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# INHIBITION OF DIHYDROFOLATE REDUCTASE FROM BACTERIAL AND VERTEBRATE SOURCES BY FOLATE, AMINOPTERIN, METHOTREXATE AND THEIR 5-DEAZA ANALOGUES

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**Abstract**—The inhibition of dihydrofolate reductases from *Escherichia coli* and chicken liver by folate, methotrexate, aminopterin and their 5-deaza analogues was investigated to examine the importance of the N-5 nitrogen in slow-binding inhibition. Methotrexate, aminopterin and their 5-deaza analogues acted as slow, tight-binding inhibitors of both enzymes. Inhibition by methotrexate and 5-deazamethotrexate conformed to a mechanism in which there is an initial rapid formation of an enzyme–NADPH–inhibitor complex followed by a slow isomerization of this complex (Mechanism B). Aminopterin exhibited the same type of inhibition with the enzyme from *E. coli*. With the chicken-liver enzyme, however, the inhibition by aminopterin conformed to another type of slow-binding mechanism which involves only the slow interaction of the inhibitor with the enzyme to form an enzyme–NADPH–inhibitor complex (Mechanism A). The inhibition of both enzymes by 5-deazaaminopterin was also described by Mechanism A. Folate behaved as a classical, steady-state inhibitor of both enzymes, whereas 5-deazafolate exhibited slow-binding inhibition (Mechanism B) with the enzyme from *E. coli* and classical, steady-state inhibition with the enzyme from chicken liver. The substitution of a carbon for a nitrogen at the 5-position of methotrexate and aminopterin did not affect the tightness of binding of these compounds. By contrast, 5-deazafolate was bound about 4000 times more tightly than folate to the enzyme from *E. coli* and about 30 times more tightly than folate to the chicken-liver enzyme. Reasons for the differences in the binding of folate and 5-deazafolate are discussed.

Dihydrofolate reductase catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. This enzyme is the site of action for a number of drugs such as methotrexate and trimethoprim which are important in chemotherapy. Because of its importance in chemotherapy, dihydrofolate reductase has been well studied [1], and X-ray crystal structures of enzyme–inhibitor complexes have been determined with dihydrofolate reductases from three sources [2–4]. Many of the studies on dihydrofolate reductase have proceeded with the aim of understanding the interaction between the enzyme and tight-binding inhibitors such as methotrexate. To facilitate rational drug design it is of interest to know which interactions between inhibitor and enzyme groups are important for tight-binding inhibition. The 2,4-diamino-pyrimidine structure seems to be essential for tight-binding [1], and a number of workers have emphasized the importance of the interaction between the N-1 nitrogen of this ring and a conserved carboxyl side chain in the enzyme [2–6]. However, little is known about the part played by the constituents of the pyrazine ring of pteridine inhibitors. This paper reports the results of studies which were designed to determine the importance of the N-5 nitrogen in the binding of folate and its analogues to dihydrofolate reductase from chicken liver and *Escherichia coli*. The investigations were performed by comparing the inhibition of the enzymes by methotrexate, aminopterin or folate with that caused by their corresponding 5-deaza analogues.

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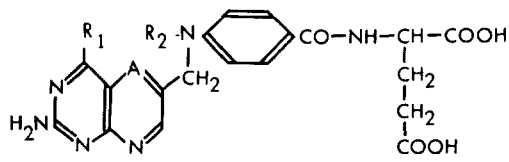
## MATERIALS AND METHODS

*Materials.* The structures of folate and its analogues which have been used in the present work are given in Table 1. Folic acid (I) was obtained from Calbiochem. Methotrexate (V) and aminopterin (III) were purchased from ICN Chemical and Radioisotope Division. The 5-deaza analogues of folate (II), aminopterin (IV) and methotrexate (VI) were prepared as described previously [7]. Dihydrofolate was prepared from folic acid by using the method of Blakley [8], and NADPH was purchased from Boehringer. Dihydrofolate reductase was purified from *E. coli* as previously described [9]. The enzyme from chicken liver was purified essentially as described by Kaufman and Kemerer [10] except that a chromatofocusing column was used in place of isoelectric focusing.

The concentrations of the substrates NADPH and dihydrofolate were determined enzymically by using a molar absorbance change for the dihydrofolate

Table 1. Structures and extinction coefficients of compounds used as inhibitors of dihydrofolate reductase

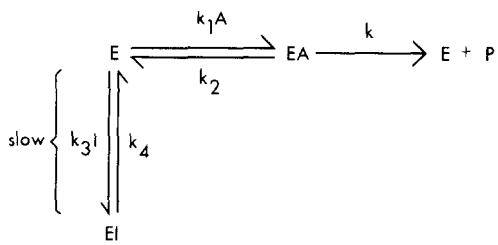


| Compound | Structure A | Structure $R_1$ | Structure $R_2$ | Extinction coefficient $\varepsilon$ ($M^{-1} \times 10^{-3}$) | Extinction coefficient $\lambda$ (nm) | pH | Ref. |
|---|---|---|---|---|---|---|---|
| Folate (I) | N | OH | H | 28.0 | 282 | 7 | 11 |
| 5-Deazafolate (II) | CH | OH | H | 24.9 | 278 | 7 | 7 |
| Aminopterin (III) | N | $NH_2$ | H | 22.1 | 302 | 13 | 12 |
| 5-Deazaaminopterin (IV) | CH | $NH_2$ | H | 23.9 | 280 | 7 | 7 |
| Methotrexate (V) | N | $NH_2$ | $CH_3$ | 22.1 | 302 | 13 | 12 |
| 5-Deazamethotrexate (VI) | CH | $NH_2$ | $CH_3$ | 25.2 | 305 | 7 | 7 |

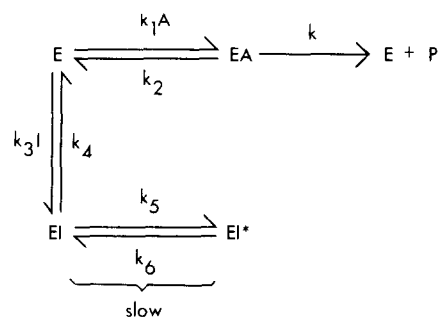
reaction of 11,800 $cm^{-1}$ at 340 nm [9]. The concentrations of the inhibitors were determined spectrophotometrically by using the extinction coefficients given in Table 1.

*Enzyme assays.* The activity of dihydrofolate reductase was determined at 30° by using a Cary 118 spectrophotometer to follow the decrease in absorbance at 340 nm due to the disappearance of dihydrofolate and NADPH. Assays were performed at pH 7.4 in a buffer mixture containing 2-(*N*-morpholino)-ethane sulphonic acid (0.025 M), sodium acetate (0.025 M), Tris (0.05 M) and NaCl (0.1 M) together with NADPH (100 $\mu$M). For experiments in which slow-binding inhibition was observed, the concentration of dihydrofolate was 50 $\mu$M. When slow-binding inhibition was not observed, the concentration of dihydrofolate was set at 10 $\mu$M.

MECHANISM A




MECHANISM B




*Data analysis.* A number of folate analogues have been shown to be slow-binding inhibitors of dihydrofolate reductase [13] and Cha [14] has described two basic mechanisms by which slow-binding inhibition can occur (Scheme 1). For Mechanism A the initial rate will be independent of the concentration of the inhibitor, whereas for Mechanism B it will be inversely proportional to the inhibitor. One inhibition constant ($K_i$), which is equal to $k_4/k_3$, will be obtained for an inhibitor conforming to Mechanism A. For Mechanism B, there will be two inhibition constants. $K_i$, equal to $k_4/k_3$, will be associated with the inhibition of the initial velocity while $K_i^*$, equal to $K_i k_6/(k_5 + k_6)$, will be associated with the inhibition of the steady-state velocity. Data from progress curves exhibiting slow-binding inhibition were fitted to equations describing either Mechanism A or Mechanism B as previously described [13]. These analyses yielded values for all the parameters associated with the inhibition. Choice between the mechanisms was made by examining the variation of the initial slope of the progress curve with inhibitor concentration. In addition, negative and/or unreasonably large values for parameters were taken as an indication that the model tested did not apply to the data. When folate analogues gave rise to classical, steady-state inhibition, they were fitted to equation 1 by using weighted, linear regression [15].

$$v = \frac{VA}{K_a(1 + I/K_i) + A}. \quad (1)$$

## RESULTS

The parameters for the inhibitions caused by folate, methotrexate, aminopterin and their 5-deaza analogues are given in Table 2. Folate caused classical, steady-state inhibition of both the *E. coli* and chicken-liver enzymes, although it was bound much more tightly to the chicken-liver enzyme ($K_i$ = 0.48 $\mu$M). By contrast, 5-deazafolate, which is not a substrate for either dihydrofolate reductase, caused slow-binding inhibition of the enzyme from *E. coli* and classical inhibition of the chicken-liver enzyme. The slow-binding inhibition by 5-deazafolate of the reaction catalyzed by the enzyme from *E. coli* con-

Table 2. Values of parameters associated with the inhibition of dihydrofolate reductase by folate and its analogues*

| Source of enzyme and compound | Type of inhibition | $K_i$ (nM) | $k_f$† | $k_r$† ($min^{-1}$) | $K_i$* (pM) | $k_5/k_6$ |
|---|---|---|---|---|---|---|
| (A) *Escherichia coli* | | | | | | |
| Folate | Classical | 22,400 ± 1,200 | | | | |
| 5-Deazafolate | Slow-binding B | 193 ± 20 | 0.33 ± 0.05 | 0.009 ± 0.005 | 5,100 ± 2,900 | 37 |
| Methotrexate | Slow, tight-binding B | 3.6 ± 0.2 | 6.9 ± 0.4 | 0.026 ± 0.002 | 13 ± 2 | 265 |
| 5-Deazamethotrexate | Slow, tight-binding B | 2.9 ± 0.2 | 11.9 ± 0.9 | 0.015 ± 0.003 | 3.9 ± 0.9 | 747 |
| Aminopterin | Slow, tight-binding B | 0.82 ± 0.21 | 2.9 ± 0.8 | 0.006 ± 0.002 | 16 ± 0.5 | 525 |
| 5-Deazaaminopterin | Slow, tight-binding A | 0.0066 ± 0.0010 | 5.9 ± 0.3 | 0.039 ± 0.006 | | |
| (B) *Chicken-liver* | | | | | | |
| Folate | Classical | 480 ± 40 | | | | |
| 5-Deazafolate | Classical | 14.2 ± 1.8 | | | | |
| Methotrexate | Slow, tight-binding B | 1.3 ± 0.2 | 2.9 ± 0.4 | 0.020 ± 0.003 | 9 ± 1 | 145 |
| 5-Deazamethotrexate | Slow, tight-binding B | 2.0 ± 0.2 | 2.5 ± 0.3 | 0.043 ± 0.009 | 34 ± 8 | 58 |
| Aminopterin | Slow, tight-binding A | 0.034 ± 0.004 | 0.95 ± 0.05 | 0.032 ± 0.004 | | |
| 5-Deazaaminopterin | Slow, tight-binding A | 0.048 ± 0.008 | 1.4 ± 0.2 | 0.069 ± 0.014 | | |

* NADPH was present at a saturating concentration of 100 μM so that the interactions of folate and its analogues were with enzyme–NADPH complexes. The concentration of the enzyme for studies on slow, tight-binding inhibition was 0.56 to 0.83 nM.

† The forward and reverse rate constants for the slow-binding inhibition are represented by $k_f$ and $k_r$ respectively. For Mechanism A, they represent $k_3$ and $k_4$, respectively, whereas for Mechanism B they represent $k_5$ and $k_6$. The units of $k_f$ are nM $min^{-1}$ for Mechanism A and $min^{-1}$ for Mechanism B.

forms to Mechanism B (Scheme 1), and the data are illustrated in Fig. 1. Both methotrexate and its 5-deaza analogue caused slow, tight-binding inhibition which also conforms to Mechanism B with the enzymes from *E. coli* and chicken liver (Table 2). The slow, tight-binding inhibition caused by aminopterin with the *E. coli* enzyme was best described by Mechanism B, whereas Mechanism A was found to apply to the slow, tight-binding inhibition caused by 5-deazaaminopterin (Fig. 2). The inhibition of the chicken-liver enzyme by both aminopterin and 5-deazaaminopterin is of the slow, tight-binding type, and the data obtained with each compound are most appropriately described by Mechanism A.

## DISCUSSION

It is becoming increasingly apparent that drugs which are useful in chemotherapy exhibit slow-binding characteristics [16]. For example, acarbose [17] and allopurinal [18] behave as slow-binding inhibitors of their respective target enzymes. The great advantage of this type of inhibition is that the inhibitor can form a tight, stable enzyme–inhibitor complex that decomposes at a rate which is independent of substrate concentration [16]. In this respect, slow-binding inhibitors differ from classical inhibitors whose effects will be readily reversed by the

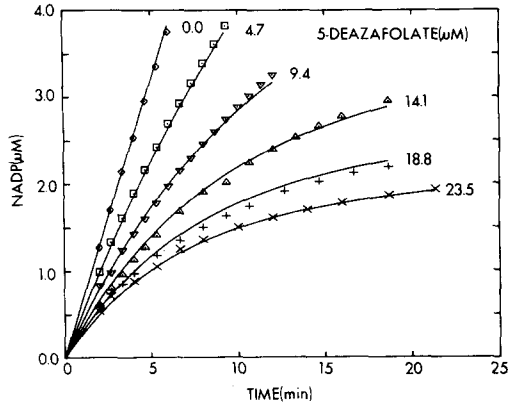



Fig. 1. Slow-binding inhibition of dihydrofolate reductase from *E. coli* by 5-deazafolate. Assays were performed and data were analyzed as outlined in Materials and Methods. The enzyme concentration was 0.72 nM. The curves represent lines of best fit to the equation which describes Mechanism B [12]. The data points represent the concentrations of NADPH formed at the times indicated. Data points collected at times of less than 2 min have not been included.

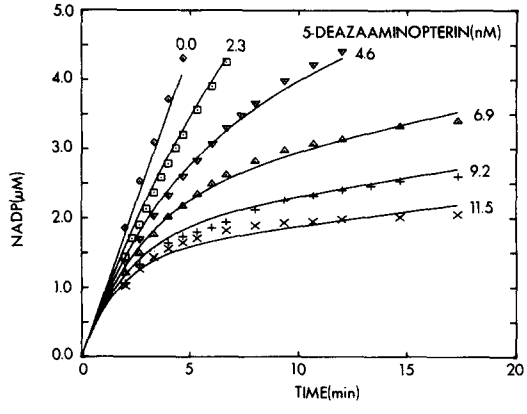



Fig. 2. Slow, tight-binding inhibition of dihydrofolate reductase from *E. coli* by 5-deazaaminopterin. Assays and data analysis were performed as outlined in Materials and Methods. The enzyme concentration was 0.73 nM. The curves represent lines of best fit to the equation which describes Mechanism A [12]. The data points represent the concentrations of NADPH formed at the times indicated. Data points collected at times of less than 2 min have not been included.

accumulation, under *in vivo* conditions, of the substrates for the inhibited enzymes. For dihydrofolate reductase, the drugs methotrexate, aminopterin and trimethoprim have been shown to be slow, tight-binding inhibitors of the enzyme from *Streptococcus faecium* [13] and the loss of potency of trimethoprim as an inhibitor of chicken-liver dihydrofolate reductase appears to be due, in part, to the loss of slow-binding characteristics (unpublished results).

Detailed studies on the inhibition of dihydrofolate reductase by folate analogues have so far been restricted to investigations with the enzyme from *S. faecium* [13]. Thus, it was of interest to determine if several of these analogues acted as slow-binding inhibitors of the enzyme from other bacteria and higher organisms. Further, with the availability of 5-deaza analogues of folate, aminopterin and methotrexate [7], the opportunity was taken to determine if the presence of the N-5 nitrogen in folate and its analogues was of importance for their interaction with the enzyme and in bringing about slow-binding inhibition.

For methotrexate the substitution of a carbon for the nitrogen at the 5-position had little effect on the behaviour of the inhibitor (Table 2). Both methotrexate and 5-deazamethotrexate caused slow, tight-binding inhibition which conforms to Mechanism B with the enzymes from *E. coli* and chicken liver. Data analysis shows that the dissociation constant for the initial EI complex ($K_i$; Scheme 1) and the overall inhibition constant ($K_i^*$) vary only slightly with the two compounds and the two enzymes (Table 2). But it is interesting to note that the 3-fold difference in the $K_i^*$ values for methotrexate and 5-deazamethotrexate was due mainly to the difference in the $k_5/k_6$ ratio. It might be mentioned that this ratio, which represents the equilibrium constant for the interconversion of EI and EI* (Scheme 1), gives a useful indication of the difference between the values for $K_i$ and $K_i^*$ as well as the degree of slow-binding inhibition. The larger the value for $k_5/k_6$, the greater is the curvature in progress curves.

Different inhibition mechanisms were exhibited by 5-deazaaminopterin and aminopterin with the enzyme from *E. coli*. Thus, 5-deazaaminopterin caused inhibition which is best described by Mechanism A, whereas the inhibition caused by aminopterin conforms to Mechanism B. In this connection it should be pointed out that Mechanism B will degenerate into Mechanism A whenever the value for the $k_5/k_6$ ratio is high. The determination of a value for $K_i^*$ requires that the inhibitor concentration be varied within the region of the $K_i^*$ value. Consequently, when the value for $K_i$ is much greater than that for $K_i^*$ because of the high $k_5/k_6$ ratio, the steady-state concentration of EI will be kinetically insignificant, and any inhibition that might be formally in accord with Mechanism B will then appear to be described by Mechanism A. The inability to make a clear distinction between two kinetic mechanisms is not peculiar to slow-binding inhibition. It can also be difficult to distinguish between non-competitive and uncompetitive inhibition when the interaction of an inhibitor with an enzyme is enhanced by the presence of a substrate on the enzyme. In addition to the theoretical aspects, there are also practical considerations to take into account when elucidating the mechanism of slow-binding inhibition. To obtain a clear distinction between Mechanisms A and B it is essential to obtain accurate data points as soon as possible after the start of the reaction. Aminopterin gave rise to only a small variation in the initial velocity of the reaction (data not shown). The conclusion that the inhibition is described by Mechanism B was dependent on the ability to obtain good data from the early stages of progress curves in the presence of the inhibitor. Analysis of two separate sets of data for 5-deazaaminopterin inhibition suggested that Mechanism A applies to the inhibition caused by this compound. Thus, under the experimental conditions, only one enzyme–inhibitor complex is present at a kinetically significant concentration. Although different inhibition mechanisms appear to apply to aminopterin and 5-deazaaminopterin with the enzyme from *E. coli*, the tightness of binding of the two compounds does not differ greatly as judged by comparison of the values for $K_i^*$ with aminopterin and $K_i$ with 5-deazaaminopterin (Table 2). With the chicken-liver enzyme, the inhibition caused by both aminopterin and 5-deazaaminopterin conformed to Mechanism A, and the parameters describing the inhibition were similar (Table 2).

The substitution of a carbon for the N-5 nitrogen of methotrexate and aminopterin had little effect on the tightness of binding of these compounds. Moreover, the 5-deaza analogues of aminopterin and methotrexate remained slow-binding inhibitors. These results are not surprising since X-ray crystallography does not indicate any interaction between the N-5 nitrogen of methotrexate and groups in bacterial enzymes [2, 3]. Similarly, if methotrexate were bound to the chicken-liver enzyme in a fashion analogous to that of phenyltriazine [4], no interaction between any enzyme group and the N-5 nitrogen of methotrexate would be expected.

In contrast to the results obtained with methotrexate and aminopterin, there was a marked difference in the inhibition caused by folate and its 5-deaza analogue. 5-Deazafolate was the more potent inhibitor of both enzymes. The difference between the two inhibitors was more pronounced with the enzyme from *E. coli* for which 5-deazafolate was about 4000-fold more inhibitory and caused slow-binding, rather than classical steady-state inhibition. There was also a marked difference in the binding of folate to the enzymes from chicken liver and *E. coli* (Table 2). Since the chicken-liver enzyme catalyzes the reduction of folate whereas the enzyme from *E. coli* does not, the question must arise as to whether folate is bound in the same way to both enzymes.

The mode of binding of folate or dihydrofolate to either enzyme has not been established by X-ray crystallography. Stereochemical considerations, however, suggest that folate and dihydrofolate are bound "upside down" relative to the way in which methotrexate is bound [2, 19, 20]. On the basis of chemical and kinetic arguments, it has been proposed that the binding and subsequent reduction of dihydrofolate involves an interaction between the N-5 nitrogen of the substrate and the $\beta$-carboxyl of

Asp-27 of the enzyme from *E. coli* (cf. Refs. 1 and 21). More recently, it has been argued from the results of model-building experiments that the N-5 nitrogen of dihydrofolate is not close to this carboxyl [3]. These experiments assume, however, that the conformation of the active site in the enzyme–substrate complex and in the enzyme–methotrexate complex is the same. Such an assumption is not necessarily valid since the slow-binding behaviour of methotrexate indicates that the enzyme–methotrexate complex undergoes an isomerization that the enzyme–substrate complex may not undergo. Moreover, if folate and 5-deazafolate are bound in the same way as that proposed for dihydrofolate in the model-building experiments, the enhanced binding of 5-deazafolate would be difficult to explain. If folate were bound with its N-5 nitrogen close to Asp-27, there would be a repulsive force between the lone pair of electrons on the N-5 nitrogen and the ionized form of the carboxyl. Substitution of carbon for nitrogen at the 5-position of folate would then eliminate the repulsion and allow 5-deazafolate to be bound more strongly than folate. The degree of repulsive force between the lone pair of electrons of the N-5 nitrogen of folate and the carboxyl group would depend on the charge associated with each group, the distance between them, and the dielectric constant. Because of the increased basicity of the N-1 and N-8 nitrogens of 5-deazafolate, it is possible that the compound is bound at the active site of dihydrofolate reductase more like methotrexate than folate. Further information on the interaction can come only from a knowledge of the three-dimensional structure of the enzyme–NADPH–deazafolate complex. The strength of binding of 5-deazafolate to the enzyme–NADPH complex may well facilitate determination of the structure of the ternary complex. In this same connection it would be of interest to determine the type of inhibition and the strength of binding to the enzyme of 8-deazafolate and the quinazoline derivative which lacks both N-5 and N-8 nitrogens. Whatever the reason for the tightness of binding of 5-deazafolate, it is, to our knowledge, the first inhibitor of dihydrofolate reductase with a nanomolar inhibition constant which does not contain a 2,4-diaminopyrimidine ring.

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