Weinstock, G. W., McEntee, K., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126–130.
Wickner, S., & Hurwitz, J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3342–3346.
Yaginuma, K., & Koike, K. (1981) *Nucleic Acids Res. 9*, 1949–1961.
Yarranton, G. T., Das, R. H., & Gefter, M. L. (1979) *J. Biol. Chem. 254*, 11997–12006.

Inhibition of Dihydrofolate Reductase: Effect of Reduced Nicotinamide Adenine Dinucleotide Phosphate on the Selectivity and Affinity of Diaminobenzylpyrimidines†

David P. Baccanari,* Susan Daluge, and R. W. King

ABSTRACT: The selectivity of benzylpyrimidines for bacterial dihydrofolate reductases was studied by using equilibrium and kinetic techniques. Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine] and a series of close structural analogues with different methoxy group substitutions on the benzyl showed in vitro *Escherichia coli* antibacterial activities that varied according to their degree of substitution. Trimethoprim, the most potent analogue tested, was 400-fold more active than benzylpyrimidine, and the monomethoxy and dimethoxy analogues were of intermediate antibacterial activity. The relative antibacterial potencies of all the compounds were directly proportional to their *E. coli* form 1 dihydrofolate reductase K_i values, as determined by classical enzyme kinetics. Inhibition of the enzyme assay is a measure of the E–I–NADPH ternary complex, and the K_i values ranged from 670 nM for the unsubstituted benzylpyrimidine to 1.3 nM for trimethoprim. However, equilibrium dialysis and fluorescence studies with the *E. coli* and *Lactobacillus casei* enzymes performed in the absence of NADPH showed that the dissociation of inhibitors from the EI binary complex did not vary as widely as kinetic K_i values and that these binary constants were not directly related to either kinetic K_i or antibacterial activity. NADPH increased the affinity of the bacterial enzymes for inhibitors, and this increased affinity in the E–I–NADPH ternary complex (cooperativity) varied with the degree of methoxy substitution (up to 230-fold for trimethoprim and the form 2 *E. coli* enzyme). Contrary to this, all the compounds were weak inhibitors of the mammalian enzyme (SR-1 rodent lymphoma), and none showed more than 8-fold binding cooperativity with NADPH. Therefore, NADPH cooperativity is an important factor in the high affinity of *E. coli* dihydrofolate reductase for trimethoprim, and the lack of cooperativity with the mammalian enzyme is important in the selectivity of trimethoprim as an antibacterial.

Dihydrofolate reductase (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolic acid. The product, tetrahydrofolate, and other reduced folates are essential for the biosynthesis of purines, thymidylate, and several amino acids (Blakley, 1969). Inhibitors of dihydrofolate reductase are effective in the treatment of cancer (methotrexate), malaria (pyrimethamine), and bacterial infections (trimethoprim).[1] Since the latter two are clinically useful because of their marked selectivity for the pathogen dihydrofolate reductases (Burchall, 1974), there is much interest in determining their mechanisms of binding. NMR spectroscopy and equilibrium and kinetic analyses have shown that inhibitor binding can be a complex process involving a variety of protein conformations with different inhibitor affinities (Baccanari & Joyner, 1981; Cayley et al., 1981; Gronenborn et al., 1981). Also, several types of cooperative ligand interactions have been observed. For example, Perkins & Bertino (1966) used fluorescence titrations to show that the L1210 dihydrofolate reductase dissociation constant for triamterene is decreased 60-fold in the presence of NADPH. Similarly, Otting & Huennekens (1972) demonstrated that the binding of methotrexate to dihydrofolate reductase is enhanced by NADPH. Cooperative interactions are also evident from the fact that the covalent binding of active site directed irreversible inhibitors is augmented in the presence of NADPH (Freudenthal et al., 1970). More recently, Birdsall et al. (1977) reported that *p*-aminobenzoylglutamate and 2,4-diaminopyrimidine (regarded as "fragments" of methotrexate) bind cooperatively in the absence of coenzyme. NADPH increases the binding of both fragments without altering the cooperativity between them. Birdsall et al. (1980) also studied the effect of $NADP^+$, NADPH, and a series of coenzyme analogues on the binding of methotrexate and trimethoprim to the *Lactobacillus casei* enzyme. The reduced coenzymes showed significantly more cooperativity than their oxidized counterparts, and dissociation rate constants for reduced 3-acetylpyridine adenine dinucleotide phosphate decreased up to 2200-fold in the ternary complex with methotrexate. Trimethoprim cooperative effects have also been observed with both isozymes of *Escherichia coli* RT 500 dihydrofolate reductase (Baccanari et al., 1981a). In the present study, equilibrium dialysis and fluorescence kinetic analyses were used to assess the effect of NADPH on the binding of trimethoprim and a series of close structural analogues to both bacterial and mammalian dihydrofolate reductases. It was shown that cooperativity (enhanced inhibitor binding in the presence of NADPH) is an important factor in the selectivity of trimethoprim as well as its tight binding to the *E. coli* enzyme and that *E. coli* dihydrofolate

† From The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709 (D.P.B. and S.D.), and the National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom (R.W.K.). *Received March 2, 1982.* Portions of this work were presented to the American Society of Biological Chemists, St. Louis, MO, May 31–June 4, 1981 (Baccanari et al., 1981b).

[1] Abbreviations: trimethoprim, 2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine; TLC, thin-layer chromatography; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-5068$01.25/0

reductase cooperativity varies according to the degree of inhibitor methoxy group substitution.

Materials and Methods

Enzymes. Dihydrofolate reductase was prepared from *L. casei* (strain MTX/R) following the method of Dann et al. (1976). *E. coli* dihydrofolate reductase (both form 1 and 2 isozymes) was prepared from strain RT500 by using the procedures previously described (Baccanari et al., 1981a; Cayley et al., 1981). Lyophilized aliquots of the purified bacterial enzymes were stored either at −15 °C sealed under nitrogen or at 5 °C in a vacuum desiccator. The enzymes were stable for at least 1 year under these conditions. Before use, the enzymes were reconstituted with water or dilute buffer (20 mM potassium phosphate, pH 7) to a protein concentration of 1 mg/mL and allowed to stand at 5 °C for 16 h. We found that in some cases the catalytic and inhibitor binding properties of the enzyme were not fully expressed immediately upon reconstitution. The overnight storage resulted in restoration of the full catalytic and inhibitor binding properties of the enzyme.

Homogeneous SR-1 rodent lymphoma dihydrofolate reductase was a gift from Dr. J. D. Dann (The Wellcome Research Laboratories, Beckenham, Kent, England). This cell line overproduces dihydrofolate reductase, and its purification was an adaptation of that used for the *L. casei* enzyme. The enzyme solution was divided into 1-mL aliquots, each containing 1.4 mg of enzyme, and stored frozen at −15 °C. Before use, the enzyme solution was thawed in a 5 °C refrigerator. This treatment resulted in no loss of enzyme activity over a storage period of 1 year.

Inhibitors. [2-^{14}C]Trimethoprim was obtained from Amersham. The other benzyl[2-^{14}C]pyrimidines and the nonlabeled compounds were prepared at The Wellcome Research Laboratories, Research Triangle Park, NC, from the appropriate benzaldehydes by the "anilino" route (Cresswell et al., 1976; Cresswell & Mentha, 1975). The 3-anilino-2-benzylacrylonitrile intermediates were characterized by elemental analyses and ^{1}H NMR. These intermediates (usually mixtures of *E* and *Z* isomers) were condensed with [^{14}C]guanidine hydrochloride (California Bionuclear Corp.) in refluxing ethanol by Dr. John Hill to give the diamino[2-^{14}C]pyrimidines (yield 55–75%, sp act. 50 mCi/mmol). Their radiochemical purity, determined by scans of silica TLC plates developed in chloroform/methanol (4/1), was >98%. Stock solutions of the nonlabeled compounds used in the fluorescence studies were made in Me_2SO/water and then diluted with the standard buffer (0.1 M sodium phosphate, pH 7.0). The final Me_2SO/water ratio was <1/500. In the other studies, inhibitors were dissolved directly in water (0.5–1 mM stock solutions) and then diluted with the appropriate buffer.

E. coli I_{50} for Growth. In vitro antibacterial activity was quantitated by determining the concentration of inhibitor needed to give a 50% reduction in the specific growth rate of *E. coli* ML-30. Bacterial cells were grown in glucose minimal medium (Harvey, 1973) at 35 °C in the presence of four to five concentrations of inhibitor. Specific growth rates of the individual cultures were calculated from plots of log optical density at 600 nm vs. time. The 50% inhibitory concentration was then calculated from plots of percent inhibition of specific growth rate vs. log [I].

Kinetic K_i Values. The catalytic activities of the *E. coli* and SR-1 enzymes were determined at 30 °C by using the spectrophotometric assay previously described [method 1 of Baccanari & Joyner (1981)]. Standard assay mixtures contained 45 μM dihydrofolate, 60 μM NADPH, 12 mM 2-mercaptoethanol, and the appropriate buffer in a final volume of 1.0 mL. The form 1 and 2 *E. coli* isozymes were assayed in 0.1 M imidazole chloride, pH 7, whereas the SR-1 enzyme was assayed in 0.1 M sodium phosphate, pH 7. The reactions were initiated with the addition of dihydrofolate to the cuvette, and the decrease in 340-nm absorbance was measured as NADPH and dihydrofolate were converted into product. In all cases, the final, steady-state velocities were used. I_{50} determinations were performed under the standard assay conditions except that various concentrations of inhibitor were preincubated (for 2 min) with the enzyme and NADPH before the addition of dihydrofolate. Plots of percentage of inhibition vs. the logarithm of inhibitor concentration in the reaction mixture were used to calculate the I_{50} value. Lineweaver–Burk plots show that trimethoprim (the tightest binding inhibitor) and unsubstituted benzylpyrimidine (the weakest binding inhibitor) are competitive with dihydrofolate (Baccanari & Joyner, 1981; D. P. Baccanari, unpublished results). Since the other inhibitors are close structural analogues, all are assumed to be competitive with dihydrofolate.

An important factor to consider when comparing the relative binding of a competitive inhibitor between two enzymes is that I_{50} values are not kinetic constants. As shown in eq 1, I_{50} is

$$I_{50} = K_i(1 + [S]/K_m) \quad (1)$$

related to the Michaelis constant of the competing substrate (K_m), the substrate concentrations ([S]), and the true kinetic inhibition constant (K_i) (Cheng & Prusoff, 1973). The I_{50} values of the SR-1 and *E. coli* form 1 enzymes for trimethoprim are approximately $28\,000 \times 10^{-8}$ and 0.8×10^{-8} M, respectively, under our assay conditions. The ratio of these I_{50} values is 35 000. However, an examination of the K_m values for these two enzymes shows this ratio to be misleading. The *E. coli* enzyme dihydrofolate K_m value is 8.9 μM (Baccanari et al., 1977), and mammalian enzyme values are commonly reported to be about 10-fold lower (Cha et al., 1981). The SR-1 enzyme also has a low dihydrofolate K_m, but our attempts to determine a precise value were hampered by substrate inhibition and nonlinear reaction velocities at low substrate concentrations where the rate increased during the assay (data not shown). The mechanisms of these anomalies were not investigated, but similar complex reaction kinetics have been observed with many other dihydrofolate reductases (Baccanari & Joyner, 1981). When an SR-1 enzyme K_i value is calculated by using eq 1, a dihydrofolate concentration of 45 μM, and a dihydrofolate K_m value of 0.6 μM [this is the average of all 17 mammalian values listed in Cha et al. (1981)], the ratio of SR-1 to *E. coli* enzyme K_i values is less than 3000 (3700 nM/1.3 nM). Therefore, the I_{50} comparison overestimates the differential binding of trimethoprim for the *E. coli* enzyme by a factor of about 10. Since many laboratories are concerned with establishing the mechanism of trimethoprim specificity, K_i comparisons (even those calculated from an approximate mammalian dihydrofolate K_m) are certainly more realistic than I_{50} comparisons. Therefore, the SR-1 enzyme K_i values used throughout this study were calculated in this manner. Form 1 and form 2 enzyme K_i values were calculated by using eq 1 and K_m values of 8.9 and 0.65 μM, respectively (Baccanari et al., 1977).

In the dihydrofolate reductase random Bi-Bi mechanism (Burchall, 1970), the competition of inhibitors with dihydrofolate for binding to both E and E–NADPH results in two dissociation constants. The binary K_D describes the affinity of E for I, and the ternary K_D describes the affinity of E–NADPH for I. The kinetic K_i calculated for such a competitive inhibitor is a composite of both these dissociation con-

stants (Spector & Cleland, 1981):

$$K_i = \frac{(\text{binary } K_D)(1 + [\text{NADPH}]/[K(\text{NADPH})])}{1 + (\text{binary } K_D/\text{ternary } K_D)([\text{NADPH}]/[K(\text{NADPH})])} \quad (2)$$

where K(NADPH) is the dissociation constant of NADPH from E–NADPH. Therefore, at saturating NADPH concentrations, the kinetic K_i equals the ternary K_D.

Equilibrium K_D Values. The dissociation constant (K_D) is the concentration of free inhibitor at which half the enzyme is in the enzyme–inhibitor complex. K_D values were measured at 5 °C in 0.1 M sodium phosphate buffer, pH 7, containing 2 mM DTT and 0.5 mM EDTA. The enzyme concentration varied between experiments but was in the range of about 20 nM to K_D. One side of each 1.0-mL dialysis cell was loaded with enzyme, bovine serum albumin (32 μg/mL), and ^{14}C-labeled inhibitor (±200 μM NADPH). The other side was loaded with buffer (±200 μM NADPH). Equilibrium across the dialysis membrane, in terms of free inhibitor, was reached after 18 h of continuous shaking. The inhibitors did not bind nonspecifically to the cell, membrane, or albumin. The concentration of free inhibitor and the sum of free inhibitor plus enzyme–inhibitor complex are measured directly from the radioactivity in each compartment. The concentration of enzyme–inhibitor complex is then calculated by difference. Therefore, free inhibitor concentration should be treated as the independent variable in regression analysis, and the familiar Scatchard equation (Scatchard, 1949) has been rearranged as follows (Scott, 1956):

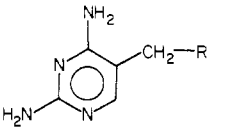
$$[\text{I}]/[\text{EI}] = K_D/(n[\text{E}]) + [\text{I}]/(n[\text{E}]) \quad (3)$$

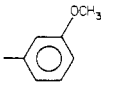
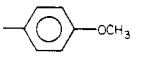
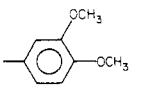
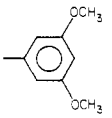
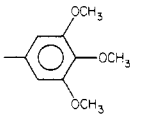
where [I], [E], and [EI] are the concentrations of free inhibitor, total enzyme, and enzyme–inhibitor complex, respectively, and n is the number of moles of binding site per mole of enzyme. Since [E] was determined independently (by methotrexate titration), K_D and n could be calculated. The maximum standard error in the K_D values determined by linear regression of the Scott equation was 30%, and in most cases, the standard error was less than 15%. Duplicate determinations of the same K_D agreed to within 35% of the mean value.

Fluorescence Studies. Equilibrium affinity constants were measured on a Perkin-Elmer MPF-44A spectrofluorometer by using the quenching of the enzyme tryptophan fluorescence. The total percentage quenching of the enzyme fluorescence is shown in Table III. Small relative volumes of the inhibitor solution were added to 3 mL of enzyme solution contained in a 1-cm square fluorescence cuvette. The resultant fluorescence, corrected for the inner filter effect by using a standard tryptophan solution, was recorded as a function of inhibitor concentration and corrected for dilution. The results were computer fitted to a single-site mass-action binding curve by using nonlinear least-squares regression analysis. The standard errors of the calculated dissociation constants varied 3–7% for the *L. casei* enzyme, 10–20% for the SR-1 enzyme, and 15–30% for the *E. coli* form 2 enzyme.

Dissociation rates were measured by using a Durrum D110 stopped-flow apparatus operating in the fluorescence mode. The inhibitor concentrations required to form the various enzyme–ligand complexes were calculated from the appropriate binary equilibrium constants. Solutions of the preformed enzyme–inhibitor or enzyme–inhibitor–NADPH complexes were placed in one delivery syringe, and a methotrexate solution was placed in the other. Methotrexate quenches the fluorescence of the enzyme in both binary and ternary complexes to a greater extent than the trimethoprim analogues.

Table I: Diaminobenzylpyrimidine Structures and *E. coli* I_{50} Values for Growth



benzyl substituent	R	*E. coli* I_{50} for growth (μM)
unsubstituted		120
3-methoxy		10.6
4-methoxy		11.8
3,4-dimethoxy		1.9
3,5-dimethoxy		1.2
3,4,5-trimethoxy (trimethoprim)		0.3

Fluorescence changes were detected by using interference filters on the emission side at wavelengths as close as possible to the wavelength where the greatest difference in the fluorescence of the complexes occurred. For the binary complexes, this was 341 nm, and for the ternary complexes, 401 nm. The excitation wavelength was 280 nm. All experiments were performed at 25 °C. The fluorescence change on mixing the two solutions (which corresponds to the displacement of bound inhibitor by methotrexate) was recorded as a function of time by using a microprocessor-controlled automatic self-scaling transient recorder (King et al., 1979). Up to 16 transients were added together to improve the signal to noise ratio. The recorded transient was then transferred to a computer for subsequent processing. The standard errors of the rate constant measurements were less than 5% in all cases.

Results

Antibacterial Activity. The relative antibacterial activity of trimethoprim and its five structural analogues was quantitated by determining the concentration of inhibitor required for a 50% decrease in the specific growth rate of *E. coli* (Table I). The antibacterial activity varied in a stepwise manner related to the degree of substitution on the benzyl ring. Trimethoprim was the most effective inhibitor in the series. It was 5-fold more active than the dimethoxy compounds, 35-fold more active than the monomethoxy compounds, and 400-fold more active than the unsubstituted diaminobenzylpyrimidine.

Equilibrium Dialysis Measurements of K_D Values. Dialysis was carried out for 18 h at 5 °C, and bovine serum albumin was included in the enzyme side of the cell to prevent inactivation of the enzyme. In most cases, 90–100% of the enzymic activity added to control cells (those lacking inhibitor) was recovered after dialysis, and concentrations of trimethoprim as low as 0.25 nM were quantitatively recovered after

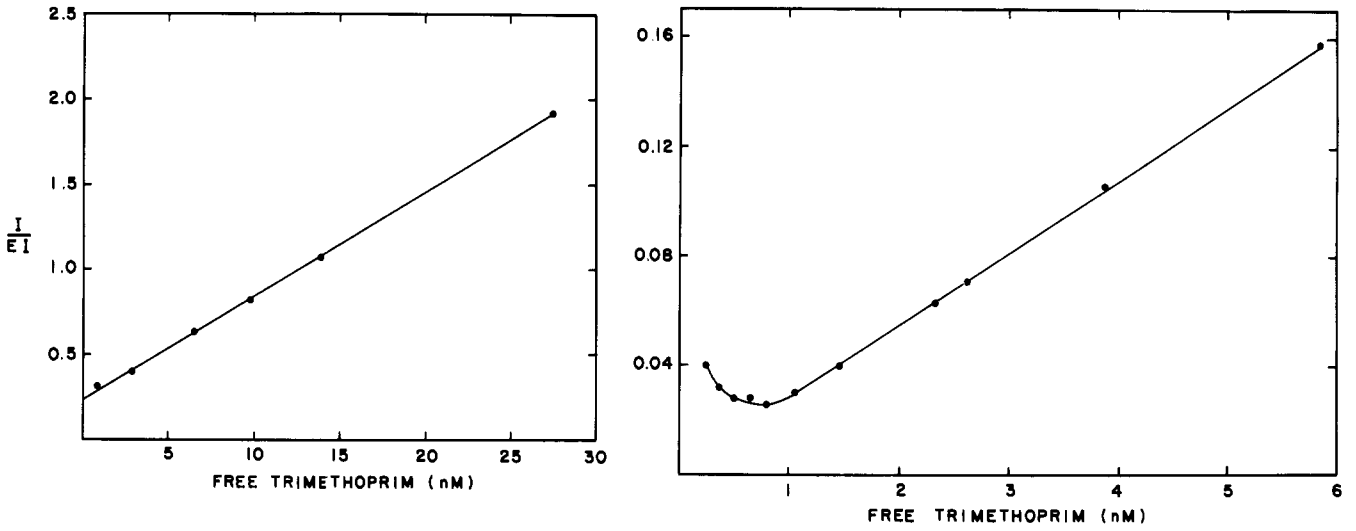


FIGURE 1: Binding of [^{14}C]trimethoprim to form 2 (left panel) and form 1 (right panel) *E. coli* dihydrofolate reductase. In both cases, equilibrium dialysis was performed in the presence of 200 μM NADPH. The enzyme concentration (19 nM) and the slope and intercept of the Scott plot were used to calculate n (0.86) and the ternary K_D (3.8 ± 0.22 mM) for the form 2 enzyme. A form 1 ternary K_D value could not be evaluated from its Scott plot because of the curvature of the line. In this experiment, the form 1 enzyme concentration was 50 nM.

Table II: Kinetic K_i and Equilibrium Binary and Ternary Dissociation Constants of *E. coli* and SR-1 Dihydrofolate Reductases

benzyl substituent	kinetic[a] K_i (nM)	binary[b] K_D (nM)	ternary[b] K_D (nM)	(binary K_D)/(ternary K_D)
		Form 1 *E. coli* Enzyme		
unsubstituted	670 (515)[c]	54	7.2	7.5
3-methoxy	73 (56)	18	0.51	35
4-methoxy	78 (60)	24	0.55	44
3,4-dimethoxy	8.3 (6.4)	9		
3,5-dimethoxy	8.6 (6.6)	18		
3,4,5-trimethoxy	1.3 (1.0)	15		
		Form 2 *E. coli* Enzyme		
unsubstituted	10400 (98)	215	1000 (263)[c]	0.22
3-methoxy	2100 (20)	158	140 (37)	1.1
4-methoxy	2530 (24)	264	180 (47)	1.5
3,4-dimethoxy	494 (4.7)	296	14 (3.7)	21
3,5-dimethoxy	426 (4.0)	101	8.0 (2.1)	13
3,4,5-trimethoxy	106 (1.0)	864	3.8 (1.0)	230
		SR-1 Enzyme		
unsubstituted	2400 (0.65)	385	130 (0.43)	3.0
3-methoxy	2500 (0.68)	218	94 (0.31)	2.3
4-methoxy	2100 (0.57)	263	100 (0.33)	2.6
3,4-dimethoxy	960 (0.26)	324	54 (0.18)	6.0
3,5-dimethoxy	2900 (0.78)	404	85 (0.28)	7.5
3,4,5-trimethoxy	3700 (1.0)	1400	300 (1.0)	4.7

[a] Calculated from I_{50} data by using eq 1 and the appropriate dihydrofolate K_m. [b] Binary and ternary K_D values were determined by equilibrium dialysis of 2-^{14}C-labeled inhibitors. [c] The numbers in parentheses are the ratios of inhibitor constant/trimethoprim constant.

equilibration across the membrane in the absence of enzyme. Figure 1 shows the results of equilibrium dialysis of the form 2 (left panel) and form 1 (right panel) *E. coli* isozymes with [^{14}C]trimethoprim. The form 2 result is representative of most dialysis experiments. Inhibitor concentrations were chosen such that [EI] ranged from 20% to 80% of n[E]. The Scott plots were linear, and n ranged from 0.7 to 0.95, indicating the system behaved as a ligand binding to a single site on a monomeric protein. Data such as these were obtained for all K_D values reported except for three form 1 ternary complexes. The form 1 ternary K_D values for trimethoprim and its two dimethoxy analogues could not be evaluated because their Scott plots exhibited the concave upward line shown in Figure 1 (right panel). Although this experiment was performed with 0.25 M KCl added to the dialysis buffer, similar results (not shown) were obtained in the absence of KCl. However, in the latter case, the enzyme was more than half saturated at 0.25 nM free trimethoprim (our lower limit of detection), and the full curvature of the Scott plot was not evident. KCl was added to weaken inhibitor binding and allow a complete titration of the enzyme such that [EI] was only 16% of n[E] at 0.25 nM trimethoprim in Figure 1 (right panel). This result does not fit the model of inhibitor binding to a single site on a monomeric protein, and its cause is presently unknown. Earlier equilibrium dialysis studies of trimethoprim binding in the form 1 ternary complex were performed at low salt and high inhibitor concentrations, and this curvature was not evident (Baccanari et al., 1981a).

The results of the equilibrium dialysis experiments with the form 1 and 2 *E. coli* isozymes and the SR-1 enzyme along with the kinetically determined inhibitor K_i values are shown in Table II. The form 1 K_i values closely parallel whole cell *E. coli* antibacterial activity in terms of the stepwise change with methoxy substitution. However, the form 1 binary K_D values do not follow this relationship. All of the inhibitors have binary K_D values similar to that of trimethoprim. The form 1 ternary

Table III: Fluorescence Quench Efficiencies and Equilibrium Binary Dissociation Constants of *L. casei*, *E. coli*, and SR-1 Dihydrofolate Reductases

	L. casei enzyme		*E. coli* form 1 enzyme		*E. coli* form 2 enzyme		SR-1 enzyme	
benzyl substituent	quench[a]	K_D (nM)	quench	K_D (nM)	quench	K_D (nM)	quench	K_D (nM)
unsubstituted	70	4200	23	<100[b]	24	1130	48	180
3-methoxy	84	260	20		20	320	54	170
4-methoxy	84	320	23		26	860	52	130
3,4-dimethoxy	90	180	31		30	510	57	430
3,5-dimethoxy	86	240	30		29	480	55	470
3,4,5-trimethoxy	98	100	31		26	260	59	1600

[a] Fluorescence quenching efficiencies are reported as the percentage of the initial fluorescence. [b] The lower limit of detection is 100 nM.

K_D data, although incomplete, show that NADPH increased the binding of the unsubstituted and monosubstituted diaminobenzylpyrimidines 7- and 40-fold, respectively. This altered inhibitor binding in the presence of NADPH is even more evident with the form 2 enzyme, where trimethoprim was bound in the ternary complex with a 230-fold lower K_D value than in the binary complex. The relative values of both the form 2 ternary K_D and kinetic K_i depend upon the degree of methoxy substitution, whereas there was little variation (a maximum of 9-fold) among the binary K_D values. A completely different result was obtained with the mammalian enzyme. All the compounds had trimethoprim-like affinity in the kinetic K_i and the binary and ternary K_D determinations. Moreover, NADPH did not greatly affect the binding of any of the inhibitors to the enzyme.

Equilibrium Fluorescence Binary K_D Measurements. Equilibrium binary dissociation constants were also determined for each of the inhibitors by measuring the ligand-induced quench of intrinsic protein fluorescence. The titration data were analyzed by assuming the ligand was binding to a single site on a monomeric enzyme. There was a good fit of the computed line to the experimental points, even in the case of the *E. coli* form 2 enzyme where the maximum fluorescence quench was only 31%. It should be noted that previous studies (Dunn et al., 1978; Cayley et al., 1981) have shown that K_D values obtained by this technique are combinations of equilibrium constants for each of the elementary steps involved in a complex reaction scheme. The results, including data with *L. casei* dihydrofolate reductase, are presented in Table III. Unsubstituted diaminobenzylpyrimidine showed weak binding to the *L. casei* and *E. coli* form 2 enzymes. There was an increase in affinity with the introduction of a methoxy substitution, but no further step increases were observed with the di- or trimethoxy inhibitors. The form 1 binary K_D values could not be evaluated accurately by this method but are clearly less than 100 nM. A completely different pattern was seen with the mammalian enzyme. The K_D values for the unsubstituted, monosubstituted, and disubstituted compounds were similar to each other and significantly less than that of trimethoprim.

Fluorescence Measurement of Rate Constants. Determinations of dissociation rate constants for trimethoprim and its analogues could serve as an independent measure of enzyme–ligand affinity. The underlying assumption is that the association rate constants for this series of close structural analogues are similar and that the relative K_D values are dependent upon differences in the dissociation rate constants for the inhibitors (Weber, 1975).

The time course for the fluorescence change which accompanies mixing an enzyme–inhibitor complex with a competing inhibitor can be fitted with precision to a single-exponential decay, as shown in Figure 2 for methotrexate reacting with the *L. casei* dihydrofolate reductase–NADPH–2,4-diamino-5-(3,4-dimethoxybenzyl)pyrimidine complex. The processes occurring on mixing a preformed complex (EL_1) with a competing ligand (L_2) can be described by eq 4. If $k_{-1} \ll k_2[L_2]$

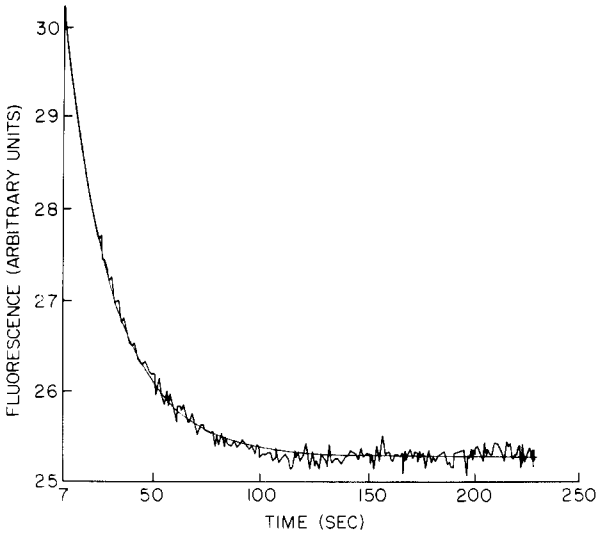


FIGURE 2: Dissociation of 2,4-diamino-5-(3,4-dimethoxybenzyl)pyrimidine from its complex with *L. casei* dihydrofolate reductase–NADPH. A solution of the ternary complex (1 μM) was mixed with an equal volume of 100 μM methotrexate, and the decrease in fluorescence at 401 nm was followed as a function of time. The experimental line is a summation of four experiments with 100-ms electronic filtering. The smooth line is an exponential decay fitted to the points by a nonlinear least-squares routine. The experiment was carried out at 25 °C and pH 7.0.

$$EL_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L_2 \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} EL_2 \qquad (4)$$
$$+$$
$$L_1$$

$\gg k_1[L_1]$, the fluorescence change due to the conversion of EL_1 to EL_2 follows single exponential with a rate constant k_{-1}. In other words, the reaction $EL_1 \rightleftharpoons E + L_1$ becomes unidirectional since free enzyme is very rapidly converted to EL_2. By using methotrexate, which has a low dissociation constant from both binary and ternary complexes, and by suitable adjustment of the initial concentration of the inhibitors, it is possible to satisfy the above conditions in a situation where at least 95% of the enzyme is complexed initially. Any remaining free enzyme will react with methotrexate, and for the *E. coli* isoenzymes, this reaction will be complete in a few milliseconds (Cayley et al., 1981), within the dead time of the instrument and consequently not detected. For the *L. casei* enzyme, approximately 40% of any free enzyme will also react rapidly while 60% reacts very slowly (Dunn et al., 1978). However, the small amplitude of the signal arising from the latter reaction means that it too is not detected. The applicability of this method to determining dissociation rate constants is easily checked by varying the concentration of the

competing ligand, and we have done this routinely.

The results obtained with the binary and ternary complexes of the four enzymes are presented in Table IV. Clearly, the values of the rate constant for dissociation are affected by the position and degree of methoxy substitution of the benzene ring, the enzyme species, and the presence or absence of the coenzyme. In general terms for the bacterial enzymes, an increase in the degree of substitution is accompanied by a decrease in the rate constant from both binary and ternary complexes. The ratio of binary to ternary rate constants shows that NADPH decreases the rate constants for all the inhibitors and that the magnitude of this change is dependent upon the extent of methoxy substitution. Quite different results were obtained with the mammalian enzyme. Overall, much faster dissociation rates were observed, and there was little difference among the various inhibitors. Also, the effect of bound NADPH was greatly reduced and similar for all compounds.

Discussion

A comparison of the form 1 dihydrofolate reductase kinetic K_i values (Table II) with their in vitro *E. coli* I_{50} for growth (Table I) shows that the kinetic constant is predictive of the antibacterial potency of these inhibitors. There was a stepwise increase in both enzyme affinity and antibacterial activity which correlates with the degree of methoxy group substitution on diaminobenzylpyrimidine. The same trend is also seen with the kinetic K_i values of the form 2 enzyme. Although the mutant form 2 isozyme is not found in wild-type *E. coli*, it serves as a useful biochemical tool because its amino acid sequence differs from the form 1 enzyme in only one position, and its poor affinity for inhibitors can be explained on this basis (Baccanari et al., 1981a). For each inhibitor, the *E. coli* I_{50} for growth was approximately 200-fold greater than the dihydrofolate reductase form 1 kinetic K_i. This ratio of I_{50} for growth of cells to dihydrofolate reductase K_i is consistent with the position of the enzyme in the folate metabolic pathway. Dihydrofolate reductase must be inhibited 95–99% before it becomes the rate-limiting enzyme in thymidylate biosynthesis (Jackson & Harrap, 1973). Also, dihydrofolate accumulates when the enzyme is inhibited, and it further competes with inhibitor for the enzyme binding site (Jackson & Harrap, 1973; Harvey, 1978).

Since dihydrofolate reductase utilizes a random Bi-Bi mechanism (Burchall, 1974), the kinetic K_i determined at saturating NADPH concentration is equal to the dissociation constant of I from the E–I–NADPH ternary complex (Spector & Cleland, 1981). In the present study, both kinetic K_i and ternary K_D were evaluated for a series of inhibitors, but the experimental conditions for these analyses differed markedly. Equilibrium dialysis was performed at 5 °C to prevent loss of enzymatic activity during the long time (18 h) needed to attain equilibrium. On the other hand, kinetic K_i values were determined at 30 °C because the interconversions of the various enzyme conformations at low temperature are too slow to attain steady state before substrate depletion and product inhibition (Baccanari & Joyner, 1981). Subramanian & Kaufman (1978) have shown that the binding of folate, dihydrofolate, and methotrexate to the chicken liver enzyme is characterized by negative entropy changes. If this is true for diaminobenzylpyrimidines, K_D values will decrease with temperature, and the value of any particular K_i would not equal its ternary K_D. However, for each enzyme, the ratio of kinetic K_i values of one inhibitor to another would be equivalent to the ratio of the inhibitor ternary K_D values. In the present case, the ratios of kinetic K_i values (compared to trimethoprim) of the form 2 *E. coli* enzyme are in close agreement with the ratios of ternary K_D values, and the same is true with the SR-1 enzyme (Table II). Although three form 1 enzyme ternary K_D values could not be measured, the ratios of kinetic K_i values for the monosubstituted compounds compared to those of the unsubstituted compounds (9) were similar to the ratios of their ternary K_D values (13). Therefore, the effect of NADPH on inhibitor binding can be assessed by comparing binary K_D values with either ternary K_D values or kinetic K_i values. For example, since all the *E. coli* form 1 binary dissociation constants fall within a 6-fold range (Table II), the marked differences in kinetic K_i values of these inhibitors must be due to enhanced binding of some compounds in their ternary complexes. This cooperativity increases as methoxy groups are substituted onto the benzyl ring. Binary and ternary K_D values can be directly compared with the form 2 and SR-1 enzymes. The form 2 results are similar to those of form 1, except that negative cooperativity is seen with the unsubstituted diaminobenzylpyrimidine. The methoxy-substituted compounds show positive cooperativity which reaches a maximum value of 230-fold with trimethoprim. Contrary to this, the SR-1 enzyme showed little cooperativity. The maximum difference between binary and ternary K_D was 7.5-fold. This poor cooperativity with the mammalian enzyme coupled to the high cooperativity with the *E. coli* enzyme plays a significant role in the specificity of trimethoprim. For example, a comparison of the trimethoprim binary K_D value of the SR-1 enzyme (1400 nM) with that of the *E. coli* form 1 enzyme (15 nM) shows an affinity difference of less than 100-fold. However, a comparison of their kinetic K_i values shows that this selectivity is increased to almost 3000-fold by NADPH.

Fluorescence studies with the *E. coli* and SR-1 enzymes corroborate these conclusions. For example, there is a close correlation between the binary SR-1 K_D values determined by equilibrium dialysis (Table II) and fluorescence (Table III). The fluorescence studies also showed that these compounds bind to the *E. coli* form 1 enzyme considerably more tightly than to the form 2 enzyme, in agreement with the equilibrium dialysis results. However, the combination of high affinity and low quenching made accurate determination of the form 1 fluorescence K_D values impossible. For the form 2 enzyme, there are up to 5-fold differences in binary K_D values determined by these two techniques. The reason for this is unclear, but, as with the kinetic K_i and ternary K_D determinations, the temperature at which the measurements were made (5 °C for equilibrium dialysis vs. 25 °C for fluorescence) may contribute to the difference. The important factor in these studies is that several independent methods were used to quantitate inhibitor binding and all showed that cooperativity augments the selectivity of diaminobenzylpyrimidines.

The fluorescence studies also include work with *L. casei* dihydrofolate reductase. Although this enzyme is not of direct therapeutic interest, it has been well characterized, and the three-dimensional structure of the ternary complex with methotrexate and NADPH has been solved by X-ray diffraction methods (Matthews et al., 1978, 1979). The *L. casei* and *E. coli* form 1 enzyme binary dissociation rate constants (Table IV) are interesting because there is a trend toward decreasing rate constants as methoxy group substitution of diaminobenzylpyrimidine increases. However, this is offset by a more pronounced decrease in ternary rate constants, and the net effect is that cooperativity increases as methoxy group substitution increases. The only exception is trimethoprim with the *E. coli* form 1 enzyme, where cooperativity is less than that seen with the 3,5-dimethoxy-substituted analogues. The reason for this discrepancy is unclear, but it could be related

Table IV: Dissociation Rate Constants of *L. casei*, *E. coli*, and SR-1 Dihydrofolate Reductases

benzyl substituent	*L. casei* enzyme			*E. coli* form 1 enzyme			*E. coli* form 2 enzyme			SR-1 enzyme		
	binary[a]	ternary[a]	binary/ ternary	binary	ternary	binary/ ternary	binary	ternary	binary/ ternary	binary	ter- nary	binary/ ternary
unsubstituted	65	3.1	21	11	2.7	4.1	17.5	15	1.2	40	20	2
3-methoxy	20	0.54	37	3.2	0.22	14.5	6.7	3.2	2.1	43	28	1.5
4-methoxy	6.8	0.20	34	3.9	0.27	14.5	12.6	4.3	2.9	47	17	2.8
3,4-dimethoxy	2.0	0.042	48	0.45	0.017	26.5	2.2	0.29	7.6	56	10	5.6
3,5-dimethoxy	7.8	0.10	78	1.2	0.016	75	8.4	0.32	26	61	21	2.9
3,4,5-trimethoxy	1.7	0.017	100	0.35	0.009	41	4.2	0.034	123	70	25	2.8

[a] Binary and ternary dissociation rate constants were determined fluorometrically by competition with methotrexate. Values are reported in units of s^{-1}.

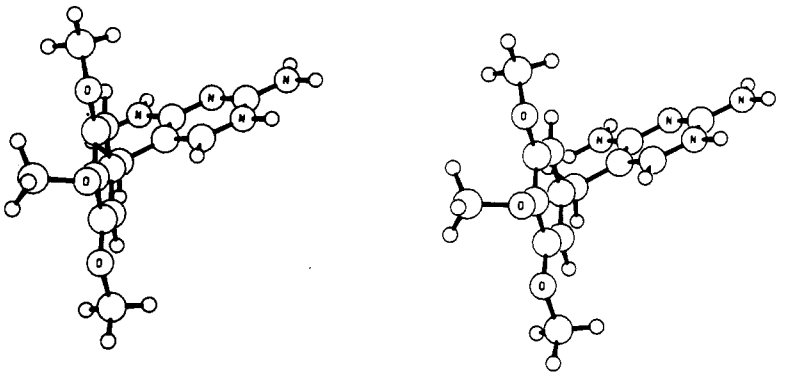


FIGURE 3: Stereo diagram of the HBr salt of trimethoprim in a conformation approximating that observed in the *E. coli* form 1 dihydrofolate reductase binary complex (Baker et al., 1981).

to the fact that the form 1 trimethoprim ternary complex behaves anomalously upon equilibrium dialysis. In general, the ratios of binary/ternary dissociation rate constants for the form 2 and SR-1 enzymes (Table IV) show fairly good agreement with their corresponding binary/ternary equilibrium K_D ratios (Table II). However, an obvious quantitative difference involves unsubstituted diaminobenzylpyrimidine, where negative cooperativity is seen with the form 2 enzyme in the equilibrium dialysis experiments and small positive cooperativity is shown in Table IV.

A large part of the selectivity and antibacterial activity of trimethoprim must be attributed to the collective interactions of the three flanking methoxy groups in the ternary complex. Realizing the complexities involved, we have made an attempt to understand the unique properties of trimethoprim at a molecular level with the information available on dihydrofolate reductase structures and on methoxybenzenes. As shown in Figure 3, the methoxy groups of trimethoprim, alone and in binary complex with *E. coli* dihydrofolate reductase, are oriented in an energetically stable staggered conformation with the *m*-methoxy groups approximately in the plane of the benzene ring and the *p*-methoxy group, by necessity, forced out of this plane (Baker et al., 1981; Koetzle & Williams, 1976). This pattern is typical of other such trimethoxybenzenes, both in solution and in the crystal (Anderson et al., 1979). The influence of the para substitutent on the conformations of meta substituents and its implications for binding to dihydrofolate reductase have been discussed by Roth et al. (1981). Although a ternary bacterial enzyme–trimethoprim complex is not yet available, NMR studies predict that no significant change in the torsion angles which define the relative orientation of the pyrimidine and phenyl rings to each other will occur on going from the binary to the ternary *E. coli* complex (Cayley et al., 1979). Since there is some evidence that trimethoprim binds similarly to the *L. casei* and *E. coli* enzymes (Cayley et al., 1979), its *E. coli* binary conformation (Figure 3) was modeled into the active site of the *L. casei*–methotrexate–NADPH structure (Kendrew model built by F. Norrington, The Wellcome Research Laboratory, Beckenham, Kent, England, from coordinates kindly provided by D. Matthews, University of California, San Diego, La Jolla, CA). The diaminopyrimidine portion was superimposed on the corresponding portion of methotrexate. One observation of possible relevance to this study is that one of the *m*-methoxy groups is positioned over the nicotinamide ring of the cofactor, presumably achieving a good hydrophobic interaction. This would be consistent with studies by Birdsall et al. (1980) showing that the nicotinamide ring of coenzyme analogues and the trimethoxybenzyl portion of trimethoprim influence cooperativity with the *L. casei* enzyme. The direct interaction of a *m*-methoxy with cofactor could be expected to be maximized in trimethoprim due to the semirigid positioning and symmetry of the *m*-methoxy groups. Matthews (1982) recently reported the first vertebrate dihydrofolate reductase structure, the chicken liver enzyme NADPH in complex with several ligands, including trimethoprim. The conformation of trimethoprim in this vertebrate structure was reported to be quite different from that observed in the *E. coli* binary structure. It was noted that, as a result of the conformational change, the trimethoxyphenyl is oriented in the chicken liver structure so as to have no close contacts or obvious interactions with the cofactor. This would then seem consistent with the low cooperativity of this series of benzylpyrimidines with the mammalian SR-1 enzyme.

It should be noted that the sources of cooperativity are probably complex. It was for this reason that we chose to study all the members of this series. For example, the 3–6-fold increase in cooperativity of the 4′-methoxy compared to the unsubstituted benzylpyrimidine for form 1 *E. coli* suggests that factors other than direct interaction of a *m*-methoxy with cofactor are also involved. Matthews et al. (1978) have predicted from a comparison of the *E. coli* binary to *L. casei* ternary methotrexate structures that the "teen loop" (residues 13–22) moves substantially on binding of NADPH. This

conformational change brings the side chain of Leu-19 into van der Waals contact with the pyrazine ring of methotrexate. Modeling of trimethoprim indicates that this movement of Leu-19 would bring it into the vicinity of the 4′-methoxy in the ternary structure. It is thus possible that the corresponding residue in *E. coli* (Met-20) accounts for the contribution of the 4′-methoxy to cooperativity on this enzyme. An added complexity in extending analysis to the mono- and dimethoxy compounds is that they have more flexibility than trimethoprim and the methoxy groups would be predicted to have several stable conformations available to them (Anderson et al., 1979). It is interesting to speculate that a detailed analysis of the *E. coli* enzyme ternary structures for this series of compounds will allow identification and quantitation of the sources of cooperativity. This information would have obvious implications for designing inhibitors with specificity for bacterial enzymes. Similarly, the variability of the cooperative effect is an important consideration in formulating quantitative structure–activity relationships. A useful equation correlating the activity of mono-, di-, and tri-substituted benzylpyrimidines would be expected to be complex. Finally, it is interesting to question whether or not cooperativity is a factor in the specificity of other classes of dihydrofolate reductase inhibitors. For example, are triazines specific for mammalian enzymes (Hitchings & Burchall, 1965) because the mammalian enzymes show cooperativity with these inhibitors while the bacterial enzymes do not, or is the lack of specificity of pyridopyrimidines (Hurlbert et al., 1968) due to similar cooperativity in both bacterial and mammalian enzymes?

Acknowledgments

We thank John Dann for supplying the homogeneous SR-1 enzyme, John Hill for preparing the radiolabeled inhibitors, Paul Ray and Rietta Dampier for performing the *E. coli* I_{50} for growth determinations, Hattie Roberts and Peter Turner for technical assistance, and J. Burchall and B. Roth for their continued interest and support.

References

Anderson, G. M., III, Kollman, P. A., Domelsmith, L. N., & Houk, K. N. (1979) *J. Am. Chem. Soc. 101*, 2344–2352.

Baccanari, D. P., & Joyner, S. S. (1981) *Biochemistry 20*, 1710–1716.

Baccanari, D. P., Averett, D., Briggs, C., & Burchall, J. (1977) *Biochemistry 16*, 3566–3572.

Baccanari, D. P., Stone, D., & Kuyper, L. (1981a) *J. Biol. Chem. 256*, 1738–1747.

Baccanari, D. P., Daluge, S., & King, R. (1981b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1748.

Baker, D. J., Beddell, C. R., Champness, J. N., Goodford, P. J., Norrington, F. E. A., Smith, D. R., & Stammers, D. K. (1981) *FEBS Lett. 126*, 49–52.

Birdsall, B., Griffiths, D. V., Roberts, G. C. K., Feeney, J., & Burgen, A. (1977) *Proc. R. Soc. London, Ser. B 196*, 251–265.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980) *Biochemistry 19*, 3723–3731.

Blakley, R. L. (1969) in *The Biochemistry of Folic Acid and Related Pteridines* (Neuberger, A., & Tatum, E. L., Eds.) North-Holland, Amsterdam.

Burchall, J. J. (1970) in *Chemistry and Biology of Pteridines* (Iwai, K., Ed.) pp 351–355, International Academic Printing Co. Ltd., Tokyo.

Burchall, J. J. (1974) in *Topics in Infectious Diseases* (Drews, J., & Hahn, F. E., Eds.) Vol. 1, pp 285–293, Springer-Verlag, New York.

Cayley, P. J., Albrand, J. P., Feeney, J., Roberts, G. C. K., Piper, E. A., & Burgen, A. S. V. (1979) *Biochemistry 18*, 3886–3895.

Cayley, P. J., Dunn, S. M. J., & King, R. W. (1981) *Biochemistry 20*, 874–879.

Cha, S., Kim, S. Y. R., Kornstein, S. G., Kantoff, P. W., Kim, K. Y., & Naguib, F. N. M. (1981) *Biochem. Pharmacol. 30*, 1507–1515.

Cheng, Y. C., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22*, 3099–3108.

Cresswell, R. M., & Mentha, J. W. (1975) U.S. Patent 3 878 252.

Cresswell, R. M., Mentha, J. W., & Seaman, R. L. (1976) U.S. Patent 3 956 327.

Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V., & Harding, N. G. L. (1976) *Biochem. J. 157*, 559–571.

Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356–2364.

Freudenthal, R., Lowe, J. K., & Hebborn, P. (1970) *Mol. Pharmacol. 6*, 617–620.

Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1981) *Nature (London) 290*, 273–274.

Harvey, R. J. (1973) *J. Bacteriol. 114*, 309–322.

Harvey, R. J. (1978) *J. Theor. Biol. 74*, 411–437.

Hitchings, G. H., & Burchall, J. J. (1965) *Adv. Enzymol. Relat. Areas Mol. Biol. 27*, 417–468.

Hurlbert, B. S., Ferone, R., Herrmann, T. A., Hitchings, G. H., Barnett, M., & Bushby, S. R. M. (1968) *J. Med. Chem. 11*, 711–717.

Jackson, R. C., & Harrap, K. R. (1973) *Arch. Biochem. Biophys. 158*, 827–841.

King, R. W., Everett, D. B., Sowden, J. M., Satchell, J. H., & Lewin, J. E. (1979) in *Techniques and Applications of Fast Reactions in Solution* (Gettins, W. J., & Wynn-Jones, E., Eds.) pp 147–149, D. Reidel, Dordrecht, The Netherlands.

Koetzle, T. F., & Williams, G. J. B. (1976) *J. Am. Chem. Soc. 98*, 2074–2078.

Matthews, D. A., & Volz, K. (1982) in *Molecular Structure and Biological Activity* (Griffin, J. F., & Daux, W. L., Eds.) pp 13–26, Elsevier Biomedical, New York.

Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946–6954.

Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N., & Kraut, J. (1979) *J. Biol. Chem. 254*, 4144–4151.

Otting, F., & Huennekens, F. M. (1972) *Arch. Biochem. Biophys. 152*, 429–431.

Perkins, J. P., & Bertino, J. R. (1966) *Biochemistry 5*, 1005–1012.

Roth, B. R., Aig, E., Rauckman, B. S., Sterlitz, J. Z., Phillips, A. P., Ferone, R., Bushby, S. R. M., & Sigel, C. W. (1981) *J. Med. Chem. 24*, 933–941.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Scott, R. L. (1956) *Recl. Trav. Chim. Pays-Bas 75*, 787–789.

Spector, T., & Cleland, W. W. (1981) *Biochem. Pharmacol. 30*, 1–7.

Subramanian, S., & Kaufman, B. T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3201–3205.

Weber, G. (1975) *Adv. Protein Chem. 29*, 59.