Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1546–1550, March 1983
Biochemistry

Biosynthesis of tetrahydrobiopterin by *de novo* and salvage pathways in adrenal medulla extracts, mammalian cell cultures, and rat brain *in vivo*

(sepiapterin/methotrexate/dihydrofolate reductase/neurotransmitter synthesis)

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Communicated by George H. Hitchings, December 13, 1982

ABSTRACT **Mammalian cells and tissues were found to have two pathways for the biosynthesis of tetrahydrobiopterin (BH_4): (*i*) the conversion of GTP to BH_4 by a methotrexate-insensitive *de novo* pathway, and (*ii*) the conversion of sepiapterin to BH_4 by a pterin salvage pathway dependent on dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) activity. In a Chinese hamster ovary cell mutant lacking dihydrofolate reductase (DUKX-B11), endogenous formation of BH_4 proceeds normally but, unlike the parent cells, these cells or extracts of them do not convert sepiapterin or 7,8-dihydrobiopterin to BH_4. KB cells, which do not contain detectable levels of GTP cyclohydrolase or BH_4 but do contain dihydrofolate reductase, readily convert sepiapterin to BH_4 and this conversion is completely prevented by methotrexate. In supernatant fractions of bovine adrenal medulla, the conversion of sepiapterin to BH_4 is completely inhibited by methotrexate. Similarly, this conversion in rat brain *in vivo* is methotrexate-sensitive. Sepiapterin and 7,8-dihydrobiopterin apparently do not enter the *de novo* pathway of BH_4 biosynthesis and may be derived from labile intermediates which have not yet been characterized.**

There is evidence that dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) can convert 7,8-dihydrobiopterin (7,8-BH_2) to tetrahydrobiopterin (BH_4) (1), the cofactor for the monooxygenases that hydroxylate tyrosine, tryptophan, and phenylalanine (2). DHFR is present in different regions of rat brain (3), and studies on the conversion of 7,8-BH_2 to BH_4 in brain preparations reinforced the proposal that DHFR mediates the final step in the *de novo* synthesis of BH_4 (4, 5). Lipophilic DHFR inhibitors, which differ from methotrexate (MTX) by their rapid, temperature-insensitive entry into cells (6, 7) and which have potential for the treatment of brain tumors, might inhibit the synthesis of BH_4, thereby impairing the formation of dopamine and serotonin.

If DHFR is required for the *de novo* biosynthesis of BH_4, it should be possible to create a drug-induced deficiency of this cofactor by treating rats with large doses of MTX. However, neither MTX nor metoprine, a lipophilic DHFR inhibitor (8), decreased BH_4 levels in rat brain, pineal, pituitary, or adrenal tissue even after treatment with large doses for 4 days (9). Also, when neuroblastoma N115 cells growing in medium supplemented with thymidine and hypoxanthine were exposed to 0.1 μM MTX, DHFR was inhibited but normal growth rates were maintained. Under these conditions, the BH_4 content of the cells was not affected but the biopterin content of the medium increased (9).

Although attempts to induce a deficiency of BH_4 *in vitro* or *in vivo* by inhibition of DHFR were unsuccessful, this evidence that the *de novo* biosynthesis of BH_4 proceeds by a MTX-insensitive pathway led to the biochemical studies presented herein. It was found that the formation of BH_4 from GTP in cell-free preparations of bovine adrenal medulla is not inhibited by MTX. Also, studies on the conversion of sepiapterin to BH_4 *in vitro* and *in vivo* indicated that a second pathway for the formation of BH_4, which is MTX-sensitive, is present in adrenal medulla, in mammalian cell cultures, and in rat brain (10).

MATERIALS AND METHODS

Materials. MTX, intestinal alkaline phosphatase, GTP, NADPH, folic acid, and pterin were obtained from Sigma; sepiapterin, biopterin, and 7,8-BH_2 were purchased from B. Schircks (Wettswill, Switzerland); Sephadex G-25 (medium) was from Pharmacia. Metoprine and leucovorin (5-formyltetrahydrofolate) were synthesized at Burroughs Wellcome. Acetonitrile and tetrahydrofuran were HPLC grade; all other chemicals were reagent grade or better.

Cell Culture. Neuroblastoma N115, KB, L1210, and L1210/R cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum, penicillin at 50 units/ml, and streptomycin at 50 μg/ml. Chinese hamster ovary (CHO) and DUKX-B11 cells, a mutant of CHO cells lacking DHFR (11), were cultured in Ham's F-12 medium containing 10% fetal calf serum, penicillin at 50 units/ml, and streptomycin at 50 μg/ml. For those studies in which growth was maintained in the presence of MTX, 20 μM thymidine and 50 μM hypoxanthine were added to the culture medium.

Pterin Analyses. The assay of BH_4 is based on its differential stability to oxidation with I_2 in acidic and alkaline solutions (12). Oxidation in acid measures total biopterin because 7,8-BH_2 and BH_4 are oxidized to biopterin under these conditions, whereas under alkaline conditions BH_4 is converted to pterin (12). Because quinonoid BH_2 (Q-BH_2) is quite unstable, rapidly rearranging to 7,8-BH_2 or being reduced to BH_4 (13, 14), the amount of base-labile biopterin serves as a measure of BH_4. Tissue preparation and pterin oxidation and isolation were done as described by Fukushima and Nixon (15). Oxidized pterins were analyzed by HPLC on a Whatman PXS 10/25 ODS column (0.46 × 25 cm) fitted with a precolumn packed with CO:PEL ODS (0.21 × 7 cm); detection was with a Perkin–Elmer model 650-10LS fluorometer using an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The mobile phase, which was filtered and degassed before use, consisted of 0.5% acetonitrile and 0.1% tetrahydrofuran in water. The flow rate was 1.5 ml/min.

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Abbreviations: DHFR, dihydrofolate reductase; 7,8-BH_2, 7,8-dihydrobiopterin; BH_4, tetrahydrobiopterin; MTX, methotrexate; CHO, Chinese hamster ovary; Q-BH_2, quinonoid BH_2.

Protein Analysis. Protein was determined by the method of Lowry *et al.* (16).

Enzyme Analyses. DHFR was analyzed as described by Duch *et al.* (3). Dihydrofolate was prepared from folic acid by the method of Futterman (17) as modified by Blakley (18).

BH_4 formation was measured in extracts of bovine adrenal medulla prepared from fresh or frozen tissue by homogenization in 3 vol of 50 mM Tris·HCl, pH 7.4/20% (vol/vol) glycerol/1 mM $MgCl_2$ and centrifugation at 28,000 × *g* for 30 min. Extracts of cells in culture were prepared by scraping the cells from four 100-mm Petri dishes into 1.5 ml of 0.1 M Tris·HCl, pH 7.5/10% glycerol/2.5 mM EDTA, sonicating for 2 min at 4°C, and centrifuging for 30 min at 28,000 × *g*. The supernatant from adrenal medulla or cultured cells (0.5 ml) was passed over a 1 × 6.4 cm column of Sephadex G-25 equilibrated and eluted with the appropriate buffer. The first 2 ml was discarded, and the next 1.2 ml collected and used for enzyme analysis. For measurement of the conversion of GTP to BH_4, reaction mixtures contained 210 μl of tissue extract, 30 μl of 20 mM GTP, 30 μl of 50 mM dithiothreitol, and 30 μl of 20 mM NADPH. Incubation was for 2 hr at 42°C. BH_4 was determined by differential iodine oxidation. For acid oxidation the reaction was stopped by the addition of 90 μl of a 5:1 mixture of 1% I_2/2% KI and 5 M HCl; the entire mixture was kept for 1 hr in the dark at room temperature and then centrifuged, and then 30 μl of 2% (wt/vol) ascorbic acid, 70 μl of 1 M K_2HPO_4, and 20 μl of 1 M acetic acid were added. For alkaline oxidation, the reaction was stopped with 100 μl of 2 M NaOH and 200 μl of a 9:1 mixture of 1% I_2/2% KI and 1 M NaOH and kept for 1 hr in the dark at room temperature. After addition of 125 μl of 2 M trichloroacetic acid and centrifugation, 25.7 μl of 1 M K_2HPO_4 and 30 μl of 1 M acetic acid were added to 450 μl of the supernatant.

For assays of sepiapterin reductase or 7,8-BH_2 reductase, 0.6 ml of the cell or tissue extract was passed over a 1 × 6.4 cm column of Sephadex G-25 equilibrated with 0.1 M potassium phosphate, pH 7.0/20% glycerol and eluted with the same buffer. The first 2 ml was discarded and the next 1.2 ml was collected and diluted to 1.6 ml with buffer. Assay mixtures contained 210 μl of Sephadex eluate, 30 μl of 50 mM dithiothreitol, 30 μl of 10 mM NADPH, and 30 μl of either 0.2 mM sepiapterin or 0.6 mM 7,8-BH_2. Incubation was for 2 hr at 25°C. The reaction was stopped by the addition of 90 μl of a 5:1 mixture of 1% I_2/2% KI and 5 M HCl. Pterins in the reaction mixtures were determined by HPLC as described above.

RESULTS

Previous studies have demonstrated that the conversion of 7,8-BH_2 to BH_4 in tissue extracts is inhibited by MTX (4, 5). If this were a DHFR-dependent process, then cells differing in their content of DHFR should have corresponding differences in their capacity to form BH_4 from 7,8-BH_2. The capacity to use 7,8-BH_2 as a substrate corresponded closely to DHFR activity (Table 1). In DUKX-B11 cells there was no detectable 7,8-BH_2 reductase activity. In L1210/R cells, which are resistant to MTX because of increased levels of DHFR (19), the increase in 7,8-BH_2 conversion relative to L1210 corresponded to the increase in DHFR activity. Although the formation of tetrahydrofolate was somewhat less in KB cells, the conversion of 7,8-BH_2 and 7,8-FH_2 was blocked by MTX, indicating dependence on DHFR. Consequently, 7,8-BH_2 reductase activity appears to be accounted for by the presence of DHFR and there is no reason to ascribe the reduction of 7,8-BH_2 to another enzyme.

The complete enzyme system for the conversion of GTP to BH_4 (*de novo* biosynthetic pathway) was found to be present in cell-free extracts of neuroblastoma N115 cells or rat and bovine adrenal medulla (10). The formation of BH_4 from GTP in the supernatant fraction of a bovine adrenal medulla homogenate proceeded in the presence or absence of 0.1 mM MTX (Table 2), indicating that DHFR activity was not required for BH_4 biosynthesis. This high concentration of MTX is sufficient to inhibit dihydropteridine reductase (20) as well as DHFR. This preparation also contained sepiapterin reductase and DHFR and readily converted sepiapterin or 7,8-BH_2 to BH_4, but their conversion to BH_4 was completely inhibited by MTX. In contrast to GTP, neither sepiapterin nor 7,8-BH_2 was converted to intermediates on the MTX-insensitive pathway, and DHFR appears to be required for their conversion to BH_4.

Table 1. 7,8-BH_2 reductase and dihydrofolate reductase activities in extracts of mammalian cells

Cell line	Reductase activity: 7,8-BH_2 → BH_4, pmol/min/mg protein	Reductase activity: 7,8-FH_2 → FH_4, nmol/min/mg protein
CHO	79	38.2
DUKX	ND	ND
L1210	93	21.4
L1210/R	498	333
KB	81	3.2

ND, not detectable. Cells were harvested from cultures in logarithmic-phase growth.

Further evidence that DHFR is not on the *de novo* pathway for BH_4 synthesis was obtained by examining CHO and DUKX-B11 cells. Endogenous synthesis of BH_4 proceeded well in both CHO and DUKX cells, and the ratio of BH_4 to total biopterin content was similar in both cell lines (Fig. 1). Thus, lack of DHFR does not prevent synthesis of BH_4 in DUKX cells. When cells grown in the presence of thymidine and hypoxanthine are treated with MTX, DHFR is inhibited but normal growth is maintained (21). Under these conditions, the presence of MTX did not decrease either total biopterin or the fraction present as BH_4 in these cells. Instead, some increase in rate of synthesis was indicated by a marked increase in the amount of total biopterin in the medium of CHO cells exposed to MTX. Because only oxidized forms of biopterin were found in the culture medium, any BH_4 excreted from the cells is apparently unstable in the absence of reducing agents. MTX had no effect on the amounts of neopterin and pterin found in the culture medium.

The conversion of sepiapterin to BH_4 in intact cells was examined by adding sepiapterin to cultures of CHO and DUKX cells and measuring the amounts of total biopterin and BH_4 in the cells 3 hr later. Sepiapterin increased the total biopterin content of both CHO and DUKX cells (Table 3). The significant comparison, however, is the fraction of cellular biopterin that was present as BH_4. Whereas CHO cells had 91% BH_4, DUKX cells had only 7%. The small amount of BH_4 present in DUKX cells probably reflects endogenous formation by the MTX-insensitive pathway. Thus, in intact DUKX cells, sepiapterin does not serve as a precursor of BH_4 via the DHFR-independent pathway. Although the supernatant fraction of DUKX cell homogenates readily converts GTP to BH_4, neither 7,8-BH_2 nor sepiapterin was converted to BH_4 by the DUKX cell extracts.

Table 2. BH_4 formation in bovine adrenal medulla extracts

Reaction*	BH_4, pmol/hr/mg protein: Control	BH_4, pmol/hr/mg protein: MTX (0.1 mM)
GTP → BH_4	161	143
7,8-BH_2 → BH_4	851	0
Sepiapterin → BH_4	300	0

*Substrate concentrations were: GTP, 2.0 mM; 7,8-BH_2, 60 μM; and sepiapterin, 50 μM.

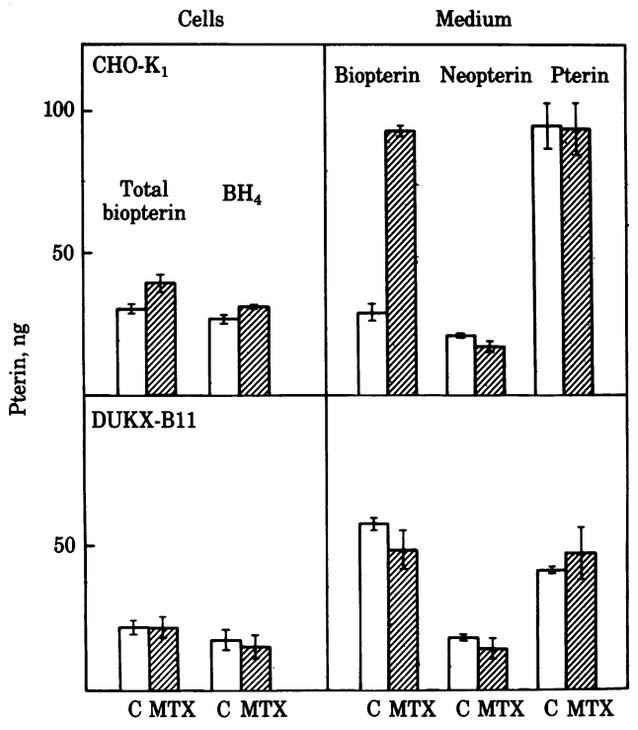


FIG. 1. Effects of MTX on BH_4 content of CHO cells and subline DUKX-B11 in culture (2×10^6 cells seeded in 100-mm Petri dishes). MTX (0.1 μM) was added 4 hr after seeding, and pterin levels in cells and medium were determined 72 hr later. C, control cells; MTX, cells treated with 0.1 μM MTX.

In contrast, both sepiapterin and 7,8-BH_2 were converted to BH_4 in similar homogenate preparations of CHO cells which do contain DHFR. Thus, the formation of BH_4 from sepiapterin and 7,8-BH_2 in CHO and DUKX cell extracts appeared to be dependent on the presence of DHFR.

KB cells differ from CHO, DUKX, and neuroblastoma N115 cells by the absence of detectable biopterins in cells or medium and by the lack of GTP cyclohydrolase (22). Sepiapterin reductase and DHFR are present in KB cells, and the formation of BH_4 from precursors can be examined without dilution by endogenous BH_4. When sepiapterin was added to the culture medium, substantial levels of BH_4 were found in the cells, and 77% of the biopterin content was BH_4. When 1 μM MTX was added to duplicate cultures 1 hr before sepiapterin, no BH_4 was formed and the biopterin content of the cells and medium reflected the formation of 7,8-BH_2 (Table 4). MTX had no effect on the activity of sepiapterin reductase in cell-free extracts. The conversion of sepiapterin to BH_4 appears to be dependent upon DHFR activity as indicated by MTX inhibition.

To study sepiapterin and 7,8-BH_2 as precursors of BH_4 *in vivo*, these compounds were injected into the brains of rats by the intracisternal route and the total biopterin and BH_4 contents of the whole brain were measured. BH_4 levels increased markedly within 30 min and reached maximal levels within 2–3 hr

Table 3. Sepiapterin metabolism in CHO and DUKX cells

	CHO-Kl		DUKX-B11	
Addition to medium	Total biopterins, ng/culture	BH_4, %	Total biopterins, ng/culture	BH_4, %
None	3.7	91	4.7	61
Sepiapterin*	30	91	14	7

*Cells were incubated with 10 μM sepiapterin for 3 hr.

Table 4. Conversion of sepiapterin to biopterins in KB cells

Additions to medium*	Total biopterins, ng/culture	BH_4, %
Control	0	—
Sepiapterin		
Cells	329	77
Medium	825	—
Sepiapterin/MTX		
Cells	162	0
Medium	2,859	—

*Cells were grown in medium supplemented with 20 μM thymidine and 50 μM hypoxanthine. Sepiapterin (10 μM) was present for 24 hr; MTX (1 μM) was added 1 hr before sepiapterin.

after injection of either sepiapterin or 7,8-BH_2. Because MTX entry into the central nervous system is known to be limited (23), MTX was also administered intracisternally at least 1 hr before injection of the pterins. Sepiapterin was a good precursor of BH_4 (Fig. 2). This conversion of sepiapterin was inhibited approximately 85% by MTX, and some accumulation of oxidized biopterins occurred in the presence of MTX. A similar experiment using 7,8-BH_2 indicated complete inhibition of BH_4 formation from this pterin by MTX. Thus, in brain, as in cells in culture, the conversion of both sepiapterin and 7,8-BH_2 to BH_4 appears to be mediated by DHFR. In contrast, as found in our preliminary studies (9), the endogenous levels of BH_4 were not decreased in rat brain by treatment for 4 days with large doses of either MTX or metoprine, a lipophilic DHFR inhibitor which readily enters the central nervous system and reaches high concentrations in rat brain 5 hr after intraperitoneal administration (8).

DISCUSSION

There is substantial evidence that GTP serves as the primary precursor for the *de novo* synthesis of BH_4 in eukaryotic cells but the reports on the properties of the enzymes involved and the structures of the pterin intermediates are conflicting (24–30). The MTX-insensitive conversion of [^{14}C]GTP to biopterins in rat brain has been reported but the likelihood that the GTP served as a source of guanosine and the evidence that BH_4 was a small fraction of the total biopterin raise questions about the experimental design and analytical procedures and limit interpretation of the data (27). Sepiapterin and 7,8-BH_2 have been identified by radioisotope studies as being derived from GTP (26, 28–30). Examination of the biosynthetic system in chicken kidney preparations led Tanaka *et al.* (29) to propose the pathway GTP → 7,8-H_2neopterin triphosphate → unstable compound X → sepiapterin → 7,8-BH_2. The biosynthesis of sepiapterin in *Drosophila* also involves an unidentified precursor (26). Dihydroneopterin triphosphate, sepiapterin, and 7,8-BH_2 have been proposed as intermediates on the pathway from GTP in *Drosophila* (26) and *Ascaris* (28) as well as in chicken kidney (29). A recent study of BH_4 formation in rat brain extracts also described sepiapterin as an intermediate on the *de novo* pathway (30). Evidence that 7,8-BH_2 could be reduced to BH_4 by brain extracts of rats and rabbits led to the proposal that the final step in the *de novo* synthesis of BH_4 in brain was the conversion of 7,8-BH_2 to BH_4 by DHFR (4, 5). Although the activity is quite low in comparison with other tissues or neonatal rat brain, DHFR is widely distributed in different regions of rat brain (3). Thus, if this were the case, then a likely consequence of inhibition of this enzyme by MTX or lipophilic DHFR inhibitors could be impairment of the synthesis of dopamine and serotonin.

The studies presented herein indicate that sepiapterin and 7,8-BH_2 depend on DHFR activity for their conversion to BH_4.

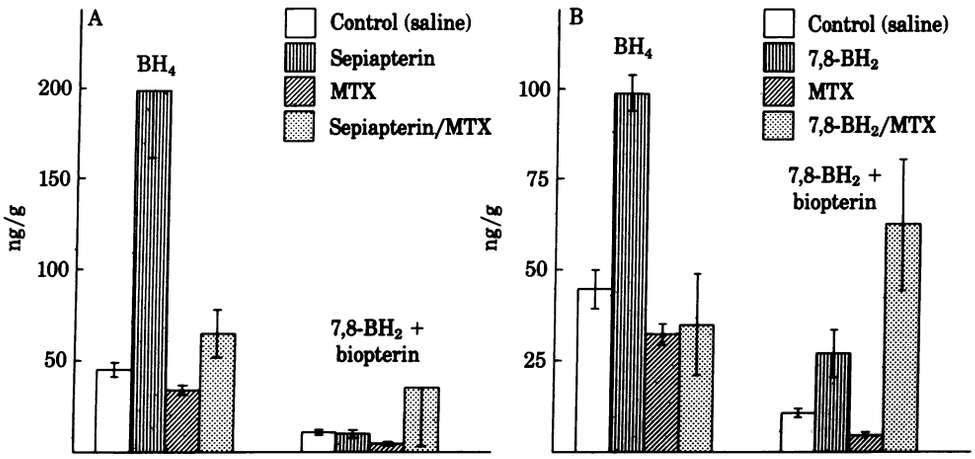


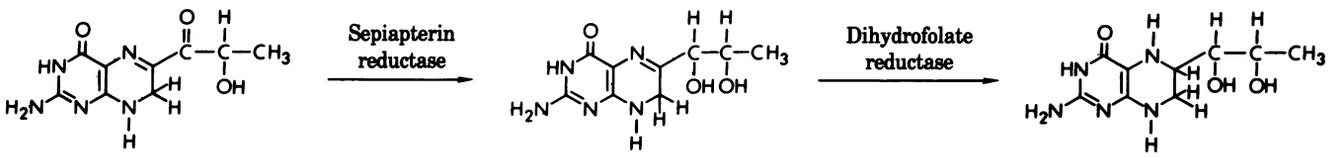
FIG. 2. Inhibition of BH_4 formation from sepiapterin and 7,8-BH_2 by MTX in rat brain. (*A*) MTX (50 μg per rat, intracisternal) was administered 1 hr before sepiapterin (10 μg/rat, intracisternal). Rats were killed 3 hr after administration of sepiapterin. (*B*) MTX (50 μg/rat, intracisternal) was administered 2 hr before 7,8-BH_2 (10 μg/rat, intracisternal). Rats were killed 3 hr after administration of 7,8-BH_2. Rats were male Sprague–Dawley (150–200 g).

Recent studies of BH_4 synthesis in bovine adrenal medulla preparations demonstrated that, unlike sepiapterin, 7,8-dihydroneopterin triphosphate does serve as a precursor of BH_4 on the *de novo* MTX-insensitive pathway (*). This designation as the "*de novo* pathway" refers to the steps in the endogenous biosynthesis of the active cofactor from nonpterin precursors, a process that is dependent upon purine nucleotide biosynthesis and the availability of guanosine, GMP, and GTP. Our present understanding of biopterin cofactor biosynthesis is comparable to when labile uncharacterized forms of the folate cofactors were converted to stable 5-formyltetrahydrofolate (31).

The present evidence indicates that sepiapterin is converted to BH_4 by a MTX-sensitive pathway. The designation of this alternate route as a "salvage pathway" is by analogy with the conservation of intact purines by their conversion to nucleotides by a route separate from *de novo* synthesis. This pterin salvage pathway is mediated by sepiapterin reductase and DHFR. During the hydroxylation of tyrosine, tryptophan, and phenylalanine, BH_4 is converted to Q-BH_2 which can be reduced to BH_4 by dihydropteridine reductase. Q-BH_2 is quite unstable and can rearrange rapidly to the more stable 7,8-BH_2 isomer which does not serve as a substrate for dihydropteridine reductase (20). Regeneration of BH_4 from 7,8-BH_2 can be mediated by DHFR, but the extent to which this occurs under physiological conditions has not been elucidated.

cursor. The increase in the rate of synthesis of biopterin in N115 and CHO cells treated with MTX, coupled with the lack of this effect in DUKX cells which lack DHFR and thus would be expected to contain little reduced folates, suggests that reduced folate cofactors may exert some control over the biosynthesis of BH_4. Kapatos and Kaufman (†) have demonstrated that exogenous folates do have an effect on the synthesis of BH_4 from GTP in neuroblastoma cells in culture.

If biopterin were a vitamin for mammals, as it is for *Crithidia fasciculata* (32), then it is likely that the biosynthesis and function of this pterin cofactor would have been elucidated long ago. Two developments during the past few years, however, have focused attention on the physiological and biochemical functions of BH_4. (*i*) Although there is no animal model for study of the effects of BH_4 deficiency, biopterin cofactor deficiency in man has been observed in atypical forms of hyperphenylalaninemia (lack of dihydropteridine reductase or enzymes at earlier steps in BH_4 biosynthesis) (33–36). Treatment with BH_4 restores hepatic metabolism of phenylalanine in these infants but neurotransmitter therapy is also required and these are serious problems in clinical management. (*ii*) Evidence that biopterin levels are less than normal in cerebrospinal fluid (37) and in brain autopsy tissue (38) from patients with Parkinson disease has directed attention to cofactor replacement as a means of therapy. A recent report that large oral doses of BH_4 allowed temporary



Sepiapterin — 7,8-BH_2 — Tetrahydrobiopterin

Pterin salvage pathway

The evidence presented herein indicates that 7,8-BH_2 is not on the *de novo* pathway. If sepiapterin reductase has a role in the *de novo* synthesis of BH_4, it is likely that it acts on a substrate different from sepiapterin *per se*. The relative contribution of the *de novo* and salvage pathways in regulating the availability of BH_4 in different tissues is not known at present but it is clear that sepiapterin can serve as a good exogenous precontrol of rigidity and tremors in patients with Parkinson disease (‡) reflects an impairment in BH_4 biosynthesis or availability deserving closer examination.

Biopterins do not seem to be needed for the growth of mam-

* Nichol, C. A., Smith, G. K. & Duch, D. S., Seventh International Symposium on Pteridines and Folic Acid Derivatives, Sept. 21–24, 1982, St. Andrews, Scotland.

† Kapatos, G. & Kaufman, S., Seventh International Symposium on Pteridines and Folic Acid Derivatives, Sept. 21–24, 1982, St. Andrews, Scotland.

‡ Curtius, H. Ch., Niederwieser, A. & Müldner, H., Seventh International Symposium on Parkinson's Disease, June 1982, Frankfurt, Federal Republic of Germany, p. 103 (abstr.).

malian cells because several cell lines which grow normally in culture have no detectable GTP cyclohydrolase and no biopterins were found in the cells or medium (22). Thus, it is likely that this cofactor is needed for specialized functions in certain tissues. In those tissues in which there is a requirement for BH_4 for the hydroxylation of tyrosine, there is evidence that the availability of the cofactor is regulated. Increased synthesis of catecholamines in the rat adrenal medulla during response to stress (insulin-induced hypoglycemia or reserpine treatment) is coordinated with induction of GTP cyclohydrolase and increased formation of BH_4. Changes in BH_4 biosynthesis, however, are not limited to tissues rich in aromatic amino acid hydroxylases. Marked increases in both GTP cyclohydrolase activity and BH_4 levels occur in the adrenal cortex in response to stress or to treatment with corticotropin (39, 40). In the adrenal cortex of spontaneously hypertensive rats, both GTP cyclohydrolase and BH_4 levels were lower than in the adrenal cortex of Wistar Kyoto rats (§). The BH_4-dependent enzymes in the adrenal cortex have not been identified. Clearly, the availability of BH_4 antagonists or inhibitors of the *de novo* synthesis of BH_4 could serve as useful metabolic probes to explore the diverse functions of this cofactor which mediates the utilization of molecular oxygen.

In a 1964 report (41) which demonstrated a role for the biopterin cofactor in the oxidation of glycerol ethers, the suggestion was made that "it may prove rewarding to attempt to develop antimetabolites that specifically affect biopterin-requiring enzymes." This is still a valid objective. Yet, so many years later, the BH_4-dependent enzymes in tissues lacking the aromatic amino acid hydroxylases are not known and characterization of the pterin intermediates and enzymes on the *de novo* pathway of BH_4 biosynthesis is still incomplete. The studies reported herein provide a means of identifying whether precursors or intermediates are on the *de novo* MTX-insensitive pathway of BH_4 biosynthesis from GTP as distinct from pterin precursors dependent on DHFR for conversion to BH_4 (pterin salvage pathway).

§ Abou-Donia, M. M., Daniels, A. J., Nichol, C. A. & Viveros, O. H., Seventh International Symposium on Pteridines and Folic Acid Derivatives, Sept. 21–24, 1982, St. Andrews, Scotland.

The authors thank Dr. Barrett Cooper for his assistance with the techniques for *in vivo* studies, Dr. Lawrence Chasin for the DUKX-B11 cells, and Dr. Kevin Scanlon for the L1210/R cells.

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