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RAPID IMPORTANT PAPER

BIOSYNTHESIS OF TETRAHYDROBIOPTERIN: POSSIBLE INVOLVEMENT OF TETRAHYDROPTERIN INTERMEDIATES

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Abstract - The biosynthetic pathway of tetrahydrobiopterin (BH_4) from dihydroneopterin triphosphate (NH_2P_3) was studied in fresh as well as heat-treated human liver extracts. The question of NAD(P)H dependency for the formation of sepiapterin was examined. NH_2P_3 was converted by fresh extracts to sepiapterin in low quantities (2% conversion) in the absence of exogenously added NADPH as well as under conditions that ensured the destruction of endogenous, free NAD(P)H. The addition of NADPH to the fresh liver extracts stimulated the synthesis of BH_4 to a much higher yield (17% conversion), and the amount of sepiapterin formed was reduced to barely detectable levels. In contrast, the heat-treated extract (enzyme A2 fraction) formed sepiapterin (1.3% conversion) only in the presence and not in the absence of NADPH. These results indicate that sepiapterin may not be an intermediate on the pathway leading to BH_4 biosynthesis under normal *in vivo* conditions. Rather, sepiapterin may result from the breakdown of an as yet unidentified intermediate that is actually on the pathway. It is speculated that NH_2P_3 may be converted to a diketo-tetrahydropterin intermediate (or an equivalent tautomeric structure) by a mechanism involving an intramolecular oxidoreduction reaction. A diketo-tetrahydropterin intermediate could be converted to 5,6-dihydrosepiapterin, which also has a tetrahydropterin ring system and can be converted directly to BH_4 by sepiapterin reductase. This proposed pathway can explain how the tetrahydropterin ring system can be formed without sepiapterin, dihydrobiopterin, or dihydrofolate reductase being involved in BH_4 biosynthesis *in vivo*.

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INTRODUCTION

Despite a number of studies on tetrahydrobiopterin (BH_4) biosynthesis in mammals, several controversial aspects have yet to be explained. BH_4 is the cofactor for phenylalanine, tyrosine, and tryptophan hydroxylases (Kaufman, 1974; Lovenberg et al., 1978) and it has been suggested that BH_4 plays an important role in regulating the synthesis of the biogenic amine neurotransmitters (Levine et al., 1983). Errors in BH_4 biosynthesis and metabolism have been extensively studied in the rare childhood disease known as BH_4-deficient hyperphenylalaninemia (also referred to as atypical phenylketonuria) (Niederwieser et al., 1982a) as well as in certain neurological and psychiatric disorders (Levine et al., 1983)). A more complete understanding of the enzymatic steps in BH_4 biosynthesis could help explain a potential involvement of altered BH_4 metabolism in certain disease processes.

Most available evidence suggests that the first reaction in mammalian BH_4 biosynthesis involves the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate (NH_2P_3) by a single enzyme, GTP cyclohydrolase I. However, Gál and co-workers (1978 a, b) have proposed that in rat brain, GTP is converted by a cyclohydrolase to a pyrimidine intermediate, which is transformed by a synthetase enzyme to the quinoid (q-) form of NH_2P_3. Our recent studies on BH_4 biosynthesis in human liver (Blau and Niederwieser, 1983) do not support a two-enzyme hypothesis for NH_2P_3 formation in mammals, since no pyrimidine intermediate could be detected after the reaction of GTP with purified human GTP cyclohydrolase I.

The enzymatic reactions involved in the formation of BH_4 from NH_2P_3 are even more controversial. Work in several laboratories using chicken kidney (Tanaka et al., 1981), rat and human liver (Häusermann et al., 1981), and Drosophila (Krivi and Brown, 1979; Dorsett et al., 1982) has suggested that NH_2P_3 is converted through some intermediate "X" to L-sepiapterin. Evidence has also been presented that sepiapterin is an intermediate in BH_4 biosynthesis in rat brain (Kapatos et al., 1982). However, whether sepiapterin synthesis requires NAD(P)H (Figure 1) or what the exact structure is for compound "X" is not clear among various laboratories. For instance, Tanaka and co-workers (1981) reported that NH_2P_3 was converted to compound "X" by a heat-stable, magnesium-dependent enzyme, A2. It was suggested that "X" was converted to sepiapterin by the heat-labile enzyme, A1, which was reported to be NAD(P)H dependent. Conversely, Otsuka and co-workers (1980) reported sepiapterin formation without the addition of NAD(P)H. Regarding the potential NAD(P)H dependency (Figure 1) of sepiapterin formation from NH_2P_3, it is puzzling that NAD(P)H should be considered necessary since there is no net difference in the redox balance between NH_2P_3 and sepiapterin. Also, it is not clear why the reported (Tanaka et al., 1981; Häusermann et al., 1981) percentage conversion of NH_2P_3 to "X" or sepiapterin is so low in most systems.

In the proposed BH_4 biosynthetic pathway having sepiapterin as an intermediate, sepiapterin can be converted to dihydrobiopterin (BH_2) by sepiapterin reductase in the presence of NAD(P)H (see references in Figure 1). According to this scheme (Kaufman, 1967), BH_2 is converted to BH_4 by dihydrofolate reductase (DHFR), a reaction that also requires NAD(P)H. Just as it seemed that sepiapterin and BH_2 were becoming established as

intermediates in BH_4 biosynthesis, novel work was presented by Duch and co-workers (1983) that demonstrated the formation of BH_4 in the presence of sufficient methotrexate (MTX) to inhibit DHFR completely. This implied that BH_4 biosynthesis could occur without sepiapterin and BH_2 as intermediates, and that there may be two possible pathways in vivo. Indeed, they have proposed (Nichol et al., 1983) that sepiapterin is not on the main biosynthetic pathway. Also, Gál and co-workers (1978 a,b) have proposed a pathway for BH_4 biosynthesis that doesn't involve sepiapterin (Figure 1). Accordingly, there is some doubt about the involvement of sepiapterin as an intermediate in BH_4 biosynthesis (Nichol et al., 1983; Curtius et al., 1983).

Therefore, the following controversial questions remain: 1) Is the formation of sepiapterin from NH_2P_3 dependent on NAD(P)H, in spite of the equivalent redox state of NH_2P_3 and sepiapterin; 2) What is a possible explanation for the very low percentage conversion of NH_2P_3 to either "X" or sepiapterin; and 3) Is sepiapterin an intermediate on the BH_4 biosynthetic pathway; if not, what is the nature of the true intermediate that

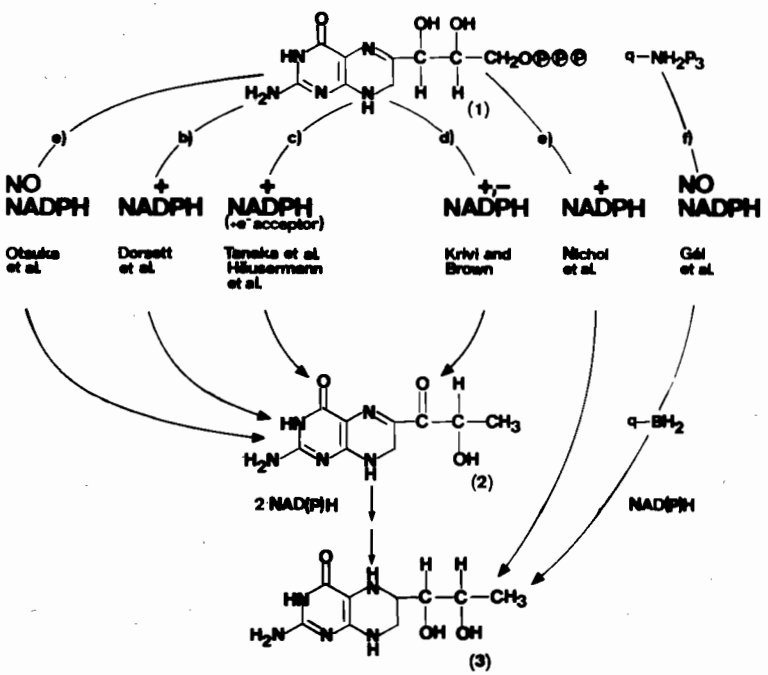

Figure 1.- Various proposals for biosynthetic pathways from dihydroneopterin triphosphate (1) to sepiapterin (2) and tetrahydrobiopterin (3). In the formation of sepiapterin, the involvement of NADPH is controversial. Tetrahydrobiopterin formation has also been shown to occur without sepiapterin as an intermediate (2 pathways on right). Gál and coworkers (1978a,b) have suggested that dihydroneopterin triphosphate exists in the quinoid form ($q\text{-}NH_2P_3$) and is converted to quinoid dihydrobiopterin ($q\text{-}BH_2$) without requiring NADPH.

could lead to sepiapterin formation. Our studies on BH_4 biosynthesis using human liver extracts and the enzyme A2 fraction have attempted to clarify some of these issues. We selected a human system for synthesis studies because of the known difference in pterin metabolism in humans as compared to lower mammals such as the rat. Specifically, neopterin has only been detected in man (Fukushima and Nixon, 1980) and monkeys but not in the lower mammals tested thus far. This suggests that there is a fundamental difference between primates and certain lower mammals in the regulation of the enzymatic steps after NH_2P_3, which is our rationale for selecting a human system for the study of BH_4 biosynthesis.

EXPERIMENTAL PROCEDURES

Materials

All pterins except dihydroneopterin triphosphate (NH_2P_3) were purchased from Dr. B. Schircks Laboratory, Wettswil A.A., Switzerland. NH_2P_3 was prepared enzymatically by incubation (90 min at 42°C in a total volume of 0.5 ml) of 0.2 mM GTP, 0.1 M NaCl, 0.01 M EDTA, 0.1 M Tris-HCl (pH 8.5) and GTP cyclohydrolase I purified from E. coli. This reaction achieved at least 90% conversion of GTP to NH_2P_3 as measured by TLC and HPLC (Blau and Niederwieser, 1983).

Old Yellow Enzyme, which catalyzes the oxidation of NAD(P)H to NAD(P) (Kaplan, 1955), was a gift from Dr. G. Wetzel, University of Constance, Federal Republic of Germany. NADase, which irreversibly hydrolyzes NAD(P) (Abramovitz and Massey, 1976), was obtained from Sigma Chemical Co.

Human liver was obtained from traffic accident victims, frozen within 1 hour, and stored at -70°C. All other reagents were commercially available.

Purification of enzymes

GTP cyclohydrolase I from E. coli was purified according to Yim and Brown (1976) and was used to generate the substrate NH_2P_3 as described above.

The "standard extract" used for biosynthesis studies was prepared in the cold by the following procedures. Human liver was homogenized in 3 volumes of 25 mM Tris-HCl (pH 7.4). After centrifugation at 27,000 x g for 45 min, the supernatant was fractionated with ammonium sulfate between 40 and 65% saturation. The supernatant was applied to a Sephadex G-25 column that was equilibrated with the same buffer. The initial fractions from the gel column were pooled to comprise the "standard extract". The combination of the ammonium sulfate and G-25 column steps should remove most of the endogenous, free NAD(P)H.

A fraction containing the heat stable enzyme, A2, was prepared according to Tanaka et al. (1981), with the following modification. The "standard extract" was heated at 80°C for 5 min. The supernatant was used as a source of the A2 enzyme.

Sepiapterin reductase was partially purified from human liver according to the method of Sueoka and Katoh (1982), except that only the first two column steps were performed.

Biochemical assays

The reaction mixture for studying NH_2P_3 transformations contained the following reaction components (final concentration) in a total volume of 0.5 ml: 25 mM Tris-HCl

(pH 7.4), 20 μM NH_2P_3, 14.0 mM $MgCl_2$ and the fresh or heat-treated "standard extract" (enzyme A2). This mixture was incubated for 15 min at 42°C at which time it was divided into 2 parts. One part was immediately treated at 100°C for 5 minutes to stop the reaction, centrifuged and analyzed for sepiapterin and "X" by high-pressure liquid chromatography (HPLC). The other part was immediately subjected to manganese dioxide (MnO_2) oxidation and analyzed for neopterin, biopterin and pterin content by HPLC, as described below.

To test for the influence of oxygen on the reaction products, certain samples were reacted under anaerobic conditions. Briefly, nitrogen was bubbled through the samples prior to starting the reaction and glucose oxidase (40 U/ml) and glucose (0.06 M, final concentration) were added to consume any residual oxygen as previously described (Fan and Brown, 1978).

Protein content was measured by the method of Bradford (1976), using bovine gamma globulin as a standard.

Pterin oxidation methods and HPLC analysis

For measuring neopterin, biopterin and pterin in the reaction products, 200 μl of each sample was acidified with 1.0 N HCl (pH should be between 1 and 2) and oxidized by exposure to 5 mg of MnO_2 for 5 min. The MnO_2 and protein were removed by centrifugation and 10 μl of the supernatant were analyzed by HPLC. Native fluorescence (excitation: 350 nm, emission: 450 nm) was determined after reverse-phase chromatography as previously described in detail (Niederwieser et al., 1982b).

For the measurement of sepiapterin and "X", a reverse-phase (Lichrosorb RP-18;5 micron, Merck) HPLC system was used with a Kontron SFM 23/8 fluorescence spectrophotometer (excitation: 425 nm, emission: 530 nm). The concentration of sepiapterin and "X" was quantitated by integration of peak area in comparison with authentic sepiapterin.

RESULTS

Formation of sepiapterin and "X" in the absence of added NAD(P)H

Incubation of NH_2P_3 with the "standard extract" (Table 1) in the absence of NADPH resulted in the formation of sepiapterin and another fluorescent compound labelled "X", as detected by fluorescence (425/530 nm) after heat inactivation of the reaction and subsequent HPLC (Figure 2). Sepiapterin production was linear during the 15 minute reaction and 2% of the NH_2P_3 was converted (Table 1). The formation of sepiapterin and "X" in this human system was totally dependent on the presence of Mg^{2+}, which has also been demonstrated in nonhuman systems (Krivi and Brown, 1979; Tanaka et al., 1981). The HPLC elution profile (Figure 2) shows that the major product eluting at 11.5 minutes has a retention time that is identical to authentic sepiapterin (standard tracing not shown). To substantiate the identity of sepiapterin formed from NH_2P_3, purified sepiapterin reductase and NADPH were added after the 15 minute reaction. After an additional 15 minutes, no sepiapterin could be detected and as expected, dihydrobiopterin was formed (Sueoka and Katoh, 1982). Thus, it is reasonable to assume that this peak at 11.5 minutes is sepiapterin. The reaction product at 15 minutes in Figure 2 has similar retention characteristics in this reverse-phase HPLC

Table 1. Effect of NADPH on the biochemical transformations of NH_2P_3 by human liver extracts.*

Incubation conditions	No MnO_2 acid oxidation after incubation (425/530 nm)		MnO_2 acid oxidation after incubation (350/450 nm)	
	Sepiapterin	"X"	Biopterin**	Pterin
I. "Standard extract"				
- NADPH	200	50	70	190
+ NADPH	10	10	1720	0
II. Heat treated "Standard extract" (enzyme A2)				
- NADPH	0	200	0	120
+ NADPH	130	120	0	0

* Concentrations are in pmoles/incubation mixture; % conversion of NH_2P_3 to each product is calculated by dividing pmoles formed by 100. Values are the mean of 3-4 determinations with several separate preparations of "standard extract". "X" is quantitated in comparison with standard sepiapterin fluorescence, although the chemical structure and molecular weight are unknown.

** The oxidation state of the biopterin formed was investigated by HPLC with electrochemical detection of reduced pterins (Niederwieser et al., 1982) as well as the differential iodine oxidation procedure to differentiate between reduced and oxidized pterins (Fukushima and Nixon, 1980). Both methods indicated that greater than 90% of the biopterin was in the fully reduced, tetrahydro form.

*** "Standard extract" was prepared in the following manner. Human liver was homogenized, fractionated with ammonium sulfate (40-65%) and subjected to G-25 column chromatography. This "standard extract was incubated with enzymatically synthesized NH_2P_3 under various conditions.

system and the same absorption characteristics as the "X" obtained by Dorsett et al. (1982) when they incubated NH_2P_3 with an ammonium sulfate fraction (40-60%) of extracts from Drosophila heads. Our "X" in Figure 2 could also be formed by heating the enzymatically synthesized NH_2P_3 for 40 minutes at 100°C, which are the same as the conditions described by Dorsett et al. (1982) for the chemical formation of their "X". In our hands, after the enzymatic formation of the reaction products, "X" could be detected when the reaction was terminated by heating at 100°C for 5 minutes; this has also been reported by Dorsett et al. (1982). However, when we terminated the reaction with methanol to precipitate protein, no "X" was detected, whereas sepiapterin was still present in the same amount (see Discussion).

Sepiapterin formation after the removal of endogenous NAD(P)H

In order to ensure that all endogenous NAD(P) and NAD(P)H in the "standard extract" was destroyed, the following experiments ware performed. After Sephadex G-25 chromatography,

which should essentially exclude small molecules not tightly bound to proteins, the "standard extract" of human liver was pretreated at 37°C for 15 minutes in the presence of 0.02 U NADase, 0.005 U Old Yellow Enzyme, 1 U superoxide dismutase, and 2 U catalase. That these enzymes were indeed active in the presence of the "standard extract" was tested according to published procedures (Kaplan, 1955; Abramovitz and Massey, 1976). The Old Yellow Enzyme, a diaphorase, should effectively catalyze the oxidation of NAD(P)H to $NAD(P)^+$, while NADase should irreversibly degrade $NAD(P)^+$. Superoxide dismutase and catalase were added as scavengers of superoxide (O_2^-) and hydrogen peroxide (H_2O_2), respectively, produced by the diaphorase reaction. Incubation of NH_2P_3 with this pretreated "standard extract" (Table 2) produced the same quantity of reaction products (sepiapterin and "X") as the incubation of the "standard extract" without pretreatment (Table 1).

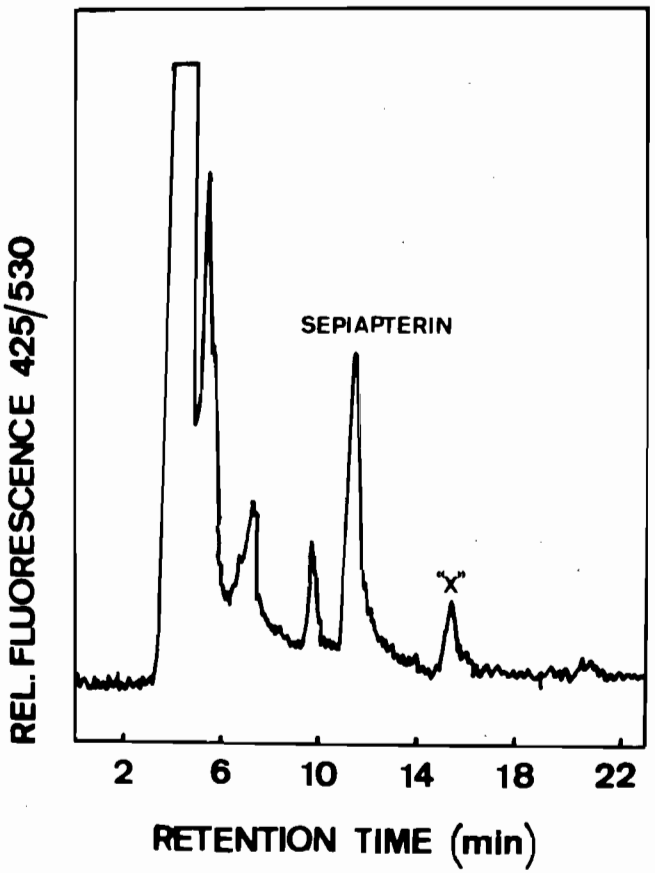

Figure 2. Fluorescence detection after HPLC separation of sepiapterin and "X". These reaction products resulted from the incubation of the "standard extract" with NH_2P_3 (Table 1, I). 50 µl out of 500 µl were injected in a 15% methanol/water mobile phase (0.8 ml/min) that was changed to 20% methanol/water at 10 minutes for a more rapid elution of "X", which has similar retention characteristics as the compound "X" proposed by Dorsett et al., (1982) (see Results).

Table 2. Effect of removal of endogenous NADPH on the formation of reaction products from NH_2P_3.*

Incubation conditions	No MnO_2 acid oxidation after incubation (425/530 nm)		MnO_2 acid oxidation after incubation (350/450 nm)	
	Sepiapterin	"X"	Biopterin	Pterin
I. "Standard extract"** + Old Yellow Enzyme + NADase	200	40	0	200
II. "Standard extract" (anaerobic conditions) - added NADPH	60	50	0	170

* Concentrations are in pmoles/incubation mixture; % conversion of NH_2P_3 to each product is calculated by dividing pmoles formed by 100. Values are the mean of 3-4 determinations with several separate preparations of "standard extract". "X" is quantitated in comparison with standard sepiapterin fluorescence, although the chemical structure and molecular weight are unknown.

** "Standard extract" was prepared in the following manner. Human liver was homogenized, fractionated with ammonium sulfate (40-65%) and subjected to G-25 column chromatography. This "standard extract was incubated with enzymatically synthesized NH_2P_3 under various conditions.

Effects of NAD(P)H and various oxidation conditions on reaction products

To quantitate biopterin formation during the "standard extract" reaction with NH_2P_3 (Table 1) a portion of the post-reaction mixture was oxidized with MnO_2 under acid conditions and measured by fluorescence (350/450 nm) after HPLC (see methods). After MnO_2 acid oxidation and HPLC, pterin formation could also be detected by fluorescence (350/450 nm) under certain reaction conditions. The results listed in Table 1 show that the incubations with the "standard extract" in the absence of NAD(P)H (where sepiapterin formation was maximal) yielded predominantly pterin and little biopterin. Essentially the same results were obtained under the conditions of NAD(P)H destruction (Table 2). However, in the presence of NAD(P)H (2.5 mM final concentration), the "standard extract" converted 17% of the NH_2P_3 to biopterin and no pterin was formed. Analysis of the reaction products in the presence of NAD(P)H using electrochemical detection for reduced pterins (Niederwieser et al., 1982b) as well as the differential iodine oxidation method of Fukushima and Nixon (1980) demonstrated that greater than 90% of the biopterin formed was in the fully reduced, tetrahydro form.

With regard to sepiapterin and "X" formation, incubation of the "standard extract" with NAD(P)H (where BH_4 synthesis is maximal) produced little or no sepiapterin and "X" (Table 1)

To obtain more information about the chemical nature of "X", the MnO_2 acid oxidation was performed after "X" was isolated from other reaction products by HPLC. After the oxidation, no pterin was detected. Furthermore neither "X" nor any other fluorescent compound could be detected.

Effect of heat treatment of "standard extract" on reaction products

The enzyme fraction A2 from human liver was formed by heating the "standard extract" in the manner described by Tanaka et al. (1981) and Yoshioka et al. (1983). The profile of the reaction products with or without the inclusion of NADPH in the reaction mixture was the opposite of the profile when the "standard extract" was used. When A2 was reacted with NH_2P_3 and $MgCl_2$ in the absence of NADPH, a significant amount of "X" was formed during the reaction and pterin could be detected after acid MnO_2 oxidation (Table 1). In contrast, when NADPH (2.5 mM final concentration) was included in the reaction, a relatively large amount of sepiapterin was formed and the amount of "X" was diminished. In this case (with NADPH), pterin could not be detected after acid MnO_2 oxidation (Table 1). No biopterin was formed in either the presence or absence of NADPH, presumably because enzymes after A2 on the BH_4 biosynthetic pathway were inactivated by heating (Tanaka et al., 1981). Therefore, it is probable that under conditions using the enzyme A2 fraction, NH_2P_3 is transformed into some intermediate(s) on the BH_4 biosynthetic pathway that cannot react further.

DISCUSSION

The different biosynthetic pathways for BH_4 biosynthesis from NH_2P_3 that have been proposed are shown in Figure 1. With the exception of the proposal by Gál et al. (1978 a,b), which involves transformation of GTP to quinoid-NH_2P_3 and subsequently into quinoid-BH_2 (Figure 1,f), the existence of NH_2P_3 as an intermediate appears to be undisputed. The conversion of NH_2P_3 to sepiapterin requires the elimination of the oxygen-triphosphate from NH_2P_3, the inversion at the secondary hydroxyl function in position 2' of the side chain, and the (formal) oxidation of the 1'-OH group to an oxo function. It is important to note that the conversion of NH_2P_3 to sepiapterin does not involve a net change in the redox balance between the two compounds, a point also noted by Krivi and Brown (1979). Nevertheless, in three of the pathways (Figure 1, b,c,d), NAD(P)H is proposed to be necessary; in one case (pathway d) it plays merely a catalytic role. Nichol et al. (1983) propose the "direct" conversion of NH_2P_3 to BH_4 without sepiapterin as an intermediate, which clearly requires reducing equivalents. The proposal of Gál and co-workers (1978 a, b) (pathway f) involves the conversion of q-NH_2P_3 to q-BH_2, and this process does require reducing equivalents, even though they reported that added NADPH was not necessary.

This controversy of NAD(P)H dependency led us to examine sepiapterin formation in our BH_4 biosynthetic system from human liver extracts. It has been proposed, but not tested directly, that the enzymes following NH_2P_3 in BH_4 biosynthesis are rate-limiting (Levine et al., 1983) because neopterin can only be detected in man and monkeys, but not in the lower mammals that have been tested thus far. Indeed, recent experiments in our laboratory have shown that NH_2P_3 can be detected in human liver but not rat liver. When using the fresh human liver extract, the conversion of NH_2P_3 to biopterin was approximately 17% (Table 1) and essentially all biopterin was in the tetrahydro form as determined by both the differential iodine oxidation method (Fukushima and Nixon, 1980) and electrochemical detection of reaction products (Niederwieser et al.,1982b) after HPLC. Thus, in the presence of NADPH, this extract is capable of synthesizing significant amounts of

BH_4 with essentially no detectable sepiapterin. When NADPH was not added to the reaction, sepiapterin formation was linear for the duration of the reaction, although only 2% of NH_2P_3 was measured as sepiapterin (Table 1). Under conditions which should ensure the elimination of any endogenous, free NADPH present in the reaction with the fresh extract, the same amount (2%) of sepiapterin was also detected (Table 2). These results indicate that sepiapterin can be formed in the absence of NADPH, however the yield is much lower than for BH_4. It is likely that in the absence of NADPH, sepiapterin is formed from the degradation of another intermediate that is generated during the reaction. The nature of this postulated intermediate is discussed below.

A remaining question is why several groups have reported that the formation of sepiapterin from NH_2P_3 is NADPH-dependent when there is no net change in the redox balance between the two compounds. A possible rationale for these reports is that reduced pyridine nucleotides might react nonenzymatically with intermediates such as those proposed by Tanaka et al. (1981) or Dorsett et al. (1982). Our experiments using the heat-treated "standard extract" that yields enzyme fraction A2 (Tanaka et al., 1981) support this concept. Enzyme fraction A2 with or without NADPH is not capable of forming BH_4, presumably because the remaining enzymes involved in BH_4 synthesis have been destroyed. Tanaka and co-workers (1981) have proposed that enzyme A2 can form the postulated intermediate, 1',2'-dioxopropyl-dihydropterin (referred to as "diketo-dihydropterin"), shown on the left side of Figure 3. They further propose that the diketo-dihydropterin can be converted by a heat labile enzyme fraction A1 to sepiapterin in a process that is NADPH dependent. In contrast to our results with the "standard extract", the reaction of enzyme A2 with NH_2P_3 in the absence of NADPH produced no sepiapterin (Table 1), whereas the inclusion of NADPH did cause the production of sepiapterin (1.3% conversion of NH_2P_3). Thus, it seems that the enzyme A2 causes the production of an intermediate that can react with NADPH to form sepiapterin. No sepiapterin was detectable when NADPH was not included in the reaction mixture. It is possible that the NADPH-dependent formation of sepiapterin that was observed by Tanaka and co-workers (1981) was the result of nonenzymatic reaction with NADPH, however the involvement of another NADPH-dependent enzyme in the formation of certain intermediates after NH_2P_3 cannot be ruled out by our studies. Based on our current results and the evidence of Nichol and co-workers (1983) that BH_4 can be formed without sepiapterin as an intermediate, the measurement of sepiapterin by Tanaka et al. (1981) and Kapatos et al. (1982) may be the result of the degradation of an intermediate directly on the BH_4 biosynthetic pathway. In the case of Kapatos et al. (1982) who studied sepiapterin formation in rat brain, it seems likely that the radioactive counts they measured as sepiapterin by HPLC may have actually been another compound that co-chromatographed with sepiapterin in their HPLC system. Later discussion on the nature of an intermediate that could degrade to sepiapterin will expand on this point.

Studies by Krivi and Brown (1979) are also relevant to the point of sepiapterin formation from an intermediate after reaction with NADPH. Their studies on the incorporation of label from either R or S NADPH into sepiapterin demonstrated a rather low selectivity for either form such that no stereochemical configuration was preferred. This lack of stereochemical specificity is not characteristic for enzyme reactions and may indicate that nonenzymatic

sensitive to oxidation by O_2. Table 2 shows that under anaerobic conditions the formation of sepiapterin and "X" are markedly reduced, indicating the possibility that oxidation of some other compound may be responsible for the appearance of these compounds.

The characteristics of "X" formed in our human liver biosynthetic system are similar to those reported by Dorsett and co-workers (1982) in their biosynthetic studies using extracts from Drosophila heads. Our "X" had similar retention characteristics on the reverse-phase HPLC system, showed an identical absorption spectrum, and could also be generated nonenzymatically by heating NH_2P_3. The fact that "X" is only formed when the reaction is terminated by heating and not when methanol precipitation is employed indicates that "X" probably results from chemical degradation of some primary product that is formed enzymatically from NH_2P_3. Since the amount of sepiapterin formed is invariant whether heating or methanol is used to terminate the reaction, "X" is probably not formed from sepiapterin.

Some additional information can be gained from studying the effects of oxidation of reaction products under acid conditions. In general, exposure of tetrahydro- or dihydropterins that have no keto function in the 1' position of the side chain to MnO_2 under acid conditions (pH between 1 and 2) causes oxidation without side chain cleavage, and the resulting products are fluorescent at 350/450 nm (Niederwieser et al., 1982b). Thus, when BH_4 is formed by the standard extract and oxidized with MnO_2 under acid conditions, only biopterin is formed and there is no side chain cleavage to form pterin (Table 1). However, when dihydropterins that have a keto function in the 1' position of the side chain (e.g., sepiapterin) are exposed to acid oxidation, no pterin or any other fluorescent compound can be detected at either of the two pairs of wavelenth settings (350/450 nm or 425/530 nm). In our experiments, the isolation of "X" by HPLC and subsequent exposure to MnO_2 acid oxidation did not produce either pterin or other fluorescent compounds. Therefore, the pterin that is formed after MnO_2 acid oxidation of the reaction products generated by the standard extract in the absence of NADPH cannot be generated by the breakdown of sepiapterin or "X", but must be generated from some other intermediate. When NADPH is included in the reaction mixture and the mixture is oxidized as above, only BH_4 is detectable and pterin is absent. This indicates that there is probably not a significant accumulation of an intermediate that can yield pterin after oxidation when NADPH is included and BH_4 is being synthesized by the "standard extract".

The question arises as to what type of intermediate could NH_2P_3 be transformed into that could be a common link for the formation of sepiapterin, "X", and pterin (after MnO_2 acid oxidation). Figure 3 depicts our proposal for the existence of tetrahydropterin intermediates in BH_4 biosynthesis and the rationale for our conclusions are presented below. It is conceivable that NH_2P_3 could be converted directly to a pterin compound having a tetrahydropterin ring system by an internal oxidoreduction reaction involving the transfer of reducing equivalents from the side chain to the pterin ring. This might occur either subsequent to or concomitant with the elimination of the triphosphate moiety. A likely structure for this compound is 1',2'-dioxopropyl-tetrahydropterin (referred to as "diketo-tetrahydropterin" in Figure 3). The 2'-position of diketo-tetrahydropterin could be reduced by a side chain reductase reaction to form 5,6-dihydrosepiapterin, which also has a tetrahydropterin ring system (the correct chemical name is 6-lactoyl-tetrahydropterin).

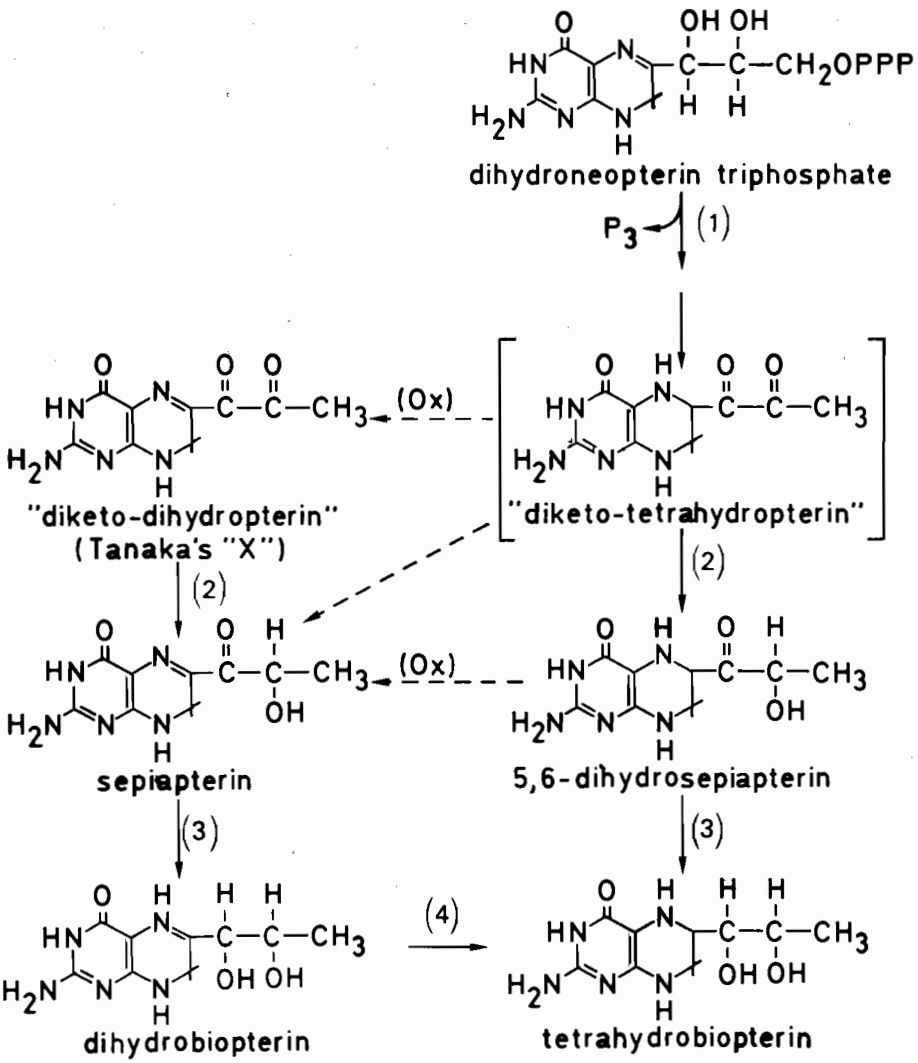

Figure 3. Proposed pathway for BH_4 biosynthesis including the existence of the novel intermediates, diketo-tetrahydropterin and 5,6-dihydrosepiapterin (6-lactoyl-tetrahydropterin). Compound "X" proposed by Tanaka and co-workers (1981) as an intermediate in BH_4 biosynthesis is depicted as a oxidative by-product (OX) of the diketo-tetrahydropterin intermediate. The enzymes that catalyze these biosynthetic reactions are indicated numbers as follows:
(1) Tanaka's proposed heat-stabile enzyme fraction, A2. (2) Tanaka's proposed heat-labile enzyme fraction, A1. (3) Sepiapterin reductase in the presence of NAD(P)H. (4) Dihydrofolate reductase in the presence of NAD(P)H. When this enzyme is blocked by methotrexate, BH_4 synthesis still proceeds (Duch et al., 1983).

Either of these compounds could yield sepiapterin through nonenzymatic reactions. Sepiapterin could be formed from diketo-tetrahydropterin by an internal rearrangement, since these compounds are of equivalent redox state. A chemical oxidation of 5,6-dihydrosepiapterin could also yield sepiapterin. It is possible that the radioactivity detected as sepiapterin by Kapatos and co-workers (1982) in their HPLC system was actually 5,6-dihydrosepiapterin, which could co-chromatograph with sepiapterin, although in our hands, the two compounds can be separated by using different chromatographic conditions from what they employed. With regard to diketo-dihydropterin (compound "X") proposed by Tanaka and co-workers (1980), it seems feasible that this compound could be an oxidative by-product of our proposed diketo-tetrahydropterin intermediate. Certainly, the exposure of this potential intermediate to MnO_2 acid oxidation could lead to side chain cleavage and pterin formation (Table 1), which was demonstrated for the postulated diketo-dihydropterin intermediate (Tanaka et al., 1981; Häusermann et al., 1981).

The previous information supports our proposal that NH_2P_3 can be converted to a diketo-tetrahydropterin intermediate by a mechanism involving an intramolecular oxidoreduction reaction. There are examples of this type of rearrangement in sugar chemistry, such as the Lobry de Bruyn-Alberda van Ekenstein transformation (Speck, 1968). This type of intramoleclular oxidoreduction reaction was originally proposed by Pfleiderer (1975,1979) to explain the chemical formation of sepiapterin from BH_4. Thus, it seems likely that diketo-tetrahydropterin and 5,6-dihydrosepiapterin are intermediates in BH_4 biosynthesis. Our proposal for the existence of these tetrahydropterin intermediates (Figure 3) may help explain some of the current controversies regarding mammalian BH_4 biosynthesis.

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