ENZYMATIC REDUCTION OF FOLIC ACID AND DIHYDROFOLIC ACID TO TETRAHYDROFOLIC ACID

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Earlier reports on the inactivation of FA in the presence of liver extracts indicated the supplements required (1, 2) and described the enzymatic conversion of FA to a labile compound possessing the properties of FAH (3). The reduction of FA by TPNH has been noted by Miller and Waelsch (4). Recently, the enzymatic reduction of FA to FAH by a bacterial extract was described by Wright and Anderson (5). The results of Blakley (6) and Greenberg (7) suggested that FA was reduced to FAH in the conversion of FA to a transformylation cofactor. Greenberg (7) also observed the reduction of FAH by DPNH.

Studies now indicate that FA and FAH are reduced to FAH with reduced pyridine nucleotides in the presence of an enzyme system partially purified from chicken liver. In the presence of the FAH-formylating system of Silverman et al. (8), the reaction product is converted to CF. The enzymatic reduction of FA and FAH is strongly inhibited by aminopterin in accord with previous results (3).

Methods

FAH was determined by measuring its decomposition product, PABGA, by the Bratton-Marshall method (9). CF was estimated microbiologically (10). Protein was measured by the method of Lowry et al. (11). α-Ketoglutarate was determined by the 2,4-dinitrophenylhydrazine method (12).

DPN and TPN were obtained from the Pabst Brewing Company. TPNH was obtained from the Sigma Chemical Company. DPNH was prepared by Na2S2O4 reduction and purified by an unpublished method of Dr. R. M. Burton. A preparation of glucose dehydrogenase (13) was

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1 Abbreviations are as follows: FA, folic acid; FAH, dihydrofolic acid; FAH, tetrahydrofolic acid; CF, citrovorum factor (N5-formyltetrahydrofolic acid); DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; PABGA, p-aminobenzoylglutamic acid.
kindly provided by Dr. B. Wright. \(N\)-Formylglutamic acid and the FAH\(_2\)-formylating enzyme were prepared and provided by Silverman et al. (8).

FAH\(_2\) was prepared as follows: 20 mg. of FA were suspended in 2 ml. of water and dissolved by the dropwise addition of \(\mathbf{N}\) KOH. 5 ml. of a solution of potassium ascorbate (pH 6.0, 100 mg. of ascorbic acid per ml.) and 200 mg. of \(\mathbf{N}\)\(_2\)S\(_2\)O\(_4\) were added. After 5 minutes at room temperature the solution was cooled to 0° and 2 \(\mathbf{N}\) HCl was added dropwise until the pH fell to approximately 2.8 (thymol blue). After several minutes at 0°, the precipitate of FAH\(_2\) was recovered by centrifugation and suspended by stirring in 5 ml. of potassium ascorbate solution. 2 \(\mathbf{N}\) HCl was added dropwise as before; the mixture was chilled and then centrifuged. The precipitate was washed four times with 10 ml. portions of cold 0.005 \(\mathbf{N}\) HCl. The freshly prepared product, a flocculent white precipitate, could be stored at refrigerator temperature as a suspension in 0.005 \(\mathbf{N}\) HCl. The dried product was a gray to gray-green powder which gradually turned yellow on storage. The spectral properties were similar to those described for FAH\(_2\) by O'Dell et al. (14). In 0.01 \(\mathbf{N}\) KOH, \(E_{283} = 21,000\) and the ratio 283:340 \(\mu\)m was 2.8 to 3.0.

A freshly prepared suspension of FAH\(_2\) in 0.005 \(\mathbf{N}\) HCl contained essentially no diazotizable amine. However, if the pH was raised to 6.0, the compound decomposed, yielding a yellow degradation product as described by Wright and Anderson (5) and diazotizable amine as noted by Zakrzewski and Nichol (15) and Blakley (16). The diazotizable amine formed at pH 6.0 on standing overnight at room temperature or upon heating at 100° for 15 to 20 minutes accounted for as much as 50 per cent of the starting material.

**EXPERIMENTAL**

The enzyme solution was prepared from a dialyzed extract of commercial chicken liver (3). Operations were carried out at 0–3°. To 800 ml. of dialyzed extract 240 gm. of ammonium sulfate were added slowly with stirring. After 30 minutes the mixture was centrifuged and the precipitate discarded. 160 gm. of ammonium sulfate were added to the supernatant fluid. After 30 minutes the precipitate was removed by centrifugation, suspended in water, and dialyzed against distilled water until free from ammonium sulfate. A small precipitate was removed by centrifugation and discarded. The dialyzed ammonium sulfate fraction (128 ml.) was stored frozen. A 20 ml. portion of the ammonium sulfate fraction (representing 125 ml. of dialyzed extract) was cooled in a \(-10^\circ\) bath and 6 ml. of ethanol were added with stirring. The precipitate was removed by centrifugation and discarded. To the supernatant fluid at \(-10^\circ\), 20
ml. of ethanol were added. The precipitate was collected by brief centrifugation, suspended in 16 ml. of water, and the insoluble protein discarded by centrifugation. The ethanol fraction was stirred with 554 mg. of calcium phosphate gel (17). The gel was recovered by centrifugation and activity was eluted by stirring for 30 minutes with 20 ml. of potassium phosphate buffer, 0.2 M, pH 5.5. The mixture was centrifuged and the gel extracted again with the same buffer. The two eluates were pooled, dialyzed against distilled water, and stored frozen (Table I).

In the ethanol precipitation step recovery of activity was variable. This step could be replaced by a preliminary treatment of the ammonium sulfate fraction with calcium phosphate gel to remove inactive protein. When this was done, more of the original activity could be recovered in the final gel eluate, but the specific activity was somewhat lower.

Previously (3), the supplements employed for the reduction of FA in the presence of a chicken liver extract were MgSO₄, citrate, DPN, and ATP. DPN and ATP could be replaced by catalytic levels of TPN. Citrate could be replaced by cis-aconitate or by a higher concentration of DL-isocitrate. It was then apparent that isocitric dehydrogenase in the liver extract was generating TPNH for the reduction of FA. In the absence of MgSO₄ and citrate, the enzymatic reduction of FA at pH 6.0 occurred with TPNH, but not with DPNH.

Although the partially purified enzyme was capable of reducing FA in the presence of citrate, MgSO₄, and TPN, activity was increased by the addition of a dialyzed extract of commercial chicken heart, indicating a partial separation of the TPNH-generating system from the FA-reducing activity. The heart extract was prepared in the same way as the dialyzed liver extract (3), except that the tissue was homogenized in 3 volumes of 

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**Table I**

<table>
<thead>
<tr>
<th>Purification of Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Dialyzed extract</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>Ethanol fraction</td>
</tr>
<tr>
<td>Gel fraction</td>
</tr>
</tbody>
</table>

* A unit is defined as the amount of enzyme which converted 1 γ of FA to FAH₄ in reaction mixtures which contained 100 μmoles of potassium phosphate buffer, pH 6.0, 10 μmoles of citrate, 10 μmoles of MgSO₄, 0.012 μmole of TPN, 0.05 ml. of heart extract, 0.227 μmole of FA, and enzyme fraction in a final volume of 1 ml. Incubation was for 1 hour at 37°.
buffer. In the assay of fractions obtained in the purification procedure, the heart extract was employed to generate TPNH in the presence of citrate, MgSO₄, and TPN and the results were corrected for the slight FA-reducing activity of the heart extract.

When either citrate or DL-isocitrate was employed to generate TPNH, the optimal pH for FA reduction was 5.5. FA was reduced in the presence of added TPNH and DPNH (Table II). In each instance maximal FA reduction was obtained at pH 5.0, but DPNH was only about one-fourth as active as TPNH. It seemed possible that the activity of DPNH might be due to the presence of a small amount of TPNH in the preparation and the presence of a transhydrogenase in the enzyme solution.

### Table II

**Reduction of FA by TPNH and DPNH**

<table>
<thead>
<tr>
<th>pH</th>
<th>TPNH μmoles</th>
<th>DPNH μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>17.6</td>
<td>1.57</td>
</tr>
<tr>
<td>4.0</td>
<td>21.1</td>
<td>2.34</td>
</tr>
<tr>
<td>4.5</td>
<td>22.4</td>
<td>5.46</td>
</tr>
<tr>
<td>5.0</td>
<td>24.6</td>
<td>7.88</td>
</tr>
<tr>
<td>5.5</td>
<td>19.3</td>
<td>6.65</td>
</tr>
<tr>
<td>6.0</td>
<td>13.7</td>
<td>5.06</td>
</tr>
</tbody>
</table>

Reaction mixtures contained 200 μmoles of acetate buffer, 28 units of enzyme, 227 μmoles of FA, and either 108 μmoles of TPNH or 130 μmoles of DPNH in a final volume of 1 ml. Incubations were for 30 minutes at 37°C.

The reduction of FAH₂ to FAH₄ by TPNH or DPNH with the liver enzyme could be demonstrated with diazotizable amine production as an indication of FAH₄ formation. However, results were complicated by the non-enzymatic formation of PABGA from FAH₂. In the presence of N-formylglutamic acid and a transformylating enzyme (8), enzymatically generated FAH₄ was converted to the stable compound CF and assayed microbiologically. The enzymatic reduction of FA and FAH₂ was compared with use of glucose dehydrogenase to generate DPNH and TPNH and N-formylglutamate-CF transformylase to trap the FAH₄ produced. Under these conditions FA was reduced only by TPNH, while FAH₂ was reduced by both DPNH and TPNH (Table III).

Attempts were made to demonstrate the stoichiometric utilization of TPNH for FAH₄ formation from FA and FAH₂ by measuring the α-ketoglutarate and PABGA produced in the presence of citrate and TPN. The α-ketoglutarate to PABGA ratio was always considerably greater than 2.
when FA was reduced and larger than 1 when FAH₂ was reduced. This suggested the occurrence of side reactions in which TPNH reacted with pterin fragments arising upon cleavage of FAH₄. This complication was overcome by enzymatically formylating the FAH₄ to CF. The α-keto-

**Table III**

*Reduction of FA and FAH₂ by DPNH and TPNH Generated with Glucose and Glucose Dehydrogenase*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nucleotide, 0.012 μmole</th>
<th>CF synthesized (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, 0.272 μmole</td>
<td>DPN</td>
<td>0.2</td>
</tr>
<tr>
<td>FAH₂, 0.272 μmole</td>
<td>TPN</td>
<td>14.4</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 40 μmoles of glucose, 38.4 units of glucose dehydrogenase, 4.57 μmoles of N-formylglutamate, 4.75 units of FAH₄-formylating enzyme, 21 units of enzyme, and DPN or TPN with FA or FAH₂ as indicated in the table in a final volume of 1 ml. Incubations were for 90 minutes at 37°C.

**Table IV**

*Stoichiometry of CF and α-Ketoglutarate Formation*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>α-Ketoglutarate* (μmole)</th>
<th>CF (μmole)</th>
<th>α-Ketoglutarate:CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, 0.274 μmole</td>
<td>0.145</td>
<td>0.0709</td>
<td>2.04</td>
</tr>
<tr>
<td>FAH₂, 0.314 μmole</td>
<td>0.259</td>
<td>0.221</td>
<td>1.17</td>
</tr>
</tbody>
</table>

Reaction mixtures containing 4 μmoles of MgSO₄, 3 μmoles of citrate, 0.012 μmole of TPN, 100 μmoles of potassium phosphate buffer, pH 6.0, 4.57 μmoles of N-formylglutamate, 4.75 units of FAH₄-formylating enzyme, 18 units of enzyme, and FA or FAH₂ as indicated, in a final volume of 0.9 ml. were incubated for 1 hour at 37°C.

* The 2,4-dinitrophenylhydrazone prepared from reaction mixtures migrated as a single spot at the same rate as the 2,4-dinitrophenylhydrazone of α-ketoglutarate in n-butanol-ethanol-0.5 N NH₃ (70:10:20), butanol-ethanol-H₂O (50:10:40), and glycine-NaOH buffer 0.1 M, pH 8.4 (18).

glutamate to CF ratios (Table IV) indicated that 2 moles of TPNH were consumed per mole of FA reduced and 1 mole when FAH₂ was reduced.

Aminopterin strongly inhibited the reduction of both FA and FAH₂ when a TPNH-generating system was used (Table V). In the presence of citrate and TPN, aminopterin did not inhibit the formation of TPNH. The reduction of both FA and FAH₂ in the presence of added TPNH or DPNH was inhibited by aminopterin.

The quantity of FAH₄ formed from FA in reaction mixtures at pH 6.0
could be calculated from the decrease in optical density at 360 μm with 7 × 10⁸ as the molar extinction coefficient for FA. The absorption of FAH₄ is negligible in this region (14). To illustrate the agreement between the decrease in optical density at 360 μm and the formation of diazotizable amine, a reaction mixture containing 300 μmoles of phosphate buffer, pH 6.0, 24 μmoles of citrate, 12 μmoles of MgSO₄, 0.03 μmole of TPN, 266 units of enzyme, and 1.25 μmoles of FA in a final volume of 9.0 ml. was incubated at 23°. From the decrease in optical density after 125 and 190 minutes it was calculated that 0.519 and 0.694 μmoles of FAH₄ were formed and 0.472 and 0.684 μmoles of diazotizable amine were present in the reaction mixture.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Aminopterin</th>
<th>FAH₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA, 0.454 μmole</td>
<td>0.0227</td>
<td>78.7</td>
</tr>
<tr>
<td>FAH₂, 0.656 μmole</td>
<td>0.0454</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Reaction mixtures containing 4 μmoles of MgSO₄, 3 μmoles of citrate, 0.012 μmole of TPN, 100 μmoles of acetate buffer, pH 5.0, enzyme (ammonium sulfate fraction, 42 units), and additions indicated in the table in a final volume of 0.85 ml. were incubated for 1 hour at 37°.

DISCUSSION

It was shown that FA was enzymatically reduced to FAH₄ by added TPNH and DPNH. However, in experiments in which catalytic amounts of reduced pyridine nucleotides were generated at pH 6.0 it appeared that TPNH was required for the reduction of FA, while the reduction of FAH₂ occurred with either DPNH or TPNH, suggesting the participation of two enzymes in the reduction of FA to FAH₄. It seemed likely that the activity of the DPNH preparation in the reduction of FA was only apparent and in reality was due to the presence of traces of TPNH and transhydrogenase in the system. In the reduction of FA occurring in the presence of reduced pyridine nucleotides, there seemed to be no requirement for ATP, cation, or phosphate.

The addition of a large excess of TPN inhibited the reduction of FA by TPNH. However, it was not possible to reverse the reaction by adding large amounts of DPN or TPN or by raising the pH of the reaction mix-
ture after enzymatic reduction of FA with ascorbate present in the reaction mixture to protect the FAH₄ formed (3).

It became apparent in the course of attempts to establish the stoichiometry for the reduction of FA and FAH₂ that TPNH was utilized in side reactions with pterin fragments arising from the cleavage of the FAH₄ formed. The availability of an enzyme which would formylate FAH₄ in the 5 position and thereby synthesize the stable compound, CF (8), made it possible to remove the FAH₄ as fast as it was formed. It was possible to carry out the reduction of FA to FAH₄ and its conversion to CF without the addition of a reducing agent. Estimation of the α-ketoglutarate formed from citrate indicated the amount of TPNH utilized in the reduction, while microbiological assay of the CF formed gave the quantity of FAH₄ produced according to the following series of reactions:

\[
\begin{align*}
(1) & \quad 2 \text{citrate} \rightarrow 2 \text{isocitrate} \\
(2) & \quad 2 \text{isocitrate} + 2\text{TPNH} \rightarrow 2\text{TPNH} + 2\text{CO}_2 + 2\alpha\text{-ketoglutarate} + 2\text{H}^+ \\
(3) & \quad 2\text{TPNH} + 2\text{H}^+ + \text{FA} \rightarrow 2\text{TPNH} + \text{FAH}_4 \\
(4) & \quad \text{N-Formylglutamate} + \text{FAH}_4 \rightarrow \text{glutamate} + \text{CF} \\
(5) & \quad 2 \text{citrate} + \text{N-formylglutamate} + \text{FA} \rightarrow \text{2CO}_2 + 2\alpha\text{-ketoglutarate} + \text{glutamate} + \text{CF} \\
(6) & \quad \text{Citrate} + \text{N-formylglutamate} + \text{FAH}_3 \rightarrow \text{CO}_2 + \alpha\text{-ketoglutarate} + \text{glutamate} + \text{CF}
\end{align*}
\]

In the absence of either the FA-reducing or -formylating enzyme, no CF activity was produced in reaction mixtures. In the absence of FA no α-ketoglutarate was formed.

The stoichiometric reduction of FAH₂ and subsequent conversion to CF indicate that the prepared compound is largely intact FAH₂ and not significantly contaminated with FA.

Aminopterin had no effect on the generation of reduced pyridine nucleotides, but effectively blocked their utilization for the reduction of FA and FAH₂. As there was no accumulation of FAH₂ when the reduction of FA was inhibited by aminopterin, it would appear that both steps in the reduction of FA to FAH₄ are blocked by the inhibitor.

**SUMMARY**

An enzyme system that reduces folic acid (FA) and dihydrofolic acid (FAH₂) to tetrahydrofolic acid has been partially purified from chicken liver. Reduced triphosphopyridine nucleotide (TPNH) appeared to be required for the reduction of FA, while FAH₂ was readily reduced by either TPNH or reduced diphosphopyridine nucleotide. The reaction product
was enzymatically formylated to CF. Aminopterin inhibited the reduction of both FA and FAH₂. FAH₂ was prepared by the reduction of FA with Na₂S₂O₄ in the presence of ascorbate.

The author wishes to thank Dr. Milton Silverman for helpful discussion and for suggesting the use of the formylating system to convert FAH₄ to citrovorum factor (CF). The author is grateful to Dr. John C. Keresztesy for advice concerning the manuscript and to Mrs. Marjorie K. Romine and Miss Rita C. Gardiner for assistance with the CF assays.

BIBLIOGRAPHY

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