The complex hepatic phenylalanine hydroxylating system consists of three essential components, two enzymes - phenylalanine hydroxylase and dihydropterin reductase (DHPR) - and the non protein coenzyme, tetrahydrobiopterin [1] whose structure is shown in Figure 1. The scheme depicted in Figure 2 shows the reactions catalyzed by phenylalanine hydroxylase and DHPR. The latter enzyme functions to regenerate tetrahydrobiopterin (BH$_4$) from its oxidation product, quinonoid dihydrobiopterin (Q-BH$_2$), thus allowing the coenzyme to function catalytically in the hydroxylation reaction [2]. The conversion of the pterin carbinolamine intermediate to the final pterin product, Q-BH$_2$, is catalyzed by a pterin dehydratase [3,4], designated in the scheme as PHS (an abbreviation for "phenylalanine hydroxylase stimulator"). An unusual aspect of this reaction is that it can occur quite rapidly non-enzymatically [3]. Because of this property, there is some uncertainty about whether or not PHS is necessary for normal phenylalanine hydroxylation in vivo. Recent evidence suggests that it does function in this way since a mild form of hyperphenylalaninemia (HPA) may be caused by a deficiency of PHS [5,6].

Subsequently, work from my own laboratory, as well as from others, showed that BH$_4$ and DHPR function with tyrosine and tryptophan hydroxylases, the enzymes involved in the synthesis of the neurotransmitters dopamine, norepinephrine and serotonin, in exactly the same way as they do with phenylalanine hydroxylase, making it likely that both of these components are important for normal brain development and function.
Shortly after the demonstration that the phenylalanine hydroxylating system consists of three essential components, BH₄, phenylalanine hydroxylase and DHPR, we predicted that there might be three different forms of phenylketonuria (PKU), each caused by the lack of one of these essential components [7]. Since patients lacking either BH₄ or DHPR would be suffering from 3 metabolic lesions, i.e., impaired hydroxylation of all 3 of the aromatic amino acids, it could also be predicted that they might be more seriously ill than patients with classical PKU, who suffer only from the consequences of a deficiency of phenylalanine hydroxylase.

During the following 10 years, we obtained liver biopsy samples from several PKU patients. Our results showed that they all lacked the same component - phenylalanine hydroxylase [8]. Although these studies did identify for the first time the missing component in the most common form of PKU - now called classical PKU - they were nonetheless disappointing in that the prediction of the existence of two other variant forms did not receive any support.

In 1974, several groups - Bartholome [9] and Smith [10] - reported briefly on PKU children who developed serious neurological symptoms despite their having been placed on the phenylalanine-restricted diet early in life. This disease, in contrast to classical PKU, was life-threatening and was described as either "malignant" or "lethal" PKU.

In that same year, we carried out an analysis of the components of the phenylalanine hydroxylating system on a liver sample from a non-responsive PKU child. We found adequate amounts of phenylalanine hydroxylase and BH₄ but no detectable levels of DHPR. This child represented the first one with a variant form of PKU caused by the lack of a component of the phenylalanine hydroxylating system other than phenylalanine hydroxylase itself [11].

We suspected that at least part of the neuropathology, which in this patient consisted of almost constant myoclonic seizures of the extremities, was caused by a deficiency of neurotransmitters secondary to impaired functioning of tyrosine and tryptophan hydroxylases as a result of the primary lack of DHPR. This suspicion was confirmed by direct measurement of metabolites of dopamine, norepinephrine and serotonin [12,13]. Accordingly, we recommended treatment with the products beyond the suspected metabolic blocks, i.e., DOPA and 5-hydroxytryptophan, together with an inhibitor of peripheral aromatic amino acid decarboxylase, as well as a phenylalanine-restricted diet [12,14]. While it was clear that this was definitely beneficial, it was also clear that it was not the final answer [13]. I will return to a discussion of other aspects of the therapy for this disease.

In 1978, following preliminary reports from several research groups describing patients who excrete abnormally low amounts of biopterin, we described the first patient with HPA caused by a demonstrated tissue lack of BH₄ due to a block in its de novo synthesis [15].

We localized the position of the metabolic block to a step between neopterin and biopterin by demonstrating that our patient had a markedly elevated urinary level of neopterin together with decreased levels of biopterin [16]. Subsequently, Niederwieser, Curtius and their colleagues, further subdivided this group of patients into those that lack GTP cyclohydrolase, the first enzyme in the de novo biosynthetic pathway for BH₄, and, the more common form, those that lack the next enzyme in the pathway, 6-pyruvoyltetrahydropterin synthase (Reviewed in Ref. 17).

Patients with hyperphenylalaninemia due to a block in BH₄ synthesis also show various neurological symptoms. In 1982, we reported the first successful treatment of one of these patients with the oral administration of relatively large (20-30 mg/kg/day) doses of BH₄ [18,19]. Subsequent experience has reported considerable success with BH₄ treatment either alone or in combination with neurotransmitter precursors. There are, however, some failures. In my opinion, the ideal therapy for HPA due to BH₄ synthesis
defects would start prenatally since it seems likely that BH₄ is necessary for normal brain development in utero.

In the remaining time, I would like to discuss an aspect of the therapy for HPA due to a deficiency of DHPR that has been somewhat neglected. This aspect deals with the important role that tetrahydrofolate (H₄-folate) derivatives appear to play in the effective treatment for this disease.

A clue that ultimately led to finding this therapeutic role for folates was provided by one of the early signs seen in the first patient with this disease i.e., lower than normal levels of folate in serum, red cells and in CSF [11]. In our attempts to try to explain why DHPR-deficient patients, in general, have low folate levels, we considered the possibility that because of the relatively low activity of dihydrofolate reductase in brain, that particularly in that tissue, DHPR may play a role in maintaining levels of H₄-folate.

To explore this possibility, we studied a reaction in which free H₄-folate is a participant. The reaction selected was that catalyzed by serine hydroxymethyl transferase, in which 5,10-methylene H₄-folate reacts with glycine to form serine and H₄-folate. In the usual assay for this enzyme, a reducing agent such as a thiol is included. Specifically, we wanted to know whether DHPR (+NADH) could function as the reducing system. The reactions that might be involved in such a system are shown in equations 1, 2, and 3 where reaction 1 is nonenzymatic and reactions 2 and 3 are catalyzed by DHPR and serine hydroxymethyl transferase, respectively.

\[
\begin{align*}
H_4\text{-Folate} + Q & \rightarrow Q-H_2\text{-Folate} \\
Q-H_2\text{-Folate} + NADH + H^+ & \rightarrow H_2\text{-Folate} + NAD^+ \\
H_4\text{-Folate} + \text{Serine} & \rightarrow 5,10\text{-methylene H}_4\text{-Folate} + \text{Glycine}
\end{align*}
\]

In preliminary experiments, we showed that in the presence of a brain homogenate, but in the absence of any reducing agent, H₄-folate, as expected, was very labile with a half-life of about 10 min. We found that NADH was as effective as mercaptoethanol in protecting against this loss [20]. Since DHPR is relatively specific for NADH, this result was consistent with the protective effect of NADH being mediated by DHPR.

In order to test whether DHPR was involved in maintaining H₄-folate levels, as measured indirectly by assaying for glycine formation from radioactive serine, we used a specific antibody against this enzyme. As can be seen in Table 1, glycine formation was stimulated about 3-fold by the addition of a reducing agent; 0.5 mM NADH was about as effective as 10 mM mercaptoethanol. And while the antiserum had no effect on the ability of the mercaptoethanol to stimulate glycine formation, it essentially completely obliterated the stimulatory effect of NADH - a result strongly suggesting that DHPR was involved.

Finally, we examined the effect of added DHPR to this system using a control human brain homogenate and an homogenate from a brain sample from a DHPR-deficient patient. As can be seen in Table 2, with the control tissue there was a stimulation by either mercaptoethanol or NADH, with little if any, further stimulation by exogenous DHPR. By contrast, with the DHPR-deficient tissue, there was a significant stimulation by added DHPR. It should be noted that there is some stimulation by NADH (not seen with NADPH) even in the absence of the reductase, either endogenous or exogenous, an indication that there is some other NADH-dependent enzyme that is able to stimulate glycine formation in this mutant homogenate.
Table 1
Effect of antiserum to dihydropteridine reductase on serine to glycine conversion in brain homogenates

<table>
<thead>
<tr>
<th>Additions</th>
<th>Control serum</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>10 mM-2-Mercaptoethanol</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>0.5 mM-NADPH</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>0.5 mM-NADH</td>
<td>3.0</td>
<td>0.9</td>
</tr>
<tr>
<td>0.5 mM-NADH + 10 mM 2-Mercaptoethanol</td>
<td>3.7</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Homogenates of rat brain were incubated with dialyzed control or immune serum for 4 hours at 4°C prior to the assay for glycine formation which was carried out for 30 min. at 37°C. The reaction mixtures contained 0.2 mM tetrahydrofolate and 0.8 mM L[U¹⁴C] serine.

Table 2
Effect of reducing agents on the conversion of serine to glycine in homogenate of brain biopsy from a patient lacking dihydropteridine reductase

<table>
<thead>
<tr>
<th>Control biopsy</th>
<th>Glycine formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reducing agent</td>
<td>1.2</td>
</tr>
<tr>
<td>10 mM-2-Mercaptoethanol</td>
<td>2.1</td>
</tr>
<tr>
<td>0.1 mM-NADPH</td>
<td>1.5</td>
</tr>
<tr>
<td>0.1 mM-NADH</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1 mM-NADH + dihydropteridine reductase</td>
<td>2.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutant biopsy</th>
<th>Glycine formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reducing agent</td>
<td>1.7</td>
</tr>
<tr>
<td>10 mM-2-Mercaptoethanol</td>
<td>3.5</td>
</tr>
<tr>
<td>0.1 mM-NADPH</td>
<td>1.6</td>
</tr>
<tr>
<td>0.1 mM-NADH</td>
<td>2.5</td>
</tr>
<tr>
<td>0.1 mM-NADH + dihydropteridine reductase</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Dihydropteridine reductase activity in the unsupplemented incubation of homogenate of the control biopsy was 0.34 µmol/min/ml while that of the mutant biopsy was less than 0.02 µmol/min/ml. There was no increased glycine formation if dihydropteridine reductase was added to incubations of homogenate of the mutant biopsy in which no reducing agent was added or 10 mM-2-mercaptoethanol was present. Incubation time, 30 min. at 37°C.

These results supported the idea that DHPR plays a role in maintaining normal brain levels of H₄-folate. Based on these results and the finding that many DHPR-deficient patients have low folate levels, we strongly recommended that a source of tetrahydrofolate, such as folinic acid (5-formyl-H₄-folate) or 5-methyl-H₄-folate be part of the treatment of DHPR-deficient patients [13].

Subsequently, several DHPR-deficient patients were treated with folinic acid with many of them showing some improvement (Reviewed in Ref. 21). In the last study, it was shown that early treatment with folinic acid in conjunction...
with neurotransmitter precursors and a phenylalanine-restricted diet led to improvement in neurological signs, which we assumed were due to increased levels of H_{4}-folate in the brains of these patients [21]. Unexpectedly, it was found that during treatment with folinic acid, CSF levels of norepinephrine and dopamine increased, whereas levels of the metabolites of these neurotransmitters and 5HIAA, the metabolite of serotonin, were either little changed or actually decreased.

Although the mechanism for this increase is not known with certainty, one possibility is that it involves the reduction of quinonoid dihydrobiopterin by CH_{3}-H_{4}-folate, a reaction that was shown by Mathews and Kaufman [22] to be catalyzed by methylene tetrahydrofolate reductase (shown below as reaction 4). That reaction, together with reaction 5, also catalyzed by the same enzyme, adds up to reaction 6, which is simply the NADPH-mediated reduction of Q-BH_{2} to BH_{4}. This net reaction is the same as that catalyzed by DHPR, but, of course, it is catalyzed by a different enzyme.

\[
\begin{align*}
Q-BH_{2} + 5\text{-Methyl-H}_{4}\text{-Folate} & \rightarrow BH_{4} + 5,10\text{-Methylene-H}_{4}\text{-Folate} & (4) \\
5,10\text{-Methylene-H}_{4}\text{-Folate} + NADPH + H^{+} & \rightarrow 5\text{-Methyl-H}_{4}\text{-Folate} + NADP^{+} & (5) \\
Q-BH_{2} + NADPH & \rightarrow BH_{4} + NADP^{+} & (6)
\end{align*}
\]

From the considerations outlined above, it is evident that the ability of CH_{3}-H_{4}-folate to reduce the Q-BH_{2} (reaction 4) may have important implications for the regulation of all BH_{4}-dependent enzymes, especially in those situations where the DHPR-catalyzed reduction of Q-BH_{2} (see Figure 2), which is probably the major route, is diminished.

Whether or not the reactions catalyzed by 5,10-methylene-tetrahydrofolate reductase, (reactions 4 and 5) underlie the beneficial effects observed when folinic acid is administered to DHPR-deficient patients, the point that may be emphasized is that H_{4}-folate derivatives play an important role in the treatment of this disease.

References


