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Tissue-Specific Irreversible Inhibitors of Dihydrofolic Reductase[1]

B. R. BAKER

Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106

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Based on their work with sulfanilamide (**1**) and *p*-aminobenzoic acid (**2**) in bacterial systems, Woods and Fildes[2] in 1940 proposed their now classic antimetabolite theory: certain substances that prevent cell growth (antimetabolites) may do so by interfering with the formation of other substances essential for cell growth (metabolites). Furthermore, they recognized that inhibition could occur if an antimetabolite structurally resembled a metabolite so that it could fit the same enzyme and yet was different enough to be devoid of growth activity. Thus replacement of the CO_2H group of **2** by SO_2NH_2 results in the antimetabolite, **1**. This concept gave impetus to a general

1 **2**

search for antimetabolites, that is, enzyme inhibitors, of therapeutic utility. The principal approach involved making minor changes in the structure of the metabolite by groups of equivalent size. This exchange by similar groups is termed *isosteric replacement* and among the known examples are OH for NH_2, NH_2 for OH, F for H, Br for CH_3, S for CH=CH, and heterocyclic rings for benzene.

Use of the antimetabolite theory by isosteric replacement has led to disappointingly few useful drugs for invasive diseases considering the efforts of many chemists over the past 28 years. In retrospect this can be attributed to the failure to achieve specificity by isosteric replacement since an antimetabolite does not have specificity *per se;* such an approach for invasive diseases has been successful only when applied to an enzyme system used by an invading organism that is not utilized by the host. Thus, in bacteria sulfanilamide inhibits the synthesis of dihydrofolate (**4**) from **2**,[3] but mammalian cells synthesize dihydrofolate (**4**) by an alternate pathway—the reduction of the B vitamin, folic acid (**3**), catalyzed by the enzyme dihydrofolic reductase. The dihydrofolate (**4**) is then further reduced by this enzyme to tetrahydrofolate (**5**), the cofactor form of the vitamin; **5** is then involved in a spate of some 17 enzyme reactions used for one-carbon transfer reactions.[4] In most cases the blockade of tetrahydrofolate (**5**) formation leads to cell death due to the inability of the cell to synthesize thymidylate (**7**) from 2′-deoxyuridylate (**6**)—a reaction requiring a stoichiometric amount of 5,10-methylenetetrahydrofolate (**8**) with generation of dihydrofolate (**4**);[4] the latter must then be recycled by reduction to tetrahydrofolate (**5**) by dihydrofolic reductase and enzymatic conversion to **8**.[4] Since thymidylate is one of the four nucleotide precursors to DNA, a lack of thymidylate (**7**) inhibits cell division and leads to cell death.[4] A shortage of tetrahydrofolate (**5**) in the cell can be created by either the block of *de novo* dihydrofolate (**4**) synthesis from *p*-aminobenzoic acid (**2**) by an antimetabolite such as sulfanilamide (**1**) or the block of dihydrofolic reductase (**4** → **5**).

In order for a bacterial cell to be inhibited by sulfa-

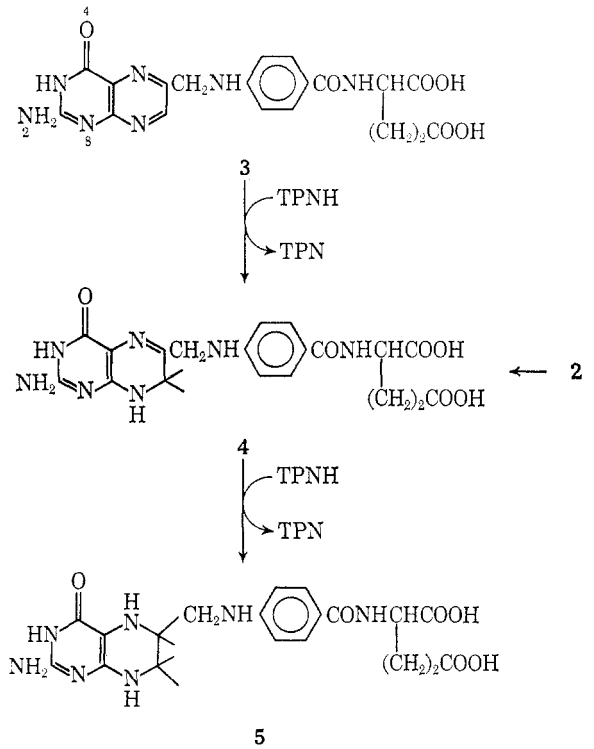
(1) Paper CXLIX in a series on Irreversible Enzyme Inhibitors. The work in this laboratory on enzyme inhibitors has been generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) (a) D. D. Woods, *Brit. J. Exp. Pathol.*, **21**, 74 (1940); (b) P. Fildes, *Lancet*, **1**, 955 (1940).

(3) (a) M. J. Griffin and G. M. Brown, *J. Biol. Chem.*, **239**, 310 (1964); (b) G. M. Brown, R. A. Weisman, and D. A. Molnar, *ibid.*, **236**, 2534 (1961).

(4) (a) T. H. Jukes and H. P. Broquist in "Metabolic Inhibitors," R. M. Hochster and J. H. Quastel, Ed., Academic Press, New York, N. Y., 1963, pp 481–534; (b) M. Friedkin, *Ann. Rev. Biochem.*, **32**, 185 (1963).

nilamide one ancillary biological requirement must be met for specificity: either the dihydrofolic reductase



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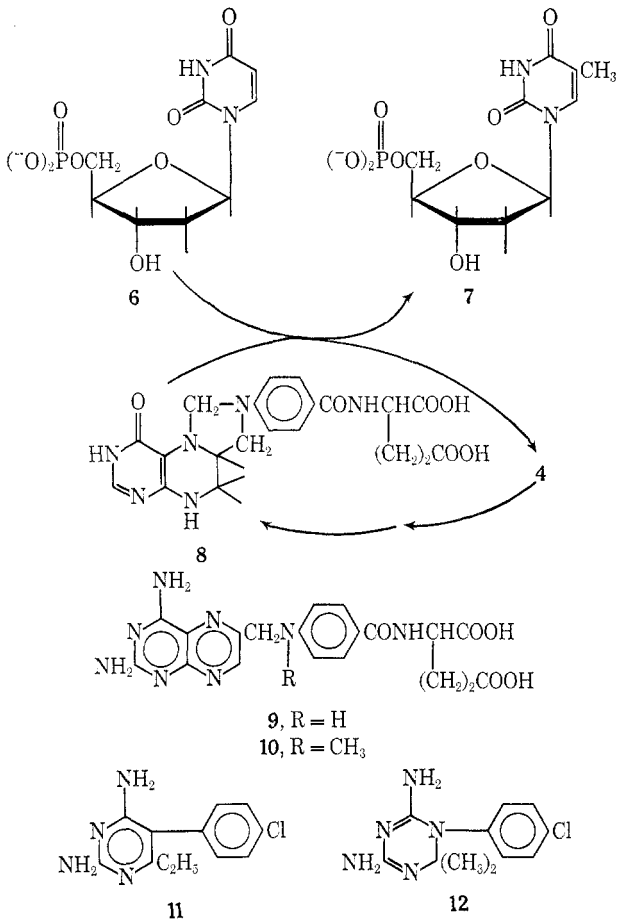
from the bacteria cannot reduce folic acid to **4** or the bacterial cell is incapable of absorbing folic acid across its cell wall from the host, that is, the pathway **3** → **5** must be inoperative in bacteria. With cancer the problem is more complicated since no metabolic pathways alternate to those used by host cells have emerged after numerous studies over the past 30 years; thus only the blockade of dihydrofolic reductase remains as a way to create a deficiency of tetrahydrofolate within the cancer cell.

When research on inhibitors of dihydrofolic reductase was started in this laboratory in 1961, the two most important classes of inhibitors of dihydrofolic reductase were the isosteric analogs, aminopterin (**9**) and amethopterin (**10**), and the nonisosteric inhibitors, pyrimethamine (**11**) and cycloguanyl (**12**);[4] **10** is used for the treatment of childhood leukemia and **11** and **12** are used for the treatment of certain forms of malaria.[5,6] The isosteric inhibitors penetrate a cell wall by active transport,[6,7] a process requiring energy and one tolerating little structural change; the nonisosteric inhibitors penetrate a cell wall by passive diffusion,[7] a process that presumably can tolerate large structural change.[8] The isosteric inhibitors most probably owe their selectivity in toxicity to the differences in active transport between leukemia cells and



normal cells, the susceptible leukemia cells having a more efficient active transport system.[8] The nonisosteric inhibitor (**11**) is more effective on susceptible malarial strains than host tissues due to a 1000-fold better binding to the malarial dihydrofolic reductase than the mammalian enzyme;[9] the reasons for this difference in binding of **11** will be discussed in more detail later.

Irreversible inhibitors of dihydrofolic reductase have been developed in this laboratory that can rapidly inactivate the enzyme from L1210 mouse leukemia with no inactivation of the enzyme from mouse liver, spleen, or intestine. The design of these compounds is based on utilization of part of the active site[10] of dihydrofolic reductase and utilization of both nonpolar and polar areas adjacent to the active site, thus taking advantage of evolutionary differences between the dihydrofolic reductases from different tissues or species; this design proceeded as follows.

Binding between Groups on Proteins and on Inhibitors or Substrates.[11] In its simplest terms an enzyme-catalyzed reaction can be depicted by eq 1 where E = enzyme, S = substrate, P = product, and

(5) For a review see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Active-Site," John Wiley & Sons, Inc., New York, N. Y., 1967, pp 197–204.

(6) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965).

(7) R. C. Wood and G. H. Hitchings, *J. Biol. Chem.*, **234**, 2377, 2381 (1959).

(8) For a review see ref 5, pp 263–266.

(9) R. Ferone, J. J. Burchall, and G. H. Hitchings, *Fed. Proc.*, **27**, 390 (1968).

(10) An active site is defined "to include the complexing regions for the substrate and coenzyme (if any) as well as the amino acid residues catalyzing the enzymatic reaction"; from ref 5, p 188.

(11) For a more complete discussion see ref 5, pp 13–21, 23–47.

E···S = an enzyme–substrate complex. Similarly,

$$E + S \rightleftarrows E \cdots S \rightleftarrows E \cdots P \rightleftarrows E + P \quad (1)$$

reversible inhibition can be depicted in its simplest terms by eq 2 where I = inhibitor, a molecule that

$$E + I \rightleftarrows E \cdots I \quad (2)$$

can form a complex (E···I) with the enzyme but cannot be converted to a product by the enzyme.

There are two simple types[12] of irreversible inhibitors that operate by forming a covalent bond between E and I that destroys the catalytic activity of the enzyme. The first is the random, bimolecular type depicted in eq 3 where X is a leaving group on the inhibitor. This type has little specificity and therefore

$$E + I\text{–}X \xrightarrow{k_B} EI + X^- \quad (3)$$

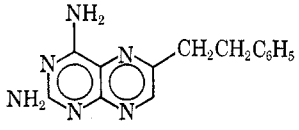
little utility for chemotherapeutic agents; for example, if acetic anhydride reacts with an ϵ-amino group of a lysine in the active site,[11] activity will be destroyed, but acetic anhydride can react randomly with any exposed amino group and thus has no specificity. Similarly, iodoacetamide or iodoacetate show little specificity from one enzyme to another. The second is the active-site-directed type depicted in eq 4 that

$$E + I\text{—}X \underset{K_i}{\rightleftharpoons} \overset{\frown}{E\cdots I\text{—}X} \xrightarrow{k_A} \overset{\frown}{E\cdots I} + X^- \quad (4)$$

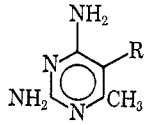
operates through a complex between the enzyme and the inhibitor; such an irreversible inhibitor can have high specificity. The reaction requires formation of a complex between E and IX which in turn can have strict structural requirements for this interaction; furthermore, if the leaving group, X, is juxtaposed to an appropriate nucleophile on the enzyme surface, then covalent bond formation can take place by a neighboring group reaction[13] that is 10^3–10^4 faster than the corresponding bimolecular reaction in eq 3.[12,14,15]

The type of inhibitor depicted in eq 4 can be sufficiently specific to inactivate a tumor enzyme with no effect on this same enzyme in normal tissue, if appropriate parameters adjacent to the active site are taken into consideration. Since eq 4 requires complex formation between enzyme and inhibitor, what are the forces that cause complex formation? These can be divided into two types:[11] (a) electron donor–acceptor complexes and (b) hydrophobic bonding and the accompanying van der Waals forces. The electron donor can be either on the enzyme or the inhibitor; such complexing can be due to anionic–cationic (Coulombic) interactions, hydrogen bonds, charge-transfer complexes, and mixed types. Hydrocarbon interactions from hydrophobic bonding and van der Waals forces are due to the affinity of two hydrocarbon moieties for each other in water; these can be extremely strong when an appropriate hydrocarbon moiety on an inhibitor complexes with a hydrocarbon moiety on a protein surface—as much as 1.4 kcal/mol of $CH_2 \cdots CH_2$ interaction.[16]

Hydrophobic Bonding to Dihydrofolic Reductase.[17] A serious discrepancy arose when the 6-phenethylpteridine (**13**)[18] was compared with the 5-phenylbutylpyrimidine (**14**)[19] as inhibitors[18] of the dihydrofolic reductase[19] from pigeon liver; **14** was a 150-fold better inhibitor than **13**. If the benzene rings of **13** and **14**



13; $I_{50} = 4.2\ \mu M$



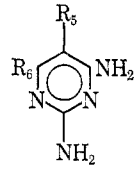
14, R = $(CH_2)_4C_6H_5$; $I_{50} = 0.027\ \mu M$
15, R = C_4H_9-*n*; $I_{50} = 2.0\ \mu M$
16, R = H; $I_{50} = 1100\ \mu M$

were complexed to dihydrofolic reductase in the same manner as the benzene ring of aminopterin (**9**), then **13** should be as good or a better inhibitor than **14**. This discrepancy was partially resolved by comparing the *n*-butylpyrimidine **15** with **16**, the former showing a 550-fold increment in binding. Since the *n*-butyl group can only complex to an enzyme by a combination of hydrophobic bonding and the accompanying van der Waals forces, it was clear that **14** was a 150-fold better inhibitor than **13** due to hydrophobic bonding by its butyl moiety.

Extensive studies were performed to locate this hydrophobic bonding region with respect to the binding of the substrate, dihydrofolate (**4**). By three lines of evidence,[20] strong support was given to the concept that this hydrophobic region was adjacent to where either the 4-oxo or the N-8 groups of dihydrofolate (**4**) resided on the enzyme and was clearly *adjacent to the active site and not part of the active site.*[10] Two additional important points should be emphasized: (a) the phenyl ring of the nonisosteric analogs (**11**, **12**) was also complexed by hydrophobic bonding in this same region[21] and (b) pH profile studies on binding of three selected inhibitors of varying pK_a's related to **11** and **12** indicated that diamino heterocycles such as **11**, **12**, **14**, and **15** could bind as a different rotamer than aminopterin (**9**) in order to give maximum binding

(12) For more complex types of irreversible inhibition and the kinetics of irreversible inhibition, see ref 5, pp 122–129.
(13) S. Winstein and R. Boschan, *J. Am. Chem. Soc.*, **72**, 4669 (1950).
(14) B. R. Baker, *Cancer Chemother. Rep.*, **4**, 1 (1959).
(15) This process has been called "affinity labeling" by L. Wofsy, H. Metzger, and S. J. Singer, *Biochemistry*, **1**, 1031 (1962).
(16) For a more complete discussion of hydrophobic bonding see ref 5, p 39.
(17) See ref 5, pp 204–241, for a more detailed discussion.
(18) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **54**, 1261 (1965).
(19) Assayed with 6 μM dihydrofolate in pH 7.4 Tris buffer; I_{50} = micromolar concentration necessary for 50% inhibition.
(20) (a) B. R. Baker, T. J. Schwan, J. Novotny, and B.-T. Ho, *J. Pharm. Sci.*, **55**, 295 (1966); (b) B. R. Baker, J. K. Coward, B.-T. Ho, and D. V. Santi, *ibid.*, **55**, 302 (1966); (c) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966); (d) ref 5, pp 222–229; (e) B. R. Baker and J. H. Jordaan, *J. Heterocycl. Chem.*, **4**, 31 (1967).
(21) B. R. Baker and B.-T. Ho, *ibid.*, **2**, 335 (1965).

by hydrophobic interaction.[22] It was therefore concluded as highly probable that **10**, **11**, **14**, and **15** were complexed to the enzyme as rotamer **17** to give maximum hydrophobic interaction rather than as rotamer



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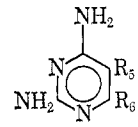

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18 for aminopterin (**9**) with its polar benzoyl-L-glutamate side chain.[20] Direct experimental support for the rotamer concept was also obtained with appropriate irreversible inhibitors.[20e]

Species Differences in the Hydrophobic Bonding Region of Dihydrofolic Reductase. That the hydrocarbon portion of **11**, **12**, **14**, and **15** was complexed to a hydrophobic region not a part of the active site had important consequences with respect to the evolutionary differences in structure of a protein with the same function.[23] The linear sequence of cytochrome *c* from 20 sources has been investigated by Margoliash and by Smith.[24] This protein with 104+ amino acid residues shows only a constancy in 35 of the amino acids in the sequence, the remainder being exchanged by one amino acid or another. They concluded that the 35 unchanged amino acids as a maximum are necessary for the function and shape of the protein, but the remainder are not. Thus little evolutionary change could be expected in the active site,[10] else an enzyme would be nonfunctional; however, evolutionary change could readily have occurred in regions not part of the active site, providing such a change does not modify the shape of the active site.

With this concept it can be predicted that isosteric analogs such as amethopterin (**10**) should show little species difference in enzyme binding since **10** binds to the active site; such is the case.[6] In contrast, complexing to the hydrophobic bonding region adjacent to the active site could show large species differences due to evolutionary changes in this hydrocarbon region; such is also the case.[25] Forty-nine selected 2,4-diamino heterocycles bearing hydrocarbon groups were compared[25] for their ability to inhibit dihydrofolic reductase from pigeon liver, *E. coli* B, and the enzyme induced when T_2 phage infects *E. coli* B; the latter enzyme is coded by the viral DNA.[26] Results with some of the compounds (**19–25**) are shown in Table I. The parent

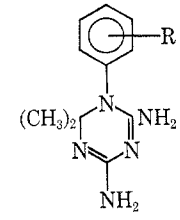
Table I

Comparative Hydrophobic Bonding to Dihydrofolic Reductase from Diverse Sources[a]

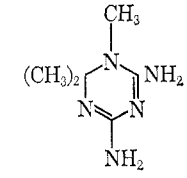
Compd	I_{50},[b] μM Pigeon liver	T_2 phage	*E. coli* B
19	0.11	0.14	3.0
20	160	11	5.8
21	1.3	0.011	1.0
22	0.0053	0.047	1.2
23	74	65	48
24	16	0.68	0.0003
25	0.041	0.066	0.21

[a] Data from ref 25. [b] Concentration necessary for 50% inhibition when assayed with 6 μM dihydrofolate.

compound of the series **19–23** is the 1-methyldihydrotriazine **23**. Note that **23**, which shows no hydrophobic bonding, has little difference in binding to the enzyme from the three sources; this result is to be



19, R = H
20, R = *p*-C_6H_5
21, R = *m*-C_6H_5
22, R = *p*-$(CH_2)_4C_6H_3Cl_2$-2,4



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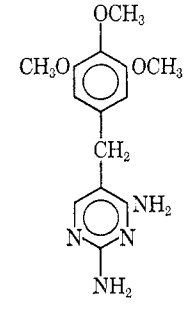

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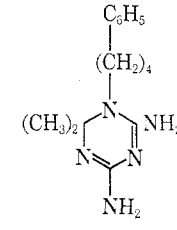

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expected if binding only to the active site occurs. Replacement of the methyl of **23** by phenyl (**19**) gives a 670-fold increment in binding to pigeon liver enzyme, but only 16-fold with the *E. coli* enzyme; the T_2 phage enzyme is similar to the pigeon liver enzyme, a 460-fold increment being observed.

Changing from **19** to its *p*-phenyl derivative **20**, in which the two benzene rings may be coplanar, has a nearly negligible effect on binding to the *E. coli* enzyme; in contrast, inhibition is decreased 1500-fold with the pigeon liver enzyme and 80-fold with the T_2-phage enzyme. An interesting contrast is also seen with the in-plane *m*-phenyl group of **21**; a 12-fold loss in binding compared to **19** occurs with the pigeon liver enzyme, but 3-fold and 13-fold increased binding occurs with the *E. coli* and T_2-phage enzymes, respectively. With the *p*-(3,4-dichlorophenylbutyl) sub-

(22) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **54**, 1740 (1965).
(23) For a more detailed discussion see ref 5, pp 184–190, 252–262.
(24) For a review see (a) E. Margoliash and E. L. Smith in "Evolving Genes and Proteins," V. Bryson and H. J. Vogel, Ed., Academic Press, New York, N. Y., 1965, pp 207–220; (b) E. Margoliash and A. Schejter, *Advan. Protein Chem.*, **21**, 91 (1966).
(25) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967).
(26) (a) C. K. Mathews and K. E. Sutherland, *J. Biol. Chem.*, **240**, 2142 (1965); (b) C. K. Mathews and S. S. Cohen, *ibid.*, **238**, PC853 (1963).

stituent (**22**) only a 3-fold increment in binding occurs with the *E. coli* and T_2-phage enzymes; in contrast, a 21-fold increment in binding is observed with the pigeon liver enzyme. The most dramatic difference in binding to the dihydrofolic reductases from *E. coli* B and pigeon liver was seen with the antibacterial agent, trimethoprim (**24**);[6] Hitchings and Burchall had previously observed[6] that **24** was complexed 50,000–70,000 times more effectively to *E. coli* dihydrofolic reductase[27] than the enzyme from rabbit, rat, or human liver. Note in the test system in Table I, trimethoprim was 53,000 times more effective on the *E. coli* enzyme than the pigeon liver enzyme. Of high significance is the closer similarity in the binding of trimethoprim (**24**) to T_2-phage-induced enzyme and pigeon liver enzyme; only a 23-fold difference in binding between the enzyme from these two sources was observed. In contrast, *E. coli* B enzyme was inhibited 2300 times more strongly by **24** than the T_2 phage enzyme. In summary, the T_2-phage-induced enzyme has an "inhibition profile"[6] closer to that of the dihydrofolic reductase from pigeon liver than *E. coli*, the organism that the T_2 phage infects.[28]

When mammalian cells in tissue culture are infected by such tumorigenic viruses as polyoma virus or SV40, the levels of some enzymes involved in DNA synthesis are increased, including dihydrofolic reductase in some cases.[29] Thus the T_2-phage–*E. coli* B system can serve as a model on whether or not the dihydrofolic reductase in a viral-induced cancer cell might have differences in the hydrophobic bonding region compared to the enzyme in a normal cell.

Even though it is not known whether the Walker 256 rat tumor was originally caused by a viral infection, the hydrophobic bonding region of the dihydrofolic reductase from this rat tumor was compared with the enzyme from normal rat liver[30] with 31 dihydrotriazines related to **19**. Five of the compounds showed 3–20-fold differences in binding to the enzyme from the two sources. The largest difference (100-fold) was observed with **25**, a 40-fold difference was noted with **22**, and no difference with **19** or **23**. These differences are not sufficient to expect chemotherapeutic effectiveness on the tumor, particularly in view of the fact that the antibacterial, trimethoprim (**24**), is about 60,000 times more effective on bacterial dihydrofolic reductase than the mammalian enzyme. Similarly, 3–10-fold dfferences in reversible binding to dihydrofolic reductase from L1210 mouse leukemia and mouse liver have been noted. These data indicate that evolutionary differences in the hydrophobic bonding region of dihydrofolic reductase had occurred. The problem was then to magnify the effect of these small differences; this was successfully achieved in the following way.

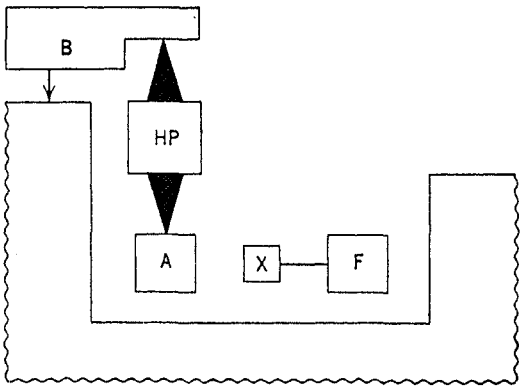


Figure 1. A schematic representation of the bridge principle of specificity with dependence on the mode of hydrophobic bonding. A is the pyrimidine binding area, HP is the hydrophobic bonding area, B is a group in a noncontact area bearing a covalent-forming group represented by the arrow, and X–F is the coenzyme or cosubstrate (from ref 5, p 248).

Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase. If a single amino acid were replaced in the hydrophobic bonding region of a tumor dihydrofolic reductase compared to the host dihydrofolic reductase, then the above 3–10-fold differences in hydrophobic bonding by inhibitors would be accounted for. This difference would be greatly magnified with an appropriately designed active-site-directed irreversible inhibitor (eq 4). It was predicted[31] that probably the greatest difference in irreversible inhibition of dihydrofolic reductase from different tissues and species would arise if the group on the inhibitor can be branched (from the hydrophobic bonding moiety) toward a hydrophilic area; if a covalent forming group is then attached to this branch, its ability to form a covalent bond is also dependent upon the effect of the hydrophobic bonding region on the bridging to the nucleophilic site. Schematically this can be represented in Figure 1.

To use this modification of the bridge principle of specificity[32] it would be necessary to know where this hydrophobic bonding region on the dihydrofolic reductase ends by emerging into a polar region. This was studied by compounds symbolized by **26**. A B_1 bridge of two to three more carbon atoms was sufficient to place the attached benzene ring between B_1 and B_2 in a polar region of the enzyme.[33] Initial work was focused on bromoacetamido and chloromethyl ketone moieties for a halogen leaving group, since these halo-

(27) It is quite possible that there is some polar interaction of the methoxy groups with the *E. coli* enzyme not present with the pigeon liver enzyme; the important point is the high probability that the trimethoxybenzyl group is not complexed in the active site regardless of whether there is both polar and nonpolar binding with this group.

(28) See ref 25 for a discussion of the paleontological significance of these observations.

(29) P. M. Frearson, S. Kit, and D. R. Dubbs, *Cancer Res.*, **26**, 1653 (1966).

(30) (a) B. R. Baker, *J. Med. Chem.*, **11**, 483 (1968); (b) B. R. Baker and M. A. Johnson, *ibid.*, **11**, 486 (1968).

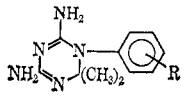
(31) See ref 5, p 248.

(32) (a) See ref 5, pp 172–183; (b) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

(33) (a) B. R. Baker, B.-T. Ho, and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967); (b) B. R. Baker and G. J. Lourens, *ibid.*, **56**, 871 (1967).

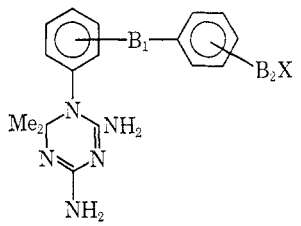
Table II

Irreversible Inhibition[a] of Dihydrofolic Reductase from Different Tissues by



Compd	R	Enzyme source[b]	$K_i \times 10^9 M$	Irreversible: Inhib concn, μM	Irreversible: % EI[c]	Irreversible: Time, min	Irreversible: % inactiv
26	m-$(CH_2)_2CONHC_6H_4SO_2F$-p	Pigeon liver	17	0.21	90	8, 60	50, 95
		W256	10	0.32	97	120	0
		Rat liver	5	0.14	97	60	0
		L1210	2	0.40	97	60	0
27	p-$(CH_2)_2CONHC_6H_4SO_2F$-p	Pigeon liver	10	0.07	87	<2	70
		W256	3	0.05	95	<1	90
		Rat liver	1	0.05	98	<2	70
		L1210	2	0.07	94	60	73
		Mouse liver	3	0.07	96	<2, 10	38, 38
		Mouse spleen		0.10		60	75
		Mouse intestine		0.10		20	85

[a] Data from ref 40 and 41. [b] W256 = Walker 256 rat tumor; L1210 = L1210 mouse leukemia. [c] Per cent total enzyme in reversible complex; calculated from $[EI] = [E_t]/(1 + K_i/I)$.[12]



26, B = bridge
X = leaving group

gen compounds have the electrophilic capacity to be able to react with any one of seven out of the total of 15 different proteinic amino acids containing a third functional group. About 20 compounds with these leaving groups and varying B_1 and B_2 bridges were synthesized and evaluated; none showed irreversible inhibition of the three vertebrate dihydrofolic reductases.[34]

The dihydrofolic reductase from chicken liver[35] has 20 serine and threonine residues out of a total of about 160 amino acid residues, and the L1210 enzyme[36] has even more; thus the enzyme surface is apt to have many serines and threonines, each having a nucleophilic hydroxyl group. The bromoacetamido group reacts too slowly[37] with a juxtaposed aliphatic hydroxyl group of the enzyme to be useful. However, a sulfonyl fluoride[38] or fluorophosphate[39] can react rapidly with serine-195 of chymotrypsin when juxtaposed in a reversible complex. Therefore structures of type **26** were synthesized with the leaving group (X) being the F of an SO_2F moiety, then evaluated;[40] some of the results are shown in Table II.

The first SO_2F compound synthesized was **26**; it was an excellent reversible inhibitor of the dihydrofolic reductase from four sources with $K_i = 2\text{–}17 \times 10^{-9}$ M.[40] When 0.21 μM **26**, sufficient to reversibly complex 90% of the enzyme to the rate-determining species, E···I, was incubated at 37° with the pigeon liver enzyme, essentially compete inactivation occurred in 60 min with a half-life of 8 min. That **26** inactivated the enzyme by the active-site-directed mechanism (eq 4) and not the random bimolecular mechanism (eq 3) was indicated by the fact that N-(*m*-aminobenzoyl)sulfonyl fluoride at 25 μM showed no inactivation of the enzyme.[12] Not too surprisingly, **26** failed to show irreversible inhibition of the dihydrofolic reductase from Walker 256 rat tumor or L1210 mouse leukemia. Since the SO_2F moiety is most probably positioned outside the active site when **26** is complexed to dihydrofolic reductase, it could be anticipated that "all or none" differences could exist in an area outside the active site from different species;[23] this is the working basis of the bridge principle of specificity.[32]

When the side chain of **26** was moved to the *para* position, the resultant **27** showed little change in its ability to complex reversibly with dihydrofolic reductase;[40] however, a dramatic change occurred with the irreversible pattern. The dihydrofolic reductases from all vertebrate sources so far tested (Table II) are irreversibly inhibited by **27**; thus **27** shows no tissue specificity,[41] although the total extent of inactivation varied among the sources for reasons to be discussed later.

As a result of extensive synthetic studies on three

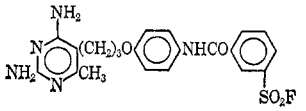
(34) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 26, 34 (1968).
(35) F. Huennekens, private communication.
(36) J. R. Bertino, Annual Medicinal Chemistry Symposium, State University of New York at Buffalo, Buffalo, N. Y., May 1967.
(37) W. B. Lawson and G. J. S. Rao, New York State Department of Health, Annual Report, Division of Laboratory Research, 1965.
(38) D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, **85**, 997 (1963).
(39) A. R. Main, *Science*, **144**, 992 (1964).
(40) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967).
(41) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969).

classes of 2,4-diamino heterocycles,[41] several compounds emerged with the ability to inactivate the L1210 leukemia enzyme with no inactivation of the enzyme from three normal tissues of the mouse;[41-43] the data with one of these compounds are summarized in Table III.

The diaminopyrimidine **28** was an equally effective reversible inhibitor of the dihydrofolic reductase from mouse L1210 leukemia and liver; its $K_i = 3 \times 10^{-9}$ *M* was about the same as that of **27**. In contrast to **27**, **28** showed high irreversible specificity toward the L1210 enzyme; **28** was able to inactivate the L1210 completely with a half-life of 2 min, but at 4–12 times the concentration showed no inactivation of the dihydrofolic reductase from liver, spleen, or intestine. This specificity in irreversible inhibition shows that the structure of the L1210 enzyme is different from the normal tissue enzyme; such structurally different, but substrate identical, enzymes are known as isozymes.

The data in Table III clearly indicate that isozyme specificity can be achieved with irreversible inhibitors

Table III
Irreversible Inhibition[a] of Dihydrofolic Reductase from Different Mouse Tissues by



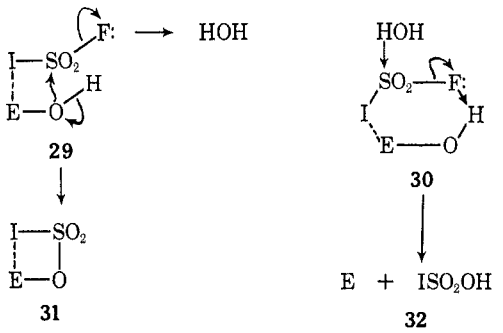
28

Enzyme source[b]	$K_i \times 10^9$ *M*	Irreversible: Inhib concn, μM	Enzyme, μM	Time, min	% inactiv
L1210	3	0.050	0.024	2, 8, 60	50, 84, 100
		0.0050	0.002	60	52
Liver	3	0.60	0.024	60	6
Spleen		0.20	0.025	60	0
Intestine		0.20	0.023	60	0

[a] Data from ref 41 and 43. [b] See Table II.

by utilizing areas adjacent to the active site of an enzyme. Although this isozyme specificity can be explained by the bridge principle of specificity,[32] another factor operates with the SO_2F moiety that aids further in specificity. The SO_2F group is stable to hot water, alcohol, or pyridine—in contrast to the SO_2Cl group;[44] furthermore the SO_2F group reacts slowly, if at all, with proteins that are not reversibly complexed with the moiety bearing the SO_2F group.[20] In contrast, when an SO_2F-bearing compound is complexed to a macromolecule such as an enzyme[45] or cellulose,[44] rapid covalent bond formation can occur. Furthermore, the enzyme can catalyze the hydrolysis of the SO_2F group, as shown kinetically in a number of cases,[40,46-48] and by product isolation in the case of chymotrypsin.[46]

Hydrolysis of acyl fluorides is assisted by hydrogen bonding of the fluorine to protic solvent.[50] It has been proposed[46] that a hydroxyl group from an enzymic serine can either form a covalent bond with the SO_2F group, or catalyze the hydrolysis of the SO_2F group, or both. Thus the complex **29** can form a covalent bond (**31**) whereas the complex **30** results in



29 **30** **31** **32**

hydrolysis to the sulfonic acid **32**. The difference in positioning of the SO_2F in the two complexes is about 3 Å. This relative positioning is in turn dependent upon the enzymic environment between the active site and the enzymic nucleophilic group undergoing covalent bond formation. The positioning of the SO_2F group of molecules such as **27** or **28** on dihydrofolic reductase would be influenced by small differences in the hydrophobic bonding region (see Figure 1); this hypothesis can also explain the specificity patterns with **26–28**.

It should be noted that even though **27** and **28** are similar in the effectiveness in inactivating L1210 dihydrofolic reductase in a broken cell system, it cannot necessarily be assumed that **27** and **28** would diffuse through the L1210 cell wall with equal effectiveness. In fact, **27** shows 2000 times the activity of **28** in L1210 cell culture; furthermore **27**—even though it is not sufficiently selective—gives a good life extension of L1210-bearing mice, but **28** does not.[41] Studies in progress are concerned with finding the best structure for rapid passive diffusion through the L1210 cell wall, then structural modification for isozyme specificity; that is, the better features of **27** and **28** must be combined in one molecule to have an effective *in vivo* agent for L1210 mouse leukemia.

Summary

Inhibitors for dihydrofolic reductase can be constructed that complex only in the active site; no differ-

(42) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 489 (1968).
(43) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 104 (1969).
(44) For a review of the chemistry of sulfonyl fluorides see ref 40.
(45) An SO_2F group attached to an appropriate inhibitor can irreversibly inhibit the appropriate enzyme such as chymotrypsin,[46] trypsin,[47] xanthine oxidase,[48] or guanine deaminase.[49]
(46) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968); **12**, 118 (1969).
(47) B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), **12**, 112 (1969).
(48) (a) B. R. Baker and W. F. Wood, *ibid.*, **11**, 650 (1968); (b) B. R. Baker and J. A. Kozma, *ibid.*, **11**, 652, 656 (1968).
(49) B. R. Baker and W. F. Wood, *ibid.*, **12**, 216 (1969).
(50) C. A. Bunton and J. H. Fendler, *J. Org. Chem.*, **31**, 2307 (1966).

ence in the enzyme from different species or tissues could be detected with this type as inhibitor. Adjacent to the active site, but not part of the active site, is a hydrophobic bonding region. This latter region has undergone considerable evolutionary change between vertebrate sources, bacteria, and the T_2-phage coded enzyme. Therefore, inhibitors that partly complex in the active site and partly complex in the hydrophobic bonding region can be constructed that show huge differences in binding between mammalian enzymes on the one hand and bacterial or protozoal enzymes on the other; these binding differences are sufficiently large for chemotherapeutic utility. Small differences in the hydrophobic bonding region between the enzyme from L1210 mouse leukemia and normal mouse liver can be detected, but these are too small for therapeutic use. These small differences can be greatly magnified with active-site-directed irreversible inhibitors composed of three moieties: a part that complexes in the active site, a part that complexes with the hydrophobic bonding region, and a part that can form a covalent bond in a hydrophilic region of the enzyme not in the active site. The formation of this covalent bond leads to rapid inactivation of the enzyme. Inhibitors can be constructed that can destroy the L1210 mouse leukemia enzyme but not destroy the enzyme from normal mouse liver, spleen, or intestine—so-called isozyme specificity. Since large differences in the ability of these inhibitors to diffuse through the L1210 cell wall have been observed, current research revolves around modifying those structures with good transport to give isozyme specificity.

All-Valence-Electron Semiempirical Self-Consistent Field Calculations

H. H. JAFFÉ

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio 45221

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Since the early 1930's, chemists have been interested in the quantum mechanical methods opened up by the work of Schrödinger, Heisenberg, and Dirac, particularly in their applications to the calculation of the electronic structure of molecules. Of the several approaches to molecular calculation that have evolved, the molecular orbital method has proved the most flexible, and has virtually displaced all others. From the early days, molecular orbital calculations have been approached in two independent ways. Both methods have almost universally been based on two assumptions: that the many-electron wave functions of the molecule required by the theory can be factored into independent one-electron functions, molecular orbitals, each of which depends only on the coordinates of one electron; and that each of these molecular orbitals (MO's) can be expressed as a linear combination of atomic orbitals, the LCAO approximation.

The first of the two approaches is an *a priori* (or *ab initio*) calculation, *i.e.*, calculations on molecules using only a limited number of fundamental constants as external input to the calculations, the charge and mass of the electron, e and m, Planck's constant, h, and the velocity of light, c. Such *a priori* calculations look relatively straightforward but always run into problems of extreme computational difficulties, even for relatively small molecules.

Under the pressure of attempting to obtain chemically interesting information short of a complete *a priori* calculation, work started very early on *semiempirical* calculations. Although even *a priori* calculations involve many approximations, the semiempirical methods require many further, sometimes extremely crude, and often unjustifiable approximations. The effect of these approximations is minimized by choosing numerical values for some or all integrals arising in the calculations by fitting results to known experimental data, and then using these same values for other calculations.

One of the earliest triumphs of this approach was the recognition that, to a fair approximation, it is possible to separate the σ and π electron systems in planar unsaturated and aromatic organic molecules, *i.e.*, to treat the electron system which has a nodal plane coincident with the molecular plane separately from the other electrons. This separation has led to the well-known Hückel molecular orbital (HMO) method.[1] The success of this method is so well known that no further discussion is needed here.

The HMO method involves, among many others, one particularly onerous approximation: all electron–electron repulsions are either neglected or averaged out through the use of empirical values for the integrals, *i.e.*, empirical parameters. The fight to eliminate this approximation has been a long and tedious one. Probably the first major success was the calculation of benzene by Goeppert-Mayer and Sklar,[2] but their

(1) A. Streitwieser, "Molecular Orbital Theory for Organic Chemists," John Wiley & Sons, Inc., New York, N. Y., 1961.

(2) M. Goeppert-Mayer and A. L. Sklar, *J. Chem. Phys.*, **6**, 645 (1938).