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TETRAHYDROBIOPTERIN BIOSYNTHESIS

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Introduction

Our laboratory has been interested in phenylketonuria (PKU) for several years, especially in light of recent demonstrations by our own and other laboratories that certain atypical forms of PKU are caused by defects in tetrahydrobiopterin (BH_4) metabolism (1,2,3,4,5,6,7,8). Approximately 1-3% of PKU cases are caused by a lack of BH_4 due to an inborn error of metabolism. It is well known that BH_4 plays an important role in regulating tyrosine and tryptophan hydroxylase in the synthesis of the biogenic amine neurotransmitters, dopamine, norepinephrine, epinephrine, and serotonin (9,10). Therefore, many atypical PKU patients suffer from a lack of proper biogenic amine synthesis in the central nervous system. These patients develop a progressive neurological illness that is unresponsive to a low phenylalanine diet. The majority of atypical PKU patients die at an early age.

We have investigated several patients that are unable to synthesize dihydrobiopterin (BH_2) as well as patients that are deficient in quinonoid dihydropteridine reductase (DHPR) activity (11,12). Recently, we have discovered one patient with a diminished GTP-cyclohydrolase activity (5). Several of these atypical PKU patients are responsive to BH_4 administration alone (12), and

others are responsive to BH_4 with supplemental neurotransmitter precursor therapy. Of course, a better knowledge of BH_4 biosynthesis in man would aid efforts to understand and treat these diseases. Our recent observation that BH_4 administration may be beneficial in certain cases of Parkinson's disease and endogenous depression (13) emphasizes the need for a further understanding of BH_4 biosynthesis and metabolism. Although our results (13,14) as well as those of others (15,16) on BH_4 administration in Parkinson's disease are only preliminary and require further investigation, they stress the importance of basic pterin research. It is with these ideas in mind that we have continued to explore BH_4 biosynthesis in certain mammals, including man.

Historical Perspectives on BH_4 Biosynthesis

Invertebrate systems

A great deal of the early investigations on BH_4 biosynthesis was accomplished on non-mammalian systems. In E.coli (17,18) and other invertebrates (19,20,21,22,23), an enzyme system was isolated that was not Mg^{++}-dependent and converted guanosine triphosphate (GTP) directly to D-erythro-dihydroneopterin triphosphate (NH_2TP) this enzyme was named GTP-cyclohydrolase I. These observations led to investigations by Krivi and Brown on the transformation of NH_2TP in BH_4 biosynthesis (see figure 1). According to their studies in *Drosophila melanogaster* (24), sepiapterin synthetase, the enzyme system responsible for the synthesis of sepiapterin from NH_2TP, consisted of 2 components referred to as enzyme A (M.W.=82,000) and enzyme B (M.W.=36,000). NADPH and a divalent cation (Mg^{++} was the most effective) were required for activity that was shown to be optimal at pH 7.4, and 30°C. The K_M for NH_2TP was 10 µM and a number of unconjugated pterins, including biopterin and sepiapterin, were in-

hibitors of the reaction. Dihydroneopterin (NH_2) would not serve as substrate in place of NH_2TP. Evidence was presented in support of a proposed reaction mechanism for the enzymatic conversion of NH_2TP to sepiapterin in which enzyme A catalyzes the production of a labile intermediate by nonhydrolic elimination of the phosphates of NH_2TP. According to this proposal, enzyme B catalyzed the conversion of this intermediate to sepiapterin in the presence of NADPH. These biosynthetic reactions are shown in figure 1. It should be noted that a similar reaction scheme was proposed by Viscontini and coworkers as early as 1973 (38,39).

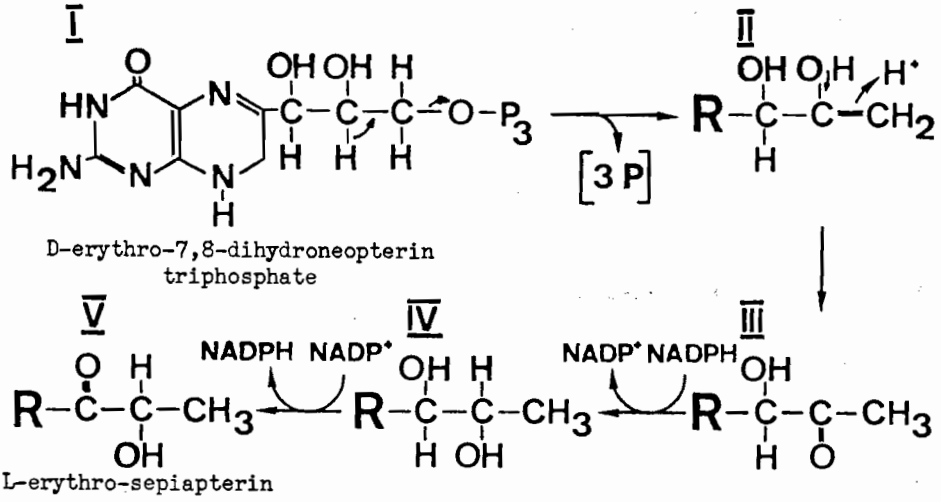

Fig. 1. Reaction scheme of Krivi and Brown. Enzymatic reaction sequence for the conversion of NH_2TP to sepiapterin proposed by Krivi and Brown. Compounds II-IV: Proposed intermediates.

Otsuka and coworkers (25) investigated BH_4 biosynthesis in *Ascaris lumbricoide suum*. According to their findings (see figure 2), GTP was converted to NH_2TP by GTP-cyclohydrolase I. NH_2TP was then converted to sepiapterin by the enzyme, sepiapterin synthetase. Their proposed synthetic pathway is shown in figure 2.

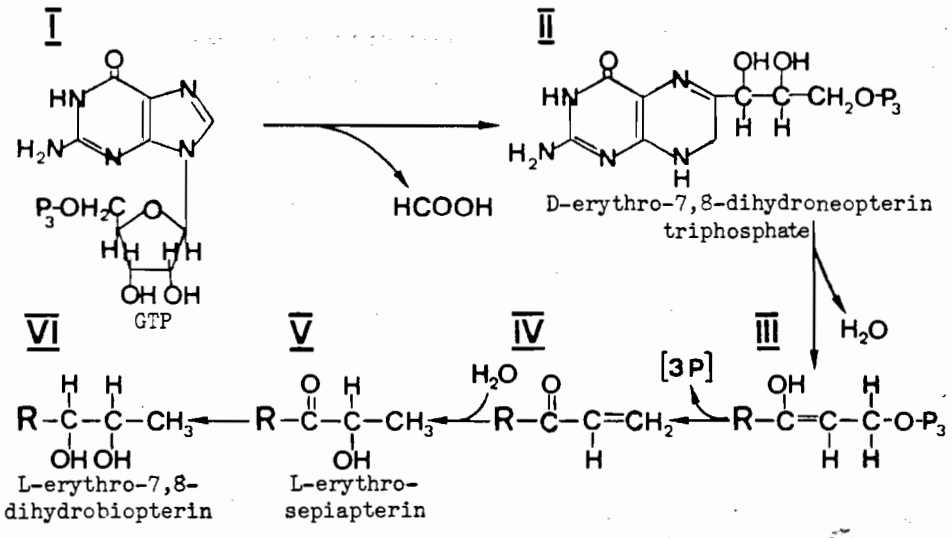

Fig. 2. The enzymatic conversions of GTP to BH_2 proposed by Otsuka and coworkers.
Compounds III and IV: Proposed intermediates.

Mammalian and avian systems

The details of the biosynthetic pathway of BH_4 in mammals are still under investigation. Gál and coworkers (26,27,28) investigated the BH_4 biosynthetic enzymes using rat brain extracts (see figure 3). They proposed that the initial conversion of GTP was catalyzed by one of two similar enzymes, GTP-cyclohydrolase A-I or Mg^{++}-dependent A-II, and that this reaction was rate-limiting in BH_4 biosynthesis. Both of these enzymes hydrolyzed GTP to 2-amino-6-(5-triphosphoribosyl)-amino-5-(or -6)-formamido-4-oxopyrimidine ($FPydP_3$). This compound was cyclized by D-erythro-7,8-dihydroneopterin triphosphate synthetase into quinonoid D-erythro-dihydroneopterin triphosphate ($q\text{-}NH_2TP$). $q\text{-}NH_2TP$ was subsequently transformed into quinonoid-L-erythro-dihydrobiopterin ($q\text{-}BH_2$) by the action of L-erythro-dihydrobiopterin synthetase. According to Gál, this reaction

was catalyzed by a single enzyme that did not require pyridine nucleotides or Mg^{++} for catalytic activity. The biosynthesis of BH_4 as proposed by Gál is shown in figure 3. The details displayed in figure 3 are contrary to results in mammalian systems that were obtained by other laboratories.

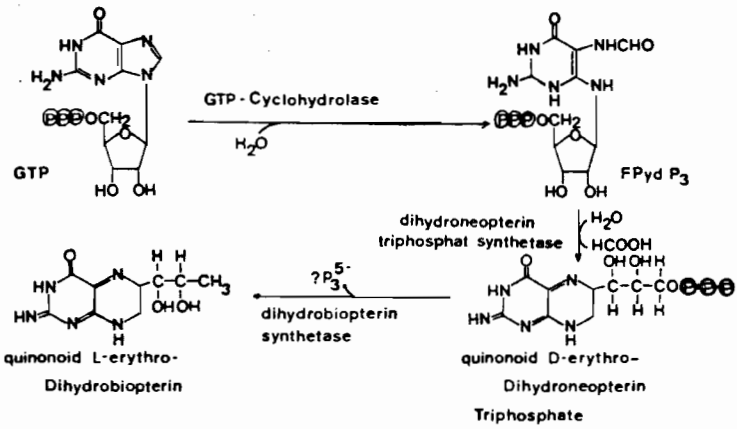

Fig. 3. Postulated biosynthesis of q-dihydrobiopterin in rat brain proposed by Gál and coworkers.

Fukushima and coworkers (29) have studied a NH_2TP synthetase (also called GTP-cyclohydrolase I) from chicken liver. This enzyme catalyzes the direct conversion of GTP to D-erythro-NH_2TP. In the process, the carbon atom from position 8 of GTP is released as formic acid. In other studies on rat liver GTP-cyclohydrolase I, Dhondt and coworkers (30) were able to fractionate 3 different forms of the enzyme. One form was heat stable and catalyzed the synthesis of NH_2TP in the presence of EDTA.

In human liver, our group has recently shown that in the presence of EDTA only one protein fraction synthesizes NH_2TP from

GTP (31). The partial purification was done by ammonium sulfate precipitation (30-50%) and column chromatography on DEAE Sephacel. This enzyme fraction is heat stable and shows no substrate inhibition.

Investigations in several laboratories on the enzymatic transformation of NH_2TP have provided generally consistent results. Tanaka and coworkers (32) obtained three protein fractions from chicken kidney, referred to as A1, A2, and B, that catalyze the conversion of NH_2TP to BH_2 in the presence of Mg^{++} and NADPH. Fraction A2, which was heat-resistant, catalyzed the conversion of NH_2TP in the presence of Mg^{++} to an unknown intermediate referred to as compound X. Fraction A1 catalyzed the subsequent conversion of compound X to sepiapterin in the presence of NADPH. Fraction B contained sepiapterin reductase (33), which catalyzed the conversion of sepiapterin to BH_2 in the presence of NADPH. Fraction A1 was demonstrated to be heat labile. These reactions are shown in figure 4.

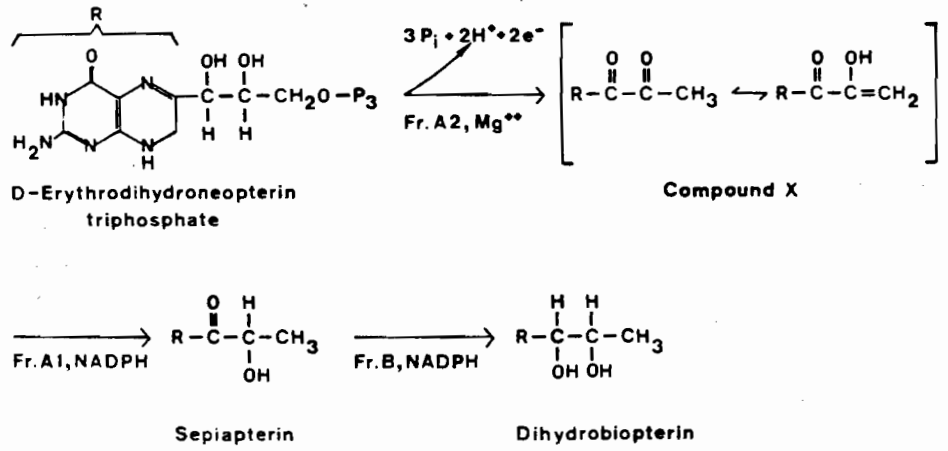

Fig. 4. Initial proposal by Tanaka and coworkers for the biosynthetic pathway of BH_2.

The pathways of Tanaka and coworkers in chicken kidney (32) and of Krivi and Brown (24) in *Drosophila melanogaster* are very similar. Both systems catalyze the first reaction from GTP to NH_2TP with only one enzyme fraction. The enzymes converting NH_2TP into sepiapterin have similar pH optima, K_M values and molecular weights. Both groups of investigators postulate a labile intermediate product and the enzymes in both systems require Mg^{++} ions as well as NADPH. But Krivi and Brown have not followed the assumption of an oxidation in the C-1' position followed by oxidation in the C-2' position. They suggested that the enzyme fraction A catalyzed the splitting of the phosphate groups via an elimination reaction that led to a labile intermediate referred to as compound III. This compound is thought to be 6-(1'-hydroxy, 2'-oxopropyl)-7,8-dihydropterin. Enzyme B is thought to reduce this intermediate to L-threo-BH_2 in the presence of NADPH, followed by an oxidation to sepiapterin.

Kapatos and coworkers described a cell-free system prepared from rat brain striatum that was capable of synthetizing BH_4 from GTP (34). They have provided evidence that sepiapterin is an intermediate in BH_4 synthesis.

Table 1 provides a summary of selected studies on BH_4 biosynthesis in a wide variety of systems.

Recent Observations on BH_4 Biosynthesis in Mammalian Systems

Experiments in this laboratory have dealt with BH_4 synthesis in liver and kidney extracts from man and rat (36,37). Following a procedure similar to that of Tanaka and coworkers, incubation of [U-^{14}C]NH_2TP with partially purified fractions of liver and kidney homogenates from man and rat yielded ^{14}C-labeled sepiapterin, BH_2, and compound X (indirectly measured),

Table 1

Comparison of different pathways of biopterin biosynthesis in mammalian and non-mammalian systems

System	Substrate	Enzyme	Products	Properties of enzyme and remarks
E.coli (18)	GTP	GTP-cyclohydrolase I	NH_2TP and formic acid	optimal pH 8-9; K_M for GTP=0.02 µM; M.W.=210,000; no cofactor req.
Comamonas sp. (19)	GTP	GTP-cyclohydrolase I	NH_2TP and formic acid	Mg^{++} req.; K_M for GTP=20 µM; M.W.=650,000
Lactobacillus plantarum (21)	GTP	NH_2TP synthetase	NH_2TP and formic acid	M.W.=200,000; stabilized by phosphate ions
Serratia indica (20)	GTP	GTP-cyclohydrolase D-I and D-II	NH_2TP	D-I: M.W.=200,000; K_M for GTP=32 µM at low GTP conc., 0.61 µM at high GTP conc. D-II: M.W.=200,000; K_M for GTP=0.44 µM at low GTP conc., 5.5 µM at high GTP conc.; inhibited by bi- and trivalent cations; K^+,

Table 1 cont (1):

				Na^+ and Li^+ are stimulating
Ascaris lumbricoides suum (25)	NH_2TP	sepiapterin synthetase	L-sepiapterin	
Drosophila melanogaster (23)	GTP	GTP-cyclohydrolase I = NH_2TP synthetase	NH_2TP and formic acid	optimal pH 8.7; K_M for GTP 22 µM; M.W.=345,000; no cofactors req.
	NH_2TP	enzyme A	compound III (see fig. 1)	req. Mg^{++}; M.W.=82,000; proposed structure is 6-(1'-hydroxy, 2'-oxopropyl)-7,8-dihydropterin
	compound III (see fig. 1)	enzyme B	sepiapterin	req. NADPH; M.W.=36,000
Chicken				
- liver (29)	GTP	GTP-cyclohydrolase I = NH_2TP synthetase	NH_2TP and formic acid	optimal pH 8.9; K_M for GTP 14 µM; M.W.=125,000; no cofactors req.

Table 1 cont (2):

- kidney (32)	NH_2TP	enzyme fraction A2	compound X (see fig. 8)	req. Mg^{++}; M.W.=77,000; proposed structure is 6-(1',2'-dioxopropyl)-7,8-dihydropterin
	compound X	enzyme fraction A1	L-sepiapterin	req. NADPH; M.W.30,000
	L-sepiapterin	sepiapterin reductase	L-dihydrobiopterin	req. NADPH
Syrian golden hamster (44)				
- kidney	NH_2TP	probably several	L-dihydrobiopterin	req. Mg^{++}, NADPH or NADH
Rat				
- brain (45)	NH_2TP	probably several	L-dihydrobiopterin	req. Mg^{++} and NADPH
- striatum (34)	GTP	probably several	L-sepiapterin	req. Mg^{++} and NADPH
- brain (26)	GTP	GTP-cyclohydrolase A-I or A-II	$FPydP_3$	A-I: optimal pH 8.0; K_M for GTP=1.2 µM; M.W.=135,000; no cofactors req. A-II: K_M for GTP=0.58 µM; M.W.=34,000; Mg^{++} req.
	$FPydP_3$	NH_2TP synthetase	NH_2TP	optimal pH 8.0; K_M for $FPydP_3$=1.4 µM; M.W.=9,080

Table 1 cont (3):

	NH_2TP	dihydrobiopterin synthetase	dihydrobiopterin	optimal pH 8.0; K_M for NH_2TP 17 μM; M.W.=124,000; no reqs for cofactors
Human				
- liver (31)	GTP	GTP-cyclohydrolase I	NH_2TP and formic acid	
- liver and kidney (37)	NH_2TP	enzyme fraction A2	compound X	req. Mg^{++}
	compound X	enzyme fraction A1	L-sepiapterin	req. NADPH
	L-sepiapterin	sepiapterin reductase	L-dihydrobiopterin	req. NADPH

depending on the particular assay conditions used. These findings are in support of the hypothesis that in rats as well as humans, BH_4 biosynthesis proceeds via sepiapterin.

Preparation of enzymes and substrates involved in sepiapterin and BH_2 synthesis in man and rat

Our procedure for studying sepiapterin synthesis is summarized in the following description: [U-^{14}C]NH_2TP was used as substrate for the enzyme assay. [U-^{14}C]NH_2TP was synthesized from [U-^{14}C]GTP via E.coli GTP-cyclohydrolase I (36) and used without further purification. Between 5 and 10 g of liver or kidney tissue from human as well as from rat were used as a source of enzymes to study sepiapterin synthesis. The tissue was homogenized, centrifuged at 27'000 x g and the supernatant was subjected to ammonium sulfate precipitation (40-65%). The precipitate was redissolved, dialyzed, and the solution was applied on an Ultrogel AcA 34 column. As described previously, Tanaka and coworkers demonstrated that components A1 and A2 of fraction A from chicken kidney are not resolved by the gel filtration step, however, the A2 component of fraction A was heat stable. Since the A1 component was heat inactivated and was necessary for sepiapterin formation, this made it possible to study the presence of the intermediate, compound X. Thus, when studying the synthesis of compound X, we heated fraction A from the Ultrogel column to remove the A1 component. To investigate the formation of sepiapterin, fraction A was not heated, so that component A1 and A2 were both active. Although Tanaka and coworkers reported a complete separation of fraction A and B (sepiapterin reductase) on Ultrogel, we consistently were unable to separate completely fraction A and B. Nevertheless, a partial separation of fractions A and B was achieved. In the following experiments, Ultrogel fractions from liver and kidney extracts were incubated with [U-^{14}C] NH_2TP and the formation of either compound X or sepiapterin and BH_2 was monitored as described below.

Assay for compound X

As mentioned previously, the labile intermediate, compound X, was formed from [U-^{14}C]NH_2TP by fraction A2 after A1 was destroyed by heat treatment (see figure 5).

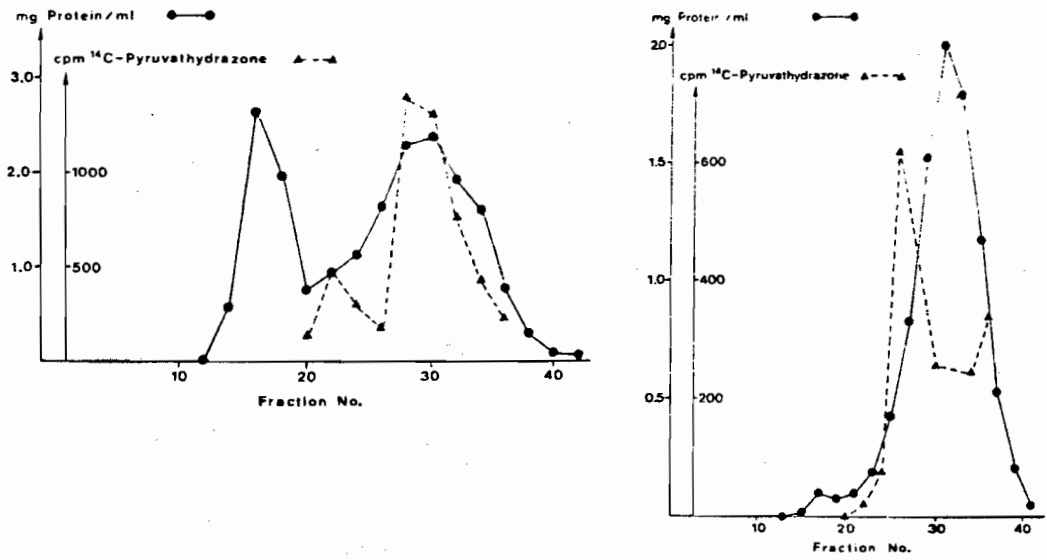

Fig. 5. Elution profile of Ultrogel AcA 34 enzyme preparation from rat liver (A) and human kidney (B). Enzyme A activity was determined by incubating the NH_2TP preparation with the Ultrogel AcA 34 fractions and measurement of the isolated [^{14}C]pyruvate hydrazone.

Following incubation of [U-^{14}C]NH_2TP with fraction A2, dinitrophenylhydrazine was added immediately to the reaction. If there is a diketo compound X, upon reaction with dinitrophenylhydrazine, a dinitrophenyl pyruvate hydrazone should be formed. This would provide indirect evidence for the existence of compound X. In fact, the pyruvate hydrazone could be demonstrated as de-

scribed below. It was extracted with chloroform and separated by TLC in 1-butanol:acetic acid:water (20:1:3, v/v) on silica gel. The zone that comigrated with authentic pyruvate hydrazone was scraped off and eluted with ethanol:water (80:20, v/v) and the radioactivity of the eluates was quantitated.

To provide evidence that the hydrazone was indeed formed from pyruvic acid, the hydrazone was cleaved under reducing conditions so that pyruvate can be converted to alanine; the dinitrophenyl pyruvate hydrazone was hydrogenated catalytically with platinum oxide in ethanol at pH 7. Identification of radioactive alanine was done by TLC in 1-butanol:acetic acid:water (4:1:1, v/v) on silica gel, by a comparison with authentic material. The ability of rat liver and human kidney extracts to form the pyruvate hydrazone is shown in figure 5.

Assay for sepiapterin and BH_2 formation

Fractions from the Ultrogel AcA 34 column were eluted as described above, however, they were not heated in order to retain component A1 enzyme activity. Since we could not achieve complete separation of fractions A and B on Ultrogel, it was necessary to test the ability of these fractions to convert [U-^{14}C]NH_2TP into either sepiapterin, BH_2, or both (see figure 6). A portion of each fraction was reacted in the dark for 1 hour at 37°C with the following mixture in a final volume of 1 ml at pH 7.4: 1) 0.05 M Tris buffer (pH 7.4); 2) 20-200 nmol [U-^{14}C]NH_2TP prepared as previously described; 3) 2.5 mM NADPH; and 4) 0.5 mM $MgSO_4$. The reaction was terminated by applying the reaction products over a Ecteola-Sephadex G-25 column (16x320 mm) equilibrated with water. Standard pterins were eluted in the following order: BH_2, sepiapterin, biopterin, and pterin. The radioactivity from samples that co-eluted with standard BH_2 and sepiapterin was collected and pooled for further purification by HPLC (see figure 7). Radioactive

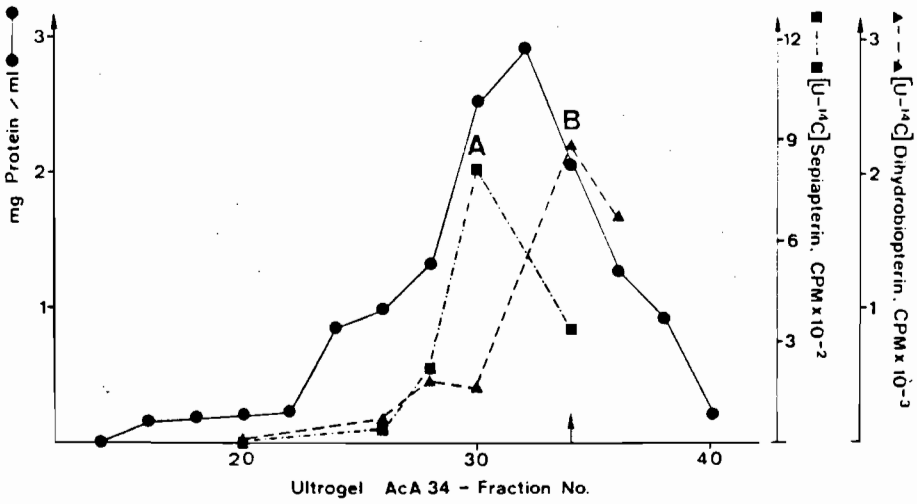

Fig. 6. Elution profile of 2 ml of a 40-65% saturated ammonium sulfate fraction of human kidney on an Ultrogel AcA 34 column (16x580 mm) in 0.05 mol/l potassium phosphate pH 6.8 . Flow rate 10 ml/h, 3 ml fractions.

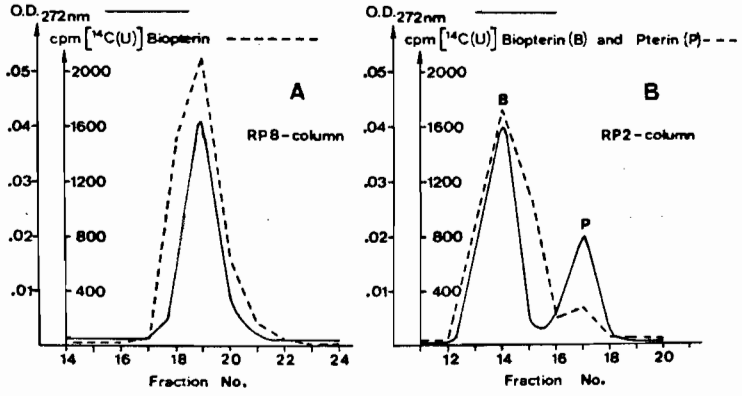

Fig. 7. HPLC purification of [U-^{14}C]B and [U-^{14}C] pterin synthesized by rat kidney extracts.
A: Second HPLC chromatography on Lichrosorb RP-8 of pooled fractions (B + pterin) from the first chromatography on RP-8.
B: Elution profile of the pooled fractions from A (18 - 21) on Lichrosorb RP-2.
B = Biopterin, P = Pterin.

fractions corresponding to the elution of BH_2 and sepiapterin were collected, concentrated and further analyzed by TLC after oxidation of BH_2 to biopterin by manganese dioxide at pH 2. The TLC system used was 1-butanol:acetic acid:water (20:3:7, v/v) on silica gel plates. After this third and final chromatographic purification, the radioactive BH_2 and sepiapterin were eluted from the scraped silica gel fractions with ethanol:3% ammonia (1:1, v/v) and radioactivity was quantitated. The results presented above are summarized in Table 2.

Table 2

Products of [U-^{14}C]GTP conversion by human kidney and rat liver *in vitro*.

Material	Ultrogel fraction	[U-^{14}C]GTP (dpm x 10^{-5})	Product	dpm/h / mg protein	% from GTP
1) Human kidney	A, 30[a]	7.3	[U-^{14}C]S[c]	367	0.05
	B, 34[a]	7.3	[U-^{14}C]BH_2	1200	0.16
2) Human kidney	A, 26[b]	4.4	[U-^{14}C]Pyr hyd[d] from compound X	2025	0.46
3) Rat liver	A, 28[b]	4.4		3720	0.85

[a] Fraction number refers to figure 6.
[b] Fraction numbers correspond to those from different experiments
[c] Sepiapterin
[d] Pyruvate hydrazone

Pterin measurements in human urine as an indicator of defects in BH_4 biosynthesis

Examination of the urinary pterin profile can often yield valuable information on the type of PKU patient and the proper method of treatment for the patient. Furthermore, clinical analyses can often substantiate results obtained from *in vitro* studies. In our PKU studies, we demonstrated relatively large amounts of 3'-hydroxysepiapterin (HS) in BH_2-deficient patients (3.9-9.6 mmol HS/mol creatinine) as compared with the low concentrations that were also detected in healthy controls as

well as in patients with dihydropteridine reductase (DHPR) deficiency (40,41). It is assumed that the excreted HS is derived from HS triphosphate by cleavage of the phosphates. It is also possible that HS might be formed from NH_2TP or NH_2 by non-enzymatic oxidation, but this is not probable for two reasons. Firstly, BH_2 can also be oxidized into sepiapterin by a non-enzymatic oxidation, although only trace amounts of sepiapterin have been detected in the urine of DHPR-deficient patients. Secondly, after loading with NH_2TP (2.5 mg/kg), a high voltage electrophoresis chromatogram of fresh urine from an adult human control showed only traces of HS, but large amounts of NH_2 and neopterin were measured. However, after storage of this urine for 9 months at -20°C, an HPLC chromatogram displayed remarkable concentrations of HS, thus, indicating that non-enzymatic oxidation had occured. Therefore it seems likely that HS triphosphate is on the metabolic pathway and an intermediate in the BH_4 biosynthesis. Thus, the most probable reaction sequence based on the above observations by Tanaka and other researchers is shown in figure 8.

Discussion

There are still many unanswered questions and several controversies regarding BH_4 biosynthesis. Following the investigations of Fukushima *et al.* in chicken liver (29), Dhondt (30) and Blau and Niederwieser (31) in human liver, there exists only one enzyme, GTP-cyclohydrolase I, synthetising NH_2TP from GTP. This is in agreement with the results of other authors investigating non-mammalian systems. On the other hand, Gál and coworkers claimed that in rat brain, there existed two distinct enzyme fractions converting GTP, first to a pyrimidine derivative ($FPydP_3$) (cyclohydrolase I and II) and a second enzyme fraction converting the pyrimidine to dihydroneopterin triphosphate (di-

(40) that large amounts of HS can be detected in the urine of a BH_2-deficient atypical PKU patient also supports the hypothesis of compound **X**. This observation further suggests that HS is an intermediate in the synthesis of BH_4.

Several groups have demonstrated that sepiapterin is converted to BH_2 by sepiapterin reductase, which indicates that sepiapterin may be an intermediate in BH_4 biosynthesis. However, the BH_2 formed appears to be in the non-quinonoid form and therefore, would not be reduced to BH_4 by quinonoid dihydropterin reductase. It has been shown *in vitro* that the conversion of BH_2 to BH_4 can be accomplished by dihydrofolate reductase (43), but whether this is a major reaction *in vivo* remains unclear.

It is noteworthy that neopterin cannot be detected in rat tissues (42), yet it exists in humans. Since the biosynthetic reactions appear to be similar in the rat and human but the pterin metabolic profile is different, this may indicate that GTP-cyclohydrolase is rate-limiting only in the rat and not in humans. The presence of neopterin in humans indicate that the conversion of NH_2TP to sepiapterin might be an important point of regulation in BH_4 synthesis in man.

Acknowledgements

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