The Mechanism of Binding of Folate Analogues by Folate Reductase*

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The first step in the conversion of folate to the functional form of the vitamin is its reduction to a tetrahydro derivative (1). The enzyme, folate reductase (also called dihydrofolate reductase or tetrahydrofolate dehydrogenase), which catalyzes this reaction has been partially purified from a variety of tissues in different laboratories (2-8). Aminopterin and amethopterin are potent inhibitors of this enzyme (2, 4, 5, 8-10). Werkheiser (11) demonstrated that both inhibitors are bound to the reductase so tightly that, from the biological point of view, the inhibition appears to be irreversible. Since at very high excess of substrate over the inhibitor, the drug could be displaced from the enzyme, the term “stoichiometric” rather than “irreversible” was suggested for this type of inhibition (11). It was estimated that aminopterin is bound to the reductase approximately 100,000 times more tightly than folate (11). It was suggested that the difference in the affinities to the reductase between folate and aminopterin is due to the stronger basicity of the latter compound (12, 13).

The purpose of the present investigation was to determine the mechanism of binding to folate reductase of folate as compared to that of aminopterin. The effect on folic acid reductase of model compounds (pteridines, purines, and pyrimidines), some of them representing fragments of the molecules of folate and aminopterin, was studied. None of the various compounds resembling the 2-amino-4-hydroxypteridine ring of folate was capable of forming a complex with the enzyme. In the case of compounds resembling aminopterin, however, the formation of a complex with the enzyme could be demonstrated even when the molecules represented only the pyrimidine portion, provided that the diamino structure was preserved. The thermodynamics of the binding of these compounds to the enzyme were also investigated. A new theory on the mechanism of binding of aminopterin and folate to the reductase which resulted from these studies will be discussed. Preliminary reports on this work have been presented (14, 15).

EXPERIMENTAL PROCEDURE

Partially purified folate reductase from chicken liver (6) was used throughout these studies. Folic acid and glucose 6-phosphate were obtained from Nutritional Biochemicals Corporation, glucose 6-phosphate dehydrogenase from California Corporation for Biochemical Research, and TPN from Pabst Laboratories.*

TPNH was prepared by reduction of TPN with glucose 6-phosphate dehydrogenase (6). Most of the inhibitors used were purchased from Nutritional Biochemicals Corporation, 2,6-Diamino-4-methylpyrimidine, 2,6-diamino-4,5-dimethylpyrimidine, and 2,4-diamino-6-hydroxypteridine were obtained by courtesy of the Wellcome Research Laboratories. 2-Amino-4-hydroxy-6-formylpteridine and 2-amino-4-hydroxy-6-methylpteridine were obtained from American Cyanamid Company, the former from the Lederle Laboratories, the latter from the Calco Division. 2,4-Diamino-6-methylpteridine (16) and 2,4-diamino-6-formylpteridine were prepared in this laboratory (17).

The enzymatic reduction of folate was measured as described elsewhere (8) by a colorimetric determination of p-aminobenzoylglutamic acid, released from tetrahydrofolate upon cleavage of the molecule.

In preliminary experiments, the inhibition constants \( K_i \) of the compounds were estimated by means of an analysis according to Lineweaver and Burk (18). These experiments were performed at pH 0.0 and TPNH served as the hydrogen donor. TPNH was regenerated by including glucose 6-phosphate and glucose 6-phosphate dehydrogenase in the reaction mixtures (19).

Modified techniques had to be used for the thermodynamic studies. Since the TPNH regenerating system does not function well at lower temperatures, this system (glucose 6-phosphate and glucose 6-phosphate dehydrogenase) was replaced by a large excess of TPNH \( \times 10^{-4} \text{m} \). To increase the initial velocity of the reaction, the pH of the reaction mixtures was lowered to pH 5.2 (6). The variation of the kinetic constants within the range of the temperatures suitable for enzymatic reactions \( 10-40^\circ \text{C} \) is relatively small. Therefore, high accuracy in the determination of the individual constants had to be assured in this study.

In order to determine accurately the Michaelis constants \( K_m \) for folate, the experimental data were analyzed in two ways: according to Lineweaver and Burk (18)

\[
\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V} \times \frac{1}{S}
\]

(1)

and according to Hoistee (20)

\[
\frac{S}{v} = \frac{K_m}{V} + \frac{1}{V} \times S
\]

(2)

where \( S = \) concentration of the substrate; \( v = \) initial reaction velocity; \( V = \) maximal reaction velocity, and \( K_m = \) Michaelis constant.

* This work was supported in part by a research grant (CY-2906) from the National Cancer Institute of the United States Public Health Service.
In Equation 1, the value of $1/V$ (ordinal intercept) can be determined with great accuracy, whereas the slope which allows the estimation of $K_m$ is less accurate. Therefore, substituting the value of $1/V$, obtained from Equation 1, into Equation 2, the values of $K_m$ (ordinal intercept in Equation 2) could be determined quite accurately.

For the determination of the inhibition constants ($K_i$) of the competitive inhibitors at different temperatures, the following procedure was used. For each analogue and each temperature, a series of incubations was performed all at the same substrate concentration ($1.09 \times 10^{-5} \text{M}$) but with different concentrations of the inhibitor. The ratio of $V$ to $v$ was then plotted against the concentration of the inhibitor according to the following equation

$$
\frac{V}{v} = 1 + \frac{K_m}{S} + \frac{K_m}{S} \times \frac{K_i}{I}
$$

where $I$ = concentration of the inhibitor.

Knowing the $K_m$ and the concentration of the substrate, $K_i$ could be calculated from the slope. This method has an advantage over the commonly used method of Lineweaver and Burk since it permits the reactions to be carried out at relatively high concentrations of the substrate.

**RESULTS**

**Preliminary Observations**—Table I lists the compounds which were tested for inhibition of folate reductase. Compounds which failed to inhibit at a concentration $4.4 \times 10^{-4} \text{M}$, usually the upper limit of solubility at the pH of the experiment, were classified as inert.

All of the compounds which have the diamino structure (2,6-diamino in the case of pyrimidines and purines, and 2,4-diamino in the case of pteridines) were inhibitors. The analysis of inhibition according to Lineweaver and Burk of these compounds is presented in Fig. 1. In all cases the inhibition was of the competitive type. The affinities of these compounds to folic acid reductase, as reflected by $K_i$ values (Table I), greatly depended on the nature of the other substituents. There is, however, no relationship between the ring structure of the inhibitors and their affinities to the enzyme. For instance, 2,6-diaminopurine is bound to the enzyme more tightly than most of the pteridines, and all 2,6-diaminopyrimidines more tightly than 2,4-diamino-6-hydroxypteridine. This indicates that the pyrazine ring of the pteridines is unlikely to be involved in the formation of the enzyme-aminopterin complex. Compounds which lack either one of the two amino groups or those in which one of the amino groups is replaced by a hydroxyl are not able to inhibit the enzyme. The only exception within this group is 2-amino-4-hydroxy-6-formylpteridine. However, this compound differs from the others in that it is a substrate for folic acid re-

<table>
<thead>
<tr>
<th>Compounds tested as inhibitors of folate reductase</th>
<th>$K_i$*</th>
<th>$S \times 10^{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Diamino-6-methyl</td>
<td>$4.7 \times 10^{-7}$</td>
<td>1.5</td>
</tr>
<tr>
<td>2,4-Diamino-6-formyl</td>
<td>$8.1 \times 10^{-8}$</td>
<td>1.5</td>
</tr>
<tr>
<td>2,4-Diamino-6-hydroxy</td>
<td>$9.0 \times 10^{-8}$</td>
<td>1.5</td>
</tr>
<tr>
<td>2-Amino-4-hydroxy-6-formyl</td>
<td>$2.9 \times 10^{-4}$</td>
<td>1.5</td>
</tr>
<tr>
<td>2-Amino-4-hydroxy-6-methyl</td>
<td>Inert</td>
<td>1.5</td>
</tr>
<tr>
<td>2-Amino-4,6-dihydroxy</td>
<td>Inert</td>
<td>1.5</td>
</tr>
<tr>
<td>2,6-Diamino</td>
<td>$1.5 \times 10^{-4}$</td>
<td>2</td>
</tr>
<tr>
<td>2-Amino-6-hydroxy</td>
<td>Inert</td>
<td>2</td>
</tr>
<tr>
<td>2-Amino</td>
<td>Inert</td>
<td>2</td>
</tr>
<tr>
<td>6-Amino</td>
<td>Inert</td>
<td>2</td>
</tr>
<tr>
<td>2,6-Diamino-4-methyl</td>
<td>$2.6 \times 10^{-8}$</td>
<td>3</td>
</tr>
<tr>
<td>2,6-Diamino-4,5-dimethyl</td>
<td>$2.8 \times 10^{-5}$</td>
<td>3</td>
</tr>
<tr>
<td>2,4,5,6-Tetraamino</td>
<td>$4.8 \times 10^{-5}$</td>
<td>3</td>
</tr>
<tr>
<td>2,4-Diamino-6-hydroxy</td>
<td>Inert</td>
<td>3</td>
</tr>
</tbody>
</table>

* Determined at 37° and pH 6.0.

† This compound is a substrate with $K_m 3.3 \times 10^{-4}$.  

![Fig. 1. Inhibition of the reduction of folate by different inhibitors.](http://www.jbc.org/)

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ductase and thus can act as a competitive inhibitor (21). 2,4-Diamino-6-hydroxypteridine did not inhibit folic acid reductase. The special structure of this compound will be analyzed further in the "Discussion."

**TABLE II**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_m$</th>
<th>$V$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$12^\circ$</td>
<td>$\times 10^9$</td>
<td>$1.97$</td>
</tr>
<tr>
<td>$22$</td>
<td>$1.83$</td>
<td>$4.17$</td>
</tr>
<tr>
<td>$31$</td>
<td>$3.13$</td>
<td>$12.5$</td>
</tr>
<tr>
<td>$40$</td>
<td>$5.69$</td>
<td>$33.4$</td>
</tr>
</tbody>
</table>

*Fig. 2. Plots of log $K$ versus $\frac{1}{T}$ for folate and some of the diamino inhibitors.*

The Michaelis constant ($K_m$) for folate ($2.3 \pm 0.3 \times 10^{-6}$ at pH 6 and $37^\circ$) was determined from the data presented in Fig. 1. The basis for the assumption that in this case the Michaelis constant approaches a true dissociation constant of the enzyme-folate complex has been discussed (21). Although the final proof cannot be presented at this time, this assumption is based on the observation that with different substrates the maximal reaction velocities ($V$) and Michaelis constants ($K_m$) vary independently from each other (21). The effect of temperature on Michaelis constant and maximal reaction velocity is shown in Table II. Between $12^\circ$ and $40^\circ$ maximal reaction velocity increases three times faster than Michaelis constant. This, as well as the fact that a plot of $K_m$ versus $\frac{1}{T}$ (Fig. 2) is a straight line, strongly supports the above concept.

**Thermodynamic Studies**—The Michaelis constant ($K_m$) for folate and inhibition constants ($K_i$) for some of the diamino inhibitors were determined at different temperatures between $10^\circ$ and $40^\circ$. The graphic presentation of the log of these constants versus the reciprocal of absolute temperatures is shown in Fig. 2. From the slopes of these lines, the thermodynamic constants for the dissociation of the enzyme-substrate or the enzyme-inhibitor complexes could be determined according to the following equations

$$-\Delta F^0 = RT \times \ln K$$

$$\ln K = \frac{\Delta H}{R} \times \frac{1}{T} + C$$

or

$$\Delta H = -\text{slope} \times 2.303 R + T \Delta S$$

Where $\Delta F^0$ = change of the standard free energy, $\Delta H$ = change of the total binding energy, $\Delta S$ = change of entropy, $K$ = dissociation constant of the enzyme-substrate, or enzyme-inhibitor complex, $T$ = absolute temperature, $R$ = gas constant, and $C$ = integration constant.

A summary of the data is presented in the upper part of Table III. Folate has an unusually high binding energy as compared with the binding energies of diaminopteridines, -purines, or

**TABLE III**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m^*$</th>
<th>$K_i^*$</th>
<th>$\Delta H$</th>
<th>$\Delta F^0$</th>
<th>$\Delta S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Folate</td>
<td>$2.3 \times 10^{-6}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 2,4-Diamino-6-methylpteridine</td>
<td>$1.8 \times 10^{-6}$</td>
<td>$5.0$</td>
<td>$7.9$</td>
<td>$9.5$</td>
<td></td>
</tr>
<tr>
<td>3. 2,4-Diamino-6-formylpteridine</td>
<td>$8.1 \times 10^{-6}$</td>
<td>$4.1$</td>
<td>$6.9$</td>
<td>$9.5$</td>
<td></td>
</tr>
<tr>
<td>4. 2,4-Diamino-6-hydroxypteridine</td>
<td>$3.7 \times 10^{-6}$</td>
<td>$1.8$</td>
<td>$4.7$</td>
<td>$9.6$</td>
<td></td>
</tr>
<tr>
<td>5. 2,6-Diamino-4-methylpyrimidine</td>
<td>$2.1 \times 10^{-6}$</td>
<td>$2.1$</td>
<td>$5.1$</td>
<td>$10$</td>
<td></td>
</tr>
<tr>
<td>6. 2,6-Diaminopurine</td>
<td>$1.8 \times 10^{-6}$</td>
<td>$6.0$</td>
<td>$7.9$</td>
<td>$6.4$</td>
<td></td>
</tr>
<tr>
<td>7. Aminopterin</td>
<td>$10^{-13}$ to $10^{-11}$</td>
<td>$1.16$</td>
<td>$13.7$ to $15.1$</td>
<td>$7$ to $11.7$</td>
<td></td>
</tr>
<tr>
<td>8. 2-Amino-4-hydroxy-6-methylpteridine</td>
<td>$1.5 \times 10^{-1}$</td>
<td>$5.0$</td>
<td>$1.1$</td>
<td>$13$†</td>
<td></td>
</tr>
<tr>
<td>9. 2-Amino-4,6-dihydroxypteridine</td>
<td>$33$</td>
<td>$1.8$</td>
<td>$2.1$</td>
<td>$13$†</td>
<td></td>
</tr>
<tr>
<td>10. 2-Amino-6-hydroxypurine</td>
<td>$2.8 \times 10^{-2}$</td>
<td>$6.0$</td>
<td>$2.1$</td>
<td>$13$†</td>
<td></td>
</tr>
</tbody>
</table>

*At pH 5.2 and $26^\circ$.

† Values assumed on basis of the reasoning presented in the "Discussion."
The replacement of the 4-hydroxyl group of folate by an amino group changes this vitamin into an antagonist of exceptional potency. A satisfactory explanation for the remarkable effectiveness of the 4-amino folate antagonists as inhibitors of folate reductase was attempted by this detailed study of compounds with closely related structures. Data presented herein show that in the case of 2,4-diaminopteridines (and 2,6-diaminopurines and -pyrimidines) both of the amino groups are essential for the binding of the analogue to the enzyme. The fact that diamino analogues are much stronger bases than the corresponding aminohydroxy compounds may suggest that the binding to the reductase is ionic in character. The following findings, however, speak against this hypothesis.

1. In most cases, there is no correlation between the basic ionization constants of the inhibitors and their inhibitory potency.

2. The binding of the antagonists to the enzyme is stronger at pH 6 than at pH 5.2.

3. The difference between the basic ionization constants of folate (pK approximately 2.5) and aminopterin (pK approximately 5.5) can hardly account for the 100,000-fold difference between the dissociation constants of folate-enzyme and aminopterin-enzyme complexes. Thus, it is suggested that the binding between the enzyme protein and the diamino inhibitors occurs by means of hydrogen bonds. As presented in Fig. 3, both amino groups as well as both ring nitrogens could participate in this binding. The differences in behavior toward the reductase of the diamino- and the aminohydroxy analogues are not surprising if the structural differences between these two groups of compounds are taken into consideration (Fig. 4). Both 6-amino- and 6-hydroxy-pteridines are potentially tautomeric compounds. Whereas the amino analogues exist predominantly in a true amino form (Fig. 4A), the equilibrium is heavily in favor of the iminol form in the case of hydroxy analogues (Fig. 4D) (22, 23); the same applies to the pteridines (24, 25). Obviously, position 6 of the pyrimidines and purines corresponds to position 4 of the pteridines. When either of the two amino groups is replaced by an hydroxyl, the binding still can take place. However, if the hydroxy analogue exists in an imido rather than iminol form, two of the four hydrogen bonds are disrupted. The inert behavior of 2,4-diamino-6-hydroxy-pteridine requires some explanation. This compound fulfills the structural requirement as an inhibitor of the reductase with respect to its two amino groups (Fig. 5), yet it does not inhibit the enzyme. This may be due to the fact that a hydrogen atom is attached to one of the ring nitrogens (N1 in Fig. 5). Thus, not only the hydrogen binding capacity of N1 is modified but also a sterical hindrance may be created.

It is reasonable to assume that the same mechanism of binding applies to folate and aminopterin. In the case of these compounds, however, additional binding seems to occur at the p-aminobenzoylglutamic acid residue. From the hypothesis presented above, it would seem to follow that those of the 4-hydroxy compounds which enter into complex formation with the reductase, such as 2-amino-4-hydroxy-6-formylpteridine and folate, must exist in an iminol rather than imido form. Another possibility is that the imido-iminol equilibrium of these compounds is shifted under the influence of the enzyme in favor of the iminol form, thus enabling the complex formation with the enzyme. The thermodynamic constants, especially the entropy changes presented in the upper part of Table II, give support to the latter possibility. The enforcement of an iminol configuration, unusual for folate, causes a decrease in the entropy of the system. Upon dissociation of the complex, on the other hand, folate spontaneously assumes its normal imido configuration with the result that the entropy increases.

It is reasonable to assume that all of the 2-amino-4-hydroxy-pteridines undergo a rearrangement similar to that described for folate under the influence of the reductase. In most of the cases, however, the energy balance is so unfavorable (ΔH too low or ΔS too high) that no binding with the enzyme can be detected. It has been demonstrated elsewhere that aminopterin is a stoichiometric inhibitor of folic acid reductase (11). In the case of stoichiometric inhibitors, the dissociation constant of the en-o

1. S. F. Zakrzewski, unpublished data.
enzyme-inhibitor complex cannot be calculated by the conventional method of Lineweaver and Burk. Also, it cannot be decided on the basis of this method whether stoichiometric inhibition is of the competitive or noncompetitive type. Thus, the exact dissociation constant for aminopterin could not be determined. Werkheiser (11) estimated, however, that if this compound is a competitive inhibitor of the reductase, its dissociation constant would be between $10^{-4}$ and $10^{-3}$ at $37^\circ$. On the basis of the competitive relationship observed between the 2,4-diaminopteridines and folate, it seems likely that aminopterin is also a competitive inhibitor of folic acid reductase, in the sense that it is attached to or obstructs the active sites of the enzyme. It may be assumed that the complex between this reductase and aminopterin has very similar binding energy ($\Delta H$) to that between the reductase and folate (+11.6 kilocalories). From the dissociation constant reported by Werkheiser (11), the change of free energy ($\Delta F^o$), occurring on dissociation of reductase-aminopterin complex was computed to be between +13.7 and +15.1 kilocalories. Substituting these two values in Equation 6, the entropy change $-7.6$ to $-11.7$ calories per degree was obtained. This value is very similar to those determined for several 2,4-diaminopteridines ($-9.5$ calories per degree). Thus, it appears that the binding of the nonpteridine part of the molecule of aminopterin (and also folate) does not contribute significantly to the change of entropy. Consequently, the positive entropy change associated with the dissociation of the reductase-folate complex seems to be related to the rearrangement of the substrate rather than the enzyme.

The assumption that the binding energies of the reductase-aminopterin and the reductase-folate complexes are similar requires some comment. It is impossible to determine quantitatively how much the replacement of one OH group by a NH$_2$ group will influence the binding energy of the analogues to the enzyme. If compounds 8, 9, and 10 were to exist exclusively in the iminol form, the binding energies ($\Delta H$) of these compounds to the reductase should be very similar to those of the corresponding diamino derivatives. Further, in each case, the change of entropy ($\Delta S$) resulting from the tautomeration of the imido to iminol form should be similar to that calculated for folate. From these assumed values, changes in standard free energies ($\Delta F^o$) and dissociation constants ($K_i$) of the enzyme-inhibitor complexes were calculated (Table III). In each of these cases, the dissociation constant is so large that no inhibition could be observed, under the conditions of the experiment.

The activity of 2-amino-4-hydroxy-6-formylpteridine as an inhibitor (and also substrate) of folate reductase requires explanation. Obviously the binding energy of this compound to the enzyme will be similar to that of 2,4-diamino-6-formylpteridine (+4.1 kilocalories) rather than to that of folic acid. It can be expected, however, that the strongly electron-withdrawing carbonyl group in resonance with the pteridine ring will have considerable effect on the imido-iminol equilibrium. Consequently, the tendency to exist in iminol form may be much greater in the case of 2-amino-4-hydroxy-6-formylpteridine than in the case of other aminohydroxy analogues.

The above hypothesis provides a satisfactory explanation for the experimental findings related to the formation of these enzyme-substrate or enzyme-inhibitor complexes.

**SUMMARY**

The effect on folate reductase of various diamino- and aminohydroxypteridines, purines, and pyrimidines has been studied. All diamino analogues, i.e. compounds structurally related to aminopterin, are bound to the enzyme. Both amino groups on the pyrimidine ring are essential for this binding. The pyrazine ring does not appear to participate in the complex formation with the enzyme. Among the aminohydroxy analogues only folate and 2-amino-4-hydroxy-6-formylpteridine are bound to the reductase. Thermodynamic studies indicated that the dissociation of folate from the enzyme is associated with the increase of entropy of the system, whereas the dissociation of all 2,4-diamino compounds is associated with a decrease of entropy. On the basis of these observations a hypothesis concerning the mechanism of binding of aminopterin and folate to the reductase is suggested which can explain why aminopterin is bound to the enzyme 100,000 times more tightly than folate.

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**REFERENCES**

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