

## Folate Deficiency Alters Melatonin Secretion in Rats<sup>1,2</sup>

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**ABSTRACT** The final step of melatonin (MLT) synthesis is methylation of *N*-acetyl-serotonin, with *S*-adenosyl-methionine as a methyl donor provided by a metabolic pathway involving sulfur-containing amino acids (homocysteine and methionine). Remethylation of homocysteine to methionine requires folate. The present study was undertaken to test the influence of folate deficiency on MLT secretion. Severe folate deficiency was induced in rats by feeding them a synthetic diet containing (per kg diet) 0 mg folate and 10 g succinylsulfathiazole. Control rats were fed the same diet containing 8 mg folate/kg. After 4 wk, erythrocyte folate concentrations were significantly lower and plasma homocysteine levels were greater in folate-deficient rats than in controls. Pineal MLT concentration and urinary excretions of MLT, 6 sulfatoxymelatonin (the main hepatic MLT metabolite) and methoxylated catechol compounds were lower in the folate-deficient group than in the controls, whereas plasma catecholamine concentrations did not differ. Decreases generally were more marked at wk 2 than at wk 4 for the urinary metabolite excretions. These findings indicate that folate deficiency dramatically alters MLT secretion in rats. *J. Nutr.* 132: 2781–2784, 2002.

**KEY WORDS:** • melatonin • folate • deficiency • rats

Melatonin (MLT)<sup>4</sup> is an indole hormone, which acts as an internal synchronizer for the timing of daily events and is of promise for treatment of circadian rhythm disturbances (1). MLT synthesis in the pineal gland undergoes large daily changes, with a peak occurring at night. This day/night rhythm of MLT synthesis is generated at the serotonin acetylation step by a large nighttime increase in the activity of a specific aryl-alkylamine-*N*-acetyltransferase (NAT, EC 2.3.1.87). The activation of NAT depends on the activity of the sympathetic nerves originating from the superior cervical ganglia (2). By contrast, hydroxy-indole-*O*-methyltransferase (HIOMT, EC 2.1.1.4), the enzyme that catalyzes the final step of MLT synthesis from *N*-acetyl-serotonin, with *S*-adenosylmethionine (SAM) as a methyl donor, displays little or no day/night change.

SAM is provided by a metabolic pathway involving sulfur-containing amino acids (homocysteine and methionine) (Fig. 1). Homocysteine is at the intersection of two pathways, i.e., remethylation to methionine, which requires folate and vitamin B-12 (or choline via betaine in an alternative reaction),

and transsulfuration to cystathionine, which requires pyridoxal 5'-phosphate as a cofactor (3). These pathways are coordinated by SAM, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase and as an activator of cystathionine  $\beta$ -synthase.

Folate or vitamin B-12 deficiency leads to mild hyperhomocysteinemia in humans, a condition that recent epidemiological studies have shown to be associated with increased risk of vascular disease (4). Although sulfhydryl radicals are considered to be strong reducing groups, some authors have suggested that homocysteine is a prooxidant that produces H<sub>2</sub>O<sub>2</sub> (3).

MLT displays marked protective effects against oxidative stress, as a free radical scavenger. This action is aided by its ability to cross all biological membranes without a receptor (5). Moreover, MLT increases gene expression for various antioxidant enzymes in rat brain (6). The present study was undertaken to address the question whether folate deficiency could alter MLT secretion in rats.

### MATERIALS AND METHODS

**Animals and diet.** The study was conducted in accordance with the guidelines approved for animal experimental procedures by the French Ethics Committee (decree 87–848). Male Sprague-Dawley rats ( $n = 24$ ;  $190 \pm 10$  g, 6 wk old; IFFA CREDO, L'Arbresle, France) were housed in a room with controlled temperature ( $21 \pm 2^\circ\text{C}$ ) and a 12:12-h light:dark (lights on at 0700 h, 300 lx at the animal level). They had free access to food (nonpurified diet A04, U.A.R., Epinay sur Orge, France) and water. After a 2-wk adaptation period, they were randomly assigned to two groups (3 rats/cage). The control group ( $n = 12$ ) received a synthetic diet (HICG) (7) con-

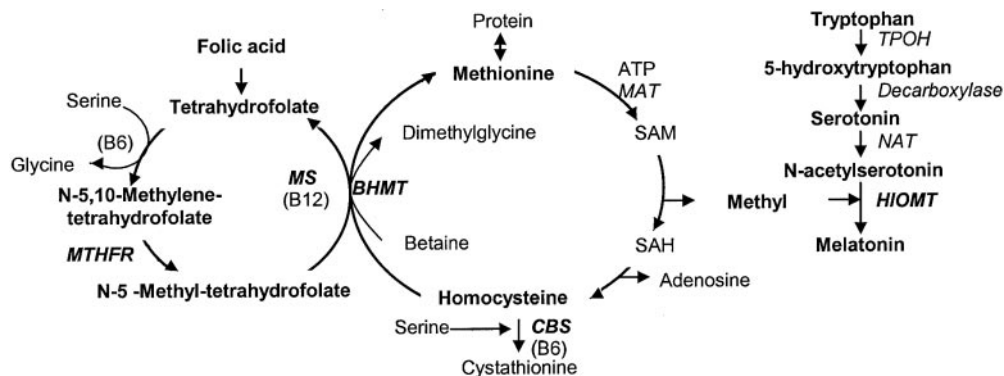
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<sup>4</sup> Abbreviations used: aMT6S, 6 sulfatoxymelatonin; E, epinephrine; HIOMT, hydroxy-indole-*O*-methyltransferase; ME, metepinephrine; MLT, melatonin; NAT, *N*-acetyltransferase; NE, norepinephrine; NME, normetepinephrine; SAM, *S*-adenosylmethionine; tHcy, total homocysteine.

**FIGURE 1** Interaction between melatonin biosynthesis and sulfur-containing amino acid metabolism (BHMT, betaine homocysteine S-methyl-transferase; CBS, cystathionine  $\beta$ -synthase; MS, methionine synthase; MTHFR, methylene-tetrahydrofolate reductase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine).



taining 20% casein, 8% gelatin, 8 mg folic acid + 10 g succinylsul-fathiazole/kg diet for 4 wk, whereas the folate-deficient group ( $n = 12$ ) was fed an identical diet with the folate omitted for the same time. Diets were supplied by INRA-UPAE (Jouy en Josas, France).

**Sample collection.** The rats were kept individually in metabolic cages (U.A.R.) to collect urine for 1 d before diet administration and at the end of wk 2 and 4 of treatment. Urine samples were collected every 3 h over a 24-h period, using a fraction collector maintained at 7°C, then weighed after collection to determine their volume and stored at -20°C until assay. Blood (0.2 mL) was collected weekly by incision of the tail for hematological evaluation. At the end of the experiment, the rats were killed by decapitation under anesthesia with diethylether at a precise time, between 0900 and 1200 h. Trunk blood was collected into plastic tubes containing EDTA and centrifuged at 4°C (2000  $\times$  g for 10 min). Plasma was stored at -20°C until assay. Brains were removed immediately after killing and stored at -70°C for further studies. Pineal glands were dissected separately.

**Analyses.** Body weight was measured twice weekly. Hematological variables including leukocyte and erythrocyte counts, blood hematocrit and hemoglobin concentration were determined using an autoanalyzer (Minos STX, ABX, Montpellier, France). Erythrocyte folate concentration was determined with an  $^{125}\text{I}$  folate kit (ICN Pharmaceuticals, Costa Mesa, CA). Plasma total homocysteine (tHcy) was measured by HPLC using the fluorometric method of Ubbink et al. (8).

The levels of 6-sulfatoxymelatonin (aMT6S), the main hepatic MLT metabolite, and/or MLT were determined in urine and/or pineal samples, using RIA developed in our laboratory (9,10). All pineal glands and urine samples from each rat were assayed in the same series to eliminate the interassay variability. Plasma epinephrine (E) and norepinephrine (NE) were determined after alumina extraction using HPLC and electrochemical detection. Metanephrines (metepinephrine, ME and normetepinephrine, NME), which represent methylated catabolites of catecholamines, were determined in urine by HPLC with electrochemical detection after ion-exchange solid phase extraction (11).

**Statistical analysis.** All results are presented as means  $\pm$  SEM. Student's  $t$  test was used to detect significant group differences in data obtained at wk 4. Urine data were tested by multiple- or two-way ANOVA for repeated measures to evaluate the influence of diet and

time on MLT, aMT6S and catechol compound levels. Post-hoc comparisons were performed with Bonferroni's multiple range test to identify the significant differences. Differences were considered significant at  $P < 0.05$  (two-tailed).

## RESULTS

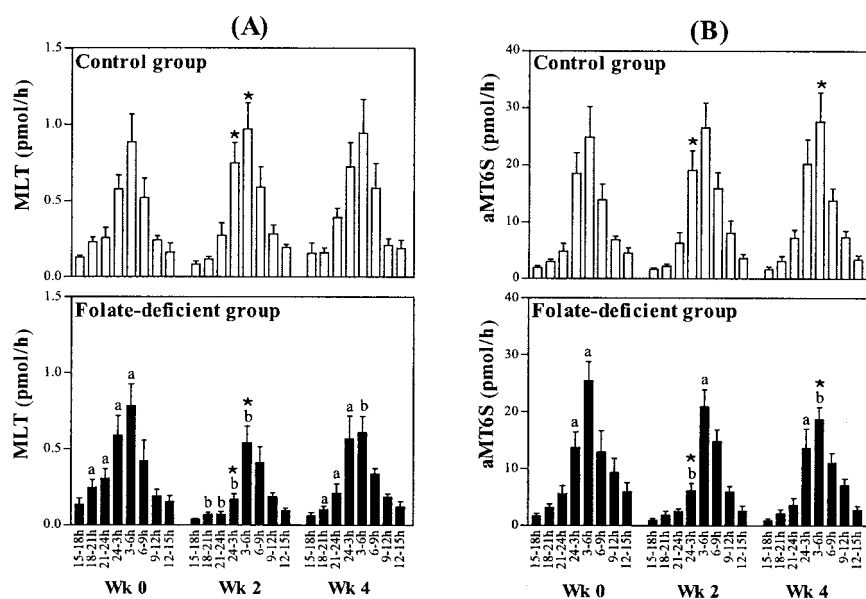
Premature death occurred during the night of d 21 in 3 folate-deficient rats housed in the same cage; there was no evidence of a causal connection with diet. Consequently, 3 rats of the control group were randomly discarded to balance the two groups in the statistical analysis. Body weights did not differ between the deficient and control rats at the end of the experiment (Table 1). At no time point did the mean weights differ between the two groups (data not shown). Erythrocyte folate concentrations were significantly lower and plasma tHcy concentrations were significantly higher in the folate-deficient rats than in the controls. Figure 2 displays the 24-h urine MLT (panel A) and aMT6S (panel B) excretions in control and folate-deficient rats. Multiple ANOVA showed daily MLT and aMT6S variations as well as effect of time (wk) and a time  $\times$  treatment interaction. Multiple comparisons performed on 3-h blocks showed that the alterations in MLT and aMT6S excretions occurred in the evening and the night and were maximal at wk 2 in the folate-deficient group. MLT and aMT6S excretions peaked at the same time. In addition, a two-way ANOVA performed on day, night and 24 h MLT and aMT6S excretions showed an effect of time and a time  $\times$  treatment interaction. Post-hoc comparisons confirmed a treatment effect that was more pronounced at wk 2 (Table 2). The 24-h urine aMT6S excretion was lower in the folate-deficient rats at wk 2 and 4 than at wk 0 (more marked at wk 2), whereas night and 24-h urine MLT and night urine aMT6S excretions were lower only at wk 2 vs. wk 0. Night and 24-h urine MLT and aMT6S excretions differed between the two groups at wk 2 and 4. Pineal MLT concentration in the

**TABLE 1**

Body weight, hematological variables, erythrocyte (Erc) folate and plasma total homocysteine (tHcy) concentrations in control (C) and folate-deficient (FD) rats<sup>1</sup>

Group	Body weight	Erythrocyte count	Hematocrit	Hemoglobin	Erc folate	Plasma tHcy
	g	$10^{12}/\text{L}$	L/L	g/L	$\mu\text{mol}/\text{L}$	
C	383 $\pm$ 8	8.57 $\pm$ 0.17	0.49 $\pm$ 0.01	163.8 $\pm$ 1.4	6.92 $\pm$ 0.32	3.26 $\pm$ 0.27
FD	378 $\pm$ 9	8.55 $\pm$ 0.12	0.50 $\pm$ 0.01	163.9 $\pm$ 1.3	1.63 $\pm$ 0.09*	12.66 $\pm$ 1.08*

<sup>1</sup> Values are means  $\pm$  SEM,  $n = 9$ . \*Different from the control group,  $P < 0.05$ .



**FIGURE 2** Daily urine melatonin (MLT, *A*) and sulfatoxymelatonin (aMT6S, *B*) excretions in control and folate-deficient rats at wk 0, 2 and 4; 3-h blocks are represented. Values are means  $\pm$  SEM,  $n = 9$ . For each 3-h block, wk 0, 2 and 4 means without a common letter differ (Bonferroni's multiple comparisons test,  $P < 0.05$ )

folate-deficient group was lower than in the control at wk 4 (Table 3). Urinary methoxylated catechol (NME and ME) excretions were lower in the folate-deficient group at wk 2 and 4 than at wk 0. By contrast, plasma NE and E levels did not differ between groups when measured at wk 4 (Table 3).

## DISCUSSION

The present report clearly establishes a marked alteration of MLT secretion in folate-deficient rats. To our knowledge, this is the first study to show a direct influence of folate deficiency on MLT biosynthesis. Both the decrease of erythrocyte folate concentration and the threefold increase in plasma tHcy concentration provided evidence of folate deficiency. The dramatic decrease in diurnal pineal MLT concentration as well as in nocturnal urine MLT and aMT6S excretions, demonstrated that folate deficiency alters MLT secretion. Although measurement of aMT6S in urine is widely used to evaluate the MLT secretion, the excretion of the native hormone, which represents  $\sim 1\%$  of the total secretion, appears to be a sensitive

marker for our purpose. Nocturnal MLT secretion is very sensitive to folate deficiency because reduced MLT excretion was observed from wk 2 of folate deficiency. The slight rise of urine MLT and aMT6S levels at wk 4 could be related to an alternative methylation of *N*-acetyl-serotonin with choline as a donor because folate and choline metabolism are interdependent. Severe folate deficiency causes secondary depletion of choline and phosphocholine in rat liver (12). By contrast, in the control rats, which were 10 wk old at the end of the experiment, there was a slight ( $P = 0.07$ ) increase in MLT excretion with time, which could be related to growth. Indeed, in young adult rats, MLT secretion increases with maturation (13).

The decreases in MLT and aMT6S excretions in the folate-deficient rats, which were more marked during the first part of the night, were probably the consequence of impaired methylation of *N*-acetyl-serotonin, the last step of MLT synthesis, rather than an alteration of the release of NE from sympathetic nerve terminals in the pineal gland. In the absence of abnor-

**TABLE 2**

Day (0700–1900 h), night (1900–0700 h) and 24-h excretions of urine melatonin (MLT) and sulfatoxymelatonin (aMT6S) in control (C) and folate-deficient (FD) rats<sup>1</sup>

	Group	wk 0	wk 2	wk 4
Urine MLT, pmol/24 h	C	8.53 $\pm$ 1.34 <sup>a</sup>	9.57 $\pm$ 1.49 <sup>a</sup>	9.71 $\pm$ 1.56 <sup>a</sup>
	FD	7.47 $\pm$ 1.18 <sup>a</sup>	4.30 $\pm$ 0.63 <sup>b,*</sup>	5.88 $\pm$ 1.00 <sup>a,*</sup>
Day urine MLT, pmol/12 h (0700–1900 h)	C	2.68 $\pm$ 0.46 <sup>a</sup>	3.25 $\pm$ 0.62 <sup>a</sup>	3.04 $\pm$ 0.72 <sup>a</sup>
	FD	2.09 $\pm$ 0.37 <sup>a</sup>	1.81 $\pm$ 0.29 <sup>a,*</sup>	1.77 $\pm$ 0.19 <sup>a</sup>
Night urine MLT, pmol/12 h (1900–0700 h)	C	5.85 $\pm$ 0.97 <sup>a</sup>	6.32 $\pm$ 1.11 <sup>a</sup>	6.67 $\pm$ 1.14 <sup>a</sup>
	FD	5.38 $\pm$ 0.93 <sup>a</sup>	2.49 $\pm$ 0.44 <sup>b,*</sup>	4.11 $\pm$ 0.87 <sup>a,*</sup>
Urine aMT6S, pmol/24 h	C	223 $\pm$ 34 <sup>a</sup>	241 $\pm$ 30 <sup>a</sup>	247 $\pm$ 34 <sup>a</sup>
	FD	232 $\pm$ 34 <sup>a</sup>	161 $\pm$ 15 <sup>c,*</sup>	177 $\pm$ 18 <sup>b,*</sup>
Day urine aMT6S, pmol/12 h (0700–1900 h)	C	69 $\pm$ 9 <sup>a</sup>	83 $\pm$ 13 <sup>a</sup>	74 $\pm$ 7 <sup>a</sup>
	FD	89 $\pm$ 14 <sup>a</sup>	69 $\pm$ 10 <sup>a</sup>	63 $\pm$ 9 <sup>a</sup>
Night urine aMT6S, pmol/12 h (1900–0700 h)	C	154 $\pm$ 29 <sup>a</sup>	158 $\pm$ 26 <sup>a</sup>	173 $\pm$ 29 <sup>a</sup>
	FD	143 $\pm$ 22 <sup>a</sup>	92 $\pm$ 14 <sup>b,*</sup>	114 $\pm$ 16 <sup>a,*</sup>

<sup>1</sup> Values are means  $\pm$  SEM,  $n = 9$ .

\*Different from the control group.

a,b,c Values in a row with different superscripts differ,  $P < 0.05$ .

TABLE 3

Levels of plasma and urine catechol compounds and pineal melatonin (MLT) in control (C) and folate-deficient (FD) rats<sup>1</sup>

	Group	wk 0	wk 2	wk 4
Pineal MLT, <i>pmol/mg tissue</i>	C	—	—	1.75 ± 0.18
	FD	—	—	1.19 ± 0.09*
Plasma NE, <i>nmol/L</i>	C	—	—	1.13 ± 0.24
	FD	—	—	1.21 ± 0.21
Plasma E, <i>nmol/L</i>	C	—	—	6.86 ± 1.46
	FD	—	—	5.29 ± 0.43
Urine NME, <sup>2</sup> homogenize <i>nmol/24 h</i>	C	7.60 ± 0.82	6.39 ± 0.49	7.43 ± 0.93
	FD	7.60 ± 1.09 <sup>a</sup>	4.26 ± 0.33 <sup>c,*</sup>	5.41 ± 0.66 <sup>b,*</sup>
Urine ME, <i>nmol/24 h</i>	C	1.52 ± 0.20	1.42 ± 0.25	1.42 ± 0.30
	FD	1.83 ± 0.41 <sup>a</sup>	0.91 ± 0.15 <sup>b,*</sup>	1.22 ± 0.20 <sup>a</sup>

<sup>1</sup> Values are means ± sem, *n* = 9. Means in a row without a common superscript letter differ, *P* < 0.05.

<sup>2</sup> Abbreviations: E, epinephrine; ME, metepinephrine; NE, norepinephrine; NME, normetepinephrine.

\*Different from the control group.

mal plasma E and NE concentrations, the decrease in urine methoxylated catechol excretions strengthens this hypothesis. Further, the inappropriate presence of disulfide-containing compounds (circulating tHcy is the sum of free, disulfide and protein-bound Hcy) could inactivate HIOMT, with the consequence of a reinforced alteration of MLT synthesis (14).

Our present data could explain, at least in part, why MLT secretion declines progressively with age in mammals, especially in humans where folate deficiency can occur in the elderly (15). Results of case studies and population-based studies have suggested that low blood folate concentrations are related to dementia and to poor cognitive function in older adults (16). Recent data indicate that serum or cerebrospinal fluid folate levels have a strong negative association with the risk of Alzheimer's disease (17,18). In addition, a decrease in the amplitude of the MLT rhythm has been described in senile dementia and in elderly people with insomnia (19,20). It would be of interest to evaluate folate status in these patients and to determine MLT levels after folate supplementation because a beneficial effect of folate treatment of insomnia has been suggested (21).

In conclusion, our findings show a close interaction between folate and MLT metabolism. They suggest the possible benefits of testing the hypothesis that folate supplementation could be an indirect and safe way to increase MLT secretion in humans.

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