Ann. Rev. Nutr. 1988. 8:185–209

INBORN ERRORS OF PTERIN METABOLISM

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CONTENTS

INTRODUCTION

The pteridines constitute a large and structurally varied group of natural compounds involved in the biosynthetic pathways of cofactors and vitamins.

0199-9885/88/0715-0185$02.00

Their occurrence in different organisms has previously been reviewed (150). The base structure of these heterocyclic compounds is the pyrimidino-[4,5-b]-pyrazine. Derivatives with the structure 2-amino-4-oxo were designated by the term "pterins" and those with the structure 2,4-dioxo by the term "lumazines." Pterins can be divided into two groups: Those derived from 7,8-dihydropteroic acid, containing a *p*-aminobenzoate group at the 6-position plus glutamate, are designated as conjugated pterins. Unconjugated pterins contain neither of these two groups; instead, substitution occurs at the 6-position of the ring nucleus.

This chapter presents a survey of the inborn errors of pterin metabolism, with the main emphasis on unconjugated pterins, in the form of a relatively brief text with few figures and with an extensive bibliography.

BIOSYNTHETIC PATHWAY OF TETRAHYDROBIOPTERIN

The known inborn errors of pterin metabolism are those involved either in the biosynthesis or in the regeneration of L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4). BH_4 is the natural cofactor of phenylalanine-4-hydroxylase (EC 1.14.16.1), tyrosine-3-hydroxylase (EC 1.14.16.2), and tryptophan-5-hydroxylase (EC 1.14.16.4), the key enzymes in the biosynthesis of biogenic amines (89). In addition to the hydroxylation of aromatic amino acids, BH_4 might play a role in ether lipid oxidation, proline hydroxylation, and mitochondrial electron transport (114). According to present knowledge (33, 117, 165, 177), BH_4 can be synthesized from guanosine triphosphate (GTP) in at least four enzymatic steps (Figure 1).

GTP cyclohydrolase I (GTPCH; EC 3.5.4.16), the first enzyme in the biosynthesis of BH_4, catalyzes the production of D-erythro-7,8-dihydroneopterin triphosphate (NH_2TP) and formic acid from GTP in a single reaction step (13). This is the rate-limiting step in many mammals (1, 9), but not in humans (12, 46).

6-Pyruvoyl tetrahydropterin synthase (PTPS) is the enzyme catalyzing the conversion of dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin (PTP) (177). This conversion is magnesium dependent and involves the elimination of triphosphate and a rearrangement reaction (29). For some time the structure of the intermediate PTP remained unclear. It was only recently recognized to be of tetrahydro form (117, 165, 177). This was confirmed by ^{1}H-NMR (65) and by fast atom bombardment mass spectrometry (156) of the isolated PTP.

6-Pyruvoyl tetrahydropterin 2'-keto reductase (PTPR) (33) and sepiapterin reductase (SR) (164) are the enzymes catalyzing the two-step reduction of

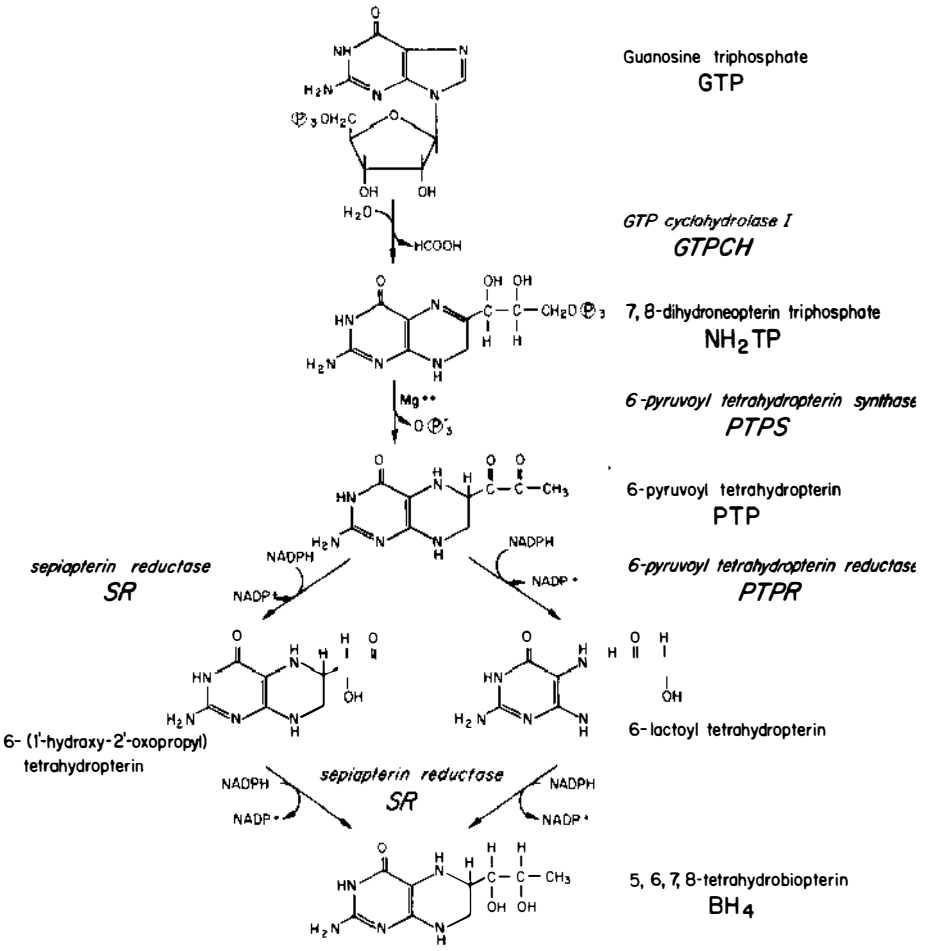

Figure 1 Proposed pathway for the biosynthesis of tetrahydrobiopterin from GTP in humans.

PTP to BH_4. It has not yet been proven that both NADPH-dependent reductases are on the BH_4 pathway in vivo. Neither has the sequence of these two steps been determined, i.e. whether reduction occurs first at C-1' or at C-2' of the side chain of PTP.

During hydroxylation of aromatic amino acids, molecular oxygen is consumed and BH_4 is oxidized to quinonoid dihydrobiopterin (84). In the presence of reduced pyridine nucleotides (28) quinonoid dihydrobiopterin is subsequently reduced back to BH_4 by dihydropteridine reductase (DHPR; EC 1.6.99.7).

HYPERPHENYLALANINEMIA DUE TO TETRAHYDROBIOPTERIN DEFICIENCY

In 1974 Bartholomé in Heidelberg (4) and Smith in London (166) described independently patients with hyperphenylalaninemia characterized by progressive neurological disease unresponsive to treatment by a low-phenylalanine diet. These children show normal phenylalanine-4-hydroxylase activity in liver biopsies but all the symptoms of classical phenylketonuria (PKU). In the following years a number of cases of atypical PKU were described and it was suggested that all these patients suffer from a defect in BH_4 metabolism (6, 7, 18, 21, 37, 38, 85, 91, 92, 97, 115, 151, 153, 167). Because all untreated patients show severe cerebral deterioration and most of them die at an early age, it was suggested that this clinical syndrome should be called malignant hyperphenylalaninemia (35, 36).

In 1975 Bartholomé & Byrd (5) showed that these patients can successfully be treated with L-DOPA and 5-hydroxytryptophan. Based on the speculation that pterins might be used in the treatment of PKU (82), Smith et al (167) proposed that patients with atypical PKU might benefit from substitution therapy with reduced pterins. Indeed, Danks et al (38) showed that intravenous administration of synthetic BH_4 decreases the serum phenylalanine level and therefore can function as a cofactor substitute for phenylalanine-4-hydroxylase in vivo. In 1978 Schaub et al (160) successfully treated an infant with atypical PKU by oral administration of chemically pure BH_4 synthesized by Schircks et al (161). Two years later Niederwieser et al (133) introduced the BH_4 loading test as a diagnostic tool in combination with analysis of urinary pterins. Meanwhile therapy with L-DOPA plus Carbidopa, and 5-hydroxytryptophan alone or in combination with BH_4 was shown to be successful (20, 36, 55, 56, 90, 113, 134, 160) and a number of enzymatic assays became available for the differential diagnosis of atypical PKU (3, 12, 136).

So far, three inborn errors of metabolism are known to cause BH_4 deficiency (Figure 2): (*a*) GTP cyclohydrolase I deficiency, (*b*) 6-pyruvoyl tetrahydropterin synthase deficiency, and (*c*) dihydropteridine reductase deficiency. These defects are discussed in the following sections. The subject has been reviewed by Niederwieser (126–128), Dhondt (40, 42), Kaufman (87), Naylor (124), and Cotton (26).

GTP Cyclohydrolase I Deficiency

In GTPCH deficiency, the enzyme catalyzing the first step in BH_4 biosynthesis by forming dihydroneopterin triphosphate from GTP is deficient (Figure 2). Although this variant of atypical PKU was detected only recently by Niederwieser et al (129, 143), four patients have already been described (47, 109, 125).

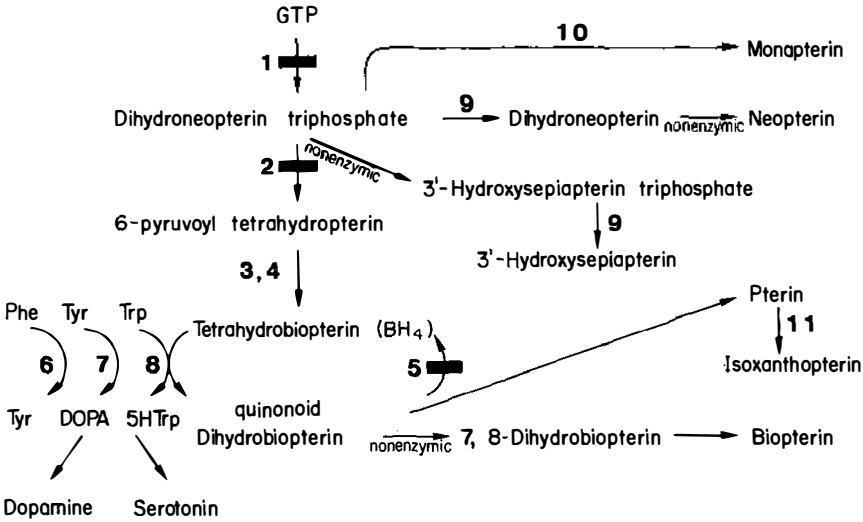

Figure 2 Metabolism and function of tetrahydrobiopterin and possible metabolic defects in hyperphenylalaninemia: *1*: GTP cyclohydrolase I, *2*: 6-pyruvoyl tetrahydropterin synthase, *3*: sepiapterin reductase, *4*: 6-pyruvoyl tetrahydropterin reductase, *5*: dihydropteridine reductase, *6*: phenylalanine-4-hydroxylase, *7*: tyrosine-3-hydroxylase, *8*: tryptophan-5-hydroxylase, *9*: phosphatase, *10*: dihydroneopterin triphosphate-2'-epimerase, *11*: xanthine oxidase.

In the first case (129, 143) hyperphenylalaninemia was recognized at the age of 20 months. The child suffered from severe developmental retardation, severe muscular hypotonia, and frequent episodes of hyperthermia without infections. Urinary excretion of neopterin, biopterin, pterin, isoxanthopterin, dopamine, and serotonin was very low, although the relative proportion of pterins was normal. In cerebrospinal fluid (CSF), neopterin and biopterin as well as the neurotransmitter metabolites, homovanillic acid and 5-hydroxyindoleacetic acid, were also low. Three other patients (47, 109, 125) developed the same biochemical abnormalities: high blood phenylalanine levels (10–20 mg/dl) and low urinary pterins (<1% of normal excretion). In the one case measured, plasma and CSF pterin levels as well as CSF 5-hydroxytryptophan, homovanillic acid, and dihydrophenylalanine were also very low (125). The BH_4 loading test was performed in three patients (47, 125, 143); in each case blood phenylalanine values fell to normal levels within 4 to 6 hours after loading. In two patients the diagnosis of GTPCH deficiency was confirmed by measurement of GTPCH activity in needle liver biopsies (125, 143). In both patients the activity measured was less than 4% of that found in controls.

6-Pyruvoyl Tetrahydropterin Synthase Deficiency

Using the *Crithidia fasciculata* assay for biopterins and thin-layer chromatography, Leeming et al (97) demonstrated in a patient with typical PKU both

low serum and urine biopterin levels. In a patient with normal phenylalanine-4-hydroxylase and dihydropteridine reductase (DHPR) in the liver, Rey et al (153) and Bartholomé et al (6) postulated a defect in biopterin biosynthesis. In patients with PTPS deficiency, low biopterin and high neopterin (30, 69, 86, 131, 132, 145, 179), high dihydroneopterin (131), and high 3'-hydroxysepiapterin (137) levels were found by high performance liquid chromatography (HPLC) measurement of pterins in urine and plasma. The enzyme defect was postulated and confirmed by Niederwieser et al (131) at the step following NH_2TP. In 1985 Niederwieser et al (136) demonstrated an absence of PTPS, the enzyme catalyzing the conversion of NH_2TP to PTP, in liver biopsies from five patients with this form of atypical PKU.

PTPS was in the past designated as "dihydrobiopterin synthetase, DHBS" (64a), "phosphate-eliminating enzyme, PEE" (71), or "sepiapterin-synthesizing enzyme-1, SSE1" (183). "Dihydrobiopterin synthetase" was a provisional name for an enzyme described by Gàl (64a), which was responsible for the biopterin biosynthesis in rat brain. It must, however, be assumed that this enzyme does not exist. "Phosphate-eliminating enzyme" and "sepiapterin-synthesizing enzyme-1" are older names for PTPS. Because of the block in the conversion of NH_2TP to PTP, NH_2TP might accumulate. Subsequently NH_2TP is dephosphorylated by phosphatase and excreted as dihydroneopterin. Dihydroneopterin is readily oxidized nonenzymatically to neopterin (Figure 2).

So far more than 80 cases of PTPS deficiency have been described (43, 130a). PTPS deficiency seems to be heterogenous (42) and one should distinguish between peripheral or partial (48, 52, 68, 72, 76, 142, 155) and classical (4–6, 24, 30, 31, 40, 41, 44, 51, 53, 56, 62, 66, 69, 70, 88, 90, 107, 113, 119, 123, 131–134, 137, 139, 145, 152–154, 162, 170, 174, 176, 178–180, 183) defects.

In the peripheral or partial form of PTPS deficiency, normal levels of neurotransmitter metabolites were measured in CSF. It is therefore of practical importance to find out whether or not the "patient" belongs to this type of deficiency (normal biopterin metabolism in the brain) and thus may not need treatment with neurotransmitter precursors. Because the peripheral types show normal levels of biogenic amine metabolites and moderately elevated levels of neopterin and biopterin in CSF, Niederwieser et al (142) proposed that this type of atypical PKU is due to an incomplete PTPS deficiency or heterozygosity. In those cases where no CSF data are available, it is not possible to differentiate between partial and peripheral types.

Transient forms of PTPS deficiency were reported in a few cases (23, 108, 147, 154, 176) and a transient maturation delay of biopterin biosynthesis was suggested.

Dihydropteridine Reductase Deficiency

In 1975 dihydropteridine reductase (DHPR) deficiency, a defect in cofactor regeneration, was described by Kaufman et al (92), Smith et al (167), Danks et al (37), and Butler et al (21). Kaufman et al (92) demonstrated that in a patient with normal phenylalanine hydroxylase in the liver biopsy no DHPR activity was detectable. The enzyme defect was confirmed by a study using antibodies against pure sheep liver DHPR. Whereas extracts from control human liver gave a single precipitin line with antiserum, extracts from this patient's liver gave no precipitin line at all (116). DHPR is expressed in fibroblastic cells, and in cells cultured from the skin of the DHPR-deficient patient activity was markedly decreased or even absent (38, 115).

Quinonoid dihydrobiopterin formed from BH_4 through the hydroxylation reaction of phenylalanine is an extremely unstable compound and tautomerizes readily to 7,8-dihydrobiopterin (Figure 2). Since DHPR catalyzes the regeneration of quinonoid dihydrobiopterin to BH_4, and since 7,8-dihydrobiopterin is not a substrate for DHPR, patients with DHPR deficiency excrete very high amounts of total biopterin. In the sum of neopterin plus biopterin, the percentage of biopterin is always more than 80% (51, 139). In addition, owing to the lack of BH_4 in these patients, there is no feedback inhibition of GTPCH and therefore pterin biosynthesis is generally activated.

Although most DHPR-deficient patients were detected by screening the urinary pterins (37, 70, 80, 118, 123, 175, 185), some newborns can be missed if the urine is collected under a low-phenylalanine diet (32, 130). But even under a normal diet some patients might not be detected. The same is true for the BH_4 loading test. Few patients with DHPR deficiency respond well to the loading test with 7.5 mg BH_4/kg (27, 30, 39, 169). Of the patients investigated by our group and others, nine responded partially with a slight decrease in plasma phenylalanine (128), two patients responded at 20 mg BH_4/kg (149), and some did not respond at all (27, 54, 102). This may be due to an absolute DHPR deficiency or more likely to the heterogeneity of the molecular defect as demonstrated by Cotton et al (27, 58). Because newborns with DHPR deficiency can escape standard screening, measuring DHPR activity in dry blood spots of Guthrie cards as described by Arai et al (3) is strongly recommended. In most cases diagnosis was confirmed by measurement of DHPR activity in erythrocytes (3, 15, 16, 40, 54, 59, 77, 80, 96, 121), leukocytes (102, 122, 185), or cultured skin fibroblasts (27, 39, 60, 67, 81, 118, 175).

Other Forms

Apart from the defects described above, one should expect at least one more form of BH_4 deficiency caused by a metabolic block in the conversion of PTP to BH_4. This conversion is catalyzed by the enzymes 6-pyruvoyl tetrahydro-

pterin reductase and sepiapterin reductase (Figure 1). However, it is not yet clear if both enzymes are on the pathway and if 6-pyruvoyl tetrahydropterin reductase might be compensated at least partially by sepiapterin reductase (33). In any case PTP would accumulate and pterin would probably be excreted in higher amounts. However, so far no such patients have been detected.

Dhondt et al (48) recently described a patient with mild hyperphenylalaninemia caused by a defect in the BH_4 biosynthetic pathway different from the defects previously reported. A boy with normal clinical examination but with tremors of the upper limbs after stimulation and moderate tendency to hypertonia, showed an increased neopterin-to-biopterin ratio in urine, normal CSF pterins and neurotransmitters, and a positive BH_4 loading test. The most interesting finding was an unknown pterin compound excreted appropriately in the same amount as biopterin. Dhondt also detected this blue fluorescing compound in the urines of both parents and one of the brothers. We recently analyzed this compound isolated from the patient's urine by gas chromatography and mass spectrometry and found it to be most probably biopterin with the side chain in the C-7 position (N. Blau & H. Ch. Curtius, unpublished observation). This finding suggests that in this patient BH_4 biosynthesis is impaired, possibly by a new mutant of GTPCH.

SYMPTOMS OF TETRAHYDROBIOPTERIN DEFICIENCY

Clinical Manifestations

BH_4-deficient patients show clinical symptoms different from those with classical PKU (36, 42). They are usually characterized by progressive neurological disease unresponsive to lowered plasma phenylalanine through restriction of the phenylalanine intake (4, 6, 25, 30, 88, 153, 167). The symptoms can begin during the first weeks of life but usually they are noted at the age of a few months. Most of these patients show clinical symptoms similar to those of infants with neurodegenerative diseases: impaired tone and posture, and hypersalivation due to difficulties in swallowing. Furthermore, feeding difficulties, hypotonia of the trunk, poor head control, hypertonia of the extremities, periods of hyperthermia without infections, and athetotic movements are particularly frequent features. In patients with total BH_4 deficiency, the mean birthweight is 500–800 g lower than that of controls, which suggests retarded growth in utero (168). The neurological disease due to dopamine and serotonin deficiency (8, 19, 20, 72, 93, 112, 169) is progressive and leads to severe myoclonic epilepsy and mental retardation, and often to early death.

Patients with a peripheral or partial defect in BH_4 biosynthesis differ from those with a total defect insofar as some of them never present clinical

symptoms and they usually show no neurological symptoms (42, 48, 72, 142; A. Ponzone, unpublished information).

Biochemical Manifestations

Neonatal hyperphenylalaninemia (>1.2 mmol/liter) is usually the first biochemical manifestation in newborns with BH_4 deficiency. Some newborns, however, show lower phenylalanine levels. Newborns with blood phenylalanine levels below 1 mmol/liter have been described in a number of cases of BH_4 deficiency (24, 30, 99, 134, 176); most of these infants were breast-fed. Substantial amounts of BH_4 in human milk (45) and a low-protein diet may be responsible for mild hyperphenylalaninemia in these neonates. Therefore screening of all newborns with 0.15 mmol/liter blood phenylalanine is recommended (40, 139).

Patients with BH_4 deficiency excrete either traces of pterins (GTPCH deficiency) or higher amounts of one of the metabolites occurring before the metabolic block on the pathway. In PTPS deficiency, neopterin accumulates and only small amounts of biopterin are excreted (86, 131, 132), while in DHPR deficiency biopterin will accumulate, although predominantly in the dihydro form (37, 118). Similar pterin patterns were found in blood (52, 67, 88, 96, 98) and in CSF (54, 74, 75, 80).

In most cases of atypical PKU the BH_4 cofactor deficiency is accompanied by low levels of the neurotransmitters dopamine and serotonin. CSF concentrations of homovanillic acid, a dopamine metabolite, and 5-hydroxyindoleacetic acid, a serotonin metabolite, are low in these patients compared with controls (19, 54, 93, 107, 112, 113, 129, 139, 175, 178, 179). Patients with peripheral and/or partial forms of PTPS deficiency without neurological symptoms show normal CSF neurotransmitter metabolite levels (48, 72, 76, 142).

LABORATORY DIAGNOSIS OF TETRAHYDROBIOPTERIN DEFICIENCY

Pterins are presented in physiological fluids and tissues in the reduced and oxidized forms. Oxidized pterins are highly fluorescent and can be detected with specificity and sensitivity in urine, blood, or CSF after high performance liquid chromatography (HPLC) (51, 63, 78, 108, 110, 123, 132, 144, 145, 185). Stea et al (171, 172) were the first to describe HPLC of oxidized pterins in urine. They used strong cation exchange HPLC and precleaning of the sample on cation and anion exchangers. Several modifications of this method were later described (51, 182). Fukushima & Nixon (63) were the first to use a reverse-phase HPLC technique for the separation of neopterin and biopterin

from other unconjugated pterins. Several investigators developed methods for the separation of pterins either by reverse-phase HPLC (2, 143a, 181) or by ion-pair reverse-phase HPLC (74, 78, 103–105), either with electrochemical or with fluorometric detection. Most of these techniques are fast and offer good resolution, using isocratic organic solvents. The method developed by Niederwieser et al (143a, 144) used an automatic HPLC system with means for column switching. This method involves minimal pretreatment of the biological samples (i.e. oxidation with MnO_2), separates complex mixtures of pterins in a short period of time, and uses fluorometric or amperometric detection. HPLC of reduced pterins was introduced by Bräutigam & Dreesen (17). Lunte & Kissinger (103–105) improved this method by the use of a dual electrode detector, which enables detection of both oxidized and reduced pterins in a single run. In a system developed by Hyland (78) and by Howells et al (74), oxidized and reduced pterins are analyzed using ion-pair reverse-phase HPLC with sequential electrochemical and fluorometric detection. Tetrahydropterins are detected by electrochemical oxidation, dihydropterins by fluorescence following post-column electrochemical oxidation, and the fully oxidized pterins by their native fluorescence.

The inherently poor selectivity of most chromatographic methods prevents identification of unknown pterins in many cases. Gas chromatography in combination with mass spectrometry can be used to overcome such problems. Kuster et al (94, 95) investigated approximately 70 trimethylsilyl derivatives of naturally occurring and synthetic pteridines by capillary gas chromatography and mass spectrometry. This method is used for the study of biopterin metabolism in humans.

Radioimmunoassays for neopterin (120, 157, 173) and BH_4 (111) as well as polarization fluoroimmunoassays of neopterin and biopterin (158) were described, although these techniques are not suitable for the screening of BH_4 deficiencies.

Analysis of Urinary Pterins

Measurement of urinary pterins is the method of choice for screening and differential diagnosis of defects in BH_4 biosynthesis. The characteristic pattern of pterins excreted in urine makes it possible to identify all three variants of BH_4 deficiency in older patients.

By plotting the percentage of total biopterin versus total biopterin/creatinine (Figure 3), classical PKU, GTPCH, PTPS, and DHPR deficiency can graphically be completely separated (138). In GTPCH-deficient patients, biopterin comprised approximately 50% of the sum of neopterin plus biopterin; the values fall near the ordinate and are separated from other variants and from controls. Total pterin excretion in these patients is extremely low. Patients with PTPS deficiency excrete very low amounts of biopterin and very

high amounts of neopterin. The values (biopterin over the sum of biopterin plus neopterin) are usually below 5% biopterin. The neopterin-to-biopterin ratio in these patients varies between 40 and 300, although it should be stressed that patients with various infections also excrete elevated amounts of neopterin in urine (135). Patients with DHPR deficiency showed the highest biopterin levels, and at elevated plasma phenylalanine levels the percentage of biopterin was always higher than 80%. Neopterin excretion was comparable to that of classical PKU.

Healthy newborns excreted relatively more neopterin than children or adults (132). In some patients a positive correlation between pterin excretion and plasma phenylalanine levels was observed. Dietary treatment corrected the plasma phenylalanine levels of newborns and at the same time decreased pterin excretion. Therefore, analysis of pterins in urine should be performed before starting with the low-phenylalanine diet.

Analysis of Pterins in Blood and Serum

In some cases of atypical PKU, pterins were analyzed in blood or serum as well as in urine (39, 67, 80, 88, 97, 98, 154). However, these methods require deproteinization prior to HPLC analysis. Dry blood spots on Guthrie cards can be used for simultaneous measurement of total biopterin and DHPR activity (96). By this method screening is possible for DHPR deficiency and PTPS deficiency. However, using the *Crithidia fasciculata* bioassay, low

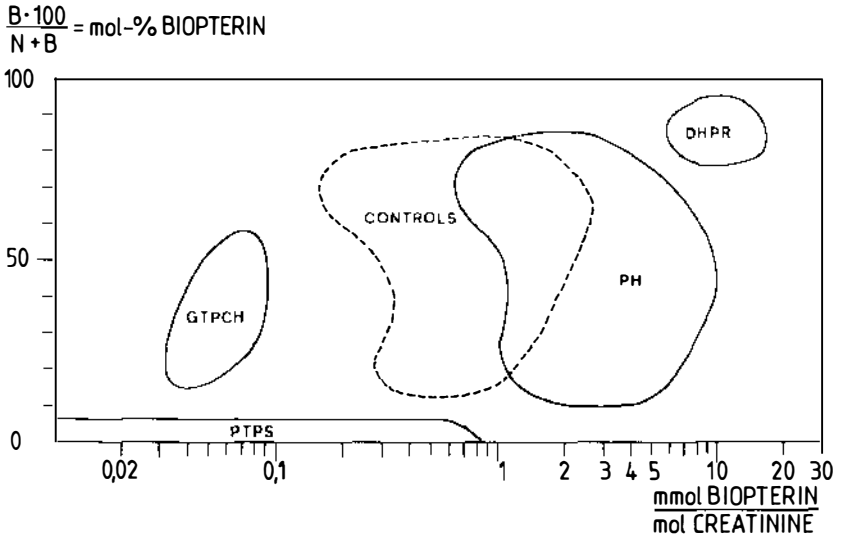

Figure 3 Differentiation of BH_4 deficiency variants by two-dimensional semilogarithmic plotting of urinary biopterin, neopterin, and creatinine.

biopterin levels in patients with a partial or peripheral defect will not be detected.

Analysis of Pterins in Tissue

The methods used for analysis of pterins in tissues depend on the oxidation state of the pterin of interest. Extraction methods used prior to HPLC or gas chromatography use protein precipitation and cleanup procedures on ion exchange or reverse-phase material. Reduced pterins should be extracted and analyzed in the presence of an antioxidant (ascorbic acid, dithioerythritol).

The pterin pattern in liver tissue from patients with atypical forms of PKU was investigated by Nixon et al (145) with HPLC and fluorescence detection. Patients with PTPS deficiency were shown to have high levels of neopterin and very low levels of biopterin in liver, and those with DHPR deficiency show high levels of biopterin and lower levels of neopterin in liver. By analysis of reduced pterins in different brain areas, Levine et al (100, 101) measured the ability of various BH_4 analogues to enter the rat brain. They used HPLC with electrochemical detection as described by Niederwieser et al (144). Generally, measurement of pterins in different tissues is not suitable for screening of BH_4 deficiency.

Neurotransmitter Metabolites and Pterins in CSF

Measurement of neurotransmitter metabolites and pterins in CSF is important for differential diagnosis of total and peripheral forms of PTPS deficiency, as well as for therapy control. Since BH_4 is an essential component not only for the phenylalanine hydroxylation but also for tyrosine and tryptophan hydroxylation, a cofactor deficiency will result in low levels of dopamine and serotonin in the brain (87). Levels of homovanillic acid and 5-hydroxyindoleacetic acid were found to be extremely low in CSF of patients with total PTPS deficiency, DHPR deficiency, and GTPCH deficiency (18–20, 24, 38–40, 42, 54, 80, 93, 112, 125, 129, 174, 175, 178, 179). Patients with peripheral PTPS deficiency show normal or even elevated concentrations of neurotransmitter metabolites in CSF (48, 72, 142). Homovanillic acid and 5-hydroxyindoleacetic acid can easily be separated by ion-pair reverse-phase HPLC and quantitated by amperometric detection (49, 79, 100, 101a, 144). CSF obtained by spinal tap is collected into EDTA vials and analyzed without pretreatment. Samples should be collected at low plasma phenylalanine levels and without therapy.

Prior to analysis, pterins should be preserved by adding an antioxidant. Particularly BH_4 autooxidizes rapidly via quinonoid dihydrobiopterin to 7,8-dihydrobiopterin. To prevent autooxidation, it is recommended that one add dithioerythritol as an antioxidant and diethylenetriaminopentaacetic acid as a chelating agent to the CSF (73, 74). In addition, for HPLC of reduced pterins

dithioerythritol should be added to the mobile phase. The ratio of total neopterin to total biopterin in CSF of newborns and children is about 1, and the percentage of biopterin of the sum of neopterin plus biopterin is between 32 and 65% (142). An age-related distribution with slow decrease of CSF biopterin and constant values of neopterin was observed up to 30 years of age (49). Patients with PTPS deficiency show high neopterin and low biopterin levels, while patients with DHPR deficiency show low to normal neopterin and high biopterin levels (128); in those with the peripheral type of PTPS deficiency both neopterin and biopterin levels were increased (142). In two patients with DHPR deficiency normal levels of BH_4 and increased levels of dihydrobiopterin in CSF were reported (75).

Enzyme Assays

Once the BH_4-deficient patient has been detected, it is necessary to differentiate between the variants of BH_4 deficiency. Not only localization of the enzyme defect but also the particular heterogeneity of PTPS deficiency and problems in the diagnosis of DHPR deficiency require additional tests. Measurement of enzyme activity as the most discriminating method allows exact localization of the metabolic defect.

The enzyme assay for DHPR activity has been used in liver biopsy (92), cultured skin fibroblasts (115), amniocytes and chorionic villi (57), and peripheral leukocytes (59, 122) for diagnosis of DHPR deficiency. Measurement of DHPR activity in dry blood spots of Guthrie cards is, however, a widely used and routine method for the diagnosis of DHPR deficiency. The method of Arai et al (3) uses the reduction of tetrahydropterin to quinonoid dihydropterin in the presence of ferricytochrome C. In the presence of NADH, quinonoid dihydropterin is reduced back to tetrahydropterin by DHPR. The formation of ferrocytochrome C is monitored spectrophotometrically. Only 5 μl of blood are required for the assay in erythrocytes.

The assay for GTPCH activity in liver biopsy (10, 12) was used for diagnosis of GTPCH deficiency in two cases (125, 129). The HPLC assay uses direct measurement of neopterin phosphates after separation from GTP and other hydrolytic products. The same assay can be used for measurement of GTPCH activity in stimulated lymphocytes (11). No GTPCH activity was detected in the liver biopsy or phytohemagglutinine-stimulated lymphocytes of the patient with GTPCH deficiency. The samples from the father and mother showed 30 and 46%, respectively, of the mean enzyme activity of control lymphocytes. These results demonstrate that heterozygotes of GTPCH deficiency can be recognized by measurement of the enzyme activity in stimulated lymphocytes.

Several enzyme assays have been developed for the measurement of enzymes converting NH_2TP to BH_4 (44, 183, 184). Niederwieser et al (136)

described an assay for PTPS activity in liver that requires only 5–10 mg of tissue. The HPLC method is based on the measurement of BH_4 derived from NH_2TP in the presence of NADPH, magnesium, and sepiapterin reductase. The same assay was used to measure PTPS activity in erythrocytes (140, 163). In patients with the typical form of PTPS deficiency no activity was detected in the liver. By measuring PTPS activity in erythrocytes it is possible to differentiate between patients and heterozygous carriers (163). Fetal erythrocytes show about four times higher activity than those of adults. This is because PTPS activity is higher in younger erythrocytes, including reticulocytes. By comparing in two dimensions the normalized values of PTPS activity versus the normalized percentage of urinary biopterin, one can separate obligate heterozygotes from homozygotes and controls (162).

Tetrahydrobiopterin Loading Test

The BH_4 loading test is another method commonly used for the differential diagnosis of almost all the variants with a defect in BH_4 biosynthesis. This test is based on the observation of Danks et al (38) that intravenous administration of 2 mg of BH_4 per kg decreases elevated serum phenylalanine levels in patients with hyperphenylalaninemia. However, at that time BH_4 was not available in sufficient amounts and purity. The oral loading test was routinely introduced by Niederwieser et al (133) in 1979. Within 4 to 6 hours after oral administration of 7.5 mg BH_4 per kg, a dramatic decrease to normal levels of phenylalanine and a rise of tyrosine were observed. Some of the patients with DHPR deficiency respond well to the BH_4 loading test, while some respond only partially, with a slight decrease in phenylalanine, and some do not respond at all. A few patients respond only at higher doses (20 mg/kg) of BH_4 (149). This may be due to mutants of DHPR. With regard to the cofactor activation of phenylalanine hydroxylase by BH_4, the degree of nonresponse may depend on intestinal absorption or on the BH_4 uptake by hepatocytes (54). This test is also useful for interpretation of urinary pterins when the results are obscured by high neopterin levels brought on by a viral infection. In addition, it should be possible to detect a hypothetical K_m mutant of phenylalanine hydroxylase by this test.

In one case of partial PTPS deficiency with borderline hyperphenylalaninemia (68), BH_4 loading was used in combination with oral administration of phenylalanine (106).

Prenatal Diagnosis

Although only 1–2% of newborns with hyperphenylalaninemia are deficient in the BH_4 cofactor, prenatal diagnosis of all three variants of BH_4 deficiency in families at risk is of considerable value (15, 141). However, in the past prenatal diagnosis was only possible for DHPR deficiency (61). Meanwhile,

prenatal diagnosis of BH_4 deficiencies was achieved mainly by measurement of pterin metabolites in amniotic fluid (50) and of enzyme activities in cultured amniotic fluid cells. In some cases fetal liver biopsy or fetal blood proved useful when the activity of the enzyme involved was not expressed in amniotic fluid cells. In selected cases chorionic villi biopsy might also be suitable. Rapid development of recombinant DNA technology will in the future enable more sensitive and more selective prenatal diagnosis of a variety of genetic disorders of BH_4 biosynthesis.

The first prenatal diagnosis of GTPCH deficiency was performed only recently (J. L. Dhondt, unpublished observation) by measurement of pterins in amniotic fluid of a pregnancy at risk. However, because of very low pterin concentrations differentiation between homozygosity and heterozygosity was not possible in this case.

PTPS deficiency can be prenatally diagnosed by measurement of neopterin and biopterin in amniotic fluid (140). High levels of neopterin and extremely low levels of biopterin have been measured. Because PTPS like GTPCH is not expressed in amniotic cells, the enzyme defect can only be confirmed by measurement of PTPS activity in fetal liver (136) or in fetal erythrocytes (140).

Since DHPR activity is expressed in normal fibroblasts and amniotic fluid cells (60, 115), as well as in blood cells (3, 59), and chorionic villi (57), DHPR deficiency can also be diagnosed prenatally. The first prenatal diagnosis of DHPR in a fetus at risk was described by Firgaira et al (61). Normal results were obtained in two cases (15, 61), and the results were confirmed by measuring DHPR activity in erythrocytes after the birth of these children. Recently, a cDNA clone for DHPR spanning the complete coding region was isolated by Dahl et al (34). Restriction fragment length polymorphisms (RFLPs), detected by using the restriction enzymes, might be useful for prenatal diagnosis of DHPR deficiency.

TREATMENT

Impaired hydroxylation of tyrosine and tryptophan in patients with BH_4 deficiency reduces the formation of the catecholamines and serotonin and results in severe neurological disorders. Therefore substitution with the neurotransmitter precursors, L-DOPA and 5-hydroxytryptophan, the BH_4 cofactor, or a combination of both, is essential (54a).

Neurotransmitter Therapy

First attempts at compensating the neurotransmitter deficiency in a patient with atypical PKU were successfully made by Bartholomé & Byrd (5). Although this therapy has since been widely used (6, 18–20, 42, 52, 66, 93,

148, 153, 170, 179), its possible risk during the period of neonatal brain development was discussed (22). However, data on long-term treatment are still scarce, and it is not known whether neurotransmitter precursors might have severe side effects. L-DOPA and 5-hydroxytryptophan are usually given in combination with an inhibitor of peripheral aromatic amino acid decarboxylase (Carbidopa) (55). Neurotransmitter replacement therapy with an initial dose of 6 mg L-DOPA per kg, 5 mg 5-hydroxytryptophan per kg, and 0.5 mg Carbidopa per day (148) favorably altered the clinical course of the disease. In a 7-year followup study (170) of a child with PTPS deficiency, mental and physical development were normal.

Tetrahydrobiopterin Therapy

In 1968 Jacobson (82) had already proposed that the defective hydroxylation of phenylalanine, tyrosine, and tryptophan in a patient with PKU may be alleviated by giving a suitable cofactor in excess. It was proposed that this would permit a less restricted phenylalanine intake. Cofactor replacement as suggested by Smith et al (167) was called into question by Kaufman (85). Danks et al (38, 39) were able to show that the elevated plasma phenylalanine levels can be lowered by BH_4 administration. Since it had been shown that peripherally administered reduced pterins do enter the brain (64, 83), replacement therapy with reduced pterins was proposed. As soon as this compound was available in a chemically pure form, a number of patients with atypical PKU were successfully treated with BH_4 alone or in combination with neurotransmitter precursors (8, 24, 30, 42, 55, 56, 62, 66, 90, 107, 113, 116, 129, 131, 133, 141, 160). A defective cofactor production can to some extent be corrected with a single oral dose of 2–5 mg BH_4 per kg. Because of the relatively poor penetration of BH_4 through the blood-brain barrier, this will, however, not always improve neurotransmitter biosynthesis in the brain; therefore orally administered BH_4 will mainly keep plasma phenylalanine levels under control. A few patients only were treated with high-dose BH_4 monotherapy (90, 99, 134). Successful treatment with 6-methyl tetrahydropterin reported by Kaufman et al (90) was not confirmed, and the results of later investigations by his and other groups were discouraging (99, 113; S. Scheibenreiter, unpublished observation). Trials with 6-hydroxymethyl tetrahydropterin, a BH_4 analogue that showed 3- to 6-fold better penetration into the rat brain and good cofactor activity for phenylalanine hydroxylase, also failed (99).

In DHPR deficiency there is no regeneration of the exogenous cofactor and administration of BH_4 will not improve the clinical symptoms. For those DHPR-deficient patients with simultaneous folic acid deficiency, therapy with 5-formyl tetrahydrofolic acid is recommended (81).

ACKNOWLEDGMENTS

This review is dedicated to the memory of Professor Alois Niederwieser, who passed away February 5, 1987. I am particularly indebted to Professor Hans-Christoph Curtius for valuable discussions and for his criticism of this report as well as to M. Killen and M. Stucki for preparing the manuscript.

This work was supported by the Swiss National Science Foundation, grant no. 3.395-0.86.

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