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THE INHIBITION OF DIHYDROFOLATE REDUCTASE BY FOLATE ANALOGUES: STRUCTURAL REQUIREMENTS FOR SLOW- AND TIGHT-BINDING INHIBITION

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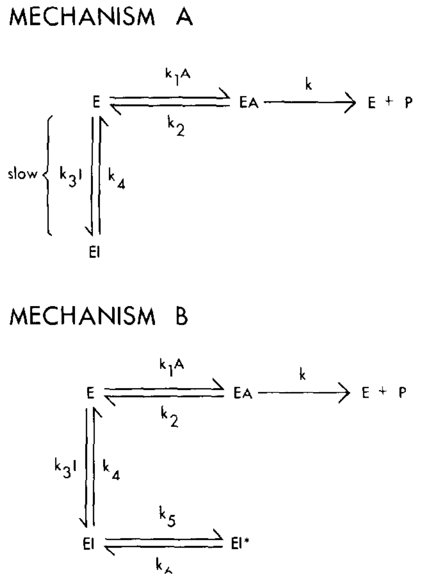
Abstract—Investigations have been made of the inhibition by 19 folate analogues of the reaction catalysed by dihydrofolate reductase from *Streptococcus faecium* A. Methotrexate, 7,8-dihydromethotrexate, aminopterin, methyl 4-amino-4-deoxy-10-methylpteroate and trimethoprim act as slow, tight-binding inhibitors, while 1-deazamethotrexate and 1-deaza-4-amino-4-deoxypteroate function as slow-binding inhibitors. Computer analysis of progress curve date indicated that the inhibition by these compounds conforms to a mechanism whereby there is an initial rapid formation of an enzyme–NADPH–inhibitor complex which subsequently undergoes a relatively slow, reversible isomerization reaction. Determinations were made of the dissociation constants for the release of each of the inhibitors from the initial ternary complex as well as of the rate constants associated with the forward and reverse isomerization reactions. The resulting values were used to calculate the overall inhibition constants for the inhibitors. 1-Deaza-2,4-diamino-6-methylpteridine and two derivatives of *p*-aminobenzoylglutamate also exhibited slow-binding inhibition. As the inhibition was parabolic, it appears that two molecules of each of these compounds combine with the enzyme–NADPH complex. Eight derivatives of 2,4-diaminopteridine and one derivative of 2,4-diaminopyrimidine yielded classical linear competitive inhibition with respect to dihydrofolate and dissociation constants were determined for their interaction with the enzyme–NADPH complex. From the type of inhibition and values for the kinetic parameters, conclusions have been drawn about the structural features of folate analogues which influence the formation of the enzyme–NADPH–inhibitor complex and its subsequent conformation change.

In the late 1950's, it was demonstrated [1] that the anti-leukaemic drugs aminopterin and methotrexate are potent inhibitors of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP oxidoreductase, EC 1.5.1.3). Since that time many compounds have been synthesized and tested as inhibitors of the enzyme [2]. These investigations have suffered from several weaknesses which include the use of partly purified enzyme preparations to determine the inhibition and the expression of the potency of an inhibitor as an I_{50} value. This value, which gives a measure of the concentration of an inhibitor required to produce 50% inhibition, does not necessarily indicate the strength of binding of an inhibitor to an enzyme. For multisubstrate reactions the relationship between I_{50} and K_i for the dissociation of the inhibitor from an enzyme–inhibitor complex is dependent on both the type of inhibition and the concentrations of fixed substrates [3, 4]. With a tight-binding inhibitor, which causes inhibition at concentrations comparable to that of the enzyme, the magnitude of the I_{50} value is also influenced by the concentration of enzyme [5]. A further weakness of the earlier work on dihydrofolate reductase has been the lack of appreciation that substrate analogues can give rise to inhibition which develops slowly. Under these circumstances, the observed inhibition will depend on the order in which the components are added to the assay [6] and the time of measurement [7]. When the reaction is started by adding enzyme, the inhibition is virtually unobservable initially, but increases as a function of time. Conversely, when the reaction is initiated by substrate after pre-incubation of enzyme and inhibitor, the pronounced initial inhibition decreases with time.

The time-dependency of inhibition has been discussed recently by Cha [8, 9], who has also developed kinetic theory for what has been termed [5] the slow-binding and slow, tight-binding inhibition of enzymes by substrate analogues. Williams *et al.* [10] have developed quantitative analyses based on this theory and have examined the interaction of methotrexate with the dihydrofolate reductase–NADPH complex. These studies revealed that the inhibition occurs in two stages: an initial rapid formation of an enzyme–NADPH–methotrexate complex and a subsequent slow isomerization of the ternary complex. It is the combination of these two steps that results in methotrexate having a very low inhibition constant of 60 pM.

The aforementioned investigations have opened the way for more definitive studies on the inhibition of dihydrofolate reductase by folate analogues, since it is now possible to determine the structural features of the inhibitors that influence their initial interaction with the enzyme–NADPH complex and their ability to induce the conformational change in the resulting ternary complex. The present report describes quantitatively the inhibition of dihydrofolate reductase by 19 folate analogues.



Scheme 1

THEORY AND DATA ANALYSIS

Cha [8] has described two basic mechanisms by which a competitive inhibitor can exhibit slow-binding characteristics (Scheme 1). In Mechanism A it is assumed that there is a slow establishment of the equilibrium between the E and EI complexes and such an assumption is a reasonable, although not an obligatory, consequence of an inhibitor being of the tight-binding type. By contrast, in Mechanism B it is postulated that there is a rapid formation of an EI complex and that the slow development of inhibition is due to a subsequent slow isomerization of the EI complex. An important difference between the two mechanisms is that the slow step of Mechanism B does not involve the binding or release of inhibitor. It follows that inhibitors do not have to be of the tight-binding type in order to cause slow-binding inhibition. Cha [8] also described a third mechanism (Mechanism C, not shown), but it will not be considered further as it is simply a variant of Mechanism A that allows free enzyme to exist as an equilibrium mixture of two different forms.

Provided that the concentration of free inhibitor is not significantly altered as a result of the formation of an enzyme–inhibitor complex, both mechanisms of Scheme 1 yield the same rate equation, viz.

$$v = v_s + (v_o - v_s)\exp(-k't), \qquad (1)$$

in which the reaction velocity (v) at any time (t) is related to the initial velocity (v_o), the steady-state velocity (v_s) and an apparent first-order rate constant (k'). This equation shows that the rate of the enzyme-catalysed reaction decays from an initial value (v_o) at $t = 0$ to a final value (v_s) as $t \rightarrow \infty$. If the reaction is initiated by the addition of enzyme, then v_o is greater than v_s, whereas the converse is true if the reaction is initiated with substrate. Cha [8] has derived expressions for the apparent rate constant, k', for the two mechanisms of Scheme 1, while Williams *et al.* [10] have described a computer program for the fitting of progress curve data to the integrated form of equation (1) which is given as equation (2):

$$P = v_s t - \frac{(v_s - v_o)}{k'}[1 - \exp(-k't)]. \qquad (2)$$

When a compound acts as a tight-binding inhibitor by causing inhibition at a concentration comparable to that of the enzyme, there will be a significant difference between the concentrations of free and total inhibitor and the rate of product (P) formation as a function of time will not be described by equation (2). In the presence of a tight-binding inhibitor, the integrated rate equation for Mechanism A is given by the more complex equation (3):

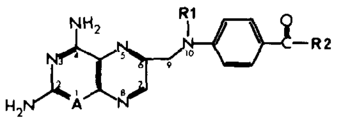
$$P = v_s t + \frac{(1-\gamma)(v_o - v_s)}{\lambda\gamma}\ln\left[\frac{1-\gamma\exp(-\lambda t)}{1-\gamma}\right], \qquad (3)$$

where λ and γ are functions of the various rate constants as well as the concentrations of substrate, inhibitor and enzyme [9]. Williams *et al.* [10] have developed a computer program for the fitting of progress curve data to this function. When allowance is made for the tight-binding inhibition of a reaction conforming to Mechanism B, it is not possible to derive an analytical expression that is analogous to equation (3). However, numerical methods can be applied to solve the equations that describe this mechanism and to analyse progress curve date [10, 11].

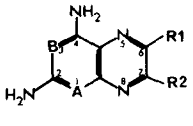
Since it has been shown previously [10] that the inhibition of dihydrofolate reductase by methotrexate can be described by Mechanism B (Scheme 1), it has been assumed that, unless there was evidence to the contrary, the same mechanism applies with all the inhibitory folate analogues. Progress curve data which conformed to Mechanism B, and which were obtained under conditions where depletion of the free inhibitor concentration was negligible, were fitted to equation (2) by using the STEPIT computer program [10]. This program was obtained from the Quantum Chemistry Program Exchange at Indiana University as part of the STEPT package (QCPE307). If, however, there was significant reduction in the concentration of free inhibitor, as with slow, tight- binding inhibitors [5], it was necessary to employ for data analysis the CRICF computer program that has been described by Chandler *et al.* [11]. Both programs yielded values for $K_i(k_4/k_3)$, k_5 and k_6. Problem dependent subroutines for both STEPIT and CRICF were written in FORTRAN in this laboratory and details may be obtained from the authors. No detailed analyses were made of data obtained with those slow-binding analogues of folate that gave inhibition which was a parabolic, rather than a linear, function of inhibitor concentration. This was due to the fact that the molecular mechanism underlying this type of inhibition is not clear. When folate analogues gave rise to steady-state, linear competitive inhibition, the data were fitted by non-linear regression analysis [12] to equation (4). All programs were run on a Univac 1100/42 computer.

$$v = \frac{VA}{K\left[1 + \frac{I}{K_i}\right] + A}. \qquad (4)$$

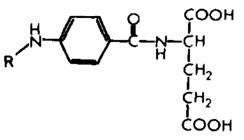
Table 1. Structures of compounds tested as inhibitors of dihydrofolate reductase



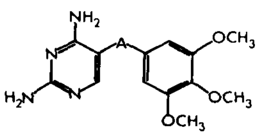
I	A=N; R1=CH_3 ; R2=glutamate
II	7,8-dihydro-I
III	A=N; R1=H; R2=glutamate
IV	A=N; R1=CH_3; R2=OCH_3
V	A=CH; R1=CH_3; R2=glutamate
VI	A=CH; R1=H; R2=OH



VII	A=N; B=N; R1=H; R2=H
VIII	A=N; B=N; R1=CH_3; R2=H
IX	A=N; B=N; R1=H; R2=CH_3
X	A=N; B=N; R1=H; R2=CH_2OH
XI	A=N; B=N; R1=CH_3; R2=CH_3
XII	A=N; B=N; R1=C_2H_5; R2=C_2H_5
XIII	A=CH; B=N; R1=CH_3; R2=H
XIV	A=N; B=CH; R1=CH_3; R2=H
XV	A=N; B=N; R1=phenyl; R2=NH_2



XVI	R=H
XVII	R=C_3H_7



XVIII	A=CH_2
XIX	A=CO

MATERIALS AND METHODS

Materials. Structures of the folate analogues that have been used in the present work are given in Table 1 and designated by Roman numerals. Folic acid was purchased from Calbiochem. Methotrexate (I) and aminopterin (III) were obtained from ICN Chemical and Radioisotope Division and further purfied by butanol extraction [13]. 7,8-Dihydrofolate and 7,8-dihydromethotrexate (II) were prepared by the reduction of folate and methotrexate, respectively, according to the method of Blakley [14]. Dihydromethotrexate migrated as a single component when subjected to thin-layer chromatography, and was used in kinetic experiments within a few hours of preparation. *N*-(*p*-aminobenzoyl)-L-glutamate (XVI,PABG) and 2,4-diamino-5(3′,4′,5′-trimethoxybenzyl) pyrimidine (XVIII, trimethoprim) were obtained from Sigma. The *N*′-propyl derivative of XVI (XVII, propyl-PABG) was prepared as described by Birdsall *et al.* [15]. 2,4-Diaminopteridine (VII), 2,4-diamino-6-methyl pteridine (VIII), 2,4-diamino-7-methyl pteridine (IX), 2,4-diamino-7-hydroxymethyl pteridine (X), 2,4-diamino-6, 7-dimethyl pteridine (XI) and 2,4-diamino-6, 7-diethyl pteridine (XII) were gifts from Dr. D. J. Brown. 1-Deaza-2,4-diamino-6-methyl pteridine (XIII), 3-deaza-2,4-diamino-6-methyl pteridine (XIV), 1-deaza methotrexate (V), 1-deaza-4-amino-4-deoxy pteroic acid (VI) and methyl 4-amino-4-deoxy-10-methylpteroate (IV) were gifts from Dr. J. A. Montgomery. 2,4-Diamino-5(3′,4′,5′-trimethoxybenzoyl) pyrimidine (XIX) and 2,4,7-triamino-6-phenyl pteridine (XV, triamterene) were received from Dr. R. L. Blakley. NADPH was supplied by P-L Biochemicals, while other chemicals were high purity preparations from commercial sources. Dihydrofolate reductase was prepared from a methotrexate-resistant mutant strain of *Streptococcus faecium* A [10].

Concentrations of methotrexate and aminopterin were determined spectrophotometrically at pH 13 using a molar extinction coefficient of 23,250 at 258 nm [16]. Since the u.v. spectrum of dihydromethotrexate is essentially identical to that of dihydrofolate [17] a molar extinction of 28,000 (282 nm, pH 7.2) for dihydrofolate [18] was used to calculate the concentration of dihydromethotrexate. The concentrations of dihydrofolate and NADPH were determined enzymically using dihydrofolate reductase and a molar extinction coefficient of 12,300 at 340 nm [19]. The enzyme concentration was determined by fluorescence titration with methotrexate [10] and concentrations of other compounds were determined by weight.

Enzyme assays. The activity of the enzyme was determined at 30° by following the decrease of NADPH and dihydrofolate by absorbance measurements at 340 nm by using a Cary 118 spectrophotometer. Experiments were performed at pH 7.4 in a buffer mixture containing 2-(*N*-morpholino)-ethane sulfonate (0.025 M), sodium acetate (0.025 M), Tris (0.05 M) and NaCl (0.1 M), as well as NADPH (90 μM) and the indicated concentration of dihydrofolate and folate analogues.

Progress curves. Progress curves were obtained as previously described [10]. The onset of inhibition

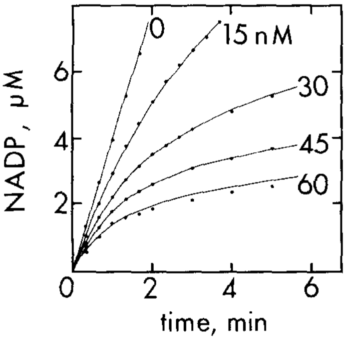


Fig. 1. Slow, tight-binding inhibition of dihydrofolate reductase by 7,8-dihydromethotrexate. Reaction mixtures contained buffer (pH 7.4), NADPH (90 μM), dihydrofolate (92 μM) and the indicated concentrations of dihydromethotrexate. Reactions were initiated by the addition of enzyme (6.0 nM) and progress curves were obtained by following the absorbance at 340 nm. Data (●) from several curves were combined and fitted, as described under Theory and Data Analysis, to the differential equations that describe Mechanism B (Scheme 1), using the CRICF program. The solid lines represent theoretical curves that were constructed using values obtained for the kinetic parameters (Table 2).

was followed by continuously monitoring the disappearance of NADPH and dihydrofolate, after initiation of the reaction with dihydrofolate reductase (6.0 nM). Experiments to measure the recovery of the activity of the enzyme after its pre-incubation with an inhibitor were performed with methotrexate (I), dihydromethotrexate (II) and IV. Dihydrofolate reductase (0.66 μM) was incubated for 10 min in the buffer mixture containing NADPH (90 μM) and inhibitor, after which an aliquot was diluted 110-fold into an assay mixture containing dihydrofolate but lacking the inhibitor. The reaction was monitored at 340 nm.

RESULTS

A variety of folate analogues has been tested as inhibitors of the dihydrofolate reductase reaction and several compounds gave rise to the time-dependent decrease in reaction rate which is characteristic of any slow-binding inhibitor. This effect is illustrated in Fig. 1 by the results obtained with dihydromethotrexate. It is apparent that both the development and extent of the inhibition are a function of the inhibitor concentration. The date of Fig. 1 were analysed by using the CRICF program and from the good fit between the data and the theoretical curves, it follows that the inhibition can be described by Mechanism B. Thus, dihydromethotrexate functions as a slow, tight-binding inhibitor of dihydrofolate reductase. Methotrexate (I), aminopterin (III), methyl 4-amino-4-deoxy-10-methylpteroate (IV) and trimethoprim (XVIII) also function as slow, tight-binding inhibitors. By contrast, 1-deazamethotrexate (V) and 1-deaza-4-amino-4-deoxy pteroate (VI) act as slow-binding inhibitors at concentrations which are much greater than that of the enzyme. All the inhibition data were analysed on the basis that the reactions conform to Mechanism B and the values for K_i, k_5 and k_6, as well as the overall inhibition constant (K_i^*), are listed in Table 2.

Slow-binding inhibition was observed with *p*-aminobenzoylglutamate (PABG, XVI) but the progress curve data could not be fitted to equation (2). The progress curves obtained over a range of inhibitor and dihydrofolate concentrations exhibited well defined steady-state rates and when these results were plotted in double reciprocal form (Fig. 2a) it appeared that PABG behaves as a competitive inhibitor with respect to dihydrofolate. However, the inhibition is not of the linear type, as a replot of the slopes of the lines of Fig. 2a against the concentration of PABG is markedly curved (Fig. 2b). A linear secondary plot was obtained when slopes were plotted against $[\text{PABG}]^2$ and such a result suggests that the inhibition by PABG is par-

Table 2. Values of the kinetic parameters for the slow-binding inhibition of dihydrofolate reductase by folate analogues

	Kinetic parameter				
Compound	K_i (nM)	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6	K_i^* (M)
Methotrexate (I)	23 ± 8	5.1 ± 1.8	0.013 ± 0.001	400	5.8×10^{-11}
Aminopterin (III)	37 ± 10	3.2 ± 1	0.012 ± 0.007	270	1.3×10^{-10}
Methyl 4-amino-4-deoxy-10-methylpteroate (IV)	33 ± 21	6.1 ± 4.3	0.049 ± 0.001	120	2.6×10^{-10}
Dihydromethotrexate (II)	11 ± 2	2.6 ± 0.5	0.080 ± 0.004	33	3.4×10^{-10}
Trimethoprim (XVIII)	4.6 ± 0.5	2.1 ± 0.4	0.58 ± 0.06	3.7	9.6×10^{-10}
1-Deaza-4-amino-4-deoxypteroate (VI)	700 ± 50	0.42 ± 0.09	0.15 ± 0.04	2.8	1.9×10^{-7}
1-Deaza-methotrexate (V)	1200 ± 80	0.36 ± 0.10	0.33 ± 0.11	1.1	5.8×10^{-7}

Values of K_i, k_5 and k_6 associated with the interaction of the slow, tight-binding inhibitors I–IV and XVIII with the dihydrofolate reductase–NADPH complex were obtained by using the CRICF program to analyse progress curve data, while those associated with interaction of the slow-binding inhibitors V and VI with the same enzyme form were determined by analysing progress curve data with the STEPIT program (cf. Theory and Data Analysis). The overall inhibition constant (K_i^*) was calculated using the relationship: $K_i^* = K_ik_6/(k_5 + k_6)$. Structures of the folate analogues are given in Table 1.

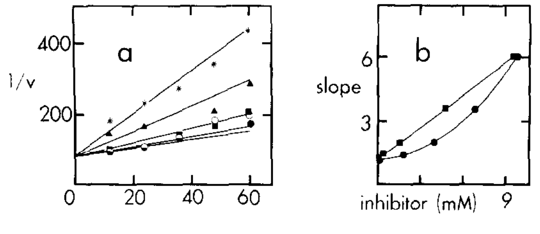


Fig. 2. Parabolic inhibition of dihydrofolate reductase by *p*-aminobenzoylyglutamate (PABG, XVI). Reaction mixtures contained buffer (pH 7.4), NADPH (90 μM), variable concentrations of dihydrofolate and the following concentrations of PABG: ●, none; ○, 1.96 mM; ■, 3.92 mM; ▲, 6.86 mM; ✱, 9.8 mM. Reactions were initiated by the addition of enzyme (6.0 nM), and after incubation of reaction mixtures for several minutes velocities determined from the steady-state regions of the progress curves. The velocity data for each PABG concentration were fitted separately to the Michaelis–Menten equation and a weighted mean value of the maximum velocity (V) was calculated from the five estimates. The combined data of panel (a) were fitted to equation (5) with I replaced by $[\text{PABG}]^2$ and with V held constant. The values of V, K_a and K_i were used to draw the lines of the figure. In panel (b), the slope of the lines of panel (a) is plotted as a function of [PABG] (●) or $[\text{PABG}]^2$ (■).

abolic competitive. Experiments were also performed by varying the concentrations of PABG and dihydrofolate in the presence of a fixed concentration (45 μM) of 2,4-diaminopteridine (VII) which acts as a linear competitive inhibitor with respect to dihydrofolate. Under these conditions the curvature of a secondary plot of slopes against PABG concentration was considerably reduced, but not eliminated. Thus it was not possible to analyse quantitatively the slow-binding inhibition by PABG. Similar slow-binding, parabolic inhibition was found with propyl-PABG (XVII) and 1-deaza-2,4-diamino-6-methyl pteridine (XIII).

A number of folate analogues did not exhibit slow-binding behaviour, but rather acted as classical linear, competitive inhibitors in relation to dihydrofolate. These compounds, together with their K_i values, are listed in Table 3.

Table 3. K_i values for folate analogues that act as linear competitive inhibitors of dihydrofolate reductase

Compound	K_i (μM)
7-hydroxymethyl-DAP (X)	11 ± 1
DAP (VII)	5.2 ± 0.5
7-methyl-DAP (IX)	1.2 ± 0.1
6,7-dimethyl-DAP (XI)	0.55 ± 0.03
3-deaza-6-methyl-DAP (XIV)	0.53 ± 0.04
6-methyl-DAP (VIII)	0.38 ± 0.04
6-phenyl-7-amino-DAP (XV)	0.084 ± 0.006
5-(3′,4′,5′-trimethoxybenzoyl)-2,4-diaminopyrimidine (XIX)	0.053 ± 0.005
6,7-diethyl-DAP (XII)	0.0053 ± 0.0004

K_i values represent dissociation constants for the interaction of folate analogues with the enzyme–NADPH complex. DAP, 2,4-diaminopteridine.

DISCUSSION

It has been established [10] that the potent inhibition of dihydrofolate reductase by methotrexate occurs as a consequence of a two-stage reaction which involves the rapid formation of an enzyme–NADPH–methotrexate complex and its subsequent slow conversion to a form that could approximate to a transition-state complex. Procedures have been described for determining the dissociation constant for the release of methotrexate from the initial ternary complex as well as the rate constants for the forward and reverse isomerization reactions. These same techniques, in addition to classical procedures, have now been applied to investigations of the inhibition of dihydrofolate reductase from *S.faecium* A by a range of folate analogues. The basic aims of the study were: (a) to identify the folate derivatives which inhibit the enzyme according to Mechanism B of Scheme 1 and those which function as classical competitive inhibitors with respect to dihydrofolate, and (b) to identify the structural features of the inhibitors which are required for their strong, rapid interaction with the enzyme–NADPH complex and for induction of the conformational change in the enzyme–NADPH–inhibitor complex. Although the present work has been restricted to studies on dihydrofolate reductase from one source, the procedures are general and can be applied to other dihydrofolate reductases or to any enzyme whose inhibition by substrate analogues can be described by Mechanism B.

The seven compounds listed in Table 2 exhibited slow-binding inhibition while only five of them function as tight-binding inhibitors. Thus tight-binding inhibition is not a pre-requisite for slow-binding inhibition. The five tight-binding inhibitors showed only a 6-fold difference in their overall inhibition constant (K_i^*) values, with methotrexate (I) being the strongest and trimethoprim (XVIII) being the weakest inhibitors of this group. The two slow-binding inhibitors, 1-deazamethotrexate (V) and 1-deaza-4-amino-4-deoxypteroate (VI) were relatively weak inhibitors with K_i^* values some 3–4 orders of magnitude higher than that for methotrexate (I). The higher K_i^* values are due largely to the weak initial binding of V and VI to the enzyme–NADPH complex, but the low values of the k_5/k_6 ratios also contribute. The K_i and K_i^* values for methotrexate (I) are considerably lower than those for 1-deazamethotrexate (V). These results confirm the importance of the N(1) nitrogen in the binding of folate analogues to dihydrofolate reductase [20–23].

The glutamate moiety of folate analogues is not essential for an enzyme–NADPH–inhibitor complex to undergo a conformational change as methyl 4-amino-4-deoxy-10-methylpteroate (IV), 1-deaza-4-amino-4-deoxypteroate (VI) and trimethoprim (XVIII), which do not possess a glutamate moiety, are capable of inducing the change (Table 2). Williams *et al.* [10] have proposed that the isomerization reaction which gives rise to EI* is analogous to the formation of the transition-state in catalysis. The finding that the glutamyl moiety is not necessary for this isomerization is consistent with this notion, as it is known that compounds such as dihydropteroate which also lack this moiety, are good substrates of dihydrofolate reductase [24]. There are only relatively small differences in the magnitude of the k_5 values for the five slow-binding inhibitors which contain a nitrogen atom at the 1 position of the pteridine ring (Table 2). However, the k_5 values for the two 1-deaza compounds, *viz.* V and VI, are some 10-fold lower. This suggests that N(1) is involved in the slow transition from EI to EI*, but is not solely responsible for initiating the isomerization. By contrast with the relative constancy of the k_5 values for slow-binding inhibitors, the values for k_6 vary as much as 50-fold. It can be considered that the magnitude of k_6 represents a measure of the degree to which the folate analogues are locked on the enzyme. Therefore, it is of interest that the k_6 value for trimethoprim (XVIII) is very much higher than those for the other slow, tight-binding inhibitors and more comparable to those for the slow-binding inhibitors (Table 2).

A more useful parameter to consider in connection with the relative stability of EI and EI* complexes (Mechanism B, Scheme 1) is the k_5/k_6 ratio which denotes the equilibrium constant for the distribution of enzyme between the two enzyme–inhibitor complexes. The larger is the ratio, the more stable is the EI* complex. The k_5/k_6 ratios (Table 2) indicate that methotrexate (I) and aminopterin (III) form the most stable EI* complexes, while the complex formed with trimethoprim (XVIII) is considerably less stable. These results suggest that the EI* complex formed by trimethoprim does not approximate the transition-state as well as does the complex formed by methotrexate.

None of the folate analogues listed in Table 3 exhibited a transient phase in their inhibition of dihydrofolate reductase and all behaved as linear, competitive inhibitors with respect to dihydrofolate. However, their ability to combine with the enzyme–NADPH complex varied by about 1000-fold. 2,4-Diaminopteridine (DAP, VII) is a relatively weak inhibitor. The inhibition is decreased by the introduction of a hydroxymethyl group at the 7-position (X), but increased by the addition of a methyl group at the same position (IX) or better still at the 6-position (VIII). The effectiveness of the 6,7 dimethyl-DAP(XI) is intermediate between the 6-methyl and 7-methyl derivatives. Since 3-deaza-6-methyl-DAP(XIV) and 6-methyl-DAP (VIII) have similar K_i values, it may be concluded that the N(3) nitrogen has little effect on the binding of the pteridine ring. There is a marked increase in the inhibition on attachment of larger hydrophobic groups to the 2,4-diamino pteridine or to the 2,4-diaminopyrimidine nucleus [cf. 25]. 6,7-Diethyl-DAP (XII) proved to be the most potent competitive inhibitor of the compounds listed in Table 3 with a K_i value of 5.3 nM. The result illustrates that slow-binding is not a necessary consequence of tight-binding, in contrast to the proposal of Cha [8].

Trimethoprim (XVIII) and its derivative (XIX) are structurally related, but one (XVIII) acts as a slow, tight-binding inhibitor while the other acts as a classical competitive inhibitor (Tables 2 and 3). This finding raises the important question of whether or not the EI complex formed by a classical competitive inhibitor undergoes a conformational change to yield an EI* complex. An answer to this question is not available as it would require the application of rapid reaction kinetic techniques to studies on the inhibition of the enzyme.

Since the dihydrofolate (and methotrexate) binding site of dihydrofolate reductase consists of pteridine and *p*-aminobenzoate sub-sites [26], it was of interest to determine whether analogues possessing only the pteridine or the *p*-aminobenzoate moieties were capable of giving rise to slow-binding inhibition. Three such compounds were identified, *viz.* XIII, XVI, XVII, but interpretation of the results was not straightforward as each of the compounds caused parabolic competitive inhibition (cf. Fig. 2). The results suggest that two molecules of each of these inhibitors are bound per active site and are in agreement with the findings of NMR studies which indicate that XVI binds to two sites on the enzyme from *Lactobacillus casei* [27]. It is perhaps significant that the three parabolic competitive inhibitors were very poor inhibitors so that the observation of parabolic effects may be a direct consequence of the high concentrations that are necessary to cause any inhibition. The data do not permit conclusions to be drawn as to whether one or both sub-sites must be occupied for induction of the slow conformational change.

It was hoped that the present investigations would yield sufficient detailed information about the properties of inhibitory folate analogues so as to permit a more rational approach to the design of inhibitors with a high affinity for dihydrofolate reductase. This aim has been partly achieved in that information has been gained about the structural features that are of importance for binding and isomerization. The results indicate that while methotrexate (I) possesses the lowest overall inhibition constant (K_i*), it does not form the most stable EI complex. Both trimethoprim (XVIII, Table 2) and 6,7-diethyl-2.4-diaminopteridine (XII, Table 3) have 4- to 5-fold lower K_i values than does methotrexate. On the other hand, methotrexate has a k_5 value as high, and a k_6 value as low, as any of the folate analogues that have been tested. If the value for k_6 of 0.013 min^{-1} as obtained with methotrexate (Table 2) represents a lower limiting value for the conversion of EI* to EI, then any enhancement in the inhibitory effect of a folate analogue must come through an increase in the value for k_5. This may be possible if the conversion of EI to EI* is analogous to, but 100 times slower than, the formation in catalysis of a transition-state complex which occurs with a net rate constant of at least 640 min^{-1}. However, it remains to be determined what features of dihydrofolate are responsible for the higher rate and how they may be incorporated into a methotrexate-like molecule so as to produce a more potent inhibitor whose initial binding to the enzyme–NADPH complex is not reduced.

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