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Biosynthesis of Tetrahydrobiopterin in the Presence of Dihydrofolate Reductase Inhibitors

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SUMMARY

Since there is no nutritional requirement for the biopterin cofactor, we attempted to create a drug-induced deficiency in rats in order to study the role of tetrahydrobiopterin in regulating the biosynthesis of dopamine and serotonin. The hypothesis that dihydrofolate reductase (EC 1.5.1.3) mediates the final step in the *de novo* synthesis of tetrahydrobiopterin was tested by treating rats with methotrexate along with leucovorin as a protective agent; there was no reduction in total biopterin or in the fraction present as tetrahydrobiopterin in adrenal medulla, adrenal cortex, pituitary, brain, or pineal glands. Similar results were obtained with metoprine, a lipid-soluble inhibitor of dihydrofolate reductase which readily enters the central nervous system. Treatment with loading doses of phenylalanine along with methotrexate reduced the level of tetrahydrobiopterin in liver. Neuroblastoma N115 cells growing in medium supplemented with thymidine and hypoxanthine continued to form normal amounts of tetrahydrobiopterin in the presence of concentrations of methotrexate which completely inhibited dihydrofolate reductase; higher concentrations of methotrexate increased the tetrahydrobiopterin content of the cells 2-fold and the total biopterin in the medium 3-fold. Although attempts to create a drug-induced deficiency were unsuccessful, the evidence indicates that the *de novo* synthesis of tetrahydrobiopterin proceeds by a pathway independent of dihydrofolate reductase and that folate antagonists such as methotrexate are unlikely to impair the hydroxylation of tyrosine and tryptophan, which is dependent upon the availability of the biopterin cofactor.

INTRODUCTION

BH_4[1] is the cofactor required for the hydroxylation of phenylalanine, tyrosine, and tryptophan (1) as well as for the cleavage of glycerol ethers (2). BH_4 deficiency does not normally occur in animals, since the biosynthesis of this cofactor from GTP occurs in kidney, liver, brain, pineal, adrenal, and probably other tissues (3–7). Indeed, the lack of any nutritional requirement for biopterin was shown by studies in which rats fed a biopterin-free diet for two generations behaved normally and excreted about 30 μg of biopterin daily in urine (8). However, biopterin cofactor deficiency in man has been observed in the atypical forms of phenylketonuria associated with lack of dihydropteridine reductase or enzymes at earlier steps in the biosynthesis of BH_4. For those individuals BH_4 is a vitamin, and cofactor replacement, as well as treatment with precursors of dopamine and serotonin, is essential for survival (9, 10). A drug-induced deficiency of BH_4 in animals would be useful in examining the role of this cofactor in the regulation of the biosynthesis of dopamine and serotonin as well as examining the role of BH_4 in those tissues which contain BH_4 yet do not synthesize these amines.

Partial purification of the enzymes on the biosynthetic pathway in *Drosophila* (11) and chicken kidney (12) indicated that sepiapterin and 7,8-BH_2 are intermediates in the conversion of GTP to BH_4. Dihydrofolate reductase (EC 1.5.1.3), which catalyzes the conversion of 7,8-dihydrofolate to tetrahydrofolate, also converts 7,8-BH_2 to BH_4, although at a much slower rate (13, 14). Dihydrofolate reductase is present in brains of rats (13, 15) and rabbits (16). Reduction of 7,8-BH_2 to BH_4 by brain extracts and inhibition of this reaction by MTX reinforced the proposal that dihydrofolate reductase mediates the final step in the *de novo* synthesis of this hydroxylation cofactor (13, 14).

If this hypothesis is valid, then it should be possible to induce a BH_4 deficiency by treating animals with dihydrofolate reductase inhibitors. Rats can be given doses of MTX or metoprine sufficient to inhibit dihydrofolate reductase completely while toxic effects can be prevented and normal growth maintained by the administration of

[1] The abbreviations used are: BH_4, *l-erythro*-5,6,7,8-tetrahydrobiopterin; 7,8-BH_2, 7,8-dihydrobiopterin; MTX, methotrexate; HPLC, high-pressure liquid chromatography.

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calcium leucovorin. Also, mammalian cells can be grown in culture without dependence on dihydrofolate reductase by supplementation of the medium with hypoxanthine and thymidine (17). Using these two test systems, we report herein the effects of dihydrofolate reductase inhibition on total biopterins and BH_4 levels in tissues of treated rats and in cultures of neuroblastoma cells. Part of this work was the subject of a preliminary report (18).

MATERIALS AND METHODS

Chemicals. MTX (*N*-{*p*-{[2,4-diamino-6-pteridinyl)methyl]-methylamino} benzoyl} glutamic acid), GTP, bacterial alkaline phosphatase, and pterin were purchased from Sigma Chemical Company (St. Louis, Mo.). Metoprine (2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine) and calcium leucovorin (5-formyl tetrahydrofolate) were synthesized at Burroughs Wellcome Company (Research Triangle Park, N.C.). Neopterin, biopterin, and sepiapterin were purchased from Dr. B. Schircks (Wettswill, Switzerland).

Cell culture. The neuroblastoma clone NIE-115 (19) was maintained in a 1:1 mixture of Ham's F12 medium and Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum, streptomycin (50 units/ml), and penicillin (50 μg/ml).

Animals. Male Sprague-Dawley rats, 150–200 g, were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). The rats had free access to food and water and were maintained in air-conditioned quarters with a 12-hr light/dark cycle. All rats were killed by decapitation, and the tissues were dissected promptly and frozen on dry ice.

Assay of biopterins. Frozen tissues were treated as described by Fukushima and Nixon (20). Neuroblastoma cells were scraped into 2 ml of 0.1 N phosphoric acid. Sufficient 2 N phosphoric acid was added to 2 ml of the culture medium to give a final concentration of 0.1 N. Total biopterin and tetrahydrobiopterin were assayed by differential iodine oxidation in acid and alkali (20, 21). In this procedure, 7,8-BH_2 and BH_4 are oxidized to biopterin in acidic solution whereas BH_4 yields pterin under the alkaline conditions. Quinonoid-BH_2 is quite unstable and rapidly rearranges to 7,8-BH_2 or is reduced to BH_4. Consequently the amount of base-labile biopterin serves as a measure of BH_4. Oxidized pterins were separated by HPLC using a Whatman PXS 10/25 ODS column (0.46 × 25 cm) and a pre-column packed with CO:PEL ODS (0.21 × 7.0 cm) and measured with a fluorometric detector (Perkin-Elmer Model 650-10LS) using an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The solvent system, which was filtered and degassed before use, consisted of 0.5% acetonitrile and 0.1% tetrahydrofuran in water.

GTP cyclohydrolase. The enzyme was assayed using a modification of the method previously published (6). Neuroblastoma N115 cells (60-mm Petri dish) were scraped into 500 μl of 0.1 M Tris-HCl (pH 7.8), sonicated for 30 sec, and centrifuged at 26,000 × *g* for 5 min. The supernatant (250 μl) was passed over a column (0.5 × 6.5 cm) of Sephadex G-25 (medium) which had been equilibrated with 0.1 M Tris-HCl (pH 7.8) containing 0.3 M KCl, 2.5 mM EDTA, and 10% glycerol. The column was washed with two 600-μl portions of the same buffer; the first portion was discarded and the second was used for assay of the enzyme. The reaction mixture, consisting of 200 μl of the Sephadex eluate and 50 μl of 10 mM GTP, was incubated for 90 min at 37° in the dark. The reaction was stopped by the addition of 25 μl of a 5:1 mixture of 1% I_2/2% KI/5 N HCl, and the mixture was centrifuged; 25 μl of 2% ascorbic acid followed by 25 μl of 1 N NaOH were then added. The neopterin triphosphate was dephosphorylated by the addition of 1.5 units of bacterial alkaline phosphatase and incubation for 1 hr at 37° in the dark. The reaction was terminated by the addition of 50 μl 1 N acetic acid. The neopterin was determined by HPLC.

GTP levels. Monolayers of cells in culture grown in 60-mm Petri dishes were scraped into 380 μl of ice-cold 5% perchloric acid. The precipitate was removed by centrifugation and the supernatant was neutralized by the addition of 230 μl 0.6 M K_3PO_4. GTP was quantitated by HPLC with UV detection using a Whatman Partisil 10 SAX anion exchange column (0.46 × 25 cm). Nucleotides were eluted isocratically using 0.4 M ammonium phosphate (pH 3.25) in 5% acetonitrile at a flow rate of 2 ml/min. GTP was measured at 262 nm using an LDC Spectromonitor III.

RESULTS

In an initial experiment, the effect of a large single dose of MTX (100 mg/kg, i.p.) on total biopterins and BH_4 content of whole adrenal and liver was examined. Although there was no significant effect on total biopterin levels in either tissue, the fraction of BH_4 was reduced in liver. A loading dose of phenylalanine, which is known to be metabolized in liver primarily by phenylalanine hydroxylase (1), further reduced the fraction of total biopterin present as BH_4 to 25% and only slightly reduced the BH_4 content of the adrenal (Table 1). Since the lack of effect on biopterin content in adrenal could be related to slow turnover of the cofactor, chronic treatment with MTX was used in subsequent experiments.

Daily administration of MTX (10 mg/kg, i.p.) for 4 days did not alter total biopterin content of pineal, pituitary, brain, or adrenal medulla (Table 2) and had no effect on the percentage of biopterin present as BH_4 in these tissues (Fig. 1). The administration of calcium leucovorin maintained a healthy appearance of the drug-treated animals and prevented loss of body weight due to the toxic doses of MTX. In adrenal cortex, an increase in total biopterin content was noted in MTX-treated animals. This change is consistent with that observed in response to insulin-induced hypoglycemia or adrenocor-

TABLE 1

Tissue content of biopterins from rats treated with a large single dose of MTX

Sprague-Dawley rats weighing about 200 g received MTX (100 mg/kg, i.p.) and were killed 4 hr later. A corresponding group received the same dose of MTX and after 3 hr were dosed with phenylalanine (1 g/kg in saline, i.p.); they were killed 1 hr later. The number of rats in each group is shown in parentheses. The results are expressed as mean ± standard error of the mean.

Tissue	Control		MTX		MTX + phenylalanine	
	Total biopterin	% BH_4	Total biopterin	% BH_4	Total biopterin	% BH_4
	μg/g tissue		*μg/g tissue*		*μg/g tissue*	
Adrenal	0.35 ± 0.03 (5)	92 ± 2	0.35 ± 0.03[a] (5)	87 ± 1[a]	0.29 ± 0.04[a] (5)	81 ± 4[b]
Liver	0.65 ± 0.08 (5)	93 ± 1	0.49 ± 0.06[a] (5)	48 ± 10[c]	0.92 ± 0.1[a] (4)	24 ± 7[d]

[a] Data not significantly different from controls.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.001$.

TABLE 2

Total biopterin content of tissues from rats treated with multiple doses of MTX

Sprague-Dawley rats weighing about 200 g received MTX (10 mg/kg, i.p.) daily for 4 days, and duplicate groups received the same dose of MTX along with calcium leucovorin (10 mg/kg, i.p.) daily for 4 days. The number of rats in each group is shown in parentheses. Control rats received saline i.p. The results are expressed as mean ± standard error of the mean.

Tissue	Total biopterins (BH_4 + BH_2 + B)		
	Control	MTX-treated	MTX + leucovorin
	μg/g tissue		
Pineal	9.7 ± 0.6 (3)	6.9 ± 1.2[a] (5)	8.5 ± 1[a] (5)
Pituitary	0.51 ± 0.09 (6)	0.50 ± 0.05[a] (6)	0.47 ± 0.04[a] (6)
Brain	0.068 ± 0.004 (6)	0.056 ± 0.002[b] (5)	0.061 ± 0.003[a] (5)
Adrenal medulla	2.98 ± 0.17 (6)	3.28 ± 0.34[a] (6)	3.26 ± 0.48[a] (5)
Adrenal cortex	0.36 ± 0.03 (6)	1.84 ± 0.36[c] (5)	0.67 ± 0.18[a] (5)
Liver	1.22 ± 0.03 (6)	0.98 ± 0.17[a] (6)	0.70 ± 0.07[d] (6)

[a] Data not significantly different from controls.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.001$.

ticotropic hormone treatment (22) and apparently reflects a response to stress. In the corresponding group treated with leucovorin and MTX, this increase was less pronounced. In each case, however, there was no reduction in the percentage of BH_4. Total biopterin was somewhat lower in livers of rats receiving MTX and leucovorin, but, likewise, there was no significant difference in percentage of BH_4 (control 89 ± 2 versus treated 87 ± 2). Because MTX does not readily cross the blood-brain barrier, a similar experiment (Table 3) was carried out using another dihydrofolate reductase inhibitor, metoprine, which attains a high brain to plasma ratio (23). As in the experiments using MTX, there was no reduction in total biopterins of pineal, pituitary, brain, and adrenal medulla. Adrenal cortical levels were increased significantly, whereas levels in liver were decreased. With the exception of liver, the fraction of total biopterins as BH_4 was not markedly affected.

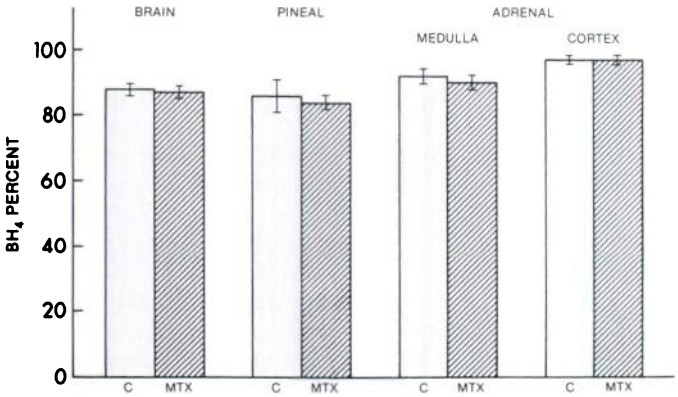

FIG. 1. *Tetrahydrobiopterin content of tissues from rats treated with multiple doses of MTX*

C, Tissues from control rats which received saline i.p. *MTX*, Tissues from rats which received MTX (10 mg/kg) daily for 4 days. Other conditions are the same as described in Table 2. Two-tailed Student's *t*-test indicated no significant difference in values between control and MTX-treated animals.

TABLE 3

Tissue content of biopterins in rats treated with multiple doses of metoprine

Rats received metoprine (10 mg/kg, i.p.) daily for 3 days, and duplicate groups received saline. The number of rats in each group is shown in parentheses. The results are the mean ± standard error of the mean.

Tissue	Control		Metoprine-treated	
	Total biopterin	% BH_4	Total biopterin	% BH_4
	μg/g tissue		*μg/g tissue*	
Pineal (ng/gland)	12.1 ± 0.5 (4)	94 ± 2	12.7 ± 0.6[a] (6)	88 ± 1[b]
Pituitary	0.71 ± 0.07 (5)	94 ± 1	0.70 ± 0.04[a] (6)	92 ± 1[a]
Brain	0.090 ± 0.001 (5)	85 ± 1	0.095 ± 0.003[a] (6)	84 ± 1[a]
Adrenal medulla	6.1 ± 1.0 (5)	90 ± 1	6.4 ± 0.3[a] (6)	87 ± 1[b]
Adrenal cortex	0.49 ± 0.06 (5)	87 ± 5	1.16 ± 0.18[b] (5)	88 ± 3[a]
Liver	1.07 ± 0.13 (5)	89 ± 5	0.73 ± 0.03[b] (7)	32 ± 5[c]

[a] Data not significantly different from controls.
[b] $p < 0.05$.
[c] $p < 0.001$.

In cell cultures, dihydrofolate reductase can be inhibited completely by MTX while normal growth is maintained by the addition of thymidine and hypoxanthine to the culture medium (17). Neuroblastoma cells (N115) synthesize BH_4, and oxidized pterins are found in the culture medium. The presence of growth-inhibitory concentrations of MTX in the culture medium of these cells, in the presence of thymidine and hypoxanthine, did not lower the cellular content of total biopterins and did not reduce the BH_4 level in the cells (Fig. 2). However, the total biopterin content of the medium was markedly increased in the presence of MTX. No significant changes were apparent in the amounts of the neopterin or pterin in the medium. Higher concentrations of MTX in the medium did not lower the levels of biopterin or the percentage of BH_4 in cells (Table 4). Indeed, at MTX concentrations of 1 μM and 100 μM the amounts of cellular

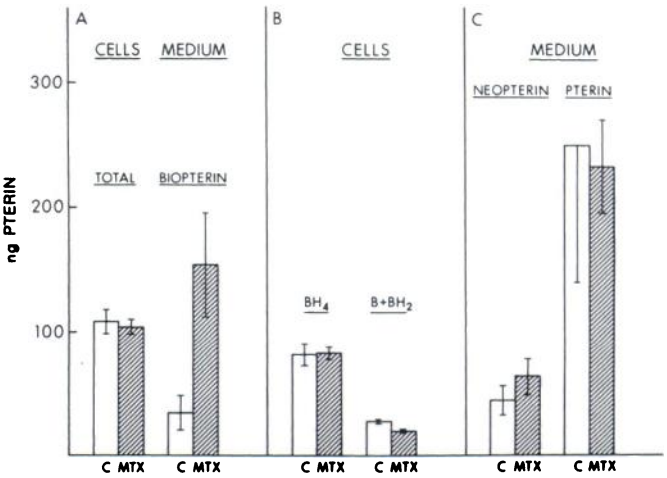

FIG. 2. *Effect of MTX on tetrahydrobiopterin biosynthesis by neuroblastoma N115 cells in culture*

MTX (0.1 μM) was added 4 hr after seeding 100-mm Petri dishes with 2×10^6 cells. The pterins in cells and medium were assayed 24 hr later. A, total biopterin content of cells and medium; B, tetrahydrobiopterin and oxidized biopterins in cells; C, Neopterin and pterin content of the culture medium.

TABLE 4

Effect of MTX on biopterin biosynthesis in neuroblastoma N115 cultures

Culture medium contained 20 μM thymidine and 50 μM hypoxanthine. MTX was added 4 hr after seeding of 2×10^6 cells into 100-mm Petri dishes and was present in the culture medium for the duration of the experiment. Biopterin levels were determined 24 hr after addition of MTX. The results are the mean ± standard error of the mean.

	Biopterin	% BH_4
	ng/culture	
Control		
Cell	38.6 ± 2.2	100
Medium	10.8 ± 0.8	—
MTX, 0.1 μM		
Cell	34.0 ± 0.2[a]	100
Medium	33.3 ± 0.4[b]	—
MTX, 1.0 μM		
Cell	53.1 ± 12.4[a]	100
Medium	33.3 ± 3.2[c]	—
MTX, 100 μM		
Cell	74.6 ± 3.6[c]	100
Medium	32.7 ± 5.0[c]	—

[a] Data not significantly different from controls.
[b] $p < 0.01$.
[c] $p < 0.05$.

biopterin were increased, indicating some acceleration in the rate of synthesis. Since there were no changes in GTP pools (Table 5) or GTP cyclohydrolase levels (Table 6), these do not appear to be factors related to the changes in biopterin levels due to MTX.

DISCUSSION

Since our laboratory is engaged in the evaluation of lipophilic dihydrofolate reductase inhibitors with potential for the treatment of brain tumors (23–26), the suggestion that dihydrofolate reductase inhibition could impair dopamine and serotonin synthesis (27) was of obvious concern. The level of dihydrofolate reductase in rat brain is quite low in comparison with the activity in liver but is present in different brain regions of rats (26) and rabbits (16). Investigation of dihydrofolate reductase in rabbit brain, which has much higher activity than rat brain, led Spector *et al.* (16) to suggest that inhibition of brain dihydrofolate reductase by MTX might explain the toxic effects of MTX in the central nervous system.

TABLE 5

Effect of MTX on GTP levels in neuroblastoma N115 cultures

Cells (7.5×10^5) were seeded in 60-mm Petri dishes; 48 hr after seeding, MTX was added and GTP levels were determined 8 hr and 24 hr later as described under Materials and Methods. Values for GTP are mean ± standard error of the mean.

Condition	GTP	
	8 hr	24 hr
	nmoles/culture	
Control	11.56 ± 0.01	12.92 ± 0.60
MTX, 0.1 μM	11.40 ± 0.32[a]	13.63 ± 0.03[a]
MTX, 1 μM	11.72 ± 0.48[a]	13.10 ± 0.32[a]
MTX, 100 μM	11.07 ± 0.26[a]	11.59 ± 0.01[a]

[a] No significant difference from controls as determined by Student's two-tailed *t*-test.

TABLE 6

Effect of MTX on GTP cyclohydrolase levels in neuroblastoma N115 cultures

Cells (7.5×10^5) were seeded in 60-mm Petri dishes; 48 hr after seeding, MTX was added and GTP cyclohydrolase levels were determined 8 hr and 24 hr later as described under Materials and Methods. Values are mean ± standard error of the mean.

Condition	GTP cyclohydrolase	
	8 hr	24 hr
	ng neopterin/90 min/culture	
Control	311 ± 13	351 ± 24
MTX, 0.1 μM	338 ± 10[a]	316 ± 32[a]
MTX, 1 μM	284 ± 2[a]	342 ± 6[a]
MTX, 100 μM	305 ± 9[a]	321 ± 15[a]

[a] No significant difference from controls as determined by Student's two-tailed *t*-test.

Further examination of the conversion of 7,8-BH_2 to BH_4 by dihydrofolate reductase in rabbit and rat brain preparations reinforced the proposal that dihydrofolate reductase mediates the final step in the *de novo* synthesis of BH_4 (13, 14) and led to speculation that MTX, which is administered intrathecally in the treatment of meningeal leukemias, could interfere with neurotransmitter synthesis dependent on BH_4 (27). Since MTX does not readily enter the central nervous system, the lack of any change in total or reduced biopterins in brain (Table 1; Fig. 1) might be due to exclusion of the drug. Consequently, metoprine, which enters the central nervous system rapidly and reaches concentrations in brain much higher than in plasma (23), was used in similar experiments (Table 3). No decrease in the BH_4 content of brain occurred after either single or multiple treatments with large doses of metoprine. The lack of effect of either MTX or metoprine on BH_4 levels in brain and other tissues indicates that syntheses of dopamine and serotonin are unlikely to be impaired as a result of cofactor deficiency induced by administration of dihydrofolate reductase inhibitors.

The quinonoid BH_2, generated during hydroxylation of the aromatic amino acids, is converted to BH_4 by dihydropteridine reductase, which does not accept 7,8-BH_2 as a substrate (28). Inhibition of this enzyme *in vitro* by MTX has been demonstrated, although the concentrations of MTX required to inhibit dihydropteridine reductase (K_i 34 μM) are several orders of magnitude higher than those required for inhibition of dihydrofolate reductase (28). Since concentrations of MTX which inhibit the growth of cells in culture (0.1 μM) might not be sufficient to inhibit BH_4 generated from quinonoid BH_2, the effects of concentrations up to 100 μM MTX on BH_4 as well as total biopterin content of neuroblastoma cells were determined. Under these conditions, not only was there no reduction in BH_4, but the cellular content of the cofactor in treated cultures was twice as high as that in control cultures, and the total biopterin content in the medium increased 3-fold (Fig. 2; Table 4). There is apparently some stimulation of BH_4 synthesis by MTX which might be due to an indirect effect of MTX on folate cofactor levels. The changes are not due to effects on either GTP pools or levels of GTP cyclohydrolase,

since there were no differences from controls in either during the course of these experiments. These data clearly indicate, however, that biosynthesis of BH_4 proceeds by a *de novo* pathway which does not involve a dihydrofolate reductase-dependent reaction.

Evidence that MTX could interfere with phenylalanine metabolism *in vivo* was reported in 1961 during a study of patients receiving MTX for the treatment of leukemia or choriocarcinoma. Goodfriend and Kaufman (29) treated such patients with oral doses of phenylalanine and found that the rate of clearance of phenylalanine from serum was much slower during MTX therapy. At that time it was unclear whether the hydroxylation cofactor was derived from folic acid. However, feeding folic acid-deficient diets failed to reduce the level of the hydroxylase cofactor in the livers of rats (29). The severe impairment of folic acid metabolism induced by toxic doses of MTX reported herein did not significantly reduce the tissue content of total biopterins, but the reduction in the percentage of BH_4 observed when phenylalanine was given along with MTX indicates interference with cofactor regeneration that would be consistent with slowing phenylalanine hydroxylation, which occurs primarily in the liver. It appears that phenylalanine loading introduces an artifical situation involving abnormal cofactor utilization in liver during which an impairment of cofactor regeneration can be demonstrated. If biopterin cofactor formation were dependent on dihydrofolate reductase, then one would expect interference with phenylalanine metabolism during MTX treatment that would mimic the genetic defect in phenylketonuria. However, this has not proven to be the case despite extensive clinical use of MTX, including high-dose therapy.

Trimethoprim, an antibacterial agent, is another inhibitor of dihydrofolate reductase which is widely used clinically, and its effect on BH_4 formation in rat liver has been studied. Using a purified dihydrofolate reductase preparation from rat liver, Stone (30) observed that the apparent K_m of 43 μM for 7,8-dihydrobiopterin was 430 times higher than the K_m for dihydrofolate, and thus the reduction of 7,8-BH_2 is more susceptible to inhibition by dihydrofolate reductase inhibitors than is the reduction of dihydrofolate. However, the administration of trimethoprim had no significant effect on the hepatic supply of BH_4 in rats (30). The lack of effect of trimethoprim or MTX on BH_4 levels in liver indicates that 7,8-BH_2 is unlikely to be involved in generation of BH_4 *in vivo* unless, as is indicated in Table 1, large doses of phenylalanine require cofactor recycling when isomerization of quinonoid BH_2 to 7,8-BH_2 may occur.

Other studies substantiate the interpretation that dihydrofolate reductase is not involved in the *de novo* biosynthesis of BH_4. Cultures of a mutant line of Chinese hamster ovary cells (DUKX-B11), a cell line which lacks dihydrofolate reductase, synthesize BH_4 to the same extent as the parent cell line (7). Also, in cell-free preparations of bovine adrenal medulla, the formation of BH_4 from GTP is not inhibited by MTX whereas the conversion of 7,8-BH_2 to BH_4 is blocked by MTX (7). Thus, dihydrofolate reductase may have a role in generating BH_4 from 7,8-BH_2 derived from labile precursors, but the evidence indicates that 7,8-BH_2 is not an intermediate in the *de novo* synthesis of BH_4 (7). The studies reported herein have directed our attention to the characterization of the enzymes and the intermediates involved with the *de novo* synthesis of BH_4. In the same way that MTX has been a useful metabolic probe with which to elucidate metabolism dependent on folate cofactors, new inhibitors of BH_4 synthesis may be useful in achieving a drug-induced deficiency of BH_4, thereby enabling studies of metabolism dependent on the biopterin cofactor.

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