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Interactions between inhibitors of dihydrofolate reductase

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The binding of substrates and inhibitors to dihydrofolate reductase was studied by steady-state kinetics and high-field ^{1}H-n.m.r. spectroscopy. A series of 5-substituted 2,4-diaminopyrimidines were examined and were found to be 'tightly binding' inhibitors of the enzyme ($K_i < 10^{-9}$ M). Studies on the binding of 4-substituted benzenesulphonamides and benzenesulphonic acids also established the existence of a 'sulphonamide-binding site' on the enzyme. Subsequent n.m.r. experiments showed that there are two binding sites for the sulphonamides on the enzyme, one of which overlaps the coenzyme (NADPH) adenine-ring-binding site. An examination of the pH-dependence of the binding of sulphonamides to the enzyme indicated the influence of an ionizable group on the enzyme that was not directly involved in the sulphonamide binding. The change in pK_a value from 6.7 to 7.2 observed on sulphonamide binding suggests the involvement of a histidine residue, which could be histidine-28.

INTRODUCTION

Dihydrofolate reductase (DHFR) (tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3) is a ubiquitous enzyme that is the target for a number of clinically useful drugs, including methotrexate (MTX), trimethoprim (TMP) and pyrimethamine and their derivatives. Structure–activity relationships of the inhibitors of DHFR have been reviewed in detail (Blaney *et al.*, 1984). Antibacterial drugs of the 'antifolate' class (of which trimethoprim is an example) are often used in conjunction with sulphonamides, following the observation that such combinations are synergistic (Greenberg, 1949). This effect has traditionally been explained on the basis of the two components binding independently to two enzymes on a linear metabolic pathway, namely dihydropteroate synthetase (EC 2.5.1.5) and DHFR (Potter, 1951). However, subsequent workers have claimed that this is not an adequate explanation of the phenomenon (Rollo, 1955; Webb, 1963; Rubin *et al.*, 1964; Wise & Aboudania, 1975), and there is evidence to suggest that the synergism may be a result of simultaneous binding of the antifolate inhibitor and the sulphonamide to DHFR (Poe, 1976; Poe & Ruyle, 1981).

The present study is designed to investigate and extend further this hypothesis by identifying the position of and residues involved in the sulphonamide-binding site of purified bacterial DHFR.

EXPERIMENTAL

Materials

Escherichia coli M.R.E. 600 was obtained as a frozen cell suspension from the Centre for Applied Microbiology and Research, Porton Down, Wilts., U.K. Folic acid, MTX and NADPH were from Sigma Chemical Co., Poole, Dorset, U.K. Dihydrofolic acid was prepared by the method of Futterman (1957) as modified by Blakely (1960), and stored as a suspension in 5 mM-HCl at −70 °C. All other reagents were of the highest purity commercially available and were used without further purification. *Lactobacillus casei* N.C.B. 6375 (MTX/R) DHFR was isolated and purified as described previously (Dann *et al.*, 1976).

Isolation of *E. coli* M.R.E. 600 DHFR

M.R.E. 600 is a wild-type strain of *E. coli*, grown in the absence of any DHFR inhibitors, thus lacking elevated concentrations of the enzyme. This strain was chosen because of the ready availability of kilogram quantities of cultured cells, in addition to the fact that changes reported to arise in enzyme sequence/structure in response to the presence of DHFR inhibitors will not have occurred (Albrecht *et al.*, 1972; Niethammer & Jackson, 1975; Flintoff *et al.*, 1976; Jackson & Niethammer, 1977).

The purification of DHFR from *E. coli* M.R.E. 600 was accomplished by using the procedure of Poe *et al.* (1972), and included gel-filtration, ion-exchange and affinity-chromatography stages (see Table 1 for more details). The MTX–aminoethyl-Sepharose affinity resin used in the isolation was prepared as suggested by Cuatrecasas (1970) and Poe *et al.* (1972). The protein concentration was measured at each stage during the purification by using the methods of Lowry *et al.* (1951) and of Warburg & Christian (1941), with bovine serum albumin as standard. Throughout the purification, the enzyme activity was assessed by using a spectrophotometric assay adapted from Baccanari *et al.* (1975). The assay was performed at 37.0 ± 0.1 °C in 50 mM-Tris/HCl buffer, pH 7.2, containing 50 μM-dihydrofolate, 60 μM-NADPH, 50 mM-KCl, 10 mM-2-mercaptoethanol, 1 mM-EDTA and enzyme in a total volume of 3.00 ml. The

Abbreviations used: DHFR, dihydrofolate reductase; MTX, methotrexate; TMP, trimethoprim; *p*-ABG, *N*-(*p*-aminobenzoyl)-L-glutamic acid.

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decrease in absorbance at 340 nm was measured with a Pye–Unicam SP.8-100 spectrophotometer. A value of 12300 $M^{-1}\cdot cm^{-1}$ for the absorption coefficient of the reaction was used (Hillcoat *et al.*, 1967). One unit of enzyme activity is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate/min under the stated conditions of pH and temperature.

Synthesis and inhibitors

MTX, TMP and *N*-(*p*-aminobenzoyl)-L-glutamic acid (*p*-ABG) were obtained from Sigma Chemical Co. 4-Nitrobenzenesulphonamide, n-propylbenzene, n-butylbenzene, 4-fluorobenzenesulphonyl chloride, 4-chlorobenzenesulphonyl chloride and 4-bromobenzenesulphonyl chloride were purchased from Aldrich Chemical Co. 4-Hydroxybenzenesulphonic acid was obtained as a 65% (w/v) solution from Fluorochem.

2,4-Diaminopyrimidine was synthesized according to the method of English & Clapp (1947). TMP and *p*-ABG were recrystallized from water before use. MTX was used as obtained from Sigma Chemical Co.

The following 2,4-diaminopyrimidine derivatives were generously given by Dr. D. Warburton, May and Baker, Dagenham, Essex, U.K., and were all used without further purification: 2,4-diamino-6-methyl-5,3′-(3-nitrophenoxy)prop-1′-yloxypyrimidine (M&B 35902A), 2,4-diamino-6-methyl-5,3′-(2-trifluoromethylphenoxy)prop-1′-yloxypyrimidine (M&B 38082A), 2,4-diamino-6-methyl-5,3′-(3-trifluoromethylphenoxy)prop-1′-yloxypyrimidine (M&B 35414), 2,4-diamino-6-ethyl-5,3′-(2-trifluoromethylphenoxy)prop-1′-yloxypyrimidine (M&B 39019), 2,4-diamino-6-ethyl-5,3′-(2-cyclohexylphenoxy)prop-1′-yloxypyrimidine (M&B 39434), 2,4-diamino-6-ethyl-5,3′-(2-trifluoromethyl-4-sulphonamidophenoxy)prop-1′-yloxypyrimidine hydrochloride (M&B 39568) and 2,4-diamino-5-hydroxy-6-methylpyrimidine (DEO 1693).

Sulphonamides and sulphonic acids were prepared by established methods (Scheifele & DeTar, 1963; Spryskov & Apar'eva, 1950; Clarke *et al.*, 1963).

RESULTS

The results for the purification of *E. coli* M.R.E. 600 DHFR are given in Table 1. The percentage recovery and purification factors shown are lower limits, as a consequence of the inability to measure enzyme activity accurately in the two initial purification steps. This is thought to arise from the presence of nucleic acids, which are known to interfere with the enzyme assay (Hänngi & Littlefield, 1974).

Electrophoresis of the purified enzyme on an SDS/polyacrylamide gel showed a single band having an R_F value of 0.74 ± 0.02. Calibration with protein molecular-mass markers gave a linear relationship between R_F and log(molecular mass). The molecular mass calculated for DHFR was 21700 ± 1300 Da. Measurements on a calibrated Sephadex G-75–50 column gave a closely similar value of 21000 ± 1000 Da. This value of approx. 21000 is in contrast with values of 17000 for DHFR from an MTX-resistant strain of *E. coli* (Poe *et al.*, 1972) and 17800 for DHFR from a TMP-resistant strain of *E. coli* (Baccanari *et al.*, 1975) among others (Mathews & Sutherland, 1965; Burchall & Hitchings, 1965; Erickson & Mathews, 1973). It seems that the differences can be ascribed to the different bacterial strains used, similar differences having been noted for DHFR from different strains of *L. casei* (Dunlap *et al.*, 1971; Dann *et al.*, 1976) and *Streptococcus faecalis* (Albrecht & Hutchinson, 1969; D'Souza *et al.*, 1972).

General kinetic properties of the enzyme

The assay system used in all measurements of substrate/inhibitor binding was similar to that used during the enzyme purification. However, to overcome the problems often encountered with non-linear initial reaction rates, the enzyme was preincubated with coenzyme (at a concentration of at least $10K_m$, i.e. 100 μM) for a period of at least 5 min. Studies indicated that this preincubation time was sufficient to allow formation of the binary complex.

Values for the K_m and $V_{max.}$ of the enzyme, as well as inhibitor K_i values, were calculated by using a weighted analysis (Cornish-Bowden, 1979), and the enzyme concentration was measured from MTX titration. The values for the various kinetic parameters are given in Table 2. The K_m values of 9.21 ± 0.25 μM for dihydrofolate and 5.07 ± 0.24 μM for NADPH are in close agreement with values of 10.0 and 8.9 μM respectively reported previously for DHFR from wild-type *E. coli* (Burchall

Table 1. Purification of DHFR from extracts of *E. coli* M.R.E. 600

For experimental details see the text.

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
Crude cell lysate	250	2650	–	–	–	–
Protamine sulphate supernatant	260	2400	–	–	–	–
Streptomycin sulphate supernatant	275	2250	8500	4	(100)	(1)
Ammonium sulphate (65–85%-satn. precipitate)	10	790	6900	9	81	2
Sephadex G-75 (3 cm × 90 cm)	160	145	6650	46	78	12
DEAE-cellulose chromatography	180	28	5900	214	69	57
MTX–aminoethyl-Sepharose chromatography	120	5	5600	1077	66	285
Sephadex G-75 (1.5 cm × 15 cm)	15	5	5450	1065	64	282

Table 2. Steady-state kinetic parameters for *E. coli* M.R.E. 600 DHFR

Conditions were as follows: 50 mM-Tris/HCl buffer, pH 7.2, containing 50 mM-KCl, 10 mM-2-mercaptoethanol and 1 mM-EDTA at 37.0 °C. The enzyme concentration was 0.63 nM.

Substrate	Second substrate	K_m (μM)	$k_{cat.}$ (s^{-1})
Dihdyrofolate	NADPH (100 μM)	9.21±0.25	122.7±20.4
NADPH	Dihydrofolate (100 μM)	5.07±0.24	117.2±10.6

& Chan, 1969). The $k_{cat.}$ (catalytic-centre activity) is significantly larger at approx. 120 s^{-1} than the value previously reported by Poe *et al.* (1972) of 10.0±0.9 s^{-1}. However, this difference may be accounted for by the different experimental conditions, namely pH 7.2 and 37.0 °C as used in the present study compared with pH 7.4 and 23 °C as used by Poe *et al.* (1972).

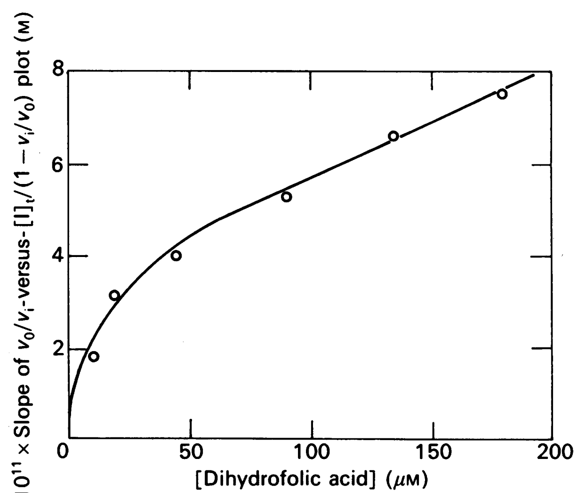


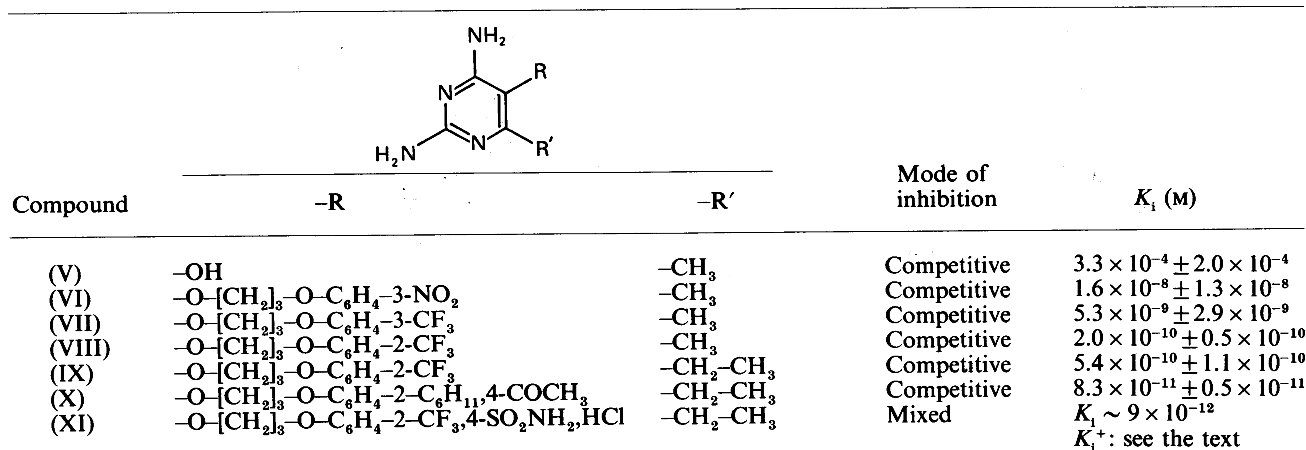
Fig. 1. Henderson plot for binding of inhibitor (XI) to *E. coli* M.R.E. 600 DHFR

Binding of inhibitors to DHFR

In order to assess the usefulness of the novel inhibitors examined in this study as potential chemotherapeutic agents, their binding to DHFR was characterized in terms of mode of inhibition (determined from Hanes–Wolff plots) and K_i values (dissociation constant of the enzyme–inhibitor–coenzyme ternary complex, calculated by using the weighted analysis mentioned above). The K_i values for 'tightly bound' inhibitors were calculated as described by Henderson (1972).

Inhibition of *E. coli* M.R.E. 600 DHFR by MTX, TMP, *p*-ABG and 2,4-diaminopyrimidine was examined and was found to be comparable with previously reported results. The results for the inhibition by the novel 2,4-diaminopyridimidine derivatives (V)–(XI) are given in Table 3. It is apparent that compounds (V)–(X) all exhibit competitive inhibition; from the structural similarity with the substrate this is presumed to arise from competition for the binding site of the dihydrofolate pyrimidine ring. Compound (XI) is anomalous in that it exhibits mixed inhibition. This is assumed to arise from a dual mode of action composed of both pure competitive and non-competitive inhibition. From the Henderson plot for compound (XI) (Fig. 1) it is apparent that the K_i^+ [dissociation constant for compound (XI) acting in its non-competitive mode] is some orders of magnitude greater than the K_i (dissociation constant for competitive inhibition). Therefore the value of K_i^+ cannot be directly measured [at concentrations of compound (XI) approaching K_i^+ the enzyme is completely inhibited owing to the competitive mode of action of compound (XI)]. It is proposed that this mixed mode of inhibition results from the inhibitor binding to the enzyme in two distinct ways: firstly, as a competitive inhibitor when the 2,4-diaminopyrimidine ring binds to the corresponding portion of the substrate-binding site, and, secondly, as a non-competitive inhibitor in which the sulphonamido moiety becomes the major influence on the binding. The findings reported by Poe (1976) indicate that K_i^+ would be expected to be several orders of magnitude greater than the value of K_i, as observed. The existence of a site on the enzyme capable of binding sulphonamides is confirmed by the results for a series of substituted benzenesulphonamides binding to the enzyme (Table 4).

Table 3. Inhibition of *E. coli* M.R.E. 600 DHFR by 2,4-diaminopyrimidine derivatives



Compound	–R	–R′	Mode of inhibition	K_i (M)
(V)	–OH	$-CH_3$	Competitive	$3.3\times10^{-4}\pm2.0\times10^{-4}$
(VI)	$-O-[CH_2]_3-O-C_6H_4$-3-NO_2	$-CH_3$	Competitive	$1.6\times10^{-8}\pm1.3\times10^{-8}$
(VII)	$-O-[CH_2]_3-O-C_6H_4$-3-CF_3	$-CH_3$	Competitive	$5.3\times10^{-9}\pm2.9\times10^{-9}$
(VIII)	$-O-[CH_2]_3-O-C_6H_4$-2-CF_3	$-CH_3$	Competitive	$2.0\times10^{-10}\pm0.5\times10^{-10}$
(IX)	$-O-[CH_2]_3-O-C_6H_4$-2-CF_3	$-CH_2-CH_3$	Competitive	$5.4\times10^{-10}\pm1.1\times10^{-10}$
(X)	$-O-[CH_2]_3-O-C_6H_4$-2-C_6H_{11},4-$COCH_3$	$-CH_2-CH_3$	Competitive	$8.3\times10^{-11}\pm0.5\times10^{-11}$
(XI)	$-O-[CH_2]_3-O-C_6H_4$-2-CF_3,4-SO_2NH_2,HCl	$-CH_2-CH_3$	Mixed	$K_i \sim 9\times10^{-12}$ K_i^+: see the text

Table 4. Inhibition of *E. coli* M.R.E. 600 DHFR by 4-substituted benzenesulphonamides

Compound	R	Mode of inhibition	$10^3 \times K_i$ (M)
(XII)	H	No inhibitory effect	–
(XIII)	CH_3	Competitive	0.39±0.05
(XIV)	C_2H_5	Competitive	11.2±1.1
(XV)	n-C_3H_7	Competitive	1.8±0.6
(XVI)	n-C_4H_9	Competitive	3.7±0.9
(XVII)	NH_2	Non-competitive	11.9±3.7
(XVIII)	NH–CO–CH_3	Non-competitive	19.9±1.4
(XIX)	NO_2	Mixed	K_i = 0.15±0.01 K_i^+ = 8.3±2.6
(XX)	OH	Competitive	0.85±0.20
(XXI)	F	Competitive	3.4±0.7
(XXII)	Cl	Competitive	16.4±4.6
(XXIII)	Br	Competitive	3.5±1.5

These results indicate that the mode of action and K_i value vary widely with substituent. However, the range of K_i values presented is broadly similar to that measured by Poe (1976) for an alternative series of sulphonamides. One noteworthy point of difference is that Poe (1976) found 4-aminobenzenesulphonamide (XVII) to be a competitive inhibitor (with respect to dihydrofolate, $K_i = 24 \times 10^{-3}$ M), whereas the present study indicated compound (XVII) to be a non-competitive inhibitor ($K_i = 12 \times 10^{-3}$ M). This finding is supported by the observation that inhibitors carrying a 4-NHR substituent [compounds (XVII), (XVIII) and (XXVI)] all exhibit non-competitive inhibition. In addition, it was found that under the assay conditions used 4-nitrobenzenesulphonamide (XIX) was partially reduced to compound (XVII). The mixed inhibition observed for compound (XIX) is thus assumed to be due to competitive inhibition arising from 4-nitrobenzensulphonamide, with a non-competitive effect due to the 4-aminobenzenesulphonamide formed in the assay. It should be noted that the K_i^+ for compound (XIX) is closely similar to the K_i for compound (XVII).

The similarity between the measured K_i values for the benzenesulphonamides and benzenesulphonic acids (Table 5) suggests that the major contribution to the binding energy of these compounds comes from the interaction of the aromatic portion of the inhibitor with the enzyme. In view of the large difference in charge state of the two classes of inhibitor at pH 7.2, an electrostatic interaction important for binding would be predicted to result in large differences in observed K_i values. This indicates that these inhibitors are not mimicking the *p*-ABG moiety of the substrate and forming an electrostatic interaction with arginine-57 (Kuyper *et al.*, 1982).

Table 5. Inhibition of *E. coli* M.R.E. 600 DHFR by 4-substituted benzenesulphonic acids

Compound	R	Mode of inhibition	$10^3 \times K_i$ (M)
(XXIV)	H	No inhibitory effect	–
(XXV)	CH_3	Competitive	1.6±0.3
(XVI)	NH_2	Non-competitive	110±25
(XXVII)	OH	Competitive	1.1±0.5

Table 6. Inhibitor combinations tested against *E. coli* M.R.E. 600 DHFR

Inhibitor A	Inhibitor B
Combinations binding simultaneously to the enzyme	
2,4-Diaminopyrimidine (III)	*p*-ABG (IV)
2,4-Diaminopyrimidine (III)	4-Methylbenzene-sulphonamide (XIII)
2,4-Diaminopyrimidine (III)	4-Aminobenzene-sulphonamide (XVII)
p-ABG (IV)	TMP (II)
M&B 35902A (VI)	4-Methylbenzene-sulphonamide (XIII)
M&B 35902A (VI)	4-Aminobenzene-sulphonamide (XVII)
M&B 39434 (X)	4-Methylbenzene-sulphonamide (XIII)
M&B 39434 (X)	4-Aminobenzene-sulphonamide (XVII)
Combinations binding mutually exclusively to the enzyme	
MTX (I)	4-Methylbenzene-sulphonamide (XIII)
MTX (I)	4-Aminobenzene-sulphonamide (XVII)
TMP (II)	4-Methylbenzene-sulphonamide (XIII)
TMP (II)	4-Aminobenzene-sulphonamide (XVII)
p-ABG (IV)	4-Methylbenzene-sulphonamide (XIII)
p-ABG (IV)	4-Aminobenzene-sulphonamide (XVII)
p-ABG (IV)	M&B 38082A (VIII)
4-Methylbenzene-sulphonamide (XIII)	4-Aminobenzene-sulphonamide (XVII)

As an aid to determining the position and extent of the sulphonamide-binding site, the effect of different combinations of two inhibitors acting on the enzyme was monitored (see Table 6 for a list of inhibitor combinations examined). Results obtained from the combination experiments were analysed according to the graphical methods described by Dixon & Webb (1979) and Yonetani & Theorell (1964) (Figs. 2 and 3 respectively). Such experiments indicate whether a particular combination is synergistic (i.e. both inhibitors can bind simultaneously to the enzyme) or if the inhibitor binding is mutually exclusive. The binding of two synergistic inhibitors to the enzyme can occur with either positive or negative co-operativity, the distinction being made according to the value of the inhibitor interaction factors, α and β, obtained from the graphical analyses mentioned (Table 7). α refers to the interaction of two competitive inhibitors (or a non-competitive inhibitor and substrate) and indicates the change in affinity of one inhibitor for the enzyme (as judged by its K_i value) caused by the binding of the second. Similarly, β refers to the interaction of a competitive with a non-competitive inhibitor [see Segel (1975) for further details].

Binding of inhibitors to *L. casei* N.C.B. 6375 (MTX/R) DHFR

In an effort to localize the sulphonamide-binding site

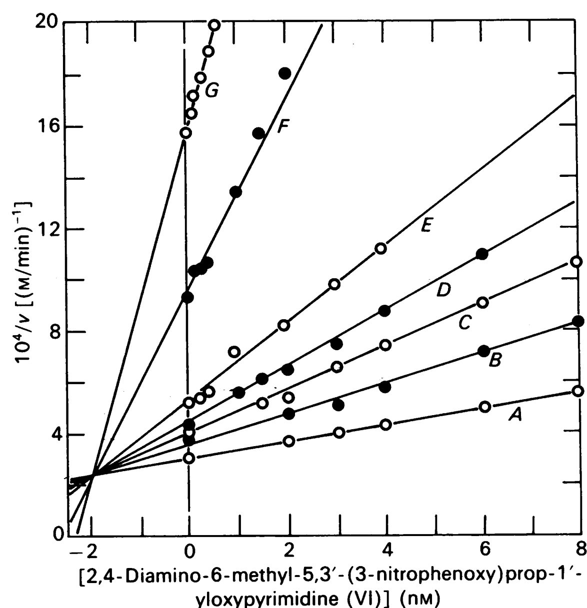


Fig. 2. Dixon plot for the binding of a combination of inhibitors (VI) and (XVII) to *E. coli* M.R.E. 600 DHFR

Concentrations of 4-aminobenzenesulphonamide (XVII) are: curve *A*, 0 M; curve *B*, 5 mM; curve *C*, 10 mM; curve *D*, 15 mM; curve *E*, 25 mM; curve *F*, 50 mM; curve *G*, 100 mM.

further, and to ascertain some of the residues involved in inhibitor binding, high-field ^{1}H-n.m.r. studies were performed. The *L. casei* N.C.B. 6375 (MTX/R) enzyme was chosen for these binding studies, since the effects of various ligands on this enzyme have already been studied

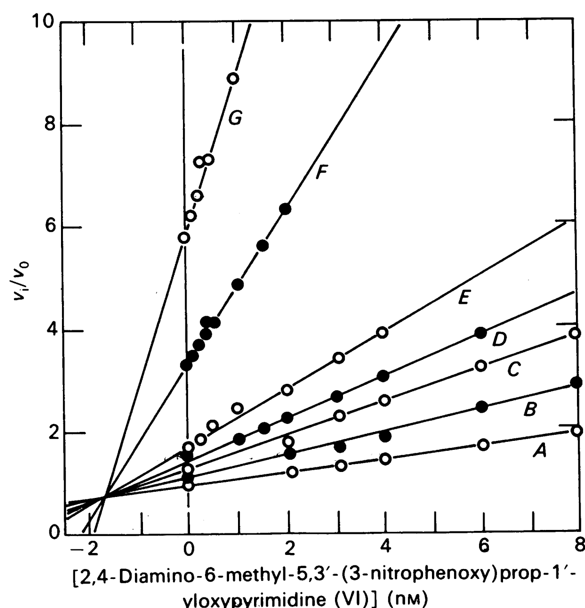


Fig. 3. Yonetani–Theorell plot for the binding of a combination of inhibitors (VI) and (XVII) to *E. coli* M.R.E. 600 DHFR

Concentrations of 4-aminobenzenesulphonamide (XVII) are: curve *A*, 0 M; curve *B*, 5 mM; curve *C*, 10 mM; curve *D*, 15 mM; curve *E*, 25 mM; curve *F*, 50 mM; curve *C*, 100 mM.

Table 7. Values for the inhibitor interaction factors, α and β, for synergistic combinations of inhibitors acting on *E. coli* M.R.E. 600 DHFR

For positive co-operativity: $\alpha < 1$, $\beta < 1$. For negative co-operativity: $\alpha > 1$, $\beta > 1$.

Inhibitor A	Inhibitor B	α	β
(III)	(IV)	0.09	–
(III)	(XIII)	2.15	–
(III)	(XVII)	3.27	2.14
(IV)	(II)	1.06	–
(VI)	(XIII)	4.37	–
(VI)	(XVII)	0.17	0.60
(X)	(XIII)	4.86	–
(X)	(XIII)	4.47	1.32

in detail by the use of n.m.r. (Roberts *et al.*, 1974; Way *et al.*, 1975; Feeney *et al.*, 1975, 1977*a*,*b*; Birdsall *et al.*, 1977*a*,*b*; Kimber *et al.*, 1977, 1978). A comparison of the effects of several inhibitors on both *E. coli* M.R.E. 600 DHFR and *L. casei* N.C.B. 6375 (MTX/R) DHFR is shown in Table 8. For each inhibitor the mode of inhibition is identical, and the K_i values are closely similar for binding to both enzymes. It would thus appear that the mode of binding to the two enzymes is comparable, suggesting that it is possible to discuss directly the results of n.m.r. studies on the *L. casei* enzyme with the inhibition studies on the *E. coli* enzyme.

In the n.m.r. binding studies portions of 4-methylbenzenesulphonamide (XIII) were added to the *L. casei* enzyme at pH 6.5 in 500 mM-KCl/50 mM-$KHPO_4$ solution. ^{1}H-n.m.r. spectra were recorded at 500 MHz on a Bruker AM500 spectrometer, and the interaction was monitored via the chemical-shift changes in the C-2–H resonances of the imidazole rings of the histidine residues (Birdsall *et al.*, 1977*a*).

Titration of the enzyme with increasing concentrations of compound (XIII) produced two effects (Fig. 4), the major effect corresponding to a large upfield shift in the resonance of H_C, and a smaller upfield shift of H_F. This is in comparison with the effect of *p*-ABG (IV) (Fig. 5), which produces a similar upfield shift in H_C but a large downfield shift in H_F. From previous work with *p*-ABG (Birdsall *et al.*, 1977*a*), it has been established that the effect on H_C (histidine-64) is due to binding of *p*-ABG (IV) in the adenine pocket of the coenzyme-binding site, whereas the effect on H_F is due to binding in the benzoylglutamate-binding site used by dihydrofolate.

The results of a competition experiment involving titration of the preformed enzyme–compound (XIII) binary complex with *p*-ABG are shown (Fig. 6). These data suggest that *p*-ABG and compound (XIII) share common binding sites in the vicinity of H_C and H_F. However, the H_F binding sites are non-identical, as evidenced by the absence of ring-current shifts on the aromatic protons of compound (XIII) when bound, compared with the substantial observed shifts in the aromatic protons of compound (IV) (Birdsall *et al.*, 1977*a*). From Fig. 6 the K_a value of compound (XIII) at the H_F site was estimated to be approx. $3.6\times10^{-3}\pm0.2\times10^{-3}$ M [compared with a value of

Table 8. Comparison of inhibitors acting on *E. coli* M.R.E. 600 DHFR an *L. casei* N.C.B 6375 DHFR

Conditions were as follows: 50 mM-Tris/HCl buffer, pH 7.2, containing 50 mM-KCl, 10 mM-2-mercaptoethanol and 1 mM-EDTA at 37.0 °C.

Inhibitor	*E. coli* M.R.E. 600 DHFR K_i (M)	*L. casei* N.C.B. 6375 DHFR K_i (M)	Mode of inhibition
(I)	6×10^{-12}	3×10^{-11}	Competitive
(II)	$1.8\times10^{-8}\pm0.2\times10^{-8}$	$1.22\times10^{-7}\pm0.03\times10^{-7}$	Competitive
(IV)	$2.6\times10^{-3}\pm0.8\times10^{-3}$	$2.9\times10^{-3}\pm1.4\times10^{-3}$	Competitive
(XIII)	$3.9\times10^{-4}\pm0.5\times10^{-4}$	$9.1\times10^{-4}\pm3.1\times10^{-4}$	Competitive
(XVII)	$1.2\times10^{-2}\pm0.4\times10^{-2}$	$4.0\times10^{-2}\pm1.4\times10^{-2}$	Non-competitive

$1.05\times10^{-3}\pm0.05\times10^{-3}$ M for *p*-ABG (Birdsall *et al.*, 1977*a*)]. The observed shifts of H_F are markedly different, despite the close similarity of the K_a values for compounds (IV) and (XIII), suggesting that compound (XIII) does not interact directly with the imidazole ring of histidine-28. This is consistent with the distinct electronic differences between compounds (IV) and (XIII). It was not possible to estimate the K_a value for compound (XIII) at site C from the data in Fig. 6. However, when 10 equivalents of either compound (IV) or compound (XIII) are added to the enzyme, H_C is shifted by similar amounts (0.215 and 0.210 p.p.m. respectively), which suggests that the two ligands are binding fairly similarly at site C (similar binding constants and similar bound shifts would explain the data). As the binding of these ligands at site C is relatively weak [K_a for *p*-ABG at this site is $4.29\times10^{-2}\pm0.16\times10^{-2}$ M (Birdsall *et al.*, 1977*a*)] compared with that for NADPH, this site can be considered to be unimportant in all measurements on inhibitor carried out in the presence of high concentrations of NADPH, as was the case in the spectrophotometric assay used in the *E. coli* binding studies.

Effect of pH on sulphonamide binding to *L. casei* N.C.B. 6375 (MTX/R) DHFR

The K_i values of compound (XIII), measured over the pH range 5.5–8.5 under conditions of ionic strength similar to those used for the n.m.r. studies, are shown (Fig. 7). From these data, the pK_a of the binding site in the free enzyme (pK_E) and in the enzyme–inhibitor binary complex (pK_{EI}) may be calculated. The values obtained were 6.67 and 7.33 respectively. These results would appear to implicate a histidine residue (or residues) in the pH-dependence of the sulphonamide binding [cf. a similar pK_a shift of H_F on the binding of compound (IV) was measured from n.m.r.: pK_E = 6.54 and pK_{EI} = 7.2 (Birdsall *et al.*, 1977*a*)]. This conclusion is supported by the n.m.r. titration of histidine C-2–H chemical shift as a

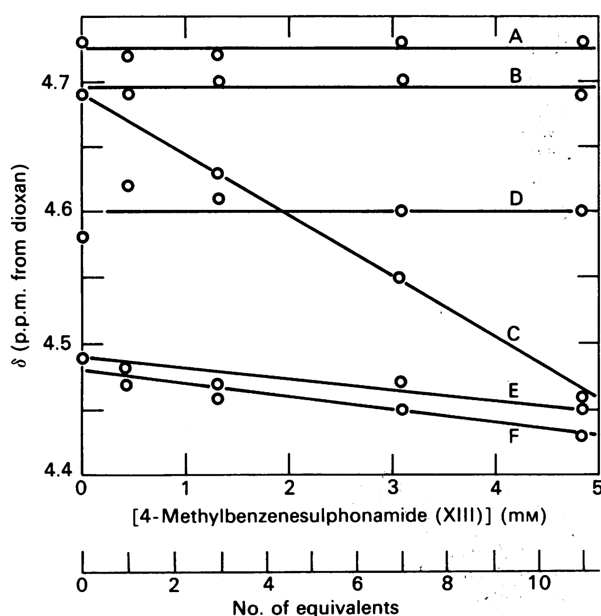


Fig. 4. Chemical shifts of six histidine residues in *L. casei* N.C.B. 6375 DHFR (1.6 mM) on titration of the enzyme with increasing concentrations of inhibitor (XIII)

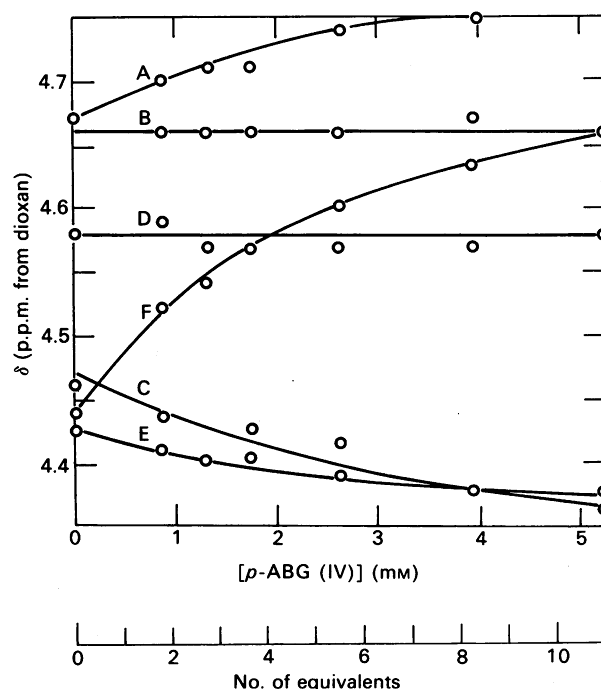


Fig. 5. Chemical shifts of six histidine residues in *L. casei* N.C.B. 6375 DHFR (1.06 mM) on titration of the enzyme with increasing concentrations of inhibitor (IV)

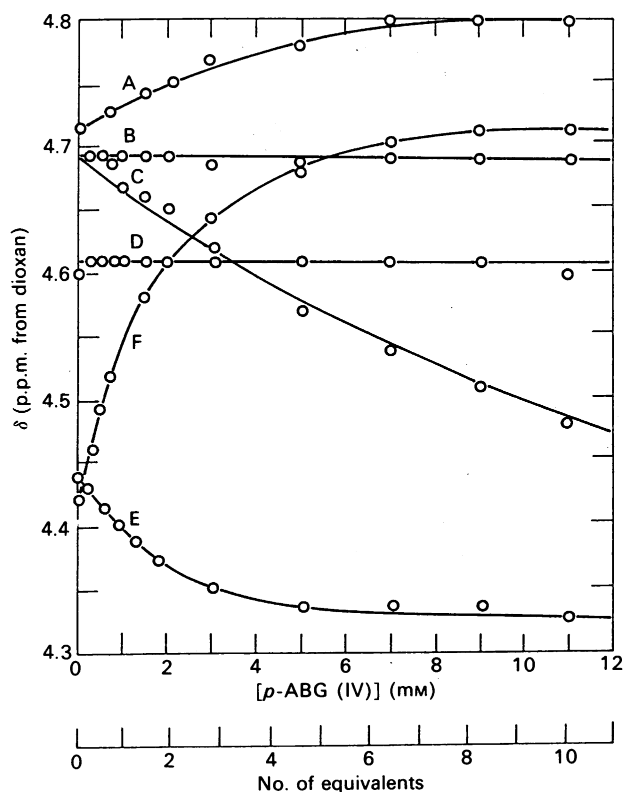


Fig. 6. Chemical shifts of six histidine residues in preformed binary complex of *L. casei* N.C.B. 6375 DHFR (0.44 mM) and inhibitor (XIII) (4.84 mM) on titration with increasing concentrations of inhibitor (IV)

function of sulphonamide concentration (Fig. 4), which indicates some small perturbations of histidine residues E and F on sulphonamide binding.

The agreement between pK_E and pK_{EI} found previously and in the present study is very close, even though different ligands were involved. This strongly suggests that, although the binding sites for compounds (IV) and (XIII) on *L. casei* DHFR are non-identical (as evidenced by differences in ring-current effects on the aromatic protons of the ligands and in the induced shifts at the C-2–H of H_F), histidine-28 forms a vital part of both sites.

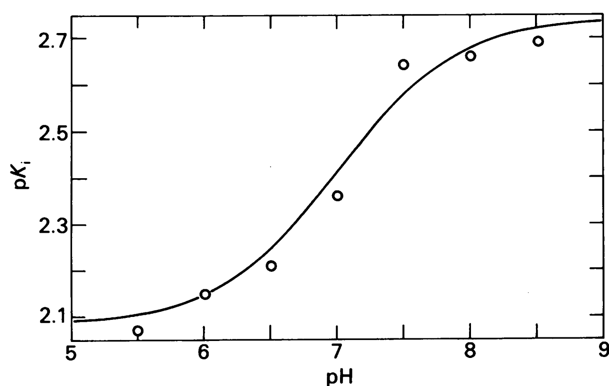


Fig. 7. pK_i values of inhibitor (XIII) measured at different pH values

CONCLUSION

The present study has confirmed the existence of a moderately potent sulphonamide-binding site on bacterial DHFR that appears to overlap the benzoylglutamate-binding site of the substrate. In order to be of the greatest use in a clinical chemotherapeutic regimen, the sulphonamide should be capable of binding to the site with positive co-operativity in the presence of other (e.g. 2,4-diaminopyrimidine-type) inhibitors. However, in the majority of cases examined here in which synergism was observed, the ligand binding showed negative co-operativity (Table 7). A knowledge of the exact position of the sulphonamide-binding site may allow the logical design of inhibitor combinations that experience no negative co-operativity in binding.

The n.m.r. studies reported in the present paper indicate that, although the sulphonamide (XIII) competes with *p*-ABG (IV), it does not form an electrostatic interaction with the charged imidazole ring of histidine-28, nor does its aromatic ring bind in the same binding pocket as the benzoyl ring of *p*-ABG. However, there are some small shifts in the C-2–H proton resonance of histidine-28 when the sulphonamide binds, which indicate that this residue is influenced to some extent by the binding.

The simplest explanation for the observed competition would be that the binding sites overlap, but are non-identical. The experiments described in the present paper were unable to probe any interactions involving the conserved arginine-57 with either the sulphonamide (XIII) or *p*-ABG (IV). Arginine-57 is known to interact with the α-CO_2^- group of the glutamate moiety of MTX (and also *p*-ABG by inference) and with TMP derivatives carrying carboxylate side chains of various lengths (Jones, 1980). Arginine-57 may interact indirectly with the sulphonamido group of compound (XIII), which would involve the aromatic ring being displaced from its binding site in the *p*-ABG–DHFR complex and being further away from phenylalanine-30, the residue responsible for the observed ring-current shifts seen for the aromatic protons of *p*-ABG. However, as the charge state of the ligand appears to have very little influence on K_i (for example, the sulphonamides and sulphonic acids have K_i values differing by only two orders of magnitude, whereas their pK_a values differ by 8 units or more), this potential interaction would appear to be relatively unimportant.

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