Evidence for the Formation of the 4a-Carbinolamine during the Tyrosine-dependent Oxidation of Tetrahydrobiopterin by Rat Liver Phenylalanine Hydroxylase*

Michael D. Davis and Seymour Kaufman

From the Laboratory of Neurochemistry, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland 20892

In the presence of phenylalanine and molecular oxygen, activated phenylalanine hydroxylase catalyzes the oxidation of tetrahydrobiopterin. The oxidation of this tetrahydropterin cofactor also proceeds if the substrate, phenylalanine, is replaced by its product, tyrosine, in the initial reaction mixture. These two reactions have been defined as coupled and uncoupled, respectively, because in the former reaction 1 mol of phenylalanine is hydroxylated for every mole of tetrahydropterin oxidized, whereas in the latter reaction there is no net hydroxylation of tyrosine during the oxidation of the tetrahydropterin.

During the course of the coupled oxidation of tetrahydrobiopterin, a pterin 4a-carbinolamine intermediate can be detected by ultraviolet spectroscopy (Kaufman, S. (1976) in Iron and Copper Proteins (Yasunobu, K. T., Mower, H. F., and Hayashi, O., eds) pp. 91-102, Plenum Publishing Corp., New York). Dix and Benkovic (Dix, T. A., and Benkovic, S. J. (1988) Biochemistry 27, 5859-5866) have postulated that the formation of this intermediate only occurs when the oxidation of the tetrahydropteridine is tightly coupled to the concomitant hydroxylation of the aromatic amino acid. However, during the tyrosine-dependent uncoupled oxidation of tetrahydrobiopterin by phenylalanine hydroxylase, we have detected the formation of a spectral intermediate with ultraviolet absorbance that is essentially identical to that of the carbinolamine. Furthermore, this absorbance can be eliminated by the addition of 4a-carbinolamine dehydratase, an enzyme which catalyzes the dehydration of the 4a-carbinolamine. Quantitation of this intermediate suggests that there are two pathways for the tyrosine-dependent uncoupled oxidation of tetrahydrobiopterin by phenylalanine hydroxylase because only about 0.3 mol of the intermediate is formed per mol of the cofactor oxidized.

Phenylalanine hydroxylase is an iron-containing monooxygenase (1, 2) which catalyzes the conversion of oxygen and phenylalanine to tyrosine and water (3) using two reducing equivalents from tetrahydrobiopterin (BH$_4$)$^*$ (4-7) (Equation 1).

Phenylalanine + tetrahydrobiopterin $\rightarrow$ tyrosine + quinonoid dihydrobiopterin + H$_2$O (1)

Quinonoid dihydrobiopterin + NADH $\rightarrow$ tetrahydrobiopterin + NAD$^*$ + H$^+$ (2)

A second enzyme, dihydropteridine reductase (5), catalyzes the NADH-dependent regeneration of BH$_4$ from its oxidized product, quinonoid dihydrobiopterin (8, 9) (Equation 2). Under normal reaction conditions the ratio of tyrosine formed to oxygen and BH$_4$ consumed is 1:1:1 (4, 10). Under different conditions, however, the oxidation of BH$_4$ can be completely (11, 12) or partially (10, 13) uncoupled from the hydroxylation of the amino acid substrate. In the presence of analogues of the substrate such as para-fluorophenylalanine (14) or para-chlorophenylalanine (15) or analogues of BH$_4$, such as 7-methyltetrahydropterin or tetrahydropterin (10, 13, 16), the hydroxylation reaction is partially coupled, whereas in the presence of lysolecithin and tyrosine (in place of phenylalanine) the enzymatic oxidation of BH$_4$ is fully uncoupled (11, 12). Under these conditions phenylalanine hydroxylase functions either partially or fully as a tetrahydropterin oxidase.

Although the precise mechanism of action of phenylalanine hydroxylase is unknown, an important clue to this process was provided by the demonstration that concomitant with the hydroxylation of phenylalanine, BH$_4$ is converted to the corresponding 4a-carbinolamine (17, 18). At neutral pH, this pteridine intermediate, whose structure is shown in Fig. 1, is unstable and rapidly breaks down to form quinonoid dihydrobiopterin (qBH$_4$) (17). Under these conditions, even with only catalytic amounts of BH$_4$, the breakdown is so rapid that this step does not limit the rate of the hydroxylation reaction. In contrast, at alkaline pH (pH 8.2-8.4), the intermediate is sufficiently stable that its conversion to qBH$_4$ becomes the rate-limiting step in the enzymatic hydroxylation of phenylalanine (17, 19). These observations led to the demonstration that an enzyme present in liver (20) which was purified to homogeneity (21), is a dehydratase that catalyzes the conversion of the 4a-carbinolamine to the corresponding quinonoid dihydrobiopterin (22).

Recently, Dix and Benkovic (23), using conditions where the hydroxylation reaction is partially uncoupled, have reported that tetrahydropterins are oxygenated to 4a-carbinolamines only during catalytic events involving substrate hydroxylation. This generalization was made based on studies of partially coupled reactions with either para-chlorophenylalanine and 6-methyltetrahydropterin, or phenylalanine with tetrahydropterin. However, in contrast to their conclusion, we have observed an intermediate during the fully uncoupled oxidation of BH$_4$ whose properties appear to be the same as

---

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

$^*$ To whom correspondence should be addressed.

$^1$ The abbreviations used are: BH$_4$, 6-(dihydroxypropyl)-L-erythro-5,8,7,8-tetrahydropterin (tetrahydrobiopterin) (the naturally occurring isomer is (6R)-BH$_4$); qBH$_4$, quinonoid dihydrobiopterin; 7,8-dihydropterin, 7,8-dihydrobiopterin plus 7,8-dihydropterin; HPLC, high performance liquid chromatography; L-Dopa, L-3,4-dihydroxyphenylalanine.

Phenylalanine hydroxylase is an iron-containing monooxygenase (1, 2) which catalyzes the conversion of oxygen and phenylalanine to tyrosine and water (3) using two reducing equivalents from tetrahydrobiopterin (BH$_4$)$^*$ (4-7) (Equation 1).
those of the carbinolamine. This result does not support the
generalization of Dix and Benkovic (23) and strongly suggests
that substrate hydroxylation is not a necessary requirement
for the formation of the carbinolamine.

EXPERIMENTAL PROCEDURES

Materials—Phenylalanine, lyssolecithin, 2,6-dichlorophenol-indo-
phenol, glucose 6-phosphate, glucose-6-phosphate dehydrogenase
(from Leuconostoc mesenteroides), L-3,4-dihydrophenylalanine, 2,4,5-
trihydroxyphenylalanine, tyrosinase (from mushroom), and NADH
were purchased from Sigma. Potassium phosphate was obtained from J.
T. Baker Co. (6R)-tetrahydrobiopterin and 6-methyl-
tetrahydropterin were purchased from Dr. B. Schirks Laboratories,
Jena, Switzerland. Thin-layer plates, MN 300 cellulose (normal), 250
μm thick, were purchased from Analtech Inc. Catalase and superoxide
dismutase were obtained from Boehringer Mannheim. Dihydropter-
dine reductase from sheep liver was purified through the calcium
phosphate gel step (24). 4a-Carbinolamine dehydratase was purified
by the method of Huang et al. (21). Phenylalanine hydroxylase was
purified by the hybrid procedures as described previously (25). The
pure enzyme was between 30 and 70% active (2, 26). L-Tyrosine
was purchased from Calbiochem and recrystallized twice from water after
recovery of the enzyme and the two substrates phenylalanine and mole-

cular oxygen combine with phenylalanine hydroxylase to form a quaternary complex (10). The next step in catalysis
is believed to be the formation of the still hypothetical 4a-
hydroperoxide-tetrahydrobiopterin Fig. 1 (16, 17), although
other plausible intermediates have been suggested (36). As a
consequence of the subsequent hydroxylation of phenylala-
nine to tyrosine, the carbinolamine 4a-hydroxytetrohydro-
biopterin is formed (17, 18). This oxidation product of the
reduced pteridine is released from the enzyme and forms qBH2
by losing a molecule of water (Fig. 1). Although this dehy-
dration occurs rapidly under physiological conditions (17), an
enzyme, 4a-carbinolamine dehydratase, catalyzes the reaction
(19-22). It is qBH2 which is the substrate for the NADH-
dependent dihydropteridine reductase, the enzyme responsible
for maintaining biotinyl in the fully reduced state (i.e.
as BH2) (8, 9). In the absence of this regeneration system,
qBH2 nonenzymatically isomerizes to form a mixture of 7,8-
dihydropterin (plus lactoyl aldehyde) and 7,8-dihydrobiot-
erin (33-35) (Fig. 1).

The spectra of the observable species of reduced biotin
"determine the course of the enzymatic conversion of phenylala-
nine to tyrosine by phenylalanine hydroxylase, in 20 mM
potassium phosphate, pH 8.2, are shown in Fig. 2. The spec-

of the log of the concentration versus time. Attempts to fit the
biphasic kinetics of the two intermediates (the carbinolamine and
qBH2) as the sum of two exponentials by computer analysis were
unsuccessful. Therefore, the first phase of these reactions is described
by a simple τo. For ease of comparison, the rates of the decrease of
the concentration of BH2 and the increase in the concentration of
tyrosine were also expressed in terms of τo.

RESULTS AND DISCUSSION

The Oxidation of Tetrahydrobiopterin by Phenylalanine Hy-
droxylase—The naturally occurring cofactor for phenylala-
nine hydroxylase is BH4 (7), (Fig. 1). During the physiological
reaction, BH4 and the two substrates phenylalanine and mol-

cular oxygen combine with phenylalanine hydroxylase to form a quaternary complex (10). The next step in catalysis
is believed to be the formation of the still hypothetical 4a-
hydroperoxide-tetrahydrobiopterin Fig. 1 (16, 17), although
other plausible intermediates have been suggested (36). As a
consequence of the subsequent hydroxylation of phenylala-
nine to tyrosine, the carbinolamine 4a-hydroxytetrohydro-
biopterin is formed (17, 18). This oxidation product of the
reduced pteridine is released from the enzyme and forms qBH2
by losing a molecule of water (Fig. 1). Although this dehy-
dration occurs rapidly under physiological conditions (17), an
enzyme, 4a-carbinolamine dehydratase, catalyzes the reaction
(19-22). It is qBH2 which is the substrate for the NADH-
dependent dihydropteridine reductase, the enzyme responsible
for maintaining biotinyl in the fully reduced state (i.e.
as BH2) (8, 9). In the absence of this regeneration system,
qBH2 nonenzymatically isomerizes to form a mixture of 7,8-
dihydropterin (plus lactoyl aldehyde) and 7,8-dihydrobiot-
erin (33-35) (Fig. 1).

Tetrahydrobiopterin by Phenylalanine Hy-
droxylase and 4a-carbinolamine tetrahydrobiopterin; qBH2
by phenylalanine hydroxylase. The following abbreviations were
used: 4a-PEROXY-BH4, 4a-peroxotetrahydrobiopterin; 4a-HY-
DROXY-BH4, 4a-carbinolamine tetrahydrobiopterin; 7,8 PH2, 7,8-
dihydropterin; 7,8 BH2, 7,8-dihydrobiopterin.

Fig. 1. The chemical structures of pterin derivatives
formed from BH4 during the oxidation of tetrahydrobiopterin
by phenylalanine hydroxylase. The following abbreviations were
used: 4a-PEROXY-BH4, 4a-peroxotetrahydrobiopterin; 4a-HY-
DROXY-BH4, 4a-carbinolamine tetrahydrobiopterin; 7,8 PH2, 7,8-
dihydropterin; 7,8 BH2, 7,8-dihydrobiopterin.
An Intermediate in the Uncoupled Oxidation of Tetrahydrobiopterin

The spectrum of BH₄ has a broad absorbance maximum centered at 297 nm ($\epsilon = 10,200 \text{ M}^{-1} \text{ cm}^{-1}$) and an even broader shoulder at about 255 nm ($\epsilon = 4,900 \text{ M}^{-1} \text{ cm}^{-1}$). The strong absorbance of the carbinolamine centered at 246 nm ($\epsilon = 19,600 \text{ M}^{-1} \text{ cm}^{-1}$) allows this intermediate to be readily identified, whereas the second broader peak centered at about 285 nm ($\epsilon = 8,500 \text{ M}^{-1} \text{ cm}^{-1}$) is easily masked by the absorbance of the other pterins. The spectrum of qBH₂ shows two extremely broad peaks centered at around 255 nm ($\epsilon = 6,500 \text{ M}^{-1} \text{ cm}^{-1}$) and 301 nm ($\epsilon = 8,700 \text{ M}^{-1} \text{ cm}^{-1}$) and a shoulder at approximately 370 nm ($\epsilon = 3,700 \text{ M}^{-1} \text{ cm}^{-1}$). This shoulder allows the identification of qBH₂, because none of the other pterins absorb beyond 380 nm. The final spectrum shown in Fig. 2 is the composite spectrum of 7,8-dihydrobiopterin and 7,8-dihydropterin (38). Both 7,8-dihydropteridines have similar spectra (34, 35), and their combined spectrum has absorbance maxima at 278 nm ($\epsilon = 9,800 \text{ M}^{-1} \text{ cm}^{-1}$) and 325 nm ($\epsilon = 5,900 \text{ M}^{-1} \text{ cm}^{-1}$).

The spectra of BH₄ and the 7,8-dihydropteridines are similar to those published previously (33, 35, 37). On the other hand, to the best of our knowledge, this is the first record of the absorbance spectra of pure qBH₂ (Fig. 2). The spectrum shown was obtained by extrapolation because of the instability of qBH₂. The absorbance of qBH₂ was found to be approximately 5 and 10% of the absorbance of BH₂ has a broad absorbance maximum centered at 297 nm ($\epsilon = 10,200 \text{ M}^{-1} \text{ cm}^{-1}$) and an even broader shoulder at about 255 nm ($\epsilon = 4,900 \text{ M}^{-1} \text{ cm}^{-1}$). The strong absorbance of the carbinolamine centered at 246 nm ($\epsilon = 19,600 \text{ M}^{-1} \text{ cm}^{-1}$) allows this intermediate to be readily identified, whereas the second broader peak centered at about 285 nm ($\epsilon = 8,500 \text{ M}^{-1} \text{ cm}^{-1}$) is easily masked by the absorbance of the other pterins. The spectrum of qBH₂ shows two extremely broad peaks centered at around 255 nm ($\epsilon = 6,500 \text{ M}^{-1} \text{ cm}^{-1}$) and 301 nm ($\epsilon = 8,700 \text{ M}^{-1} \text{ cm}^{-1}$) and a shoulder at approximately 370 nm ($\epsilon = 3,700 \text{ M}^{-1} \text{ cm}^{-1}$). This shoulder allows the identification of qBH₂, because none of the other pterins absorb beyond 380 nm. The final spectrum shown in Fig. 2 is the composite spectrum of 7,8-dihydrobiopterin and 7,8-dihydropterin (38). Both 7,8-dihydropteridines have similar spectra (34, 35), and their combined spectrum has absorbance maxima at 278 nm ($\epsilon = 9,800 \text{ M}^{-1} \text{ cm}^{-1}$) and 325 nm ($\epsilon = 5,900 \text{ M}^{-1} \text{ cm}^{-1}$).

The spectra of BH₄ and the 7,8-dihydropteridines are similar to those published previously (33, 35, 37). On the other hand, to the best of our knowledge, this is the first record of the absorbance spectrum of "pure" qBH₂ (Fig. 2). The spectrum shown was obtained by extrapolation because of the instability of qBH₂. The absorbance of qBH₂ was found to be essentially the same for all three pteridines, the peak at 278 nm ($\epsilon = 9,800 \text{ M}^{-1} \text{ cm}^{-1}$) and 325 nm ($\epsilon = 5,900 \text{ M}^{-1} \text{ cm}^{-1}$).

The simplest interpretation of these spectral changes is based on those derived from the first observation of the carbinolamine (17). Thus, the initial spectra are a composite of the absorbance of BH₄ and the carbinolamine, with a small.
contribution centered at 278 nm due to the concomitant conversion of phenylalanine to tyrosine. The increase in absorbance at 246 nm is a function of the formation of the carbinolamine, whereas the lack of any significant absorbance changes above 340 nm for the initial spectra indicates that during the early part of the reaction, little, if any, qBH₂ is being formed. The long wavelength absorbance in the final spectrum suggests that subsequent to the formation of the carbinolamine, qBH₂ is formed. Although we have not included the subsequent spectra, the peak at 246 nm did decline with time while the absorbance above 340 nm increased before it too decreased.

If 4a-carbinolamine dehydratase is added to an otherwise identical reaction mixture, the spectral changes observed are quite different (Fig. 3b). The initial spectrum now more closely resembles that of (6R)-BH₄ and the peak at 246 nm never appears (17). Instead, a steady increase in absorbance between 235 and 280 nm is observed. The peak at 297 nm still declines, but a second broader absorbance maximum centered at around 300 nm forms along with a peak at 278 nm. There is also an increase in the absorbance beyond 320 nm (Fig. 3b).

In comparison with Fig. 3a, the spectral changes observed in Fig. 3b are consistent with the catalytic properties attributed to the dehydratase (19, 20, 22). Thus, a rapid build-up of qBH₂ is demonstrated by the large amount of absorbance observed above 320 nm, by the new spectral peak found at 300 nm, as well as by the increase in absorbance between 235 and 280 nm (see Fig. 2). On the other hand, the absence of a peak at 246 nm is strong evidence against any significant accumulation of the carbinolamine. Finally, the peak at 278 nm can again be attributed to the spectral differences between the substrate, phenylalanine, and the product, tyrosine (31).

Formation of the Carbinolamine in the Tyrosine-dependent Uncoupled Oxidation of (6R)-BH₄—As mentioned earlier, in the presence of lysoleculinic acid and tyrosine (and in the absence of phenylalanine) phenylalanine hydroxylase catalyzes a completely uncoupled reaction in which BH₄ is oxidized without any net hydroxylation of the amino acid (11, 12). At pH 6.9 the uncoupled reaction proceeds at approximately 10% of the rate of the coupled reaction (Table I) (11, 12). As the pH is raised from 6.9 to 8.2, the rate of the uncoupled reaction decreases somewhat but remains approximately one-tenth of the rate of the coupled reaction (Table I), which decreases in a parallel manner (40). Although this result is the opposite of that reported by Dix and Benkovic (23), who found that in contrast to the coupled reaction, the uncoupled reaction proceeds at a more rapid rate as the pH is raised to 8.4, the discrepancy may be more apparent than real because these workers were studying the partially uncoupled oxidation of synthetic tetrahydropteridines. In any case, the data in Table I indicate that the fully uncoupled oxidation of (6R)-BH₄ at pH 8.2 is rapid enough to allow the observation of the carbinolamine.

Dix and Benkovic (23) were unable to detect the formation of the carbinolamine during the uncoupled portion of the phenylalanine hydroxylase-catalyzed oxidation of 6-methyltetrahydropterin or tetrahydropterin. Based on their findings, they drew the general conclusion that substrate hydroxylation was a necessary prerequisite for the formation of carbinolamine. However, when the experiment described in Fig. 3a was repeated with the substitution of tyrosine for phenylalanine as the only change, a similar, although smaller increase in absorbance at 246 nm was observed (Fig. 4a). The absorbance at 297 nm (due to BH₄) also decreased. There were, however, two features of this kinetic experiment that differed from that in Fig. 3a. First, no new peak at 278 nm was formed. Second, the long wavelength absorbance began to increase immediately (Fig. 4a).

The formation of a new spectral peak at 246 nm during the oxidation of BH₄ is diagnostic for the carbinolamine (Fig. 1), although it should be noted that the amount of absorbance found at 246 nm is significantly less than would be expected if all of the BH₄ were rapidly converted to this species, as was essentially true in the reaction with phenylalanine present (compare Fig. 4a with Fig. 3a). The lack of a significant increase in absorbance at 278 nm (which is mainly due to tyrosine formation in the coupled reaction) paralleling the decrease at 297 nm is consistent with the reaction being uncoupled and totally devoid of phenylalanine. At the very least, we can conclude that the ultraviolet absorbance spectrum of the aromatic amino acid (in this case tyrosine) is not being detectably altered during the oxidation of BH₄. Finally, the rapid increase in absorbance above 340 nm is strong evidence that qBH₂ is being formed at the very early stages of this reaction.

Further evidence for the formation of the carbinolamine during the tyrosine-dependent uncoupled oxidation of BH₄ is presented in Fig. 4b. The spectra shown are for an experiment which followed the identical protocol as that in Fig. 4a except that the 4a-carbinolamine dehydratase was included. First, the peak at 246 nm, which was observed in Fig. 4a, is missing and in its place is a broad and smaller increase in absorbance between 235 and 280 nm. Second, the decrease in absorbance at 297 nm is accompanied by a slight shift in the center of this broad peak to about 300 nm, and there is a significant amount of increase in absorbance above 320 nm even at the earliest of times. Finally, as in Fig. 4a, there is no evidence for a change in the spectrum of tyrosine. In fact, excluding this last spectral characteristic, Fig. 4b is essentially identical to Fig. 3b. Thus, the addition of the dehydratase to the fully uncoupled reaction causes the oxidation of (6R)-BH₄ to rapidly form qBH₂ without the accumulation of the carbinolamine. As a corollary, it appears that a detectable amount of carbinolamine is formed during the uncoupled oxidation of

<table>
<thead>
<tr>
<th>Aromatic amino acid</th>
<th>pH 6.8</th>
<th>pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>5.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*The phenylalanine hydroxylase was approximately 50% active (26).
An Intermediate in the Uncoupled Oxidation of Tetrahydrobiopterin

**Fig. 4.** The formation of the carbinolamine during the tyrosine-dependent uncoupled oxidation of tetrahydrobiopterin by phenylalanine hydroxylase. a, as described in Fig. 3a except 740 μM tyrosine was added to the reaction mixture in place of the phenylalanine and the concentration of phenylalanine hydroxylase was increased to 2.9 μM (~50% active (26)). The spectra were recorded at the following times after the final addition: 5 s (---); 10 s (---); 15 s (---); 20 s (---); 25 s (---); and 30 s (---). b, as above, except 56 μg/ml 4a-carbinolamine dehydratase was included in the reaction mixture. The spectra were recorded at the following times after the final addition: 5 s (---); 10 s (---); 15 s (---); 20 s (---); 25 s (---); 30 s (---); and 40 s (---).

(6R)-BH4 when the dehydratase is excluded from the reaction mixture.

Additional support for this last conclusion was provided by the results of experiments which allowed the initial build-up of the carbinolamine prior to the addition of the dehydratase. In this case, NADH and dihydropteridine reductase were added to regenerate BH4 from qBH2 (5), and glucose 6-phosphate and glucose-6-phosphate dehydrogenase were included to regenerate the NADH (17). For the fully coupled oxidation of BH4, a rapid increase in absorbance at 245 nm is observed following the addition of phenylalanine hydroxylase (Fig. 5a). The absorbance change reaches a maximum and then very slowly begins to decline. This decrease is greatly accelerated if the dehydratase is added to the reaction mixture. These results are identical to those found in an earlier study (17), and can be interpreted in a similar manner. Thus, the carbinolamine is formed during the early part of the reaction and rapidly reaches a steady-state level. The subsequent addition of the dehydratase catalyzes the breakdown of this intermediate and the loss in absorbance at 245 nm. The slow decay of the carbinolamine (in the absence of the dehydratase) following the formation of the steady-state concentration of this species may be due to the slow irreversible inactivation of phenylalanine hydroxylase which occurs during catalysis (41). The resulting decrease in active enzyme would then slow down the rate of carbinolamine formation which, in turn, lowers the steady-state concentration of this intermediate. The parallel study carried out with the fully uncoupled system appears to be identical to that found with the coupled system (Fig. 5b), a result consistent with the carbinolamine being formed in the completely uncoupled reaction.

Further support for our assignment of the transient absorbance peak at 246 nm as being due to the carbinolamine is obtained from the physical separation of the pterin intermediates by HPLC. Haavik and Flatmark (29) have reported the resolution of the reaction intermediates formed during the oxidation of BH4 by phenylalanine hydroxylase in the presence of phenylalanine. After reproducing their findings (data not shown), we have gone on to perform the analogous experiment for the tyrosine-dependent reaction. As can be seen in
Fig. 6a, when tyrosine, BH₄, and lysolecithin are combined in the absence of phenylalanine hydroxylase, two peaks are observed. By performing the chromatography of each of these constituents individually (data not shown) we can assign the first peak to tyrosine, the second to BH₄. The addition of phenylalanine hydroxylase (Fig. 6b) results in the formation of a new peak which has a retention time which is similar to that of the carbinolamine formed in the fully coupled reaction (29), whereas the peak due to BH₄ is significantly diminished. The large excess of tyrosine necessary for the tyrosine-dependent uncoupled reaction masks the position on the chromatogram where qBH₂ elutes.² The peak assigned to the carbinolamine diminished in the time-frame of the breakdown of this intermediate as the reaction was monitored by ultraviolet spectroscopy (data not shown). Furthermore, if 4a-carbinolamine dehydratase (phenylalanine hydroxylase stimulating enzyme) is added to the enzyme-catalyzed oxidation of BH₄, the additional peak seen in Fig. 6b essentially disappears (Fig. 6c). The relationship between the new peak and the dehydratase is consistent with our assignment of this peak to the carbinolamine. Finally, the ultraviolet spectrum of this chromatographic peak is essentially the same as that of the carbinolamine obtained during the fully coupled reaction (Fig. 7) and is furthermore essentially identical to the spectra published previously (17, 29) and to that shown in Fig. 2. From the results outlined in Figs. 4–7, we conclude that the carbinolamine is indeed formed during the tyrosine-dependent uncoupled oxidation of (6R)-BH₄ catalyzed by phenylalanine hydroxylase.

Evidence against the Hydroxylation of Tyrosine by Phenylalanine Hydroxylase—Fisher and Kaufman (12) have shown that there is no net hydroxylation of tyrosine catalyzed by phenylalanine hydroxylase at pH 6.9. However, these workers did not examine this reaction under conditions which are optimized for monitoring the formation of the carbinolamine, i.e. pH 8.2 (17, 19). We therefore set out to determine if phenylalanine hydroxylase could catalyze the hydroxylation of tyrosine at alkaline pH. In Table IIa we list the Rᵢ values of phenylalanine and tyrosine, as well as those of all of the plausible end products of their oxidation by phenylalanine hydroxylase. As can be seen, all of the compounds can be resolved by thin-layer chromatography (27). The results of the application of this method to the analysis of the products of the tyrosine-dependent oxidation of (6R)-BH₄ in the presence of NADH and dihydropteridine reductase are also shown. The reaction was monitored for the conversion of NADH to NAD⁺ and allowed to proceed until about 90% of this cofactor had been oxidized. This was equivalent to approximately 95% of tyrosine at alkaline pH. In Table IIb we list the values of phenylalanine and tyrosine, as well as those of all of the plausible end products of their oxidation by phenylalanine hydroxylase.

Fig. 7. Ultraviolet spectra of the 4a-carbinolamine formed in the coupled and uncoupled oxidations of (6R)-BH₄ by phenylalanine hydroxylase. The spectra were obtained by resolving individual peaks by HPLC and using a photodiode array detector (29). The protocol is the same as that outlined for Fig. 6. The coupled reaction contained 25 mM potassium phosphate, pH 8.3, 100 μg/ml catalase, 1.1 mM phenylalanine, 70 μM (6R)-BH₄ and 0.2 μM phenylalanine hydroxylase (70% active). The uncoupled reaction contained 25 mM potassium phosphate, pH 8.3, 100 μg/ml catalase, 1.0 mM tyrosine, 70 μM (6R)-BH₄, 0.2 mM lysolecithin, and 3.1 μM phenylalanine hydroxylase (70% active).

²The close proximity of the elution positions of qBH₂ and tyrosine under the chromatographic conditions used has been shown by Hauvik and Flatmark (29). We have confirmed this observation by monitoring the elution of qBH₂ (prepared by the method of Matsurra et al. (32)) at 400 nm (data not shown), a wavelength where none of the other pterin intermediates absorb (Fig. 2).
of the [14C]tyrosine present in the reaction mixture. A control experiment was performed which did not contain phenylalanine hydroxylase. Samples for both experiments were chromatographed and analyzed for 14C-labeled products. As can be seen in Table IIA, both in the presence and absence of phenylalanine hydroxylase, more than 90% of the tyrosine was recovered unchanged. Furthermore, the recovery of tyrosine varied by less than 1% between the enzymatic and nonenzymatic incubations. In a similar experiment, the final reaction solutions were analyzed for tyrosine by monitoring its phenylthiohydantoin derivative on a Waters Picotag HPLC system. Again the concentration of tyrosine in the minus phenylalanine hydroxylase blank was within 2% of the reaction which contained phenylalanine hydroxylase (data not shown). Additional evidence consistent with phenylalanine hydroxylase not functioning as a tyrosine hydroxylase is found in Table IIB. In this case, aliquots of the above reaction mixtures were assayed for [14C]Dopa (28). (To validate this assay, separate aliquots were pre-incubated with tyrosinase, which converts tyrosine to Dopa, prior to the Dopa determination.) Thus, within the limits of our analysis (1-2%) our results are consistent with the conclusion that tyrosine is not hydroxylated by phenylalanine hydroxylase.3

Optimal Conditions for the Formation of the Carbinolamine—Until now, we have reported that the increase in absorbance at 246 nm, due to the formation of the carbinolamine during the tyrosine-dependent oxidation of BH4 by phenylalanine hydroxylase, is significantly lower than the amount observed during the fully coupled reaction without trying to explain this difference (compare Fig. 3a with Fig. 4a). At first this smaller spectral change was thought to be the consequence of the slower rate of the uncoupled oxidation compared to the fully coupled reaction (Table 1). Because the rate of breakdown of the carbinolamine should be independent of the amino acid present during its formation, the accumulation of this intermediate during the steady-state would be dependent on its rate of formation. And indeed, in a reaction mixture similar to that described for Fig. 5b (i.e. enzymatically regenerating both BH4 and NADH) varying the amount of active phenylalanine hydroxylase between 0.27 and 1.1 μM showed a proportional increase in the steady-state absorbance at 246 nm (data not shown). However, in experiments in which BH4 was not regenerated (reaction conditions which are more amenable to quantitating the concentration of the carbinolamine), the ratio of carbinolamine (obtained by extrapolation as described under "Methods") to BH4 (initially added to the reaction) was approximately 0.3, even under what we believe are optimal conditions for the formation of this intermediate. This result is consistent with the observation made earlier (Fig. 4a) that an increase in absorbance above 360 nm (characteristic of the formation of qBH3) is observed at the onset of the fully uncoupled reaction. Thus, it appears that in contrast with the fully coupled reaction, during the tyrosine-dependent oxidation of BH4 by phenylalanine hydroxylase under our experimental conditions, only 30% of the oxidized BH4 passes through the carbinolamine intermediate.

There is further evidence for this conclusion. Under certain reaction conditions, with a low concentration of BH4, and an alkaline pH, the breakdown of the carbinolamine can become the sole rate-determining step during the hydroxylase-catalyzed, tightly coupled oxidation of BH4. In this case, the addition of the 4a-carbinolamine dehydratase has been reported to increase the rate of the coupled reaction as much as 5-fold (19). However, although we were able to confirm this published result (data not shown), the addition of the dehydratase during the analogous, fully uncoupled oxidation of BH4 resulted in less than a 2-fold activation. Thus, whereas the dehydratase does affect the rate of the tyrosine-dependent oxidation of the pterin cofactor by phenylalanine hydroxylase, this effect is far less dramatic than in the coupled reaction.

Kinetic traces for the tyrosine-dependent oxidation of BH4 in the presence of NADH and dihydropteridine reductase are shown in Fig. 8. As can be seen, there is a clear difference in the reaction rate when the dehydratase is included, most notably in the first minute of the reaction. Whereas the kinetic trace is essentially linear when the dehydratase is present, in its absence a rapid deceleration is observed. After these initial changes, both rates appear to deviate from linearity in a similar fashion, although the rate of the decrease in absorbance continues to be greater for the reaction containing the dehydratase (Fig. 8). The rapid deceleration in reaction velocity observed in the absence of the dehydratase is probably

---

**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>cpm</th>
<th>% total</th>
<th>cpm</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>1,430</td>
<td>1,670 cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>62,300</td>
<td>76,660 cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>±Tyrosinase</td>
<td>2.29%</td>
<td>2.37%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*4,5,6-Trihydroxyphenylalanine (6-hydroxydopa) migrated as a smear from the origin to 0.18.

---

3 It is conceivable that tyrosine is transiently hydroxylated at the para-position by phenylalanine hydroxylase at some time during the short time period after the binding of BH4 and before the release of the carbinolamine from the catalytic site. The product, para-dihydroxydihydrophenylalanine, could then rapidly breakdown to form tyrosine and OH-. Furthermore, it is possible that this intermediate would be undetected by our spectral analysis, and because the final product is tyrosine we suspect our analysis of the product (e.g. as in Table II) would be uninformative. To test this hypothesis, the tyrosine-dependent uncoupled oxidation of BH4 was performed in an O2 atmosphere. After incubation with the complete phenylalanine-hydroxylating system, the tyrosine was isolated and analyzed by mass spectrometry. The results showed no incorporation of the heavy oxygen into the aromatic amino acid. Thus, unless the oxygen added to the para-position of tyrosine is stereospecifically removed while this putative intermediate is still bound to the enzyme, this hypothesis is inconsistent with the experimental findings (H.-U. Siegmund and S. Kaufman, unpublished results).
An Intermediate in the Uncoupled Oxidation of Tetrahydrobiopterin

**Fig. 8.** The effect of 4a-carbinolamine dehydratase (PHS) on the rate of the tyrosine-dependent oxidation of (6R)-BH$_4$ by phenylalanine hydroxylase. The uncoupled oxidation of 7.5 mM (6R)-BH$_4$, by 2.2 mM phenylalanine hydroxylase (40% active) was performed in the presence or absence of 20 µg/ml 4a-carbinolamine dehydratase. The reaction was run in 0.05 M potassium phosphate, pH 8.2, at 25 °C and contained 1.3 mM tyrosine, 100 µM l-lyssolecithin, 100 µM NADH, 50 µg/ml catalase, 40 µg/ml superoxide dismutase, and an excess of dihydropteridine reductase.

Due to the gradual build-up of a steady-state concentration of the carbinolamine, which is not a substrate for dihydropteridine reductase. It seems likely that the smaller stimulation by the dehydratase of the tyrosine-dependent uncoupled oxidation of BH$_4$, as compared to the tightly coupled reaction in the presence of phenylalanine, is due to the alternate pathway for qBH$_4$ formation in the uncoupled reaction.

Although not all of the BH$_4$ oxidized by phenylalanine hydroxylase during the tyrosine-dependent reaction appears to proceed via the carbinolamine, there are reasons to believe that this intermediate is formed along the main reaction pathway. Thus, no carbinolamine is observed if the oxidation of tetrahydrobiopterin by phenylalanine hydroxylase is attempted in the presence of tyrosine but in the absence of l-lyssolecithin (an activator of phenylalanine hydroxylase (42)), indicating that the formation of this intermediate requires the enzyme to be activated. A similar requirement had been reported earlier for the tyrosine-dependent uncoupled oxidation (12). Furthermore, the concentration of the carbinolamine found during the steady-state is dependent on the concentration of the phospholipid (data not shown), and this dependency is in reasonable agreement with the $K_m$ for l-lyssolecithin that had been determined by monitoring the oxidation of BH$_4$ during the fully uncoupled reaction (43). Similarly, the carbinolamine is not observed in the absence of a substrate or substrate analog and the concentration of the intermediate found during the steady-state shows a correlation with the concentration of tyrosine (data not shown), and this correlation is in reasonable agreement with the $K_m$ determined previously for tyrosine (43).

**Quantitation of the Reactants, Intermediates, and Products—**Quantitation of the carbinolamine formed during the tyrosine-dependent oxidation of BH$_4$ was carried out with the use of the spectra shown in Fig. 2. Although the spectrum of the carbinolamine was derived from experiments involving the coupled reaction, this spectrum can also be used to describe that portion of the uncoupled reaction as well (Fig. 9). The data shown in Fig. 9 were obtained during the fully uncoupled oxidation of BH$_4$, in the presence of regenerating systems for both NADH and BH$_4$ (17), and represent a point in time when the level of the carbinolamine had reached a maximum. At this point in the oxidation, it was estimated that 30% of the initial BH$_4$ added to the reaction mixture was in the form of the carbinolamine. The dotted line represents the experimental data, whereas the solid line was derived by the spectral deconvolution of the experimental results and then summing the values determined, using the spectra in Fig. 2. As can be seen, the spectra in Fig. 9 have two broad absorbance maxima centered at about 245 and 295 nm, respectively, and are strikingly similar to the initial spectrum seen in Fig. 3a, which was obtained under quite different reaction conditions. Apparently these spectra are composites of very similar quantities of the same spectral species.

The deconvolution of spectra from experiments which lack the two regenerating systems are shown in Fig. 10. In this case, all of the newly formed spectral species can be observed. During the coupled reaction, a decrease in the concentration of BH$_4$ with time is accompanied by an increase in the carbinolamine along with the concomitant conversion of phenylalanine to tyrosine (Fig. 10a). After about 40 s, the oxidation of BH$_4$ is essentially complete, the maximum concentration of carbinolamine has been reached and tyrosine formation has reached a maximum. As the reaction continues over the next minute or so, the carbinolamine begins to decrease and a new species, qBH$_4$, begins to accumulate. With further time, the qBH$_4$ also decreases while the 7,8-dihydropteridines begin to become the predominant form of reduced pterin. These results are as predicted (17). However, there are some slight discrepancies in the data, which are probably due to the errors inherent in our analysis. First, there appears to...
be slightly too much tyrosine formed between 40 and 80 s. Second, the last 10% of the BH₄ decays at a slower rate than expected for a simple first-order decay. And, finally, the concentration of the carbinolamine appears to be decreasing to about 7% of the initial BH₄, rather than to zero. These anomalies deal with relatively small percentages of the reactants and, overall, the analysis appears to be internally consistent.

The results of the above experiment are tabulated in Table III. As can be seen, the stoichiometry of the pteridine species supports the model which has a linear progression of one species being converted to the next without a branched pathway. The ratio of tyrosine formed to BH₄ oxidized is consistent with this reaction being tightly coupled.

A comparison of the half-time of oxidation of BH₄ with those for the formation of tyrosine and the carbinolamine indicates that these processes occur at the same time (Table III). In contrast, the half-life for the build-up of qBH₂ is 4–5 times longer than these initial events, consistent with qBH₂ being formed at a subsequent time. Furthermore, although the rate of decay of the carbinolamine is somewhat slower than that for the formation of qBH₂, the two values are well within a factor of 2, suggesting that qBH₂ is indeed the product of the dehydration of the carbinolamine. Finally, the rate of disappearance of qBH₂ is equivalent to the rate of appearance of the 7,8-dihydropteridines. Thus, the kinetic analysis agrees with the stoichiometric determinations; they are also fully consistent with results of our previous study (17).

The spectral analysis of the tyrosine-dependent uncoupled oxidation of BH₄ is shown in Fig. 10b. The decrease in concentration of BH₄ is again accompanied by the formation of the carbinolamine, although in this case, as expected for the uncoupled reaction, there is no detectable change in the aromatic amino acid. Furthermore, qBH₂ appears to be increasing at a similar rate, suggesting that some of this pteridine is formed independently of the carbinolamine. By the end of the first 50 s, almost all of the BH₄ has been oxidized and the maximal concentrations of the two intermediates, qBH₂ and carbinolamine, have been reached. For the remainder of the reaction, the two intermediates decrease as the final products, 7,8-dihydropterin and 7,8-dihydrobiopterin, accumulate.

The stoichiometry for the uncoupled reaction is shown in Table III. Again, the amount of qBH₂ and 7,8-dihydropteridines formed is equivalent to the initial concentration of BH₄, which is consistent with the final products (i.e. the 7,8-dihydropteridines) arising from the rearrangement of qBH₂. On the other hand, only one-third of the initial concentration of BH₄ appears to form the carbinolamine intermediate, suggesting that at least two-thirds of the qBH₂ is a direct product of the oxidation of BH₄. The kinetic analysis is also consistent with this interpretation. As can be seen in Table III, the half-time of the decrease in the concentration of BH₄ is exactly in between the rates of formation of the carbinolamine and qBH₂. These values differ by less than a factor of 2, a difference that is probably within the experimental error of the analysis, and therefore for the present discussion will be considered to be the same. The rate of decay of the carbinolamine is approximately 4 times slower than the rate of formation of qBH₂, indicating that most of the qBH₂ was not derived from the carbinolamine. On the other hand, the rate of decrease of the concentration of qBH₂ is approximately equal to that of the increase of the 7,8-dihydropteridines, suggesting that these two chemical processes are derived from the same event.
An Intermediate in the Uncoupled Oxidation of Tetrahydrobiopterin

Table III
Quantitative description of the coupled and uncoupled oxidations of (6R)-BH₄ by phenylalanine hydroxylase

The experimental protocols are the same as described for the coupled reaction in Fig. 3a and for the uncoupled reaction in Fig. 4a. The values listed were determined as described under “Methods.”

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration*</th>
<th>% total</th>
<th>t₅₀</th>
<th>Formation</th>
<th>k</th>
<th>Decay</th>
<th>Formatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coupled</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH₄</td>
<td>14.4</td>
<td>100</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>13.4</td>
<td>93</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbinolamine</td>
<td>14.8</td>
<td>103</td>
<td>9</td>
<td>0.5 (80)</td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>qBH₄</td>
<td>14.5</td>
<td>101</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,8-Dihydrobiopterin + 7,8-dihydropterin</td>
<td>12.3</td>
<td>85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncoupled</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH₄</td>
<td>13.7</td>
<td>100</td>
<td>15</td>
<td></td>
<td></td>
<td>0.6</td>
<td>70</td>
</tr>
<tr>
<td>Carbinolamine</td>
<td>4.4</td>
<td>32</td>
<td>10</td>
<td></td>
<td></td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>qBH₄</td>
<td>14.1</td>
<td>103</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,8-Dihydrobiopterin + 7,8-dihydropterin</td>
<td>12.2</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The values listed were obtained from plots of the log of the concentration versus time as described under “Methods.” These quantities represent the total individual amounts of these substrates, products, and intermediates either added to or formed during these reactions.

** The numbers in parentheses are the half-times (t₅₀) in seconds.

Fig. 11. Proposed scheme for the phenylalanine hydroxylase-catalyzed reactions in the presence of either tyrosine or phenylalanine. Phenylalanine hydroxylase must be activated in order to catalyze the sequence of reactions shown in the scheme. The completely uncoupled reaction is catalyzed by lysocellitin-activated phenylalanine hydroxylase in the presence of tyrosine (12, 43). By contrast, because phenylalanine can serve as both activator and substrate, the normal tightly coupled hydroxylation of phenylalanine (sequences 1, 2, and 5) requires the addition of no other activators (41).

The most noticeable difference between the coupled and uncoupled reactions is the relative maximal concentrations of qBH₄ and the carbinolamine. During the coupled reaction, the carbinolamine is the more abundant intermediate (Fig. 10a), whereas in the uncoupled reaction it is qBH₄ which appears to accumulate in large quantities (Fig. 10b). On the other hand, with the exception of one reaction resulting in the hydroxylation of an aromatic acid, and the other having two means of forming qBH₄, the two sets of results are quite similar. Thus, the reaction conditions were set so that the oxidation of BH₄ proceeded at the same rate, and the half-times for the formation of the carbinolamine are equivalent. Similarly, the rates of the breakthrough of the carbinolamine and qBH₄ are not changed by the presence or absence of phenylalanine or tyrosine.

A Summary of the Uncoupled Oxidation of BH₄—A scheme that can account for the present results is shown in Fig. 11, where XH₄ is a tetrahydropterin, qXH₂ is quinonoid dihydropterin, XH₂OH is the pterin 4a-carbinolamine, XH₃OOGH is the putative pterin 4a-hydroperoxide, and Fe=O is the postulated hydroxylating species, an enzyme-bound iron-oxo intermediate (44). Because the exact electronic configuration of this last species has not been established, the oxidation state of this intermediate has not been specified. It has been reported that the isolated enzyme must be reduced prior to catalysis (45, 46). Furthermore, Benkovic and coworkers (46) have presented evidence that this reduction results in the Fe²⁺ form of the enzyme being converted to the Fe³⁺ form. However, since the precise valence state of the protein-bound iron in each of the remaining steps in catalysis is unknown, we have not specified the valence of the iron in our scheme.

Nonetheless, our earlier observation that phenylalanine hydroxylase can catalyze the hydroxylation of an alkane carbon (44) suggests, as we have noted previously, that an iron-oxo intermediate is involved in the hydroxylation reaction. Although we are discussing the uncoupled reaction, for the sake of completeness, the scheme also includes the normal coupled hydroxylation reaction (reaction 5) where an amino acid that is capable of being hydroxylated, RH, is converted to its hydroxylated product, ROH. Not shown is the nonenzymatic rearrangement of qXH₂ to the corresponding 7,8-dihydropteridine (with quinonoid dihydropterin, the rearrangement yields a mixture of two 7,8-dihydropteridines: 7,8-dihydrobiopterin and 7,8-dihydropterin (35)).

We propose that the uncoupled reaction proceeds through the same enzyme-bound oxygenated intermediate that we have suggested earlier (16, 17) for the coupled reaction, a 4a-tetrahydropterin hydroperoxide (see Fig. 1). Although there is no direct evidence for its formation, the demonstration that the 4a-carbinolamine is a product of the completely uncoupled reaction supports, as we have noted previously for the coupled reaction, the postulate that a pterin hydroperoxide is the precursor of the carbinolamine (17). This proposal is also in accord with mechanisms that have been suggested for flavin monooxygenase-catalyzed hydroxylation reactions in which a flavin hydroperoxide intermediate has been directly observed (48-51). Nonetheless, it should be emphasized that

*Consistent with our proposal, Hill et al. (47) have presented indirect evidence for an enzyme-bound reduced-oxygen intermediate during the reaction of phenylalanine hydroxylase with 6-methyl-5,6,7,8-tetrahydropterin and 13-hydroxypropionic acid. Furthermore, these workers (47) suggest that this intermediate could be a ferrous reduced oxygen species.
pathways for the formation of the carbinolamine that do not go via the pterin hydroperoxide can be envisioned.

Our present results indicate that the enzyme-catalyzed uncoupled oxidation of BH$_4$ does not proceed through a linear sequence of reactions but rather that the pathway is a branched one. Thus, we have presented evidence that approximately two-thirds of the qXH$_2$ formed does not pass through the 4a-carbinolamine intermediate. The sequence that does go through the carbinolamine is shown as reactions 1, 2, and 4. In the coupled reaction, according to this formulation, the other product of reaction 2, Fe=O, would be consumed in the hydroxylation reaction (reaction 5). In the uncoupled reaction, we propose that Fe=O is dissipated through a reductive reaction involving another molecule of XH$_4$ as the electron donor (reaction 4); in this sequence, XH$_4$ is also converted to qXH$_2$. Without considering any other source of qXH$_2$, reactions 3 (catalyzed by 4a-carbinolamine dehydratase) and 4 predict that one-half of the qXH$_2$ formed proceeds through the carbinolamine.

We have previously reported that at pH 6.8 the tyrosin-dependent uncoupled oxidation of BH$_4$ catalyzed by lysolecithin-activated phenylalanine hydroxylase results in the formation of H$_2$O$_2$ (12). Under conditions used in the present study (i.e. pH 8.2), we have found in preliminary experiments that 30–40% of the oxidizing equivalents of molecular oxygen are consumed in a pathway that leads to H$_2$O$_2$ formation. To account for these results, we propose that during the uncoupled reaction the pterin hydroperoxide intermediate can also break down to H$_2$O$_2$ and qXH$_2$ (reaction 6) in a reaction analogous to the decomposition of the carbinolamine to H$_2$O$_2$ and qXH$_2$. Because there is no detectable H$_2$O$_2$ formed during the analogous coupled oxidation of XH$_4$ (12), the alternative suggestion that the H$_2$O$_2$ observed during the uncoupled reaction is formed by a pathway which does not proceed through the common intermediate, XH$_4$OOH, seems less likely. Whatever the precise mechanism of the enzyme-dependent formation of H$_2$O$_2$, this pathway, which does not go via the carbinolamine, together with the consequences of reactions 3 and 4, can account for our observation that two-thirds of the qXH$_2$ that is formed in the completely uncoupled reaction does not pass through the carbinolamine.

The 4a-carbinolamine has been shown to be an intermediate in the oxidation of BH$_4$ by phenylalanine hydroxylase purified either from rat liver (17, 52) or from Chromobacterium violaceum (53), as well as in the analogous reaction with tyrosine hydroxylase purified from phaeochromocytoma cells (29, 54). Recently, it has been stated (23) that the carbinolamine is only formed during catalytic events involving substrate hydroxylation (23). This generalization was based on experiments using partially uncoupled conditions (13–15). Since we have observed the formation of the carbinolamine during the completely uncoupled oxidation of BH$_4$, our results are not consistent with this generalization.

The heterogeneity of the reaction intermediates observed in our studies mandates a minimum of two pathways for the fully uncoupled oxidation of BH$_4$ only one of which apparently involves the carbinolamine. Thus, it is conceivable that the partially uncoupled portion of the oxidation of the tetrahydropteridines described by Dix and Benkovic (23) follows the pathway that does not involve the formation of the carbinolamine. In any case, our present results show that the carbinolamine can indeed be formed in the absence of any net hydroxylation of the aromatic amino acid substrate.

Acknowledgments—We would like to thank Dr. Peter S. Backlund, Jr. for performing the tyrosine determination on the Waters PicoTag HPLC system, Dr. Kasturi Srinivasaschar for aid in the use of his HPLC instrument equipped with a diode array detector, Mary Carpenter for expert technical assistance in preparing phenylalanine hydroxylase, Dr. Sheldon Milstein for helpful discussions, and Dr. John Giovanni for permission to read this manuscript. We are grateful to Dr. Gerald Cohen of the Mount Sinai School of Medicine for the generous gift of 2,4-dihydroxyphenylalanine. We would also like to thank Kathy Carter for her expert preparation of this manuscript.

REFERENCES
An Intermediate in the Uncoupled Oxidation of Tetrahydrobiopterin