Comparison of the Inhibition of Methotrexate-Sensitive and -Resistant *Lactobacillus casei* Cell Cultures with Purified *Lactobacillus casei* Dihydrofolate Reductase by 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-*s*-triazines. Use of Quantitative Structure-Activity Relationships in Making Inferences about the Mechanism of Resistance and the Structure of the Enzyme in Situ Compared with the Enzyme in Vitro[1]

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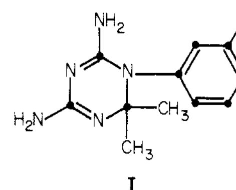
The inhibitory action of a set of 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(3-substituted-phenyl)-*s*-triazines on *Lactobacillus casei* dihydrofolate reductase is compared with their action on methotrexate-resistant and methotrexate-sensitive cell cultures by means of quantitative structure–selectivity analysis. The analysis uncovers major differences in the steric and hydrophobic interactions of the substituents X with the three different systems. Correlation analysis is used to define the hydrophobic binding site for 3-X in the isolated enzyme. This is shown to be similar to that of the sensitive cells but different from that in the resistant cells, which have a larger hydrophobic binding site. When X has the general structure 3-$CH_2ZC_6H_4$-Y (Z = O or NH), it is shown that Y does not interact with the isolated enzyme, but in the living cells, Y interacts with a molecular barrier in a way that can be quantitatively related to the molar refractivity of X. The methotrexate-resistant cells are resistant to highly hydrophilic inhibitors such as methotrexate but are not able to resist hydrophobic inhibitors. The results with the inhibition of *L. casei* dihydrofolate reductase are compared with the inhibition of enzyme from bovine liver.

In recent years, X-ray crystallographic studies have enormously increased our understanding of enzyme structure and of how certain ligands combine with these macromolecules. At the same time, there has been much discussion about whether such studies yield an accurate representation of the combination of enzyme and ligand in solution or in vivo. Such knowledge is not only important in the fields of biochemistry and molecular biology, it is of increasing importance in many of the modern approaches to drug and pesticide design. Initial studies are being made on purified enzymes in the search for potent inhibitors which can then be modified to make suitable drugs or pesticides. There is often a failure of correlation between data obtained with isolated enzymes and inhibitors and data obtained in vivo with the inhibitors. There has been surprisingly few careful studies of sets of substrates or inhibitors on purified enzymes which can then be compared with the in situ action of ligands with enzyme. We are initiating a systematic study of inhibitors of dihydrofolate reductase (DHFR) using correlation analysis[4] to produce quantitative structure–activity relationships (QSAR) to help us better understand differences in response of enzymes in vivo[5,6] and in vitro.[7-9] In order to obtain a comprehensive view of effective drug design, we believe that studies must be conducted on at least three levels: isolated enzyme, enzyme in cells, and enzyme in whole animals.

Studying the action of ligands on isolated enzyme, one can circumvent the problems of membrane penetration as well as cellular side reactions, while working with cells mitigates the more complex metabolic and distribution problem faced in whole-animal studies.

In the present study we compare QSAR for a set of triazines, I, acting on methotrexate-resistant and metho-



I

trexate-sensitive *L. casei* cells with QSAR obtained for the same compounds acting on purified DHFR from *L. casei*.

Previous studies[6] produced eq 1 for the 50% inhibition

$$\log 1/C = 0.53 (\pm 0.10)\, \pi_3 - 0.67 (\pm 0.35) \log (\beta \cdot 10^{\pi_3} + 1) + 0.79 (\pm 0.25)\, MR' + 3.13 (\pm 0.15) \quad (1)$$

$$n = 28;\ r = 0.949;\ s = 0.302;\ \pi_3 = 4.03;\ \log \beta = -3.46$$

in vitro of *L. casei* DHFR. In eq 1, π_3 is the hydrophobic constant[10] for substituents in the 3 position of I and β is a disposable parameter evaluated by an iterative process in the Kubinyi bilinear model[11] for structure–activity relationships; this model is essentially two straight lines dependent on the hydrophobicity of X as modeled by the hydrophobic parameter π (or log *P*) connected by a small region of curvature. The initial dependence of log 1/*C* on π in eq 1 has a slope of 0.53; a change in mechanism occurs at π_0, and the slope becomes –0.14 (0.53–0.67) for larger π values. The number of congeners in this expression on

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which the equation is based is represented by n, r is the correlation coefficient, s is the standard deviation from regression, and π_0 is the optimum π value producing maximum activity. It has recently been found that using the bilinear model to account for the dependence of activity on π often yields a better fit of the data than the so-called parabolic model ($\pi + \pi^2$).[12] Such is the case with the data used to derive eq 1, where C is the molar concentration of I causing 50% inhibition of purified *L. casei* DHFR in vitro (i.e., $C = K_{i\,app}$).[13,14] The term in MR′ is a special one which applies only to three congeners, where X of I = $-CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$; $-CH_2NHC_6H_4$-4′-SO_2NH_2, and $CH_2OC_6H_4$-3′-$NHCONH_2$. MR′ is the molar refractivity[10] of the substituents (Y) attached to the $-CH_2ZC_6H_4$-Y moiety and it is scaled by 0.1 to make it more nearly equiscalar with π, at least for apolar substituents. For these three examples, π for $CONH_2$, SO_2NH_2, and $NHCONH_2$ has been replaced by MR′. The positive coefficient with this term in eq 1 indicates that these compounds are more active than their hydrophobicity alone would lead one to expect. It was assumed that they are projecting beyond the hydrophobic pocket into which 3-X groups appear to bind.

Leaving these three congeners aside, what one finds with the remaining 25 is a simple relationship in which inhibitory power first increases linearly (slope 0.53) up to about $\pi = 4$. At this point a break occurs in the relationship and a new dependence of negative slope (−0.14) develops. This slope is so close to 0 that it was assumed that part of the substituents with π above 4 projected beyond the hydrophobic pocket into an aqueous phase and thus do not contact the enzyme.

Since three congeners are too few to support a firm conclusion, we have now made 11 more congeners of the type $-CH_2OC_6H_5$-3′-Y with which to further explore this problem. In addition, this extended QSAR is compared with QSAR obtained from the action of I on intact *L. casei* cells.

Experimental Section

Enzyme Assay. The procedure for obtaining the $K_{i\,app}$ values for *L. casei* DHFR is a modification[13] of our earlier procedure.[15] The 95% confidence limits on log $1/K_{i\,app}$ have been evaluated using the jackknife procedure.[14] None of the triazine inhibitors in this report are of the very tight binding type, so that the formation of E–I and E–NADPH–I complexes significantly changes the concentration of free inhibitor, such as those recently reported by Williams et al.,[16] nor do they approach stoichiometric inhibition. All of the evidence indicates that they are simple competitive inhibitors.[13] The DHFR from *Lactobacillus casei* was obtained from the New England Enzyme Center, Tufts University School of Medicine, as a lyophilized powder. The methotrexate-resistant strain of *L. casei* from which this enzyme is isolated was generously supplied by R. Kisliuk of Tufts University.

Bacterial Growth Inhibition. The procedures employed are a modification of those reported.[17] Methotrexate-sensitive *L. casei* (ATCC7469) was maintained by weekly passage in yeast extract peptone agar. The resistant strain was maintained by weekly passage in Flynn[18] agar containing 5 μg/mL methotrexate. Standard folic acid assay medium was prepared as previously described with the exception that the glucose and folic acid were sterilized separately and added aseptically at the time of the test. Basal medium (8 mL) was placed in each culture tube and sterilized by autoclaving at 121 °C for 10 min. Folic acid, diluted in 25% sterile glucose solution, was added in 1 mL quantities to give a final concentration of 0.001 μg/mL. Test compounds were dissolved in deionized water where possible or, alternatively, in 0.1 N HCl or Me_2SO. These stock solutions were diluted in sterile deionized water to give desired concentrations and added to the culture tubes in 1 mL quantities to give a final volume of 10 mL. The test inoculum was prepared by transferring twice in test medium. The first transfer was incubated for 24 h and the second one for 16–18 h at 37 °C. The resulting culture was centrifuged, washed twice, resuspended in 0.85% saline, and diluted to give a reading of 50 klett units (Klett–Summerson photoelectric colorimeter, 660-nm filter). To each tube of test medium was added 0.1 mL of this suspension, providing an initial concentration of approximately 3.5×10^5 to 6.0×10^5 cells per milliliter as determined by plate count. The cultures were incubated at 37 °C for 22 h, and turbidity was read in klett units.

Preliminary tests were made with each inhibitor to determine the range of activity. A second test was then run using five to six inhibitor concentrations, in triplicate, to establish the dose–response relationship. Activities as percent growth inhibition were plotted against concentration to establish linearity in the range of 20–80% inhibition. A dose–response equation was then calculated by the method of least squares and used to compute molar I_{50} values, which were then converted to the form log $1/C$ for correlation analyses. The 95% confidence intervals were derived from the confidence intervals associated with each dose–response equation in a manner similar to that used previously.[15]

Correlation Analysis. Our previously discussed general approach to the formulation of correlation equations was followed.[19] Stepwise regression analysis was not employed; instead, all possible equations were derived and considered.

The molar refraction parameter MR was first introduced into structure–activity analysis sometime ago[20] but received little attention until recently.[4,7–9] The values of MR (scaled by 0.1 to make them more nearly equiscalar with π), as well as σ and π constants, were taken from our previous work[4] or from our recent compilation.[10] Pauling and Pressman[20] viewed MR as a parameter for modeling dispersion forces, and this would of course be reasonable if the coefficient with MR had a positive sign. When such a coefficient has a negative sign, as in the present work, almost the only way to interpret the meaning is as some kind of steric effect due to the volume of the substituent

$$\mathrm{MR} = \left(\frac{n^2 - 1}{n^2 + 2}\right)\left(\frac{\mathrm{MW}}{d}\right)$$

where n is the index of refraction, MW is the molecular weight, and d is the density. The major contribution to MR comes from the volume part of the expression, MW/d.

Triazine Inhibitors. The syntheses for most of the inhibitors have been reported.[15] The details for compounds **31–41** will be published elsewhere. The purity of each inhibitor was established by thin-layer chromatography and carbon–hydrogen analysis.

Results

QSAR for Triazines Inhibiting Isolated DHFR. Equations 2–6 for the action of inhibitors I on purified

$$\log 1/K_{i\,app} = 0.28\ (\pm 0.14)\ \pi' + 3.75\ (\pm 0.34) \quad (2)$$

$$n = 44;\ r = 0.54;\ s = 0.844;\ F_{1,42} = 17.4$$

$$\log 1/K_{i\,app} = 0.29\ (\pm 0.07)\ \pi' + 1.56\ (\pm 0.26)\ I + 3.24\ (\pm 0.18) \quad (3)$$

$$n = 44;\ r = 0.918;\ s = 0.403;\ F_{1,41} = 14.3$$

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$$\log 1/K_{i\,app} = 0.46 (\pm 0.09)\, \pi' - 0.57 (\pm 0.25) \log (\beta \cdot 10^{\pi'} + 1) + 1.38 (\pm 0.23)\, I + 3.16 (\pm 0.16) \quad (4)$$

$n = 44$; $r = 0.947$; $s = 0.333$; $\pi_0 = 4.39$ (3.67–5.11); $\log \beta = -3.75$; $F_{2,39} = 10.5$

$$\log 1/K_{i\,app} = 0.53 (\pm 0.11)\, \pi' - 0.64 (\pm 0.25) \log (\beta \cdot 10^{\pi'} + 1) + 1.49 (\pm 0.25)\, I + 0.70 (\pm 0.65)\, \sigma + 2.93 (\pm 0.26) \quad (5)$$

$n = 44$; $r = 0.953$; $s = 0.319$; $\pi_0 = 4.31$ (3.71–4.91); $\log \beta = -3.66$; $F_{1,38} = 4.73$

$$\log 1/K_{i\,app} = 0.52 (\pm 0.10)\, \pi' - 0.64 (\pm 0.24) \log (\beta \cdot 10^{\pi'} + 1) + 1.80 (\pm 0.40)\, I + 0.68 (\pm 0.63)\, \sigma - 0.27 (\pm 0.28)\, MR_Y + 2.94 (\pm 0.25) \quad (6)$$

$n = 44$; $r = 0.958$; $s = 0.308$; $\pi_0 = 4.33$ (3.72–4.94); $\log \beta = -3.68$; $F_{1,37} = 3.75$

DHFR from the methotrexate-resistant strain of *L. casei* bacteria have been formulated from the data in Table I. Log $1/K_{i\,app}$ in these expressions is equivalent to log $1/C$ used in our previous work,[6] π' is the normal π value for aromatic substituents[10] except for those congeners of the type $-CH_2Z-C_6H_5-Y$ (**25**, **29**, and **30–41**, Table I) where π_Y is set equal to 0 and, hence, π' in these 14 examples refers to π for either $-CH_2NHC_6H_5$ or $-CH_2OC_6H_5$. The indicator variable I is given the value of 1 for the congeners containing the $-CH_2Z-$ bridge. Except for **41** (Y = 3-$NHCSNH_2$), all of these congeners are reasonably well fit, regardless of whether the bridge is $-CH_2NH-$ or $-CH_2O-$ or whether Y is hydrophilic or hydrophobic. Equation 5 is a marginal improvement over eq 4. The last term to enter eq 6 in the stepwise development is MR_Y which is very weak—note the F value and large confidence limits. The simplest conclusion is that Y of $-CH_2Z-C_6H_4-Y$ does not contact the enzyme in either a hydrophobic or steric sense.

The rather large coefficient (1.38) with the indicator variable in eq 4 shows that, other factors being constant, the introduction of the two types of bridges, $-CH_2O-$ and $-CH_2NH-$, between two phenyl groups increases the inhibitory power by a factor of 25 (antilog of 1.38). This is especially interesting in view of the fact that the reverse order $-OCH_2-$ compounds (**20** and **31**) do not show such activity.

A substituent whose inhibitory power we normally underestimate[6] with both bacterial and mammalian DHFR is 3-CN. This group appears to have a specific interaction with DHFR.

QSAR for Triazines Inhibiting Methotrexate-Resistant *L. casei* Cell Culture. Equations 1–6 were formulated from data obtained using DHFR isolated from *L. casei* cells resistant to methotrexate. It is of interest to compare the QSAR from the isolated enzyme with that obtained from the living organism. As yet there is little clear evidence that isolated enzyme in buffer solution and enzyme in situ in the living cell show the same reaction pattern with a set of inhibitors. Equations 7–9 have been developed from the data of Table I. C in these expressions is the molar concentration which produces 50% inhibition of growth in the resistant *L. casei* cell culture measured at the end of 22 h, and π refers to the complete 3-X substituent, including Y. Using π' as in eq 2–6 does not give as good results nor would one expect it to since in the whole-cell study the drugs must make their way through the lipophilic bacterial membranes to the sites where the DHFR resides. Two extremely interesting differences between eq 9 and eq 4 are evident: the MR_Y term is now highly significant and it bears a negative coefficient; the dependence on π is linear, not bilinear. Neither the addition of a π^2 nor bilinear term in π to eq 8 or 9 resulted in an improved correlation, which indicates that π_0 is at least >5.9 (π for the most hydrophobic substituent). These results are similar to those found by Walsh et al.;[21] using their data for triazines of type I, we found[6] $\pi_0 = 5.79$ for *Staphylococcus aureus* cells and 5.07 for *Escherichia coli* cells. More recently, Wooldridge,[22] in a study of both 3- and 4-substituted I, derived eq 10, where C is the MIC for action against *S. aureus*.

$$\log 1/C = 0.42 (\pm 0.06)\, \pi + 3.57 (\pm 0.17) \quad (7)$$

$n = 38$; $r = 0.911$; $s = 0.401$; $F_{1,36} = 176$

$$\log 1/C = 0.44 (\pm 0.05)\, \pi + 0.51 (\pm 0.22)\, I + 3.36 (\pm 0.17) \quad (8)$$

$n = 38$; $r = 0.946$; $s = 0.320$; $F_{1,35} = 21.8$

$$\log 1/C = 0.45 (\pm 0.05)\, \pi + 1.05 (\pm 0.33)\, I - 0.48 (\pm 0.24)\, MR_Y \quad (9)$$

$n = 38$; $r = 0.964$; $s = 0.264$; $F_{1,34} = 17.1$

$$\log 1/C = 0.60\pi - 1.89 \log (\beta \cdot 10^{\pi} + 1) + 2.84 \quad (10)$$

$n = 66$; $r = 0.963$; $s = 0.344$; $\pi_0 = 5.86$

The slope of the π term in eq 9 is not much different from the slope of the corresponding π term in eq 4; that is, the membranes of the intact *L. casei* do not cause a change in the dependence of activity on hydrophobicity. The fact that π_0 for intact cells is greater than π_0 for isolated enzyme suggests that long hydrophobic groups [e.g., $O(CH_2)_{11}CH_3$] extend beyond the enzyme to make hydrophobic contact with other cellular material.

The coefficients with the term in I in eq 4 and 9 are similar, bringing out the similar role for the $-CH_2Z-$ bridge in vitro and in vivo. The $-CH_2Z-$ bridge in the purified enzyme makes variations of I about twice as potent as in vivo.

The intriguing feature of eq 9 is the term in MR_Y with its negative coefficient. Equations 2–4 lead us to believe that Y of $-CH_2Z-C_6H_4-Y$ does not contact the enzyme in its purified form in buffered solution. If indeed they do, the effect is very slight. The negative coefficient with MR_Y in eq 9 reflects a steric effect of Y in the binding of congeners with this function to enzyme in the cell. That is, we are assuming that MR_Y is primarily a measure of molar volume.[10] Whereas Y in the isolated enzyme appears to make no contact with the enzyme (eq 5), Y inhibits the binding with enzyme in situ. Inhibition could be caused by a macromolecule or membrane loosely bound at the point where Y projects beyond the DHFR. Inhibition might also be the result of a conformational change in the reductase, which could loosely block the access of Y to the region beyond the enzyme.

Another perspective of the data upon which eq 9 is based can be had by factoring it into two groups which I in eq 9 has allowed us to merge. Equation 11 correlates con-

$$\log 1/C = 0.45 (\pm 0.07)\, \pi - 0.48 (\pm 0.16)\, MR_Y + 4.40 (\pm 0.24) \quad (11)$$

$n = 14$; $r = 0.983$; $s = 0.160$

$$\log 1/C = 0.43 (\pm 0.06)\, \pi + 3.38 (\pm 0.17) \quad (12)$$

$n = 24$; $r = 0.958$; $s = 0.301$

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Table I. Parameters Used for Deriving Equations 4-17

		L. casei DHFR: $K_{i\,app}$ [a]		*L. casei* cells: log 1/*C*							
				methotrexate sensitive		methotrexate resistant					
no.	X	obsd [b]	calcd [c]	obsd [b]	calcd [d]	obsd [b]	calcd [e]	π'	π	*I*	MR_Y
1	3-SO_2NH_2	1.82 (1.77-1.87)	2.31	2.35 (1.22-2.81)	2.63			−1.82	−1.82	0	0
2	3-$CONH_2$	2.47 (2.42-2.51)	2.47	2.96 (2.26-3.50)	3.01			−1.49	−1.49	0	0
3	H	2.64 (2.61-2.66)	3.16	4.25 (3.50-5.07)	4.47	3.31 (1.38-4.79)	3.37	0.00	0.00	0	0
4	3-$COCH_3$	2.87 (2.82-2.91)	2.90	4.05 (3.78-4.34)	3.99	3.31 (1.02-4.66)	3.14	−0.55	−0.55	0	0
5	3-CH_3	3.07 (3.04-3.10)	3.42			3.82 (3.64-3.99)	3.62	0.56	0.56	0	0
6	3-OCH_3	3.12 (3.09-3.15)	3.15	4.46 (4.23-4.67)	4.46	2.98 (2.86-3.10)	3.37	−0.02	−0.02	0	0
7	3-OH	3.19 (3.16-3.22)	2.85	3.54 (3.26-3.79)	3.88	3.43 (3.28-3.57)	3.08	−0.67	−0.67	0	0
8	3-$C(CH_3)_3$	3.20 (3.16-3.23)	4.07					1.98	1.98	0	0
9	3-$COOC_2H_5$	3.21 (3.18-3.23)	3.39	4.51 (4.11-4.83)	4.87	3.06 (2.98-3.13)	3.60	0.51	0.51	0	0
10	3-SO_2F [f]	3.21 (3.18-3.24)	3.18	3.16 (2.47-3.75)	4.52			0.05	0.05	0	0
11	3-F	3.29 (3.23-3.35)	3.22	4.91 (4.63-5.22)	4.59	3.32 (2.87-3.67)	3.44	0.14	0.14	0	0
12	3-CF_3	3.29 (3.23-3.34)	3.57	4.86 (4.58-5.15)	5.11	3.22 (3.05-3.39)	3.76	0.88	0.88	0	0
13	3-Cl	3.45 (3.40-3.51)	3.49	5.01 (4.91-5.23)	5.00	3.94 (3.67-4.24)	3.68	0.71	0.71	0	0
14	3-NO_2	3.56 (3.54-3.59)	3.03	4.37 (2.78-5.12)	4.24	2.97 (2.79-3.14)	3.25	−0.28	−0.28	0	0
15	3-CN	3.68 (3.64-3.72)	2.89	4.89 (4.41-5.47)	3.97	3.70 (2.81-4.32)	3.13	−0.57	−0.57	0	0
16	3-Br	3.69 (3.66-3.72)	3.56	5.01 (4.65-5.97)	5.10	3.86 (0.03-4.98)	3.75	0.86	0.86	0	0
17	3-I	3.73 (3.69-3.76)	3.68	5.11 (4.92-5.38)	5.26	3.58 (3.36-3.84)	3.86	1.12	1.12	0	0
18	3-$O(CH_2)_2OC_6H_5$	3.74 (3.69-3.78)	3.94					1.68	1.68	0	0
19	3-$O(CH_2)_2OC_6H_4$-3′-CF_3	4.10 (4.07-4.13)	4.33	5.73 (5.60-5.85)	5.87	4.27 (4.12-4.41)	4.48	2.56	2.56	0	0
20	3-$OCH_2C_6H_5$	4.20 (4.18-4.23)	3.93	5.43 (4.94-5.92)	5.55	4.34 (2.44-5.07)	4.09	1.66	1.66	0	0
21	3-$O(CH_2)_3CH_3$	4.20 (4.16-4.23)	3.89	5.77 (5.71-5.91)	5.51	4.05 (2.95-4.68)	4.06	1.59	1.59	0	0
22	3-$OCH_2C_6H_3$-3′,4′-Cl_2	4.41 (4.33-4.47)	4.47			4.73 (4.49-4.97)	4.64	2.91	2.91	0	0
23	3-$O(CH_2)_{11}CH_3$	4.63 (4.58-4.67)	4.67	5.51 (5.45-5.57)	5.57	5.69 (5.49-5.78)	5.70	5.91	5.91	0	0
24	3-$O(CH_2)_8CH_3$	4.71 (4.69-4.73)	4.78	5.96 (5.85-6.15)	6.01	5.48 (4.79-5.60)	5.23	4.29	4.29	0	0
25	3-$CH_2NHC_6H_3$-3′,5′-$(CONH_2)_2$	4.74 (4.70-4.78)	5.00	4.60 (4.46-4.73)	4.20	2.76 (1.74-3.51)	2.90	1.00	−1.34	1	1.96
26	3-$O(CH_2)_4OC_6H_5$	4.79 (4.76-4.82)	4.39	5.94 (5.68-6.20)	5.91	4.50 (3.99-4.85)	4.55	2.71	2.71	0	0
27	3-$O(CH_2)_4OC_6H_4$-3′-CF_3	4.79 (4.76-4.81)	4.69					3.59	3.59	0	0
28	3-$(CH_2)_5CH_3$	4.96 (4.93-5.00)	4.58	6.45 (6.29-6.62)	6.00	5.02 (4.63-5.13)	4.77	3.21	3.21	0	0
29	3-$CH_2NHC_6H_4$-4′-SO_2NH_2	5.04 (5.00-5.07)	5.00	4.85 (4.39-5.52)	4.91	3.44 (−20.30-5.45)	3.48	1.00	−0.82	1	1.23
30	3-$CH_2OC_6H_4$-3′-$NHCONH_2$	5.36 (5.28-5.43)	5.30	5.49 (5.15-5.82)	5.12	4.30 (2.70-5.03)	3.93	1.66	0.36	1	1.37
31	3-$CH_2OC_6H_5$	5.44 (5.42-5.46)	5.30	6.00 (5.98-6.01)	6.36	5.07 (4.82-5.24)	5.11	1.66	1.66	1	0.10
32	3-$CH_2OC_6H_4$-3′-Cl	5.41 (5.39-5.43)	5.30	6.20 (6.18-6.21)	5.87	5.24 (5.16-5.32)	5.17	1.66	2.37	1	0.60
33	3-$CH_2OC_6H_4$-3′-CN	5.31 (5.28-5.35)	5.30	5.42 (5.16-5.73)	5.84	4.60 (4.09-4.93)	4.61	1.66	1.09	1	0.63
34	3-$CH_2OC_6H_4$-3′-OCH_3	5.56 (5.52-5.56)	5.30	5.79 (5.61-5.97)	5.69	4.85 (4.65-5.01)	4.77	1.66	1.64	1	0.79
35	3-$CH_2OC_6H_4$-3′-CH_2OH	5.45 (5.41-5.48)	5.30			4.41 (4.32-4.50)	4.36	1.66	0.63	1	0.72
36	3-$CH_2OC_6H_4$-3′-CH_3	5.33 (5.29-5.38)	5.30			4.93 (4.79-5.06)	5.12	1.66	2.22	1	0.57
37	3-$CH_2OC_6H_4$-3′-C_2H_5	5.31 (5.28-5.35)	5.30	6.06 (6.06-6.07)	5.45	5.02 (4.77-5.20)	5.10	1.66	2.68	1	1.03
38	3-$CH_2OC_6H_4$-3′-$CH(CH_3)_2$	5.41 (5.40-5.43)	5.30			5.07 (4.89-5.24)	5.10	1.66	3.21	1	1.50
39	3-$CH_2OC_6H_4$-3′-$C(CH_3)_3$	5.44 (5.41-5.47)	5.30			5.28 (4.99-5.58)	5.08	1.66	3.64	1	1.92
40	3-$CH_2OC_6H_4$-3′-$NHCOCH_3$	5.45 (5.47-5.51)	5.30	4.54 (4.37-4.72)	5.01	3.91 (3.70-4.17)	4.01	1.66	0.69	1	1.49
41	3-$CH_2OC_6H_4$-3′-$NHCSNH_2$	4.36 (4.34-4.39)	5.30	3.79 (3.65-3.93)	4.30	3.32 (−61.07-5.65)	3.47	1.66	0.26	1	2.22
42	3-$O(CH_2)_{10}CH_3$	4.54 (4.52-4.57)	4.72	5.51 (5.20-5.61)	5.78	5.96 (5.93-5.98)	5.70	5.37	5.37	0	0
43	3-$O(CH_2)_{12}CH_3$	4.81 (4.79-4.83)	4.61	5.31 (5.12-5.41)	5.29	6.02 (6.02-6.02)	6.17	6.45	6.45	0	0
44	3-$O(CH_2)_{13}CH_3$	4.50 (4.46-4.54)	4.56	5.11	4.96	6.06 (6.06-6.07)	6.40	6.99	6.99	0	0

[a] See Hansch and Leo[10] and Kubinyi[11] for procedure used to calculate $K_{i\,app}$. [b] Figures in parentheses are 95% confidence limits. [c] Calculated using eq 4. [d] Calculated using eq 16. [e] Calculated using eq 9. [f] This point not used in the derivation of eq 9.

geners where X = $-CH_2ZC_6H_5$-Y and eq 12 correlates the remaining 24 congeners. In each of these results we find only a linear dependence of activity on π with essentially the same slope. MR_Y in eq 11 has the same importance as in eq 9. These results justify our merging of the two sets of data to obtain eq 9.

QSAR for Triazines Inhibiting Methotrexate-Sensitive *L. casei* Cells. We have derived correlation eqs 13–17 from the data in Table I. Equations 16 and 17 are the two best equations.

$$\log 1/C = 0.30\ (\pm 0.13)\ \pi'_3 + 4.57\ (\pm 0.33) \quad (13)$$

$$n = 34;\ r = 0.595;\ s = 0.753;\ F_{1,32} = 17.5$$

$$\log 1/C = 0.79\ (\pm 0.18)\ \pi'_3 - 0.10\ (\pm 0.03)\ \pi'^2_3 + 4.42\ (\pm 0.22) \quad (14)$$

$$n = 34;\ r = 0.862;\ s = 0.482;\ F_{1,31} = 47.1;\ \pi_0 = 3.91\ (3.4\text{–}4.6)$$

$$\log 1/C = 0.59\ (\pm 0.16)\ \pi'_3 - 0.16\ (\pm 0.03)\ \pi'^2_3 - 0.45\ (\pm 0.25)\ MR_Y + 4.55\ (\pm 0.20) \quad (15)$$

$$n = 34;\ r = 0.907;\ s = 0.407;\ F_{1,30} = 13.5;\ \pi_0 = 3.69\ (3.4\text{–}4.1)$$

$$\log 1/C = 0.82\ (\pm 0.14)\ \pi'_3 - 0.11\ (\pm 0.02)\ \pi'^2_3 - 0.97\ (\pm 0.38)\ MR_Y + 0.91\ (\pm 0.51)\ I + 4.47\ (\pm 0.17) \quad (16)$$

$$n = 34;\ r = 0.937;\ s = 0.343;\ F_{1,29} = 13.3;\ \pi_0 = 3.81\ (3.5\text{–}4.3)$$

$$\log 1/C = 0.80\ (\pm 0.15)\ \pi' - 1.06\ (\pm 0.27)\ \log(\beta \cdot 10^{\pi'} + 1) - 0.94\ (\pm 0.39)\ MR_Y + 0.80\ (\pm 0.56)\ I + 4.37\ (\pm 0.19) \quad (17)$$

$$n = 34;\ r = 0.929;\ s = 0.371;\ \pi_0 = 2.94\ (1.77\text{–}4.11);\ \log \beta = -2.45$$

Although eq 17 is not as good a fit of the data, it has been included for comparison with eq 4 and 9. These equations resemble eq 4 for the isolated enzyme more than eq 9 for the inhibition of methotrexate-resistant *L. casei* cells. Using the modified π' values in these two equations gives a better correlation than using π for all of 3-X. If π is used in eq 16 in place of π', the resulting equation has $r = 0.900$, $s = 0.431$, and $\pi_0 = 3.98$ (3.47–4.84). If the same substitution is made in eq 17, the resulting equation has $r = 0.900$, $s = 0.435$, and $\pi_0 = 3.48$ (2.42–4.54). All of these results indicate π_0 for the sensitive cells to be much lower than for the resistant cells. Equations 16, 17, and 9 all have a highly significant negative term in MR_Y.

Discussion

Considering the complexity of the molecular modifications of I in Table I, the heterogeneity of the amino acid residues which constitute the region around the binding sites of the DHFR and how these may differ in vitro and in vivo, it is gratifying that by means of a few variables we can develop correlation equations to account for the variations in the inhibitory power of a wide variety of inhibitors. It is clear both from this work and earlier studies[6] that the hydrophobic interactions of 3-X are obviously of great importance, both in the union of inhibitor with DHFR and in the penetration to the point of location of the DHFR. An important point of theoretical as well as practical difference is the much higher π_0 with the methotrexate-resistant cells, which is in sharp contrast to the limited hydrophobic pockets uncovered by π_0 of about 4 or less for both the isolated enzyme and the enzyme in situ in the methotrexate-sensitive cells. With the sensitive cells, initial increases in hydrophobicity over that of the parent compound increases potency until the exponential term ($-0.11\pi^2$) becomes significant. A maximum potency due solely to hydrophobicity of 3-X alone occurs with the sensitive cells at 3.81 (which is close to 4 for the isolated enzyme). Introduction of this figure into eq 4 shows that the maximum increase (compared to $\pi = 0$) in $\log 1/C$ would be 1.6 log units. With resistant cells, π_0 is about 5.9 so that the maximum contribution would be about 2.5. Inspection of the data in Table I shows that this is actually realized. The sensitive cells interact with the inhibitor much more like isolated enzyme, despite the fact that the isolated enzyme comes from resistant cells! The slope of the positive π term for the sensitive cells is greater than that for the isolated enzyme, suggesting the importance of lipophilic character for penetration of the lipophilic membranes. Nevertheless, one soon reaches the maximum hydrophobic effect at about $\pi = 4$.

If, in the living cells, all of the inhibitory effect can be attributed only to the triazines blocking the action of DHFR, then the assumption of a larger hydrophobic space in which 3-X can interact in the resistant cells is in order. Of course it is possible that some side effect in the resistant cells (possibly another enzyme) accounts for the activity of congeners I with large hydrophobic groups.

A likely explanation for the larger effective hydrophobic region in the resistant cells is that DHFR is bound to membrane or a lipophilic macromolecule in such a way that large groups extend beyond the hydrophobic pocket of the enzyme and into a hydrophobic region of the adjacent macromolecule.

What makes cells resistant to methotrexate and certain other 2,4-diaminopyrimidines? This is a point of great interest in both bacterial and cancer chemotherapy. Schimke and his colleagues have shown[23,24] that growing cells in methotrexate leads to huge increases in the production of DHFR by gene amplification. It has often been assumed that it is this large excess of DHFR which protects tumor cells from DHFR inhibitors; however, it is also known that folic acid, methotrexate, and some of its analogues enter *L. casei* cells as well as mammalian cells via active transport.[26,27] It may be that resistance to methotrexate occurs by changes in the active-transport system. Our results now clearly show that the hydrophobic character of methotrexate-resistant cells differs from that of methotrexate-sensitive cells and that the surroundings of the DHFR molecule may differ in the sensitive and resistant cells in *L. casei*. Thus, there appear to be a number of mechanisms by which resistant cells could protect themselves from DHFR inhibitors.

It is of interest to note the great resistance of cells adapted to methotrexate. Log $1/C$ for the resistant cells is 2.85 (2.51–3.39), while log $1/C$ for the sensitive cells is 10.90 (10.908–10.914). One needs an increase of 10^8 in methotrexate concentration to obtain a 50% inhibition of the resistant cells compared to the sensitive cells. It seems highly unlikely that this resistance could be due solely to the increase in DHFR (on the order of a few hundred times that in sensitive cells) produced by resistant cells. Lipophilicity alone could play an important role. Log P for

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methotrexate at pH 7.4 is ca. −3.00, while log P for a potent inhibitor (**23**) in Table I is 2.84. Assuming no stereoelectronic differences affecting inhibitory potency between methotrexate and the triazines, one can calculate a difference in potency of 0.45 (3.00 + 2.84) = 2.63. Even adding 2 log units for the extra DHFR, this represents a potency difference of about 5 log units, rather far from the 8 observed. Hence, it would seem to us that lack of an effective transport mechanism in resistant cells must also be involved in the explanation for the amazing resistance to methotrexate.

Equation 9 shows that hydrophobicity is the most important parameter in determining activity of I against resistant cells. The most weakly active compounds are the examples where X = H or $COCH_3$ (with log P values of −1.08 and −1.63). The congener X = SO_2NH_2 (log P = −2.90) was too inactive to obtain reliable results. However, our results show that quite potent congeners active against resistant cells can be made simply by making X very lipophilic. The most hydrophobic congeners have a log $1/C$ of 6 against resistant cells. The most potent congener active against the sensitive cells has a log $1/C$ only a little higher (6.5). No doubt even more potent congeners for the resistant cells could be made by the introduction of additional substituents in position 4.

This insight into the role of hydrophobicity in increasing the toxicity of 4,6-diamino-*s*-triazines to methotrexate-resistant cells will be, we believe, of value in the design of better antitumor agents. Hydrophobicity alone is not the complete answer, since Walsh[21] noted that very lipophilic variations of I highly active against bacteria in vitro were not active on bacteria in mice, presumably because such lipophilic congeners bind too tightly to serum protein. We have found that organic compounds bind to serum albumin in proportion to their log P values.[28] Still, we believe that the QSAR paradigm can be employed to surmount this difficulty.

Greco and Kakala[29] have discussed the problem of tumor cell resistance to methotrexate and noted that such cells take up the drug very slowly. They showed that such resistant cells are attacked by lipophilic DHFR inhibitors and that there is a correlation between drug potency and log P. Rosowsky et al.[30] have also found that a human tumor cell line which is transport resistant to methotrexate retains its sensitivity to lipophilic derivatives of methotrexate. Thus, it would seem that combination chemotherapy in cancer using methotrexate and a more lipophilic DHFR inhibitor might be advantageous.

The importance of the indicator variable I, which takes the value of 1 for compounds with the $-CH_2Z-$ bridge between the two phenyl rings and 0 for all other compounds, is clear in every instance. This structural feature increases the potency of I by about tenfold in the case of the methotrexate-resistant cells and by a factor of 25 in the purified enzyme reacting in buffer solution. Moreover, the geometry of the bridge (possibly with the help of the lone-pair electrons on O) places Y in such a position that it does not appear to contact the enzyme in its purified form in solution, despite the fact that π_0 would indicate a hydrophobic pocket large enough for these congeners. In the living cells the bridges place Y so that it appears to meet with a barrier, the effect of which is brought out by the negative MR term in eq 9, 16, and 17. The effect of MR is three times greater in the sensitive cells than it is in the resistant cells. The I term produces its maximum contribution in the isolated enzyme where Y does not run into steric hindrance.

A bridge which would provide almost the same geometry as $-CH_2O-$ is $-OCH_2-$. Nevertheless, this latter bridge does not require I to produce a good fit. This is especially noticeable in the case of eq 4 for isolated enzyme, where the coefficient with I is large (1.38). Two congeners, 3-$OCH_2C_6H_5$ and 3-$OCH_2C_6H_5$-3′,4′-Cl_2, are both well fit by eq 4 . These same two congeners are well fit by eq 9 for the resistant cells; however, only one of these (3-$OCH_2C_6H_5$) was tested on the sensitive cells. Since the geometry between the two phenyl moieties would be much the same using either $-OCH_2-$ or $-CH_2O-$, the greater potency associated with the latter suggests a special role for the O or NH between the phenyl rings. We plan molecular graphics studies to uncover the reasons behind the striking difference in the two similar bridges.

The present results indicate that our previous hypothesis about the significance of MR in eq 1 is wrong. It is the $-CH_2Z-$ bridge which is the root cause of the unusual activity of congeners **25**, **29**, and **30**–**41** of Table I. The bridge places the phenyl moiety in such a position that its substituents cannot contact the hydrophobic pocket; hence, these substituent groups have essentially no effect in the case of the isolated enzyme. In contrast with DHFR in situ, in the living cells, groups on this second phenyl ring have a negative effect regardless of whether they are hydrophobic or hydrophilic in character. It is difficult to interpret the origin of this effect as being anything other than steric in nature. In the living cell, either the conformation of DHFR is changed to restrict the fit of Y to the enzyme or DHFR is bound to a membrane or other macromolecule in such a way that Y encounters a barrier in place of the open space found with DHFR in vitro.

This was a surprising finding, since a π_0 of about 4 in eq 4 certainly establishes the presence of a rather large apolar binding space with capacity to accommodate most of the groups 3-$CH_2OC_6H_5$-Y. Groups of the type 3-O-$(CH_2)_nCH_3$ bind hydrophobically up to $\pi \sim 4$ in the sensitive cells and up to π of almost 6 in the resistant cells. Also, groups with more flexible bridges, such as $-O(CH_2)_2OC_6H_5$, $-O(CH_2)_2OC_6H_4$-3′-CF_3, and $-O(CH_2)_4OC_6H_5$, are well fit without the help of I.

Since it is tacitly assumed that the DHFR in sensitive and resistant cells has the same structure, our results embodied in eq 4, 9, 16, and 17 show that the environment in which the enzyme is placed has an important effect on how it interacts with inhibitors and, by implication, with substrates. These differences may be due to contiguous macromolecules with which portions of the inhibitors come into contact and/or actual changes in the geometry of the enzyme by cellular components.

It has of course been clear from many studies that one does not expect or get the same response from an inhibitor on an isolated enzyme that one obtains from a cell culture. Much of the difference between results from two such different systems has often been attributed to problems of penetration of the inhibitor to the location of the enzyme in the cell. A large amount of evidence has now been accumulated to show that differences in rates of penetration by a set of congeners into cellular material can be rationalized in terms of the hydrophobic parameters π or log P. It is assumed for substituents of moderate size that penetration is independent of MR; this is particularly true in the case of the resistant cells in this study where π and

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Table II. Log $1/C$ for 3-$CH_2OC_6H_4$-3′-R against Methotrexate-Resistant *L. casei* Cells

group	log $1/C$ obsd	log $1/C$ (eq 9) calcd	π
CH_3	4.93	5.12	0.56
C_2H_5	5.02	5.10	1.02
$CH(CH_3)_2$	5.07	5.10	1.55
$C(CH_3)_3$	5.28	5.10	1.98

MR are reasonably orthogonal and activity depends linearly solely on π (especially eq 12). The π term is composed of two components: membrane penetration and hydrophobic interaction with the DHFR. These processes must either be rather similar or the system must be at quasi-equilibrium, since the coefficient with π in eq 12 is close to that of the positive π term in eq 4. Thus, we believe the term in MR brings to light a specific steric effect for a wide variety of substituents present in vivo but absent in vitro.

Biochemists have long discussed the possibility that enzymes in situ behave differently from those studied in purified form in buffer solution. Frieden and Colman[31] speculate about "The ability to specifically bind large protein molecules either from the same enzyme unit as in self-association or another protein molecule which results in an alteration of the kinetic properties of the enzyme itself. This alteration may reflect changes in the ability of the enzyme to bind substrate molecules or modifier ligands or a change in a rate-limiting step of the reaction or any combination of these possibilities." More recently, attempts have been made to circumvent the problem of membrane crossing by ligands. Aragón et al.[32] conclude from their studies that phosphofructokinase is more active in situ than in vitro.

We believe that the use of ligands as molecular probes (either as inhibitors or as substrates) and QSAR offer great promise in establishing differences and similarities between enzymes or other macromolecules in vitro and in the cell.

A substituent, SO_2F, which B. R. Baker[4] regarded as having great potential value in the design of active-site-directed irreversible enzyme inhibitors gives the expected results with *L. casei* enzyme (**10**, Table I) as well as with bovine and *E. coli* DHFR (**6** and S. W. Dietrich et al.[15]); however, this congener is much less active than expected with whole cells, suggesting that a nucleophilic reaction is apt to occur with cellular material and the SO_2F group. A similar lower than expected activity for this function is apparent in the study by Walsh et al.[21]

Bioisosterism[33] is often defined as two compounds producing the same response in a given system. The concept has often been used in the design of drugs as well as other molecules of biological interest. An interesting example of nonisometric bioisosterism[34] is apparent in the activities of the bridged congeners 3-$CH_2OC_6H_4$-3′-R, where R is an alkyl group. Against resistant cells, these compounds show almost identical observed and calculated log $1/C$ values (Table II).

The increasing activity which one expects from eq 9 for increasing hydrophobicity of R is almost exactly canceled by the negative term of the collinear (for this subset) MR parameter. This is a nice example of the power of QSAR to rationalize what may at first glance seem a rather strange group of bioisosteres.

Comparison of the QSAR for the inhibition of bacterial enzyme with mammalian enzyme shows some significant differences. Equation 18 has been obtained with inhibitors

$$\log 1/K_{i\,app} = 1.08 (\pm 0.19) \pi'_3 - 1.19 (\pm 0.25) \log (\beta \cdot 10^{\pi'_3} + 1) + 7.27 (\pm 0.13) \quad (18)$$

$$n = 38;\ r = 0.903;\ s = 0.288;\ \pi_0 = 1.61\ (1.24\text{–}2.06);\ \log \beta = -0.656$$

of type I for bovine liver enzyme at pH 7.20.[35] In eq 18, π'_3 has the same meaning as in eq 4; that is, $\pi_Y = 0$. The size of the hydrophobic pocket of bovine DHFR as defined by π_0 is about half that of the *L. casei* DHFR; however, since the slope of the positive π term in eq 18 is about twice that of eq 4, about the same potential exists for inhibition by hydrophobic substituents in each enzyme.

A dramatic difference between *L. casei* and bovine DHFR is the lack of importance of the bridge ($-CH_2Z-$), as shown by the fact that adding such a term to eq 18 does not result in an improved correlation. Twelve examples with $-CH_2O-$ bridges and two examples with $-CH_2NH-$ bridges are very well fit by eq 18 without the use of the I variable.

There is a range of 3.74 log units in the log $1/K_i$ value for the *L. casei* DHFR inhibitor between the least active and the most active congener, while the range is 10 times smaller with the bovine enzyme: 2.76. Although the correlation coefficient for eq 4 is higher than that of eq 18, the standard deviation is also higher, so that the quality of fit is actually a little better with bovine DHFR.

The major difference between the mammalian bovine liver DHFR and the bacterial *L. casei* DHFR is seen in the intercepts of eq 4 and 18. For congeners with 3-X substituents of moderate size and π values near 0, one expects the triazines to be about 10 000 times more active against bovine enzyme. The difference increases somewhat as π is increased up to a π_0 of 1.6 for the bovine enzyme. We hope that before long the reasons for this great difference will become apparent from X-ray crystallographic studies.

In conclusion, we can say that there are enough studies now in hand to show that one can formulate good QSAR for many kinds of ligands (both substrates and inhibitors) interacting with isolated purified enzymes. This report shows that QSAR can be formulated with ligands acting on the enzyme in vivo and that from a comparison of in vitro and in vivo studies, inferences may be made about the in vivo situation. Of course caution must be taken about placing too much weight on such inferences until more experience is in hand. As of the present, QSAR is the *only* way to gain information about the enzyme in the living cell. In effect, one produces a set of perturbations in the cell with a well-designed set of probes and, from a growing variety of computerized analytical techniques, one attempts to sort out noise and spurious information to obtain a picture of the interaction of the ligand and enzyme. While this might seem to be a hopelessly complex problem, we believe that the increasingly large number of publications in this area are showing that much insight can be gained about how organic compounds affect biological systems.

An essential kind of study which must be done to back up inferences made in QSAR studies with enzymes is to make extensive X-ray crystallography analyses of crucial

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members of sets of congeneric inhibitors bound to the enzyme. These studies, which are now possible, will firm up our QSAR-derived conclusions and enable us to more confidently refine QSAR techniques. Such a project is now under way between our laboratory and that of Kraut and Matthews in La Jolla employing the triazines used to develop the QSAR in this report for dihydrofolate reductase.

Structure-Activity Relationships of the Cycloalkyl Ring of Phencyclidine

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In order to investigate the structural requirements for a cycloalkyl moiety in the potent hallucinogen 1-(1-phenylcyclohexyl)piperidine (PCP, **1**), a series of structural analogues was synthesized in which the size of the cycloalkyl ring was varied from three carbons to eight carbons. Biological activities of these compounds were assessed in an in vitro assay (phencyclidine binding assay) and an in vivo assay (discriminative stimulus assay). As the cycloalkyl ring size decreased from that of cyclohexane (PCP), PCP-like activity declined in both assays, but as the cycloalkyl ring size became larger than cyclohexane, a sharp decline in PCP-like activity was observed in the in vivo assay, while activity in the in vitro assay was only slightly less than that of PCP. 1-(1-Phenylcyclooctyl)piperidine (**8**) had potent competitive binding properties in the in vitro binding assay but produced no observable PCP-like effects in the in vivo assay. The importance of the cycloalkyl ring in the structure of PCP was demonstrated by testing benzylpiperidine (**2**), which had almost no measurable activity in either assay.

Phencyclidine (PCP, **1**) is an easily synthesized and thus readily available drug that has become a substance of major abuse, particularly among teenagers. Agitated, hostile, and aggressive behavior, depressive states, a toxic psychosis, and a number of deaths, including suicides and murders, have been associated with PCP abuse.[2] PCP (Sernyl) was introduced more than 15 years ago as an anesthetic medication in man, but the high incidence of undesirable side-effects during recovery from anesthesia has precluded its further use in man.[3] In spite of PCP's many negative aspects, the euphorigenic properties of PCP have made the drug popular among recreational users.

PCP appears to represent a unique class of drugs whose spectrum of action is readily distinguishable from that of other classes of psychoactive drugs, including narcotics, LSD-like hallucinogens, amphetamine-like stimulants, barbiturate-like depressants, and cholinergic agonists.[4] Although the pharmacology of PCP has been extensively studied, little is known about the mechanism of action of PCP at the molecular level. In order to gain some understanding of the mechanism of action of PCP, we have undertaken studies to explore the structural requirements for PCP-like activity. The first of these studies is reported in this paper.

An examination of the PCP molecule reveals a semirigid structure in which the interatomic distance between the benzene and piperidine rings is governed by the internal bond angles of the cyclohexane ring. The flexibility of the cyclohexane ring allows these bond angles to conform closely to the theoretical 109.5° bond angles found for normal sp^3 hybrid bonding. This relationship was confirmed by Argos et al.[5] in a study of the molecular structure of PCP·HCl by X-ray crystallography. They found the angle θ (see Table I) to be 109.0°. Also, the distance between the bonding carbon (C7) of the cyclohexyl group and the nitrogen atom (N13) of the piperidine group was found to be 1.550 Å, while the distance between C7 and the bonding carbon of the phenyl group (C1) was 1.543 Å. Since the value for θ and the distances between C7–C1 and C7–N13 have been experimentally measured, the distance (a) between the nitrogen atom of the piperidine ring and the C1 atom of the phenyl ring can be calculated by utilizing a simple trigometric relationship ($a^2 = b^2 + c^2 - 2bc \cos \theta$, where b and c are the lengths of the adjacent sides of the angle θ). The distance a was calculated to be 2.518 Å.

PCP has been shown to exhibit anticholinergic activity.[6] In the case of acetylcholine, the distance that separates the areas of minimum potential between the negatively charged ester oxygen and the positively charged trimethylammonium group has been recognized as a critical parameter in determining cholinergic activity.[7,8] Since the PCP·HCl molecule also can be visualized as having an area of localized positive charge (the nitrogen atom of the piperidine ring) at a fixed distance (2.518 Å) from an area of negative charge (the delocalized electron cloud of the benzene ring),[8] it has been suggested that this distance might be a critical parameter in determining PCP-like activity.[8,9] In order to test whether the distance between the benzene and piperidine ring is critical for PCP-like activity, a series of compounds was prepared in which the angle θ, and consequently the distance a, was systematically altered while leaving the composition of the benzene and piperidine rings unaltered. Assuming that the distances between C7–N13 and C7–C1 of PCP are independent of bond angles and remain constant, the distance a should be directly dependent upon the value of θ. For saturated cycloalkyl ring systems of six carbons or less, the bond angles are dependent upon the ring size. By altering the size of the cycloalkyl ring in PCP, the value for θ and, consequently, the distance between the benzene and piperidine ring can be altered. For rings larger than six

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