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CHEMICAL CONSIDERATIONS ON THE BIOSYNTHESIS OF TETRAHYDROBIOPTERIN

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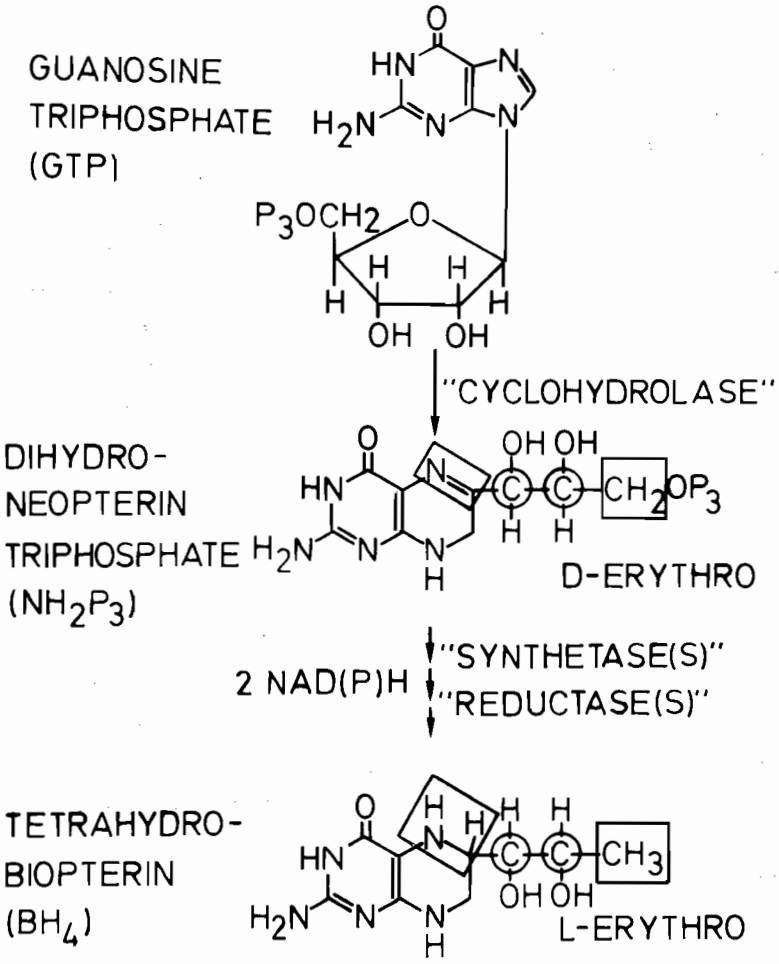
Introduction

Tetrahydrobiopterin (BH_4) is the cofactor of several aromatic amino acid hydroxylases (1) and in this regard may also fulfill an important regulatory role in biogenic amine neurotransmitter synthesis (2). It has also recently been observed that the BH_4 biosynthetic pathway exists in cellular elements involved in immune system function; 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 (3). It is now generally accepted that biosynthesis of BH_4 starts from guanosine triphosphate (GTP), which is converted to 7,8-dihydroneopterin triphosphate (NH_2P_3) (3) (Scheme 1). As already discussed in several previous articles and reviews (3,4), NH_2P_3 is converted in several steps to the final product, BH_4 (Fig. 1). While the conversion of GTP to NH_2P_3 is catalysed by a single enzyme in mammals (5), the further transformation of NH_2P_3 to BH_4 must involve several different proteins (3). It should be pointed out that in the biosynthesis of BH_4, only the structures of GTP, NH_2P_3 and BH_4 as drawn in scheme 1 have been

established beyond doubt. Current discussions and controversies deal mainly with the steps leading from NH_2P_3 to BH_4 (6-12). The purpose of the present contribution is to discuss the conceivable chemical alternatives regarding structures, modes of formation, and the reactions of intermediates occurring in the biosynthesis of BH_4.

Scheme 1



Scheme 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.

Chemical processes required for the conversion of NH_2P_3 to BH_4

A comparison of the chemical structures of NH_2P_3 and BH_4 (Scheme 1) reveals that three distinct chemical transformations are required for their interconversion. They are:

a) ELIMINATION of the (tri)phosphate group at C(3')

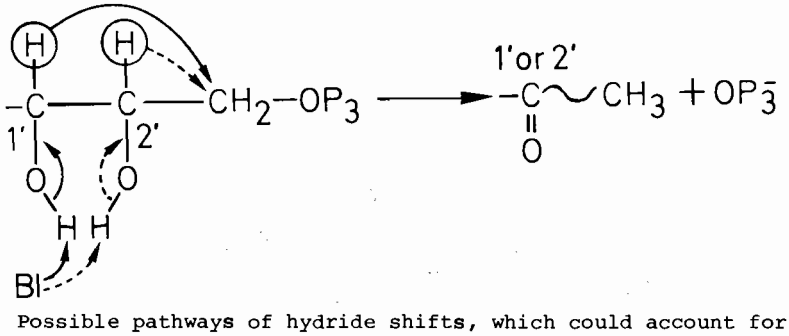
b) INVERSION at C(1'), C(2') and

c) REDUCTION with two hydride equivalents at the positions C(6)=N(5) and C(3').

The enzymatic reactions involved in these conversions will be discussed below:

a) Elimination: The elimination of a leaving group as (tri)phosphate is a facile chemical and biochemical reaction requiring a base, which catalyzes the abstraction of the C(2')-H (as a proton). Several enzymes catalysing phosphate elimination reactions are known (13), and the requirement of Mg^{2+} in reactions involving organic phosphates has been documented. However, the present case cannot be compared directly with simple elimination reactions since the final product is not at the oxidation level of an enol (or of the corresponding methylketone), but at that of a hydroxyethyl group. Its formation requires a concomitant or subsequent reduction step. A direct reductive elimination of a (tri)phosphate by formal substitution with a hydride from an exogenous donor at C(3') would be unprecedented, and appears very unlikely. On the other hand, a concerted elimination involving a 1-2 or a 1-3 hydride shift is also conceivable as shown below.

Scheme 2

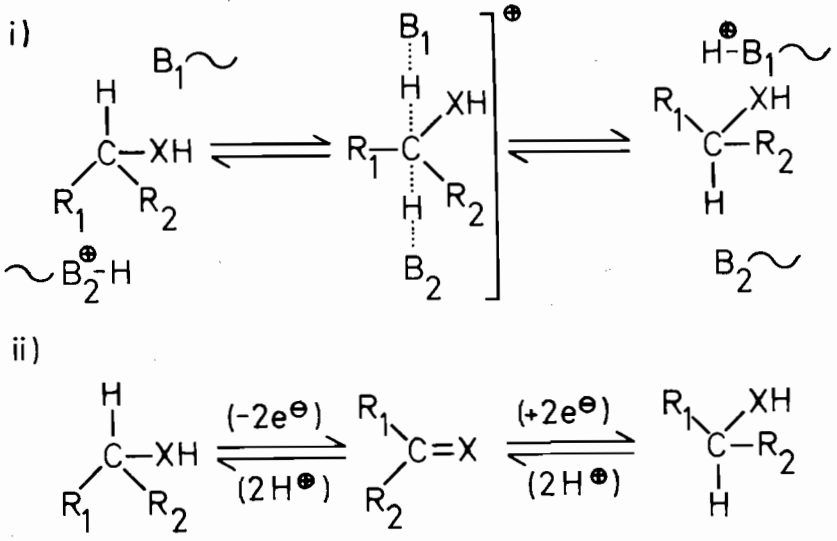


Scheme 2 Possible pathways of hydride shifts, which could account for reductive elimination of triphosphate from NH_2P_3.

Glyoxylase has been suggested (14) to function by such a mechanism. However, a proton shift occurring at an active site shielded from H^+ exchange with solvent has been proposed as the better alternative (14). Whether in our case such a shift occurs or not could 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 (labelled) solvent hydrogen at C(3') would clearly exclude this type of mechanism. A possible enzyme catalysing this conversion has been proposed by Tanaka et al. (8), and referred to as enzyme A_2, which has been shown to require Mg^{2+} for activity.

b) Inversion: Two basically distinct types of biochemical inversions at chiral centers are known, and have been discussed extensively by Walsh (15). One type of inversion can be induced directly by (two) enzyme active center bases and proceed over a carbanionic intermediate (Scheme 3, (i)). Alternatively, and as is likely to occur in our case, redox catalysis might be involved and inversion could proceed via a planar sp^2 carbon center (ii):

Scheme 3



Scheme 3 Biochemical mechanisms of inversion at chiral centers.

Pyridine nucleotides, and pyridoxal phosphate are the two organic cofactors which have been reported so far as being required in this type of reaction (16). Comparison of the types of classical racemase and isomerase reactions with the present one (i.e. the conversion of NH_2P_3 to BH_4) reveals a peculiarity. In the classical type reaction, the conversion of one enantiomer to a racemic mixture occurs, whereas the conversion of NH_2P_3 to BH_4 requires a complete conversion to the opposite enantiomer, at least with respect to the configuration of the C(1') and C(2') centers. Clearly, the thermodynamics of the overall process requires that inversion must be coupled to a chemical event that can provide the driving force for the exclusive generation of the L-erythro configuration. Possible combinations of such reactions will be considered below.

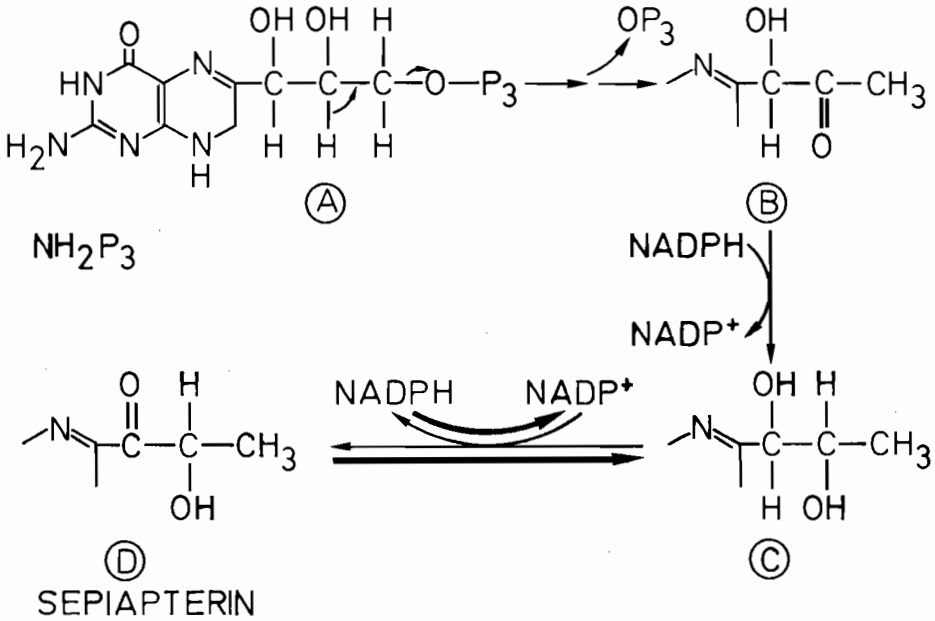
c) Reduction: The difference in redox state between NH_2P_3 and BH_4 is $4e^-$. Reduction of the C(6)=N(5) imine might, at first sight, be considered a normal hydrogenation reaction, such as those catalysed by pyridine nucleotide or flavin enzymes. The formal reduction at C(3'), on the other hand might involve a more complicated set of events, as already pointed out in section a). In addition, this case also requires the reduction of a secondarily formed 2'-keto (enol) function, which might be carried out by a nicotinamide or flavin cofactor. A further point regards the sequence of these events (elimination, inversion, and reduction). Elimination can reasonably be assumed to be an early event and has been proposed by Krivi and Brown (10) to initiate the conversion. Heintel et al. (4) as well later Milstien and Kaufman (17), start the sequence with an internal redox reaction. The implications of the sequence of these events will be discussed below.

Critical comments on some pertinent biochemical literature

A survey of recent literature on the transformations of NH_2P_3 reveals a series of inconsistencies. A major one regards the proposed requirement of pyridine nucleotide cofactors for the conversion of NH_2P_3 to sepiapterin, (and further to dihydrobiopterin and BH_4). We recently have discussed this point extensively (4) and have shown that the conversion of NH_2P_3 to sepiapterin, formerly a presumed intermediate in BH_4 biosynthesis, does not require reducing equivalents (NAD(P)H), which is in contrast to some

literature reports (7,8). On the other hand, overall conversion of NH_2P_3 to the final product BH_4 requires $4e^-$ (donated by reduced pyridine nucleotides). The proposal that sepiapterin is an intermediate in BH_4 biosynthesis has been put forward by a number of investigators, including Krivi and Brown (10), who studied the D. Melanogaster system, in which various pterins have been found that serve as pigments. This work was one of the first attempts to propose a detailed pathway for BH_4 biosynthesis. The essential steps of this sequence are shown in Scheme 4:

Scheme 4



Scheme 4 Biosynthesis of sepiapterin according to Krivi and Brown (10). In this symplified scheme NADPH is proposed to play a catalytic role in the formal transfer of hydride from C(2') to C(1').

In this scheme, elimination of the triphosphate moiety followed by ketonisation of the enolate, initiates the transformation of NH_2P_3. This step chemically poses no problems in that the incorporation of one H^+ could come from the solvent (10), or, if H^+ exchange at the enzyme active center does not occur, incorporation of the 2'H into 3' will result. Both events should be accessible to experimental verification.

The next steps involve a formal hydride shift from 2' to 3' and have been proposed to consist in an oxidation/reduction by NADPH/NADP (10). Some of the interpretations of these authors (10), however, can be questioned: Although a yield of up to 10 % sepiapterin (calculated from NH_2P_3) is reported in the presence of a $\sim$1000 fold excess of added NADPH, some sepiapterin ($\sim$1.5 %) is also formed in the absence of this coenzyme. Whether this is a clear cut argument in favour of NADPH requirement can at least be questioned, in particular in view of the contradictory findings mentioned above (4). However, the possibility cannot be dismissed that in Drosophila and in mammals different BH_4 biosynthetic pathways exist. Puzzling is also the lack of stereospecificity of label incorporation from either (R) or (S) NADP^{3}H. A rationale suggested by those authors (10) is that some "scrambling" will occur due to the formation of (unlabelled) NADPH in the step from (C) to sepiapterin (D) (Scheme 4). However, the ratio (labelled) NADPH/NH_2P_3 used by these authors was $\sim 10^3$. NADPH formed by this route should be stoichiometric with sepiapterin formed, i.e., of the order of a few % of selectively labelled NADP^{3}H added to the system. The alternative, that the NADPH formed in step (C)$\rightarrow$(D) is extremely tightly bound by the enzyme and does not dissociate between two catalytic events, is rather unlikely.

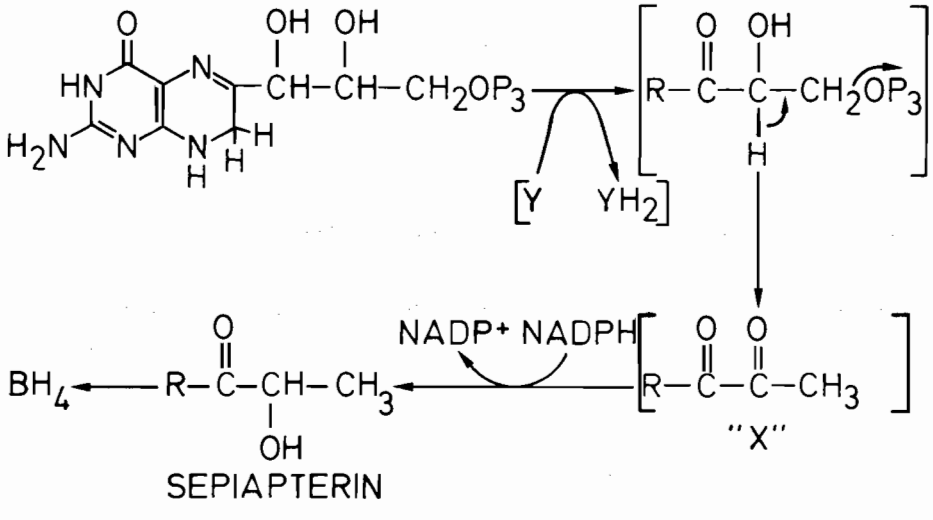
In the presence of catalytic amounts of NADPH/$NADP^+$, sepiapterin formation (D) (Scheme 4) is thermodynamically unfavoured, the equilibrium constant K being in favour of keto group reduction. Thus, from the values of Sueoka and Katoh (2), and the corresponding equation

$$K = \frac{(BH_2)(NADP^+)}{(\text{Sepiapterin})(NADPH)(H+)} = 2 \times 10^9$$

at pH 8 and at equimolar concentrations of NADPH/$NADP^+$ an equilibrum ratio of 20 ~ BH_2:Sepiapterin can be calculated. Consequently, under the conditions of Krivi and Brown (10), i.e. pH 7.5 and a large excess NADPH, only (C) should be formed to an appreciable extent (Scheme 4). In this context it is also interesting to note that Parisi et al. (24) have formulated primary formation of BH_2 from NH_2P_3 and its subsequent conversion to sepiapterin in melanogaster.

A somewhat different BH_4 biosynthetic pathway was proposed in 1981 by Tanaka et al. (8). While this work clearly stands out in its quality among recent publications on the subject, some particular points are still unclear. First, sepiapterin, which was proposed as an intermediate, also was obtained in comparatively small yields, and one might again wonder whether it is not the product of a side reaction, as has recently been suggested by several laboratories (17,3). One focal point in the work by Tanaka et al. (8) is the occurrence of a compound X as an intermediate, which is characterized by its 2',3'-diketopropyl side chain.

Scheme 5



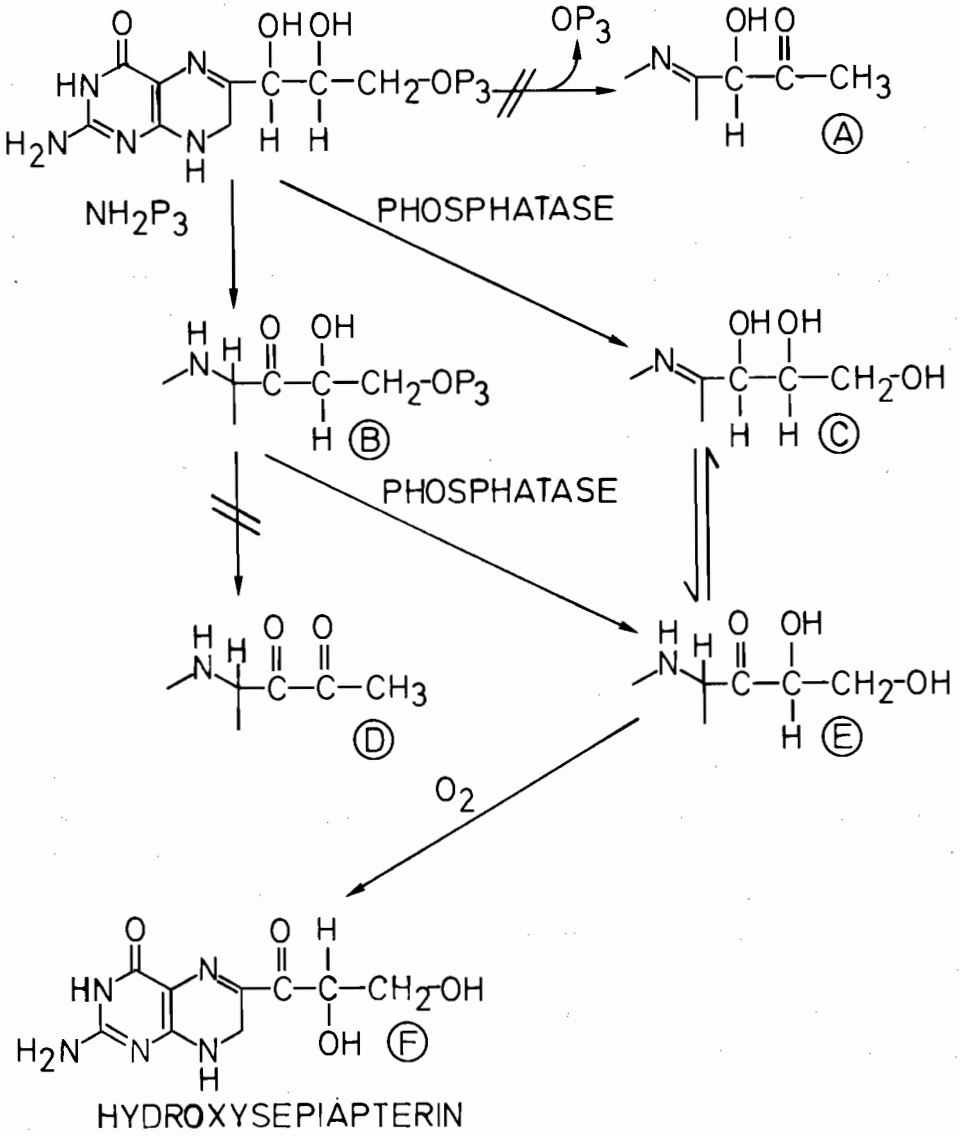
Scheme 5 Biosynthesis of BH_4 according to Tanaka et al. (8)(simplified scheme). This proposal is characterized by the occurrence of a postulated diketo function in the C(6)-side chain.

The evidence in support of structure "X" (Scheme 5), consists of the trapping of pyruvic acid with dinitrophenylhydrazine in 2 N HCl at 80° upon cleavage of the side chain. Under these conditions, oxidation of a primary intermediate cannot be excluded. The proposal (8) might nevertheless contain the proverbial grain of truth. In fact, a 2',3'-diketo-tetrahydropterin intermediate as proposed more recently by others (4,11,17) might undergo oxidation under the trapping conditions described in (8) to yield "X". Further, in the proposal of Scheme 5, the occurence of an acceptor Y is suggested, the function of which would be the oxidation of the 1'-OH group. Enzymes known to catalyse such conversion in higher organisms are pyridine nucleotide, flavin, or under specific conditions, metal containing enzymes. We consider it unlikely (although not impossible) that in this specific case an enzyme other than those mentioned above might be operative. In either case, however, reduced Y (YH_2, Scheme 5) has to donate its redox equivalents to a further acceptor since it would be present only in catalytic amounts. A logical candidate for this role is oxygen, since no further acceptors were present in their system (8). However, recent results (4) indicate that sepiapterin can be formed also under anaerobic conditions, thus it is not likely that O_2 is directly involved.

Information arising from metabolic studies

Recently, Niederwieser et al. (25), identified **3'-hydroxysepiapterin** (F) in relatively large quantities in the urine of patients having a metabolic defect in the biosynthesis of BH_4 occurring subsequent to NH_2P_3. In the same patients, considerable quantities of monapterin were also observed (26). Clearly, while such information has to be interpreted with caution, it is tempting to speculate, that one specific function (or enzyme) is being affected by the metabolic defect, and that this is the elimination of (tri)phosphate (Scheme 6, NH_2P_3 to (A)). In contrast to this, a second enzyme might still be functional and affect the chemistry at C(2'). Which enzyme first attacks NH_2P_3 is difficult to predict. At first sight it might be speculated, that in metabolically defective individuals the conversion of NH_2P_3 to (B) is still functioning, while the elimination ((B)→(D)) does not occur. Formation of 3'-hydroxysepiapterin can be envisaged as

Scheme 6



Scheme 6 Scheme showing the possible pathways leading to formation of hydroxysepiapterin in individuals having specific metabolic diseases. In this scheme (F) would be formed via side reactions of intermediates, which accumulate due to the lack of enzymatic activity leading to phosphate elimination, i.e. formation of (A).

occurring after phosphatases have converted NH_2P_3 to (C), which, due to its analogy to the postulated primary intermediate (A), might be a substrate of the second still active enzyme and be converted to (E). The latter, in turn, can be envisaged as yielding hydroxysepiapterin (F) upon autoxidation if phosphatases could also convert (B) to (E). Clearly, while the pathways of Scheme 6 should be regarded as speculative, results from metabolic diseases have to be accomodated in valid biosynthetic pathways. It is felt that the study of these rare patients will contribute to the solution of the overall "puzzle" of BH_4 biosynthesis.

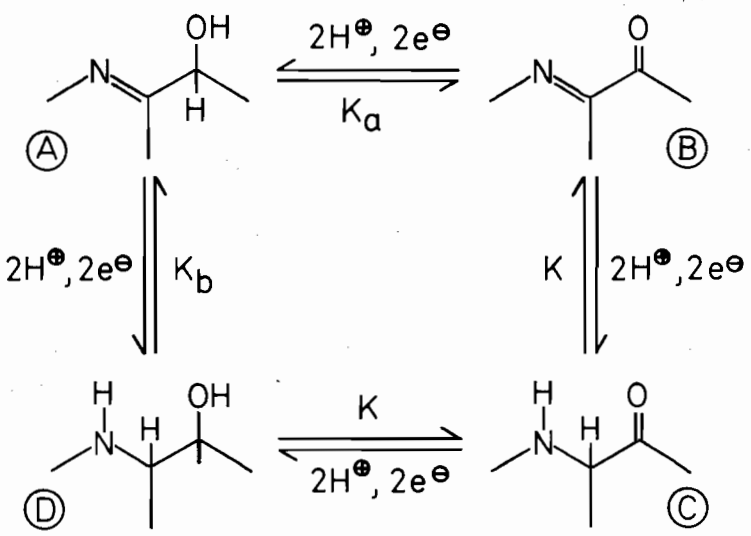
Recently proposed alternative biosynthetic pathways

As pointed out above, realizing the inconsistencies in the literature requirements of pyridine nucleotide cofactors, recently Heintel et al. (4) could show that using partially purified human liver extracts, minor quantities of sepiapterin are indeed formed in the absence of these cofactors and of O_2. The yield of sepiapterin was lowered in the presence of NADPH, while that of BH_4 was increased considerably, indicating a partitioning reaction. This led to the proposal of an alternate pathway, in which sepiapterin is bypassed and a tetrahydro-2',3'-diketo intermediate occurs (4). Similar conclusions were reached independently by Smith and Nichol (11) who have shown, that BH_4 biosynthesis in bovine adrenal medulla is feasible also in the presence of sufficient methotrexate to inhibit dihydrofolate reductase, the enzyme supposed to convert BH_2 to BH_4. They concluded, that BH_4 can be synthesized by a pathway not involving sepiapterin, and proposed that two alternate pathways can lead to BH_4 (11). Following these ideas, Kaufman's group, who in the past affirmed the involvement of sepiapterin (27), and now also state that "tetrahydro-sepiapterin is an intermediate in BH_4 biosynthesis" (17). In conclusion, the three publications mentioned in this section all postulate that new intermediates, probably of the tetrahydro type are likely to occur. They might be formed via an internal redox reaction and elimination as will be detailed in the next section.

Is an internal redox transfer involving reduction to the tetrahydropterin and oxidation of the side chain feasible?

The essence of this mechanism is outlined in Scheme 7:

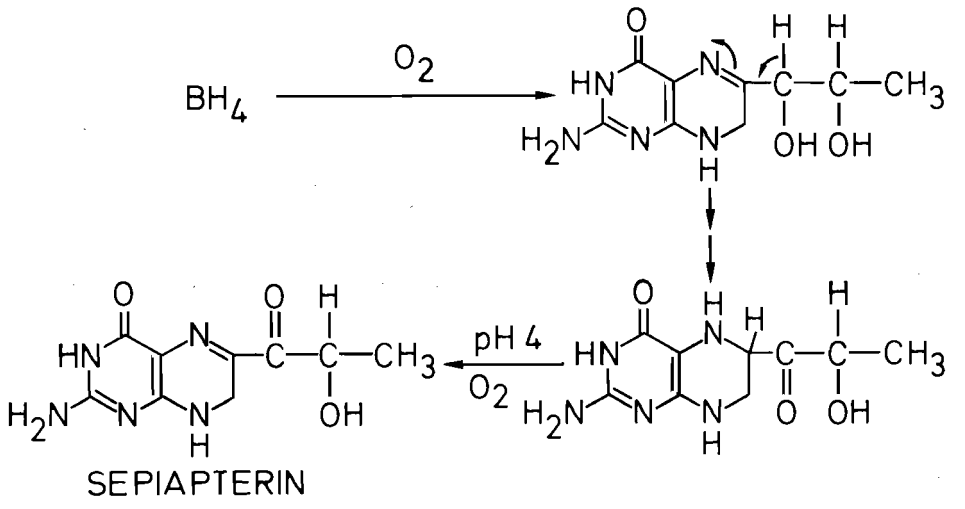
Scheme 7



Scheme 7 Combination of single redox steps leading to exchange of redox equivalents between the dihydropteridine nucleus and the C(6) side chain.

The basic question regarding this type of rearrangement is the position of the equilibrium between species (A), and (C) that is induced by the enzyme. Such reactions are well known in organic chemistry. Pertinent examples are keto/eneamine-enol/imine shifts observed in sugar chemistry (28). In the context of pteridine chemistry, a remarkable reaction was published in 1979 by Pfleiderer (22), in which BH_4 is oxidized to sepiapterin in good yields by O_2 in neutral to weakly acidic media (Scheme 8).

Scheme 8



Scheme 8 Sequence of reactions proposed to be involved in the formation of sepiapterin from BH_4 according to Pfleiderer (22).

Pfleiderer proposes (22) that BH_4 is first oxidized to BH_2, which then undergoes a Lowry de Bruyn-Alberda van Eckenstein-type transformation to generate 6-lactoyl-tetrahydropterin. The latter is oxygen sensitive, and can readily oxidize to sepiapterin (22) (Scheme 8). However, when BH_2 is allowed to stand anaerobically under analogous pH conditions, spectral changes that would be indicative of formation of a tetrahydropterin cannot be observed (Heintel, D., Curtius, H.-Ch., unpublished). In such a redox "rearrangement", applicable thermodynamics principles must be examined. The overall reaction of scheme 7 might be subdivided in two half-cells, the first (a) being an oxidation of a hydroxyacetone (or 1,2-diol) to the dioxopropyl (or to the lactoyl) moiety, and the second (b) dealing with the reduction of a dihydrobiopterin to the tetrahydro form.

$$-\underset{\underset{OH}{|}}{CH}-CHR \overset{(a)}{\rightleftharpoons} -\underset{\underset{O}{\|}}{C}-CHR + 2\ H^+ + 2\ e^- \qquad (R = H_2, H+OH \text{ or } =O)$$

$$2\ e^- + 2\ H^+ + -N=C-R \overset{(b)}{\rightleftharpoons} -\underset{\underset{H}{|}}{N}-\underset{\underset{H}{|}}{C}-R$$

From the equilibrium constant for the reduction of sepiapterin with NADH (20),

$$K = \frac{(BH_2)(NADP^+)}{(\text{Sepiapterin})(NADPH)(H^+)} = 2\times10^9$$

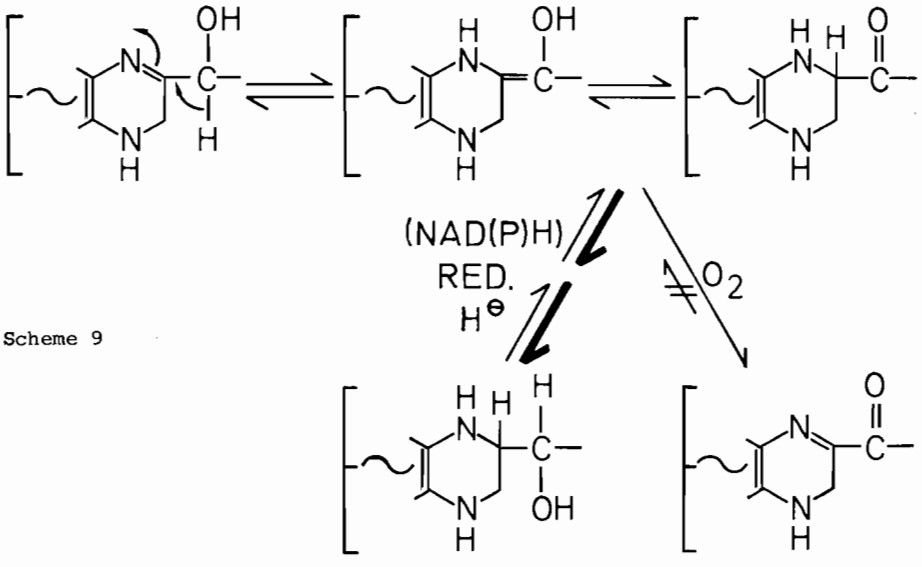
the potential change, $\Delta E^{\circ\prime}$ may be estimated at pH 7 from the relation $\Delta E' = (RT/nF)\ln(K) - 110$ mV. Combination of this value with the redox potential of the $NADH/NAD^+$ couple ($E^{\circ\prime} = -320$ mV (23)), allows the estimation of the redox potential for the sepiapterin reduction as - 210 mV. The equilibrium of the reduction of dihydropterin to tetrahydropterin, $K_{"b"}$ has been estimated (29,30,31) as 5×10^{11} for dihydrofolate/tetrahydrofolate, which corresponds to a redox potential of the couple $\sim$ -180 mV. It is reasonable to assume that the redox potential for a couple dihydropterin/tetrahydropterin will be similar in the case of similar substituents at C(6), and in fact folate reductase is able to catalyze the reduction of BH_2 to BH_4. If now one makes the assumption that no reciprocal influence exists between the two halves of the molecule, then $K_b \simeq K_c$ and reactions (a), and (b) can be combined to yield a pH independent equilibrium:

$$K_{eq} = \frac{(\text{lactoyl})(\text{tetrahydropterin})}{(\text{diyhdroxy})(\text{dihydropterin})} = \frac{K_{"b"}}{K_a} = 250$$

The value of K_{eq} corresponds to a difference in redox potential $\Delta E' \simeq 30$ mV. This clearly would indicate that the equilibrium (A) $\rightleftharpoons$ (C) of scheme 7 is in favour of species (C). It should be stated that the neglecting a reciprocal influence of the redox state of the side chain on the redox potential of the dihydro/tetrahydro couple (and vice versa) is entirely arbitrary. However, a rough estimation of the effect on the redox potential induced by variation of an α-substituent can be attempted by comparison with analogous systems. Thus for the oxidoreduction of =O/OH, the following values are known (23):

Hydroxypyruvate/Glycerate	E°' = - 160 mV
Oxaloacetate/Malate	E°' = - 165 mV
Pyruvate/Lactate	E°' = - 190 mV
Acetaldehyde/Ethanol	E°' = - 195 mV

This comparison indicates that the reciprocal effect might alter the redox potential of the couples of Scheme 7 by up to + 50 mV and more probably by approximately 20-30 mV. In any event, the conclusion from this discussion is that such an internal conversion is feasible. Binding to an enzyme active center might shift equilibria such as those on scheme 7 by several orders of magnitude. Trapping of such a species at the enzyme active center by reduction with NAD(P)H clearly would facilitate the conversion by shifting the equilibria (Scheme 9).



Scheme 9

Scheme 9 Modes of trapping of the metastable intermediates in a biosynthetic pathway not requiring the involvment of sepiapterin.

Predictions on the incorporation of labelled hydrogen during biosynthesis of BH_4

The different mechanistic alternatives outlined above might lead to different patterns of incorporation of labelled hydrogen either from H_2O or from NAD(P)H.

In all proposals, with exception of direct hydride shifts mentioned above, one *H from H_2O will be incorporated into C(3'). In Tanaka's Scheme (Scheme 5) as well as in Krivi and Brown's proposal (10) (Scheme 4), C(6), C(1'), and C(2') should each incorporate one *H provided reduction occurs directly from NAD(P)*H. On the other hand, were biosynthesis to proceed via a 6-dioxopropyl-tetrahydro intermediate, then the hydrogen at C(6) might be incorporated from water (Scheme 9).

In such a case, direct transfer of label to C(1') and C(2') might also result. Alternatively, the reduction might also occur at the level of an enolate intermediate (Scheme 9). In this case, attack of hydride could occur at any of the centers N(5), C(6), C(1'), and C(2'). In the case of hydride addition at N(5), no incorporation of label will result, as protons at this center will exchange readily. On the other hand, only two hydride equivalents might be introduced into the system via N(5). In any case the last proton originating from a hydride must end up at a nonexchangeable carbon atom. Finally the possibility also must be considered, that NADPH does not reduce directly, but via some intermediary such as a flavin enzyme, as in the case of methyltetrahydrofolate reductase.

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