ANALYTICAL BIOCHEMISTRY 200, 68–73 (1992)

Mixtures of Tight-Binding Enzyme Inhibitors. Kinetic Analysis by a Recursive Rate Equation

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Received July 8, 1991

When two or more tight-binding inhibitors are present in an enzyme assay, the equation that relates the initial velocity v to the concentration of reactants cannot be written in an algebraically explicit form. Rather, for n inhibitors it is an implicit polynomial equation of degree $n + 1$ with respect to v. The complexity of the polynomial coefficients dramatically increases with each added inhibitor. Solving the transcendental rate equation by traditional methods of numerical mathematics has proven tedious because of the sensitivity of these methods to initial estimates and because of the existence of multiple roots. However, the equation can be rearranged into a convenient *recursive* form, one in which the velocity appears on both sides and the solution is found iteratively. The algebraic form of the recursive rate equation is remarkably simple and differs from the rate equation for classical rather than tight-binding inhibition only by an added term. The numerical stability and the speed of convergence were tested on the case of two competitive inhibitors. Initial estimates of velocity that spanned 12 orders of magnitude converged within five iterations. The velocities computed with the recursive method for a single tight-binding inhibitor were identical with the values predicted by the Morrison equation. The method is used to analyze experimental data for the inhibition of rat liver dihydrofolate reductase by mixtures of the anticancer drug methotrexate and its metabolic precursor form, methotrexate-α-aspartate (a prodrug).

It is often desirable to analyze the kinetic behavior of substances that are either suspected or known to be mixtures of several tight-binding enzyme inhibitors. For example, in order to optimize the synthetic effort in the design of an inhibitory drug, a racemic rather than optically pure compound may be used in preliminary screening. In other circumstances, a metabolic conversion of a prodrug—a tight-binding inhibitor itself—to its superactive form may be studied. While the kinetic theory for a single tight-binding inhibitor has been well established, most notably in the works of Morrison (1), Henderson (2), and Cha (3), an extension to the area of multiple inhibitors poses some fundamental challenges. In this paper we propose a nontraditional algebraic form of the catalytic rate equation, one in which the velocity appears on both sides, as in Eq. [1]. We show that this form of the rate equation represents a convenient tool for the formal analysis of multiple tight-binding inhibition.

$$^{i+1}v = f(^{i}v, [S]_0, [E]_0, [I_1]_0, [I_2]_0, \ldots, [I_n]_0, K_m, k_{cat}, K_{i1}, K_{i2}, \ldots, K_{in}) \quad [1]$$

For an arbitrary set of total analytic concentrations, indicated in Eq. [1] by square brackets with lower index zero, and for the corresponding kinetic constants, the reaction velocity is calculated recursively. An ith estimate ^{i}v is effectively treated as one of the independent variables and is used to calculate the $(i + 1)$th estimate ^{i+1}v as an improved value; that value is in turn considered as a starting point in the next round of the calculation. The recursion is terminated when the difference between two successive values of the reaction velocity decreases below a predetermined convergence criterium (e.g., 0.1% relative change). The most important advantage of using the recursive form of the rate equation is that it retains remarkable algebraic simplicity for an arbitrary number of inhibitors present in the mixture and for arbitrary kinetic mechanism of inhibition (competitive, uncompetitive, mixed-type). The recursive formula is computationally very stable. In contrast, the traditional analysis of n tight-binding inhibitors yields a rate equation which contains an $(n + 1)$th degree poly-

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0003-2697/92 $3.00

nomial with respect to velocity. Consequently the only case that can be treated analytically is represented by the single tight-binding inhibitor, which gives rise to a quadratic equation. Higher polynomial equations can only be solved numerically, but the solution is sensitive to the choice of initial estimates and the convergence is often poor.

THE RECURSIVE RATE EQUATION

In the absence of inhibitors, the enzymatic rate law that predicts the dependence of reaction velocity on the concentration of the enzyme, the substrate, and the products can be written in the form of Eq. [2]. Given a particular kinetic mechanism, the numerator N and the denominator D in Eq. [2] can be derived by using any of the three currently established methods: the net-rate constant method of Cleland (4), the intuitive graphic method of King and Altman (5), or most recently the elegant method of Chou (6). In the presence of n inhibitors, the reaction velocity follows Eq. [3], in which N_{ij} is the numerator in the distribution equation for jth inhibitor in the ith enzyme form whose total number is m, and K_{ij} is the associated dissociation constant (for the terminology used here see the papers of Cleland (7) and Morrison (1)). Under practical conditions, when the affinity of an inhibitor towards the enzyme is relatively low, the uncomplexed form $[I_j]$ is present in very large excess compared to the enzyme bound forms. In this case the concentration of the bound forms $\Sigma_i[E_{ij}I_j]$ in the mass balance Eq. [4] can be neglected, $[I_j]$ in Eq. [6] can be replaced with $[I_j]_0$, and the derivation of the rate equation for the "classical" inhibition is complete. In the case of the tight-binding inhibition, however, the affinity of the inhibitor toward the enzyme is so high that there is a significant mole fraction of the inhibitor bound in the enzyme–inhibitor complex.

$$v_0 = [E]_0 N/D \qquad [2]$$

$$v = [E]_0 N/(D + \Sigma_j[I_j]\Sigma_i N_{ij}/K_{ij}) \quad j = 1, 2, \ldots, n \qquad [3]$$

$$[I_j] = [I_j]_0 - \Sigma_i[E_{ij}I_j] \quad i = 1, 2, \ldots, m \qquad [4]$$

Tight-binding inhibition of enzymes was first comprehensively analyzed by Morrison (1). In the original treatment of a single tight-binding inhibitor ($j = 1$), rate equations [3] and [5] were combined with the mass balance equation [4] and the equilibrium equation [6]. In this manner the unknown concentrations of the free enzyme $[E]$, the free inhibitor $[I]$, and the enzyme–inhibitor complex $[EI]$ were eliminated. The resulting rate equation is quadratic with respect to velocity so that it can be solved analytically to give Eq. [9] (see below). Adopting a similar approach in the case of multiple inhibitors ($j = 1, 2, \ldots, n$), from [3] we obtain the rate equation [7]. Further algebraic transformations provide different results for different numbers of tight-binding inhibitors. Thus, for one inhibitor the rearranged formula [7] yields a second-degree polynomial equation as indicated above (1). For two tight-binding inhibitors a third-degree polynomial in velocity is obtained, and so forth. The fundamental difference between the case of a single inhibitor and multiple inhibitors lies in that quadratic equations have an analytic solution, while higher order polynomial equations do not. Therefore, an explicit version of the Morrison equation for two or more inhibitors cannot be obtained. Instead, these equations can only be solved by the application of numerical analysis.

$$[E] = v/N \qquad [5]$$

$$[E_{ij}I_j] = [E][I_j]\Sigma_i N_{ij}/K_{ij} \qquad [6]$$

$$v = [E]_0 \frac{N}{D + \Sigma_j[I_j]_0 \dfrac{\Sigma_i N_{ij}/K_{ij}}{1 + (v/N)\Sigma_i N_{ij}/K_{ij}}} \qquad [7]$$

An obvious possibility to arrive at the numerical solution would be to rearrange [7] and to solve the resulting polynomial by a standard method for equations of one variable (e.g., the bisection method, the Newton–Raphson method, or the method regula falsi). This approach has two significant disadvantages. First, there is the problem of multiple roots. For example, in the case of two inhibitors present in the mixture, the corresponding cubic rate equation has three possible roots, and special precautions need to be taken to avoid the unwanted ones. Second, the algebraic complexity of the polynomial coefficients dramatically increases with each additional tight-binding inhibitor and this problem is compounded in numerical methods which use differential terms (as does for example the Newton–Raphson method). Moreover, in preliminary stages of this work we found that the convergence properties of the Newton–Raphson algorithm in solving the polynomial equations vary greatly, depending on the number of inhibitors, on the kinetic mechanism, and on the choice of the initial estimates.

An alternative approach adopted here preserves the form of the rate equation as indicated in [7], with the initial velocity v appearing on both sides. This particular algebraic form represents a suitable recursive computational formula. An initial estimate of v is used to evaluate the expression on the right-hand side of [7], and the new value is compared with the original one. When the ith and the $(i + 1)$th values of velocity become reasonably close, the calculation is terminated. An important feature of these recursive calculations is their numerical stability as measured by the speed of convergence and by the sensitivity to the initial estimates. The numerical stability of formula [7] was tested on a hypo-

thetical unimolecular enzymatic reaction (*uni-uni* in Cleland's nomenclature (7)) inhibited by the presence of two competitive tight-binding inhibitors. The concentration of the product at the beginning of the reaction was assumed equal to zero, so that the numerator and the denominator of the rate equation can be written as $N = k_{cat}[S]_0/K_m$, $D = 1 + [S]_0/K_m$, where k_{cat} and K_m are the turnover number and the Michaelis constant of the substrate. The two inhibitors have inhibition constants K_{i1} and K_{i2}, and for competitive inhibition both N_1 and N_2 are equal to unity. Under these circumstances Eq. [7] has the form of [8]. It is noteworthy that [8] bears remarkable similarity to the expression for a mixture of two classical competitive inhibitors, the only difference being in the additional term A in the denominator that also contains the velocity.

$$v = [E]_0 \times \frac{k_{cat}[S]_0/K_m}{1 + [S]_0/K_m + [I_1]_0/(K_{i1} + A) + [I_2]_0/(K_{i2} + A)}, \quad \text{where } A = vK_m/k_{cat}[S]_0. \quad [8]$$

The results of a series of test calculations are summarized in Table 1. The proposed computational method shows a remarkable speed of convergence, as well as very little sensitivity to the initial estimates. Values of the initial estimate ^0v, at the top of each column in Table 1, that span 12 orders of magnitude were found acceptable. In contrast, solving the cubic equation obtained from [8] by the Newton–Raphson method required computation time that was four to five times higher depending on the initial estimate (data not shown). The Newton–Raphson method was also found much less robust than the recursive method, and divergence in the calculation often occurred.

TABLE 1

Calculation of the Initial Reaction Velocity for a Mixture of Two Competitive Tight Binding Enzyme Inhibitors by Using the Recursive Formula of Eq. [8]

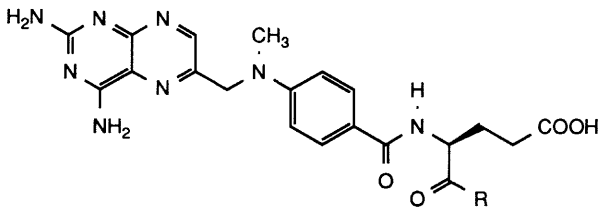
$K_{i2} = \infty$		$K_{i2} = 0.1$ nM					
i[a]	^iv[b]	i	^iv	i	^iv	i	^iv
0	1.000	0	10^6	0	1.000	0	10^{-6}
1	8.699	1	9.091	1	6.234	1	6.061
2	8.726	2	7.104	2	6.873	2	6.857
3	8.726[c]	3	6.950	3	6.929	3	6.928
		4	6.936	4	6.934	4	6.935
		5	6.935	5	6.935		

Note. Parameters: $[S]_0 = 100\ \mu$M, $K_m = 10\ \mu$M, $k_{cat} = 10\ s^{-1}$, $[E]_0 = 1$ nM, $[I_1]_0 = 0.5$ nM, $[I_2]_0 = 0.5$ nM, $K_{i1} = 1$ nM.

[a] Iteration number.

[b] Velocity (nM/s).

[c] Correct value is 8.726 nM/s as calculated by using the Morrison's equation (Ref. (1)).



SCHEME 1

APPLICATION TO DIHYDROFOLATE REDUCTASE

Methotrexate (N^{α}-[4-[[(2,4-diamino-6-pteridinyl) methyl]methylamino]benzoyl]-glutamic acid, MTX,[2] 1) is one of the most important anticancer drugs currently used in the clinical practice. It is a specific inhibitor of dihydrofolate reductase, an enzyme that plays a crucial role in the division of cancer cells. As part of a study designed to investigate the metabolism of methotrexate-α-aspartate (MTX-α-Asp, 2, Scheme 1), a potential prodrug form of methotrexate, we wished to follow the hydrolytic conversion of methotrexate-α-aspartate to methotrexate in a culture of cancer cells by using an enzyme kinetic assay. Aliquots of the mixture of both inhibitors could be rapidly assayed for the degree of metabolic conversion by using added dihydrofolate reductase, if a standard curve could be obtained that interrelates the observed initial velocity and the composition of the mixture. In an ideal case, the prodrug form would not at all inhibit the enzyme, and the liberated drug would titrate the enzyme stoichiometrically, having an infinitely small inhibition constant. In this ideal case, at the initial prodrug concentration equal to the enzyme concentration, the standard curve would be represented by a straight line connecting 100% velocity at 0% metabolic conversion and 0% velocity at 100% conversion, relative to a blank rate measured in the absence of inhibitors. That is, the pure prodrug would have no effect, at 50% metabolic conversion the velocity would decrease by one-half, and exactly at the total conversion to the active drug the enzyme would be completely inhibited. In reality, however, the prodrug may already produce nonnegligible inhibition of the en-

[2] Abbreviations used: MTX, methotrexate; MTX-α-Asp, methotrexate-α-aspartate; DHF, dihydrofolic acid.

zyme at a concentration where the completely liberated drug still leaves a residual enzymatic activity. Moreover, there is no reason a priori to expect that the decrease in the observed residual velocity should be directly proportional to the metabolic conversion. And what further complicates the metabolic model system is that the concentration of the enzyme also influences the geometry of dose–response curves (1). A rather laborious approach to the design of the metabolic experiment would rely on measuring the enzymatic velocities at varied drug/prodrug ratios for several concentrations of the enzyme and both inhibitors. From these experimental standard curves, one would select the total concentration of inhibitors that provides optimum sensitivity ("steepness") in the expected metabolic range. The method proposed here obviates the necessity to establish the standard curves experimentally. Instead, it allows the construction of such curves from the inhibition constants of pure components by using Eq. [8].

The kinetic mechanism for dihydrofolate reductase involves two substrates and two products, a *bi-bi* mechanism according to the nomenclature introduced by Cleland (7). However, when the enzyme is preequilibrated with a large excess of NADPH as was the case in this study, the kinetic mechanism effectively simplifies to the *uni-uni* type. Both methotrexate and methotrexate-α-aspartate were first examined separately in order to determine their inhibition constants and the kinetic mechanism. For both compounds, several series of assays were conducted at concentrations of both substrates constant in each series (NADPH 150 μM; dihydrofolate between 10 and 100 μM). The concentration of inhibitors varied between zero and a maximum value that produced approximately 90% inhibition. The initial reaction velocities were determined by linear regression of time vs absorbance data within the first 50% conversion of the substrate. This simple analysis was permissible because the dihydrofolate concentrations used (10 to 100 μM) were very much higher than the corresponding Michaelis constant (0.11 μM, as determined by Jarabak and Bachur (8)) so that the enzyme remained highly saturated, and thus the velocity remained constant, up to rather high degrees of substrate conversion. The statistical analysis of the initial velocities was performed by nonlinear least-squares fit (9) to the corresponding theoretical model, the Morrison equation [9]. Wang and Werkheiser (10) previously determined that methotrexate is a purely competitive inhibitor of dihydrofolate from rat liver. Therefore, in the nonlinear least-squares fit of methotrexate data the apparent inhibition constant K_i' in Eq. [9] was assumed equal to $K_i\,(1 + [S]_0/K_m)$, where K_i is the true inhibition constant (3). The optimal value of K_i was 3.6 ± 1.2 pM, in good agreement with the published value of 4.3 pM (8). On the other hand, the kinetic mechanism of inhibition for MTX-α-Asp was not previously known.

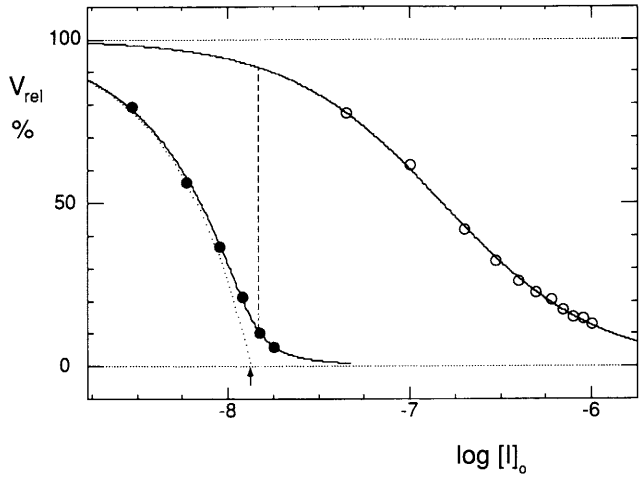


FIG. 1. Inhibition of rat liver dihydrofolate reductase (4.0 mU/ml, 13.5 nM) by methotrexate (solid circles) and methotrexate-α-aspartate (open circles) at pH 7.4. Both datasets were fitted to Eq. [9] by using the Marquardt nonlinear least-squares method (9). Constant parameters: $[S]_0$ 100 μM, K_m 0.11 μM, $[E]_0$ 13.5 nM. Optimized parameters: Methotrexate K_i 3.6 ± 1.2 pM (k_{cat} 15.3 ± 0.5 s^{-1}); methotrexate-α-aspartate K_i 1.57 ± 0.22 nM (k_{cat} 15.7 ± 0.2 s^{-1}). The dotted line (Top) corresponds to a hypothetical irreversible inhibitor ($K_i = 0$). For explanation of the broken line at 18.8 nM see text.

Therefore we used a more general formula for the apparent inhibition constant, one that corresponded to a mixed-type (noncompetitive) inhibition mechanism; the exact algebraic form for the mixed-type tight-binding inhibition constant can be found in the literature (3). Both the competitive component and the uncompetitive component of the overall inhibition constant were allowed to vary during the nonlinear least-squares fit, but only the competitive component was sufficiently defined by the experimental data (K_{is} 1.57 ± 0.22 nM). Any arbitrary value of K_{ii} higher than approximately 1 μM was acceptable, but the associated error (11) was at least 100%. From these results we conclude that MTX-α-Asp is a purely competitive inhibitor of dihydrofolate reductase from rat liver (pH 7.4).

$$v = \frac{k_{cat}[E]_0}{2([S]_0 + K_m)}\left([E]_0 - [I]_0 - K_i' + \sqrt{([E]_0 - [I]_0 - K_i')^2 + 4[E]_0K_i'}\right) \quad [9]$$

Two representative sets of data obtained in the kinetic studies of the pure drug and the pure prodrug are shown in Fig. 1. In order to accommodate large differences in the effective concentrations between the two compounds, the data are shown in logarithmic coordinates. The curves drawn through the two sets of data points represent the best nonlinear least-squares fit to Eq. [9]. The dotted curve corresponds to a hypothetical inhibitor with infinitely small inhibition constant that

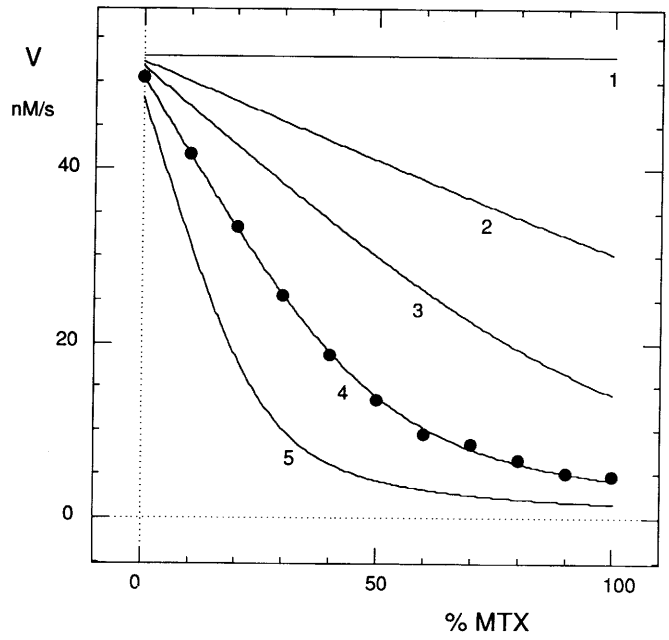


FIG. 2. Inhibition of rat liver dihydrofolate reductase by mixtures of methotrexate and methotrexate-α-aspartate. The curves were simulated by using Eq. [8] with the following parameters: $[S]_0$ 100 μM, K_m 0.11 μM, k_{cat} 15.5 s^{-1}, K_{i1} 1.57 nM (MTX-α-Asp), K_{i2} 3.6 pM (MTX), the sum of $[I_1]_0$ plus $[I_2]_0$ 0 nM (curve 1), 1.75 nM (curve 2), 3.5 nM (curve 3), 7.0 nM (curve 4), and 14.0 nM (curve 5). Experimentally determined velocities (7.0 nM total inhibitors) are indicated by solid circles.

would stoichiometrically titrate the enzyme whose concentration is indicated by the arrow on the concentration axis. In fact, at pH 6.0 methotrexate behaves very close to a stoichiometric titrant (8), and that property was utilized in determining the active site concentration. At pH 7.4, however, methotrexate does not completely inhibit the enzyme at the point of molar equivalence (13.5 nM). The broken line in Fig. 1 indicates that at approximately 20 nM methotrexate there still remains about 10% of enzymatic activity relative to the blank measured in the absence of inhibitors. The same line also indicates that an identical concentration of the prodrug will already produce about 10% inhibition. Thus, at 13.5 nM enzyme and at 20 nM total drug and prodrug, we observe about 10% nonideality both at the beginning and at the end of the hypothetical metabolic experiment; the pure prodrug is already inhibitory at the outset, and the fully liberated drug is not a stoichiometric titrant. In order to predict the inhibitory potency of various binary mixtures of MTX and MTX-α-Asp along the metabolic pathway, we need to use the recursive rate equation [8].

The curves in Fig. 2 represent simulated velocities obtained by evaluating the recursive rate equation [8] at various total drug and prodrug concentrations (0 to 14 nM) and at a constant enzyme concentration (3.5 nM). These are the desired standard curves which, arranged into a nomogram, could be used in the determination of the metabolic conversion degree from the observed residual enzymatic velocity. With the exception of curve 1 (1.75 nM total inhibitors), all calculated standard curves in Fig. 2 are markedly nonlinear. The shape of these simulated curves can be used to design the actual metabolic experiment, given a particular expected conversion degree. For example if the maximum expected conversion of the prodrug was 20%, the optimum inhibitor concentration would be about 14 nM (curve 5 in Fig. 2) because it shows maximum steepness (sensitivity) and covers the entire expected range. On the other hand, this inhibitor concentration would not be optimal if the expected maximum metabolic conversion was 50% because in that region curve 5 in Fig. 2 is rather flat (low sensitivity). In order to verify the predictions of the theory, the inhibitory activity of various mixtures of methotrexate and methotrexate-α-aspartate was measured experimentally at a constant total concentration of both components (7 nM). The experimental results, as indicated by the solid circles in Fig. 2, are in good agreement with the theoretical predictions (curve 4). In a study of methotrexate-α-aspartate metabolism, the conversion degree can be in principle determined from a single accurate measurement of the residual enzymatic activity by using a standard curve such as those depicted in Fig. 2.

EXPERIMENTAL

Rat liver dihydrofolate reductase (EC 1.5.1.3.) was obtained from Sigma Chemical Co. (St. Louis, MO) as a solution in 50% glycerol containing 0.7 M ammonium sulfate and 0.05 M potassium phosphate (pH 6.5). Methotrexate-α-aspartate was a generous gift from Dr. J. R. Piper (Southern Research Institute). Methotrexate was purchased from Sigma. Freshly reduced dihydrofolic acid (DHF) was prepared by using the method of Blakley (12).

Active site titration with methotrexate (pH 6.0). In determining the active site concentration of the enzyme (typically about 10 nM total protein in each assay), we used earlier reports of Jarabak and Bachur (8) that methotrexate at pH 6.0 is effectively an irreversible deactivator of rat liver dihydrofolate reductase. A stock solution of the enzyme (10 mU/ml) and NADPH (1.50 mM) was prepared in the assay buffer (100 mM sodium phosphate containing sodium chloride to give osmolality 290 mmol/kg, pH 6.0). The enzyme/NADPH solution (0.10 ml, final concentrations 1 mU/ml enzyme, 150 μM NADPH) was added to a spectrophotometric cuvette containing a varied amount of methotrexate (final concentration between 0 and 20 nM) dissolved in the assay buffer (0.88 ml). The solution was mixed, and after 10 min of preincubation at 37°C, the reaction was

started by the addition of 1 mM dihydrofolic acid (0.02 ml) in the buffer (final concentration 20 μM). Absorbance data were collected over 10 min by using a computer-interfaced spectrophotometer Cary-14 and commerical data-acquisition software (OLIS, On Line Instrument Systems Inc., Jefferson, GA). Reaction velocities were obtained by linear least-squares regression of an initial segment of each progress curve at less than 50% conversion. No deviations from linearity were observed within the indicated range (a typical correlation coefficient r^2 was 0.99). The background air oxidation rates, obtained under identical conditions except for the absence of the enzyme, were subtracted from the rate data prior to further statistical analysis. The active site concentration was determined by nonlinear least-squares optimization of $[E]_0$ and k_{cat} in Eq. [9] by using the Marquardt algorithm (9); the fixed parameters were the Michaelis constant for dihydrofolate (K_m 0.11 μM, Ref. (8)) and the arbitrarily chosen inhibition constant (K_i 0.3 pM). The error of optimized parameters was estimated from the final curvature matrix by using standard methods (11). Inhibition constants in the range 0.05 to 0.5 pM satisfactorily described the almost stoichiometric inactivation of the enzyme. The results of the active site titration for different batches of the rat liver dihydrofolate reductase over a period of 1 year varied within 3.25 ± 0.13 and 3.45 ± 0.11 nM per mU/ml.

Determination of inhibition constants (pH 7.4) and assays of inhibitor mixtures. The enzyme was assayed at pH 7.4 as described above in the presence of varied amounts of MTX (final concentration 0–30 nM) or MTX-α-Asp (final concentration 0–1 μM) in the preincubation mixture. Inhibition constants were obtained by nonlinear least-squares fit of the initial rate data to equation [9] by using the Marquardt algorithm (9). The varied parameters were K_i (MTX 3.6 ± 1.2 pM, MTX-α-Asp 1.57 ± 0.22 nM) and k_{cat} (15.5 ± 0.5 s^{-1} for both inhibitors); the errors of optimized parameters were estimated from the final curvature matrix (11). The constant parameters were $[S]_0$ 100 μM, K_m 0.11 μM, and $[E]_0$ 13.5 nM. In a model discrimination analysis, MTX-α-Asp was also assayed at varied substrate concentrations (10–100 μM) and the data were fitted to the rate equation for mixed-type tight binding as described above. Mixtures of MTX and MTX-α-Asp (total 7.0 nM) were assayed under identical conditions at 3.5 nM enzyme.

CONCLUSION

The recursive rate equation [7] can be conveniently used to analyze inhibitory activity of mixtures of tight-binding enzyme inhibitors. For an arbitrary number of inhibitors, the equation retains a remarkably simple algebraic form, resembling the usual expression for classical rather than tight-binding inhibition. Further applications in the area of biochemical pharmacology and analysis may include the accurate theoretical description of a three-inhibitor system (e.g., of the type prodrug → drug → metabolite), the detection of tight-binding impurities present in inhibitors of therapeutically important enzymes, or the determination of inhibition constants for an unknown component of a binary mixture, when the inhibition constant for the known component was established independently. Biochemists interested in applying the above method to their own particular system simply need to incorporate the recursive rate equation into their favorite computer program for statistical analysis of experimental data, one that is most likely based on the nonlinear least-squares model estimation. A potential difficulty with this step is that most available software packages only allow explicit rate equations in which the velocity is entirely extracted on the left-hand side but does not appear on the right-hand side as in the case of recursive equations. Some degree of redesigning most of these computer programs will be necessary. Alternatively, interested readers can obtain information about the availability of a complete enzyme kinetic software package from the author.

ACKNOWLEDGMENTS

Thanks are due to Dr. J. R. Piper (Southern Research Institute) for a gift of methotrexate-α-aspartate and to Dr. John McGuire (Rosewell Park Memorial Institute) for providing valuable advice on the preparation of freshly reduced dihydrofolic acid. P.K. is greatly indebted to Professor Daniel H. Rich for his long-time encouragement and generous support and to Professor Dexter B. Northrop for his ardent criticism, which helped to clarify substantially an early version of the manuscript. This work was supported by Grant Number EY03228 from the National Institutes of Health, National Eye Institute (to T.D.H.).

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