Further Studies on the Phenylalanine-hydroxylation Cofactor

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The partial purification and some of the properties of the phenylalanine-hydroxylation cofactor have been described (1). Although the structure of the compound remains to be determined, the recent finding that tetrahydrofolate (2) and some other tetrahydropteridines (3) have cofactor activity in the enzyme system which catalyzes the conversion of phenylalanine to tyrosine, raises the obvious possibility that the natural cofactor is a pteridine-like compound.

In the present paper, this possibility is considered in the light of recent chemical and enzymatic studies carried out on both the natural cofactor and the synthetic tetrahydropteridines. Procedures are described for the further purification of the cofactor from rat liver, and of the two enzymes involved in the hydroxylation reaction.

EXPERIMENTAL

Materials and Methods

Assays—The cofactor was assayed as previously described (1). The rat and sheep liver enzymes were assayed according to the published procedures (4), the only modification being the addition of the cofactor to the standard assay mixture. The cofactor preparation used for this purpose was 0.07 ml of the concentrated aqueous fluid after precipitation with ethanol and ether (1). Protein was determined spectrophotometrically (5) except during the calcium phosphate gel step in the rat liver enzyme preparation, in which the presence of phenylalanine interferes. The biuret method (6) was used to follow protein concentration during that particular step.

Further Purification of Cofactor—The published method for the preparation of the cofactor from rat liver (1) includes the following steps: organic solvent fractionation, and ion exchange chromatography on Dowex 1 Cl− and Dowex 50 Na+ columns. This procedure has now been extended to include precipitation with phosphotungstic acid and partition chromatography on silica gel columns. The advantages of carrying the material through these two additional steps are a-fold: a further purification is achieved and the product is much more stable than it is during that particular step.

The active fractions from the Dowex 50 column (1) were combined. For each 100 ml of combined eluates, 2.75 ml of 1 N H2SO4 were added with stirring followed by the addition of 2.90 ml of freshly prepared 20% phosphotungstic acid. The mixture was stirred for 2 to 3 hours, and then centrifuged at 1500 \( \times g \) for 20 minutes. The supernatant fluid was discarded. The pale yellow precipitate was suspended in 15 ml of cold 0.1 N H2SO4, and the suspension was extracted 2 times with 2 volumes of 37% n-butanol in ethyl ether and once with 2 volumes of ethyl ether. The aqueous layer was brought to pH 5 to 5.5 with the dropwise addition of 1 N KOH.

The aqueous layer was extracted 3 times with three volumes of n-butanol and the butanol layers were combined and concentrated under reduced pressure to several milliliters. The silica gel column was prepared in the following manner: 6.5 g of silicic acid were ground in a mortar with 3.9 ml of water. Then 23 ml of 15% chloroform in n-butanol were added to the silica-water mixture and ground until a smooth slurry was formed. This slurry was poured into a 1-cm chromatography column, allowed to settle under gravity for about 20 minutes, and finally compressed with nitrogen gas until no further change in the column height occurred. Under these conditions, a height of 14 to 16 cm was usually achieved. The butanol solution of the cofactor was added to the column and the elution was started with water-saturated 15% chloroform in n-butanol. A rate of about 0.7 ml per minute was maintained by applying pressure to the top of the column from a tank of nitrogen gas. Fractions, 5 ml, were collected; the first 45 to 50 ml of effluent fluid were discarded, the cofactor activity ordinarily being found in the second 40 to 50 ml. The organic solvents were removed from the active fractions by distillation under reduced pressure and the residue was dissolved in water. The cofactor activity in these fractions usually was quite stable for several weeks when stored at 4°.

Further Purification of Rat Liver Enzyme—All of the steps in the purification of both the rat and the sheep liver enzyme were carried out in the cold at 2–4° unless specified otherwise. Mechanical stirring was used during all additions. Ammonium sulfate precipitates were collected by centrifugation for 20 minutes at 18,000 \( \times g \) and ethanol precipitates were generally obtained by centrifugation for 15 minutes at 4000 \( \times g \). Glass-distilled water was used throughout the procedure.

The extraction, the first ethanol fractionation and the first ammonium sulfate fractionation were carried out as already described (4). The ammonium sulfate fraction was taken up in 0.033 M Tris1 buffer pH 6.8.

Adsorption and Elution from Calcium Phosphate Gel—To each 100 ml of the undialyzed ammonium sulfate fraction, 10 ml of cold 0.1 M L-phenylalanine were added, followed by 100 ml of cold 1 N H2SO4 and the suspension was extracted 2 times with 2 volumes of 37% n-butanol in ethyl ether and once with 2 volumes of ethyl ether. The aqueous layer was brought to pH 5 to 5.5 with the dropwise addition of 1 N KOH.

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cold water. Over a period of 15 minutes 73 ml of calcium phosphate gel (0.35 volume) containing approximately 20 mg of solid per ml were added, and the stirring was continued for another 10 minutes. The gel was collected by centrifugation and eluted successively with 210 ml of 0.02 m and 160 ml of 0.1 m potassium phosphate buffers pH 6.8. The latter eluate contained most of the activity.

Second Ammonium Sulfate Fractionation—The 0.1 m gel eluate was concentrated and freed of phosphate by refractionation with ammonium sulfate. Ammonium sulfate, 18.2 g, was added to each 100 ml of eluate. The precipitate was discarded. For each 100 ml of starting solution, an additional 7.35 g of ammonium sulfate were added. The precipitate obtained after centrifugation was dissolved in 0.3 volume of 0.033 m Tris buffer pH 6.8.

Adsorption and Elution from Alumina Gel—To each 100 ml of the preceding ammonium sulfate fraction, 15 ml of alumina Cy gel (dry weight = 15 mg per ml) was added. The gel was collected by centrifugation and eluted with 100 ml of 0.01 m potassium phosphate buffer pH 6.8; and then with an equal volume of 0.05 m potassium phosphate buffer pH 6.8, the latter eluate containing most of the activity.

The over-all procedure usually results in about a 70- to 100-fold purification with a 15 to 20% yield of units. The quantitative significance of these figures is uncertain, however, because some loss in activity is encountered during the time interval between each of the purification steps. In the other direction, there is usually some gain in units after the first ammonium sulfate step. The activity of the enzyme is almost completely dependent on the addition of the cofactor after the calcium phosphate gel step. Because the two gel steps show some variability from one batch of gel to another, these steps were repeated. The precipitate was dissolved in approximately 12.5 mg of protein per ml and treated with 0.135 volume of 0.2 M zinc acetate.

Enzymatic Determination of Phenylalanine by Conversion to Tyrosine—On several occasions during this work the need has arisen for a sensitive and specific method for the determination of phenylalanine. We have used the following method to determine the blood concentration of phenylalanine in the experiment which will be described later, in which the effect of amethopterin administration was studied. The method has proved useful also for determining phenylalanine blood concentration in phenylketonuric patients. The standard assay system for the rat enzyme was used (4), the only modifications being the incubation time, which was 120 minutes, and the addition of the cofactor. Rat and sheep liver enzymes which had been carried through the first ammonium sulfate step were found to be sufficiently pure for this purpose. An internal standard, in which a known amount of phenylalanine was added to the serum, was also included in every determination. When the serum phenyl-
TABLE I
Purification of sheep liver enzyme
865 g of frozen sheep liver.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Enzyme units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>2420</td>
<td>33,000</td>
<td>41,200</td>
<td>0.80</td>
<td>100</td>
</tr>
<tr>
<td>1st ammonium sulfate fractionation</td>
<td>460</td>
<td>20,200</td>
<td>13,400</td>
<td>1.50</td>
<td>61</td>
</tr>
<tr>
<td>Zinc-ethanol fractionation</td>
<td>144</td>
<td>10,900</td>
<td>3,050</td>
<td>3.60</td>
<td>33</td>
</tr>
<tr>
<td>2nd ammonium sulfate fractionation</td>
<td>75</td>
<td>8,150</td>
<td>1,050</td>
<td>7.90</td>
<td>25</td>
</tr>
<tr>
<td>Calcium phosphate gel eluate, 0.1 M phosphate</td>
<td>85</td>
<td>4,650</td>
<td>118</td>
<td>40.0</td>
<td>14</td>
</tr>
<tr>
<td>Alumina C\textsubscript{7} eluate, 0.1 M phosphate</td>
<td>73</td>
<td>4,200</td>
<td>56</td>
<td>75.0</td>
<td>13</td>
</tr>
</tbody>
</table>

TABLE II
Enzymatic determination of L-phenylalanine
The conditions of the method are described in the text.

<table>
<thead>
<tr>
<th>Phenylalanine added</th>
<th>Tyrosine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{mmol} )</td>
<td>( \text{mmol} )</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>0.40</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Alanine concentration was determined, the sample of serum was added directly to the enzyme assay mixture, without removal of the protein. The results shown in Table II demonstrate the useful range of the determination.

The preparation of the reduced pteridines has been described (3). The silicic acid which was used for the column chromatography of the cofactor was obtained from Mallinckrodt and was labeled “Suitable for chromatographic analysis by the method of Ramsey and Patterson.” Alumina gel C\textsubscript{7} was prepared by the method of Bauer (7). The other materials used, including the pteridines, were obtained from sources which have already been described (3).

RESULTS AND DISCUSSION

Specificity Studies with Synthetic Pteridines—Three tetrahydropteridines have been prepared which show cofactor activity: tetrahydrofolate, 6,7-dimethyltetrahydropteridine, and 6-methyltetrahydropteridine. The parent, nonreduced pteridines such as folic acid or 2-amino-4-hydroxy-6,7-dimethylpteridine, and the corresponding dihydropteridines are inactive.

It can be seen from the activity-concentration curves in Fig. 1 that the monomethyltetrahydropteridine is the most active compound.

The data in Fig. 2 show that monomethyltetrahydropteridine is also more active in stimulating the phenylalanine-dependent oxidation of TPNH. The complete system contained the following components (in micromoles): potassium phosphate buffer pH 6.8, 100; L-phenylalanine, 2.0; TPNH, 0.50; glucose, 125; rat liver enzyme, 0.6 mg of protein; sheep liver enzyme, 0.07 mg of protein; glucose dehydrogenase, 80 units. Final volume, 1.0 ml. Temperature, 25°C.

![Fig. 1. Comparison of tetrahydrofolate, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine (6,7 dimethyl), and 2-amino-4-hydroxy-6-methyltetrahydropteridine (6-methyl) in stimulating the conversion of phenylalanine to tyrosine. The system contained the following components (in micromoles): potassium phosphate buffer pH 6.8, 100; L-phenylalanine, 2.0; TPNH, 0.50; glucose, 125; rat liver enzyme, 0.6 mg of protein; sheep liver enzyme, 0.07 mg of protein; glucose dehydrogenase, 80 units. Final volume, 1.0 ml. Temperature, 25°C.](http://www.jbc.org/)

![Fig. 2. Comparison of 2-amino-4-hydroxy-6,7-dimethyl and 2-amino-4-hydroxy-6-methyltetrahydropteridine in stimulating the phenylalanine-dependent oxidation of TPNH. The complete system contained the following components (in micromoles): potassium phosphate pH 6.8, 100; L-phenylalanine, 2.0; TPNH, 0.15; rat liver enzyme, 0.1 mg of protein; sheep liver enzyme, 0.07 mg of protein; glucose dehydrogenase, 80 units; tetrahydropteridines, 0.05. In each case, a control in which phenylalanine was omitted was included, and the reported values have been corrected for any oxidation of TPNH which occurred in the absence of phenylalanine. Silica cells of 1.0-cm light path were used.](http://www.jbc.org/)
greater stability. More specifically, their degradation is apparently not complicated by such complex reactions as cleavage of the side chain which occurs rapidly with tetrahydrofolate (8).

Almost all of the naturally occurring pteridines have amino and hydroxyl groups in positions 2 and 4, respectively (9). It therefore seems significant that the compound 2-hydroxy-4-amino-6-methyltetrahydropteridine, differing from the active 2-amino-4-hydroxy-6-methyltetrahydropteridine only in that the amino and hydroxy groups on the pteridine ring are reversed, is completely inactive. In view of this specificity within the series of tetrahydropteridines, it would be rather surprising to find another group of structurally unrelated compounds which would show cofactor activity.

Because the tetrahydropteridines are oxidized at neutral pH, other reducing agents were tested for cofactor activity. Anoxic acid, dihydroxyfumaric acid, glutathione, 2-mercaptoethanol, and cysteine were found to be inactive. The inactivity of dihydroxyfumaric acid clearly distinguishes this system from the peroxidase-catalyzed hydroxylation of aromatic compounds (including phenylalanine) in which dihydroxyfumaric acid is active (10).

Chromatic Studies on Cofactor—Many quantitative chemical tests were carried out on the purified cofactor and most of these were negative. In dealing with a compound of unknown purity, the significance of a negative test is not always clear. To provide a basis for comparison, therefore, all of the results were related to the N content of the cofactor solution since the purest preparations of the cofactor consistently show a high N content. A test was considered to be negative when the results were less than 1% of the N content of the solution. For example, in the ester determination, the cofactor solution contained 5 μmoles of N per ml and there was 0.03 μ mole of ester per ml; this was regarded as a negative test. On this basis, the following tests were found to be negative: ninhydrin (11), total phosphate after ashing (12) hydroxamic acid test for esters (13), formimino (14), ureido (15), guanidino (16), creatine (17), creatinine (18), sugar (phloroglucinol (19), cysteine-carbazole (20), orein (21), diphenylamine (22, 23)), and aromatic amine (24). In view of the cofactor activity of tetrahydrofolate in the system, the last-mentioned test for aromatic amines was also carried out, and found to be negative, after aerobic alkaline hydrolysis (25) and after treatment with zinc dust and acid (26). Both of these procedures have been reported to liberate an aromatic amine from folic acid. From these results it is apparent that almost all of the N in samples of the highly purified cofactor is in a form which has not yet been determined.

Fractions of the purified cofactor have consistently given two positive tests which seem to be correlated with cofactor activity. One of these is an atypical Pauly test (27, 28). The test is atypical in that the incubation with diazotized sulfanilic acid must be carried out above pH 6; this results in the formation of a red-pink color which fades to a yellow color in a few seconds. A similar reaction has been reported for certain pyrimidines (29); with these pyrimidines, however, a stable red color is produced by carrying out the reaction in the presence of 10% NaOH (29). With the cofactor, stronger alkali does not lead to this stabilization of the color. The only compound which has been found to give the test in a manner which is indistinguishable from that given by the cofactor is dihydrofolinic acid. Although knowledge of the specificity of the diazo test when carried out in this way is obviously still incomplete, it may be fairly specific for certain dihydropteridines or products derived from them.

In addition, it has been found that treatment of the purified cofactor with alkali at 100° (conditions which destroy the cofactor activity) leads to the formation of highly fluorescent compound with an activation peak at 380 to 400 mp and a fluorescent peak at 470 to 480 mp, fluorescent characteristics which are not too different from those found for folic acid, folinic acid (30), and other pteridines.

Studies with Folic Acid Antagonists

Experiments in Vitro—Evidence which is pertinent to a consideration of whether the natural cofactor is a pteridine-like compound has been obtained through the use of folic acid antagonists. It has been found that aminopterin is a fairly potent inhibitor of the enzymatic conversion of phenylalanine to tyrosine. The results of the experiment, summarized in Table III, show that the reaction is more sensitive to this inhibitor when the rat liver cofactor was used rather than tetrahydrofolate. Recently it was demonstrated that at least one site of aminopterin inhibition is the reduction of dihydrofolate to tetrahydrofolate by TPNH (31), a finding which can possibly be generalized to include analogous reduction reactions for other pteridines. If so, the observation that the hydroxylation of phenylalanine is more sensitive to inhibition by aminopterin when the rat liver cofactor is used, would be consistent with the idea that the cofactor must be reduced by TPNH before it is active in the hydroxylation system. The same conclusion had previously been reached from kinetic studies (1, 4).

In the presence of the dimethyltetrahydropteridine, the conversion of phenylalanine to tyrosine has been formulated in terms of an initial reaction in which the tetrahydropteridine is utilized stoichiometrically for tyrosine formation, followed by a second reaction, catalyzed by the sheep liver enzyme, in which an oxidized product of the tetrahydropteridine is reduced back to the tetrahydro level by TPNH (3). From the finding that the dependence on the sheep liver enzyme was a function of the TPNH concentration, it was concluded that the second reaction could take place nonenzymatically, in addition to its catalysis by the sheep liver enzyme. According to this scheme, it should be possible to study separately the aminopterin sensitivity of the two steps, since in the absence of TPNH, the second reaction cannot take place. It has been found (Table IV) that under these conditions, tyrosine formation is much less sensitive to aminopterin inhibition, a finding which is consistent with the idea that the TPNH-mediated reaction is the one which is most

\[ S. \text{Kaufman, unpublished experiments.} \]

<table>
<thead>
<tr>
<th>Aminopterin concentration</th>
<th>Rat liver cofactor</th>
<th>Tetrahydrofolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 × 10^{-8}</td>
<td>48</td>
<td>10</td>
</tr>
<tr>
<td>6 × 10^{-4}</td>
<td>62</td>
<td>50</td>
</tr>
</tbody>
</table>
sensitive to amethopterin. Since this step may take place non-
enzymatically, and because it is unlikely that the nonenzymatic
reaction would be sensitive to the inhibitor, the effect of ameth-
opterin was studied as a function of TPNH concentration. It was
found that as the TPNH concentration was decreased (and the
dependence on sheep liver enzyme was increased (33)), the reac-
tion became more sensitive to inhibition by amethopterin.

Experiments in Vivo—These studies were carried out to deter-
mine whether the conversion of phenylalanine to tyrosine could
be inhibited by a folic acid antagonist in the whole animal. Since
it is known that when this enzyme system is not functional, as
in the disease phenylketonuria (32), this inactivity is reflected
by a markedly increased phenylalanine blood level (33), the
latter quantity was followed in this experiment as a gross measure
of the enzyme activity in vivo.

Amethopterin, which was shown to be as effective an inhibitor
of the reaction as aminopterin when tested in vitro, was ad-
ministered to a group of five rats intramuscularly at a level of 1
mg per day. A control group of five animals received an equal
volume of 0.85% sodium chloride solution. Both groups of rats
were allowed to eat and drink ad libitum. The blood samples
were collected 5 to 6 hours after the last injection; food was
withheld during this period. The blood was allowed to coagu-
late and a sample of the pooled sera for each group was used
directly for the determination of phenylalanine by the enzymatic
method already described.

The results of three separate experiments are shown in Table
V. In each case, the amethopterin treatment resulted in an
increased level of phenylalanine in the blood. Although there
is some very indirect evidence that folic acid may be involved in
tyrosine oxidation (34), it is unlikely that the higher blood con-
centrations of phenylalanine observed in these experiments were
due to an inhibition of tyrosine metabolism. Since the method
used for determining the phenylalanine involves the enzymatic
conversion to tyrosine, the "zero time" controls are proportional
to the tyrosine concentration of the sample. In all of the experi-
ments, the tyrosine level was unchanged or lowered as a result
of the amethopterin treatment.

After the blood had been collected for the experiments which
have just been described, the animals were killed, and the livers

were removed and pooled. Extracts prepared in the usual
manner for the rat liver enzyme (1) were assayed for phenyl-
alanine hydroxylase activity. The data in the last column of
Table V shows that the extracts obtained from the rats which
had received amethopterin were only 25% as active as those
from the control groups of animals. From the enzyme activity
and the blood phenylalanine data, it can be concluded that
amethopterin can lead to an inhibition in vivo of the conversion
of phenylalanine to tyrosine.

SUMMARY

1. The further purification of the rat and sheep liver enzymes
has been described. When carried through these procedures,
both enzymes were essentially completely resolved with respect
to the phenylalanine-hydroxylase cofactor.

2. The specificity of the tetrahydropteridines in the system
has been studied: 2-amino-4-hydroxy-6-methyltetrahydropteri-
dine was the most active compound tested, whereas the analogue,
2-hydroxy-4-amino-6-methyltetrahydropteridine, was inactive.

3. An extended purification procedure for isolating the co-
factor from rat liver has been described. The product gave
negative tests for a large series of functional groups. With the
best preparations, an atypical Pauly test and a characteristic
fluorescence after alkaline treatment were associated with the
cofactor activity.

4. The folic acid antagonists, aminopterin and amethopterin,
have been shown to inhibit the conversion of phenylalanine to
tyrosine in vitro and in vivo.

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work.

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TABLE IV
Inhibition of tyrosine formation by aminopterin at various concentrations of TPNH

The conditions of the experiment were the same as those described for the complete system in Fig. 1 except that the time of incubation was 30 minutes; 0.075 μmole of dimethyltetrahydropteridine was used. The final concentration of aminopterin was 5 x 10^-4 M.

<table>
<thead>
<tr>
<th>TPNH</th>
<th>Uncorrected</th>
<th>Corrected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmole</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>0.010</td>
<td>48</td>
<td>61</td>
</tr>
<tr>
<td>0.025</td>
<td>39</td>
<td>45</td>
</tr>
<tr>
<td>0.100</td>
<td>35</td>
<td>41</td>
</tr>
</tbody>
</table>

* The "corrected" per cent inhibition was calculated by subtracting the amount of tyrosine formed in the absence of TPNH from the other values.
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