Irreversible Enzyme Inhibitors. CLXXV.[1,2] Irreversible Inhibition of Purified Dihydrofolic Reductase

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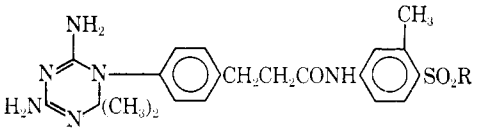
Twenty-six active-site-directed irreversible inhibitors of dihydrofolic reductase of the SO_2F type have been previously shown to inactivate the enzyme from L1210 mouse leukemia or Walker 256 rat tumor with <30% inactivation of the crude enzyme from mouse liver, rat liver, or rat kidney. Since this tissue specificity might have been due to the hydrolysis of the SO_2F group to SO_3H by the "sulfonyl fluoridase" recently shown to be present in rat liver, the irreversible inhibition of affinity column purified dihydrofolic reductase free of "fluoridase" was investigated. With the purified enzyme from these normal tissues, irreversible inhibition was as extensive as with the tumor enzymes; therefore the tissue specificity is due to rapid detoxification of the SO_2F type of inhibitor in normal liver and kidney by the "fluoridase." This newly found major biochemical difference between L1210 or Walker 256 and some normal rat and mouse tissues, namely the absence of the "sulfonyl fluoridase" in the tumor and its presence in liver and kidney, is being further investigated for possible cancer chemotherapeutic utility.

A study on the metabolism of the sulfonyl fluoride (**1**)[4] in the rat was reported in the previous paper;[2] the only detectable metabolic product was the sulfonic acid **2**, 20% of the total radioactivity being excreted in the urine and 57% in the feces. Investigation of two tissues showed that rat serum slowly converted **1** into **2**, but a rat liver extract could rapidly perform this enzymatic hydrolysis. These studies raised the question whether the tissue specific inactivation of dihydrofolic



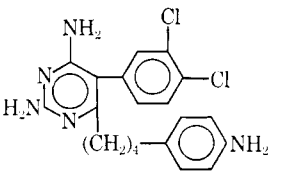
1, R = F
2, R = OH

reductase from Walker 256 rat tumor and rat liver by **1** and other irreversible inhibitors was due to a difference in the structure of the enzyme as previously supposed[5,6] or was due to the relative rates of inactivation of dihydrofolic reductase and the "sulfonyl fluoridase" catalyzed hydrolyzed of inhibitors such as **1**.[2]

The dihydrofolic reductase from rat liver, rat kidney, and mouse liver has now been separated from the "sulfonyl fluoridase" by use of an affinity column[7,8] designed for this purpose. A series of irreversible inhibitors previously shown to give rapid irreversible inhibition of either the enzyme from L1210 mouse leukemia or Walker 256 rat tumor, but little inactivation of the enzyme from rat liver, rat kidney, or mouse liver were then reinvestigated with the enzyme purified with the affinity column. All of the compounds were now highly effective irreversible inhibitors of the enzyme from normal tissues. Thus the tissue specificity seen earlier, *which is real*, is due to the relative rates of destruction of sulfonyl fluorides such as **1** by the "sulfonyl fluoridase" in normal tissues compared with the rate of inactivation of dihydrofolic reductase by **1**.

Affinity Columns.—An affinity column for enzyme purification consists of a solid polymeric support to which is attached by covalent linkage an inhibitor specific for the enzyme for which purification is desired.[7] The selection of the correct inhibitor for covalent linkage to the polymer has the same problem as the design of active-site-directed irreversible enzyme inhibitors;[7] that is, a large group must be attached to the inhibitor at a position where it does not interfere with complex formation with the enzyme. The first example of an affinity column for enzyme purification was described by Lerman in 1953[9] who attached phenylazophenol to cellulose and used the column to purify tyrosinase; this column was not completely satisfactory since it merely slowed the movement of tyrosinase down the column, but did not absorb it sufficiently tightly. A major break-through in this area was achieved by Cuatrecasas, *et al.*,[8] when they employed a cross-linked dextran, agarose (Sepharose 4B), as the polymer support; Sepharose has the big advantage over cellulose that the former has a loose, porous network that allows easy entry and exit of macromolecules resulting in greater affinity for the enzyme.

Three inhibitors (**3**–**5**) of dihydrofolic reductase containing an aniline moiety were considered for attachment to the BrCN-activated Sepharose.[8] The synthe-



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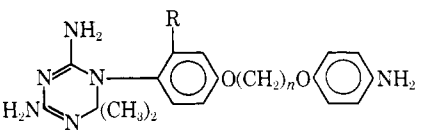


4, R = H; $n = 3$
5, R = CH_3; $n = 2$

(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 in this series see A. J. Ryan, N. M. J. Vermeulen, and B. R. Baker, *J. Med. Chem.*, **13**, 1140 (1970).

(3) (a) To whom correspondence should be addressed. (b) N. M. J. V. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(4) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 677 (1968), paper CXXIX.

(5) B. R. Baker, N. M. J. Vermeulen, W. Ashton and A. J. Ryan, *ibid.*, **13**, 1130 (1970), paper CLXXIII.

(6) B. R. Baker, *Accounts Chem. Res.*, **2**, 129 (1969), paper CXLIX.

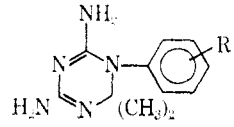
(7) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N. Y., 1967, Chapter XIII.

(8) P. Cuatrecasas, M. Wilcheck, and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U. S.*, **61**, 636 (1968).

(9) L. S. Lerman, *ibid.*, **39**, 232 (1953).

TABLE I

IRREVERSIBLE INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



No.	R	Enzyme source[b]	I_{50},[c] μM	Inhib, μM	Time, min[d]	% inactvn
1	*p*-$(CH_2)_2CONHC_6H_3$-3-CH_3-4-SO_2F	W256 (A)[e]	0.013	0.065	60	100
		Rat liver (A)[e]	0.012	0.065	60	28
		Rat liver (C)		0.065	60	93
		Rat kidney (A)[e]		0.065	20	7
		Rat kidney (C)		0.065	20	94
		L1210/FR8 (A)[f]		0.047	60	78
		Mouse liver (A)[f]		0.047	60	15
		Mouse liver (C)		0.047	60	90
6	*m*-Cl-*p*-$O(CH_2)_3OC_6H_4SO_2F$-*p*	W256 (A)[e]	0.057	0.18	60	94
		Rat liver (A)[e]	0.052	0.18	60	52
		Rat liver (C)		0.18	60	100
7	*m*-Cl-*p*-$O(CH_2)_2NHCONHC_6H_4SO_2F$-*p*	W256 (A)[e]	0.012	0.050	60	77
		Rat liver (A)[e]	0.014	0.050	60	0
		Rat liver (C)		0.050	60	100
		Rat kidney (A)[e]		0.050	20	16
		Rat kidney (C)		0.050	20	65
		L1210/DF8 (A)[g]		0.072	60	86
		Mouse liver (A)[g]		0.16	60	18
		Mouse liver (C)		0.16	60	100
8	*m*-$(CH_2)_4C_6H_4SO_2F$-*p*	W256 (A)[e]	0.014	0.050	60	85
		Rat liver (A)[e]	0.018	0.050	60	95
		Rat kidney (A)[e]		0.050	20	15
		Rat kidney (C)		0.050	20	94
9	*m*-Cl-*p*-$OCH_2CONHC_6H_4SO_2F$-*p*	W256 (A)[e]	0.025	0.074	60	64
		Rat liver (A)[e]	0.027	0.11	60	50
		Rat kidney (A)[e]		0.11	20	11
		Rat kidney (C)		0.11	20	72
		L1210/DF8 (A)[h]	0.037	0.074	60	76
		Mouse liver (A)[h]		0.16	60	0
		Mouse liver (C)		0.16	60	68
10	*p*-$(CH_2)_4C_6H_4SO_2F$-*p*	W256 (A)[e]	0.014	0.050	60	62
		Rat liver (A)[e]	0.022	0.060	60	12
		Rat liver (C)		0.060	60	88
		Rat kidney (A)[e]		0.060	20	8
		Rat kidney (C)		0.060	20	77
		L1210/DF8 (A)[h]	0.020	0.10	60	69
		Mouse liver (A)[h]		0.10	60	27
		Mouse liver (C)		0.10	60	76
11	*m*-Cl-*p*-$(CH_2)_2C_6H_4SO_2F$-*p*	W256 (A)[e]	0.0075	0.050	60	86
		Rat liver (A)[e]	0.018	0.050	60	86
		Rat kidney (A)[e]		0.050	20	43
		Rat kidney (C)		0.050	20	71
		L1210/DF8 (A)[h]	0.014	0.070	60	93
		Mouse liver (A)[h]		0.070	60	17
		Mouse liver (C)		0.070	60	92
12	*m*-Cl-*p*-$(CH_2)_2CONHC_6H_4SO_2F$-*p*	W256 (A)[e]	0.015	0.050	60	86
		Rat liver (A)[e]	0.015	0.050	60	16
		Rat liver (C)		0.050	60	71
		Rat kidney (A)[e]		0.050	20	25
		Rat kidney (C)		0.050	20	100
		L1210/DF8 (A)[h]	0.024	0.096	60	95
		Mouse liver (A)[h]	0.015	0.096	60	30
		Mouse liver (C)		0.096	60	98
13	*p*-$(CH_2)_2CONHC_6H_4SO_2F$-*p*	L1210/DF8 (A)[i]	0.025	0.070	60	73
		Mouse liver (A)[i]	0.015	0.070	60	30
		Mouse liver (C)		0.070	60	83

TABLE I (*Continued*)

No.	R	Enzyme source[b]	I_{50},[c] μM	Inhib, μM	Time, min[d]	% inactvn
14	*m*-$(CH_2)_2CONHC_6H_4SO_2F$-*p*	Pigeon liver (A)[j]	0.10	0.21	8[k]	50[k]
		L1210/FR8[j]	0.078	0.40	60	0
		Mouse liver (C)		0.14	60	15
		W256 (A)[j]		0.32	60	0
		Rat liver (C)		0.14	60	0
15[l]	*m*-Cl-*p*-$O(CH_2)_2OC_6H_4SO_2F$-*p*	W256 (A)		0.072	60	86
		Rat liver (A)		0.072	60	22
		Rat liver (C)		0.072	60	100
		Rat kidney (C)		0.072	20	100
		L1210/DF8 (A)	0.036	0.12	60	97
16	*m*-Cl-*p*-$CH_2NHCONHC_6H_4SO_2F$-*p*	W256 (A)		0.050	60	71
		Rat liver (A)		0.050	60	0
		Rat liver (C)		0.050	60	96
		L1210/DF8 (A)[i]	0.012	0.060	60	85
		Mouse liver (A)[i]		0.060	60	30
		Mouse liver (C)		0.060	60	83
17	3,5-Cl_2-4-$OCH_2CONHC_6H_4SO_2F$-*p*	L1210/DF8 (A)[m]	0.018	0.050	60	65
		Mouse liver (A)[m]		0.050	60	0
		Mouse liver (C)		0.050	60	63

[a] The technical assistance of Julie Leseman is acknowledged. [b] W256 = Walker 256 rat tumor; L1210 = mouse leukemia resistant to amethopterin; C = column purified, A = 45–90% ammonium sulfate fraction. [c] I_{50} = concn for 50% inhibn when assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described.[15] [d] Incubated with 60 μM TPNH in pH 7.4 Tris buffer as previously described.[15] Runs for 60 min at 37°; runs for 20 min at 25°. [e] Data from ref 5. [f] Data from ref 4. [g] Data from ref 13. [h] Data from ref 14. [i] Data from ref 15. [j] Data from ref 16. [k] $t_{1/2}$ for inactivation. [l] See ref 14 for synthesis. [m] Data from ref 17.

sis of the amine **3**,[10] has been previously described; the syntheses of **4** and **5** by a general route[11] previously described are in the Experimental Section.

Cuatrecasas, *et al.*,[8] attached inhibitors to Sepharose with K_i's varying from 10^{-4} to 10^{-6} M. Since **3**[10] and **4**[11] would have K_i's on the order of 10^{-8} M, **5** was selected as a weaker inhibitor of dihydrofolic reductase that would have a $K_i \sim 10^{-6}$ M.[12]

A Sepharose column prepared by BrCN activation,[8] then coupling with **3**, was prepared (Sepharose–**3**). This Sepharose–**3** column had strong affinity for dihydrofolic reductase; in fact, the affinity was so strong that the enzyme could not be eluted with 0.1 M HOAc.[8] Elution was achieved with the substrate, dihydrofolate, in pH 7.4 Tris buffer, but elution was not sharp and the enzyme was spread through a relatively large elution volume.

A Sepharose column was then prepared by coupling with the much weaker inhibitor **5**. Again the Sepharose–**5** column had strong affinity for dihydrofolate reductase, but the enzyme was readily eluted with 0.1 M HOAc[8] with a sharp elution peak. The Sepharose–**5** column gave ~400-fold purification and could be performed in 1–2 hr. Since the Sepharose–**5** column was elegantly satisfactory and since a Sepharose–**4** column could be expected to give the same elution difficulty as a Sepharose–**3** column, the Sepharose–**4** column was not investigated in detail.

Two difficulties were encountered. First, the enzyme appreciably denatured after 1 hr at 0° in 0.1 M HOAc; this problem was alleviated by immediate neutralization of the eluate fractions with an equiv of Tris base solution. The recovery was 40–60% of the enzyme when the 45–90% $(NH_4)_2SO_4$ fraction from 3 g of rat liver or kidney was purified; the recovery with a mouse liver preparation was 58–76%.

The second difficulty was a more serious one and was not adequately resolved. The synthesis of the Sepharose–**5** column material could not always be duplicated, that is, sometimes a batch of column material did not work satisfactorily; however once a satisfactory column was made, it could be used repeatedly with no difficulty. The nature of the covalent bond formed by activating Sepharose with BrCN, then reaction with an amine, is unknown.[8] It is known that an alcohol gives ROCN with BrCN in basic solution and that an alkyl cyanate can be rearranged to an isocyanate; such a rearrangement is apt to be difficult on a glucose unit of a polysaccharide and therefore it is likely that ROCN reacts directly with the arylamine to give a yet unknown product which could be a carbamate. In view of the mysterious nature of the covalent linking of arylamines to the activated Sepharose, it is not surprising that sometimes the reaction failed.

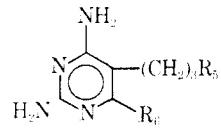
Enzyme Inhibition Results.—The triazine sulfonyl fluorides (**1**, **6**–**12**) (Table I) were extensively investigated *in vivo* against Walker 256 ascites in the rat.[5] Varying degrees of specificity against dihydrofolic reductase in crude tissue extracts were observed.[5] For example, **1** at 0.065 μM showed 100% irreversible inhibition of the Walker 256 enzyme with less than 30% irreversible inhibition of crude enzyme from rat liver, kidney, spleen, or intestine.[5] When the liver and kidney dihydrofolic reductases were purified by an affinity column to remove the "sulfonyl fluoridase" which catalyzed hydrolysis of SO_2F to SO_3H, the dihydrofolic reductase was then inactivated >90%. Similarly, the specificity seen with **1** against the L1210 enzyme *vs.* mouse liver enzyme[4] was not seen with the column-purified enzyme from mouse liver (Table I).

The remainder of the 4,6-diamino-1,2-dihydro-*s*-

(10) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 684 (1969), paper CLVIII.
(11) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 26 (1968), paper CIX.
(12) B. R. Baker and M. A. Johnson, *ibid.*, **11**, 486 (1968), paper CXVIII.

TABLE II

IRREVERSIBLE INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



No.	R_6	R_5	Enzyme source[b]	I_{50},[c] μM	Inhib, μM	Time, min	% inactvn
18	NH_2	*p*-$OC_6H_4NHCOC_6H_4SO_2F$-*m*	W256 (A)[d]	1.1	1.1	60	94
			Rat liver (A)[d]		1.1	60	0
			Rat liver (C)		1.1	60	100
			L1210/FR8 (A)[d]	6.5	1.3	60	96
			Mouse liver (A)[d]		1.3	60	0
			Mouse liver (C)		1.3	60	100
19	NH_2	*p*-$OC_6H_4NHCONHC_6H_4SO_2F$-*m*	L1210/FR8 (A)[d]	3.8	3.8	60	87
			Mouse liver (A)[d]		3.8	60	0
			Mouse liver (C)		3.8	60	100
20	CH_3	*p*-$OC_6H_4NHCOC_6H_4$-4-CH_3-3-SO_2F	L1210/DF8 (A)[e]	0.020	0.040	60	82
			Mouse liver (A)[e]		0.10	60	0
			Mouse liver (C)		0.10	60	90
21	CH_3	*p*-$CH_2C_6H_4NHCOC_6H_4SO_2F$-*m*	L1210/DF8 (A)[f]	0.011	0.070	60	95
			Mouse liver (A)[f]		0.070	60	6
			Mouse liver (C)		0.070	60	72

[a–c] See Table I. [d] Data from ref 18. [e] Data from ref 19. [f] Data from ref 20.

triazines[4,5,13–17] in Table I, except **14**, showed specificity between Walker 256 and normal rat tissues or between L1210 and mouse tissue when the irreversible inhibition was measured with crude enzyme preparation; however, with column-purified dihydrofolic reductase, the inactivation was greatly increased to where no difference between tumor enzyme and normal tissue enzyme of significance was observed.

The exception, **14**,[15] was not an irreversible inhibitor of the dihydrofolic reductase in crude extracts of L1210 or W256, nor was it an irreversible inhibitor of column-purified dihydrofolic reductase from mouse or rat liver. In contrast **14** was a good irreversible inhibitor of the enzyme from pigeon liver; thus, it is still highly probable that a species difference in the structure of dihydrofolic reductase from pigeon liver and rodent liver exists.[16]

A second class of irreversible inhibitors of dihydrofolic reductase showing differences in inactivation of the enzyme in crude extracts were the 2,4-diaminopyrimidines containing the covalent forming SO_2F group on the 5 position (**18–21**)[18–20] (Table II). Again, column-purified dihydrofolic reductase from liver was inactivated to a far greater extent than with crude enzyme preparations, showing that the lack of inactivation of the dihydrofolic reductase in crude liver extracts is due to the rapid detoxification of the sulfonyl fluorides to the corresponding sulfonic acid due to the action of the sulfonyl fluoridase.

A third class of irreversible inhibitors of dihydrofolic reductase were derived from 2,4-diaminopyrimidines having the SO_2F group bridged to the 6 position (**22–28**)[4,15,21–23] (Table III). Again the column-purified enzymes were strongly inactivated when the enzymes in crude extracts were not.

Discussion

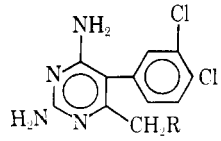
In all of our work on enzyme inhibitors we have deliberately chosen to use crude enzyme preparations in order to approximate more closely the *in vivo* situation a good inhibitor would have to face; exceptions were commercially purified enzymes such as trypsin, chymotrypsin, and xanthine oxidase. The use of crude enzymes will give the same amount of reversible inhibition as purified enzymes[24] since a reversible assay is usually performed in less than 5 min. In contrast, from the data in this paper, it is clear that differences in irreversible inhibition of crude *vs.* purified enzymes can be observed; the slower irreversible assay of 5–60 min allows possible destruction of the inhibitor in crude enzyme preparations. Alternately, if we had chosen to use purified enzymes at the beginning of this program on irreversible inhibitors we would have missed the tissue specificity—which is still real—even though the specificity is due to the presence or absence of the sulfonyl fluoridase. This sulfonyl fluoridase is apparently low or absent in L1210/DF8 mouse leukemia or in Walker 256 rat tumor; however, it is probable that the Dunning leukemia contains appreciable sulfonyl fluoridase since its dihydrofolic reductase in crude extracts is not inactivated as completely as in the other two tumors.[5]

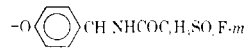
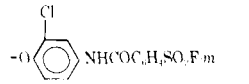
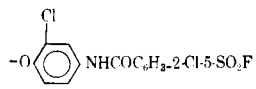
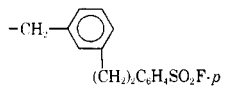
This major biochemical difference between some tumors and normal rat or mouse tissues—namely, the presence or absence of a sulfonyl fluoridase—has as much or more chemotherapeutic utility as isozyme spec-

(13) B. R. Baker and E. E. Janson, *J. Med. Chem.*, **12**, 672 (1969), paper CLV.
(14) B. R. Baker and G. J. Lourens, *ibid.*, **12**, 95 (1969), paper CXL.
(15) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII.
(16) B. R. Baker and G. J. Lourens, *ibid.*, **10**, 1113 (1967), paper CV.
(17) B. R. Baker and W. T. Ashton, *ibid.*, **12**, 894 (1969), paper CXLIX.
(18) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **11**, 489 (1968), paper CXIX.
(19) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 108 (1969), paper CXLIII.
(20) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 224 (1969), paper CLI.
(21) B. R. Baker and P. C. Huang, *ibid.*, **11**, 495 (1968), paper CXX.
(22) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 82 (1969), paper CXXXVI.
(23) B. R. Baker and M. M. J. Vermeulen, *ibid.*, **13**, 82 (1970), paper CLXVI.
(24) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965).

TABLE III

IRREVERSIBLE INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



No.	R	Enzyme source[b]	I_{50},[c] μM	Inhib, μM	Time, min	% inactvn
22		L1210/DF8 (A)[d]	0.37	0.70	60	88
		Mouse liver (A)[d]	0.29	0.70	60	0
		Mouse liver (C)		0.70	60	100
23		L1210/FR8 (A)[d]	1.4	1.4	60	80
		Mouse liver (A)[d]		1.4	60	0
		Mouse liver (C)		1.4	60	86
24		L1210/DF8 (A)[d]	0.82	0.50	60	94
		Mouse liver (A)[d]		1.0	60	0
		Mouse liver (C)		1.0	60	93
25		L1210/DF8 (A)[e]	0.53	0.53	60	82
		Mouse liver (A)[e]		1.1	60	0
		Mouse liver (C)		1.1	60	100
26		L1210/DF8 (A)[e]	0.16	0.32	60	80
		Mouse liver (A)[e]		0.32	60	16
		Mouse liver (C)		0.32	60	84
27		W256 (A)[f]	0.088	0.11	60	68
		Rat liver (A)[f]	0.046	0.11	60	33
		Rat liver (C)		0.11	60	90
28	-$OC_6H_4SO_2F$-*p*	L1210/DF8 (A)[g]	0.021	0.10	60	43
		Mouse liver (A)		0.10	60	8
		Mouse liver (C)		0.10	60	64
29	-$CH_2C_6H_4SO_2F$-*p*	L1210/DF8 (A)[g]		0.080	30	89
		Mouse liver (A)[g]	0.040	0.12	60	0
		Mouse liver (C)		0.12	60	87
30		L1210/DF8 (A)[g]	0.024	0.050	60	82
		Mouse liver (A)[g]		0.070	60	6
		Mouse liver (C)		0.070	60	92

[a-c] See Table I. [d] Data from ref 15 and 21. [e] Data from ref 22. [f] Data from ref 5. [g] Data from ref 23.

ificity of irreversible inhibition where the structure of a given enzyme differs between tissues. On the negative side, we have so far failed to demonstrate with compounds **1** and **6–12** that irreversible inhibitors should show greater specificity than reversible inhibitors *in vivo*.[5] Nevertheless, this major biochemical difference is worthy of further exploration for *in vivo* effectiveness.

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. All anal. samples had proper uv and ir spectra, moved as a single spot on Brinkman silica gel, and gave combustion values for C, H, and N within 0.4% of theoretical.

β-Bromo-3-methyl-4-nitrophenetole (31).—A mixture of 15.3 g (0.1 mole) of 3-methyl-4-nitrophenol, 13.8 g (0.1 mole) of K_2CO_3, 118 g (0.7 mole) of $(BrCH_2)_2$, and 50 ml of DMF was stirred in a bath at 70° for 20 hr. The mixture was added to 500 ml of ice water and extd with four 100-ml portions of CH_2Cl_2. The combined extracts were washed with 10% aq Na_2CO_3, then H_2O. Dried with $MgSO_4$, the solution was spin-evaporated *in vacuo*, finally at 1 mm to remove $(BrCH_2)_2$. The residue was extd with 50 ml of boiling EtOH and the hot soln filtered. Chilling gave 15.5 g (58%) of product that showed one spot on tlc in 4:1 $CHCl_3$–petroleum ether and was suitable for the next step. For anal. a sample was recrystd again from EtOH to give light yellow crystals, mp 51–53°. *Anal.* ($C_9H_{10}BrNO_3$), CHN.

1-(*p*-Acetamidophenoxy)-2-(3-methyl-4-nitrophenoxy)ethane (32).—A mixture of 15 g (58 mmoles) of **31**, 8.0 g (58 mmoles) of K_2CO_3, 9.1 g (58 mmoles) of *p*-acetamidophenol, and 50 ml of DMF was stirred in a bath at 70° for 20 hr, then processed as described for **31**. The residue remaining after removal of CH_2Cl_2 was recrystd from EtOH–THF; yield, 9.0 g (42%), mp 158–160°. *Anal.* ($C_{17}H_{18}N_2O_5$), CHN.

1-(*p*-Acetamidophenoxy)-3-(*p*-nitrophenoxy)propane (33) was prepd in 33% yield, mp 126–127°, as described for **32**. *Anal.* ($C_{17}H_{18}N_2O_5$), CHN.

1-[4-(*p*-Acetamidophenoxyethoxy)-2-methylphenyl]-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine·HCl (34).—A mixture of 3.3 g (10 mmoles) of **32**, 100 ml of EtOH, and 10 ml of Raney Ni was shaken with H_2 at 2–3 atm for 30 min when reduction was complete. The mixture was filtered through a Celite pad, then the filtrate spin-evapd *in vacuo*. The residue was dissd in 10 ml of MeOEtOH, treated with 1 ml of 12 *N* HCl, then immediately spin-evapd *in vacuo*. To the resultant arylamine·HCl were added 25 ml of Me_2CO, 25 ml of EtOH, and 1.0 g (12 mmoles) of cyanoguanidine; the soln was refluxed with stirring for 20 hr, then cooled. The product was collected by filtration, washed with Me_2CO, and recrystd from EtOH; yield, 1.20 g (26%), mp 230–232° dec. *Anal.* ($C_{22}H_{28}N_6O_3$·HCl) CHN.

4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-[*p*-(*p*-nitrophenoxy)-

TABLE IV

PURIFICATION OF RAT LIVER DIHYDROFOLIC REDUCTASE WITH A SEPHAROSE–5[a] COLUMN

Fraction	Vol ml	Total protein, mg	Total activity units[b]	Specific activity, units/mg	Recovery %	Estd % purity[c]
45–90% $(NH_4)_2SO_4$	9	90[d]	7	0.078		0.047
Affinity column[a]	4	0.1[e]	3	30	44[f]	18

[a] Prepared with a 3-ml Sepharose-**5** column; see Experimental Section. [b] One unit = 1 μmole of dihydrofolate reduced per min.[26] [c] Estimated from a specific activity of 170 μmoles/min per mg for pure enzyme.[26] [d] From absorbance at 260 and 280 mμ according to H. M. Kalckar, *J. Biol. Chem.*, **167**, 461 (1947). [e] By the Lowry method with a bovine serum albumin standard and corrected for an 0.05 *M* Tris acetate blank.[27] [f] In other runs recovery was 40–60%.

propoxy)phenyl]-*s*-triazine (35).—A mixture of 1.65 g (5 mmoles) of **33**, 15 ml of EtOH, and 15 ml of 12 *N* HCl was refluxed for 2 hr, then cooled. The arylamine·HCl was collected on a filter and washed with 20 ml of EtOH; yield, 1.37 g (82%) that showed one spot on tlc and gave a positive Bratton–Marshall test. This arylamine·HCl was converted into the triazine as described for **34**; yield, 0.80 g (45%), mp 200–203° dec. *Anal.* ($C_{26}H_{24}N_6O_4 \cdot HCl$), CHN.

1-[4-(*p*-Aminophenoxyethoxy)-2-methylphenyl]-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine (5)·2HCl.—A mixture of 0.90 g (1.95 mmoles) of **34**, 10 ml of EtOH, and 10 ml of 12 *N* HCl was refluxed for 6 hr, then spin-evapd. Recrystn of the residue from *i*-PrOH–H_2O gave 0.30 g (32%), mp gradually decomposes <195°; this material gave a positive Bratton–Marshall test. *Anal.* ($C_{20}H_{26}N_6O_2 \cdot 2HCl \cdot 2H_2O$), CHN.

1-[*p*-(*p*-Aminophenoxypropoxy)phenyl]-4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazine (4)·HCl.—A mixture of 0.67 g (1.5 mmoles) of **35**, 95 ml of MeOEtOH, 5 ml of H_2O, and 0.1 g of PtO_2 was shaken with H_2 at 2–3 atm for 1 hr when reduction was complete. The filtered soln was spin-evapd *in vacuo* and the residue recrystd from EtOH; yield, 0.48 g (76%), mp 188–193° dec. *Anal.* ($C_{20}H_{26}N_6O_2 \cdot HCl \cdot 0.5H_2O$), CHN.

Affinity Columns.—Sepharose 4B was activated with BrCN as described by Cuatrecasas, *et al.*[8] The solid was collected on a Buchner funnel and washed with 0.1 *M* $NaHCO_3$. The wet, activated Sepharose 4B was suspended in DMF (1 ml/ml of original Sepharose 4B) contg 20 mg/ml of inhibitor; in the case of **5**·2HCl, 1 equiv of Et_3N was also added. The mixture was stirred at 4° for 24 hr, then the Sepharose was collected on a Buchner funnel and thoroughly washed with DMF to remove the excess inhibitor. The solid was then washed with 0.1 *M* $NaHCO_3$ and H_2O. The wet solid was suspended in 0.05 *M* Tris buffer (pH 7.4).

A suspension from 3 ml of the original Sepharose was packed in a column of 8-mm diameter; the column material was ~5 cm high. The column was then surrounded by an ice bath. A 45–90% $(NH_4)_2SO_4$ fraction of rat or mouse liver in 0.05 *M* Tris buffer (pH 7.4) (1 ml/g of original liver) was prepd as previously described,[15] then dild with 2 vol of the buffer. This ice-cold solution was passed through the column and a 10-ml fraction collected that contained all the red hemoglobin.[25] The column was then washed with ice-cold 0.05 *M* phosphate buffer (pH 8.0) (10–15 ml) until no more protein was removed as detected by uv. The dihydrofolic reductase was eluted with ice-cold 0.1 *M* HOAc; six 2-ml fractions were collected and were immediately neutralized with cold 0.1 *M* Tris base; the enzyme usually appeared in fraction 2 and all fractions were colorless. This soln of enzyme was stable at 4° for >2 weeks when neutralized but rapidly decompd over a few hours if the soln was not neutralized. About 90 min is required for a run. After being washed with Tris buffer, the column can be reused many times.

For the inhibitor work (Tables I–II), usually 27 ml of extract from 9 g of liver was processed. Recovery of enzyme activity was 40–60% from rat liver or kidney and 58–76% from mouse liver. Purification was about 400-fold. The details of a specific run are shown in Table IV. The dihydrofolic reductase had a specific activity of 30; pure dihydrofolic reductase from L1210 has a specific activity of 170 μmoles/min per mg;[26] therefore the purity of the affinity column purified enzyme is about 20%. The purity may be higher since the amount of protein detectable by the Lowry method[27] is at the edge of the sensitivity of the method.

(25) This fraction contained the bulk of the protein and could be used for isolation of other enzymes by affinity chromatography. For example, this soln could be used for purification of guanine deaminase by a suitable affinity column; B. R. Baker and H. U. Siebeneick, to be published.

(26) J. P. Perkins, B. L. Hillcoat, and J. R. Bertino, *J. Biol. Chem.*, **242**, 4771 (1967).

(27) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *ibid.*, **193**, 265 (1951).