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## Pineal indoleamine metabolism in pyridoxine-deficient rats

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Pyridoxine deficiency causes physiologically significant decrease in brain serotonin (5-HT) due to decreased decarboxylation of 5-hydroxytryptophan (5-HTP). We have examined the effect of pyridoxine deficiency on indoleamine metabolism in the pineal gland, a tissue with high indoleamine turnover. Adult male Sprague-Dawley rats were fed either a pyridoxine-supplemented or pyridoxine-deficient diet for 8 weeks. Pyridoxine deficiency did not alter the pattern of circadian rhythm of pineal 5-HT. 5-hydroxyindoleacetic acid (5-HIAA), *N*-acetylserotonin (NAS), and melatonin. However the levels of these compounds were significantly lower in the pineal glands of pyridoxine-deficient animals. Pineal 5-HTP levels were consistently higher in the pyridoxine-deficient animals and a conspicuous increase was noticed at 22.00 h. Increase in pineal NAS and melatonin levels caused by isoproterenol (5 mg/kg at 17.00 h) were significantly lower ( $P < 0.05$ ) in the pyridoxine-deficient animals. Treatment of pyridoxine-deficient rats with pyridoxine restored the levels of pineal 5-HT, 5-HIAA, NAS, and melatonin to values seen in pyridoxine-supplemented control animals. These results suggest that 5-HT availability could be an important factor in the regulation of the synthesis of pineal NAS and melatonin.

### INTRODUCTION

The synthesis of the neurotransmitters dopamine and serotonin involves a pyridoxal phosphate-dependent decarboxylation step. Pyridoxine deficiency in young rats has been shown to cause physiologically significant decrease in serotonin (5-HT) in various brain regions without altering the concentrations of dopamine and norepinephrine at these sites<sup>9,19</sup>. Such a decrease in brain 5-HT is due to decreased decarboxylation of 5-hydroxytryptophan (5-HTP)<sup>7,23</sup>.

The highest concentration of 5-HT in the body is in the pineal gland<sup>20</sup> where 5-HT is converted to *N*-acetylserotonin (NAS) by the enzyme *N*-acetyltransferase (NAT)<sup>27</sup>. *N*-Acetylserotonin is converted to melatonin, a pineal hormone, by hydroxyindole-*O*-methyltransferase<sup>2</sup>. The rate limiting step in the pathway of melatonin synthesis is believed to be the production of NAS<sup>16</sup>. However, under certain conditions, a decrease in the formation of 5-HT could affect melatonin synthesis<sup>1,3,13,18</sup>.

5-Hydroxytryptophan decarboxylase activity in the pineal is very high<sup>24</sup> and requires pyridoxal 5-

phosphate as a cofactor<sup>12</sup>. Since pyridoxine deficiency decreases decarboxylation of 5-HTP in other brain areas<sup>23</sup>, a similar or even more enhanced effect could be expected in the pineal gland because of the high indoleamine turnover in this tissue. We have examined the effect of a moderate deficiency of pyridoxine on indoleamine metabolism in the pineal gland of adult male rats.

### MATERIALS AND METHODS

Adult male Sprague-Dawley rats (150-160 g) were fed either a pyridoxine-supplemented or pyridoxine-deficient diet<sup>8</sup>. Experiments were performed after maintaining the animals on these diets for 8 weeks at which time moderate deficiency of pyridoxine was seen in rats fed the pyridoxine-deficient diet<sup>10</sup>. The animals were exposed to light:dark cycles of 14:10 h. Lights were off from 18.00 to 04.00 h.

#### *Circadian rhythm of indoleamines*

In order to study the effect of pyridoxine deficiency on the circadian rhythm of indoleamines in the

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pineal gland, groups of rats ( $n = 5$ ) were killed at 06.00, 12.00, 18.00, 22.00 and 02.00 h. A dim red light was used when animals were killed in the dark. The pineal glands were removed, frozen, and stored at  $-70^{\circ}\text{C}$  for later analysis of indoleamines.

#### *Effect of isoproterenol administration*

The purpose of this experiment was to test whether the increase in NAS and melatonin synthesis induced by  $\beta$ -adrenergic stimulation was affected by pyridoxine deficiency. Groups of animals ( $n = 6$ ) were given intraperitoneal injections of either isoproterenol bitartrate (Sigma) dissolved in saline (5 mg/kg) or saline alone. The injections were given one hour before the onset of the dark period<sup>14</sup>, a time when the maximum response in pineal NAS and melatonin synthesis could be expected<sup>21,22</sup>. The animals were maintained in the light for 2 h after the injection and then killed. Their pineal glands were removed and kept frozen until assayed for indoleamines.

#### *Effect of pyridoxine administration*

In order to test whether pyridoxine administration could restore the altered indoleamine levels seen in pyridoxine-deficient rats, pyridoxine hydrochloride (Sigma) was injected intraperitoneally into pyridoxine-deficient rats at a dose of 10 mg/kg on two consecutive days at 15.00 h. Other deficient animals received saline at this time. After the second injection, groups of animals ( $n = 6$ ) were killed at 22.00 h and at 12.00 h. Pineal glands were removed and frozen for analysis.

#### *Pineal NAT and 5-HTP decarboxylase activities*

Pineal NAT activity was assayed in pyridoxine-deficient and control rats ( $n = 6$ ) killed in the dark at 22.00 h, when high NAT activity could be expected. Groups of pyridoxine-deficient and control animals ( $n = 6$ ) were also killed at 15.00 h for the measurement of 5-HTP decarboxylase activity in the pineal gland. The activity of this enzyme, in contrast to that of NAT, does not exhibit circadian variation<sup>25</sup>.

#### *Assays and statistical analysis*

Pineal 5-HTP, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), and NAS were measured using HPLC with electrochemical detection according to Kilpa-

trick et al.<sup>15</sup>. The glands were sonicated in 200  $\mu\text{l}$  of cold 0.1 M perchloric acid and centrifuged. The clear supernatant (20  $\mu\text{l}$ ) was injected into a liquid chromatograph consisting of Altex 210 sample injector, Beckman 100A pump, HR 80 column ( $4.6 \times 75$  mm, 5  $\mu\text{m}$  C18; ESA, Bedford, MA), ESA Coulochem controller (model 5100A) with a conditioning cell (model 5021; set at +0.05 V) and an analytical cell (model 5011; applied potential at detector 1 was 0 V and at detector 2 was +0.4 V). The mobile phase, 0.09 M sodium acetate, 0.035 M citric acid, 0.13 mM EDTA, 0.23 mM sodium octyl sulphate, and 10.5% methanol (pH 4.35), was pumped at a flow rate of 1 ml/min. The peaks were identified by relative retention times compared to standards and concentrations were determined by comparing peak areas using an integrator (Hewlett Packard: Model 3390A) interfaced with the detector.

Melatonin was determined by radioimmunoassay according to the method described by Brown et al.<sup>4</sup>. Ultraspecific rabbit anti-melatonin serum (CIDtech Res. Inc., Mississauga, Ontario, Canada) and [<sup>3</sup>H]melatonin (79 Ci/mmol; Amersham) were used in the assay. The separation of bound [<sup>3</sup>H]melatonin was done according to Viswanathan et al.<sup>26</sup>.

Pineal NAT activity was measured using the radioenzymatic method described by Champney et al.<sup>9</sup> which involves the acetylation of tryptamine with acetyl-[1-<sup>14</sup>C]-coenzyme A (54 mCi/mmol; Amersham)<sup>11</sup>. Pineal glands were sonicated in 100  $\mu\text{l}$  of 0.05 M phosphate buffer (pH 6.8) and 10  $\mu\text{l}$  aliquots were used for the determination of NAT activity.

Pineal 5-HTP decarboxylase activity was assayed as described previously<sup>23</sup>, except that 0.4  $\mu\text{Ci}$  of D,L-5-hydroxy-[G-<sup>3</sup>H]tryptophan (5.3 Ci/mmol; Amersham) was used in place of D,L-5-[3-<sup>14</sup>C]hydroxytryptophan as substrate. Pineal glands were sonicated in 200  $\mu\text{l}$  of 0.32 M sucrose and 40  $\mu\text{l}$  aliquots were used for the decarboxylase assay. The incubation medium also contained 75 mM Tris-HCl (pH 8.3), 0.3 mM pyridoxal 5'-phosphate, 10 mM  $\beta$ -mercaptoethanol and 0.1 mM pargyline hydrochloride. The reaction was performed at 37  $^{\circ}\text{C}$  for 1 h and the radioactivity of the product serotonin, separated from 5-HTP by solvent partition using 1-butanol, was determined.

Data were analysed using Student's unpaired *t*-test or one-way analysis of variance followed by Newman-Keuls multiple range test.

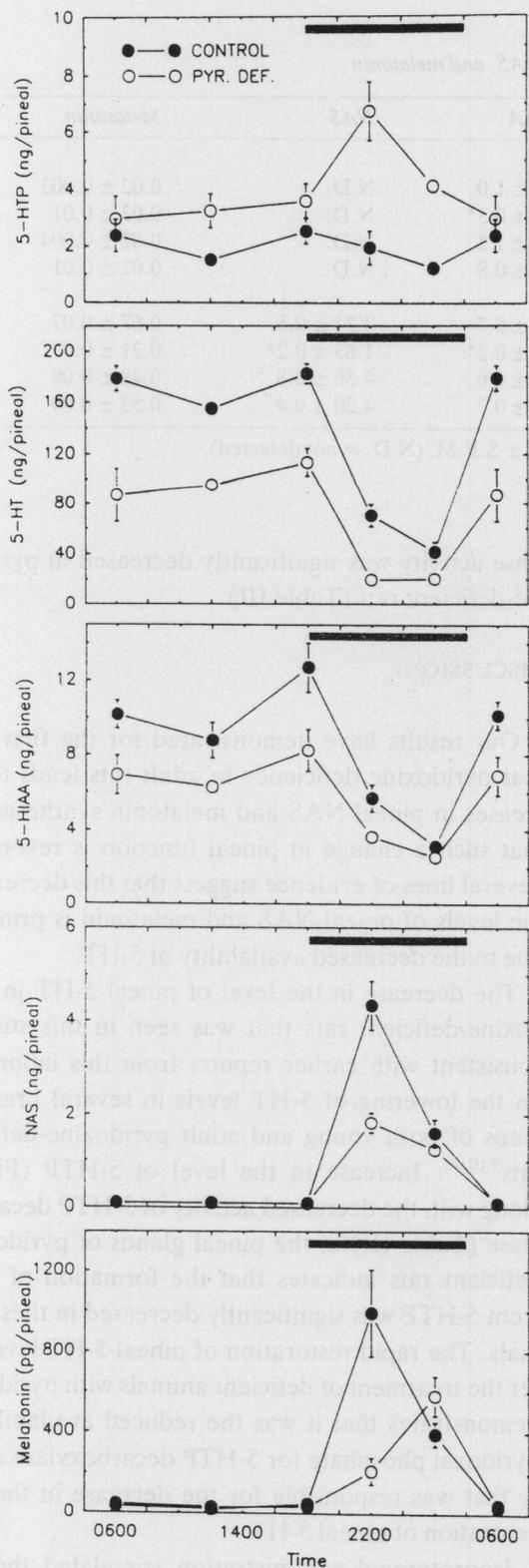


Fig. 1. Effect of pyridoxine deficiency on the circadian variation in rat pineal 5-HTP, 5-HT, 5-HIAA, NAS, and melatonin. Black bar indicates duration of darkness. Values are means  $\pm$  S.E.M.

## RESULTS

### Circadian rhythm of indoleamines

The circadian rhythm of pineal indoleamines in control animals was similar to that reported earlier<sup>17,29</sup>. Pineal melatonin and NAS showed significant circadian variation, in both pyridoxine-deficient and control animals, and their rhythms were not affected by pyridoxine deficiency (Fig. 1). However, the peak nighttime levels of pineal melatonin and NAS were significantly lower ( $P < 0.05$ ) in pyridoxine-deficient animals. Significant variations were also seen in pineal contents of 5-HT and 5-HIAA levels in both pyridoxine-deficient and control animals over the 24 h observation period. The rhythm of these compounds was not affected by pyridoxine deficiency. Pineal 5-HT and 5-HIAA levels were significantly lower ( $P < 0.05$ ) in pyridoxine-deficient animals throughout the 24 h period except for 5-HIAA levels at 02.00 h. 5-HIAA levels paralleled the 24 h rhythm in pineal 5-HT levels in both pyridoxine-deficient and control rats. Significant circadian variation in pineal 5-HTP levels was seen only in pyridoxine-deficient animals. In these animals significantly higher levels were maintained at 12.00 h ( $P < 0.05$ ), 22.00 h ( $P < 0.01$ ), and 02.00 h ( $P < 0.001$ ).

### Effect of isoproterenol administration

Isoproterenol administration stimulated the synthesis of pineal NAS and melatonin in both control and pyridoxine-deficient animals (Table I). However, the increase in NAS and melatonin were signifi-

TABLE I

Effect of isoproterenol administration on the levels of pineal 5-HT, NAS, and melatonin

	5-HT	NAS	Melatonin
Control + saline	255.63 $\pm$ 21.5	N.D.	0.06 $\pm$ 0.01
Pyridoxine-deficient	98.65 $\pm$ 12.9	N.D.	0.05 $\pm$ 0.01
Control + isoproterenol	142.92 $\pm$ 11.8*	5.70 $\pm$ 0.8	0.65 $\pm$ 0.13*
Pyridoxine-deficient + isoproterenol	98.76 $\pm$ 11.3**	3.51 $\pm$ 0.5**	0.44 $\pm$ 0.08**

\*  $P < 0.05$  compared to saline injected control; \*\*  $P < 0.05$  compared to isoproterenol injected control rats. Values in ng/pineal are mean  $\pm$  S.E.M.; N.D., not detected.

TABLE II

Effect of pyridoxine administration on pineal content of 5-HT, 5-HIAA, NAS, and melatonin

	5-HT	5-HIAA	NAS	Melatonin
Day (12.00 h)				
Control	155.08 ± 5.0	8.77 ± 1.0	N.D.	0.02 ± 0.003
Pyridoxine-deficient	95.17 ± 6.7*	6.25 ± 0.3*	N.D.	0.03 ± 0.01
Control + pyridoxine	164.54 ± 5.8	12.47 ± 1.2	N.D.	0.02 ± 0.004
Pyridoxine-deficient + pyridoxine	169.95 ± 3.0	13.12 ± 0.8	N.D.	0.02 ± 0.01
Night (22.00 h)				
Control	70.33 ± 9.0	5.69 ± 0.7	4.34 ± 0.5	0.67 ± 0.07
Pyridoxine-deficient	19.61 ± 1.7*	3.65 ± 0.2*	1.83 ± 0.2*	0.21 ± 0.07*
Control + pyridoxine	55.21 ± 9.8	5.89 ± 0.6	4.56 ± 0.8	0.48 ± 0.09
Pyridoxine-deficient + pyridoxine	76.52 ± 15.3	5.95 ± 0.7	4.20 ± 0.4	0.53 ± 0.09

\* $P < 0.05$  compared to the control animals. Values in ng/pineal are mean ± S.E.M. (N.D. = not detected).

cantly lower ( $P < 0.05$ ) in pyridoxine-deficient rats. Pineal 5-HT levels decreased significantly in isoproterenol-treated control rats. Pyridoxine-deficient animals had significantly decreased levels of pineal 5-HT when compared to saline-treated control animals. Unlike in control animals the pyridoxine-deficient animals maintained the lower pineal 5-HT level after isoproterenol administration.

#### Effect of pyridoxine administration

Treatment of pyridoxine-deficient rats with pyridoxine restored the levels of pineal 5-HT, 5-HIAA, NAS, and melatonin to values seen in pyridoxine-supplemented control animals (Table II). Such a reversal of the effect of pyridoxine deficiency was evident both during the day (12.00 h) and night (22.00 h).

#### Pineal NAT and 5-HTP decarboxylase activities

There was no significant difference in pineal NAT activity between control and pyridoxine-deficient animals (Table III). However, pineal 5-HTP decarboxylase activity was significantly decreased in pyridoxine-deficient rats (Table III).

TABLE III

Effect of pyridoxine deficiency on rat pineal 5-HTP decarboxylase and NAT activities

Values represent mean ± S.E.M. ( $n = 6$ ). Enzyme activities are expressed in nmol/pineal/h.

	5-HTP decarboxylase activity	NAT activity
Control	19.37 ± 0.79	12.80 ± 5.2
Pyridoxine-deficient	6.88 ± 0.77*	13.04 ± 3.1

\* $P < 0.001$  compared to control by Student's *t*-test.

ylase activity was significantly decreased in pyridoxine-deficient rats (Table III).

#### DISCUSSION

Our results have demonstrated for the first time that pyridoxine deficiency in adult rats leads to decreases in pineal NAS and melatonin syntheses and that such a change in pineal function is reversible. Several lines of evidence suggest that this decrease in the levels of pineal NAS and melatonin is primarily due to the decreased availability of 5-HT.

The decrease in the level of pineal 5-HT in pyridoxine-deficient rats that was seen in this study is consistent with earlier reports from this laboratory on the lowering of 5-HT levels in several brain regions of both young and adult pyridoxine-deficient rats<sup>9,10,19</sup>. Increase in the level of 5-HTP (Fig. 1) along with the decreased activity of 5-HTP decarboxylase (Table III) in the pineal glands of pyridoxine-deficient rats indicates that the formation of 5-HT from 5-HTP was significantly decreased in these animals. The rapid restoration of pineal 5-HT levels after the treatment of deficient animals with pyridoxine demonstrates that it was the reduced availability of pyridoxal phosphate for 5-HTP decarboxylase activity that was responsible for the decrease in the concentration of pineal 5-HT.

Isoproterenol administration stimulated the synthesis of pineal NAS and melatonin in both control and pyridoxine-deficient rats. This finding indicates that  $\beta$ -adrenergic receptor-mediated regulation of melatonin synthesis was not affected by pyridoxine

deficiency. However, isoproterenol-induced increases in the levels of NAS and melatonin were significantly lower in pyridoxine-deficient rats. The lower pineal 5-HT levels in the pyridoxine-deficient animals could account for such a response. The conversion of pineal 5-HT to NAS and melatonin was reflected in the reciprocal lowering of the level of 5-HT in control animals. This finding is similar to that of Brownstein et al.<sup>5</sup> who showed that pineal 5-HT levels are regulated by  $\beta$ -adrenergic receptors. In the present study, we see that pyridoxine-deficient animals, unlike the control animals, maintain the level of 5-HT in the pineal gland after isoproterenol administration, although significant conversion of 5-HT to NAS and melatonin had occurred in these animals (Table I). Isoproterenol administration also stimulates the activity of pineal 5-HTP decarboxylase (unpublished observation). This could explain the maintenance of pineal 5-HT levels even after isoproterenol-stimulated synthesis of NAS and melatonin. Whether the isoproterenol-induced stimulation in the activity of pineal 5-HTP decarboxylase was mediated by  $\beta$ -adrenergic receptors or was caused by the isoproterenol-induced decrease in pineal 5-HT concentration<sup>25</sup> is not known.

The acetylation of 5-HT to NAS is considered to be the rate limiting step in the pathway of melatonin synthesis<sup>16</sup>. However, it has been suggested that the availability of 5-HT may be involved in the regulation of pineal indoleamine rhythms<sup>28</sup>. Several studies have revealed that administration of drugs which lower the level of 5-HT can reduce pineal melatonin synthesis.

In vivo administration of benzerazide, an inhibitor of aromatic amino acid decarboxylase, significantly reduced pineal 5-HT and melatonin levels without altering pineal NAT activity<sup>1,13</sup>. There is also evidence that monofluoromethyl dopa, another potent inhibitor of aromatic amino acid decarboxylase, is capable of reducing the synthesis of pineal 5-HT and consequently the level of nighttime serum melatonin<sup>3</sup>. Niles et al.<sup>18</sup> demonstrated that depletion of 5-HT by *p*-chlorophenylalanine depressed pineal melatonin levels. In the present study, pyridoxine deficiency caused significant decrease in pineal 5-HTP decarboxylase activity with concomitant decreases in NAS and melatonin concentrations. At the same time, activity of pineal NAT was not altered due to pyridoxine deficiency.

In summary, our findings demonstrate that pyridoxine deficiency in adult rats causes significant decreases in pineal NAS and melatonin syntheses due to decreased decarboxylation of 5-HTP. These results therefore suggest that 5-HT availability, in addition to other known factors, could be important in the regulation of the synthesis of pineal NAS and melatonin. The impairment in pineal function caused by pyridoxine deficiency may have pathophysiological consequences.

#### ACKNOWLEDGEMENTS

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