Naturally Occurring Forms of Folic Acid

II. ENZYMATIC CONVERSION OF METHYLENETETRAHYDROFOLIC ACID TO PREFOLIC A-METHYLTETRAHYDROFOLATE*1

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WITH THE TECHNICAL ASSISTANCE OF MARJORIE K. RONING AND TRESVANT B. GOODWIN

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Previously (1, 6), it was shown that prefolic A, in crude form, was converted to N4-formyltetrahydrofolic acid by two separate enzymatic reactions. First, in the presence of a suitable electron acceptor, prefolic A is converted to tetrahydrofolic acid by a flavin adenine dinucleotide-linked enzyme system. Then, in the presence of transformylase and formyl-L-glutamate, tetrahydrofolic acid is formylated to N4-formyltetrahydrofolic acid. The conversion of prefolic A to tetrahydrofolic acid was postulated to be an oxidative reaction, mediated through a flavin-linked enzyme system with the dye as the electron acceptor.

More recently, it has been shown that formaldehyde is one of the products formed during the oxidation of prefolic A to tetrahydrofolic acid (7). The experiments leading to this observation were prompted by the reported synthesis of a new folic acid intermediate in methionine biosynthesis by Larrabee and Buchanan (8). These investigators showed that this folic acid derivative contained 1 mole of N-methyl group per mole of compound. During the discussion which followed the presentation of "Interrelationship of Prefolic A and Tetrahydrofolic Acid" (9), Buchanan suggested the possibility that prefolic A and the newly discovered intermediate of methionine biosynthesis might be the same compound.

In the present investigation, results have been obtained with purified natural prefolic A, and in some experiments with chemically synthesized2 prefolic A (10), which confirm those obtained with the crude material (1). Also, it will be shown that menadione is a more effective electron acceptor than the dyes used previously in the oxidation of prefolic A to tetrahydrofolic acid, and that for every mole of tetrahydrofolic acid formed from prefolic A, 1 mole of formaldehyde is released. In addition, evidence will be presented for the enzymatic synthesis of prefolic A from tetrahydrofolic acid and formaldehyde (or methanol) in the presence of flavin adenine dinucleotide and reduced diposphopyridine nucleotide.

* Paper I of this series is (1).
1 Methylenetetrahydrofolic acid may be prepared by the simple chemical admixing of an excess of HCHO with folate-H4 (2-5). On the basis of theoretical and experimental studies, these investigators have suggested that methylenetetrahydrofolic acid is the N4,N10-bridge compound.
2 Unless otherwise specified, natural prefolic A was used in all experiments.
as the amount of prefolic A synthesized. These results were confirmed occasionally by Method II.

Formaldehyde Determination—Formaldehyde was determined by the method of Kossiakoff (2). Protein Determination—Protein was determined by the method of Lowry et al. (12), with bovine serum albumin as the standard.

Preparation of Enzyme—The enzyme solution was prepared by a modification of the method used previously (1). All operations were performed at 0° unless otherwise specified. The crude hog liver extract obtained by the previous method was adjusted to 55% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation, dissolved in water, and adjusted to pH 4.5 with 4 N acetic acid. After 15 minutes, the precipitate was collected by centrifugation, suspended in 50 ml of 0.1 M phosphate buffer, pH 7.5, with the aid of a TenBroeck glass homogenizer, and adjusted to pH 7.6 with NH₄OH. This fraction was kept at -15° overnight and then thawed, and solid ammonium sulfate was added to 30% saturation. After 15 minutes, the inert protein was removed by centrifugation, and the supernatant solution was adjusted to 55% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation, suspended in 50 ml of water, and adjusted to pH 7.0. The final enzyme is purified approximately 20-fold and is stable for several months at -15°.

Enzyme Assay. Enzyme activity was assayed as indicated previously (1), with menadione as electron acceptor instead of indigo disulfonate.

Menadione Reductase—This enzyme was obtained by adjusting the first 55% ammonium sulfate supernatant fraction (see above) to 75% saturation. After dissolving in 100 ml of water, the enzyme solution was adjusted to pH 7.0 and assayed by the method of Woelkeit and Nason (13).

RESULTS

Of the various dyes tested previously as electron acceptors in the conversion of prefolic A to tetrahydrofolic acid, indigo disulfonate was the most effective (1). However, at low concentration (2 x 10⁻⁸ M), menadione is more than 3 times as effective as indigo disulfonate and almost twice as effective at higher concentrations (Fig. 1). Vitamins K₁ and K₂ (Table I) show some activity in this reaction, although they are not as active as menadione. The lower activity observed with these naturally occurring vitamins may be due to the fact that they are present in aqueous suspensions rather than in true solution.

A study of the requirements for the formation of tetrahydrofolic acid from prefolic A indicated that the process is completely dependent on all components of the "complete system" defined in Table II. The activity observed when menadione or FAD is omitted is due to the presence of endogenous FAD bound to the enzyme. These values were lowered substantially by removing the bound FAD from the enzyme with ammonium sulfate at acid pH essentially as described by Horecker (14).

Ability of Various Quinones to Replace Menadione—Menadione was used as the electron acceptor in these studies. However, other quinones may be used as electron acceptor, although less effectively. The relative activity of the quinones tested is shown in Table III. 1,4-Naphthoquinone showed significant activity, being 71% as active as menadione for equimolar amounts. Phthiocol at higher concentration (6 x 10⁻⁴ M) shows significant activity. Juglone is slightly active at low concentrations, but strongly inhibitory at higher concentrations (6 x 10⁻⁴ M). DPN and TPN at concentrations of 3 x 10⁻⁴ M are completely inert for this reaction.

Evidence for Formation of Formaldehyde During Oxidation of Prefolic A to Tetrahydrofolic Acid—Evidence for the formation of

![Fig. 1. Effect of menadione and indigo disulfonate on the rate of folate-H₄ formation from prefolic A. The reaction was carried out in an atmosphere of nitrogen at 37° for 1 hour. The reaction mixture contained, per ml of m/6 phosphate buffer, pH 6.6, 5 mg of sodium ascorbate, 0.05 µmole of each electron acceptor, 10 µg of Ba-prefolic A, 0.24 mg of enzyme protein, and electron acceptor as indicated; final volume, 2 ml.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of vitamin K and indigo disulfonate as electron acceptors in folate-H₄ formation from prefolic A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron acceptor</td>
<td>Folate-H₄ formed</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>None</td>
<td>0.90</td>
</tr>
<tr>
<td>Menadione</td>
<td>3.60</td>
</tr>
<tr>
<td>Vitamin K₁</td>
<td>1.60</td>
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<tr>
<td>Vitamin K₂</td>
<td>1.20</td>
</tr>
<tr>
<td>Indigo disulfonate</td>
<td>2.00</td>
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<table>
<thead>
<tr>
<th>Table II</th>
<th>Requirements for formation of folate-H₄ from prefolic A</th>
</tr>
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<tbody>
<tr>
<td>Omission</td>
<td>Folate-H₄ formed</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>None</td>
<td>3.05</td>
</tr>
<tr>
<td>Prefolic A</td>
<td>0.02</td>
</tr>
<tr>
<td>Menadione</td>
<td>0.78</td>
</tr>
<tr>
<td>FAD</td>
<td>0.77</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.14</td>
</tr>
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</table>
formaldehyde during the oxidation of prefolic A to tetrahydrofolic acid was obtained with prefolic A-C\textsuperscript{14} prepared enzymatically from tetrahydrofolic acid and C\textsuperscript{14}-labeled formaldehyde as described below under "Biosynthesis of Prefolic A."

The labeled prefolic A was converted to tetrahydrofolic acid by the enzymatic reaction mixture described in Table II. The tetrahydrofolic acid was formylated to citrovorum factor with N\textsubscript{6}-formyltetrahydrofolic acid-forming enzyme by menadione reductase. However, further investigation proved that this was not the reason for the inhibition which was produced by menadione. In the absence of menadione, menadione reductase was without effect on the reaction rate. The requirements for the synthesis of prefolic A from tetrahydrofolic acid and formaldehyde are shown in Table VI. The data show that the reaction is dependent on all components added to the system. The inhibitory effects of menadione on the system will be dealt with later. In an earlier report (9), it was stated that the reaction showed an absolute requirement for menadione and menadione reductase, and no mention was made of the requirement of a 1-carbon entity. This was due to the fact that the menadione had been made up in a 0.3% aqueous methanol solution. Further investigation revealed that menadione can replace formaldehyde in this system since the reaction rate with methanol is less than 20% of that observed when formaldehyde is used, and an electron donor is still an absolute requirement, it has been tentatively assumed that the methanol is being converted to formaldehyde in the reaction mixture.

**Evidence for Incorporation of Formaldehyde into Prefolic A**

To show the incorporation of C\textsuperscript{14}-labeled formaldehyde into prefolic A, the reaction for the biosynthesis of prefolic A, as described above, was scaled up to prepare approximately 1 mg of

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menadione</td>
<td>100</td>
</tr>
<tr>
<td>Coenzyme Q\textsubscript{s}</td>
<td>4</td>
</tr>
<tr>
<td>P-Quinone</td>
<td>18</td>
</tr>
<tr>
<td>1,4-Naphthoquinone</td>
<td>71</td>
</tr>
<tr>
<td>Phthiocol</td>
<td>31</td>
</tr>
<tr>
<td>2,5-Dimethyl-1-benzoquinone</td>
<td>8</td>
</tr>
<tr>
<td>Juglone</td>
<td>32</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menadione</td>
<td>100</td>
</tr>
<tr>
<td>Coenzyme Q\textsubscript{s}</td>
<td>4</td>
</tr>
<tr>
<td>P-Quinone</td>
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<td>71</td>
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<tr>
<td>Phthiocol</td>
<td>31</td>
</tr>
<tr>
<td>2,5-Dimethyl-1-benzoquinone</td>
<td>8</td>
</tr>
<tr>
<td>Juglone</td>
<td>32</td>
</tr>
</tbody>
</table>

**TABLE IV**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Prefolic A added mg</th>
<th>Folate-H\textsubscript{4} formed μ mole</th>
<th>Formaldehyde formed μ mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.94</td>
<td>0.303</td>
<td>0.300</td>
</tr>
<tr>
<td>2</td>
<td>1.98</td>
<td>0.400</td>
<td>0.375</td>
</tr>
</tbody>
</table>

* The samples were assayed in triplicate by the *P. cerevisiae* assay with N\textsuperscript{14}-formyltetrahydrofolic acid as the standard, as described previously (1). From these values, the amount of tetrahydrofolic acid formed was calculated.

**TABLE V**

<table>
<thead>
<tr>
<th>System</th>
<th>Folate-H\textsubscript{4} formed μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>2.85</td>
</tr>
<tr>
<td>+ Menadione</td>
<td>3.10</td>
</tr>
<tr>
<td>+ DPNH</td>
<td>1.15</td>
</tr>
<tr>
<td>+ DPNH + menadione</td>
<td>&lt;0.25</td>
</tr>
</tbody>
</table>
TABLE VI

Requirements for biosynthesis of prefolic A

The reaction was carried out in an atmosphere of helium at 37°C for 30 minutes. The reaction mixture contained, per ml of 0.1 M phosphate buffer, pH 7.0, 5 mg of ascorbic acid neutralized to pH 6.5 and, where indicated, 5 μg of FAD, 25 μg of formaldehyde, 1 mg of DPNH, 1.2 mg of enzyme protein, and 120 μg of dl-folate-H₄; final volume, 2 ml.

<table>
<thead>
<tr>
<th>System</th>
<th>Prefolic A synthesized (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>77</td>
</tr>
<tr>
<td>- FAD</td>
<td>32</td>
</tr>
<tr>
<td>- Formaldehyde</td>
<td>6</td>
</tr>
<tr>
<td>- DPNH</td>
<td>0</td>
</tr>
<tr>
<td>- Enzyme</td>
<td>0</td>
</tr>
<tr>
<td>- Folate-H₄</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 2. Radioactivity and biological activity of prefolic A synthesized enzymatically from tetrahydrofolic acid and formaldehyde-C¹⁴. The reaction was carried out in an atmosphere of helium. The reaction mixture contained, per ml of 0.1 M phosphate buffer, pH 7.0, 100 μg of FAD, 24 mg of enzyme protein, 1.5 mg of dl-folate-H₄, 147.5 μg of p-formaldehyde-C¹⁴ (5 μCi), and 3 mg of DPNH; final volume, 5 ml. After 1 hour at 37°C, the reaction mixture was treated as described in the text and chromatographed on DEAE-cellulose. Fractions of 4 ml each were collected. Aliquots of the fractions showing the typical ultraviolet spectrum of prefolic A were counted for radioactivity and assayed for prefolic A. O—O, prefolic A; △—△, radioactivity.

prefolic A (Fig. 2). After 60 minutes at 37°C, the reaction was stopped by heating to 80°C in an atmosphere of helium. The coagulated protein was removed by centrifugation and washed with 5 ml of water. The combined supernatant solution and wash were concentrated to 5 ml under reduced pressure below 20°C. After chromatography on diethylaminoethyl (DEAE) cellulose (10), aliquots from the fractions with the ultraviolet spectrum of prefolic A were checked for radioactivity and assayed for radioactivity. The results in Fig. 2 show that the radioactivity and the microbiological activity of the fractions are in excellent agreement. The radioactivity observed by Fraction 32 corresponds with the position on the column occupied by methylenetetrahydrofolic acid (synthetic mixture of a 3:1 molar ratio of HCHO and folate-H₄). Since these fractions show no microbiological activity, it is assumed that they represent the inactive isomer of methylenetetrahydrofolic acid. The low radioactivity in these fractions is due to destruction of methylenetetrahydrofolic acid during concentration of the reaction mixture under reduced pressure before chromatography. Prefolic A is not affected by this treatment.

Identification of Reaction Product—The product of the reaction was shown to be identical with the prefolic A isolated from horse liver with respect to several criteria. These include similar activity for both test organisms before and after enzymatic conversion to tetrahydrofolic acid (Table VII) and similar elution volumes from a DEAE-cellulose column with 10% Na₂HPO₄ (6). In addition, the ultraviolet spectra of both preparations are identical in every respect. At alkaline pH, both the synthesized and the naturally occurring prefolic A were converted to prefolic AB (9, 10) at the same rate.

Properties of Prefolic A-synthesizing System—The effect of increasing DPNH concentration is shown in Fig. 3. DPNH could be replaced by TPNH or by DPN in the presence of ethanol and alcohol dehydrogenase. The rate of prefolic A formation is linear with increasing enzyme concentration (Fig. 3) and proceeds at a linear rate with increasing time up to 60 minutes (Fig. 4).

Table VII

Comparison of microbiological activity of natural and biosynthetic prefolic A

<table>
<thead>
<tr>
<th>Assay organism</th>
<th>Horse liver</th>
<th>Biosynthetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.D.₃₆₅</td>
<td>Before enzyme treatment</td>
<td>After enzyme treatment</td>
</tr>
<tr>
<td></td>
<td>P. cerevisiae</td>
<td>L. casei</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>μg</td>
</tr>
<tr>
<td>Horse liver</td>
<td>0.76</td>
<td>0.25</td>
</tr>
<tr>
<td>Biosynthetic</td>
<td>0.73</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* The reaction mixture for enzymatic treatment was the same as that in Table II.

Fig. 3. Effect of increasing DPNH and enzyme concentrations on the rate of prefolic A formation. The reaction was carried out in an atmosphere of helium at 37°C for 30 minutes. The reaction mixture contained, per ml of 0.1 M phosphate buffer, pH 6.6, 5 mg of ascorbic acid, pH 6.5, 5 μg of FAD, 120 μg of dl-folate-H₄, 25 μg of formaldehyde, 1 mg of DPNH for increasing enzyme protein curve (△—△), 1.2 mg of enzyme protein for DPNH saturation curve (O—O), and the designated amount of DPNH or enzyme protein for the respective curves. Final volume, 2 ml.
The time course of the reaction. The reaction was carried out in an atmosphere of helium at 37°C for the designated time periods. The reaction mixture contained, per ml of 1/6 phosphate buffer, pH 6.6, 5 mg of sodium ascorbate, pH 6.5, 5 μg of FAD, 0.75 mg of DPNH, 100 μg of dl-tetrahydrofolic acid, 330 μg of methanol, and 1.2 and 2.4 mg of enzyme protein; final volume, 2 ml.

The specificity of the reaction with respect to tetrahydrofolic acid derivatives other than the 5,10-methylene compound is shown in Table VIII. Neither anhydroleucovorin, N1-formyl-, nor N1-formyltetrahydrofolic acid will serve as substrate for this enzyme.

Effects of Inhibitors—The structural similarity of dicumarol to menadione, as well as its anti-vitamin K properties, prompted an examination of the effect of this compound on this enzyme. Preliminary experiments had demonstrated that dicumarol does not serve as electron acceptor in the conversion of prefolic A to tetrahydrofolic acid. In addition, the effects of the uncoupling agents, 2,3-dimercapto-1-propanol and 2,4-dinitrophenol, were also investigated. The effects of these compounds were tested on the reaction in both directions. The results in Table IX show that all three compounds are potent inhibitors of this enzyme. Further investigation revealed that dicumarol inhibits the formation of tetrahydrofolic acid from prefolic A in a non-competitive manner, as demonstrated by the Lineweaver-Burk plot (16) shown in Fig. 6. As stated earlier, menadione inhibits the conversion of methylenetetrahydrofolic acid to prefolic A. Further investigation revealed that this inhibition is of the competitive type (Fig. 7).

An investigation of the inhibition of prefolic A oxidation by DPNH revealed that this is of the competitive type (Fig. 8).

Distribution of Enzyme—Results from a survey of a limited number of laboratories is shown in Table IX. The reactions were carried out in an atmosphere of helium at 37°C for 30 minutes. For the oxidation of prefolic A, the reaction mixture contained, per ml of 1/6 phosphate buffer, pH 7.0, 5 mg of sodium ascorbate, pH 6.5, 2.5 μg of FAD, 7.5 μg of menadione, 10 μg of Ba-prefolic A, 0.24 mg of enzyme protein, and the designated amount of each inhibitor; final volume, 2 ml.

For the formation of prefolic A, the reaction mixture contained, per ml of 1/6 phosphate buffer, pH 6.5, 5 mg of ascorbic acid, pH 6.6, 2.5 μg of FAD, 1.2 mg of enzyme protein, 100 μg of dl-tetrahydrofolic acid, 330 μg of methanol, and the designated amount of each inhibitor; final volume, 2 ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Prefolic A oxidized</th>
<th>Inhibition</th>
<th>Prefolic A formed</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.47</td>
<td>38.75</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dicumarol</td>
<td>8 X 10⁻⁶</td>
<td>2.9</td>
<td>16</td>
<td>20.5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>4 X 10⁻⁵</td>
<td>2.1</td>
<td>40</td>
<td>9</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>1.6 X 10⁻⁴</td>
<td>1.14</td>
<td>67</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>8 X 10⁻⁵</td>
<td>2.83</td>
<td>18</td>
<td>32.50</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4 X 10⁻⁵</td>
<td>2.73</td>
<td>21</td>
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<td></td>
<td>1.6 X 10⁻⁴</td>
<td>2.06</td>
<td>41</td>
<td>10</td>
<td>74</td>
</tr>
<tr>
<td>2,3-Dimercapto-1-propanol</td>
<td>8 X 10⁻⁶</td>
<td>2.6</td>
<td>25</td>
<td>30.50</td>
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<tr>
<td></td>
<td>4 X 10⁻⁵</td>
<td>2</td>
<td>42</td>
<td>7</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>1.6 X 10⁻⁴</td>
<td>1.36</td>
<td>60</td>
<td>4</td>
<td>90</td>
</tr>
</tbody>
</table>
FIG. 6. Noncompetitive inhibition of enzyme by dicumarol as demonstrated by the Lineweaver-Burk plot (16). The reaction was carried out in an atmosphere of helium at 37° for 30 minutes. The reaction mixture contained, per ml of m/6 phosphate buffer, pH 7.0, 5 mg of ascorbate, 5 μg of FAD, 10 μg of Ba-prefolic A, 0.12 mg of enzyme protein, and menadione and dicumarol as indicated; final volume, 2 ml.

FIG. 7. Competitive inhibition of prefolic A formation from tetrahydrofolic acid and formaldehyde by menadione. The reaction was carried out in an atmosphere of helium at 37° for 30 minutes. The reaction mixture contained, per ml of m/6 phosphate buffer, pH 7.0, 5 μg of ascorbate adjusted to pH 6.5, 5 μg of FAD, 1.2 mg of enzyme protein, 1.5 mg of DPNH, and increasing concentration of an equimolar mixture of tetrahydrofolic acid and formaldehyde; final volume, 2 ml.

FIG. 8. Competitive inhibition of tetrahydrofolic acid formation from prefolic A by DPNH. The reaction was carried out in an atmosphere of helium at 37° for 30 minutes. The reaction mixture contained, per ml of m/6 phosphate buffer, pH 7.0, 5 μg of FAD, 5 mg of ascorbic acid adjusted to pH 6.5, 7.5 μg of menadione, 0.12 mg of enzyme protein, 1.5 mg of DPNH where designated, and the indicated amounts of Ba-prefolic A; final volume, 2 ml.

The oxidation of prefolic A to tetrahydrofolic acid and formaldehyde and the observation that this compound contains 1 mole of N-methyl group per mole of compound4 supports the view that prefolic A is a methyl-substituted derivative of tetrahydrofolic acid. Further support is obtained by the reversal of the reaction, i.e. the biosynthesis of prefolic A by reduction of methylenetetrahydrofolic acid by DPNH, and the chemical synthesis of prefolic A by NaBH₄ reduction of methylenetetrahydrofolic acid (10).

The location of the methyl group on prefolic A has not been established. However, the N⁵-position of tetrahydrofolic acid is the most likely one. In support of this is the greater stability of prefolic A (10) when compared with other 10-substituted reduced folic acid derivatives. Kisliuk reported that the nitrogen atoms at both the 5- and 10-positions of tetrahydropteridines must be unsubstituted in order for these compounds to bind formaldehyde (2). However, Blakley (3, 4) reported that N⁰-substituted hydropteridines bind significant amounts of formaldehyde, whereas the N⁵-substituted derivatives bind little or no formaldehyde. These observations support the view that prefolic A is 5-methyltetrahydrofolic acid, since prefolic A will not bind formaldehyde. Sakami and Uksitis (17) have postulated that the major KBH₄ reduction product of a mixture of tetrahydrofolic acid and formaldehyde is 5-methyltetrahydrofolic acid.

If prefolic A is 5-methyltetrahydrofolic acid, then enzymatic oxidation should result in an equilibrium mixture of N⁵-hydroxymethyltetrahydrofolic acid, N⁵,N¹⁰-methylenetetrahydrofolic acid, and free tetrahydrofolic acid, as is postulated by Blakley for a mixture of tetrahydrofolic acid and formaldehyde (3). Therefore, the product of the enzymatic oxidation of prefolic A, like an equimolar mixture of tetrahydrofolic acid and formaldehyde, may be assayed as tetrahydrofolic acid or formylated to N⁵-formyltetrahydrofolic acid. Prefolic A is oxidized enzymatically to methylenetetrahydrofolic acid with menadione as the electron acceptor. DPN will not serve as electron acceptor in the reaction. The requirement for DPNH and not for reduced menadione for the reduction of methylenetetrahydrofolic acid to prefolic A might be explained by the following reaction scheme.

4 Unpublished observations.
In Reaction I, prefolic A, which is represented as 5-methyl-
tetrahydrofolic acid, is reversibly oxidized to 5,10-methylenetetra-
hydrofolic acid by the FAD-bound enzyme. The reduced
FAD-enzyme is then reoxidized by menadione (Reaction II).
In Reaction III, DPNH serves as the proton donor for the
reduction of the FAD-enzyme, which is the direct proton donor for
the reduction of 5,10-methylenetetrahydrofolic acid to 5-methyl-
tetrahydrofolic acid.

From the proposed scheme, one would predict that DPNH
should inhibit Reaction I in the forward direction, competitively,
and that menadione should inhibit the same reaction in the
reverse direction, competitively. The data in Figs. 7 and 8 show
that these predictions are justified.

From these results it may be added that the enzyme pos-
sesses DPNH-menadione reductase activity. Assay for this en-
zyme (13) shows an activity of 20 units per mg of protein. That
this activity is not due to contamination with a menadione reduc-
tase was confirmed by the fact that the specific activity was
essentially unchanged by retraction at acid pH and with
ammonium sulfate. In addition, the inhibitory effects of
DPNH and menadione on the forward and reverse reactions are
inconsistent with the behavior of the menadione reductase de-
scribed by Wosilait and Nason (13). However, both enzymes are
inhibited by dicumarol and other uncoupling agents.

Prefolic A will replace folic acid as a growth factor (10) for L.
casei and for the chick;4 however, its mode of action is unknown
at this time. Buchanan has suggested that prefolic A may be the
same as the intermediate for methionine biosynthesis which
was recently prepared in his laboratory (8, 18). This suggestion
is supported by the observation that prefolic A, like the methio-
nine cofactor, is prepared enzymatically from the same substrate;
the enzyme(s), although from different sources, require the same
cofactors. In addition, both prefolic A and the methionine cof-
actor contain 1 mole of N-methyl group per mole of compound.4

Further support for the role of prefolic A as the methyl donor
in methionine biosynthesis stems from the recent publication by
Sakami and Ukstins (17). These investigators have synthe-
sized a methylfolate by reduction of an equimolar mixture of
tetrahydrofolic acid and formaldehyde with KBH₄ and showed that
this compound is the intermediate in methionine biosynthesis.
In a recent publication, we have shown that prefolic A was syn-
thetized by the N₅H₄ reduction of a mixture of tetrahydrofolic
acid and formaldehyde (10).

Wilms, Rucker, and Jaenicke (10) have suggested an inter-
upal molecular rearrangement of N₅,N₁₀-methylenetetrahy-
drofolic acid to form N₅-methylidihydrofolic acid, which they
have postulated to be the methyl donor in methionine biosynthesis.

It is not unlikely that prefolic AB (9, 10) is an N-methylidihy-
drofolic acid. Noronha and Silverman (20) have observed that
prefolic A, one of the major folic acids in the liver of normal rats,
is greatly elevated when these animals are kept on a diet which
is low in methionine and vitamin B₁₂. However, when rats
from this same group are placed on a diet which is rich in methio-
nine, the level of prefolic A in the livers of these animals is
lowered substantially, and the major folic acid in these animals
is now N₅₉-formyltetrahydrofolic acid. From these experiments,
these authors predicted that prefolic A should be a methyltetra-
hydropteridine (20). Now that prefolic A has been prepared chemically (1), its role in methylation and other biological sys-
tems can be more fully evaluated.

SUMMARY

Prefolic A is enzymatically oxidized to hydroxymethyl tetra-
hydrofolic acid. The partially purified enzyme is flavin adnine
dinucleotide-linked and requires menadione as electron acceptor.
Other quinines and certain low potential dyco will also serve as
electron acceptor. Reduced diphosphopyridine nucleotide competi-
tively inhibits the formation of tetrahydrofolic acid from pre-
folic A. Neither diphosphopyridine nucleotide nor triphospho-
pyridine nucleotide will serve as electron acceptor.

In the presence of the same enzyme preparation, prefolic A is
synthesized from tetrahydrofolic acid and formaldehyde (or methanol) with reduced diphosphopyridine nucleotide or re-
duced triphosphopyridine nucleotide as electron donor. Mena-
dione competitively inhibits the biosynthesis of prefolic A. Mechanisms for the forward and reverse reactions are discussed.
Prefolic A is characterized as an N₅-methyltetrahydrofolic acid.

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Naturally Occurring Forms of Folic Acid: II. ENZYMATIC CONVERSION OF METHYLENETETRAHYDROFOLIC ACID TO PREFOLIC A-METHYLTETRAHYDROFOLATE
Kenneth O. Donaldson, John C. Keresztesy and With the technical assistance of Marjorie K. Romine and Tresvant B. Goodwin


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