6-Benzylaminouracil (16). Method B.—A solution of 0.73 g (5 mmoles) of **24**[18] and 1.07 g (10 mmoles) of benzylamine in 100 ml of water was refluxed for about 18 hr. The cooled solution was filtered and the product was washed with water; yield 0.35 g (32%), mp 313–314° dec. Three recrystallizations from aqueous AcOH gave white crystals, mp 316–317° dec. The compound moved as a single spot on tlc in 1:5 AcOH–C_6H_6. See Table II for additional data.

6-Phenylthiouracil (13). Method C.—To a solution of 1.10 g (10 mmoles) of thiophenol and 10 mmoles of NaOH in H_2O (50 ml) were added 0.73 g (5 mmoles) of **24**[18] and 50 ml of 2-methoxyethanol. After being refluxed for 12 hr, the solution was spin evaporated *in vacuo*. To the residue was added 50 ml of water, then the mixture was acidified (AcOH), and again spin evaporated *in vacuo*. The residue was heated to boiling with 100 ml of water, then cooled. The product was collected on a filter and washed with hot water; yield 1.00 g (90%), mp 267–270°. Recrystallization from EtOH gave white crystals, mp 270–272°, that moved as a single spot on tlc in 1:5 AcOH–C_6H_6. See Table II for additional data.

Method D used for **9** was the same except the product separated directly on cooling the reaction mixture; in the case of **14**, the reaction mixture was merely acidified with HCl to precipitate the product.

6-Benzoyluracil (11).—A mixture of 300 mg (1.5 mmoles) of **1**, 330 mg (3 mmoles) of SeO_2, and 50 ml of AcOH was refluxed for 2 hr, then filtered to remove Se. The filtrate was spin evaporated *in vacuo*. The residue was dissolved in 50 ml of water, then the solution was clarified by filtration; the product separated on cooling. Two more recrystallizations from water gave 100 mg (31%) of light yellow needles: mp 250–252°; λ_{max} 277 mμ (pH 1), 257 mμ (pH 13). The compound moved as a single spot on tlc in 5:1 C_6H_6–EtOAc. Langley[20] has recorded mp 252–253° for this compound prepared by a different route.

6-(α-Hydroxybenzyl)uracil (12).—A mixture of 300 mg (1.5 mmoles) of **1**, 165 mg (1.5 mmoles) of SeO_2, and 50 ml of AcOH was refluxed 1 hr, then filtered to remove Se. The residue remaining after spin evaporation of the filtrate *in vacuo* was dissolved in 50 ml of water. The hot solution was filtered, then cooled. Filtration removed 50 mg (16%) of **11**, mp 250–252°. The filtrate was concentrated to about 20 ml, then allowed to stand at 3°. The product was collected on a filter; yield 120 mg (38%); mp 224–226°; λ_{max} 264 mμ (pH 1), 257 mμ (pH 13). The compound moved as a single spot on tlc in 5:1 C_6H_6–EtOAc. Langley[20] recorded mp 224–226° for this compound prepared by an alternate route.

6-(*p*-Methylbenzyl)uracil (5).—A mixture of 1.1 g (5 mmoles) of **25**,[4,19] 0.40 g of chloroacetic acid, and 50 ml of water was refluxed for 48 hr with stirring. The cooled mixture was filtered and the product was washed with water. Recrystallization from EtOH gave 0.85 g (84%) of white crystals, mp 269–271°. See Table II for additional data.

Irreversible Enzyme Inhibitors. CV.[1,2] Differential Irreversible Inhibition of Vertebrate Dihydrofolic Reductases by Derivatives of 4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-phenyl-*s*-triazines Substituted with a Terminal Sulfonyl Fluoride[3]

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Derivatives of 4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine bridged from its **1** position to sulfanilyl fluoride with six different bridges have been synthesized; these compounds have been evaluated as reversible and irreversible enzyme inhibitors of the dihydrofolic reductases from Walker 256 rat tumor, rat liver, L1210/FR8 mouse leukemia, and pigeon liver. For each compound little difference in reversible binding to the four dihydrofolic reductases were seen. In contrast, dramatic differences in irreversible inhibition were seen. Four of the six compounds that irreversibly inhibited pigeon liver dihydrofolic reductase failed to irreversibly inhibit the dihydrofolic reductases from Walker 256 rat tumor and L1210 mouse leukemia. The two compounds containing a *p*-benzoyl (**15d**) and a *p*-phenylpropionyl (**15f**) bridge irreversibly inhibited the two tumor enzymes and the pigeon liver enzyme. However, **15d** inactivated the rat tumor >70 times as fast as the mouse leukemia enzyme. Furthermore, **15f** inactivated the rat tumor enzyme eight times as fast as the rat liver enzyme. The dihydro-*s*-triazine moiety of **15** is believed to complex within the active site of the enzyme, but the sulfonyl fluoride is believed to form a covalent bond outside the site; it is the latter area where evolutionary differences are more apt to have occurred. Thus, the differences in irreversible inhibition of these enzymes can be accounted for if these compounds are operating by the active-site-directed *exo* mechanism of irreversible inhibition, such a mechanism accounting for the specificity pattern by the bridge principle of specificity.

The discovery[5,6] of a potent hydrophobic bonding region on dihydrofolic reductase considerably complicated the successful design of the first active-site-directed irreversible inhibitors[5,7] for this enzyme.[8,9] Once it had been established that the hydrophobic bonding region was outside the active site,[10] near where either the 4 or 8 position of dihydrofolate (**1**) resides on the enzyme, two active-site-directed irreversible inhibitors soon followed;[8,9] for example, the 5-phenylbutyl group of **2** complexes with the hydrophobic bonding region, thus allowing the 6-phenethyl group to project back into the active site.[8]

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and W. Rzeszotarski, *J. Med. Chem.*, **10**, 1109 (1967).

(3) For the previous paper on inhibitors of dihydrofolic reductases see B. R. Baker and M. A. Johnson, *J. Heterocyclic Chem.*, in press.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(5) For a review on the mode of binding of inhibitors to dihydrofolic reductase, see B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapter X.

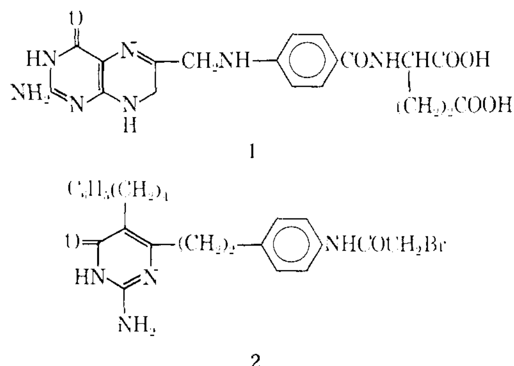
(6) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

(7) B. R. Baker, *ibid.*, **53**, 347 (1964).

(8) B. R. Baker and J. H. Jordaan, *ibid.*, **55**, 1417 (1966); paper LXVII of this series.

(9) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 1422 (1966); paper LXVIII of this series.

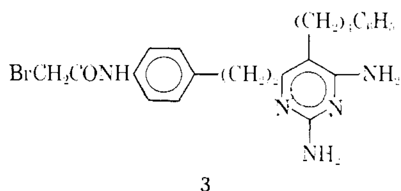
(10) B. R. Baker, T. J. Schwan, J. Novotny, and B.-T. Ho, *ibid.*, **55**, 295 (1966).



1

2

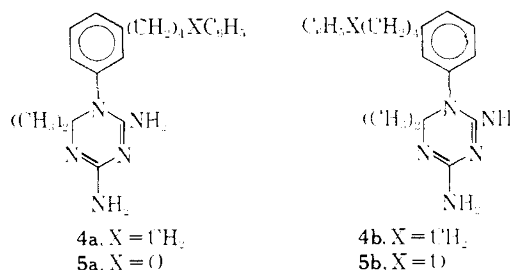
Since a concentration of 10^{-5} *M* of **2** would be needed to be effective *in vivo*, attention was turned to the 2,4-diaminopyrimidine type of inhibitor. 2,4-Diaminoheterocycles are 300–3000-fold more potent reversible inhibitors of dihydrofolic reductase than the corresponding 2-amino-4-hydroxyheterocycles;[5] since the rate of inactivation by an active-site-directed irreversible enzyme inhibitor is dependent upon the concentration of reversible complex between the enzyme and irreversible inhibitor,[11] these 2,4-diaminoheterocycles should be able to operate at 10^{-7} to 10^{-8} *M*. The solution to this problem was not expected to be the simple conversion of a 2-amino-4-hydroxypyrimidine irreversible inhibitor such as **2** to **3**;[8] the evidence[12] that compounds of type **2** bind as a different



3

rotomer than compounds of type **3** was strong. When **3** was synthesized, it was found to be a good reversible inhibitor, but not an irreversible inhibitor of dihydrofolic reductase.[13] This result supported the contention that **2** and **3** were complexed in different rotomeric conformations as indicated by their two-dimensional structures as drawn. This difference in rotomeric configurations for binding was also confirmed in the 6-phenylpyrimidine series.[9,14]

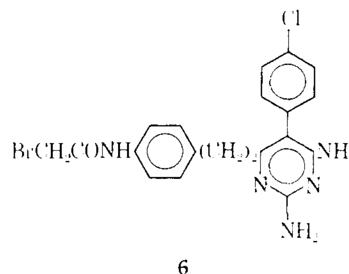
A possible solution to this dilemma was the following. At some point the hydrophobic bonding region on the enzyme should end and return to a polar region. If a leaving group could then be placed at the end of the hydrophobic bonding group where it emerges into a polar region, then a potent active-site-directed irreversible inhibitor should be obtained. Diagrammatically, this can be represented by structure **4a** where the terminal phenyl group could be positioned to a polar region on the enzyme. Two divergent studies were then embarked upon: (a) How far does the hydrophobic bonding region extend as detected by molecules such as **4** and **5**? (b) How much does the hydrophobic bonding region differ from species to species



4a, X = CH_2 **4b**, X = CH_2
5a, X = O **5b**, X = O

or tissue to tissue due to evolutionary changes having taken place?

The second problem will be discussed first. Since the hydrophobic bonding region is adjacent to, but not part of, the active site, evolutionary changes could have occurred readily without destroying the integrity of the active site. That large species differences could exist was shown by study of the enzymes from pigeon liver, *E. coli* B, and induced by T_2 phage.[15,16] In contrast, only relatively small differences (10–100-fold) in binding to the hydrophobic bonding region of dihydrofolic reductase from rat liver and Walker 256 rat tumor could be found;[17] these differences are not sufficient to be utilizable for chemotherapy with reversible inhibitors.[16] That small differences in hydrophobic bonding could be greatly magnified by introducing the extra dimension of active-site-directed irreversible inhibition was then demonstrated.[18] The 5-(*p*-chlorophenyl)pyrimidine (**6**) was an active-site-directed irreversible inhibitor of the dihydrofolic reductase from *E. coli* B; when the dimensions between the leaving group and diaminopy-



6

rimidine of **6** were kept constant, but the hydrophobic bonding group was changed to phenylbutyl (**3**), the compound could no longer irreversibly inhibit the *E. coli* B enzyme.

The second of the simultaneous studies was concerned with the length of the hydrophobic bonding region. Compounds of type **4** and **5** were selected since the extra phenylalkyl group could complex to the enzyme, either to the right (as in **4a**) or to the left as in **4b**. The phenylamyl group of **4** gave a 16-fold increment in binding compared to the parent 1-phenyl-*s*-triazine;[19] the contribution by the butyl part of **4** was only about threefold. That this terminal phenyl group of **4** was complexed in a relatively polar region on the enzyme was indicated by the nearly equal complexing ability of the phenylamyl group of **4** and

(11) For the kinetics of irreversible enzyme inhibition see ref 5, Chapter VIII.

(12) B. R. Baker and H. S. Shapiro, *J. Pharm. Sci.*, **55**, 308 (1966).

(13) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **4**, 31 (1967); paper LXXXIII of this series.

(14) B. R. Baker and R. B. Meyer, Jr., *J. Pharm. Sci.*, **56**, 570 (1967); paper LXXXIV of this series.

(15) B. R. Baker and B.-T. Ho, *ibid.*, **55**, 470 (1966).

(16) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967); paper XCVII of this series.

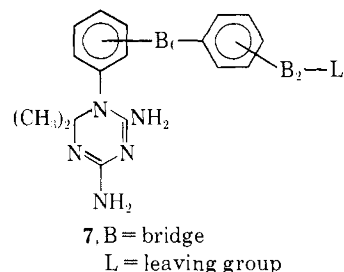
(17) B. R. Baker, to be published.

(18) B. R. Baker and J. H. Jordaan, *J. Pharm. Sci.*, **56**, 660 (1967); paper LXXXVIII of this series.

(19) B. R. Baker, B.-T. Ho, and G. J. Lourens, *ibid.*, **56**, 737 (1967); paper LXXXVI of this series.

phenoxybutyl group of **5**.[20] It had previously been demonstrated that when a benzyl group was complexed to the hydrophobic bonding region of dihydrofolic reductase, the more polar phenoxy group gave a 30-fold loss in binding.[6]

The above two studies were then reconverged onto one objective diagrammatically indicated in **7**; if the proper bridge lengths, B_1, between the two phenyl groups, and B_2, between the outside phenyl and the leaving group, L, and if the proper leaving group could be found, then an active-site-directed irreversible



7, B = bridge
L = leaving group

inhibitor should emerge which could utilize the small differences in the hydrophobic bonding region of dihydrofolic reductases from different tissues. Initial work was focused on the bromoacetamido and chloromethyl ketone groups for the L group of **7**, since these two groups have the electrophilic character 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 two leaving groups and varying B_1 and B_2 groups were synthesized and evaluated; none showed irreversible inhibition of the dihydrofolic reductases from pigeon liver, Walker 256 rat tumor, or L1210 mouse leukemia.[21]

The dihydrofolic reductase from chicken liver[22] has 20 serines and threonines and the L1210 enzyme[23] has even more. The bromoacetamido group reacts too slowly[24] with a neighboring aliphatic hydroxyl group on the protein to be useful. However, a sulfonyl fluoride[25] or fluorophosphate[26] can react rapidly with serine-195 of chymotrypsin when juxtaposed in a reversible complex. Therefore, structures of type **7** where L is a sulfonyl fluoride were then synthesized; some of these compounds were excellent active-site-directed irreversible inhibitors and compose the subject of this paper.

Chemistry.—Aromatic compounds bearing the SO_2F group are used to fix various dyes to fibers. Sulfanilyl fluoride (**10**) can be converted to a diazonium salt then coupled to give a suitable azo dye; these azo dyes having the SO_2F group are used for fast dying of cotton, vinylon, silk, cellulose acetate, and Nylon-6.[27] It was further shown that dyestuffs containing an SO_2F group are fixed on cellulose by formation of a covalent sulfonate ester; about 75% of the sulfonate is linked to the primary hydroxyl groups and the remainder to secondary hydroxyl groups.[27e]

The first extensive studies on the chemistry of aromatic sulfonyl fluoride were made by Steinkopf[28] and by Davies and Dick.[29] The sulfonyl fluoride group is quite stable to a variety of reagents providing they are not strongly basic; it is much more stable than the sulfonyl chloride group. Whereas the sulfonyl chloride rapidly reacts with pyridine or hot ethanol, the sulfonyl fluoride is stable to these reagents. Yet the sulfonyl fluoride will rapidly form a covalent bond with an alcohol group of cellulose when the sulfonyl fluoride is attached to a dye that is reversibly complexed with the cellulose. These two parameters, stability to bimolecular reaction but fast reaction within a complex, are exactly what are needed for an active-site-directed irreversible inhibitor.

Three other points in the chemistry of aromatic sulfonyl fluorides were important for synthesis of dihydrofolic reductase inhibitors of type **15**. The nitro group of *p*-nitrobenzenesulfonyl fluoride can be reduced to give **10** with a Raney nickel catalyst,[30] but palladium leads to hydrogenolysis of the fluoro group. Secondly, the aromatic sulfonyl fluoride group is relatively stable to hot acid as shown by the successful acid hydrolysis of N-acetylsulfanilyl fluoride to **10**.[30] Thirdly, the *p*-amino group of sulfanilyl fluoride (**10**) can be made to react with acid chlorides in boiling xylene.[31] Thus, a set of reaction conditions are available that are compatible with the conversion of sulfanilyl fluoride (**10**) to inhibitors of type **15**.

Sulfanilyl fluoride (**10**) was acylated in boiling toluene or xylene with *m*- or *p*-nitrocinnamoyl chloride to give **12** and **13** (Scheme I); similarly, *m*- and *p*-nitrobenzoyl or phenylacetyl chloride afforded the amides **14**. Catalytic reduction of the nitro group of **14** with Raney nickel proceeded rapidly in 15–30 min, but 1–2 hr were required for further reduction of the double bond of **12** and **13**; most of the resultant amides **16** were readily crystallized in good yield. Reaction of the amines **16** with cyanoguanidine in acetone and hydrochloric acid or ethanesulfonic acid by the three-component method of Modest[32] afforded the desired enzyme inhibitors **15**. Thus, only three simple steps were necessary for synthesis of these potent active-site-directed irreversible inhibitors of the dihydrofolic reductases (Table I).

Earlier attempts to use *p*-nitrobenzaldehyde to form **11** followed by reduction of C=N linkage, as previously employed for other aromatic amines,[33] were unsuccessful. The *p*-amino group of **10** was too inert to

(20) B. R. Baker and G. J. Lourens, *J. Pharm. Sci.*, **56**, 871 (1967); paper LXXXVII of this series.

(21) B. R. Baker and G. J. Lourens, to be published.

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(25) D. E. Fahrney and A. M. Gold, *J. Am. Chem. Soc.*, **85**, 997 (1963).

(26) A. R. Main, *Science*, **144**, 992 (1964).

(27) (a) H. Z. Lecher, R. P. Parker, and C. M. Hofmann, U. S. Patent 2,436,697 (1948); *Chem. Abstr.*, **42**, P3969*e* (1948); (b) R. P. Parker and C. M. Hofmann, U. S. Patent 2,427,995 (1947); *Chem. Abstr.*, **42**, P375*i* (1948); (c) T. Muruyama, T. Kitao, I. Kushiro, N. Kuroki, and K. Konishi, *Kogyo Kagaku Zasshi*, **63**, 997 (1960); *Chem. Abstr.*, **60**, 16013*e* (1964); (d) T. Muruyama, T. Kitao, and K. Konishi, *Bull. Univ. Osaka Prefect., Ser. A*, **8** (2), 77 (1960); *Chem. Abstr.*, **55**, 11854*f* (1961); (e) B. Krazer and H. Zollinger, *Helv. Chim. Acta*, **43**, 1513 (1960).

(28) W. Steinkopf, *J. Prakt. Chem.*, **117**, 1 (1927).

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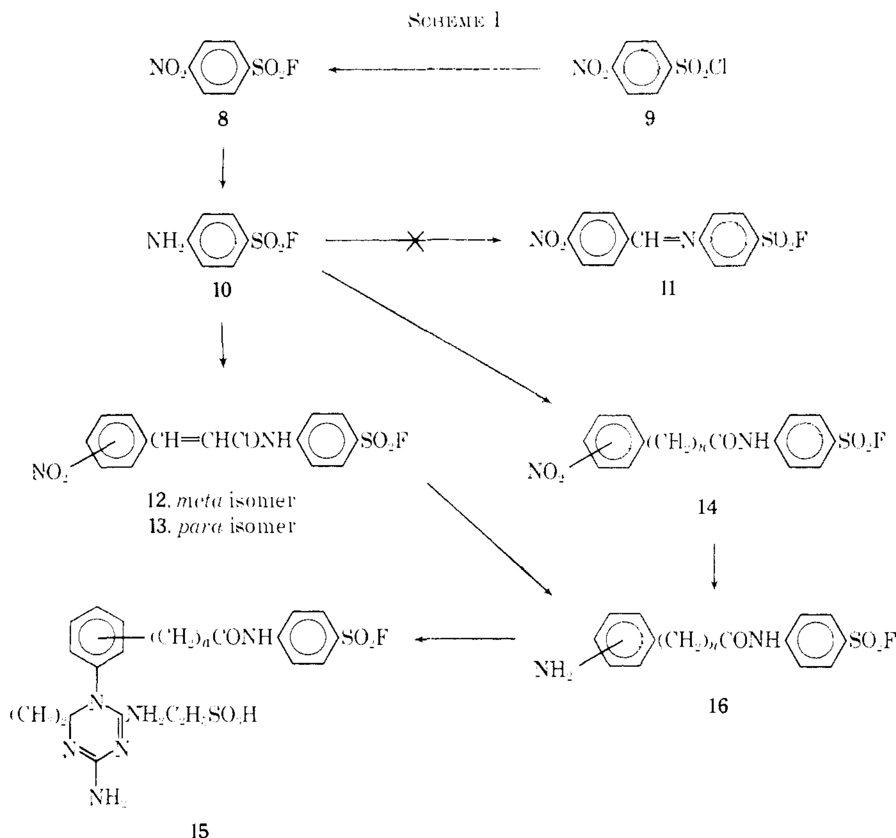
(32) E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

(33) (a) B. R. Baker and C. E. Morreal, *J. Pharm. Sci.*, **52**, 840 (1963); (b) B. R. Baker, D. V. Santi, P. I. Almaula, and W. C. Werkheiser, *J. Med. Chem.*, **7**, 24 (1964); (c) B. R. Baker and J. H. Jordaan, *ibid.*, **8**, 35 (1965); (d) B. R. Baker, T. J. Schwan, and B.-T. Ho, *J. Pharm. Sci.*, **56**, 38 (1967); (e) B. R. Baker, G. D. F. Jackson, and R. B. Meyer, Jr., *ibid.*, **56**, 566 (1967).

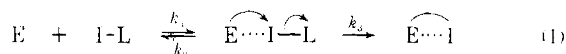
SCHEME I



meta series	*para* series
a, $n = 0$	d, $n = 0$
b, $n = 1$	e, $n = 1$
c, $n = 2$	f, $n = 2$

react with an aldehyde due to inactivation by the electron-withdrawing sulfonyl fluoride group; this lowered reactivity was previously noted with some sulfanilamide derivatives.[34]

Inhibitor Evaluation.—An active-site-directed irreversible inhibitor operates by first forming a reversible complex with the enzyme. If a nucleophilic group on the enzyme and a leaving group (L) are juxtaposed within the complex, then a fast irreversible reaction takes place that inactivates the enzyme[35,36] (see eq 1).



$$E + I\text{-}L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdots I\text{-}L \xrightarrow{k_3} E\cdots I \qquad (1)$$

Thus, two kinetic parameters are involved,[11,36] the reversible dissociation constant, $K_i = k_2/k_1$, and the rate of inactivation within the complex, k_3. Our method for measuring the reversible dissociation constant (K_i) for inhibitors of dihydrofolic reductase—or its equivalent,[6] the I_{50}[37]—has been previously described.[38] Also, our method for measuring the rate of inactivation (k_3) of dihydrofolic reductase has been described previously.[39] These methods were satisfactory for irreversible inhibitors[8,9] that inactivated with a half-life of 10–120 min and satisfactory "rate-saturation" kinetics[11,36] could be observed.

With some of the compounds in Table I, the rate of irreversible inhibition was so rapid that the previously employed methods[6,39] did not effectively separate reversible from irreversible inhibition; however, that irreversible inhibition was taking place was still detectable, but the kinetic calculations were not valid due to the difficulty of determining with reasonable accuracy the I_{50} and the rate of inactivation. Therefore these procedures have now been modified.

Dihydrofolic reductase preparations[15,38] frequently contain an extraneous TPNH oxidase. This is most easily compensated for by running a base line with TPNH, enzyme, inhibitor, and buffer; the enzyme reaction is then initiated by final addition of dihydrofolate. When a Gilford 2000 spectrophotometer is employed, the extraneous base line can either be subtracted from the over-all rate or can be compensated for by use of a double-beam measurement where one cuvette has no dihydrofolate. Such a procedure could give an erroneous I_{50} for a fast irreversible inhibitor since the enzyme may be in contact with the inhibitor as long as 5 min before starting the reaction by addition of dihydrofolate. This error can be eliminated by adding the enzyme last; a correction is then applied if necessary by running the TPNH extraneous oxidation separately, or more preferably, by destruction of the

(34) B. R. Baker, D. V. Santi, J. K. Coward, H. S. Shapiro, and J. H. Jordaan, *J. Heterocyclic Chem.*, **3**, 425 (1966).

(35) B. R. Baker, W. W. Lee, E. Tong, and L. O. Ross, *J. Am. Chem. Soc.*, **83**, 3713 (1961).

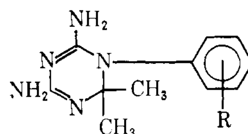
(36) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(37) I_{50} = μM concentration of inhibitor giving 50% inhibition in the presence of 6 μM dihydrofolate, the substrate. Thus $K_i \simeq I_{50}/6$, since $K_m = 1 \times 10^{-6}$ M; this equation is valid when the substrate concentration is at least four times greater than K_m.

(38) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964).

(39) B. R. Baker and J. H. Jordaan, *ibid.*, **2**, 21 (1965).

TABLE I
INHIBITION OF DIHYDROFOLIC REDUCTASES BY



			Reversible		Irreversible					
Compd	R	Enzyme source[a]	I_{50}, µM	Estd $K_i \times 10^6$ M	Inhibitor concn, µM	TPNH concn, µM	% EI[b]	Time, min	% inactiv	$t_{1/2}$, min[c]
15a	m-CONH-C_6H_4-SO_2F	W256	1.1	0.2	5.6	60	97	120	0	
					5.6	0		120	0	
		L1210	3.5	0.6	18	60	97	60	0	
					6.5	30	91	120	0	
		Pigeon liver	1.3	0.2	1.3	60	87	<2	100	<2
					0.15	60	42			<2
15b	m-CH_2CONH-C_6H_4-SO_2F	W256	0.046	0.008	0.18	60	97	60	0	
		Rat liver	0.060	0.01	0.30	0		60	0	
					0.30	60	97	60	0	
		L1210	0.30	0.05	1.3	60	96	60	0	
					1.6	30	97	60	0	
		Pigeon liver	0.31	0.05	0.35	0				<2
					0.35	60	88	<2	80	<2
15c	m-$(CH_2)_2$CONH-C_6H_4-SO_2F	W256	0.064	0.01	0.32	60	97	120	0	
					0.21	0		120	0	
		Rat liver	0.028	0.005	0.14	60	97	60	0	
					0.14	0		60	0	
		L1210	0.078	0.01	0.40	60	97	60	0	
		Pigeon liver	0.10	0.02	0.21	60	90	8	50	8
					0.21	0		10	50	10
15d	p-CONH-C_6H_4-SO_2F	W256	21	4	25	60	88	<1	100	<1
		L1210	600	100	25	60	20	60	45	68
		Pigeon liver	144	24	25	60	50	60	90	<5
15e	p-CH_2CONH-C_6H_4-SO_2F	W256	0.020	0.003	0.10	60	97	120	0	
		L1210	0.097	0.016	0.50	60	98	60	0	
					0.40	30	97	60	0	
		Pigeon liver	0.054	0.009	0.26	0		20	50	20
					0.26	60	97	15	50	15
15f	p-$(CH_2)_2$CONH-C_6H_4-SO_2F	W256	0.020	0.003	0.15	0		120	100	
					0.15	60	98	120	100	
					0.05	60	95	<1	90	<1
					0.02	30	87			2[d]
		Rat liver	0.0060	0.001	0.05	60	98	<2	70	<2
					0.02	30	95			8[e]
		L1210	0.080	0.01	0.4	30	97	<1	80	<1
					0.07	30	84			<2
		Pigeon liver	0.07	0.01	0.07	60	87	<2	70	<2
					0.1	30	90	4	90[d]	
					0.05	30	83			4[d]
16a	(NH_2-C_6H_4)CONH-C_6H_4-SO_2F	W256		Large	25	60	~0	60	0	
		L1210		Large	25	60	~0	60	0	
		Pigeon liver		Large	25	60	~0	60	0	
19 and 16a	m-CH_2CONH-C_6H_5	Pigeon liver	0.34	0.06	1.7[f]	60	97	30	0	
				Large	1.7[f]		~0			

[a] W256 = Walker 256 rat tumor, L1210 = mouse leukemia L1210/FR8. [b] Per cent total enzyme in reversible complex; calculated from $[EI] = [E_t]/(1 + K_i/[I])$. [c] $t_{1/2}$ = half-time for inactivation at 37°; see Experimental Section for procedure. [d] At 28° in the presence of 6 µM dihydrofolate; the per cent inactivation is approximate. [e] At 28° in the absence of dihydrofolate. [f] Incubated **19** and **16a** together in same solution.

TPNH oxidase by a 2-hr preincubation of the enzyme preparation at 37°.

An example of the difficulty encountered in the older procedure is that of **15a** (Table I); an I_{50} of 0.03 µM was observed with the pigeon liver enzyme which is equivalent to $K_i = 5 \times 10^{-9}$ M. By use of this K_i, a "rate-saturation" effect was not seen, that is, the relative rates were not correlatable with the amount of reversible E···IL complex, the rate-limiting species. The amount of enzyme complexed is given by eq 2,[11,36] where $[E_t]$ = total enzyme concentration.

$$[E\cdots IL] = \frac{[E_t]}{1 + \frac{K_i}{[IL]}} \quad (2)$$

By adding enzyme last to a cuvette containing inhibitor, TPNH, dihydrofolate, and buffer, then measurement of the velocity within 30–60 sec, an I_{50} of 1.3 μM was obtained equivalent to $K_i = 2 \times 10^{-7}$ M; besides the short contact time between enzyme and inhibitor, this procedure has another advantage; namely, the substrate will decrease the rate of inactivation by 50% when the inhibitor is present at the I_{50} concentration.

The second difficulty to be overcome was in the method for determining the rate of inactivation. In order to cut down aliquoting errors, the inhibitor is normally mixed with enzyme at 37°, then aliquots are removed starting 1–2 min after mixing. This first point taken at 1–2 min is satisfactory for inhibitors with a half-life of inactivation greater than 10 min, since with sufficient points the plot can be extrapolated back to zero time. However, **15a** gave total inactivation of the pigeon liver enzyme in 2 min at 37°. Therefore, a more laborious but better method was devised to obtain the zero-time point.

A zero-time aliquot was removed from the enzyme solution at 37°, then the appropriate amount of inhibitor was added to the remainder of enzyme at 37° and aliquots were removed at intervals and chilled to 0° until ready for assay. The inhibitor was added to the zero-time aliquot cooled to 0° just before assay. Since the assay method dilutes the incubation aliquot tenfold, no appreciable reversible inhibition is seen when the inhibitor is used at an incubation concentration equal to the I_{50}; if $5 \times I_{50}$ is used in the incubation, then about 20% reversible inhibition is seen in the zero point. By this method, near 100% activity was obtained as a zero point with an I_{50} of 1.3 μM incubation concentration of **15a**. The second point at 2 min showed that total inactivation of the pigeon liver enzyme had taken place in this time. This procedure was satisfactory for all the cases in Table I except one.

When dihydrofolic reductase is assayed with 6 μM dihydrofolate and a 2–5-fold excess of TPNH, the reaction is linear in rate throughout the reaction to completion due to the low K_m of dihydrofolate and lack of binding by the product, tetrahydrofolate; for 6 μM dihydrofolate, the total optical density change is near 0.06 unit. If curvature in the rate line in the absence of inhibitor is seen before completion, a new TPNH solution will alleviate this condition. When a reversible inhibitor or a relatively slow irreversible inhibitor is added, the slope of the line is decreased, but it is still straight. In the case of **15f** and the dihydrofolic reductase from pigeon liver or Walker 256 rat tumor, the rate of enzyme reaction at an I_{50} concentration of inhibitor showed rapid curvature (at 28°). In fact, nearly total inactivation occurred in about 5 min with only about 30% of the dihydrofolate having been reduced. From the rate of change in slope, it could be estimated that the half-life of inactivation of this dihydrofolic reductase at 28° with an I_{50} concentration of inhibitor and 6 μM dihydrofolate was about 2 min; in the absence of dihydrofolate, the half-life can be estimated to be about 1 min. Therefore, a tangent to the initial slope was used for the velocity of reversible inhibition.

When the rate of inactivation of the dihydrofolic reductase from Walker 256 rat tumor or pigeon liver with **15f** at the I_{50} concentration was determined by the new method, the zero point already showed 60% inactivation. The proper zero point could only be obtained when the zero-time aliquot was added to a cuvette containing TPNH, dihydrofolate, buffer, and $I_{50}/10$ of inhibitor, the latter corresponding to the concentration from other incubation aliquots. Inactivation was almost total in less than 1 min at 37°. This difficulty with the zero point can obviously be avoided by using this more cumbersome technique. However, in routine screening of compounds, such a fast inactivation is seldom encountered, but is readily detectable by any curvature in the velocity line when the I_{50} is being determined. Therefore, for compounds not showing curvature in the I_{50} velocity line, we prefer the first modified method where the inhibitor is added to the zero-time aliquot at 0°.

Experimental Section[40]

N-(*m*-Nitrobenzoyl)sulfanilyl Fluoride (14a). Method A.—A mixture of 1.75 g (10 mmoles) of sulfanilyl fluoride, 1.86 g (10 mmoles) of *m*-nitrobenzoyl chloride, and 50 ml of xylene was refluxed for 2 hr during which time about 15 ml of the xylene was allowed to distil and HCl was evolved. The mixture was cooled, the product was collected by filtration, then washed with xylene. Recrystallization from EtOAc–petroleum ether (bp 30–60°) gave 2.32 g (72%) of buff crystals, mp 216–217°. See Table II for additional data.

N-(*m*-Aminobenzoyl)sulfanilyl Fluoride (16a). Method B.—A suspension of 1.95 g (6 mmoles) of **14a** and 1 g of Raney nickel (Grace Co.) in 100 ml of EtOH was shaken with H_2 at 2–3 atm until 18 mmoles of H_2 was absorbed (20 min). The solution was filtered through a Celite pad and the filter cake was washed (EtOH). The combined filtrate and washings were spin-evaporated *in vacuo* to about 10 ml, then diluted (H_2O) and cooled. The product was collected and recrystallized from aqueous EtOH; yield, 1.00 g (57%) of buff crystals, mp 200–204° dec. For analytical data and for additional compounds prepared by this method see Table II.

In the cases where a hydrochloride or ethanesulfonate salt were isolated, the appropriate acid was added to the solution after removal of the catalyst. The solution was spin-evaporated *in vacuo* and the residue was recrystallized.

N-[*m*-(4,6-Diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl)-benzoyl]sulfanilyl Fluoride (15a) Hydrochloride. Method C.—A mixture of 589 mg (2 mmoles) of **16a**, 185 mg (2.2 mmoles) of cyanoguanidine, 15 ml of reagent acetone, and 0.18 ml of 12 N HCl was refluxed with stirring for 24 hr, during which time the product crystallized from the solution. The cooled mixture was filtered and the product was washed (Me_2CO). Recrystallization from EtOH gave 380 mg (42%) of white crystals, mp 231–233° dec. See Table II for analytical data and similar preparations.

For preparation of an ethanesulfonate such as **15e**, ethanesulfonic acid was added instead of HCl.

Enzyme Assay Methods[41]

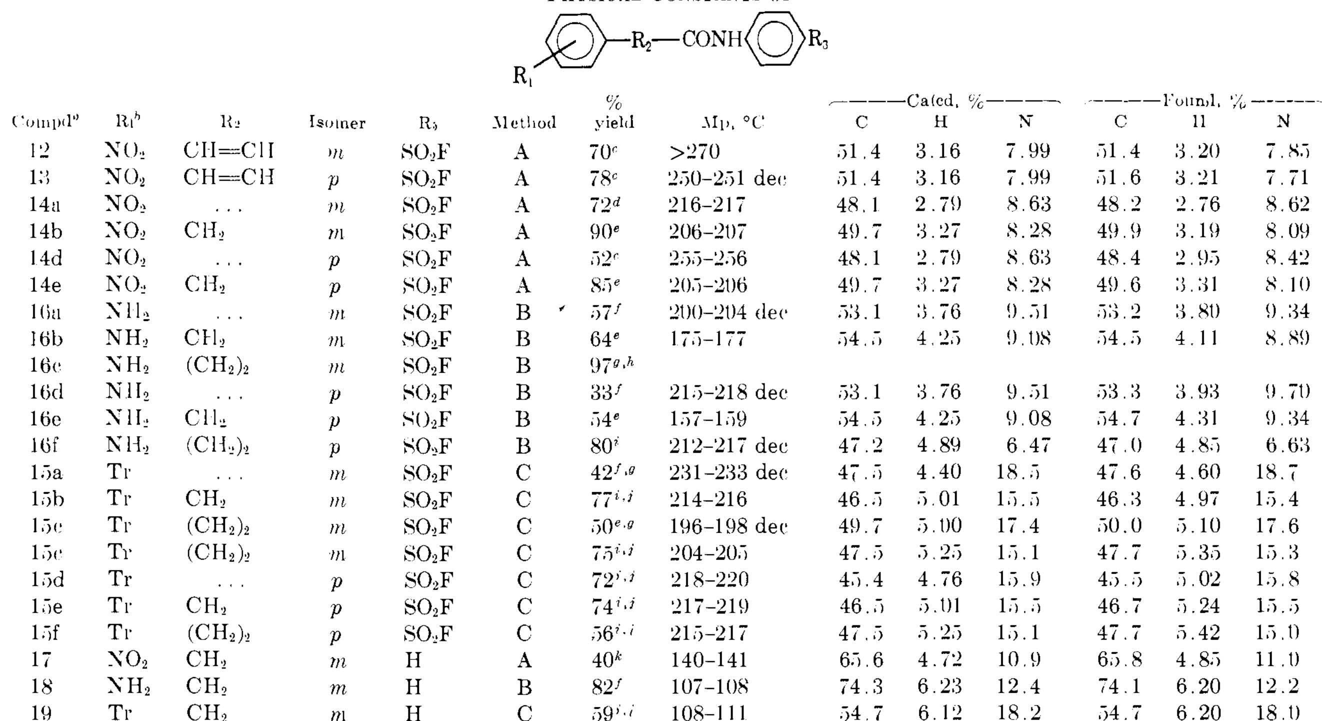
Materials.—Tris hydrochloride, Tris (trishydroxymethylaminomethane), folic acid, and TPNH were purchased from Sigma Chemical Co. Pigeon liver acetone powder was purchased from General Biochemicals Corp. Walker 256 rat tumors in albino rats were excised from untreated control groups used in the CCNSC screen 7 days after intramuscular transplantation, livers were excised from the same group of rats and both tissues were stored at −15° until ready for use.[42] Mouse leukemia

(40) Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected.

(41) The technical assistance of Barbara Baine, Ann Janda, and Jean Reeder with these assays is acknowledged.

(42) We wish to thank Dr. John Venditti of the CCNSC and Dr. T. J. McBride of Chas. Pfizer and Co. for these materials from the CCNSC screen.

TABLE II
PHYSICAL CONSTANTS OF



Compd[a]	R_1[b]	R_2	Isomer	R_3	Method	% yield	Mp, °C	Calcd, % C	H	N	Found, % C	H	N
12	NO_2	CH=CH	*m*	SO_2F	A	70[c]	>270	51.4	3.16	7.99	51.4	3.20	7.85
13	NO_2	CH=CH	*p*	SO_2F	A	78[c]	250–251 dec	51.4	3.16	7.99	51.6	3.21	7.71
14a	NO_2	. . .	*m*	SO_2F	A	72[d]	216–217	48.1	2.79	8.63	48.2	2.76	8.62
14b	NO_2	CH_2	*m*	SO_2F	A	90[e]	206–207	49.7	3.27	8.28	49.9	3.19	8.09
14d	NO_2	. . .	*p*	SO_2F	A	52[c]	255–256	48.1	2.79	8.63	48.4	2.95	8.42
14e	NO_2	CH_2	*p*	SO_2F	A	85[e]	205–206	49.7	3.27	8.28	49.6	3.31	8.10
16a	NH_2	. . .	*m*	SO_2F	B	57[f]	200–204 dec	53.1	3.76	9.51	53.2	3.80	9.34
16b	NH_2	CH_2	*m*	SO_2F	B	64[e]	175–177	54.5	4.25	9.08	54.5	4.11	8.89
16c	NH_2	$(CH_2)_2$	*m*	SO_2F	B	97[g,h]							
16d	NH_2	. . .	*p*	SO_2F	B	33[f]	215–218 dec	53.1	3.76	9.51	53.3	3.93	9.70
16e	NH_2	CH_2	*p*	SO_2F	B	54[e]	157–159	54.5	4.25	9.08	54.7	4.31	9.34
16f	NH_2	$(CH_2)_2$	*p*	SO_2F	B	80[i]	212–217 dec	47.2	4.89	6.47	47.0	4.85	6.63
15a	Tr	. . .	*m*	SO_2F	C	42[f,g]	231–233 dec	47.5	4.40	18.5	47.6	4.60	18.7
15b	Tr	CH_2	*m*	SO_2F	C	77[i,j]	214–216	46.5	5.01	15.5	46.3	4.97	15.4
15c	Tr	$(CH_2)_2$	*m*	SO_2F	C	50[e,g]	196–198 dec	49.7	5.00	17.4	50.0	5.10	17.6
15c	Tr	$(CH_2)_2$	*m*	SO_2F	C	75[i,j]	204–205	47.5	5.25	15.1	47.7	5.35	15.3
15d	Tr	. . .	*p*	SO_2F	C	72[i,j]	218–220	45.4	4.76	15.9	45.5	5.02	15.8
15e	Tr	CH_2	*p*	SO_2F	C	74[i,j]	217–219	46.5	5.01	15.5	46.7	5.24	15.5
15f	Tr	$(CH_2)_2$	*p*	SO_2F	C	56[i,j]	215–217	47.5	5.25	15.1	47.7	5.42	15.0
17	NO_2	CH_2	*m*	H	A	40[k]	140–141	65.6	4.72	10.9	65.8	4.85	11.0
18	NH_2	CH_2	*m*	H	B	82[f]	107–108	74.3	6.23	12.4	74.1	6.20	12.2
19	Tr	CH_2	*m*	H	C	59[i,j]	108–111	54.7	6.12	18.2	54.7	6.20	18.0

[a] All compounds had ir and uv spectra in agreement with their assigned structures. [b] Tr = 4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazin-1-yl. [c] Recrystallized from 2-methoxyethanol. [d] Recrystallized from EtOAc–petroleum ether (bp 30–60°). [e] Recrystallized from aqueous 2-methoxyethanol. [f] Recrystallized from EtOH–H_2O. [g] Hydrochloride. [h] Amorphous, but essentially homogeneous on tlc. [i] Ethanesulfonate. [j] Recrystallized from aqueous 2-PrOH. [k] Recrystallized from C_6H_6.

L1210/FR8 was an amethopterin-resistant strain[43] with enhanced dihydrofolic reductase level grown as local subcutaneous tumors.[44] Dihydrofolate was prepared by dithionite reduction of folic acid,[45] then stored at 3–4° as a homogenized 1.82 m*M* suspension in 5 m*M* HCl containing 0.1 *M* mercaptoethanol.

Buffers.—Buffer A was 0.05 *M* Tris hydrochloride, pH 7.4. Buffer B was the same as A, but with the addition of 10 m*M* mercaptoethanol and 1 m*M* EDTA.

Dihydrofolic Reductases. A. Pigeon Liver.—An extract of pigeon liver acetone powder with buffer A was precipitated between 45–90% saturation by $(NH_4)_2SO_4$ as previously described,[38] except that each insoluble fraction was collected by centrifugation for 10 min at 20,000 rpm in a No. 40 rotor of a Spinco L centrifuge. In order to remove[39] an extraneous TPNH oxidase, the final solution was incubated at 37° for 1 hr, then chilled and filtered through a Celite pad. The final volume from 10 g of acetone powder was 38 ml. For assay the solution was diluted 1:3.5; 100 μl of this diluted solution in a total volume of 1.00 ml gave an OD change of about 0.10 OD unit/min when assayed as described below.

B. Mouse Leukemia L1210/FR8.—In a prechilled small head of a Waring blendor was placed 1.6 g of L1210/FR8 acetone powder,[44] 32 ml of ice-cold buffer A, and a trace of GE-60 antifoam. The mixture was blended at high speed for 2 min, then centrifuged for 30 min at 20,000 rpm in a no. 40 rotor of a Spinco L centrifuge. The supernatant (24 ml) was stored at 3° and was stable for months. For assay, it was diluted 1:20; 100 μl of this dilute solution in a total of 1.00 ml gave an OD change of about 0.01 OD unit/min when assayed as described below.

C. Walker 256 Rat Tumor.—A mixture of 30 g of frozen tumor[42] and 60 ml of ice-cold buffer A was blended for 2 min at high speed in a prechilled small head of a Waring blendor, then centrifuged for 20 min at 20,000 rpm in a no. 40 rotor of a Spinco L centrifuge. To the stirred supernatant (73 ml) cooled in an ice bath was added 4.3 ml of 5% aqueous streptomycin. After being stirred an additional 10 min, the mixture was centrifuged for 10 min at 20,000 rpm. To the stirred supernatant (65 ml) cooled in an ice bath was added 17.9 g of enzyme grade $(NH_4)_2SO_4$ over a period of 1–2 min (45% of saturation). After being stirred 10 min more, the mixture was centrifuged for 10 min at 20,000 rpm. To the stirred supernatant (62 ml) cooled in an ice bath was added 21 g of $(NH_4)_2SO_4$ (90% of saturation). After being stirred for an additional 10 min, the mixture was centrifuged for 20 min at 20,000 rpm. The supernatant was rejected and the pellets were dissolved in buffer A giving a final volume of 40 ml; this solution could be stored at 3° with little or no loss in activity over 3 months. For assay, the solution was diluted 1:2, then 50 μl gave an OD change of about 0.01 unit/min under the conditions described below. If an extraneous TPNH oxidase was present, the solution was incubated at 37° for 1 hr, then chilled and filtered.

D. Rat Liver.—The enzyme was prepared as described for the rat tumor. The final volume was 35 ml; this solution was stable at 3° over 6 months. For assay, the solution was diluted 1:3; 50 μl of the diluted solution gave an optical density change of about 0.01 unit/min when assayed as described below.

Reversible Inhibition of Dihydrofolic Reductases.—A 3.72 m*M* solution of TPNH in 0.01 *M* NaOH was sufficiently stable to be stored only 4 days at 3°. For enzyme assay 0.20 ml was diluted with 1.04 ml of buffer B to give a 600 μ*M* solution; this solution should be stored at 0° and renewed every 4 hr.

Dihydrofolate was the 1.82 m*M* suspension above diluted with buffer B to give a 120 μ*M* solution. This solution should be stored at 0° and renewed every 4 hr.

In a 1-ml cuvette were placed 0.85 ml of buffer B, 50 μl of 600 μ*M* TPNH, and 50 μl of enzyme. After the system had balanced, 50 μl of 120 μ*M* dihydrofolate was added and the decrease in optical density at 340 mμ was followed on a 0–0.1 slide wire range of a Gilford 2000 or Cary 11 spectrophotometer. Sufficient enzyme should be used to give 0.008–0.010 OD unit change/min. Any small decrease l optical density prior to addition of dihydrofolate is subtracted from the enzyme rate. This rate without inhibitor is termed V_0; the cuvette concentrations were 6 μ*M* in dihydrofolate and 30 μ*M* in TPNH.

Inhibitors were dissolved in 1 m*M* HCl; if not soluble, an equal volume of DMF was added, then the solution was diluted with 1 m*M* HCl. Fresh solutions were prepared daily. To de-

(43) M. Friedkin, E. Crawford, S. R. Humphreys, and A. Goldin, *Cancer Res.*, **22**, 600 (1962).

(44) An acetone powder of this subcutaneous L1210/FR8 was kindly provided by Dr. A. W. Schrecker of the CCNSC.

(45) S. Futterman, *J. Biol. Chem.*, **228**, 1031 (1957).

termine the rate, V_1, in the presence of inhibitor, in a 1-ml cuvette were placed 0.75 ml of buffer B, 100 μl of inhibitor, 50 μl of 120 μM dihydrofolate, and 50 μl of 600 μM TPNH. When the system had balanced, 50 μl of enzyme was added and the rate of decrease in optical density followed. If the rate was linear for 5 min, then no measurable irreversible inhibition had occurred. Several inhibitor concentrations were run to give V_1/V_0 = 1.5-2.5; the concentration giving V_1/V_0 = 2 is the I_{50}.[46] If the rate with inhibitor present is not linear and gradually decreases, but the rate in the absence of inhibitor is linear, then both reversible and irreversible inhibition are occurring simultaneously; only in the case of **15f** with Walker 256 and pigeon liver was this nonlinearity seen with the compounds and isozymes in Table I.

Irreversible Inhibition of Dihydrofolic Reductase.—The enzymes from pigeon liver, rat liver, and rat tumor were stable at 37° for 2 hr in the presence or absence of TPNH; all three enzymes were also stable in the presence of 5% DMF. The L1210 mouse leukemia was unstable at 37° in the absence of TPNH, but with 30 μM TPNH it was stable for 1 hr; 5% DMF caused rapid inactivation, but 5% DMSO was satisfactory. The assay conditions below were shown to give a rate dependent upon the enzyme concentration.

Into two tubes were placed 0.40 ml of buffer A, 0.50 ml of enzyme solution (sufficient to give a velocity of 0.008-0.012 OD unit/min when assayed below), and 50 μl of 600 μM TPNH. The two tubes were placed in a 37° bath; after 5 min, 50 μl of solvent without inhibitor was added to the first tube which served as the enzyme control and the time was noted. From the second tube was removed 0.48 ml which was placed in a tube at 0°, then labeled I_1. To the remainder of the second tube (I_2) was added 25 μl of inhibitor and the time was noted. After 2 min, 0.50 ml was removed from the control tube and placed in an ice bath with the label C_1. The two original tubes (C_2 and I_2) were left in the 37° bath for 1-2 hr, then chilled in an ice bath until ready for assay.

The C_1, C_2, and I_2 tubes were assayed as follows: in a 1-ml cuvette were placed 0.80 ml of buffer B, 50 μl of 240 μM dihydrofolate, and 50 μl of 300 μM TPNH. When the base line had become constant, the reaction was initiated by adding 100 μl of incubation aliquot. For the I_1 point, 25 μl of inhibitor solution was added just before assay. This procedure is satisfactory for a routine assay for ± irreversible inhibition when run at I_{50} × 5, providing the I_{50} velocity measurement was linear; if it was not linear, then the appropriate amount of inhibitor solution was added to the cuvette rather than to the enzyme aliquot for the I_1 point.

If the compound showed irreversible inhibition, then a time study could be performed by increasing the amounts in the I tube so that four to six aliquots could be removed at chosen time intervals.

Results and Discussion

Pigeon Liver Enzyme.—The first two compounds of this series synthesized for enzymic evaluation bridged the dihydro-*s*-triazine to sulfanilyl fluoride with *m*-benzoyl (**15a**) and *m*-phenylpropionyl (**15c**) groups. Both showed irreversible inhibition of pigeon liver dihydrofolic reductase (Table I); **15a** completely inactivates the enzyme in less than 2 min at 37° at a concentration equal to the I_{50} which reversibly complexes about 87% of the enzyme. The new methods for measuring the I_{50} of fast irreversible inhibitors were then devised with **15a**, as discussed earlier and described in the Experimental Section.

Not too surprisingly, **15a** and **15c** failed to irreversibly inhibit the dihydrofolic reductases from Walker 256 rat tumor and L1210 mouse leukemia. Since the sulfonyl fluoride group is most probably positioned outside the active site where it attacks a nucleophilic group on the enzyme surface,[5,12] it can be anticipated that "all or none" differences could exist in this area outside of the active site of the enzyme from different species;[47] this is the working basis of the bridge principle of specificity.[47,48] Therefore, the remaining four compounds of series **15** were synthesized to see if a different bridge length between the *s*-triazine that is complexing in the active site and the leaving group that is attacking outside the active site could irreversibly inhibit the tumor enzymes; as will be discussed below, the *p*-benzoyl and *p*-phenylpropionyl bridges gave effective irreversible inhibitors of the two tumor enzymes.

Of the six compounds of structure **15**, all showed irreversible inhibition of the pigeon liver enzyme at a concentration equal to or 30 times K_i; the rate of reaction is dependent upon the amount of enzyme reversibly complexed with the enzyme. When the concentration of inhibitor is equal to its K_i, then 50% of the enzyme is reversibly complexed as the rate-determining species [E···I] according to eq 2. When the concentration of inhibitor is 30 times K_i, the amount of enzyme reversibly complexed is 97% of the total enzyme. Since the rate of inactivation is dependent upon the amount of reversible complex, [EI], the difference in rate between a K_i concentration and a 30 × K_i concentration should only be twofold, a so-called rate-saturation effect.[11,36] Thus in order to compare two irreversible inhibitors with different dissociation constants (K_i), the two compounds should be compared at equal [EI] concentration, not equal [I] concentration.[13] Note that in the presence of TPNH, **15c** inactivated the enzyme with a half-life of about 10 min and **15e** with a half-life of about 15 min but the other compounds inactivated the enzyme in less than 2 min; the difference in these rates can be rationalized on the basis of bridge principle of specificity, that is, at equal [EI] concentration, the rate of inactivation is dependent upon the nucleophilic ability of the amino acid group being attacked and upon the closeness of juxtaposition of the enzyme nucleophilic group and the inhibitor leaving group.[47,48]

Further experiments were then performed to support the concept that these inactivations occurred by the active-site-directed mechanism.

(a) That the inactivation did not proceed by a random bimolecular process, shown in eq 3, was indicated by the different rates of inactivation of **15a-f** at varying concentrations. This bimolecular mechanism

$$E + I \xrightarrow{k_B} EI \quad (3)$$

was definitely eliminated by the fact that N-(*m*-aminobenzoyl)sulfanilyl fluoride (**16a**) at the high concentration of 25 μM showed no inactivation of the enzyme, whereas **15a** at 0.15 μM could inactivate the enzyme with a half-life of less than 2 min. Note that the only difference between **15a** and **16a** is the lack of the diamino-*s*-triazine ring in **16a** necessary for forming a reversible complex in order for the active-site-directed mechanism to operate (eq 1).

(b) It was possible that trace metals originally present in the commercial sulfanilyl fluoride could have been carried through the synthesis and caused the inactivation. The identical sample of **16a** showing

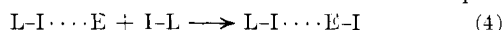
(46) B. R. Baker, W. W. Lee, W. A. Skinner, A. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633 (1960).

(47) See ref 5, Chapter IX.

(48) B. R. Baker, *J. Med. Pharm. Chem.*, **5**, 654 (1962); B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

no inactivation of the enzyme was converted to the diamino-*s*-triazine, **15a**, which could rapidly inactivate the enzyme; therefore **15a** could not have contained trace metals arising from the sulfanilyl fluoride, else **16a** should also have inactivated the enzyme. Further verification was obtained by synthesis of the ethanesulfonate salt of **15c**, the latter showing the same rate of inactivation as the hydrochloride salt of **15c**; since the solubilities of these two salts are quite different, it is unlikely they would have an identical quantity of a given trace metal after purification.

(c) A third mechanism for enzyme inactivation by irreversible inhibitors has been observed with trypsin;[11,49] when the appropriate inhibitor is reversibly complexed to the enzyme, a conformational change can sometimes occur which exposes an enzymic nucleophilic group to bimolecular attack as shown in eq 4.



$$\text{L-I}\cdots\text{E} + \text{I-L} \longrightarrow \text{L-I}\cdots\text{E-I} \quad (4)$$

That this mechanism was not operational was shown by incubating the enzyme with a combination of **19**, which does not have the sulfonyl fluoride group, and **16a**, which does not have the diamino-*s*-triazine moiety. Since no inactivation was seen, it is highly improbable that **15b** formed a reversible complex that was then attacked bimolecularly by a second molecule of **15b**.

Thus, the active-site-directed mechanism is the only possible mechanism yet thought of that is compatible with the data in Table I.

Walker 256 Enzyme.—Of the six different bridges (**15**) between the diamino-*s*-triazine binding to the active site and the sulfanilyl fluoride group, only the *p*-benzoyl (**15d**) and *p*-phenylpropionyl (**15f**) bridges gave molecules that allowed irreversible inhibition of the dihydrofolic reductase from this rat tumor (Table I). A relatively high concentration of 25 μM of **15d** had to be employed due to the relatively poor K_i of this compound. That **15d** did not inactivate the enzyme by the random bimolecular mechanism of eq 3 was shown by the lack of inactivation of this tumor enzyme by 25 μM **16a**, which lacks the diamino-*s*-triazine needed for complexing to the active site, but is still capable of bimolecular attack.

There are two requirements for an active-site-directed irreversible inhibitor to be effective on an enzyme system, as can be seen from eq 1: (a) the lower the reversible dissociation constant, the less concentration of inhibitor is needed to give an effective amount of the rate determining [E···I] species, and (b) the faster the rate of covalent bond formation within the complex, the more effective the inhibitor. If both parameters are good, the amount of random reaction with other proteins or isozymes in the host will be decreased. Note that the *p*-benzoyl-bridged compound (**15c**) meets only one of these two requirements; **15c** can rapidly inactivate the enzyme in less than 1 min, but a relatively high concentration of 4–25 $\times$ 10^{-6} M would be needed to form the required amount of [E···I] complex. In contrast, the *p*-phenylpropionyl bridged compound (**15f**) meets both requirements; it can inactivate this rat tumor enzyme in less than 2 min at an inhibitor concentration of 2–5 $\times$ 10^{-8} M.

The next requirement for an active-site-directed irreversible inhibitor to be effective on a tumor *in vivo* is to have sufficient isozyme specificity so that little or no attack occurs on the target enzyme in normal host tissues. The effect of **15f** on the dihydrofolic reductase from the liver of rats bearing the Walker 256 tumor was investigated. Note first that **15f** differed in reversible binding to the dihydrofolic reductase from the two tissues about threefold. Secondly, rat liver enzyme was also rapidly inactivated by **15f** at 37°. When the inactivation rate was slowed at 28° so that it could be measured, the rat liver enzyme was half-inactivated in 8 min in the absence of dihydrofolate, but the tumor enzyme was half-inactivated in 2 min in the presence of 6 μM dihydrofolate; since dihydrofolate at this concentration would protect against inactivation of **15f** by a factor of two, it can be estimated that **15f** inactivates the tumor enzyme about eight times as fast as the liver enzyme. This eightfold difference is insufficient for effective chemotherapy, but does indicate that the bridge principle of specificity is partially operational. By further modification of the bridge of **15f**, of the hydrophobically bonded 1-phenyl group, of the environment of the leaving group, or of combinations of these, it should be theoretically possible to separate these rates a 1000-fold or more. Note that a higher degree of specificity is already present with the same tissue from two different species; namely, rat liver and pigeon liver. At near equal concentration, **15b** can inactivate pigeon liver enzyme with a half-life of less than 2 min, but the rat liver enzyme shows no inactivation in 60 min. Since 15% irreversible inhibition is readily detectable, the half-life of inactivation of rat liver is greater than 250 min; therefore, **15b** inactivates the pigeon liver isozyme more than 120 times as fast as the rat liver isozyme.

L1210 Enzyme.—This isozyme had an irreversible inhibition profile coincidentally (?) similar to the rat tumor isozyme with the seven compounds in Table I; that is, the L1210 dihydrofolic reductase was inactivated by **15d** and **15f**, but not by the other analogs of **15** or by **16a** lacking a triazine moiety. However, the rates of inactivation by **15d** were different; corrected for the same amount of [E···I] complex, **15d** inactivated the rat tumor enzyme greater than 15 times as fast as L1210 enzyme. With the same 25 μM concentration of **15d**, the rat tumor enzyme was inactivated greater than 68 times more rapidly than L1210 enzyme, one-fourth of this difference being due to the relative abilities of the two isozyme to complex reversibly with **15d**.

The diamino-*s*-triazine bridged by *p*-phenylpropionyl (**15f**) was a good irreversible inhibitor of L1210 enzyme; at a concentration of 4 $\times$ 10^{-7} M, **15f** inactivated the enzyme with a half-life of less than 1 min. Again note that this inactivation is not due to a random bimolecular reaction since 250 $\times$ 10^{-7} M **16a** failed to show irreversible inhibition of the L1210 isozyme.

Other Observations.—Little difference in the rates of inactivation could be seen whether or not TPNH was present; this apparent independence of TPNH concentration is convenient for chemotherapeutic purposes since higher or lower TPNH concentrations in the target cell will not effect the rate of inactivation. However, this independence of TPNH concentration is rather surprising in view of the difference in reversible binding

(49) T. Inagami, *J. Biol. Chem.*, **240**, PC3453 (1965).

to dihydrofolic reductase by diamino heterocycles in the presence or absence of TPNH; diamino heterocycles are complexed as much as 100-fold better in ternary complex with enzyme and TPNH than in binary complex without TPNH.[50] Since the I_{50} values are determined kinetically, they represent binding in ternary complex; furthermore, since the rate of inactivation described by eq 1 is dependent upon the amount of $[E \cdots I]$ reversible complex, less of the enzyme is complexed in the absence of TPNH.

Consider the inactivation of pigeon liver enzyme by **15c** (Table I); there is little difference in rate of inactivation in the presence or absence of TPNH. If the binary binding constant of **15c** is only $1/100$ of the ternary binding constant of 2×10^{-8} M, then the binary constant would be 2×10^{-6} M; thus in the experiment without TPNH, the amount of $[E \cdots I]$ reversible complex would be 16% of the total enzyme concentration and the rate of inactivation should be 16/90 $\simeq$ 1/5 as fast as in the presence of TPNH, if everything else is equal. However, everything else may not be equal. First, TPNH might cause a conformational change that slows the rate of covalent bond formation; that is, if k_2 in eq 1 is five times as fast in binary complex than in ternary complex, the observed rates of **15c** would be equal in the presence or absence of TPNH. Secondly, the difference in binary and ternary binding could change if hydrophobic bonding is present.[51] If the difference in binary or ternary binding were only tenfold, then the binary K_i of **15c** would be 2×10^{-7} M, the amount of enzyme in reversible binary complex would be 50%, and the relative rates of inactivation in binary and ternary complex would be $90/50 = 1.8$-fold, which is not too different from the observed rates.

The only functional group on an enzyme that is more susceptible to attack by a sulfonyl fluoride than a bromoacetamido group is the alcohol function of serine or threonine. Some bromoacetamides attached to **7**, which have the same dimensions from the 1-phenyl-*s*-triazine moiety to the leaving group, fail to inactivate pigeon liver dihydrofolic reductase; therefore, it is probable that serines or threonines of the enzyme are involved in covalent bond formation with the sulfonyl fluorides **15**. That these sulfonyl fluorides (**15**) and their intermediates are stable to water and alcohol is shown by their methods of preparation; yet these sulfonyl fluorides, when properly juxtaposed to an enzymic nucleophilic group within the enzyme-inhibitor complex, are completely covalently bonded in 60 sec or less. The question then arises as to how fast these sulfonyl fluorides in high dilution react with other proteins by the random bimolecular process of eq 3; **15c** was selected for study with pigeon liver dihydrofolic reductase.

When 2×10^{-7} M **15c** was preincubated at 37° with bovine serum albumin at 2.5 mg/ml for 1 hr, then pigeon liver dihydrofolic reductase was added, inactivation of the latter still occurred at the same rate as with no pretreatment; thus, **15c** did not react appreciably with serum protein in 1 hr. It is therefore highly probable that none of the sulfonyl fluorides (**15**) would react appreciably with serum proteins in 1 hr by the bimolecular mechanism. A drug-like nitrogen mustard is inactivated in serum with a half-life of about 2 min, but still has an effect on responsive tumors. It is, therefore, most likely that a compound such as **15f** would completely inactivate Walker 256 rat tumor dihydrofolic reductase before any measurable reaction with serum proteins occurred by a random bimolecular process. Furthermore, **15c** was stable to a 1-hr preincubation at 37° with 0.05 M Tris buffer at pH 7.4, or with 25 mg/ml of $(NH_4)_2SO_4$. In contrast, when the usual 45–90% $(NH_4)_2SO_4$ fraction of pigeon liver dihydrofolic reductase was incubated with 2×10^{-7} M of **15c** for 1 hr, all of the dihydrofolic reductase was inactivated; however, when a second aliquot of dihydrofolic reductase was added, no further inactivation of the enzyme occurred showing that the inhibitor had been destroyed. That this destruction was not due to foreign protein, ammonium sulfate, or Tris buffer was shown earlier; thus something in the enzyme extract other than these three types of materials had destroyed the inhibitor. Further work will be necessary to determine if the destruction was caused by macromolecules such as nucleic acid, or by small molecules, or even by some reaction catalyzed by dihydrofolic reductase. Nevertheless, the pigeon liver dihydrofolic reductase was completely inactivated by 2×10^{-7} M **15c** before **15c** was destroyed.

The following requirements for an active-site-directed irreversible inhibitor useful for chemotherapy of a specific tumor (Walker 256) have so far been met by **15f**: (a) the inhibitor will inactivate the enzyme at a concentration of 10^{-7} to 10^{-9} M (actually 10^{-8} M), (b) the inactivation should be rapid (actually less than 1 min), (c) the enzyme attacked should be important for cell growth and division, (d) the compound should be relatively inert toward proteins (actually no observable reaction in 1 hr) other than the target enzyme, and (e) the compound should be able to penetrate the necessary cell membranes (actually compounds of type **15** penetrate by passive diffusion).

The following requirements have not yet been met: (a) the dihydrofolic reductases from host tissues other than the tumor should not be attacked (actually **15f** inactivates tumor enzyme eight times more rapidly than liver), and (b) the compound should have an observable chemotherapeutic effect on the tumor in the living host system. Since parameter a has not yet been met, it is unlikely that parameter b will be met efficiently. Therefore studies are being vigorously pursued to modify **15f** by the bridge principle of specificity[47] so that the resultant compound will inactivate the Walker 256 rat tumor enzyme and not the rat liver enzyme or will inactivate the mouse leukemia L1210 enzyme and not the mouse liver; a variety of ways to accomplish isozyme specificity have been previously described with lactic dehydrogenase[5,47] and additional ways will probably emerge from further studies underway. When an active-site-directed irreversible inhibitor for the tumor is found that will not inactivate the liver enzyme, then parameter b, the effect in an intact animal, will be worthy of study. At that time it will then be necessary to observe which normal tissue other than liver, if any, is still susceptible to irreversible inhibition to the inhibitor; then further modification of the compound to build in additional specificity between the tumor and susceptible normal tissue could be pursued.

(50) J. P. Perkins and J. R. Bertino, *Biochemistry*, **5**, 1005 (1966).
(51) B. Hillcoat and J. R. Bertino, to be published.