Tetrahydrobiopterin Uptake in Supplemental Administration: Elevation of Tissue Tetrahydrobiopterin in Mice Following Uptake of the Exogenously Oxidized Product 7,8-Dihydrobiopterin and Subsequent Reduction by an Anti-folate-Sensitive Process

Keiko Sawabe1,2,*, Kazunori Osuke Wakasugi3, and Hiroyuki Hasegawa1,2

1Department of Biosciences and 2Biotechnology Research Center, Teikyo University of Science and Technology, Uenohara, Yamanashi 409-0193, Japan
3Internal Medicine Clinical Immunohaematology, Hachioji Medical Center, Tokyo Medical University, Hachioji, Tokyo 193-0944, Japan

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Abstract. In order to increase the tissue level of tetrahydrobiopterin (BH$_4$), supplementation with 6R-tetrahydrobiopterin (6RBH$_4$) has been widely employed. In this work, the effectiveness of 6RBH$_4$ was compared with 7,8-dihydrobiopterin (7,8BH$_2$) and sepiapterin by administration to mice. Administration of 6RBH$_4$ was the least effective in elevating tissue BH$_4$ levels in mice while sepiapterin was the best. In all three cases, a dihydrobiopterin surge appeared in the blood. The appearance of the dihydrobiopterin surge after BH$_4$ treatment suggested that systemic oxidation of the administered BH$_4$ had occurred before accumulation of BH$_4$ in the tissues. This idea was supported by the following evidences: 1) An increase in tissue BH$_4$ was effectively inhibited by methotrexate, an inhibitor of dihydrofolate reductase which reduces 7,8BH$_2$ to BH$_4$. 2) When the unnatural diastereomer 6SBH$_4$ was administered to mice, a large proportion of the recovered BH$_4$ was in the form of the 6R-diastereomer, suggesting that this BH$_4$ was the product of a dihydrofolate reductase process by which 7,8BH$_2$ converts to 6RBH$_4$. These results indicated that the exogenous BH$_4$ was oxidized and the resultant 7,8BH$_2$ circulated through the tissues, and then it was incorporated by various other tissues and organs through a pathway shared by the exogenous sepiapterin and 7,8BH$_2$ in their uptake. It was demonstrated that maintaining endogenous tetrahydrobiopterin in tissues under ordinary conditions was also largely dependent on an methotrexate-sensitive process, suggesting that cellular tetrahydrobiopterin was maintained both by de novo synthesis and by salvage of extracellular dihydrobiopterin.

Keywords: dihydrofolate reductase, tetrahydrobiopterin, sepiapterin, methotrexate

Introduction

6-(R)-L-erythro-5,6,7,8-Tetrahydrobiopterin (6RBH$_4$) is an essential cofactor of the aromatic amino acid hydroxylases, phenylalanine- (1), tyrosine- (2), and tryptophan-hydroxylase (3, 4). The biosynthesis of classic monoamines such as dopamine, noradrenaline, adrenaline, and serotonin is dependent on cofactor BH$_4$. Furthermore, it is also a cofactor of NO-synthase (NOS) (5, 6). Three NOS isoforms, nNOS, eNOS, and iNOS, are known to function in tissues and organs of neural, circulatory, and immune systems, respectively (7, 8). Since all NOS isoforms require BH$_4$ as an essential cofactor and regulator, BH$_4$ might be a key substance in ensuring that these enzymes maintain their role in various bodily functions. Therefore, an insufficient supply of BH$_4$ might result in various disorders related to regulatory systems in the body, as mentioned above. Indeed, many studies have appeared demonstrating that such disorders have been effectively diminished by BH$_4$ supplementation employed in the treatment of a monoamine or NO deficiency.
Many patients with a severe deficiency of BH$_4$ have received BH$_4$ therapy and have had their quality of life greatly improved. In these cases, however, the efficiency of BH$_4$ uptake has not been appreciable (9–20). What is known concerning the pharmacodynamics of administered BH$_4$ cannot sufficiently explain the poor uptake of the compound by these recipients. There has been little basic research on the permeability and the metabolic fate of exogenously given BH$_4$ as related to various organs of the body. Biosynthesis of tetrahydrobiopterin from guanosine 5’-triphosphate (GTP) requires at least three enzymes: GTP-cyclohydrolase I, 6-pyruvoyl tetrahydropterin synthase, and sepiapterin reductase (21, 22). Sepiapterin, an oxidation product of the biosynthetic intermediate 6-lactoil-tetrahydropterin, and 7,8-dihydrobiopterin (7,8BH$_4$) has long been known to be salvaged by sepiapterin reductase and dihydrofolate reductase, respectively (23). In our in vitro work using cultured RBL2H3 and PC12 cells, uptake of BH$_4$ was shown to be hampered by a cellular process resulting in a very low level of incorporation, while sepiapterin was incorporated and converted to BH$_4$ in a very efficient manner (24, 25). In this research, we observed an increase in the BH$_4$ content of tissues when we gave mice BH$_4$, 7,8BH$_4$, and sepiapterin. In the present study, we compared 6RBH$_4$, 7,8BH$_4$, and sepiapterin as to how they are incorporated and delivered to animal organs, and we found that sepiapterin and 7,8BH$_4$ can efficiently elevate tissue BH$_4$, to a greater extent than 6RBH$_4$. A new pathway was proposed by which administered 6RBH$_4$ was somehow oxidized to 7,8BH$_4$ and probably after circulation, it was converted to BH$_4$, resulting in appreciable elevation of tissue BH$_4$.

Materials and Methods

6RBH$_4$ was donated by Daiichi Suntory Pharma (Tokyo), and 6-(5)-L-erythro-5,6,7,8-tetrahydrobiopterin (6SBH$_4$), sepiapterin, and 7,8BH$_4$ were purchased from Schircks Laboratories (Jona, Switzerland). Methotrexate (MTX) was purchased from Wako (Osaka). CM-cellulose (medium viscosity, sodium salt) was obtained from Fluka (Buchs, Switzerland). C57BL/6J mice were from Japan SLC (Hamamatsu) and adult males 8–10 weeks of age were used in the present experiments. The animals were maintained on an ordinary laboratory chow and tap water ad libitum, under a constant 12-h light-dark cycle. The experiments were conducted in accordance with the ethical guidelines of the Teikyo University of Science and Technology Animal Experimentation Committee and the guidelines of The Japanese Pharmacological Society.

Pterins were given to animals at a dose of 10 mg/kg body weight irrespective of the pterin species, sepiapterin, 7,8BH$_4$, or 6RBH$_4$. The pterin compounds were dissolved in 0.05 N HCl or 0.9% NaCl just prior to oral administration (p.o.) or intraperitoneal injection (i.p.), respectively. When MTX was given, the reagent was suspended in aqueous 2% CM-cellulose and was administered 90 min before the pterins. Whole blood was collected by heart puncture from mice under anesthesia, and then the brain and other organs were dissected. The proximal third of the small intestine was taken and opened longitudinally, washed with cold saline, and then blotted three times with filter paper to remove the mucus coat. The collected blood was oxidized immediately for pterin quantitation as described below. The other organs were weighed, frozen immediately in liquid N$_2$, and then stored at −80°C until determination.

Frozen tissues were partially thawed and homogenized immediately in 5 vol of 0.1 N HCl. An aliquot (100 μL) of the homogenate was mixed with a 0.5 vol of an acid-iode solution (2% I$_2$, 3% KI in 0.1 N HCl) or with alkaline-iode (2% I$_2$, 3% KI in 0.2 N NaOH) for oxidation under acid or alkaline conditions, respectively (26). Aliquots of freshly drawn blood (50 μL each) were added to a 10:4 mixture of PBS(−) and the above acid-iode or alkaline-iode solutions for the differential oxidation as above. The oxidation reaction was allowed to proceed for 1 h at room temperature. After addition of a 0.5 vol of ascorbic acid (2.5%) dissolved in perchloric acid (0.4 M), a clear solution was obtained by centrifugation at 10,000 × g for 10 min. Biopterin and pterin were determined by HPLC equipped with a fluorescence detector (Ex = 350 and Em = 450 nm). The solid phase was Fine-SIL C18T-5 and the mobile phase was 7% methanol. Since biopterins of various redox states, such as tetrahydro-, quinonoid dihydro-, and 7,8-dihydrobiopterin, convert to fully oxidized biopterin stoichiometrically by I$_2$-oxidation under acidic conditions, biopterin measured after acid-I$_2$ oxidation was taken as the “total biopterin”. Tetrahydrobiopterin, and quinonoid dihydrobiopterin as well if present, convert to pterin in a selective and stoichiometric manner (26); hence, the difference between the pterin quantity after acid-oxidation and that after alkaline-oxidation was termed “BH$_4$” throughout this paper. Since the amount of fully oxidized biopterin detected in fresh tissue extracts was insignificant, the biopterin calculated from the “total biopterin” minus “BH$_4$” was termed “BH$_3$” or referred to as “oxidized biopterin”. When specified in the form of 7,8-dihydrobiopterin, “7,8BH$_3$” was used instead of “BH$_3$”. For example, the substrate of dihydrofolate reductase was “7,8BH$_3$”, rather than “BH$_3$”, and a working solution of the reagent BH$_4$ for administration to mice was termed “7,8BH$_3$.”. In order to distinguish
6RBH$_4$ from 6SBH$_4$ in tissue extracts, cation exchange chromatography was employed according to Bailey and Ayling (27). Freshly dissected tissue was homogenized in 0.1 N HCl. Proteins were removed by centrifugation after addition of perchloric acid (final concentration of 0.1 M). The supernatant was subjected to HPLC with UV detection (254 nm). The solid phase was Whatman, Partisil-10 SCX (HICHROM, England), and the mobile phase was 30 mM NaH$_2$PO$_4$ (pH 3.3). Terms for BH$_4$ of known C6-stereo configuration, that is, BH$_4$ in solutions of the purchased 6RBH$_4$ and 6SBH$_4$ as well as those determined by SCX-HPLC, carried the prefixes 6RBH$_4$ and 6SBH$_4$, respectively.

Results

BH$_4$ administration was less effective in elevating the tissue BH$_4$ level than sepiapterin or 7,8BH$_2$

We compared sepiapterin to 6RBH$_4$ with respect to the effectiveness of how they elevate the tissue BH$_4$ level when administered to mice. C57BL/6J male mice received a 10 mg/kg dose of sepiapterin, 7,8BH$_2$, or 6RBH$_4$ and the tissue level of biopterin in liver, kidney, and blood was monitored for 6 h after the administration. A remarkable rise was observed after both oral administration (p.o.) and intraperitoneal injection (i.p.) of the pterins as shown in Fig. 1. Sepiapterin administration was most effective at increasing the biopterin level in respective organs. In the case of the oral route (upper panels), the biopterin level continued to rise for 2 h in the liver (up to 10-fold) and for 1 h in both the kidney (10-fold) and blood (3-fold). After tissue biopterin reached the peak values, it decreased quasi-exponentially. Administration of 6RBH$_4$ resulted in a similar rise in the tissue biopterin level, but the highest level was only about half that of sepiapterin in the liver (53%) and blood (64%), while it was comparable to sepiapterin with respect to the kidney. In addition, the peak rise in tissue biopterin in the liver after 7,8BH$_2$ administration was not as high as after sepiapterin or BH$_4$ and the level of biopterin reached merely 1.6-fold that of the initial level. In contrast, increases in tissue biopterin in kidney and blood after the oral treatment with 7,8BH$_2$ were comparable to those with 6RBH$_4$. However, the levels of biopterin that were significantly higher than at the starting point lasted for 6 h after the oral administration, and in the liver and kidney, they were 122% and 140% ($P<0.05, n = 6$), respectively. At this time point, levels had already returned to the initial values in the case of sepiapterin or 6RBH$_4$ treatment. We also compared intraperitoneal injection (i.p., lower panels in Fig. 1) to the above p.o. experiment. The overall profile of data

![Graph](image-url)
from i.p. injection of sepiapterin or 6RBH₄ was similar to that for p.o. administration, but the quantity of biopterin detected in the respective organs was 2- to 3-fold greater than with oral administration. It was noted that BH₄ administration i.p. lead to a much lower elevation of liver biopterin compared to after sepiapterin injection. The biopterin level in the brain was quite stable after 6RBH₄ administration while a slight elevation of biopterin was observed after both sepiapterin and 7,8BH₂ loading; roughly 1.5-fold (i.p.) at the peak (1 h) and 1.2-fold (p.o.) at 2 h (P<0.05) (data not shown). As to the redox state of the biopterin measured in the liver and kidney, BH₄ consistently comprised around 95% of the total biopterin regardless of whichever of 6RBH₄, 7,8BH₂, or sepiapterin was administered. It was noted that virtually no oxidized biopterin (BH₂) was detected in the brain throughout the experiment. In contrast, the relative amount of BH₂ in the blood changed in response to pterin administration as described below.

The BH₂ surge appeared in the blood after BH₄ administration as well as after sepiapterin or 7,8BH₂.

The percentage of BH₄ in the blood was 80% to 85% under ordinary conditions. After oral administration of sepiapterin, the net increase in blood biopterin was 0.81 ± 0.43 nmol/g over the initial 0.33 ± 0.02 nmol/g. As shown in Fig. 2a, a great surge in oxidized biopterin (BH₂, 49% of the biopterin increase) appeared at around 30 min. The administered sepiapterin was converted to BH₂ by sepiapterin reductase and then to BH₄ by the action of dihydrofolate reductase (DHFR), but it was possible that the latter enzyme was not powerful enough to prevent accumulation of the substrate 7,8BH₂. Therefore, administration of BH₂ was not expected to be followed by a BH₂ surge. However, a remarkable amount of BH₂ (0.22 ± 0.03 nmol/g blood, 50% of the net increase in biopterin) appeared 30 min after the oral 6RBH₄ treatment (Fig. 2c). Intraperitoneal injection of 6RBH₄ also caused a BH₂ surge (0.41 ± 0.03 nmol/g blood, 27.9% of the net increase, not plotted in the figure). This indicated that 6RBH₄ administered either p.o. or i.p., was oxidized somewhere in the animal and was then delivered throughout the body through the blood circulation. The appearance of the BH₂ surge in the blood after 6RBH₄ administration strongly suggested the systemic oxidation of 6RBH₄. In contrast, oral administration of 7,8BH₂ appeared to cause the least BH₂ surge (Fig. 2b). As a consequence, the drop in the percentage of BH₄ after loading with 7,8BH₂ was smallest among the three pterins examined (inset panels of Fig. 2). Following the BH₂ surge, a proportion of BH₂ was recovered to its original percentage of 82% within 1 h, and the relative amount then continued to increase at least for the following 5 h, reaching around 94% (see the insets). The concomitant decrease in BH₂ suggested either that conversion of BH₂ to BH₄ catalyzed by DHFR occurred throughout the experimental period or that BH₂ selectively passed beyond our detection by such means as through urinary

![Fig. 2](image)
Distribution of exogenous BH₄ involved an MTX-sensitive pathway

The possible involvement of DHFR was examined for accumulation of BH₄ in the liver, kidney, intestinal mucosa, and blood following administration of the pterins. Mice were pretreated with MTX (10 mg/kg, p.o.) for 90 min; then 6RBH₄, 7,8BH₂, or sepiapterin was given orally, and 30 min later, the tissue distribution of biopterin was measured with special regard to fully reduced biopterin BH₄ (Fig. 3). Sepiapterin administration without MTX treatment raised tissue BH₄ up to 30.4 ± 0.61 nmol/g (liver, 4.2-fold over endogenous BH₄; Fig. 3a), 13.0 ± 1.44 nmol/g (kidney, 10.5-fold; Fig. 3b), 38.9 ± 15.3 nmol/g (intestinal mucosa, 24.5-fold; Fig. 3c), and 0.77 ± 0.15 nmol/g (blood, 3.5-fold; Fig. 3d). Furthermore, administration of 7,8BH₂ led to similar results (liver, 14.4 ± 2.8 nmol/g; kidney, 10.8 ± 3.4 nmol/g; intestinal mucosa, 5.97 ± 1.84 nmol/g; blood, 0.66 ± 0.09 nmol/g), but elevation of the tissue BH₄ level was lower than with sepiapterin. Pretreatment of animals with the DHFR inhibitor MTX blocked BH₄ accumulation, but a very large accumulation of oxidized biopterin (BH₂) was observed in the examined organs after administration of sepiapterin and 7,8BH₂ as was expected. For example, 7,8BH₂ administration resulted in a 10.7-fold (13.3 ± 1.2 nmol/g) increase in tissue BH₄ in the kidney, and sepiapterin treatment caused a 15.0-fold (18.6 ± 1.9 nmol/g) increase in this organ, but neither brought about an increase in BH₄. Interestingly, MTX inhibited an increase in BH₄, even after 6RBH₄ administration. In the case of the liver, 6RBH₄ administration without MTX doubled the tissue BH₄ level (12.5 ± 1.4 nmol/g), and although there was no increase in BH₄ (6.41 ± 0.70 vs endogenous 7.20 ± 0.28 nmol/g) with MTX, tissue BH₂ rose considerably. The observed increase in BH₄ after 6RBH₄ administration without MTX treatment was, therefore, presumed to be derived from 7,8BH₂ that was reduced back to BH₄ by the action of DHFR. The kidney and the intestinal mucosa both contain relatively low levels of endogenous BH₄. Without MTX, administration of 6RBH₄ strongly increased tissue BH₄.

Fig. 3. Methotrexate (MTX) inhibition of BH₄ accumulation in organs administered with 6RBH₄, 7,8BH₂, and sepiapterin. MTX (10 mg/kg) was given 90 min before oral administration of the pterins. Pterins were administered at the same dose of 10 mg/kg body weight. Organs were dissected out at 30 min after the pterins were administered. Tissue biopterin levels were measured in liver (a), kidney (b), intestinal mucosa (c), and blood (d). The mucosa was collected from the proximal 1/3 of the small intestine. The height of bars represents the total biopterin. BH₂ (closed columns) and BH₄ (open columns) were calculated as described in Materials and Methods. Data are mean ± S.E.M. values (n = 5). *P < 0.05, **P < 0.01, in comparison between with and without MTX.
in these tissues, and with MTX, this increase was only reduced by half, suggesting the MTX-resistant increase was due to direct accumulation in the form of BH$_4$ without having to take the BH$_2$ route. With MTX administration, a huge increase in BH$_2$ was observed in the blood after administration of any of the pterins including 6RBH$_4$. Simultaneously, the BH$_4$ content was even decreased by MTX. These results suggested that the steady state level of BH$_4$ had been maintained largely by the catalytic action of DHFR.

An MTX-sensitive process was also involved in maintaining the endogenous BH$_4$ level.

It was noted that the DHFR-catalyzed conversion of BH$_2$ to BH$_4$ seemed to have been involved in maintaining BH$_2$ at the endogenous level to a considerable degree (compare the two leftmost bars in the panels of Fig. 3). Hence, no pterin supplement was used in examining the effect of MTX on the steady state level of tissue BH$_4$ in liver, kidney, and blood (Fig. 4). The MTX treatment increased the amount of BH$_2$, presented as the total bipterin minus BH$_4$, in all organs tested. The BH$_2$ content in the liver was the least altered by MTX administration among all tissues examined, suggesting that the large pool of liver BH$_4$ might be maintained by de novo biosynthesis which does not include DHFR in the pathway. In contrast, the BH$_4$ level in the kidney dropped to 46.2% within 1 h and the level did not recover even at the end of the experiment (6 h after MTX administration), while the total bipterin level did not show any significant change, indicating that the oxidized bipterin increased instead of being reduced back by the function of DHFR. MTX treatment raised the bipterin concentration in the blood almost 2-fold (0.27 ± 0.02 vs 0.37 ± 0.05 nmol/g) within 1 h. It was interesting that the increase in the blood bipterin was caused almost exclusively by oxidized bipterin not by BH$_4$.

The raised BH$_4$ level in the blood did not disappear during our experimental period as was the case with kidney. Since blood bipterin might be in equilibrium in tissues throughout the body, the BH$_2$ increase might be a reflection of its facilitated excretion from tissues where it might have been salvaged back to BH$_4$ by the action of DHFR in the absence of MTX.

The unnatural diastereomer 6SBH$_4$ was converted to 6RBH$_4$, additional evidence that exogenous BH$_4$ was oxidized to 7,8BH$_2$ and then reduced back by DHFR.

The increase in BH$_4$ after 6RBH$_4$ administration suggest be mediated by an enzyme reaction catalyzed by DHFR which would likely convert 7,8BH$_2$ to the 6R-diastereomer of BH$_4$. For confirmation of the involvement of DHFR, we administered 6SBH$_4$ to mice intraperitoneally. As shown in Fig. 5, administration of 6SBH$_4$ (10 mg/kg, i.p.) resulted in a large increase in BH$_4$ (47.1 ± 5.2 nmol/g, 30 min) compared to the

**Fig. 5.** Administration of 6SBH$_4$ increased the levels of 6RBH$_4$. Mice were injected with 6RBH$_4$ or 6SBH$_4$, i.p. at a dose of 10 mg/kg. Tissue extracts after 30 min were applied to a PartiSil-10 SCX column equipped with a UV-detector (254 nm) for separation of 6R- and 6SBH$_4$ (27). The open bar represents 6RBH$_4$ and the dotted bar, 6SBH$_4$. Data are mean ± S.E.M. values (n = 6).
vehicle control (1.24 ± 0.09 nmol/g). The major portion of the accumulated BH₄ (73%) consisted of the 6R-diastereomer which must have been reduced by DHFR after oxidation of exogenous 6SBH₂ to 7,8BH₂. The remainder was in the form of 6SBH₄.

Discussion

Phenylketonuria resulting from a hereditary deficiency in phenylalanine hydroxylase causes severe mental retardation as well as acute nervous disorders. As a malignant variation of phenylketonuria, BH₄-deficiency is known to lead to neuronal disorders related to the lack of effective biosynthesis of catecholamines and serotonin. BH₄ is a common and essential cofactor of tryptophan and tyrosine hydroxylases as well as of phenylalanine hydroxylase (1–4). Unlike the classic phenylketonuria, marked by a lack of the holoenzyme phenylalanine hydroxylase, BH₄-deficiency, so-called malignant phenylketonuria or hyperphenylalaninemia, was dramatically reversed by the administration of BH₄. Consequently, supplementation with BH₄ has drawn the attention of many clinical specialists. In an attempt to supply the pteridine cofactor, chemically reduced 5,6,7,8-tetrahydro-6-methylpterin and chemically reduced tetrahydrobiop terin were examined for their effects (11, 28). In a classic research study to explore the biosynthetic pathway of BH₄, sepiapterin was shown to be efficiently converted to biop terin (23). It was long accepted that the first pteridine in BH₄ biosynthesis from GTP was dihydropteridine-triphosphate, the product of GTP-cyclohydrolase I (29–31). Sepiapterin and 7,8BH₂ were proven to be the intermediate precursors of BH₄ in a vertebrate system using bullfrog (23). Actually, patients of BH₄-deficiency were reported to have been relieved from abnormal urinary excretion of high phenylalanine and low serotonin by an experiment in which mice received 6SBH₂ (32). Shortly after, researchers discovered another biosynthetic pathway from dihydropteridine-triphosphate to BH₄, a routing other than through 7,8BH₂ (for review: (33)). Just prior to the finding of that, the de novo pathway of BH₄ biosynthesis, the naturally occurring C₆-diastereomer of BH₄ was determined to be 6RBH₄ (34, 35). Therefore, 6RBH₄ was viewed as being more “natural” than sepiapterin or 7,8BH₂. Within a short while, 6RBH₄ became commercially available for the therapy of BH₄-deficient patients (the compound is currently available from Daiichi Suntory Pharma Co., Ltd., Tokyo, and Schircks Laboratory). Based on finding from most in vitro or in vivo studies, as well as from practical therapy of BH₄-deficiency, 6RBH₄ appeared to be the only choice among agents that elevate the cellular or tissue BH₄ level.

In 1996, the first scientific evaluation appeared comparing 6RBH₄ and sepiapterin with regard to cellular uptake, and it was revealed that sepiapterin taken up by cells in culture elevated the cellular BH₄ concentration much more effectively than did 6RBH₄ (24). Mechanistic studies on sepiapterin and BH₄ incorporation have been ongoing using various culture cell lines. In brief, when cells were incubated with BH₄, most cells rejected its uptake, resulting in little increase in the cellular BH₄ level (36–38). However, sepiapterin more effectively elevated the BH₄ level by means of the so-called salvage pathway composed of sepiapterin reductase and DHFR, which are sensitive to N-acetylserotonin and MTX, respectively. In RBL2H3 cells, for example, a 100-μM sepiapterin feeding elevated the cellular BH₄ (base line level: 30 pmol/10⁶ cells) by 20-fold or more within 60 min while 6RBH₄ raised it by merely 1.5–2.0-fold at the same concentration. In some cells such as erythrocytes, however, BH₄ appeared to be incorporated in its reduced form. We were interested in whether 6RBH₄ was ineffective in elevating the tissue BH₄ level in the whole body as was observed with the cellular systems. In this study, administration of sepiapterin, 7,8BH₂, and even 6RBH₄ led to a BH₄ surge, as shown in Fig. 2. The emergence of the surge after a 6RBH₄ feeding suggested that most of the 6RBH₄ given was first oxidized and thereafter reduced back to BH₄. The reducing process following putative and systemic oxidation of exogenous BH₄ was supported first by the effective inhibition of BH₄ elevation by MTX. The involvement of a DHFR reaction was further suggested by an experiment in which mice received 6SBH₂. Injection of the mice with 6SBH₂ led to a dramatic increase in BH₄ in the kidney, 74% of which was in the 6R-diastereomer form (Fig. 5). Since the substrate specificity of DHFR strictly disallows reduction of quinonoid-BH₃, the inevitable oxidation intermediate of biop terin, DHFR reduces 7,8BH₂ and provides BH₄ with a 6R-configuration (34, 35, 39–41) (see Fig. 6). It was clear that the conversion of 7,8BH₂ to 6RBH₄ took place inside the cells by the action of the cytosolic enzyme DHFR. The precursor 7,8BH₂ might have formed from (6S)quinonoid-BH₃ by spontaneous intramolecular rearrangement, and the pterin lost C₆-chirality. Since dihydropteridine reductase (DHPR) is abundant in most cells, this might take place in some region outside of the area accessible by the enzyme, such as in non-cytosolic cellular compartments and...
extracellular spaces; otherwise the enzyme DHPR would have reduced quinonoid-BH$_2$ back to BH$_4$ preserving the 6$S$-configuration. The formed 7,8BH$_2$ must have been taken up by the cells so that the cytosolic enzyme DHFR created the 6$R$-diastereomer of BH$_4$ as quantified in the kidney 30 min after i.p. injection of 6$S$BH$_4$. Therefore, the major portion of accumulated BH$_4$ in the organs we examined might have entered as 7,8BH$_2$ from other cells, presumably from other organs, via the bloodstream. A significant portion of the accumulated BH$_4$ remained as the original 6$S$-diastereomer (26% of the increase, Fig. 5). The 6$S$-isomer might have been preserved inside the cells because either it was maintained in the tetrahydro-form or was reduced back to BH$_4$ by DHPR immediately after oxidation to quinonoid-BH$_2$. The C6-chirality is preserved through conversion between quinonoid-BH$_2$ and BH$_4$ (H. Hasegawa, unpublished results) but is lost when converted to 7,8BH$_2$ (see Fig. 6). Furthermore, the remaining 6$S$BH$_4$ could have directly moved into the tissue in its original form since the reducing reaction to produce 6$S$BH$_4$ never occurs in animals. The other portion of BH$_4$ (74% of tissue BH$_4$ in the kidney) was the salvaged product of 7,8BH$_2$. Our data did not allow us to calculate the percentage of exogenous BH$_4$ salvaged after oxidation in the entire body. We did make a rough estimation below based on a study published by Hayashi and others (14).

In their experiments, in which rats were fed 6RBH$_4$ (10 mg/kg, p.o.), they found that tissue BH$_4$ was elevated in the liver (1.5-fold) and kidney (3.4-fold), both of which showed a peak rise around 2 h following administration. In the same study in which they fed rats the same dose of radioactive 6RBH$_4$ ((6$R$)-[6-T]-BH$_4$) and performed whole-body autoradiography 2 h after treatment, virtually no significant radioactivity was noted in organs such as liver and kidney, but remained only in the gastric contents. Based on these results, they claimed that 6RBH$_4$ was scarcely absorbed by the digestive tract. In fact, their autoradiogram depicted almost no deposition of radioactivity; however, our interpretation is that almost all of the radioactive 6RBH$_4$ administered was cumulatively oxidized to non-radioactive 7,8BH$_2$, releasing most of the C6-tritium within 2 h. Tritium labeling at C6 of 6RBH$_4$ might have been lost when the [6-T]-BH$_4$ was oxidized to 7,8BH$_2$ while BH$_4$ retrieved by the subsequent reduction might not have been visualized by the autoradiography. All their results were strongly consistent with those of this present study showing that most exogenous BH$_4$ was oxidized and delivered throughout the tissues and then reduced back to BH$_4$. Their report further presented a table without any comment about BH$_2$- and BH$_4$-concentrations in the blood separate from those in the plasma over a time course of 24 h after the administration of...
Although they did not refer to BH₄ over the endogenous level) following administration. This was most likely because the administered BH₄ was as effective a supplement as 7,8BH₂ or sepiapterin (Fig. 1). This was most likely because the administered BH₄ was first oxidized to BH₂, a form relatively easily taken up by the cells and a precursor of the salvage pathway. Whatever pterins would be chosen for administration, the BH₂ surge was shown to be accompanied by sepiapterin, 7,8BH₂, or even 6RBH₄. The DHFR-catalyzed reaction was involved in the delivery and subsequent retrieval of BH₂. Furthermore, this antifolate-sensitive process was also shown to be involved in maintaining the endogenous BH₂ level to a considerable degree under ordinary conditions (Fig. 4). When anti-folate is prescribed to a patient, a chronic increase in blood BH₂ could last for a long time. At a high level, BH₂ may bind to NOS in the place of BH₄, resulting in the production of active oxygens (6, 42 – 45) which are potentially detrimental to tissues. Actually, the possible relationships between an elevated BH₂ level and various circulatory disorders have been suggested in high fructose-induced diabetes model rats (46, 47). In the present study, administration of 6RBH₄ was demonstrated to evoke a BH₂ surge and that use of MTX results in a long lasting rise in BH₂ levels in blood. Since many patients would have to receive long term treatment with 6RBH₄ or with anti-folate reagents, a BH₂ surge or long lasting rise in BH₂ levels should be carefully monitored for any deleterious effects on the body.

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