Review

# Vitamin B-6, kynurenines, and central nervous system function: developmental aspects

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## Vitamin B-6 nutritional status in early development

Vitamin B-6 refers collectively to six metabolically interconvertable water-soluble vitamers *(Figure 1)*. The physiologically active forms, pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP), are generated from their dietary precursors, pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), by the action of pyridoxine oxidase and pyridoxine kinase *(Figure 1)*. Formation of the major excretory product, pyridoxic acid, is catalyzed by various non-specific phosphatases and/or aldehyde oxidase, depending on the particular precursor vitamer.

Pyridoxal phosphate acts as a cofactor at the active sites of enzymes catalyzing a great number of reactions critical to the metabolism of amino acids. These include transamination reactions, decarboxylations, and α- and β- addition and elimination reactions. In 1968, Sauberlich[1] catalogued over 50 enzymes, most involved in amino acid metabolism, which require PLP as a cofactor. There are now over 100 enzymes requiring vitamin B-6 as a cofactor.

In view of its central role in intermediary metabolism, it is not surprising that vitamin B-6 is present in all animal and in many plant tissues.[2,3] Although there are no particularly rich sources of this vitamin, it is widely available in foods.[4,5] Clinical presentation of frank vitamin B-6 deficiency is rare. However, many investigations have indicated that vitamin B-6 nutrition is suboptimal in significant portions of the United States population. A study of the dietary intake of vitamin B-6 of 11,658 individuals found a mean intake of 70% of the recommended daily allowance (RDA) for this nutrient.[5] This and other studies[6,7] have indicated that suboptimal vitamin B-6 intake may be quite common among women of childbearing age. Mean intake among women of childbearing age was roughly 50% of the RDA, and about 15% of women surveyed reported intakes amounting to less than 25% of the RDA.[5] In addition, mean dietary intake was reported to be about 60% of the RDA in pregnant and lactating women.[5] Similar results have been obtained using biochemical indices of vitamin B-6 nutritional status.[6,8] Several reports have suggested that the RDA for vitamin B-6 during pregnancy and lactation may be too low.[9-11]

Although fetal vitamin B-6 status may be maintained during gestation by placental uptake of vitamin B-6 from the maternal circulation,[12,13] vitamin B-6 levels in cord blood were found to be highly correlated with those measured in maternal plasma.[14] Studies have shown that the vitamin B-6 content of human milk[15] and PLP levels in breast-fed infants[9] vary with maternal vitamin B-6 status. Borschel et al.[9] measured very low plasma PLP levels in two infants breast fed by mothers receiving vitamin B-6 supplements equivalent to the RDA. These data suggest that some degree of vitamin B-6 deficiency may be common in human infants.

Adequate vitamin B-6 nutrition is essential for the normal function and development of the immature central nervous system (CNS). While adults may endure long periods of vitamin B-6 deprivation without overt signs of vitamin deficiency,[16-18] vitamin B-6–deficient neonates may suffer severe neurological impairment[17,19] with acute signs including ataxia, tremor, and seizures. In addition, recent investigations have described deficiency-related alterations in CNS morphology[20] and behavioral changes that persist well into maturity.[21] Despite increasing knowledge of the metabolic and neurochemical alterations associated with vitamin B-6 deficiency in neonates, the mechanisms underlying the various deficiency-induced impairments are poorly understood. In particular, the biochemical bases for the contrasting vulnerabilities of neonatal and adult organisms remain unexplained.

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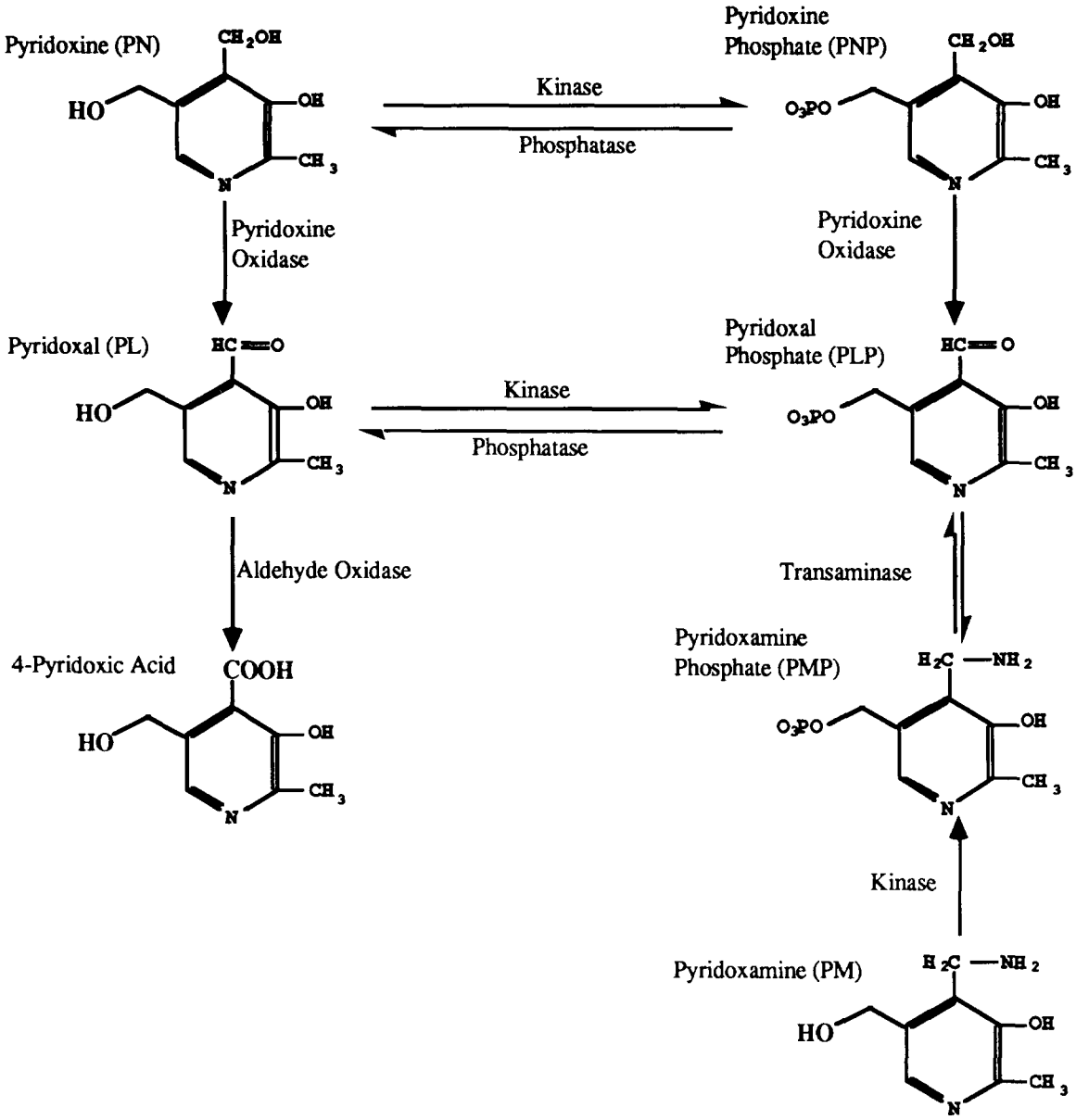


**Figure 1** Metabolic interconversion of B-6 vitamers.

A dietary deficiency of vitamin B-6 results in the abnormal functioning of a number of enzymes that participate in the metabolism of a wide variety of neurotransmitters and neuromodulators including glutamate, gamma-aminobutyric acid (GABA), dopamine, serotonin, and taurine. Among other metabolic pathways that may be affected by vitamin B-6 deficiency, the kynurenine pathway may be of particular interest. Alterations of flux through this pathway have formed the basis of diagnostic tests of functional vitamin B-6 status,[22] and the kynurenine pathway incorporates several neuroactive intermediates.[23-25] The elevation of CNS levels of 3-hydroxykynurenine (3HK) in neonatal vitamin B-6 deficiency[17] is of particular interest because 3HK was among the more potent of several kynurenine pathway metabolites shown to induce seizures upon intracerebroventricular (i.c.v.) administration in rodents.[24] In vitamin B-6–deficient neonatal rats, the elevation of 3HK was observed to occur approximately coincidently with the onset of neurological signs. No such rise in CNS levels of 3HK was measured in adult rats, even after prolonged dietary deprivation of vitamin B-6.[17] These results suggest that the kynurenine pathway may be particularly sensitive to altered vitamin B-6 nutritional status during the neonatal period.

## Neonatal vitamin B-6 deficiency

In view of the widespread involvement of PLP-dependent enzymes in the metabolism of CNS neurotrans-

mitters, it is surprising that adult organisms are rarely reported to manifest neurological signs as a result of dietary deprivation of vitamin B-6. While there have been several reports of convulsions in adult rats deprived of vitamin B-6 for periods in excess of 4 months,[26,27] vitamin B-6 deficiency in adult life is more often associated with anemia, peripheral neuropathy, and lesions of the skin (acrodynia).[28] However, a recent report indicated electroencephalographic abnormalities in two of eight women depleted of vitamin B-6 for only 12 days.[29] In contrast, vitamin B-6–deficient human infants[30,31] and neonates of several species[32] manifest severe neurological impairment including ataxia, tremor, irritability, and seizures.

The requirement of human infants for vitamin B-6 was dramatically demonstrated in the early 1950s as a result of the use of an infant formula containing insufficient quantities of this vitamin. Affected infants exhibited marked irritability and recurrent seizures, but none of the signs associated with vitamin B-6 deficiency in adulthood.[30,31,33] The seizures were poorly controlled by anticonvulsant treatments but rapidly reversible upon administration of vitamin B-6.[31] It is noteworthy that the convulsive syndrome resulted not from complete avitaminosis, but from a borderline deficiency.[31] The vitamin B-6 content of the infant formula was estimated to be roughly one-half that of the mean content of human milk from unsupplemented mothers.[31]

In experimental animals, the effects of neonatal vitamin B-6 deficiency have been examined in the progeny of dams maintained on defined vitamin B-6–restricted diets from weaning, conception, or parturition through lactation. Maternal health is generally reported to be adequately supported by the vitamin-restricted diets for the duration of the study. Early studies[34,35] generally employed diets essentially free of pyridoxine, while more recent investigations[36,37] often employed a range of experimental diets containing graded levels of pyridoxine. Maternal dietary vitamin B-6 restriction generally has little effect on birthweight or brain weight of progeny at birth,[37,38] although small differences in both parameters have been reported.[39,40] Control and deficient progeny experience similar weight gain in the first week of life, after which growth is retarded in vitamin B-6–deficient neonates. Decreased brain weight is also observed in deficient animals after 10–15 days of age.

Overt signs of neonatal vitamin B-6 deficiency in experimental animals include abnormal gait, ataxia, tremor, and seizures.[40] In rats, onset of overt neurological signs is commonly reported to occur at 10–18 days of age regardless of the duration of maternal deprivation.[20,34,41] Guilarte[36,42] has reported that some of the neurological deficits subside as the animals mature despite continued maintenance on diets providing marginal levels of vitamin B-6. However, a recent report[21] indicates that neonatal vitamin B-6 restriction results in a pattern of hyperactivity that becomes evident after weaning and persists well into adulthood. In contrast, overt neurological impairment does not result when vitamin restriction is initiated after weaning.[43,44]

These observations would suggest that fetal vitamin B-6 status is adequately maintained in utero despite maternal deficiency, or that the requirement for vitamin B-6 is particularly critical during the second 2 weeks of life. The former suggestion is supported by studies of the vitamin B-6 content of neonatal rat brain tissue and maternal milk. When measured in brain tissue from 2-day-old rats, vitamin B-6 status, as assessed by microbiological vitamin B-6 assay and percent alanine aminotransferase saturation, did not differ among litters of rats born to dams maintained from weaning on diets providing 1.2–19.2 mg PN/kg diet.[37] On the other hand, the vitamin B-6 content of maternal milk reflected maternal vitamin B-6 nutritional status.[45] However, by 12 days of age the vitamin B-6 measured in brain tissue from neonates reared by dams receiving the 1.2 mg PN/kg diet were less than one-third of those measured in other experimental groups.[37] These results are consistent with the suggestion of Coburn[46] that existing tissue can efficiently conserve vitamin B-6, but that growing tissue will need approximately 15 nmol of vitamin B-6 to supply each gram of new tissue. Therefore, in growing vitamin B-6 deficient animals, the animals retain most of their initial level of vitamin B-6, but the tissue concentrations are reduced relative to controls because the animals have grown.

## Neurochemical and neuropathological changes in neonatal vitamin B-6 deficiency

Vitamin B-6 deficiency has been shown to produce widespread changes in CNS levels of various amino acids,[36,42,44,47] altered monoaminergic,[36,48] GABAergic,[39,42,49] and glutamatergic neurotransmission.[42,50,51] It is likely that these changes contribute to the motor abnormalities and convulsive activity associated with neonatal vitamin B-6 deficiency. In addition, histological examination of CNS tissue from vitamin B-6–deficient neonatal rats has revealed abnormalities in myelination,[52] and other pathological changes suggestive of altered or delayed development or of decreased neuronal longevity.[41]

In vitamin B-6–deficient rat pups, a myelination deficit is evident at 15 days of age.[20,52] Significantly fewer myelinated axons were observed in the mediodorsal portion of the pyramidal tract in vitamin-deficient pups relative to controls. In accord with this morphological finding, neurochemical studies have revealed deficiency-induced changes in CNS lipid metabolism. The cerebroside content of brain lipid fractions from vitamin B-6–deficient rat pups was decreased relative to controls at 12 and 21 days of age, and cholesterol was also decreased in 21-day-old rats.[53,54] In addition, the cerebellar content of certain long-chain fatty acids was shown to be decreased in 15-day-old vitamin B-6–deficient rat pups relative to age-matched controls.[54] Other investigators have demonstrated deficits of microsomal fatty acid elongation[55] and incorporation of [$^3$H]-acetate into brain lipids.[56]

Neuropathological changes induced by neonatal vitamin B-6 deficiency have been described in a series of reports by Kirksey et al. Gross changes in CNS morphology include a decrease in neocortical and cerebellar cortical area and a decrease of cerebellar molecular and granular layers.[57] Further, decreases in dendritic arborization have been described in cerebellar Purkinje cells[58] and in neocortical pyramidal and stellate neurons.[41] Synaptic density has been reported to be diminished in striata[47] and cerebral cortices[41] from vitamin B-6–deficient rat pups. In addition, neonatal vitamin B-6 restriction resulted in an increase in histologically abnormal shrunken neocortical neurons.[41,59] The authors have interpreted this observation as indicative of reduced neuronal longevity.

Perinatal vitamin B-6 deficiency has been shown to produce global changes in parameters of GABAergic neurotransmission. The activity of the PLP-dependent, GABA biosynthetic enzyme, glutamic acid decarboxylase (GAD), is significantly lower in brain homogenates from vitamin-deficient rat pups relative to controls.[39,60] While there was no difference between control and deficient pups at birth, a slowing of the normal postnatal increase in CNS GAD activity resulted in a significant decrement in GAD activity in deficient pups after the first week of life.[39] The decrement in GAD activity was due to PLP cofactor depletion, because the measured levels of GAD apoenzyme were significantly higher in deficient pups.[39,60] The deficiency-induced decrement in GABA biosynthetic activity was reflected in a decrease of brain GABA. GABA levels measured in brain tissue from vitamin B-6–deficient rats at 2 weeks of age were reported to be about 50% of control levels.[39,42] Guilarte[36,42] has shown that the deficit in CNS levels of GABA is transient, and that GABA is normalized by 56 days of age despite continued maintenance on a diet providing marginal levels of vitamin B-6. Normalization of GABA level was not noted at 5–6 weeks of age in the progeny of dams fed a pyridoxine-free diet from parturition.[60] This disparity may reflect the more nearly adequate levels of vitamin B-6 intake provided by the vitamin B-6–restricted diet used in Guilarte's laboratory. Perinatal vitamin B-6 deficiency also results in changes in indicators of GABAergic synaptic function. Guilarte[50] has demonstrated a decrease (relative to controls) of both basal and potassium-stimulated release of GABA from cortical and hippocampal slices prepared from vitamin B-6–deficient neonatal rat brain. An increase in GABA receptor binding has been demonstrated in vitamin B-6–deficient cerebellum.[49] This may reflect an adaptive upregulation of the GABA receptor in response to the deficiency-induced depletion of GABA. However, despite the importance of GABAergic neurotransmission in vitamin B-6 deficiency-induced neurological changes there has been a paucity of studies on the effect of vitamin B-6 deficiency on the GABA/benzodiazepine receptor complex during development.

The effects of neonatal vitamin B-6 deficiency on monoaminergic systems appear to be highly dependent on dietary protocol and analytical methods. In 3–8-week-old rats reared by dams fed a pyridoxine-free diet from parturition, there was no difference between controls and deficient rats in CNS levels of dopamine and norepinephrine.[61] In contrast, striatal dopamine was markedly decreased in rat pups reared by dams fed a pyridoxine-free diet during the last week of gestation and early lactation,[48] and in rats reared by dams fed marginal diets throughout gestation, lactation, and after weaning.[36] In the latter study, the difference in striatal dopamine levels between control and rats pups maintained on vitamin B-6 marginal diets increased with age. Furthermore, the deficiency-induced deficit in dopamine levels appeared to be unrelated to either precursor availability or to the activity of the PLP-dependent biosynthetic enzyme, DOPA decarboxylase.[36]

While these reports from the two laboratories appear in conflict, a recent report from Dakshinamurti et al.[62] clearly indicates a substantial dopaminergic deficit in cerebral cortex, thalamus, and hypothalamus of vitamin B-6–deficient rats. A potential explanation for the differences in dopamine measurements reported by Dakshinamurti in 1976[61] and 1990[62] may be related to the different methods employed for dopamine analysis. The high pressure liquid chromatography with electrochemical detection method used in the more recent investigation[62] is more sensitive and selective than the fluorescence method used previously.[61]

Similarly, serotonergic function has been reported to be decreased in the cerebral cortex of 3–8-week-old rats reared by dams maintained on pyridoxine-free diets from parturition[61] and increased in 14-day-old neonatal rats reared by dams fed a vitamin B-6–free diet in the last week of gestation and early lactation *(Figure 2)*. In the latter study, the observed increase

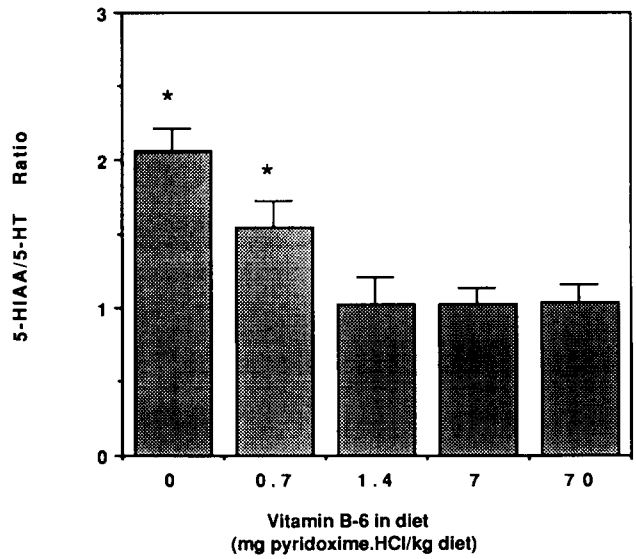


**Figure 2** Serotonergic activity as measured by 5-HIAA:5-HT ratios in the frontal cortex of 14-day-old neonatal rats from dams fed different levels of vitamin B-6 in the diet. *Significantly different from control at $P < 0.05$.

in serotonergic function at 14 days of age was attributed to increased precursor availability because CNS levels of tryptophan also were increased by vitamin B-6 deficiency.[63] The degree of the increase in serotonergic activity in the frontal cortex of 14-day-old rat pups as measured by the ratio of the major serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) to serotonin (5-HT) was highly dependent on the levels of vitamin B-6 in the diet *(Figure 2)*. The disparity between these reports on serotonergic activity in vitamin B-6 deficiency are most likely related to differences in dietary protocols, the level of vitamin B-6 in the diet, and/or the age of the experimental animals at the time of sacrifice.

The level of brain glutamate has been shown to be reduced in vitamin B-6 deficiency.[42,47] The interpretation of this finding with regard to glutamatergic neurotransmission is unclear because glutamate is present in both metabolic and neurotransmitter pools, and assessment of the effect of vitamin B-6 deficiency must rely on more direct indicators of glutamatergic function. Two recent reports have described changes in both presynaptic and post-synaptic glutamatergic function. The spontaneous and potassium-evoked release of glutamate by cortical and hippocampal tissue has been studied in brain slices from normal and vitamin B-6–deficient neonatal rats.[50] Consistent with the observation of reduced tissue levels of glutamate, basal release of glutamate from hippocampal slices and potassium-evoked release from cerebral cortex were both lower in vitamin B-6–deficient neonatal tissue. Deficiency-induced alterations in the modulation of the N-methyl-D-aspartate (NMDA)-preferring subtype of excitatory amino acid receptor have also been recently described.[51] Glutamate-stimulated [$^3$H]MK-801 binding, an indicator of NMDA receptor ion-channel function,[64] was reduced in both hippocampal and cortical membranes prepared from vitamin B-6–deficient neonatal rat brain. Glycine-stimulated [$^3$H]MK-801 binding also was significantly reduced in cortical membranes from vitamin B-6–deficient animals. Measured differences in $B_{max}$ for glutamate and glycine-dependent [$^3$H]MK-801 binding indicate that the observed reductions reflect a relative paucity of glutamate- and glycine-sensitive MK-801 binding sites in vitamin B-6–deficient brains. These deficiency-induced alterations in the activation of the NMDA glutamate receptor subtype and in glutamate release may have important implications to the developing young because they play an important role in the regulation of neuronal morphology and synaptogenesis,[65–67] and in the induction of long-term potentiation (LTP), a synaptic phenomenon thought to be involved in some forms of learning and memory processes in the mammalian brain.[68,69]

In addition to the changes observed in classical neurotransmitter systems, neonatal vitamin B-6 deficiency results in a dramatic increase in CNS levels of 3HK,[17,48] a tryptophan metabolite reported to have convulsant properties.[24,70] The increase of 3HK in CNS tissue from vitamin B-6–deficient neonatal rats occurs approximately coincidently with the onset of neurological signs. Increased levels of 3HK were observed in vitamin B-6–deficient pups at 14 days of age, and CNS levels in excess of 200 nmol/g tissue were measured in frontal cortices from 18-day-old rats reared in pyridoxine-free diets.[17] In contrast, CNS levels of 3HK were below the limits of detection (1 nmol/gm tissue) in vitamin B-6–deficient pups at birth and 7 days of age, and in adult rats maintained on a pyridoxine-free diet for up to 56 days.[17]

## Neonatal vitamin B-6 deficiency and the kynurenine pathway

The kynurenine pathway of tryptophan metabolism mediates the catabolism of tryptophan and its utilization for the biosynthesis of nicotinamide coenzymes. Both biochemical and nutritional aspects of kynurenine pathway metabolism have recently been reviewed by Bender.[71,72] The following discussion will emphasize issues relating to the control of circulating and CNS levels of 3HK in vitamin B-6 deficiency, and in particular, factors that may contribute to the special vulnerability of the kynurenine pathway to disruption by vitamin B-6 deficiency in neonatal life. A schematic diagram of the kynurenine pathway is shown in *Figure 3*.

The initial step of kynurenine pathway metabolism, the oxidative cleavage of the indole ring of tryptophan to produce N-formylkynurenine, may be catalyzed by either of two enzymes with distinct organ distributions, cofactor requirements, substrate specificities, and modulatory controls. Tryptophan dioxygenase (TDO) activity is restricted to liver[71] and its catalytic activity has a rather strict specificity for L-tryptophan as a substrate.[71] Indoleamine dioxygenase (IDO) has a wider tissue distribution and a broader substrate specificity. While absent in liver, IDO activity has been measured in a number of tissues including intestine, kidney, lung, stomach, placenta, and brain. N-formylkynurenine is rapidly hydrolyzed by a formamidase to form kynurenine, a substrate for three kynurenine pathway enzymes.

The PLP-dependent enzymes kynureninase and kynurenine aminotransferase catalyze the formation of anthranilic acid and kynurenic acid, respectively. Alternately, kynurenine may undergo a kynurenine-3-monooxygenase catalyzed hydroxylation of its aromatic ring to produce 3HK. The further metabolism of 3HK is mediated by kynurenine aminotransferase and kynureninase. Kynurenine aminotransferase catalyzes a cyclizing internal aminotransferase reaction resulting in the formation of xanthurenic acid. Kynureninase, an enzyme that is exquisitely sensitive to depletion of its pyridoxal phosphate cofactor, catalyzes the elimination of an alanine moiety to produce 3-hydroxyanthranilic acid. A branch point in the pathway is reached with the formation of 3-acroleyl-3-amino fumarate from the oxidative cleavage of the aromatic ring of 3-hydroxyanthranilic acid. 3-Acroleyl-3-amino fumarate may be decarboxylated to enter the catabolic

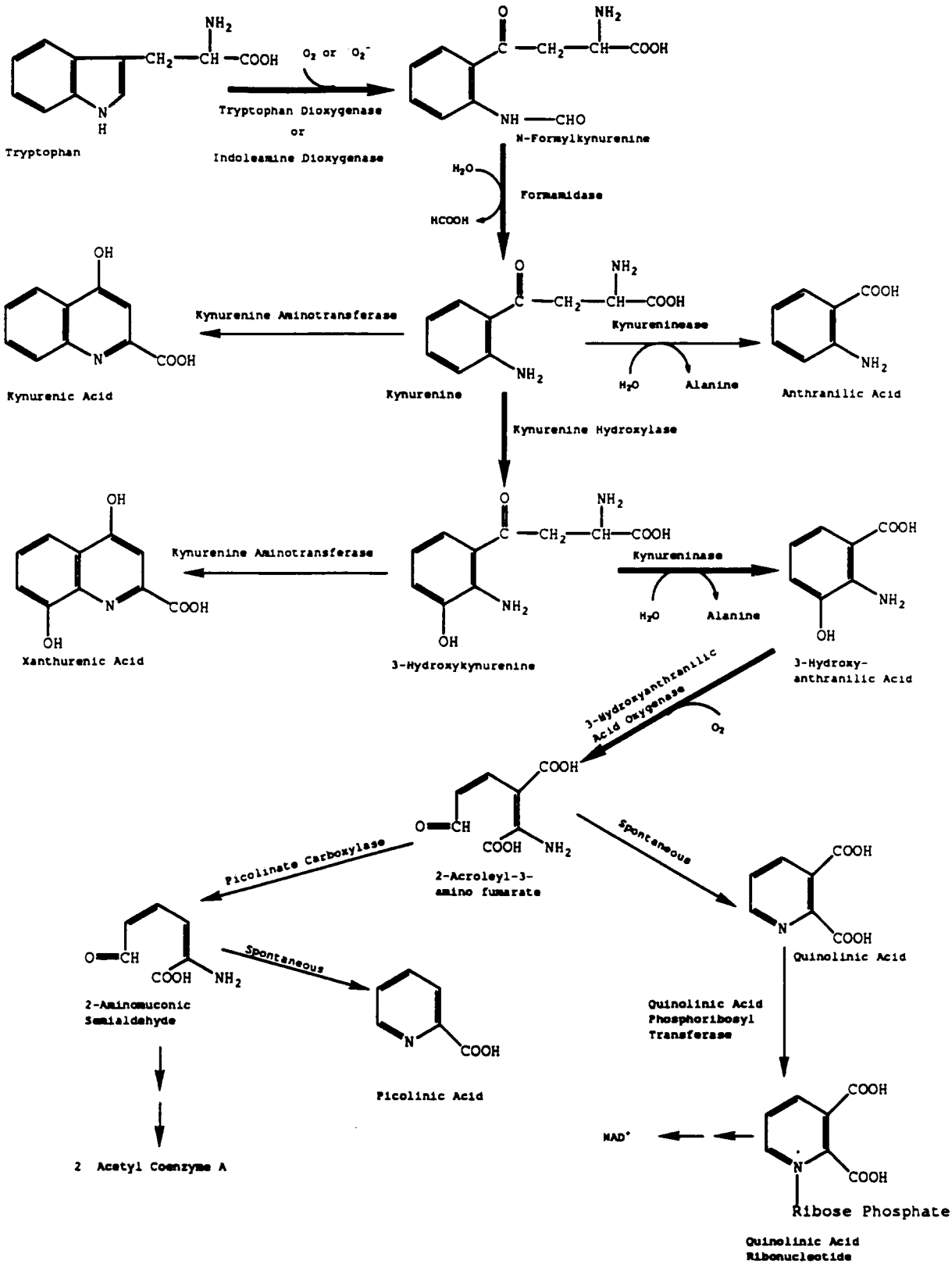


**Figure 3** The kynurenine pathway of tryptophan metabolism. Major fluxes are indicated with bold arrows.

branch of the pathway, or may spontaneously cyclize to form quinolinic acid, a pyridine nucleotide precursor.

Liver and kidney are undoubtedly the most quantitatively important sites of kynurenine pathway metabolism. Most components of the kynurenine pathway have been directly demonstrated or inferred to be present in the brain. Kynurenine,[73,74] kynurenic acid,[75] 3HK,[73] 3-hydroxyanthranilic acid,[76,77] and quinolinic acid[78] have all been measured in CNS tissue. The appearance of [$^{14}$C]-kynurenine and 3HK following intracerebroventricular (i.c.v.) administration of [$^{14}$C]-tryptophan strongly suggests that the catalytic activities associated with IDO, formamidase, and kynurenine hydroxylase must be available within the CNS.[73] Kynurenine hydroxylase activity has, in addition, been more directly assayed in mitochondrial preparations from mouse cerebral cortex.[79] The biosynthesis of quinolinic acid from its precursor, 3-hydroxanthranilic acid, has been demonstrated in the brain using in vivo microdialysis.[80] 3-hydroxyanthranilic acid oxygenase and quinolinic acid phosphoribotransferase, the enzymes catalyzing the synthesis and metabolism of quinolinic acid, have been demonstrated to be present in the brain using immunohistochemical techniques.[81] Kynureninase activity has also been detected in rat brains but its activity appears to be very low.[82] In accord with this finding, but in contrast to what is observed in liver, anthranilic acid has been shown to be a far better substrate than 3HK for the synthesis of 3-hydroxyanthranilic acid in CNS tissue.[76,77]

While the catalytic activities required for the biosynthesis of 3HK have been demonstrated in brain, it is likely that a substantial portion of the 3HK accumulated in vitamin B-6–deficient rat brain may be derived from peripheral metabolism of tryptophan. Plasma 3HK levels are also markedly elevated as a result of neonatal vitamin B-6 deficiency[17] and the enzymes of the kynurenine pathway are far more concentrated in liver and kidney than in the brain. On the basis of experiments in which the appearance of labeled kynurenine and 3HK was measured in CNS following systemic or i.c.v. injection of [$^{14}$C]-tryptophan, Gal and Sherman[73] estimated that about 40% of the CNS content of these metabolites is derived from local synthesis. Labeled kynurenine and 3HK also appear in CNS after systemic administration of [$^{14}$C]-kynurenine. The accumulation of kynurenine in brain after systemic administration has been confirmed by others.[83,84] Data from our laboratory using neonatal rats indicate that 3HK also accumulates in the brain after intraperitoneal injection *(Figure 4)*. Thus, it is evident that mechanisms exist for the transfer into CNS of kynurenine pathway intermediates released into the circulation from liver and kidney.

The control of kynurenine pathway metabolism has been investigated primarily in liver. The first enzyme in the pathway, TDO, is generally regarded to be rate limiting because, under basal conditions, its activity is lower than those of enzymes catalyzing downstream steps in the metabolism of tryptophan. It has been suggested, however, that following induction of TDO,

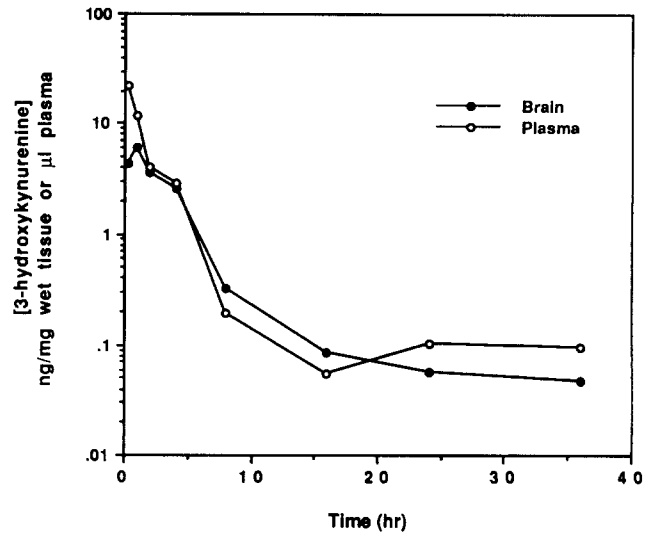


**Figure 4** Time course of accumulation of 3HK in rat brain following intraperitoneal administration. 3HK was measured by HPLC-EC in 0.1 N perchloric acid extracts from brain and plasma taken at the indicated intervals following intraperitoneal administration of 25 mg/kg 3-hydroxy-D,L-kynurenine to 14-day-old rat pups. Data represent means of measurements taken from three rats from different litters.

the activities of kynurenine hydroxylase and kynureninase may be no less than those of TDO, and that these downstream enzymes may exert significant control over flux through the pathway.[85] Thus, steady-state levels of kynurenine and 3HK might be expected to rise. This hypothesis has been directly evaluated using isolated hepatocytes.[86] Flux control coefficients (the proportion of control over a metabolic flux that can be attributed to an individual step) were determined for several catalytic activities in hepatocytes isolated from uninduced or dexamethasone-treated livers. Under basal conditions the control coefficient of TDO for flux through TDO was 0.75, while that for tryptophan into the hepatocytes was 0.25. In dexamethasone-induced hepatocytes, the control coefficient for transport had risen to 0.75, while that for TDO fell to 0.25. In either case, the control coefficients for kynurenine hydroxylase and kynureninase were negligible. In accord with these results, Takikawa et al.[87] found that plasma kynurenine levels were insensitive to induction of hepatic TDO activity with hydrocortisone.

It is evident from the abnormal excretion of tryptophan metabolites following a tryptophan load that kynurenine pathway metabolism is compromised as a result of vitamin B-6 deficiency. After administration of a loading dose of tryptophan, both human subjects[88] and rodents[89] excrete large quantities of kynurenine, 3HK, and xanthurenic acid in the urine, suggesting a restriction of metabolic flux through kynureninase. In hepatocytes isolated from vitamin B-6–deficient rats, the flux control coefficients for the control by kynureninase of fluxes through kynureninase and TDO were estimated as 0.42 and 0.28, respectively.[90] The difference between these coefficients suggests that a portion

of the flux through TDO does not pass through kynureninase. In view of the increased excretion of xanthurenic acid by vitamin B-6–deficient organisms and the increased levels of this metabolite accumulated in the incubation medium bathing vitamin B-6–deficient hepatocytes, it is likely that a portion of this diverted flux passes through kynurenine transaminase. This enzyme may also exert significant control over metabolic flux in vitamin B-6 deficiency.

Although flux through kynureninase is impaired in vitamin B-6–deficient hepatocytes, the impact of impaired flux on circulating levels of kynurenines in intact adult animals appears to be minimal in the absence of a tryptophan load. No appreciable difference was observed between vitamin B-6–deficient and –adequate rats with respect to the levels of kynurenine and 3HK measured in liver, kidney, and plasma prior to the administration of a tryptophan load.[91,92] Further, the basal levels of kynurenine and 3HK excretion in vitamin B-6–deficient rodents is not markedly increased relative to vitamin B-6–adequate controls.[89]

While systematic comparative investigations have not yet been reported, the results obtained in studies of neonatal rats would appear to be in marked contrast to what may be observed in adults. Levels of 3HK measured in plasma and CNS tissue from vitamin B-6–deficient rat pups were observed to be dramatically elevated in the absence of a tryptophan load.[17,48] In contrast, 3HK levels remained below the limits of detection in adult rats deprived of vitamin B-6 for 56 days.[17] This would suggest that the degree of control over metabolic flux exerted by kynurenine aminotransferase and/or kynureninase in vitamin B-6–deficient neonates is substantially greater than in adults.

The much greater accumulation of 3HK in neonatal, as compared with adult, vitamin B-6 deficiency must result from a relative imbalance of fluxes directed toward (through IDO and TDO) or away (through kynureninase and kynurenine aminotransferase) from 3HK. This could reflect a more severe deficiency induced by neonatal vitamin B-6 restriction protocols, the sequence of development of the various 3HK catabolic and biosynthetic activities, or other factors unique to infancy.

The possibility that the dietary protocols employed in the study of neonatal vitamin B-6 may induce a more severe deficiency state is difficult to evaluate on the basis of available data. In general, only CNS levels of vitamin B-6 are reported in connection with studies of neonatal vitamin B-6 deficiency, while these are of little interest to those examining parameters of vitamin B-6 deficiency in adults. Some comparative measurements of brain regional vitamin B-6 levels in control and vitamin B-6–deficient neonatal and adult rats[17] illustrate another difficulty in determining the relative severity of vitamin B-6 deficiency in adults versus neonates. While the degree of vitamin B-6 depletion evident in adult rats deprived of vitamin B-6 for 56 days is equivalent to that observed in vitamin B-6–deficient neonates when expressed as a percentage of age-matched controls, tissue levels of vitamin B-6 measured in vitamin B-6–deficient neonates were about one-third of those measured in vitamin B-6–deficient adults. In addition, it is possible that the elevation of 3HK could affect the phosphorylation and metabolic trapping of B-6 vitamers, because 3HK has been shown to inhibit pyridoxal kinase with an $IC_{50}$ of 100 μmol/L.[93]

The early postnatal development of the kynurenine pathway has not been extensively studied. However, developmental changes in several key enzymes have been reported in various organs. Hamon and Burgoin[94] have shown that synaptosomal uptake of tryptophan exhibits a transient increase, reaching a peak level 2–3 times that measured in adult brain prior to the third week of life. Rat hepatic TDO activity is undetectable during the first week of life.[95] Hepatic TDO synthesis begins approximately 10 days after birth, and TDO activity reaches adult levels by 22 days of age.[95] In addition, rat brain kynurenine hydroxylase activity was found to increase 10-fold during the second week of life, and the magnitude of this increase was augmented in pups rendered hypothyroid at birth.[79] Circulating levels of thyroid-stimulating hormone, thyroxine, and triiodothyronine have been shown to be reduced in vitamin B-6–deficient rat pups relative to controls.[96] Thus, it is likely that the robust early postnatal increase in kynurenine hydroxylase may be enhanced as a result of vitamin B-6 deficiency. In contrast, the kynureninase activity measured in rat liver and kidney increased only two-fold in the first 3 weeks of life.[82] While these data are clearly inadequate to provide a coherent view of the development of kynurenine pathway metabolism, they suggest that the activity of enzymes related to 3HK biosynthesis may increase more rapidly in early postnatal life than does kynureninase. In particular, it is of interest that the initiation of hepatic TDO biosynthetic activity appears to closely precede the elevation of 3HK and the onset of neurological signs in vitamin B-6–deficient neonatal rats.

An increase in precursor availability may also contribute to the abnormal elevation of 3HK in neonatal vitamin B-6 deficiency. CNS levels of tryptophan have been reported to be somewhat higher during the second and third weeks of life than in adults,[97] and CNS levels of tryptophan are further elevated as a result of vitamin B-6 deficiency.[63] Because the TDO holoenzyme is stabilized in the presence of tryptophan, hepatic kynurenine pathway metabolism may be augmented during neonatal vitamin B-6 deficiency both by mass action and by induction of TDO activity.

Finally, kynurenine aminotransferase activity may be more vulnerable to cofactor depletion in the developing organism than in adults. Its Kd for PLP is similar to that of kynureninase,[98] an enzyme known to be very sensitive to cofactor depletion. The observed resistance of kynurenine aminotransferase to cofactor depletion is thought to be due to its intramitochondrial localization.[88,99] However, it is possible that the diffusional barrier provided by the mitochondrial membranes may not provide the same degree of resistance to cofactor depletion in neonatal and adult organisms.

Rat brain mitochondria increases three-fold in the first 3 weeks of postnatal life,[100] and PLP levels within mitochondria formed in a vitamin B-6–poor environment may reflect ambient levels of PLP.

## Convulsant properties of kynurenines

The convulsant properties of several kynurenine pathway metabolites, including 3HK, have been demonstrated in a series of studies from the laboratory of Lapin.[24, 101-103] Intracerebroventricular injection of quinolinic acid (1 μg), kynurenic acid (5 μg), 3HK (10 μg), kynurenine (25 μg), and nicotinic, xanthurenic, and 3-hydroxyanthranilic acids (50 μg) were all reported to produce seizures in mice.[24] Kynurenine and 3HK were also reported to produce seizures following i.c.v. administration in rats.[70] While the $ED_{50}$ reported in rats for the production of seizures by 3HK and kynurenine were similar, the duration of 3HK-induced seizures was significantly longer than those induced following administration of kynurenine.[104] Consistent with observations of species differences in sensitivity toward the convulsant effects of kynurenines,[24,103] substantially higher doses of both metabolites were required to precipitate seizures in rats as compared with mice.

While data relating to the behavioral effects and neuroactive properties of 3HK are scarce, other kynurenine metabolites, such as quinolinic acid, kynurenic acid, and kynurenine in particular, have been more thoroughly examined. Quinolinic acid and kynurenic acid have been the focus of intense research interest because they act as agonist and antagonist, respectively, at the NMDA-preferring subtype of excitatory amino acid receptor.[105] Quinolinic acid has excitotoxic properties and the axon sparing lesions produced by its focal microinjection into striatum[106] mimic subtle neurochemical features of the lesions associated with Huntington's disease.[107,108] Investigation of the neuroactive properties of kynurenine has been largely restricted to the Soviet Union. This work, much of which is unavailable in the English-language scientific literature, has been detailed in a recent review.[109] In addition, related work published in Western journals has been more critically reviewed by Stone and Connick.[25]

Although the convulsant properties of kynurenine have been repeatedly demonstrated, the mechanistic bases of kynurenine-induced seizures are presently unknown. With the exception of quinolinic acid, none of the convulsant kynurenine metabolites that have been tested (including kynurenine and 3HK) have exhibited neuroexcitant activity when applied to neocortical neurons[110] or to hippocampal slices.[111] However, it was recently demonstrated that L-kynurenine, like glycine, may modulate NMDA receptor activity.[112] Studies of the interactions of quinolinic acid and kynurenine with various convulsants and anticonvulsants also suggest that these compounds induce seizures through different mechanisms. For example, kynurenine-induced seizures were antagonized by pretreatment with glycine[113] or taurine,[114] while the convulsant activity of quinolinic acid was unaffected or enhanced. Kynurenine, in contrast to quinolinic acid, was observed to potentiate strychnine-induced seizures.[115]

Because kynurenine appears to lack neuroexcitatory activity, several investigators have explored interactions with inhibitory systems. Pinelli et al.[104] reported that the binding of [$^3$H]-GABA to membranes prepared from rat brain is slightly more potently inhibited by L-kynurenine than by bicuculline. However, bicuculline is the more potent convulsant,[104] and the action of GABA as assessed electrophysiologically in hippocampal slices was unaffected in the presence of up to 5 mmol/L L-kynurenine.[116] Zarkovsky[117] examined the effect of kynurenine on [$^3$H]-flunitrazepam binding to the benzodiazepine receptor in rat brain membranes. L-kynurenine produced a stereoselective inhibition of pentobarbital-stimulated, but not basal, [$^3$H]-flunitrazepam binding that was attributable to a decrease in the affinity of the receptor for its benzodiazepine ligand. The effects of kynurenine metabolites on [$^3$H]-flunitrazepam binding have also been examined by Guilarte et al.[118] These studies showed that basal binding of [$^3$H]-flunitrazepam to rat brain membranes was inhibited by both L-kynurenine and 3HK with potencies similar to purines, which have been proposed as endogenous benzodiazepine receptor ligands. 3HK also inhibited the GABA enhancement of [$^3$H]-flunitrazepam binding with a calculated Ki in the range of 3HK concentrations measured in CNS tissue from vitamin B-6–deficient neonatal rats.

## Neurotoxic properties of 3-hydroxykynurenine

An evaluation of the neurotoxicity of 3HK was undertaken in our laboratory as a first step toward assessing its role in the neuropathological changes associated with vitamin B-6 deficiency. The poor solubility of 3HK in a wide range of suitable injection vehicles renders this compound a poor candidate for microinjection studies. Therefore, its toxicity was initially examined in an in vitro cell culture system. A mouse neuroblastoma × embryonic rat retinal neuron hybrid cell line (N18-RE-105) was selected for in vitro toxicity studies. This cell line is neuronally derived, expresses a neuron-like morphology, and is easily maintained in culture. Moreover, the homogeneous cell population provided by this clonal cell line is well suited to mechanistic studies.

The primary findings on the cytotoxicity of 3HK are that this kynurenine metabolite is toxic to a neuronal cell line,[119] that the accumulation of $H_2O_2$ in an intracellular compartment is critical to the observed toxicity,[120] and that mechanisms subserving the uptake of 3HK exist both in the N18-RE-105 cell line in which toxicity has been demonstrated and in the mammalian brain.[121] The in vitro cytotoxic effects of 3HK are evident at concentrations in the range of those that have been previously measured in CNS tissue from vitamin B-6–deficient neonatal rats. While it is likely that $H_2O_2$ is produced as a result of the oxidation of 3HK, it is not clear whether 3HK is oxidized intra-

cellularly or in the culture medium. However, experiments demonstrating the accumulation of [$^3$H]3HK[121] suggest that the uptake and subsequent intracellular oxidation of 3HK could contribute to the inferred cytosolic pool of $H_2O_2$.

The critical involvement of $H_2O_2$ in 3HK toxicity is indicated by comparison of the concentration dependence of $H_2O_2$ and 3HK-mediated cytotoxicity and by the protective effects of $H_2O_2$ scavengers.[119,120] Both $H_2O_2$ and 3HK kill N18-RE-105 cells with steep dose-response functions that reach maxima within 1 log unit. Because the cytolytic potency of $H_2O_2$ was determined to be one to two orders of magnitude greater than that of 3HK, the oxidation of a fraction of the toxic concentrations of 3HK could account for the observed toxicity. The observation that 3HK-induced cell lysis is abolished both in the presence of catalase and following pretreatment of cell cultures with horseradish peroxidase provides further support for the participation of $H_2O_2$ in 3HK toxicity.[120] In contrast, scavengers of other reactive oxygen species had no effect on 3HK-induced cell lysis. Superoxide dismutase and the hydroxyl radical scavenger, mannitol, both failed to modify 3HK toxicity, and superoxide dismutase + catalase was no more effective than catalase alone. Thus, superoxide and hydroxyl radicals either play no significant role in the observed cytotoxic effects of 3HK, or are produced in a cellular compartment inaccessible to exogenously added superoxide dismutase or mannitol.

The data also indicate that 3HK is potentiated in the presence of ascorbate[122] and inhibited by the iron chelator, desferrioxamine.[120] The toxicity of the autoxidizable neurotoxin, 6-hydroxydopamine, is similarly potentiated by ascorbate,[123,124] and the potentiation has been attributed to the reductive recycling of the quinone oxidation product in the presence of ascorbate.[125] $H_2O_2$ generation is markedly enhanced and quinone accumulation suppressed when dopamine or 6-hydroxydopamine is incubated in the presence of ascorbate.[125,126] The protective effect of desferrioxamine suggests a role for iron in 3HK toxicity: either in catalyzing the oxidation of 3HK or in promoting the reduction of $H_2O_2$ to the highly reactive hydroxyl radical. Alternatively, desferrioxamine may function as an hydroxyl radical scavenger.[127,128] The millimolar concentrations of desferrioxamine required to attenuate the in vitro toxicity of dopamine, dihydroxyphenylalanine,[129] and 3HK[120] seem rather high in view of the very high affinity of this chelator for iron,[130] but are in the range in which desferrioxamine has been demonstrated to act as an effective hydroxyl radical scavenger.[128] On the other hand, the cellular uptake of desferrioxamine is thought to be mediated by fluid phase pinocytosis,[131] and high extracellular concentrations of desferrioxamine may be required to attain intracellular levels sufficient to scavenge intracellular iron.

Results obtained in the study of 3HK cytotoxicity bear sufficient resemblance to data from investigations of the toxicity of catecholamines and related compounds to suggest that the underlying mechanisms of toxicity may be very similar. Dopamine,[132,133] its 6-hydroxy-[134,135] and 6-amino-[136] analogues, and norepinephrine[137] have all been shown to autoxidize and to exhibit cytotoxic properties in various in vitro systems. Like 3HK, the dose response curves for toxicity are often reported to be steep,[134,137,138] although this may be somewhat dependent on target cell type.[124,139] 6-hydroxydopamine has, in addition, been widely employed as a dopaminergic neurotoxin in vivo[140]; and the oxidative lability of dopamine has been proposed as having a role in the etiology of Parkinson's disease.[141,142]

noted that most of the neurochemical and neuroanatomical alterations measured in vitamin B-6 deficiency are preceded by the derangement of tryptophan metabolism.

hydroquinone all were observed to have no effect on the number of C-1300 neuroblastoma cells surviving for 24 hours after a 1-hour exposure to 6-hydroxydopamine.[139] The inability of superoxide dismutase to modify 6-hydroxydopamine toxicity has been confirmed in several investigations.[137,138]

The uptake of 3HK was demonstrated in the neuronal cell line in which its toxicity has been demonstrated, and it was more fully characterized in rat brain slice preparations.[121] Like the accumulation of kynurenine in rat brain slices,[143] the uptake of 3HK could be resolved into two components on the basis of the requirement for sodium. A brain regional analysis of the two transport components revealed a moderate degree of heterogeneity in the regional distribution of both transport processes. In addition, these data strongly suggest that the two processes are independently distributed. Both transport components were sensitive to temperature and to metabolic inhibitors. Sodium-dependent 3HK transport was virtually abolished in the presence of ouabain, cyanide, and azide. In contrast, the sodium-independent process was unaffected by ouabain and only moderately, but significantly, suppressed in the presence of azide and cyanide.

The studies in our laboratory have demonstrated the in vitro cytotoxicity of 3HK and have shown that at least two systems subserving the intracellular accumulation of 3HK exist in mammalian brain. While the in vitro cytotoxicity of 3HK is evident at concentrations similar to those measured in CNS tissue from vitamin B-6–deficient neonatal rats, its capacity to damage CNS tissue in vivo remains to be demonstrated. Studies are currently underway to test this possibility. On the other hand, the in vivo neurotoxicity of another autoxidizable aminophenolic kynurenine pathway metabolite, 3-hydroxyanthranilic acid, has recently been described.[144] Moreover, the conditions of CNS exposure to 3HK in neonatal vitamin B-6 deficiency may be more severe than those employed in the in vitro studies. Although the time course of 3HK elevation has not been fully delineated, it is likely that CNS levels of 3HK may be substantially elevated for a

period of several days,[17] to a week, or more. Several studies have indicated that the activities of the major antioxidant enzyme systems are relatively low in the developing CNS[145,146] and there is recent evidence that vitamin B-6 deficiency may have a "pro-oxidant" effect independent of any elevation of 3HK. Both basal and induced lipid peroxidation was increased in livers from vitamin B-6–deficient rats relative to pair-fed controls.[147] In addition, the activities of catalase and glutathione peroxidase were reduced in vitamin B-6–deficient liver relative to controls.[147]

The relationship between elevation of CNS levels of 3HK and the observed neuropathological sequelae of neonatal vitamin B-6 deficiency remains to be determined. Groziak and Kirksey[41] have reported that maternal dietary vitamin B-6 restriction confined to the gestational period produced neocortical pathology indistinguishable from that produced by a dietary restriction imposed throughout gestation and lactation. These data are difficult to reconcile with previous reports indicating that vitamin B-6 status is adequately maintained in utero.[37] Nonetheless, because 3HK levels are unlikely to rise substantially in pups receiving adequate vitamin B-6 nutrition during the first weeks of postnatal life, these data are inconsistent with a causative role for 3HK in the observed neocortical pathology.

Neuropathological changes, including a decrease in synaptic density, have also been described in striata from vitamin B-6–deficient neonatal rats.[47] In accord with these data, Guilarte reported a deficiency-induced decrease in striatal dopamine levels. In contrast to other deficiency related neurochemical changes,[42] the deficit in striatal dopamine developed after the second week of life and increased with age.[36] Thus, the onset of the dopaminergic deficit appears to occur approximately coincidently with the elevation of CNS levels of 3HK. In view of the hypothesized vulnerability of dopaminergic neurons to oxidative injury,[141,142] the possible involvement of 3HK in dopaminergic terminal degeneration merits further study.

## Summary

Dietary intakes of vitamin B-6 have been reported to be significantly lower than the RDA in large segments of the U.S. population, and they are particularly low in women of childbearing age. This nutrient is essential for the normal function and development of the immature CNS, and inadequate maternal intake, even at levels sufficient to support maternal health, may have grave consequences for the suckling young. In human infants and in neonates of several species, vitamin B-6 deficiency results in ataxia, tremor, and seizures that subside upon administration of vitamin B-6. In addition to these striking acute neurological signs, marginal intakes of vitamin B-6 during early development may produce subtle but persistent or permanent changes in CNS function, some of which may not be currently known. For example, a fundamental question may be whether learning capacity is affected by a nutritional inadequacy of vitamin B-6 in early life. The recent evidence that neonatal vitamin B-6 deficiency results in a decreased level of glutamate release[50] and in the activation of the NMDA glutamate receptor subtype[51] may have important implications for learning and memory processes in these animals. The activation of the NMDA receptor in early development plays an important role in brain plasticity.

Despite increasing knowledge of the neurochemical alterations associated with neonatal vitamin B-6 deficiency, the mechanistic bases of both the acute and long-term behavioral changes, as well as the observed alterations in CNS morphology, remain poorly understood. The identification of vitamin B-6 deficiency-induced increases in the levels of the putative endogenous neurotoxin and convulsant, 3HK, provides a starting point from which a working hypothesis on the biochemical bases of the neuropathological changes observed can be tested. In this context, it should be noted that most of the neurochemical and neuroanatomical alterations measured in vitamin B-6 deficiency are preceded by the derangement of tryptophan metabolism.

The convulsions that comprise the most striking feature of neonatal vitamin B-6 deficiency have been widely attributed to the decrease in GABA levels, although the evidence for this is largely circumstantial.[148] While it is likely that the deficit in GABAergic neurotransmission plays an important role in the deficiency-induced seizure disorder, several other factors may contribute to seizures. Taurine, which has been shown to have anticonvulsant properties,[149] is also decreased in CNS tissue from B-6–deficient neonatal rats.[42] In addition, the observed increases in glycine and 3HK may also tend to promote seizures. Glycine has been shown to potentiate the effects of agonists at the NMDA-preferring excitatory amino acid receptor, and 3HK has been shown to induce convulsions after i.c.v. administration. Although the precise role of 3HK in vitamin B-6 deficiency-induced seizures is not presently known, recent studies in children with infantile spasms have shown significantly higher levels of cerebrospinal fluid 3HK relative to normal controls.[150] This is especially important because recent reports have described the successful treatment of infantile spasms with vitamin B-6.[151,152]

Persistent or permanent alterations of CNS function are suggested by studies of locomotor behavior, dopaminergic neurochemistry, and CNS morphology. Vitamin B-6–deficient neonatal rats exhibit a pattern of hypoactivity on measures of spontaneous locomotor activity prior to weaning, and increasing hyperactivity after weaning.[21] Dopamine levels measured in striata from control and deficient rats are similar at 14 days of age, but differ increasingly at 28 and 56 days of age. Thus, striatal dopamine levels measured in tissue from 56-day-old vitamin B-6–deficient rats were less than 70% of those measured in striata from control rats. While it has not been demonstrated whether either the progressive hyperactivity or the increasing deficit of striatal dopamine is reversible upon vitamin B-6 sup-

plementation, histological data suggest that such changes may have morphological substrates. Decreases in dendritic arborization and synaptic density have been demonstrated in both cortices and striata from vitamin B-6–deficient neonatal rats.

In rats, the second and third weeks of life appear to represent a critical period of development, at least with respect to vulnerability to inadequate vitamin B-6 nutrition. This period of development in the rat brain approximates the maturational level of a term human neonate. Overt neurological signs of neonatal vitamin B-6 deficiency, including seizures and abnormalities in locomotion, are commonly reported to develop between 10 and 18 days postnatally. The time of onset of neurological signs does not appear to be strongly influenced by the duration or severity of maternal vitamin B-6 restriction, and neurological impairment does not commonly result from vitamin B-6 deprivation imposed after weaning. In addition, it is during this period that most of the deficiency-induced alterations in CNS neurochemistry appear most pronounced.

One of the most striking neurochemical changes occurring in the second and third weeks of life is the dramatic elevation of CNS levels of 3HK. Elevation of 3HK was not observed in control pups, or in adult rats deprived of dietary vitamin B-6 for 8 weeks. This metabolite is normally present in rat brain at levels below 1 nmol/gm tissue. During the second week of life, 3HK increases, reaching concentrations in excess of 150 nmol/gm in CNS tissue from vitamin B-6–deficient rat pups at 18 days of age. This elevation of CNS levels of 3HK begins approximately coincidently with the onset of overt neurological signs, and appears to precede a selective depletion of dopamine in vitamin B-6–deficient striatum.

The persistent depletion of striatal dopamine and the neuropathological data indicating decreased neuronal longevity are consistent with an oxidative insult of the type indicated by the in vitro studies on the cytotoxicity of 3HK. The involvement of an "oxidative stress" type of neuronal damage resulting from the generation of $H_2O_2$ due to 3HK autoxidation may be operational in neonatal vitamin B-6 deficiency. Increased oxidative stress in vitamin B-6–deficient animals with decreased levels of enzymes essential for the maintenance of oxidative homeostasis may result in early neuronal aging and death.

The long-term effects of marginal dietary intakes of vitamin B-6 during development are still essentially unknown. However, as this review suggests, inadequate intakes of vitamin B-6 during neuronal development results in the improper functioning of a number of enzymes essential for the synthesis of basic components of cells, neurotransmitters, and for the removal of potentially toxic endogenous metabolites. The association of changes in synaptic neurochemistry with behavior resulting from long-term intakes of vitamin B-6–inadequate diets is one of the challenges for the next generation of nutritional biochemists in order to understand the relationship between diet and brain function.

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