Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 385–389, January 1991
Biochemistry

Conversion of 6-substituted tetrahydropterins to 7-isomers via phenylalanine hydroxylase-generated intermediates

(hyperphenylalaninemia/tetrahydrobiopterin)

MICHAEL D. DAVIS*, SEYMOUR KAUFMAN, AND SHELDON MILSTIEN

Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, MD 20892

Contributed by Seymour Kaufman, October 1, 1990

ABSTRACT **A new variant form of hyperphenylalaninemia has recently been discovered in which the patients characteristically excrete 7-biopterin in their urines in addition to the natural 6-biopterin (Curtius, H. Ch., Kuster, T., Matasovic, A., Blau, N. & Dhondt, J.-L. (1988) *Biochem. Biophys. Res. Commun.* 153, 715–721). This isomer had not been found previously in humans, and although its origin was not established, preliminary evidence suggested that it might be produced from 6-biopterin. We have now found that 7-biopterin can be formed *in vitro* from (6*R*)-tetrahydrobiopterin during the hydroxylation of phenylalanine catalyzed by phenylalanine hydroxylase [L-phenylalanine, tetrahydrobiopterin:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1]. The resulting 7-biopterin was unequivocally identified by the following criteria: preparative isolation and conversion to 7-hydroxymethylpterin following periodate oxidation and borohydride reduction, quantitative conversion to pterin-7-carboxylic acid after oxidation with permanganate, and liquid chromatography/thermospray mass spectrometry. Addition of 4a-carbinolamine dehydratase, an enzyme involved in the regeneration of tetrahydrobiopterin from the pterin carbinolamine intermediate (also called 4a-hydroxytetrahydrobiopterin) formed in the phenylalanine hydroxylase reaction, greatly decreased the amount of the 7-biopterin formed. This result implies that the *in vitro* formation of 7-biopterin occurs via the nonenzymatic rearrangement of the unstable substrate of the dehydratase, 4a-hydroxytetrahydrobiopterin, and suggests that this new variant of hyperphenylalaninemia may be caused by a lack of 4a-carbinolamine dehydratase activity. A mechanism for the rearrangement is proposed that predicts that other 6-substituted tetrahydropterin substrates of the aromatic amino acid hydroxylases could also give rise to rearranged products from an opening of the pyrazine ring of the corresponding 4a-hydroxytetrahydropterin intermediate.**

Mammalian aromatic amino acid hydroxylases require a tetrahydropterin cofactor (1). The naturally occurring cofactor, tetrahydrobiopterin (BH_4), a fully reduced 6-substituted pterin (2), was first discovered because of its role in the hydroxylation of phenylalanine to tyrosine catalyzed by hepatic phenylalanine hydroxylase [L-phenylalanine, tetrahydrobiopterin:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] (3, 4). A genetic lack of phenylalanine hydroxylating activity results in phenylketonuria (PKU) (5). The most prevalent form of this disease, called classical PKU, has been shown to be caused by a lack of phenylalanine hydroxylase (6). Variant forms of PKU have also been described that are caused by either a deficiency of BH_4 (7–14) or a defect in the pathway required to reduce this cofactor, which is oxidized during catalysis (15). All of these variant forms of PKU result in a BH_4 deficiency, hyperphenylalaninemia, and a biogenic amine neurotransmitter deficiency, since BH_4 is also required for the conversion of tyrosine and tryptophan to catecholamines and serotonin, respectively (16).

A new type of hyperphenylalaninemia has recently been discovered in which the patients have only mild hyperphenylalaninemia and excrete in their urine a pterin that had not been observed previously in mammals (17, 18). This pterin has now been identified as 7-biopterin (19, 20), an isomer of biopterin [2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)pteridine or 6-biopterin]. The origin of this new isomer has been the subject of some speculation (21). Although the pathway of formation of 7-biopterin is not known, there have been two key findings that appear to provide important clues to its source. The first is that this variant form of PKU appears to be hereditary; and the second is that the amount of 7-biopterin found in these patients' urine increases when 6-BH_4, 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropteridine], is administered orally (20). A logical hypothesis that links these two observations is that the formation of 7-biopterin and the hyperphenylalaninemia are due to the same missing gene product.

Although their initial search for the origin of the 7-biopterin in the urine of their hyperphenylalaninemic patients was focused on the biosynthetic pathway of BH_4, Curtius and coworkers (22) have also recently suggested the possibility that this new pterin could arise from the breakdown of a short-lived tetrahydropterin intermediate formed during the hydroxylation of aromatic amino acids by phenylalanine (23) and tyrosine hydroxylases (24, 25). This intermediate, 4a-hydroxytetrahydrobiopterin (4a-carbinolamine), is actually the pterin product of the hydroxylation reaction and is rapidly dehydrated by an enzyme, 4a-carbinolamine dehydratase, forming quinonoid dihydrobiopterin (26, 27). Thus, if patients exhibiting this new variant form of PKU were missing 4a-carbinolamine dehydratase, the oxidation of BH_4 to the unstable 4a-carbinolamine by the aromatic amino acid hydroxylases would give rise to an increased concentration of this intermediate, which would result in increased conversion of the carbinolamine to 7-BH_4. In this communication, we report that 7-biopterin is indeed formed from BH_4 *in vitro* during the hydroxylation of phenylalanine to tyrosine catalyzed by phenylalanine hydroxylase when the reaction is carried out in the absence of 4a-carbinolamine dehydratase, suggesting that the 7-isomer arises by a nonenzymatic rearrangement of the pterin carbinolamine.

MATERIALS AND METHODS

L-Phenylalanine, NADH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenter-*

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BH_4, tetrahydrobiopterin; 6- or 7-BH_4 isomers, 2-amino-4-hydroxy-(6 or 7)-[1,2-dihydroxypropyl-(L-*erythro*)-5,6,7,8-tetrahydropteridine]; PKU, phenylketonuria.

*To whom reprint requests should be addressed.

oides) were purchased from Sigma. Beef liver catalase was purchased from Boehringer Mannheim. Sheep liver dihydropteridine reductase was purified through the calcium phosphate gel step (28). 4a-Carbinolamine dehydratase was purified from rat liver by the method of Huang *et al.* (29). Phenylalanine hydroxylase was purified from rat liver by a combination of two methods (30, 31) in what has been described as the hybrid procedure (32). (6*R*)-BH_4, 6-biopterin, 7-biopterin, pterin-6-carboxylic acid, and pterin-7-carboxylic acid were purchased from B. Schirks' laboratory (Jona, Switzerland). All other reagents were of the highest quality available. The HPLC apparatus consisted of a Waters M6000 pump, a Waters WISP autosampler, and a Perkin–Elmer LS-40 fluorescence detector interfaced with a Nelson analytical data analysis system. The 6- and 7-biopterins and 6- and 7-hydroxymethylpterins were analyzed on a Spherisorb C_{18} column (5 μm, 25 cm, PhaseSep) eluted with 5% methanol/95% water at a flow rate of 1.5 ml/min (20). Column temperature was usually maintained at 32°C with a SysTec (Minneapolis) column heater.

The conversion of 6-biopterin, 7-biopterin, and the unknown to their corresponding hydroxymethylpterins was performed by incubating 7.5–8.3 μM pterin in 0.1 M potassium bisulfate (pH 2.3) containing 13 μM potassium periodate for 2 hr. An excess of glycerol was then added to react with the remaining periodate. This was followed by the addition of potassium hydroxide (to 0.1 M) and sodium borohydride (in 0.1 M KOH) to a final concentration of 3.5 mM. After standing at room temperature overnight, the products were analyzed by HPLC.

The various pterins were quantitatively oxidized to the corresponding pterin carboxylic acids by the alkaline permanganate method. Samples (75 μl) were mixed with 20 μl of 1 M NaOH and 10 μl of saturated potassium permanganate. After the samples were boiled for 30 min, they were cooled and incubated for 20 min at room temperature with 70 μl of methanol. After centrifugation, an aliquot of the supernatant was analyzed by HPLC with the use of an Econosphere C_{18} column (5 μm, 15 cm; Alltech Associates) eluted with 0.1 M sodium phosphate (adjusted to pH 3 with H_3PO_4) containing 5% methanol at a flow rate of 1 ml/min.

Reverse-phase HPLC/mass spectroscopy was performed on a Finnigan TSP-46 LCMS with a Spherisorb C_{18} column equilibrated with 50 mM ammonium acetate/4% methanol, which delivered the sample (2–3 nmol) or the standards via a thermospray interface to the mass spectrometer. Both authentic 6- and 7-biopterin and the 7-biopterin isolated from phenylalanine hydroxylase reactions all had identical mass spectra, showing that an M+1 molecular ion at 238 mass units was eluted at the appropriate retention time.

RESULTS AND DISCUSSION

The origin of 7-biopterin has recently been the subject of some speculation (20–22). The discovery of increased excretion of this isomer in the urine of some hyperphenylalaninemic patients (17–19), its increased excretion after a load of natural tetrahydrobiopterin (20), and the demonstration that the stereochemistry of the dihydroxypropyl side chain is identical with that of the natural cofactor (33) have led to the suggestion that 7-biopterin may be produced from 6-biopterin as a result of a genetic alteration in the phenylalanine hydroxylase system (22).

The hepatic phenylalanine hydroxylase system is complex, consisting of three enzymes and two cofactors (1):

$$\text{phenylalanine} + \text{tetrahydrobiopterin} + O_2 \rightarrow \text{tyrosine} + \text{4a-carbinolamine tetrahydrobiopterin}, \quad [1]$$

$$\text{4a-carbinolamine tetrahydrobiopterin} \rightarrow \text{quinonoid dihydrobiopterin} + H_2O, \quad [2]$$

$$\text{quinonoid dihydrobiopterin} + \text{NADH} + H^+ \rightarrow \text{tetrahydrobiopterin} + NAD^+. \quad [3]$$

Reaction 1 is catalyzed by phenylalanine hydroxylase (1). Reaction 3 is catalyzed by dihydropteridine reductase (4). The essential coenzyme, BH_4 is synthesized *de novo* from GTP (34), and as mentioned above, genetic defects caused by the lack of enzymes in the BH_4 biosynthetic pathway as well as deficiencies in phenylalanine hydroxylase (reaction 1) and dihydropteridine reductase (reaction 3) all lead to hyperphenylalaninemia (35). To date, however, no patients with hyperphenylalaninemia have been identified who are lacking the enzyme that catalyzes reaction 2, the 4a-carbinolamine dehydratase. Since the reaction catalyzed by the dehydratase also proceeds nonenzymatically (23), a deficiency of the dehydratase might be expected to result in only a mild form of hyperphenylalaninemia (36).

Several different mechanisms for the formation of 7-biopterin have been proposed by Curtius and co-workers (21, 22) involving possible rearrangements of 6-BH_4 biosynthetic intermediates. These hypothetical pathways seem unlikely since they do not provide any obvious links to any genetic event. These workers have also suggested the intriguing possibility that these patients may have a defect in the dehydratase pathway (reaction 2) which would allow the carbinolamine intermediate to accumulate and, by a hydrolytic pyrazine ring-opening pathway, to form an imino alloxan derivative that could then close the ring in two ways to form a mixture of the 6- and 7-substituted pteridine isomers (22).

To test this hypothesis, we prepared the 4a-carbinolamine of (6*R*)-BH_4 with pure rat liver phenylalanine hydroxylase. Reaction conditions were utilized that maximized the steady-state concentration of the 4a-carbinolamine. The reaction mixture included: pure phenylalanine hydroxylase, NADH, a regeneration system for the NADH, dihydropteridine reductase, a low concentration of BH_4, and no 4a-carbinolamine dehydratase. Aliquots of the reaction mixture were removed at different times and treated with manganese dioxide in acid to fully oxidize all reduced pterin intermediates to the corresponding nonreduced parent pterins, which were then analyzed by HPLC. The 6- and 7-isomers of biopterin can be resolved by reverse-phase HPLC (20), with the 6-biopterin eluted just prior to the 7-isomer (Fig. 1*a*). At approximately 1 min after mixing the reaction components, the amount of carbinolamine reached a steady-state level (data not shown). At this time, all of the biopterin was still present as the 6-isomer (Fig. 1*b*). The reaction mixture was then divided into two separate reaction vessles, 4a-carbinolamine dehydratase was added to one, and the incubations were continued. In the control reaction mixture, there was a significant amount of a previously unobserved blue fluorescing HPLC peak that had the same retention time as authentic 7-biopterin (Fig. 1*c*). However, there was much less of this compound formed in the phenylalanine hydroxylase reaction mixture to which the dehydratase was added (Fig. 1*d*). The kinetics of the formation of the new pterin, tentatively identified as 7-biopterin based on its fluorescence and elution time, in the absence and presence of the dehydratase is shown in Fig. 2. As can be seen, the rate of formation of this new fluorescent pterin during the phenylalanine hydroxylase-catalyzed hydroxylation of phenylalanine is substantially lower in the presence of the dehydratase. These results are consistent with the hypothesis that 7-biopterin can be formed from the 4a-carbinolamine of 6-BH_4, which is the substrate of the dehydratase.

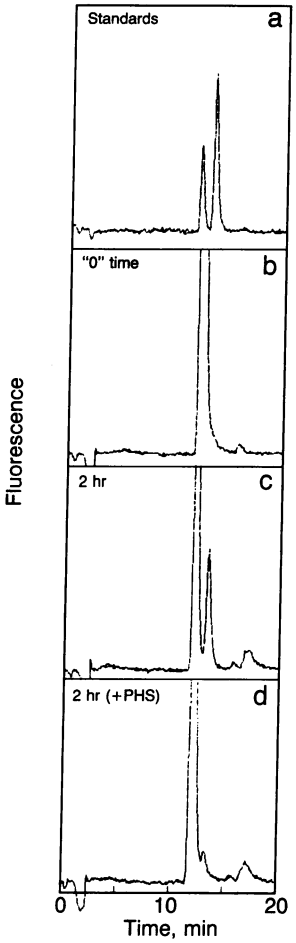

FIG. 1. Reverse-phase HPLC analysis of the pterin products of the oxidation of (6*R*)-BH_4 by phenylalanine hydroxylase in the presence and absence of 4a-carbinolamine dehydratase. (*a*) Authentic 6- and 7-biopterin. The 6-biopterin is eluted prior to the 7-biopterin. (*b–d*) Products formed in the absence or presence of 4a-carbinolamine dehydratase. (6*R*)-BH_4 (22 μM) was added to a 3-ml cuvette containing 30 mM potassium phosphate (pH 8.2), 0.1 mg of catalase per ml, 85 μM NADH, 10 mM glucose 6-phosphate, 1 mM L-phenylalanine, 0.21 μM phenylalanine hydroxylase (≈50% active), and an excess of dihydropteridine reductase and glucose-6-phosphate dehydrogenase. The reaction was carried out at 25°C and monitored by ultraviolet absorption spectroscopy for 4a-carbinolamine formation (23). Approximately 1 min after mixing all components, the concentration of 4a-carbinolamine reached a steady state. An aliquot was removed, oxidized as described, and analyzed by HPLC using a fluorescence detector (*b*). The reaction mixture was then divided into two flasks, and 0.03 mg of the 4a-carbinolamine dehydratase was added per ml to one of the samples, and the incubations were continued. After 2 hr, aliquots from both flasks were removed and analyzed separately as described above. There was more 7-biopterin formed in the absence (*c*) than in the presence (*d*) of the dehydratase.

To confirm the identify of this new pterin, we scaled up reaction mixtures 30-fold and isolated this pterin in its oxidized form by preparative HPLC. Its structure was deduced by the results of several different specific chemical modifications as compared with the same reactions with synthetic 6- and 7-biopterin. Treatment with periodate, followed by borohydride reduction, was used to convert the putative 7-biopterin to 7-hydroxymethylpterin. As shown in Fig. 3*a*, authentic 6- and 7-hydroxymethylpterins can only be partially separated by reverse-phase HPLC and have similar retention times. Conversion of putative 7-biopterin to 7-hydroxymethylpterin resulted in the formation of a single fluorescent HPLC peak that was eluted with the same retention

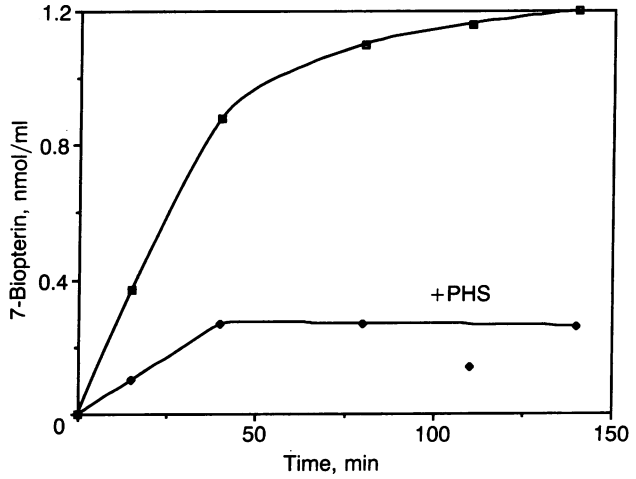

FIG. 2. Time course of the formation of 7-biopterin during the phenylalanine-dependent oxidation of (6*R*)-BH_4 catalyzed by phenylalanine hydroxylase. The conditions for this experiment were the same as described for Fig. 1. Aliquots from the reaction mixture were removed at the indicated times, and the pterins were analyzed as described. PHS, phenylalanine hydroxylase-stimulating enzyme, the generic name for the 4a-carbinolamine dehydratase.

time as that of the derivative obtained from authentic 7-biopterin (not shown). As a more definitive demonstration of the identity, mixtures of the putative 7-hydroxymethylpterin and standard 6- and 7-hydroxymethylpterins were analyzed by HPLC. The putative 7-hydroxymethylpterin was separated from 6-hydroxymethylpterin standard, whereas it was coeluted with the 7-hydroxymethyl standard (Fig. 3 *b* and *c*).

Treatment with alkaline permanganate oxidizes the side chain of pterins to the corresponding carboxylic acid. Permanganate oxidation of the purified 7-biopterin, isolated from the reaction mixture described above, gave a quantitative yield of pterin-7-carboxylic acid (Fig. 4). Interestingly, we found that the relative fluorescent intensitites of the 6- and 7-isomers differed by a factor of 10 in the HPLC buffer system (pH 3), with the 6-substituted carboxylic acid having the greater fluorescence. This was an important character-

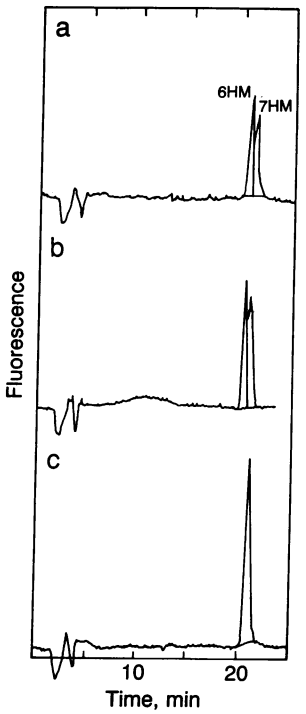

FIG. 3. Analysis of 6- and 7-hydroxymethylpterins (6HM and 7HM) by reverse-phase HPLC. The 6- and 7-hydroxymethylpterins were prepared from 6- and 7-biopterin, respectively, by periodate oxidation followed by borohydride reduction and were chromatographed as described. (*a*) Authentic 6- and 7-hydroxymethylpterin. Each isomer alone gave a single peak eluted at the indicated times. (*b*) Mixture of the hydroxymethylpterins derived from authentic 6-biopterin and the unknown compound. The unknown compound gave a single fluorescent peak eluted at the same time as 7-hydroxymethylpterin. (*c*) Mixture of the hydroxymethylpterins derived from authentic 7-biopterin and the unknown compound.

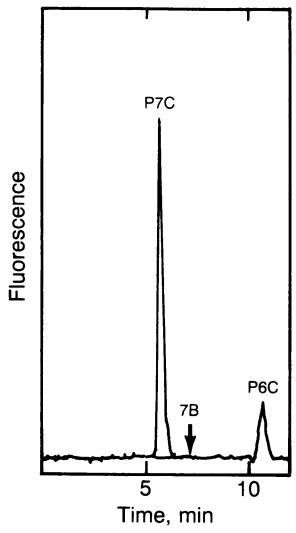

FIG. 4. Reverse-phase HPLC analysis of the pterin products formed after alkaline permanganate oxidation of the putative 7-biopterin. The unknown pterin was treated with alkaline permanganate and chromatographed as described. Standard 6- and 7-biopterin (7B) gave quantitative yields of the corresponding carboxypterins. The retention times of authentic pterin-6-carboxylic acid (P6C) and pterin-7-carboxylic acid (P7C) were identical to those prepared by permanganate oxidation.

istic, since our isolated putative 7-biopterin was contaminated with ≈1% 6-biopterin. As can be seen in Fig. 4, after permanganate oxidation of the new compound, the resulting product gave two peaks on the HPLC chromatogram; the first peak comigrated with the pterin-7-carboxylic acid standard, whereas the second was coeluted with standard pterin-6-carboxylic acid. There was no detectable 7-biopterin remaining. The relative fluorescence intensities of the two peaks indicate that the amount of pterin-6-carboxylic acid produced by the oxidation was, as expected, ≈1% of the 7-isomer. Furthermore, liquid chromatographic analysis coupled with thermospray mass spectrometry confirmed that the compound eluted with the same retention time as 7-biopterin had the same mass spectrum (data not shown). Taken together, these analyses unequivocally prove that our identification of this new HPLC peak as 7-biopterin is correct.

The original mechanism proposed by Curtius and coworkers (22) for the conversion of the 4a-carbinolamine of BH_4 to 7-biopterin only sketched possible intermediates. In fact, the complete hydrolysis of the pyrazine ring as suggested would lead to two products that would not likely react with each other at the very low concentrations in which they are generated as enzymatic intermediates. Our proposed mechanism for the conversion of the fully reduced 6-BH_4 to a 7-substituted pterin via the phenylalanine hydroxylase-generated carbinolamine intermediate is shown in Fig. 5. The first step is described above by reaction **1**, with BH_4 being enzymatically converted to the corresponding 4a-carbinolamine derivative by phenylalanine hydroxylase, which then dissociates from the enzyme. When 4a-carbinolamine dehydration is rapid, the dihydroxypropyl side chain is normally retained in the 6-position, allowing the cofactor to cycle catalytically. In the absence of the dehydratase, which catalyzes reaction **2** and results in the formation of the quinonoid dihydro derivative of 6-biopterin [(6)-qBH2], we suggest that the increased lifetime of the carbinolamine allows significant amounts of its pyrazine ring to open, yielding a 4a-keto and a 5-amino moiety (as shown immediately below the 6-4a-carbinolamine in Fig. 5). The 5-amino constituent can attack the 8a-carbon forming a five-membered ring containing two equivalent secondary amines (Fig. 5). This ring can then reopen in two ways, with either of the ring nitrogen atoms giving rise to a primary amine. When the primary amine is formed *ortho* to the dihydroxypropyl side chain, the original 6-substituted pterin is reformed, simply reflecting the reversibility of the proposed ring openings and closings. If the five-membered ring opens in the other direction, converting the original N^8 nitrogen to the primary amine, subsequent addition to the 4a-carbonyl will generate the 7-substituted carbinolamine isomer. Dehydration of either carbinolamine gives rise to the respective quinonoid dihydrobiopterin isomers with a retention of side-chain configuration.

The ring-opening of a reduced form of biopterin had been suggested earlier by Hamilton (37), although for an intermediate (a hypothetical tetrahydrobiopterin hydroperoxide) that in his scheme occurred just prior to the formation of the 4a-carbinolamine. Subsequent studies by Ayling and coworkers (38–40) demonstrated that severing of the analogous carbon–nitrogen bond of pyrimidinone cofactor analogues could occur during the catalytic hydroxylation of phenylal-

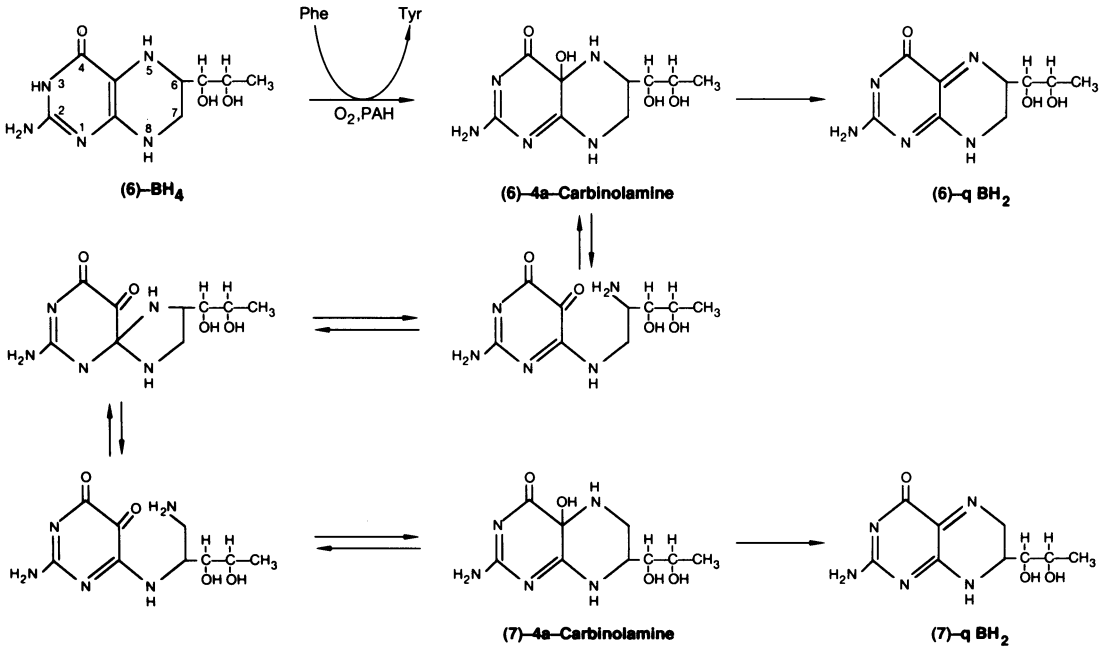

FIG. 5. Proposed mechanism for the synthesis of 7-substituted reduced pterins from 6-substituted reduced pterins via the carbinolamine intermediate generated by phenylalanine hydroxylase. (6)-BH_4, natural (6*R*)-BH_4; (6)-4a-carbinolamine, 4a-hydroxytetrahydrobiopterin; PAH, phenylalanine hydroxylase; Phe, phenylalanine; Tyr, tyrosine; (6)-qBH_2, quinonoid dihydrobiopterin; (7)-4a-carbinolamine, 4a-hydroxy-7-dihydroxypropyltetrahydropterin; (7)-qBH_2, 7-dihydroxypropyl quinonoid dihydropterin.

anine by phenylalanine hydroxylase—a result consistent with the proposed hypothesis of the opening of the pyrazine ring of a reduced form of biopterin. Pike *et al.* (40) pointed out that this result was equally consistent with the ring-opening occurring just subsequent to the formation of the carbinolamine, which is what has been drawn in our scheme (Fig. 5). In addition, these workers were able to demonstrate that the ring-opened species could recyclize nonenzymatically. Furthermore, they demonstrated that the recyclization of ring-opened homologues of pterin cofactors derived from pyrimidodiazepines was aided by 4a-carbinolamine dehydratase (40).

In the present study, we have found that the percentage of BH_4 that is converted to 7-substituted pterins per catalytic turnover of phenylalanine hydroxylase is relatively small as shown by the slow rate of formation of 7-biopterin. Interestingly, the total amount of biopterin (6- plus 7-isomers) recovered from reaction mixtures decreased significantly with time in the absence of the dehydratase (data not shown), without the appearance of additional fluorescent pterin species, suggesting that the opening of the pyrazine ring may lead to a number of not yet identified products. Pike *et al.* (40) have also found appreciable losses of starting material during the phenylalanine hydroxylase-catalyzed cyclization of a ring-opened form of 6-methyltetrahydropterin, giving rise to a number of products that only absorb in the low ultraviolet region.

Finally, in our proposed mechanism for the rearrangement of 6-BH_4 to 7-BH_4, the dihydroxypropyl side chain does not play any role in the reaction. Thus, it is expected that any tetrahydropterin substrate for phenylalanine hydroxylase that forms a carbinolamine with a significant lifetime should also undergo the same rearrangement. It has been demonstrated that the commonly used BH_4 analogue, 6-methyltetrahydropterin, is also converted to a carbinolamine intermediate during the phenylalanine hydroxylase-catalyzed hydroxylation of phenylalanine (27). Analysis of the pterins remaining after phenylalanine hydroxylase reactions with 6-methyltetrahydropterin as the cofactor, carried out as described above, demonstrated that 7-methylpterin was indeed slowly formed in the absence of the dehydratase (data not shown). Further work is necessary to determine whether other cofactors also undergo this rearrangement, but this result provides further support for the proposed mechanism.

The observation and identification of a side-product of the *in vitro* oxidation of BH_4 by phenylalanine hydroxylase as 7-biopterin were unexpected but did provide a new perspective on the role of an enzyme discovered and characterized in this laboratory, the 4a-carbinolamine dehydratase (23, 41). Heretofore, it remained somewhat of a puzzle why the dehydration of the 4a-carbinolamine, which at physiological pH proceeded nonenzymatically at a pace rapid enough not to be rate-limiting, still required an enzyme to catalyze the reaction. Our results suggest that the role of the dehydratase may not be only to increase the rate of the overall phenylalanine hydroxylase reaction but also to minimize the conversion of the pterin cofactor to the 7-isomer. This latter function may become more important in the absence of the dehydratase, since cofactor concentration may actually decrease because of loss of open-ring forms of the carbinolamine intermediate. Furthermore, the presence of 7-BH_4 itself could also have deleterious effects on phenylalanine hydroxylase activity. Preliminary studies in our laboratory (M.D.D. and S.K., unpublished data) indicate that even though 7-BH_4 is a cofactor for phenylalanine hydroxylase, its competition with the natural cofactor would lead to a decreased hydroxylation rate, since the reaction with 7-BH_4 is partially uncoupled, producing tyrosine inefficiently.

We are grateful to Dr. H. Fales (National Heart, Lung, and Blood Institute) and Dr. L. Pannell (National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases) for their assistance with mass spectral analyses.

1. Kaufman, S. & Fisher, D. B. (1974) in *Molecular Mechanisms of Oxygen Activation*, ed. Hayaishi, O. (Academic, New York), pp. 285–369.
2. Kaufman, S. (1963) *Proc. Natl. Acad. Sci. USA* **50**, 1085–1093.
3. Kaufman, S. (1957) *J. Biol. Chem.* **226**, 511–524.
4. Kaufman, S. (1958) *J. Biol. Chem.* **230**, 931–939.
5. Jervis, G. A. (1953) *Proc. Soc. Exp. Biol. Med.* **82**, 514–515.
6. Kaufman, S. (1958) *Science* **128**, 1506.
7. Kaufman, S., Milstien, S. & Bartholomé, K. (1975) *Lancet* **i**, 708.
8. Smith, I., Clayton, B. E. & Wolff, O. (1975) *Lancet* **i**, 328–329.
9. Rey, F., Blandin-Savoja, F. & Rey, J. (1976) *N. Engl. J. Med.* **295**, 1138–1139.
10. Leeming, R. J., Blair, J. A. & Rey, F. (1976) *Lancet* **i**, 99.
11. Rey, F., Harpey, J. P., Leeming, R. J., Blair, J. A., Aicardi, J. & Rey, J. (1977) *Arch. Fr. Pediatr.* **34**, 109–120.
12. Niederwieser, A., Leimbacher, W., Curtius, H. Ch., Ponzone, A., Rey, F. & Leupold, D. (1985) *Eur. J. Pediatr.* **144**, 13–16.
13. Niederwieser, A., Ponzone, A. & Curtius, H. Ch. (1985) *J. Inherited Metab. Dis.* **8**, Suppl. 1, 34–38.
14. Niederwieser, A., Blau, N., Wang, M., Jorrer, P., Atares, M. & Cardesa-Garcia, J. (1984) *Eur. J. Pediatr.* **141**, 208–214.
15. Kaufman, S., Holtzman, N. A., Milstien, S., Butler, I. J. & Krumholz, A. (1975) *N. Engl. J. Med.* **293**, 785–790.
16. Kaufman, S. (1981) in *Function and Regulation of Monoamine Enzymes*, eds. Usdin, E., Weiner, N. & Youdim, M. B. H. (MacMillan, Woodbridge, IL), pp. 165–173.
17. Dhondt, J. L., Guibaud, P., Rolland, M. O., Dorche, C., Andre, S., Forzy, G. & Hayte, J. M. (1988) *Eur. J. Pediatr.* **147**, 153–157.
18. Curtius, H. Ch., Kuster, T., Matasovic, A., Blau, N. & Dhondt, J. L. (1988) *Biochem. Biophys. Res. Commun.* **153**, 715–721.
19. Blau, N., Curtius, H. Ch., Kuster, T., Matasovic, A., Schoedon, G., Dhondt, J. L., Guibaud, T. & Blascovics, M. (1989) *J. Inherited Metab. Dis.* **12**, 335–338.
20. Curtius, H. Ch., Matasovic, A., Schoedon, G., Kuster, T., Guibaud, P., Guidici, T. & Blau, N. (1990) *J. Biol. Chem.* **265**, 3923–3930.
21. Curtius, H. Ch., Adler, C., Matasovic, A. & Akino, M. (1990) in *Chemistry and Biology of Pteridines 1989*, eds. Curtius, H. Ch., Ghisla, S. & Blau, N. (de Gruyter, Berlin), pp. 274–285.
22. Adler, C., Curtius, H. Ch. & Pfleiderer, W. (1990) in *Pterins and Biogenic Amines in Neuropsychiatry, Pediatrics and Immunology*, eds. Blau, N., Curtius, H. Ch. & Levine, R. A. (Lakeshore, Grosse Pointe, MI), in press.
23. Kaufman, S. (1976) in *Iron and Copper Proteins 1976*, eds. Yasunobu, K. T., Mower, H. F. & Hayaishi, O. (Plenum, New York), pp. 91–102.
24. Haavik, J. & Flatmark, T. (1987) *Eur. J. Biochem.* **168**, 21–26.
25. Dix, T. A., Kuhn, D. M. & Benkovic, S. J. (1987) *Biochemistry* **26**, 3354–3361.
26. Huang, C. Y. & Kaufman, S. (1973) *J. Biol. Chem.* **248**, 4242–4251.
27. Lazarus, R. A., Benkovic, S. J. & Kaufman, S. (1983) *J. Biol. Chem.* **248**, 4235–4241.
28. Craine, J. E., Hall, S. & Kaufman, S. (1972) *J. Biol. Chem.* **247**, 6082–6091.
29. Huang, C. Y., Max, E. E. & Kaufman, S. (1973) *J. Biol. Chem.* **248**, 4235–4241.
30. Kaufman, S. & Fisher, D. B. (1970) *J. Biol. Chem.* **245**, 4745–4750.
31. Shiman, R., Gray, D. W. & Pater, A. (1979) *J. Biol. Chem.* **254**, 11300–11306.
32. Kaufman, S. (1987) *Methods Enzymol.* **142**, 3–17.
33. Viscontini, M. (1990) *Helv. Chim. Acta* **73**, 1064–1067.
34. Nichol, C. A., Smith, G. K. & Duch, D. S. (1985) *Annu. Rev. Biochem.* **54**, 729–764.
35. Kaufman, S. (1976) *Adv. Neurochem.* **2**, 1–132.
36. Kaufman, S. (1983) in *Advances in Human Genetics*, eds. Harris, H. & Hirschhorn, K. (Plenum, New York), pp. 217–297.
37. Hamilton, G. A. (1974) in *Molecular Mechanisms of Oxygen Activation*, ed. Hayaishi, O. (Academic, New York), pp. 405–451.
38. Bailey, S. W. & Ayling, J. E. (1980) *J. Biol. Chem.* **255**, 7774–7781.
39. Bailey, S. W., Weintraub, S. T., Hamilton, S. M. & Ayling, J. E. (1982) *J. Biol. Chem.* **257**, 8253–8260.
40. Pike, D. C., Hora, M. I., Bailey, S. W. & Ayling, J. E. (1986) *Biochemistry* **25**, 4762–4771.
41. Kaufman, S. (1970) *J. Biol. Chem.* **245**, 4751–4759.