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Biosynthesis of Tetrahydrobiopterin in Man

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The biosynthesis of tetrahydrobiopterin (BH_4) from dihydroneopterin triphosphate (NH_2P_3) was studied in human liver extract. The phosphate-eliminating enzyme (PEE) was purified ~750-fold. The conversion of NH_2P_3 to BH_4 was catalyzed by this enzyme in the presence of partially purified sepiapterin reductase, Mg^{2+} and NADPH. The PEE is heat stable when heated at 80 °C for 5 min. It has a molecular weight of 63 000 daltons. One possible intermediate 6-(1′-hydroxy-2′-oxopropyl)5,6,7,8-tetrahydropterin(2′-oxo-tetrahydropterin) was formed upon incubation of BH_4 in the presence of sepiapterin reductase and $NADP^+$ at pH 9.0. Reduction of this compound with $NaBD_4$ yielded monodeutero threo and erythro-BH_4, the deuterium was incorporated at the 2′ position. This and the UV spectra were consistent with a 2′-oxo-tetrahydropterin structure. Dihydrofolate reductase (DHFR) catalyzed the reduction of BH_2 to BH_4 and was found to be specific for the pro-*R*-NADPH side. The sepiapterin reductase catalyzed the transfer of the pro-*S* hydrogen of NADPH during the reduction of sepiapterin to BH_2. In the presence of crude liver extracts the conversion of NH_2P_3 to BH_4 requires NADPH. Two deuterium atoms were incorporated from (4*S*-^{2}H)NADHP in the 1′ and 2′ position of the BH_4 side chain. Incorporation of one hydrogen from the solvent was found at position C(6). These results are consistent with the occurrence of an intramolecular redox exchange between the pteridine nucleus and the side chain and formation of 6-pyruvoyl-5,6,7,8-tetrahydropterin(tetrahydro-1′-2′-dioxopterin) as intermediate.

Despite the numerous studies carried out on tetrahydrobiopterin (BH_4) biosynthesis in mammals, several controversial aspects still have to be clarified (Levine *et al.*, 1983a; Ghisla *et al.*, 1984). BH_4 is a cofactor for phenylalanine, tryptophan and tyrosine hydroxylases, and it has been suggested that it also plays an important role in the regulation of the synthesis of biogenic amine neurotransmitters (Levine *et al.*, 1983b). Errors in BH_4 biosynthesis and metabolism have been extensively studied in the rare childhood disease BH_4-deficient hyperphenylalaninaemia, alternatively referred to as atypical phenylketonuria (Niederwieser *et al.*, 1982), as well as in certain neurological and psychiatric disorders (Curtius *et al.*, 1982, 1983). The urinary excretion of neopterin (a metabolite of an intermediate in BH_4 biosynthesis) has been shown to be elevated in certain diseases where there is an alteration in the status of the immune system (Wachter *et al.*, 1983). A complete understanding of the enzymatic steps in the BH_4 biosynthesis in man is therefore of great importance.

The first reaction in mammalian BH_4 biosynthesis involves the conversion of GTP to dihydroneopterin triphosphate (NH_2P_3) by a single enzyme, GTP cyclohydrolase I. This has been reported in non-mammalians by Fan and Brown (1976) in *Drosophila* and by our group in humans (Blau and Niederwieser, 1983). We developed an enzyme assay for GTP cyclohydrolase I in human liver biopsies and lymphocytes and reported the first patient with GTP cyclohydrolase deficiency (Niederwieser *et al.*, 1984a). While the conversion of GTP to NH_2P_3 is catalyzed by a single enzyme in mammals, the further transformation of NH_2P_3 to BH_4 is likely to involve several enzymes. It should be pointed out that up to now in the biosynthesis of BH_4, only the structures of GTP, NH_2P_3 and BH_4 have been established beyond doubt, as shown in Figure 1. Current discussions and controversies deal mainly with the steps leading from NH_2P_3 to BH_4.

Tanaka *et al.* (1981) reported that in chicken kidney, NH_2P_3 is converted through some intermediate "*x*" to L-sepiapterin by a heat stable, magnesium-dependent "fraction A_2" It was suggested that "*x*" was converted to

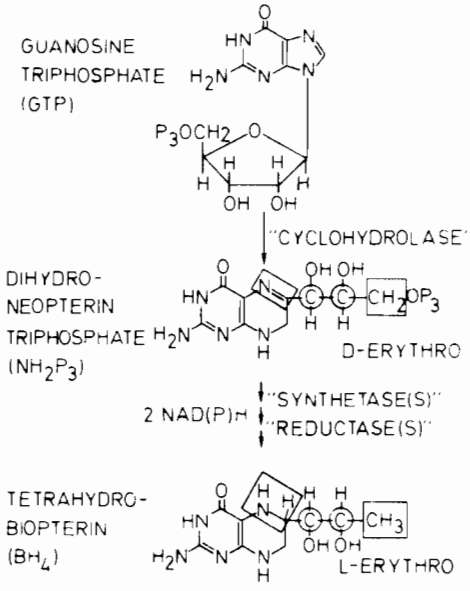

Figure 1 Biosynthesis of tetrahydrobiopterin from guanosine triphosphate. *Note* that only the structure of the molecules shown have been established beyond doubt. The parts of the molecules of NH_2P_3 which are subjected to reduction are denoted by squares, those which undergo inversion by circles

sepiapterin by the heat labile "fraction A_1" which was reported to be NADPH-dependent, and that "*x*" was a diketo-dihydropterin.

Regarding the potential of NADPH dependency of the sepiapterin formation from NH_2P_3, reported by some authors, it is puzzling that NADPH should be considered necessary since there is no net difference in the redox balance between NH_2P_3 and sepiapterin (Heintel *et al.*, 1984). Recently, however, we showed that NADPH is not necessary in human and vertebrates for sepiapterin formation. NH_2P_3 was shown to be converted to sepiapterin without addition of NADPH as well as under conditions that ensured the destruction of endogenous, free NADPH (Heintel *et al.*, 1984). Our results have also indicated that sepiapterin may not be an intermediate on the pathway leading to BH_4 biosynthesis under normal *in vivo* conditions.

Duch *et al.* (1983) demonstrated the formation of BH_4 in the presence of sufficient methotrexate to inhibit dihydrofolate reductase (DHFR) (EC 1.6.99.7) completely. This implied that sepiapterin is not on the biosynthetic pathway. This was also confirmed by Milstien and Kaufman (1983) and ourselves (Heintel *et al.*, 1984). Together with the groups of Kaufman, Nichol and Brown, we suggested the occurrence of internal oxidoreduction reactions leading to tetrahydropterins without the need of NADPH (Heintel *et al.*, 1984; Levine *et al.*, 1983a). This process appears to be thermodynamically feasible (Ghisla *et al.*, 1984).

A comparison of the chemical structures of NH_2P_3 and BH_4 reveals that three distinct chemical transformations are required for their interconversion (see Figure 1): (a) elimination, (b) inversion, (c) reduction.

(a) *Elimination:* The elimination of a leaving group such as (tri)phosphate is a facile chemical reaction requiring a base which catalyzes the abstraction of the C(2′)-H as a proton. Several enzymes catalyzing phosphate elimination reactions are known, and the requirement for Mg^{2+} in reactions involving organic phosphates has been documented. During the reaction one proton from the solvent is introduced in the 3′ position. However, a proton shift occurring at an active site shielded from H^+ exchange with solvent has also been proposed. This is shown in Figure 2. Whether, as in our case, such a shift occurs or not can be verified experimentally. Conservation of the 1′ or of the 2′ hydrogens in the 3′-CH_3 of BH_4 would be consistent with such hypothesis. Conversely, incorporation of (labeled) solvent hydrogen at C(3′) would clearly exclude this type of mechanism. A possible enzyme catalyzing this conversion has been proposed by Tanaka *et al.* (1981), and is referred to as "fraction A_2" which has been shown to require Mg^{2+} for activity.

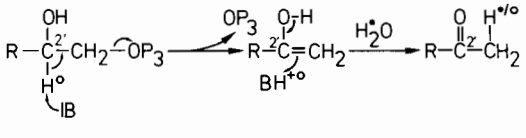

Figure 2. Elimination of triphosphate

(b) *Inversion:* Two basically distinct types of biochemical inversions at chiral centers are known. One type of inversion can be induced directly by two enzyme active center bases and proceeds over a carbanionic transient. Alternatively, and as is likely to occur in our case, redox catalysis is involved and inversion could proceed via a planar sp^2 carbon center (Figure 3).

(c) *Reduction:* The difference in redox state between NH_2P_3 and BH_4 is $4e^-$. Reduction of the C(6)–N(5) imine might, at first sight, be considered a normal hydrogenation reaction, such as those catalyzed by pyridine nucleotide or flavin enzymes. In the case when an intramolecular rearrangement reaction takes place, a keto-group in the 1′ position must be reduced (see Figure 10). The formal reduction at C(3′), on the other hand, might involve a more complicated set of events, i.e. triphosphate elimination to form an enol, ketonization of the latter and finally reduction of the 2′-keto group thus formed. The sequence of these events, elimination, intramolecular oxidoreduction reaction, inversion, and reduction of the keto-groups, is not established. Elimination can be an early event and has been proposed by Krivi and Brown (1979) to initiate the conversion. Milstien and Kaufman (1983), on the other hand, proposed that the sequence starts with the internal redox reaction.

Concerning biosynthesis, the following questions are still unclear:

(1) How is L-biopterin formed from D-neopterin with inversion of two chiral centers at 1′ and 2′? (see Figure 1).

(2) Are quinonoid intermediates or dihydropteridine reductase (DHPR) involved in the biosynthesis?

(3) Is the tetrahydro form of the pterin nucleus formed by an intramolecular redox rearrangement?

(4) What is the mechanism of the reduction steps? Does direct transfer of NADPH hydride occur? If yes, from which (*R* or *S*) side?

(5) What is the role of sepiapterin reductase in the biosynthesis of BH_4. Can it be confirmed that folate reductase is not involved?

To clarify these questions we investigated the biosynthesis with stereospecific deuterium labeled pyridine nucleotides.

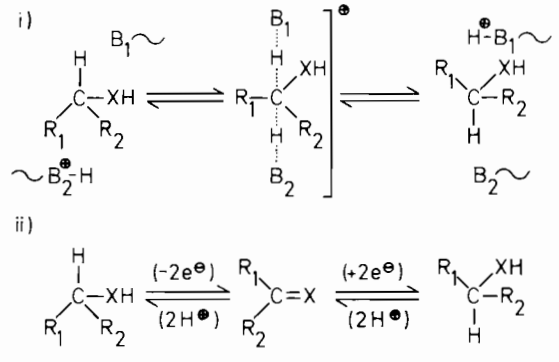

Figure 3 Biochemical mechanisms of inversion at chiral centers. (i) Inversion over a carbanionic intermediate; (ii) inversion via a planar sp^2 carbon center

RESULTS AND DISCUSSION

(1) Isolation of "phosphate-eliminating enzyme"

We have isolated PEE from human liver by $(NH_4)_2SO_4$ fractionation and subsequent purification by several chromatographic columns. This enzyme was capable of synthesizing BH_4, in the presence of partially purified sepiapterin reductase, Mg^{2+} and NADPH. PEE is heat stable at 80 °C for 5 min and has a molecular weight of about 63 000 daltons, as determined by gel chromatography. The enzyme was purified ~ 750-fold. Our results are shown in Table 1.

In an experiment using labeled [α-^{32}P]GTP, we could show that PEE eliminates the phosphates of NH_2P_3, and thus confirms the results of Tanaka *et al.* (1981) obtained with chicken kidney preparations. This suggests that the enzymes are similar in both cases. We succeeded in scaling down the assay of the PEE in order to carry out measurements with liver biopsy material. In controls from adult traffic accident victims we found an activity of the order of 1 nU mg protein^{-1}. In the liver biopsy of a patient with "BH_2-synthetase deficiency" no PEE activity could be detected (Niederwieser *et al.*, 1985).

These experiments allow us to conclude that PEE + Mg^{2+} are necessary for the elimination and sepiapterin reductase + NADPH for the reduction processes. A third factor or enzyme might also be necessary for biosynthesis.

(2) Synthesis of 6-(1′-hydroxy-2′-oxopropyl)5,6,7,8-tetrahydropterin (2′-oxo-tetrahydropterin)

In the search for the intermediate in the biosynthetic pathway, many speculations have been published. Krivi and Brown (1979) proposed a dihydro-2′-keto compound; Suzuki and Goto (1973), and later on Tanaka *et al.* (1981) suggested a dihydro-diketo compound (compound *x*); our group (Heintel *et al.*, 1984), Smith and Nichol (1983), Milstien and Kaufman (1983), as well as Brown (Switchenko *et al.*, 1984) suggested a tetrahydromono or diketo pterin as intermediate. Recently, Smith and Nichol (1984) and Switchenko *et al.* (1984) published electrochemical data and a u.v. spectrum in favour of a tetrahydro compound without, however, proposing any chemical structure. They also claimed that there might be two NADPH-dependent reductases to reduce the keto compounds (Switchenko *et al.*, 1984; Smith and Nichol, 1984). In analogy with the oxidation of BH_2 to sepiapterin in the presence of NADP and sepiapterin reductase at pH 9.0, we attempted the dehydrogenation of BH_4 with sepiapterin reductase from human liver and rat erythrocytes as well as with crude homogenate from human liver (Figure 4). The formation of a new product was demonstrated by HPLC and with electrochemical detection (Figure 5). The u.v. spectrum of the corresponding HPLC fraction was in accordance with that of a tetrahydropterin. Upon reduction with sodium borohydride both erythro and threo BH_4 were formed. When sodium borodeuteride was used instead, a single deuterium was incorporated in the 2′ position of the side chain of BH_4, as shown by GC–MS. These experiments prove the structure of the compound to be 2′-oxo-tetrahydropterin. The reactions of sepiapterin reductase are shown in Figure 6. The further oxidation of the 2′-oxo-tetrahydropterin by sepiapterin reductase and NADP to the postulated tetrahydro-diketo compound (6-pyruvoyl tetrahydropterin) has not yet been observed.

(3) Incubation studies with deuterated (*R*-or-*S*-^{2}H)-NADPH in H_2O and ^2H_2O

Recently, we studied the biosynthesis of tetrahydrobiopterin from either dihydroneopterin triphosphate,

Table 1 Yield of BH_4 after incubation of NH_2P_3 with PEE and sepiapterin reductase after various purification steps

	*PEE, human liver** (pmol BH_4 min^{-1})		
	Hydroxyapatite	*Gel filtration AcA 44*	*Heat treatment after AcA 44* (5 min, 80 °C)
SR, human liver*			
Hydroxyapatite		68	112
Gel filtration AcA 44		58	88
Blue Sepharose CL-6B	148	‡	25
SR, rat erythrocytes†			
Matrex Red A		‡	15

The assay contained the following reaction components (final concentrations) in total volume of 125 µl: NH_2P_3 (0.02 mmol l^{-1}), $MgCl_2$ (8 mmol l^{-1}), NADPH (1 mmol l^{-1}), 10 µl sepiapterin reductase (in excess) and 20 µl PEE in 0.1 mol l^{-1} Tris HCl pH 7.4. After incubation for 1 h at 37 °C the produced BH_4 was determined by HPLC and electrochemical detection (Niederwieser *et al.*, 1984b)

*Sepiapterin reductase was prepared as described by Sueoka and Katoh (1982). On the hydroxyapatite column, PEE could be separated from sepiapterin reductase. Both enzymes were further purified on 2.6 × 70 cm column of AcA 44 in 50 mmol l^{-1} potassium phosphate buffer, pH 6.0. Sepiapterin reductase was afterwards applied to a Blue Sepharose CL-6B column and eluted with NADPH (1 mmol l^{-1}) (Sueoka and Katoh, 1982)

†Sepiapterin reductase from rat erythrocytes was purified according to Sueoka and Katoh (1982)

‡BH_4 formation was ≲ the detection level. More recent experiments (Heintel and Curtius, unpublished) carried out under optimized conditions indicate substantial BH_4 production using highly purified PEE and sepiapterin reductase

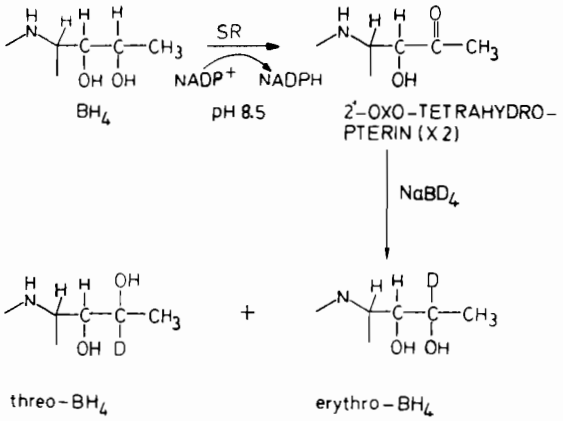

Figure 4 Reverse reaction of BH_4 to X2 with sepiapterin reductase. The reaction mixture contained (final concentrations) in a total volume of 2 ml, *R*-BH_4 (0.08 mmol l^{-1}), $NADP^+$ (0.4 mmol l^{-1}), 60 mU human sepiapterin reductase after purification on AcA 44 in 0.2 mol l^{-1} Tris–HCl pH 9.0. After 30 min at 25 °C, the mixture was acidified to pH 7.0 and the protein separated over Sephadex G 25. The column was equilibrated in degassed H_2O which contained DTE (1 mmol l^{-1}). The fractions which contained the BH_4 and X2 were lyophilized. Afterwards X2 was isolated by HPLC (system described by Niederwieser *et al.*, 1984b). Reduction of the isolated X2 was performed with NaB^2H_4 for 1 min at pH ~8.0 followed by adjustment to pH ~1 with HCl. The produced erythro-BH_4 was purified for GC–MS analysis

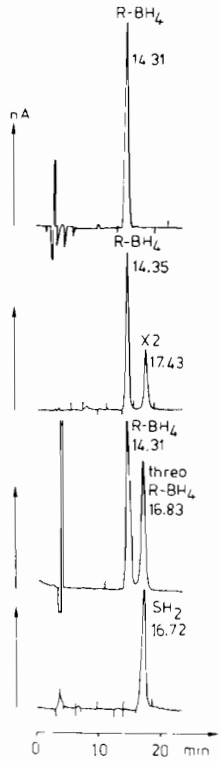

Figure 5 HPLC chromatography of tetrahydropterins with EC-detection. HPLC conditions as described elsewhere (Niederwieser *et al.*, 1984), except that the eluent contained 5% methanol

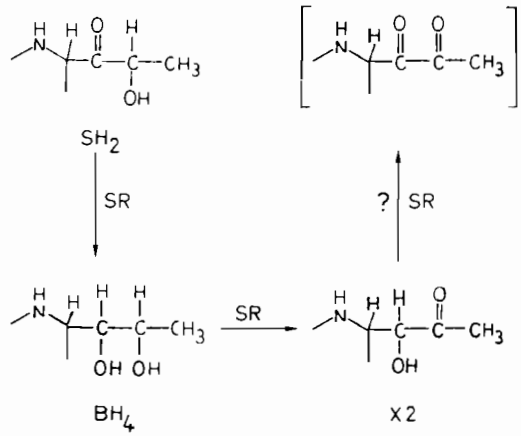

Figure 6 Reactions of sepiapterin reductase

sepiapterin or dihydrobiopterin using extracts from human liver, purified sepiapterin reductase from human liver, rat erythrocytes and dihydrofolate reductase (Curtius *et al.*, 1985). The incorporation of hydrogen in tetrahydrobiopterin was studied in either ^2H_2O or H_2O using deuterated (*R*- or *S*-^{2}H)NADH and deuterated (*R*- or *S*-^{2}H)NADPH. In NADPH-dependent enzyme reactions, the transfer of a hydride ion is stereospecific. Either the pro-*R* or pro-*S* hydride is transferred to the substrate (Figure 7).

Dihydrofolate reductase (DHFR)

In the formation of tetrahydrobiopterin from dihydrobiopterin in the presence of dihydrofolate reductase (DHFR), the pro-*R* hydrogen of NADPH was transferred to the C(6) position of the ring moiety of BH_4. Therefore DHFR is specific for the pro-*R* NADPH hydrogen (Figure 8).

Sepiapterin reductase

Sepiapterin reductase catalyzed the transfer of the pro-*S* hydrogen of NADPH during the reduction of sepiapterin at the side chain position C(1′) to yield dihydrobiopterin (Figure 9).

Conversion of NH_2P_3 to BH_4

During the conversion of NH_2P_3 to BH_4 in the presence of human liver extract, arsenite to inhibit the H/D exchange of NADPH with solvent (see below), NADPH and ^2H_2O, one deuterium was incorporated in the 6-position of the ring. This is consistent with an intramolecular oxidoreduction reaction which is envisaged to occur via tautomerization in the C(6) position of the ring moiety and the C(1′), as shown in Figure 10.

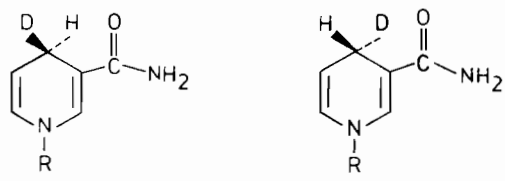

Figure 7 Transfer of either the pro-*R* or pro-*S* hydride in NADPH-dependent enzyme reactions

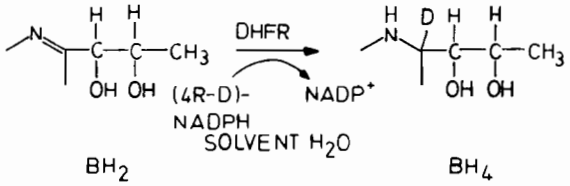

Figure 8 Hydrogen transfer from (4*R*-^{2}H)NADPH by dihydrofolate reductase (substrate BH_2, product BH_4)

During the conversion of NH_2P_3 to BH_4 with crude extract from human liver and deuterated (*R*- or *S*-^{2}H)-NADPH in H_2O, two deuterium atoms were incorporated in the 1′ and 2′ positions, respectively, from the *S* side of deuterated NADPH. This could only be observed after inhibition of diaphorases which were present in the extract. The flavoprotein α-lipoyl dehydrogenase, a diaphorase present in liver tissue, can catalyze a hydrogen exchange between NADPH and H^+ of H_2O (Ernster *et al.*, 1965).

CONCLUSIONS

(1) DHFR has been shown to be specific for the pro-*R* NADPH side in the reduction of BH_2 to BH_4. During the conversion of NH_2P_3 to BH_4 with crude human liver extracts, no deuterium transfer to the ring moiety of BH_4 was found from either deuterated (*S* or *R*) NADPH. This indicates that DHFR is not involved in the biosynthesis of BH_4 from NH_2P_3 and that BH_2 is not an intermediate, which is in accordance with the conclusions of others (Duch *et al.*, 1983).

(2) Our finding of the incorporation of one deuterium from the solvent into position 6 is consistent with the occurrence of an intramolecular oxidoreduction (see Figure 10).

(3) Our results show that during the conversion of NH_2P_3 to BH_4 with deuterated (*S*-^{2}H)NADPH in H_2O, one deuterium atom is incorporated each at the 1′ and the 2′ position of the side chain. This requires that during biosynthesis, the intermediates must carry a diketo side chain at C(6).

(4) The dehydrogenation of BH_4 by sepiapterin reductase to form 2′-oxo-tetrahydropterin indicates that the reverse reaction can also be catalyzed by this enzyme and that sepiapterin reductase could indeed take place in the reduction of the 1′ and the 2′ keto functions.

(5) The PEE appears to be involved in the elimination of phosphate. The exact locus at which

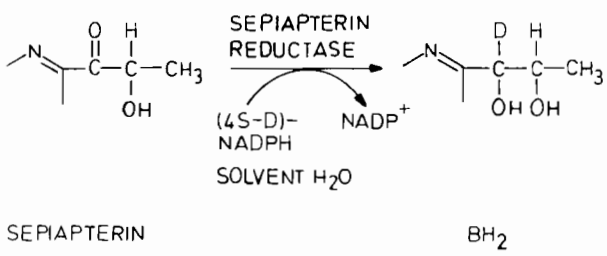

Figure 9 Hydrogen transfer from (4*S*-^{2}H)NADPH by sepiapterin reductase from rat erythrocytes (substrate sepiapterin, product BH_2)

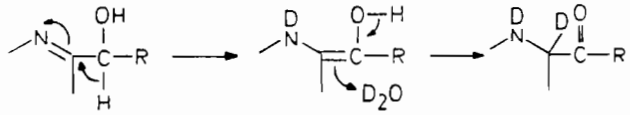

Figure 10 Intramolecular oxidoreduction reaction leading to tetrahydropterins without the need of NADPH

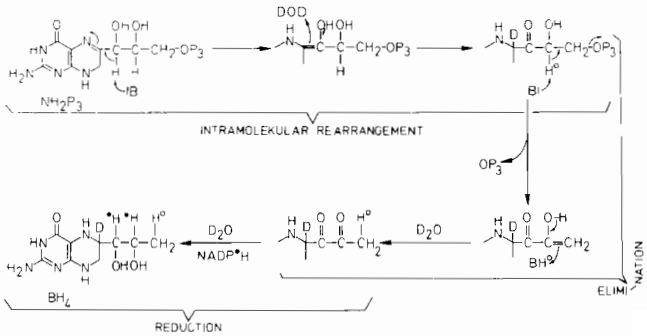

Figure 11 Possible pathway of BH_4 biosynthesis including recent GC–MS results of deuterated NADPH and D_2O studies

elimination occurs in the sequence in Figure 11 cannot be determined by the present experiments.

From these results it can be calculated that the metabolic scheme of Figure 11 may be the most likely.

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