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Tetrahydrobiopterin biosynthesis

Studies with specifically labeled (^{2}H)NAD(P)H and ^2H_2O and of the enzymes involved

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The biosynthesis of tetrahydrobiopterin from either dihydroneopterin triphosphate, sepiapterin, dihydrosepiapterin or dihydrobiopterin was investigated using extracts from human liver, dihydrofolate reductase and purified sepiapterin reductase from human liver and rat erythrocytes. The incorporation of hydrogen in tetrahydrobiopterin was studied in either ^2H_2O or in H_2O using unlabeled NAD(P)H or (*R*)-(4-^{2}H)NAD(P)H or (*S*)-(4-^{2}H)NAD(P)H. Dihydrofolate reductase catalyzed the transfer of the *pro-R* hydrogen of NAD(P)H during the reduction of 7,8-dihydrobiopterin to tetrahydrobiopterin. Sepiapterin reductase catalyzed the transfer of the *pro-S* hydrogen of NADPH during the reduction of sepiapterin to 7,8-dihydrobiopterin. In the presence of partially purified human liver extracts one hydrogen from the solvent is introduced at position C(6) and the 4-*pro-S* hydrogen from NADPH is incorporated at each of the C(1′) and C(2′) position of BH_4. Label from the solvent is also introduced into position C(3′). These results suggest that dihydrofolate reductase is not involved in the biosynthesis of tetrahydrobiopterin from dihydroneopterin triphosphate. They are consistent with the assumption of the occurrence of a 6-pyruvoyl-tetrahydropterin intermediate, which is proposed to be formed upon triphosphate elimination from dihydroneopterin triphosphate, and via an intramolecular redox reaction. Our results suggest that the reduction of 6-pyruvoyl-tetrahydropterin might be catalyzed by sepiapterin reductase.

Tetrahydrobiopterin (BH_4) is the cofactor of mammalian aromatic amino acid hydroxylases [1, 2], important enzymes involved in neurotransmitter biosynthesis. A pterin-derived cofactor is common to molybdenum-containing enzymes [3] and it is speculated that pterins may fulfil further important regulatory functions [4, 5].

The discovery of inherited diseases caused by metabolic defects in BH_4 biosynthesis [6 – 8] and the treatment of neurological and affective disorders with BH_4 [9 – 11] has increased the interest in this field. Nevertheless major steps in BH_4 biosynthesis are still unclear. While it is generally accepted that the first reaction in mammalian BH_4 biosynthesis is catalyzed by a single enzyme, GTP cyclohydrolase I, converting GTP to dihydroneopterin triphosphate (NH_2P_3) [12], the pathway from NH_2P_3 to BH_4 is controversial. Earlier reports claimed that sepiapterin is an obligate intermediate [13 – 15]; newer evidence, however, questions this. Based on different experimental approaches, it has been suggested by several groups [16 – 19] that BH_4 biosynthesis might instead proceed via tetrahydropterin intermediates. Some of these aspects have been reviewed recently [20]. It is likely that more than one enzyme is involved in the formation of BH_4 from NH_2P_3, including the elimination of the $-OP_3$ group of NH_2P_3, the formal reduction at the side-chain position C(3′) and the reduction of the pterin nucleus 7,8-double bond. These reactions occur under inversions at C(1′) and C(2′) [20].

The conversion of NH_2P_3 to BH_4 thus involves the rupture and formation of four kinetically stable C-H bonds, those at C(6), C(1′), C(2′) and C(3′). We have studied the requirement and specifity of incorporation of label from NAD(P)H or from solvent during the synthesis of BH_4 from NH_2P_3. In particular the following questions have been investigated. (a) What is the mechanism of the reduction steps? Does direct transfer of NAD(P)H hydride occur? If yes, from which (*R* or *S*) side? (b) What is the role of sepiapterin reductase and dihydrofolate reductase in the biosynthesis of BH_4? (c) Is the mechanism consistent with intermediate formation of a tetrahydro form of the pterin nucleus by an intramolecular redox rearrangement?

MATERIALS AND METHODS

Materials

All pterins except NH_2P_3 and dihydrosepiapterin (SH_2) were purchased from Dr. B. Schircks Laboratories (CH-8645 Jona, Switzerland). NH_2P_3 was prepared as described by Heintel et al. [19]. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Regis Chemical Co.

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Abbreviations. BH_4, 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin or tetrahydrobiopterin; BH_2, 6-(L-*erythro*-1,2-dihydroxypropyl)-7,8-dihydropterin or dihydrobiopterin; NH_2P_3, 7,8-dihydroneopterin triphosphate; S, 6-lactoyl-7,8-dihydropterin or sepiapterin; SH_2, 6-lactoyl-5,6,7,8-tetrahydropterin or dihydrosepiapterin; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/MS, gas chromatography/mass spectrometry; HPLC, high-pressure liquid chromatography.

Enzymes. Dihydrofolate reductase (EC 1.5.1.3); alcohol dehydrogenase (EC 1.1.1.1); alcohol dehydrogenase (NADP) (EC 1.1.1.2); glutamate dehydrogenase (EC 1.4.1.3); glutathione reductase (EC 1.6.4.2); dihydropteridine reductase (EC 1.6.99.7); sepiapterin reductase (EC 1.1.1.153).

^2H_2O (99.75% ^2H) was obtained from Merck (Darmstadt, FRG). All other chemicals are commercially available. Human liver was obtained from traffic accident victims. It was stored at −70°C. SH_2 was prepared by reduction of 0.042 mM sepiapterin with 1 mM NADPH in 0.05 M potassium phosphate buffer, pH 6.5, containing 5 mM dithioerythritol and 98 mU dihydrofolate reductase, essentially as described by Milstien and Kaufman [16] and Katoh et al. [21]. This reaction achieved at least 80% conversion of sepiapterin to SH_2 as monitored by absorbance at 420 nm, and then dihydrofolate reductase was removed by ultrafiltration with Centriflow C25 (Amicon).

Enzymes and extracts

Bovine liver dihydrofolate reductase was from Sigma Chemical Co. Sepiapterin reductase from rat erythrocytes was partially purified by the method of Sueoka and Katoh [22], except that only the first two column steps were performed. Partially purified human liver extracts were prepared by ammonium sulfate fractionation and Sephadex G-25 chromatography as described earlier [19]. A partially purified sepiapterin reductase from human liver (= hydroxyapatite fraction) was prepared by an adaptation of the method of Katoh and Sueoka [23].

Specifically deuterated pyridine nucleotides

(R)-(4-^2H)NADH was synthesized from NAD^+ (Boehringer, Mannheim, FRG), using 500 mg NAD^+ in 100 ml 0.05 M Tris/HCl buffer, pH 8.7, and ($^2H_6$)ethanol (> 99% ^2H, Merck, Sharp and Dhome), in the presence of horse liver alcohol dehydrogenase (Boehringer); the product was purified by fractional precipitation of the barium salt: 1.25 g barium acetate/500 mg $NAD(P)^+$ with 220 ml ethanol. (R)-(4-^2H)NADPH was obtained analogously starting from $NADP^+$ (Boehringer) and using alcohol dehydrogenase from *leuconostoc* (Boehringer). (4,4′-$^2H_2$)NADH was prepared by dehydrogenation of (R)-(4-^2H)NADH with liver glutamate dehydrogenase (Boehringer) followed by reduction of the obtained (4-^2H)NAD^+ with ($^2H_6$)ethanol and liver alcohol dehydrogenase. (S)-(4-^2H)NADPH was obtained by two alternative pathways. In the first one, (R)-(4-^2H)NADPH, obtained as described above, was dehydrogenated to (4-^2H)-$NADP^+$ using glutamate dehydrogenase and 2-oxoglutarate in 0.05 M Tris/HCl, pH 7.6 containing 0.01 M NH_4^+. (4-^2H)$NADP^+$ was then reduced with the $NADP^+$-dependent alcohol dehydrogenase from *Leuconostoc* using unlabeled ethanol. The final product was precipitated as the barium salt and purified by HPLC (Si-100 Poliol-DEAE column, 7.5 × 500 mm, Serva, Heidelberg, FRG) with a 0.05 – 0.25 M NaCl gradient in 1 mM pyrophosphate, pH 8.4. This rather cumbersome method produces (S)-(4-^2H)NADPH of isotopic purity > 95%, and probably > 98%, as estimated from ^1H-NMR analysis (cf. below). Alternatively (S)-(4-^2H)NADPH was obtained starting from $NADP^+$ using glutathione reductase in ^2H_2O (99.7%, Roth, Freiburg, FRG), 0.05 M Tris/HCl buffer, pH 8.7, reduced glutathione and dithiothreitol (both recrystallized twice from ^2H_2O/acetone). The product was precipitated as the barium salt and purified by HPLC. In our hands the (S)-(4-^2H)NADPH obtained by this second method was of about ~ 90% isotopic purity, indicating that glutathione reductase was probably only selective for the *si* side. The isotopic and stereochemical purity of the selectively labeled compounds was investigated by 250-MHz ^1H-NMR spectroscopy (Bruker), according to the method of Arnold et al. [24]. With (R)-(4-^2H)NADH, (S)-(4-^2H)NADH, 4,4′-$^2H_2$)NADH, (R)-(4-^2H)NADPH, and (S)-(4-^2H)NADPH (prepared by the first procedure above), signals of (contaminating) ^1H at the position in question were below the detection level (< 5%).

Assays involving the incorporation of ^2H label in BH_4 from either ^2H_2O or from (selectively) labeled (^2H)NAD(P)H

The conditions for the enzymatic synthesis of BH_4 or BH_2 were optimized to obtain at least 1 µg of product after incubation and sample purification. For the experiments in ^2H_2O, all substrates and chemicals were dissolved in buffers made in this solvent. NH_2P_3 and SH_2 were lyophilized after synthesis and then dissolved in ^2H_2O. Sepiapterin reductase and dihydrofolate reductase were used without lyophilization (^1H_2O content was irrelevant for the assay, < 1%). The human liver extract was concentrated by ultrafiltration (Amicon) to a fifth, diluted to the initial volume with 0.1 M Tris/HCl, pH 7.4, in ^2H_2O, and the procedure repeated three times. The exchangeable H^+ concentration should then be < 1%.

Synthesis of BH_4 from BH_2 by dihydrofolate reductase. 0.050 mM BH_2, 0.3 mM dithioerythritol, 0.4 mM (R)-(4-^2H)NADH, dihydrofolate reductase (100 mU) and 50 mM potassium phosphate buffer, pH 6.5, were incubated for 1 h at 25°C in a total volume of 2 ml. The incubation in ^2H_2O was carried out analogously except that 0.2 mM NADPH in a total volume of 1 ml was used. The conditions for the incubation with 0.2 mM (R)-(4-^2H)NADPH were the same.

Synthesis of BH_2 from sepiapterin by sepiapterin reductase from rat erythrocytes. 0.05 mM sepiapterin, 0.2 mM (R)-(4-^2H)NADPH or (S)-(4-^2H)NADPH, sepiapterin reductase (9 mU) and 300 mM potassium phosphate buffer, pH 6.4, were incubated for 10 min at 25°C in a total volume of 1 ml.

The assay in ^2H_2O was similar except that 0.1 M Tris/^{2}HCl, p^{2}H 7.4, was used.

Synthesis of BH_4 from SH_2 by sepiapterin reductase from rat erythrocytes. SH_2 was produced as described in Materials using (S)-(4-^2H)NADPH. The solution containing the product was used directly after ultrafiltration with Amicon. SH_2 (1.0 ml) obtained as above, sepiapterin reductase (22 mU) and 0.1 mM methotrexate (to inhibit dihydrofolate reductase) were then incubated for 1 h at 37°C in a total volume of 1.15 ml. For the reaction in ^2H_2O with NADPH, SH_2 was lyophilized and then redissolved in ^2H_2O. The conditions for the incubation of SH_2 were the same as mentioned above.

Synthesis of BH_4 *from NH_2P_3 by human liver extract.* 0.020 mM NH_2P_3, 6.4 mM $MgCl_2$, 5 mM sodium pyrophosphate, 1 mM NADPH in ^2H_2O, or 0.5 mM (R)-(4-^2H)-NADH, or 2.5 mM (4,4′-$^2H_2$)NADH, or 1 mM (R)-(4-^2H)-NADPH, or 1 mM (S)-(4-^2H)NADPH, 0.1 M Tris/HCl (pH 7.4) and human liver extract (≈ 100 mg protein) were incubated in the absence of diaphorase inhibitor for 2 h with NADPH and 5 h with NADH at 37°C in a total volume of 5 ml. For minimization of label exchange the incubation was carried out with 5 mM NADPH and in the presence of 1 mM $NaAsO_2$ for 60 min (cf. also below).

Synthesis of BH_2 or BH_4 from sepiapterin by human liver extract. 0.075 mM sepiapterin, 0.3 mM (4,4′-$^2H_2$)NADH, 0.1 M Tris/HCl, pH 7.4, 0.3 ml hydroxyapatite fraction of human liver were incubated for 5 h at 37 °C in a total volume of 1 ml. For synthesis in ^2H_2O, 0.025 mM sepiapterin, 2.5 mM NADH, human liver extract (≈ 70 mg protein) and 0.1 M Tris/^{2}HCl, p^{2}H 7.4, were incubated for 2 h at 37 °C in a total volume of 2 ml. For incubation with (*S*)-(4-^{2}H)-NADPH: 0.025 mM sepiapterin, 1 mM NADPH, human liver extract (≈ 40 mg protein) and 0.1 M Tris/HCl, pH 7.4, were incubated for 2 h at 37 °C in a total volume of 2 ml. The incubation in ^2H_2O with NADPH was analogous.

Determination of NADPH label exchange with solvent

To measure ^{2}H exchange from (*S*)-(4-^{2}H)NADPH, NADPH (1 mM) was incubated in ^2H_2O at 37 °C in the presence of human liver extract (cf. above), and 100-µl aliquots were inactivated by boiling (≈ 10 – 20 s) after 10, 30, 60 and 120 min. The samples were centrifuged and the supernatant was incubated with sepiapterin (0.05 mM) and 4.5 mU sepiapterin reductase for 1 h at 25 °C in 0.1 M Tris/HCl pH 7.4. The samples were analyzed for BH_2 by GC/MS, and ^{2}H incorporation of 20%, ≈ 80%, > 80% and > 90%, respectively, was found. In the presence of 0.1 mM $NaAsO_2$ the exchange was reduced to 55% after a 60-min incubation; in the presence of 5 mM or 10 mM NADPH and 1 mM $NaAsO_2$ only ≈ 20% and > 5% exchange was found. For practical reasons (interference of excess NADPH during GC/MS analysis) 5 mM NADPH and 1 mM $NaAsO_2$ were selected for the standard incubations; the exchange was then ≈ 20%, in some cases however also higher, possibly due to the origin of the liver extract or to the presence of salt or residual glutathione in the NADPH used.

As a control after 60 min an aliquot of the incubations listed in Table 3 was treated as described in this section and the extent of exchange in (*S*)-(4-^{2}H)NADPH was estimated from the incorporation of ^{2}H in BH_2. When (*R*)-(4-^{2}H)-NADPH was used, the control was carried out similarly using dihydrofolate reductase and BH_2 by analyzing the ^{2}H content of BH_4. The same type of controls were carried out when NADPH in ^2H_2O was used.

HPLC analysis

To obtain optimal conditions for the particular incubation mixtures, the samples were first analyzed for BH_4 or BH_2 with HPLC by electrochemical detection. HPLC was performed on a pre-column (40 × 4.6 mm) of Lichrosorb RP 18 (Merck, 5 µm) connected in series with an analytical column (250 × 4.9 mm) of Spherisorb S5 ODS (Kontron). Eluent: citrate/phosphate buffer (13.3 mM citric acid and 6.6 mM Na_2HPO_4), pH 3.3 containing 60 µM EDTA, 1.4 mM 1-octanesulfonic acid, 0.16 mM dithioerythritol and 10% methanol [25]. Detection was performed on Bioanalytical Systems LC-4B amperometric detector. The applied potential was 0.25 V for BH_4 and BH_2. At this potential, a tenfold higher concentration of BH_2, as compared to BH_4, is necessary for detection. (*R*)-BH_4 and (*S*)-BH_4 are resolved under the chromatographic conditions used.

Purification of enzymatically synthesized BH_4 and BH_2

After incubation, all samples which contained human liver extract were ultrafiltered, acidified with 2 M HCl to pH < 2 and then applied to a 0.5-ml Lewatit SP 1080 (H^+, 60–150 mesh) column. For BH_4 samples, the column was equilibrated with 1 mM dithioerythritol and 0.1 mM ascorbic acid in H_2O. Upon application of the sample, the column was washed with 4 ml of the same solvent, and BH_4 was eluted with 5.5 ml of acetonitrile/pyridine/0.1 M NH_4OH/water (60:3:4:57, v/v) containing 1 mM dithioerythritol and 0.1 mM ascorbic acid, pH 10. The fraction at 0.5 – 5.5 ml containing the product was dried *in vacuo*. The procedure for the isolation of BH_2 was the same except that the buffers contained no dithioerythritol or ascorbic acid. For derivatization, a mixture of 100 µl acetonitrile/BSTFA (1:1, v/v) was added to dry samples containing at least 1 µg BH_4 or BH_2 and heated for 1 h at 100 °C.

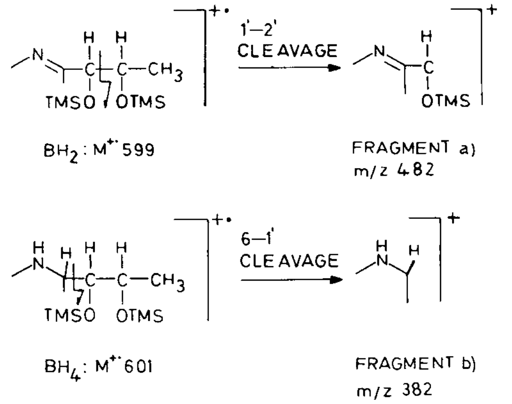
Gas chromatography/mass spectrometry (GC/MS)

The trimethylsilylated BH_4 or BH_2 samples were analyzed for deuterium content with GC/MS, as described by Kuster and Niederwieser [26]. The column was an SE-54 glass capillary (20 m × 0.3 mm; H. Jaeggi, Trogen, Switzerland), injector temperature was 275 °C and carrier gas helium at 1.4 bar (140 MPa). Temperature program: 5 min at 180 °C, then to 270 °C at a rate of 5 °C/min. For GC/MS 1 µl of derivatized sample was injected at an inlet split ratio of 1:5. The deuterium content of the samples was calculated in comparison to a BH_4 or BH_2 standard.

RESULTS

Interpretation of the GC/MS spectra

The electron impact mass spectra of trimethylsilylated BH_2 and BH_4 [26] yield intense molecular ions (*m/z* 599 and 601) together with ions at *m/z* 482 and 382 which are characteristic for BH_2 and BH_4, respectively. Scheme 1 shows how the fragment (a) *m/z* 482 results by cleavage of the C(1′)-C(2′) bond from the molecular ion of BH_2; fragment (b) *m/z* 382 is formed by the cleavage of the C(6)-C(1′) bond from the molecular ion of BH_4. The error in the determination of deuterium incorporation was 5 – 20% and in general < 10%. This was estimated from the scatter of results obtained by measuring repeatedly (three times) standard samples of BH_4 under variation of the concentration in the range expected. The error was dependent primarily on the amount of material available.



Scheme 1. *Mass spectral fragmentation of BH_2 and BH_4* yielding the fragments (a) and (b), respectively. TMS = trimethylsilyl

to be incorporated into the side chain, one of them in the C(1′) position which could be deduced from the mass *m/z* 482 of BH_2 (cf. Scheme 1). The relative degree of deuteration of BH_4 could be estimated as 53% $^2H_4$, 31% $^2H_3$, 9% $^2H_2$, 4% $^2H_1$ and 3% $^2H_0$. During derivatization with BSTFA, BH_4 was partially oxidized to BH_2. Therefore, BH_2 was also analyzed in the same sample when possible. In BH_2, the molecular ion contained three ^2H, the fragment *m/z* 482 approximately one ^2H ($\approx$ 80% $^2H_1$). Two basically different types of mechanism might, a priori, be responsible for this rather surprising result. First, the hydrogen from NADPH could be transferred indirectly, e.g. via another cofactor like a flavin. On the other hand, exchange of the NADPH label with solvent might compete with incorporation. While the (rapid) exchange of label from NAD*H is well documented (*H = ^2H or ^3H), literature on NADPH is scarce [28]. We found that label exchange from $NADP^2H$ indeed proceeds at rates comparable to those of BH_4 biosynthesis in human liver extracts and that it has a $t_{1/2}$ of the order of 15–20 min under our standard incubation conditions.

This exchange was followed using sepiapterin reductase and sepiapterin and monitoring incorporation of ^2H in BH_2 after denaturation of samples as described in Materials and Methods. The exchange was effectively inhibited in the presence of $NaAsO_2$; it was further reduced by increasing the concentration of NADPH to 5 mM. Under these optimized conditions, the exchange was reduced to < 20% in the standard incubation assays (60 min). It should be noted that such an extent of exchange should have only a secondary effect on the incorporation of label into product, since under initial conditions, i.e. when product formation is maximal, NADPH will be present in its unmodified form. It is probable that the exchange or scrambling is due to diaphorase-type enzymes such as lipoic acid dehydrogenase and glutathione reductase. In agreement with this is our observation that during synthesis of (*S*)-(4-^2H)NADPH from $NADP^+$ using glutathione reductase in 99.7% ^2H_2O, the ^2H label at the *S* position was of the order of 90–95% indicating a partial lack of stereospecificity.

Incubation of sepiapterin with human liver extract (substrate S, product BH_2)

In contrast to the results observed with the purified sepiapterin reductase from rat erythrocytes, no deuteride was transferred from (4,4′-$^2H_2$)NADH to sepiapterin by human liver extract to yield BH_2 in the absence of $NaAsO_2$. In ^2H_2O, in the presence of NADH, the BH_2 produced contained one deuterium (70–80% $^2H_1$) at the C(1′) position, the label prob-

Table 3. *Incorporation of deuterium into BH_4 from solvent and from NADP(P)H using human liver extracts and NH_2P_3 as the substrate*

NAD(P)H	Solvent	Inhibition of exchange by diaphorases	Expts	^2H in		Incorporation			
				BH_4	BH_2	$M^{+\bullet}$-ion		fragment	
						BH_2	BH_4	BH_2	BH_4
						%			
(4,4′-$^2H_2$)NADH	H_2O	no	1	0	–[d]	–[d]	~ 0	–[d]	≈ 0
(*R*)-(4-^2H)NADH	H_2O	no	1	0	–[d]	–[d]	~ 0	–[d]	≈ 0
(*R*)-(4-^2H)NADPH	H_2O	no	1	0	0	0	~ 0	0	≈ 0
(*R*)-(4-^2H)NADPH	H_2O	yes[a]	1	0	0	< 20 ($^2H_1$)	< 20 ($^2H_1$)	–[d]	–[d]
(*S*)-(4-^2H)NADPH	H_2O	no	3	0	–[d]	≈ 15 ($^2H_1$)	0–15 ($^2H_1$)	–[d]	0
(*S*)-(4-^2H)NADPH	H_2O	yes[b]	1	–[d]	2	> 40 ($^2H_2$)	–[d]	50 ($^2H_1$)	–[d]
NADPH	^2H_2O	no	3	≈ 4	≈ 3	≈ 50 ($^2H_3$)	40–70 ($^2H_4$)	80 ($^2H_1$)	90–100 ($^2H_1$)
NADPH	^2H_2O	yes[c]	2	≈ 2	≈ 1	60 ($^2H_1$)	> 40 ($^2H_2$)	20 ($^2H_1$)	> 85 ($^2H_1$)

[a–c] Exchange of label in NADPH was determined at the end ot the incubation as a control (cf. Materials and Methods for details). Despite inhibition (a) 10%, (b) 70%, (c) 23% exchange of label was found.

[d] Not measured.

Table 4. *Incorporation of deuterium in BH_2 using human liver extracts and sepiapterin*

Substrate/ product	NAD(P)H	Solvent	Expts	^2H in BH_2	Incorporation	
					$M^{+\bullet}$-ion	fragment
					%	
S/BH_2	(4,4′-^2H)NADH	H_2O	1	0	0	0
S/BH_2	NADH	^2H_2O	2	1	80	73
S/BH_2[a]	(*S*)-(4-^2H)NADPH	H_2O	1	1	55	–[b]
S/BH_2[a]	(*S*)-(4-^2H)NADPH	H_2O[c]	1	1	> 60	> 60
S/BH_2[a]	NADPH	^2H_2O	2	0	0–15	0–15

[a] In the presence of NADPH some formation of BH_4 is also observed due to reduction of BH_2 probably by dihydrofolate reductase.

[b] Not measured.

[c] In the presence of $NaAsO_2$ to inhibit exchange.

ably originating from NADH which has incorporated ^2H from the solvent. In contrast to this, with NADPH in ^2H_2O no deuterium incorporation took place. These results indicate that exchange of the NAD*H label in the liver extracts is considerably faster than that of NADP*H. They also indicate that sepiapterin reduction is probably as fast or faster than NADPH hydrogen exchange. Thus, under the conditions used, and in the presence of $NaAsO_2$ as inhibitor of exchange, incorporation of ^2H from (*S*)-(4-^2H)NADPH in H_2O was only slightly increased from ≈ 55% to > 60% (Table4).

DISCUSSION

The technique of GC/MS peviously described by our group [26] not only allows analysis of the total incorporation of deuterium into BH_4 and BH_2 but also information can be obtained on the position at which the molecules are labeled, by measuring the four ions with *m/z* 599, 601, 482 and 382. In addition, the method of selected ion monitoring (SIM) has the advantage of increased sensitivity by a factor of 100 – 1000, since only the relevant ions are monitored. The sensitivity is also increased compared with other chromatographic technics like HPLC. This is essential in samples with a high biological background. Thus fragment (a) (Scheme1) from BH_2 permits a differentiation between incorporation of deuterium into the nucleus including C(1′) of the side chain and into the position C(2′) and C(3′). The fragment (b) from BH_4 allows differentiation between the ring and the side-chain hydrogens. This technique, however, does not allow differentiation between the label in position C(2′) and the one at C(3′).

The conversion of sepiapterin or dihydrosepiapterin to BH_2 or BH_4 using purified sepiapterin reductase from rat erythrocytes leads to deuterium incorporation from (*S*)-(4-^2H)NADPH into the C(1′) position of the side chain (Table2). Experiments with purified sepiapterin reductase from human liver yield the same results (purification procedure to be published).

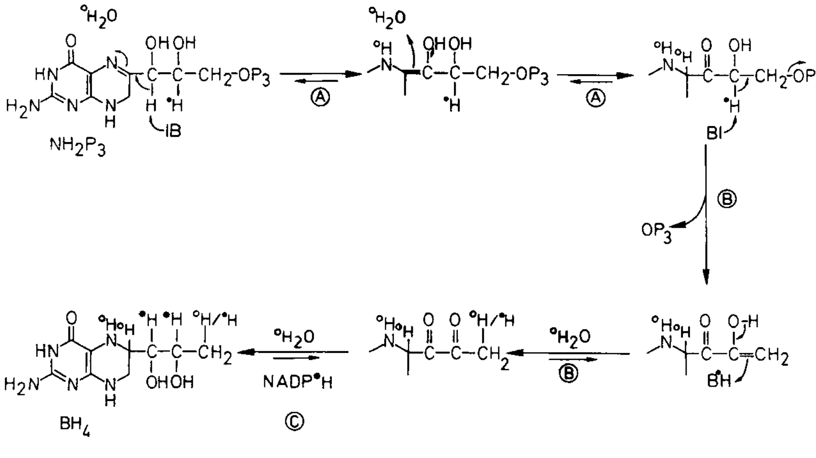
During the conversion of BH_2 to BH_4 with purified dihydrofolate reductase and (*R*)-(4-^2H)NADPH, one deuterium atom was incorporated into the C(6) position of (*6R*)-*BH*$_4$. Therefore, this reductase is specific for the *pro-R* NADPH hydrogen.

During the conversion of NH_2P_3 to BH_4 with human liver extracts, no label transfer to the ring position C(6) of BH_4 from either (4*S*)- or (4*R*)-(4-^2H)NADPH was found (Table3). This indicates that dihydrofolate reductase is not involved in the biosynthesis of BH_4 from NH_2P_3, which is in accordance with the conclusions of others [29], whose results were obtained by a different experimental approach.

On the other hand, the finding of incorporation of solvent hydrogen into position C(6) of the pterin is of mechanistic significance. It indicates that the tetrahydro ring system of BH_4 is not generated by direct reduction from an agent such as NADPH. Thus a plausible mechanistic pathway would be that of steps (A) in Scheme2, involving an internal redox shuttle. This is a chemically feasible reaction of the Amadori type [30], which should be thermodynamically feasible as estimated from the ratio of the redox potentials for the oxidoreduction of the C(1′) keto group and of the N(5)-C(6) double bond, respectively [20]. Furthermore, such a mechanism would require the generation of an intermediate C(1′) carbonyl and the reduction of this by NADP*H would lead to incorporation of *H label into the C(1′) position of BH_4, as indeed was found experimentally (Table3).

Elimination of triphosphate to form a C(2′) carbonyl function (step B, Scheme 2) is also a facile chemical process requiring a base to abstract the C(2′) proton. The finding of incorporation of solvent label into (3′)CH_3 is consistent with this mechanism. The substoichiometric incorporation of label in experiments carried out in ^2H_2O suggest that the base presumed to abstract the C(2′) proton either exchanges this proton only partially with solvent, or that it is a trifunctional one (lysine) and does not exchange with solvent (steps B, Scheme2). Which of these possibilities applies cannot be deduced from the present results since the error in the quantification of the ^2H label at position (3′)CH_3 is large. On the other hand, a secondary exchange of (3′)CH_3 of BH_4 via enolization of the (2′) keto function (step B, Scheme2) is improbable since BH_4 incubated with liver extracts remained unchanged.

The label originating from (*S*)-(4-^2H)NADPH found at C(2′) of BH_4 is possibly introduced via sepiapterin reductase. The reduction of model dicarbonyl compounds by this enzyme has been demonstrated recently [31]. Furthermore, we have been able to dehydrogenate the C(2′)OH group of BH_4 to the (2′) keto function at pH 8 – 9 in the presence of excess $NADP^+$. In fact, the product of this reaction yields the expected 50% mixture of *erythro* and *threo* (2′-^2H)BH_4 upon reduction with borodeuteride (Heintel, D., in press [32]). This finding, together with the observation that sepiapterin re-



Scheme2. *Proposed pathway of BH*$_4$ *biosynthesis.* (A) Intramolecular rearrangement; (B) elimination; (C) reduction

ductase can mediate the reduction of the C(1′) keto group of 6-lactoyl-tetrahydropterin (dihydrosepiapterin, Table 2) [16], indeed strongly suggests that this enzyme is a dicarbonyl reductase with wide specifity and that its (main?) natural substrate might not be sepiapterin itself. Its name should then be '6-pyruvoyl-tetrahydropterin reductase'. In conclusion, we consider the reactions of Scheme 2 as mechanistically consistent with the results of our group, and also with those of others [16 – 18], which are based on different approaches. Two ambiguous points remain. While steps (C) are logically the last ones in the overall biosynthesis of BH_4, we do not have information on the sequence of steps (A) and (B). Chemical considerations, i.e. the activation of C(2′)-H upon introduction of a keto group at C(1′), which would facilitate elimination, argue in favour of (A) preceding (B). Also the sequence of reduction of the C(2′), and C(3′) keto groups of the postulated intermediate, and whether 'sepiapterin reductase' is the only enzyme catalyzing these events, are still unclear. Studies with purified enzymes aiming to clarify this specific point and the question of whether one or two enzymes are involved in the catalysis of steps (A) and (B) are currently in progress.

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