animals.

Results of three-way and two-way ANOVA for total KYNase activity were nearly identical to the holoenzyme results: significant three-way main effects of diet, $F(4, 154) = 44.6$, and pregnancy, $F(1, 154) = 70.1$; for each strain, signif-

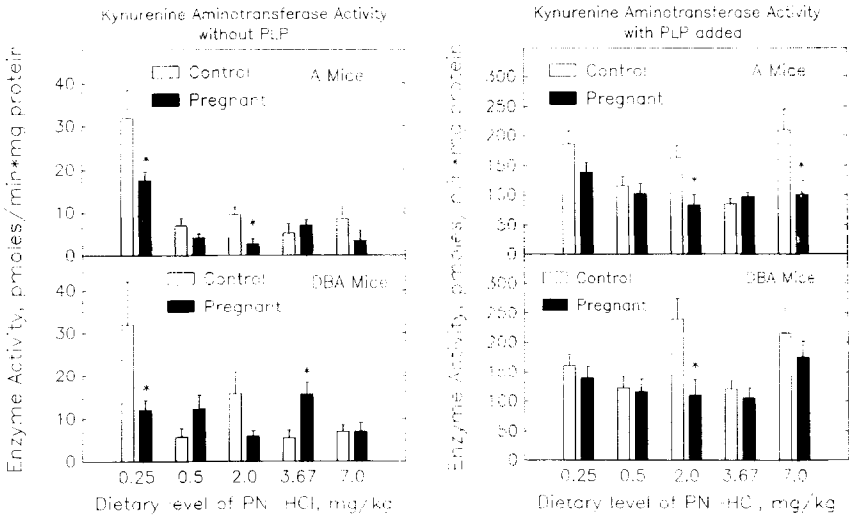

FIG. 2. Kynurenine aminotransferase activity in control and pregnant A/Ibg and DBA/2Ibg mice fed five different levels of pyridoxine-HCl. Apoenzyme activity (−PLP) is shown in the left-hand panels and total enzyme activity is shown in the right-hand panels. *Significantly different from respective control group, $p < 0.05$.

icant two-way main effects of diet [$F(4, 80) = 21.4$, A mice; $F(4, 74) = 25.8$, DBA mice] and pregnancy status [$F(1, 80) = 43.3$, A mice; $F(1, 74) = 28.3$, DBA mice], and significant diet by pregnancy interactions [$F(4, 80) = 5.57$, A mice; $F(4, 74) = 4.69$, DBA mice]. In addition, there were significant strain by diet, and strain by diet by pregnancy interactions. Total enzyme activity was similar for all dietary levels in control mice, except in those fed the lowest amount of PN-HCl. The total enzyme activity was significantly lower in virtually all of the pregnant groups, indicating that pregnancy had a more pronounced effect on enzyme levels than did dietary restriction of vitamin B-6.

DISCUSSION

The results of this study show that the two vitamin B-6-requiring enzymes in the kynurenine pathway of tryptophan metabolism, KAT and KYNase, are differentially responsive to both pregnancy and dietary restriction of vitamin B-6. Kynureninase activity was significantly reduced in pregnant animals, and this pregnancy-induced reduction was increased by vitamin B-6 restriction. Kynurenine aminotransferase activity, on the other hand, remained fairly constant in all but the lowest dietary level of PN-HCl, where a significant increase in activity was observed. The differences between the two inbred

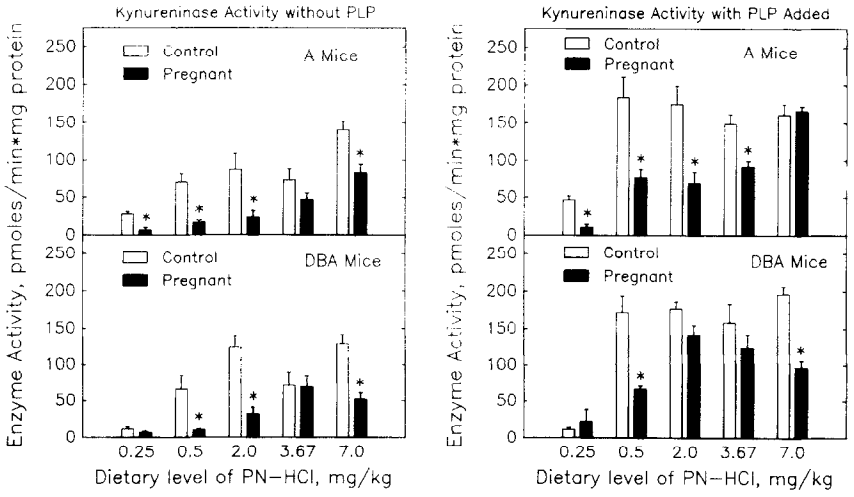

FIG. 3. Kynureninase activity in control and pregnant A/Ibg and DBA/2Ibg mice fed five different levels of pyridoxine-HCl. Apoenzyme activity (−PLP) is shown in the left-hand panels and total enzyme activity is shown in the right-hand panels. *Significantly different from respective control group, $p < 0.05$.

strains in response was small and most probably without physiological significance, suggesting that the strain difference in pregnancy-associated seizure susceptibility is not mediated at the level of expression of KAT or KYNase activities.

Most of tryptophan is metabolized through the kynurenine pathway. Two metabolites, KYN and 3-HK, occupy pivotal positions because they can be further metabolized via two alternative routes. Under normal conditions, the primary metabolites of tryptophan are *N*-methylnicotinamide and *N'*-methyl-2-pyridone-5-carboxamide, which are products of the KYNase branch of the kynurenine pathway (Fig. 1). Products of the KAT branch of the pathway, XANTH, KYNA, and anthranilic acid (5,10), are normally found in very low concentrations. In vitamin B-6 deficiency states and in pregnancy, KAT metabolites (XANTH and KYNA) and intermediates (KYN and 3-HK) predominate, suggesting a block in the pathway at the level of KYNase (5,14,34). Although both enzymes require PLP, KYNase has been shown to be more sensitive to PLP depletion than KAT (20).

Our data show little effect of dietary vitamin B-6 on KAT activity. Neither KAT holoenzyme nor KAT total enzyme activity was markedly reduced by vitamin B-6 restriction. This is in agreement with an earlier report (20), which showed that KAT activity in rats fed a diet devoid of vitamin B-6 for 30 days was not markedly affected. To our knowledge, the effect of pregnancy on KAT activity has not been reported, although tryptophan metabolism studies have indicated that it would remain fairly constant (10,34). We found that pregnancy resulted in an overall reduction of enzyme activity; however, the degree of reduction was minimal, and pregnancy did not enhance the effect of B-6 restriction. The KAT activity in the presence of PLP was nearly 10 times greater than that in the absence of PLP, suggesting that the majority of the enzyme is present in the apoenzyme state in vivo. The similarity of enzyme activities across all the dietary groups was indicative of a high degree of stability of the apoenzyme. In the lowest dietary groups, there was a marked increase in the holoenzyme activity in both strains, control and pregnant.

Although vitamin B-6 deficiency is usually associated with decreased activity of PLP-requiring enzymes, paradoxical increases in activity have been described. Sampson and coworkers (23) have reported that ornithine decarboxylase (ODC) activity in the kidney, liver, and small intestine was significantly increased in vitamin B-6-deficient rats, but that levels of ODC mRNA were unchanged in the tissues. They suggested that deficiency of vitamin B-6 caused an increase in ODC activity by a posttranslational mechanism. Oka and coworkers (21) reported a differential regulation of glycogen phosphorylase isozymes in vitamin B-6-deficient rats. The activity of the muscle isozyme was markedly reduced by vitamin B-6 deficiency, but the activity of the liver enzyme was unaffected. They determined that the phosphorylase mRNA was reduced to 40% of control in muscle, but increased fivefold in liver of the vitamin B-6-deficient rats. β-Actin mRNA and the activities of DNA-dependent RNA polymerases I and II were also increased in the liver, but not the muscle of the deficient rats. These investigators suggested that vitamin B-6 may modulate transcriptional activation of these and other genes in a tissue-specific manner. Whether the increase in KAT activity in the most deficient group of mice in our study involves increased mRNA synthesis is not known.

A very different pattern of response to diet was seen with KYNase activity. Both KYNase holoenzyme and total enzyme activities showed clear dependences on dietary level of vitamin B-6, in agreement with an earlier study (20). In addition, pregnant mice had significantly lower activity than nonpregnant controls for each dietary level of PN-HCl. Within each group (pregnant or control) the enzyme activity was similar in all but the most deficient group of animals. This indicates that with intakes of 0.5 mg PN-HCl/kg diet or higher the total amount of enzyme available to the cell is approximately constant, with the difference in metabolic activity being due to the degree of saturation of the enzyme with PLP. In contrast to the increased KAT activity in the lowest dietary group, KYNase activity was significantly depressed, indicating a reduced level of synthesis, or an increased amount of degradation.

The effect of pregnancy on the reduction KYNase activity was much greater than the effect of vitamin B-6 restriction. Pregnant mice fed the highest level of PN-HCl (7.0 mg/kg) had KYNase activities equivalent to controls fed a deficient diet (0.5 mg/kg). The recommended level of PN-HCl for mice is 7 mg PN-HCl/kg diet (1); however, we (13) and others (19) have suggested that the requirement for mice is in the range of 1 or 2 mg PN-HCl/kg diet. Thus, the 7.0-mg/kg group was receiving a three- to sevenfold supplementary level of vitamin B-6, which was still not sufficient to normalize KYNase activity in the pregnant mice. Similarly, pregnant women require supplementation with vitamin B-6 at levels three- to fivefold or more over the Recommended Dietary Allowance to normalize tryptophan metabolism (10,33). Our data provide direct evidence that the alteration in the tryptophan–kynurenine pathway in pregnancy is due to a reduction in hepatic KYNase activity that is resistant to alleviation by vitamin B-6 supplementation. The mechanism underlying the reduction of KYNase activity in pregnancy remains unknown.

In summary, we have shown that pregnancy has a more pronounced effect on KYNase activity than does dietary restriction of vitamin B-6 alone, and that the effects of pregnancy and dietary restriction are additive. The metabolic consequences of the alteration of the tryptophan–kynurenine pathway may be important. As discussed previously, several of the metabolites, including 3-HK, quinolinic acid, and picolinic acid, are neurotoxins, and others, such as KYNA, are modulators of neurotransmitter systems. Studies are in progress to determine the effects of pregnancy and dietary restriction of vitamin B-6 on the steady-state concentrations of these tryptophan metabolites.

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