Potential Folic Acid Antagonists. I. The Antitumor and Folic Acid Reductase Inhibitory Properties of 6-Substituted 2,4-Diamino-5-arylazopyrimidines[1]

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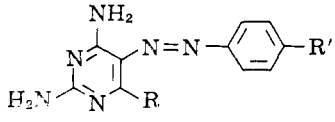
A number of 6-substituted 2,4-diamino-5-arylazopyrimidines (I) have been synthesized by conventional techniques. Their folic acid reductase and tumor-inhibitory potencies have been determined. The most potent inhibitor of folic acid reductase was found to be 2,4,6-triamino-5-phenylazopyrimidine. A structure–activity relationship is advanced for the folic reductase inhibitory activities of these compounds. No correlation could be observed between the folic reductase and tumor-inhibitory properties of the 5-arylazopyrimidines studied.

As part of a general program intended to study substituted pyrimidines as antitumor agents, a number of 6-substituted 2,4-diamino-5-arylazopyrimidines (I) have been synthesized.



Ia, R = NH_2; R′ = SO_2NH_2
b, R = NH_2; R′ = SO_3H
c, R = NH_2; R′ = CO_2H

There have been a number of previous reports of the synthesis and biological properties of 5-arylazopyrimidines. The laboratories of Timmis,[2] Modest,[3] and Tanaka[4] independently synthesized and evaluated a number of 5-arylazopyrimidines and, on the basis of growth inhibitory properties in a number of microbiological systems (*Streptococcus faecalis*, *Lactobacillus casei*, and *Escherichia coli*), concluded that this group of compounds had antifolic properties. More recently, Roy-Burman and Sen[5] have examined additional members of this series and have also concluded that the 5-arylazopyrimidines are folic acid antagonists. Tanaka, *et al.*,[4c] reported the antitumor action against the Yoshida sarcoma of several 5-arylazopyrimidines and found significant growth inhibition with Ia–c. *In vitro* studies with a chick liver preparation demonstrated the inhibitory effect of some 5-arylazopyrimidines on the conversion of folic acid to citrovorum factor, probably through the inhibition of formation of tetrahydrofolate.

The 5-arylazopyrimidine structure thus appeared to offer interesting possibilities for the design of inhibitors of one or more stages of folate metabolism and, by the appropriate substitution of alkylating groups, also to offer scope for the design of selectively and specifically[6] acting antitumor agents. The general method of preparation of compounds listed in Table I was that described by Timmis and co-workers and involved coupling the diazotized amine with 2,4,6-triamino- or 2,4-diamino-6-chloropyrimidine in aqueous solution at pH 6–7. The reaction was found to proceed satisfactorily,[7] although III and VII proved difficult to prepare in analytically pure condition, and substantial losses occurred with the necessary repeated recrystallization. The 6-N-substituted compounds were prepared from the 6-chloropyrimidines (III and IV) and the amine in ethanolic solution at 100°. On cooling, the desired compounds crystallized in a high state of purity.

Results and Discussion

Enzyme Studies.—A summary of the inhibitory effects of the 5-arylazopyrimidines on folic acid reductase (from rat liver) is given in Table II. The wide range of activities of these compounds and the low activity of 2,4,5,6-tetraaminopyrimidine indicates that reductive cleavage of the 5-azo linkage is not a prerequisite for biological activity. In fact, preliminary data indicate that, in contrast with azobenzene analogs, 5-arylazopyrimidines are resistant to reduction by rat liver homogenates.[8]

The [I]/[S] ratios for 50% inhibition provide a convenient numerical manner for denoting changes in the affinity of an antagonist with structure since, under appropriate conditions, $([I]/[S])_{50} = K_i/K_m$, where K_i is the binding constant for the inhibitor and K_m is the binding constant for the substrate (in this case folic acid). The validity of the above expression has been discussed by Webb[9] who has pointed out that the $([I]/[S])_{50}$ ratio is dependent upon substrate concentration, but that the ratio approaches K_i/K_m at high substrate concentrations ($[S] > 5K_m$). In the studies reported here the concentration of folic acid employed was 8×10^{-5} *M* and the K_m for folic acid was found to be 6.2×10^{-6}. We are thus justified in employ-

(1) This work was supported by Public Health Service Grant No. CA 06645-02 from the National Cancer Institute.

(2) G. M. Timmis, D. G. I. Felton, H. O. J. Collier, and P. L. Huskinson, *J. Pharm. Pharmacol.*, **9**, 46 (1957).

(3) E. J. Modest, H. N. Schlein, and G. E. Foley, *ibid.*, **9**, 68 (1957).

(4) (a) K. Tanaka, K. Kaziwara, Y. Aramaki, E. Omura, T. Araki, J. Watanabe, M. Kawashina, T. Sugawa, Y. Sanno, Y. Sugino, Y. Ando, and K. Imac, *Gann*, **47**, 401 (1956); (b) K. Tanaka, E. Omura, T. Sugawa, Y. Sanno, Y. Ando, K. Imai, and M. Kawashina, *Chem. Pharm. Bull.* (Tokyo), **7**, 1 (1959); (c) K. Tanaka, E. Omura, M. Kawashina, J. Watanabe, H. Yokotani, H. Ito, Y. Sugino, M. Ishidata, T. Araki, Y. Aramaki, and K. Kaziwara, *ibid.*, **7**, 7 (1959); (d) M. Kawashina, *ibid.*, **7**, 13 (1959); (e) *ibid.*, **7**, 17 (1959).

(5) P. Roy-Burman and D. Sen, *Biochem. Pharmacol.*, **13**, 1437 (1964).

(6) D. J. Triggle, *J. Theoret. Biol.*, **7**, 241 (1964).

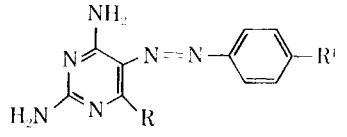
(7) M. Israel, H. K. Protopapa, H. N. Schlein, and E. J. Modest, *J. Med. Chem.*, **7**, 792 (1964).

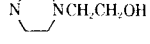
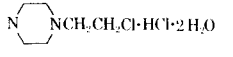
(8) M. Chadwick, P. Hebborn, D. J. Triggle, and S. Vickers, unpublished data.

(9) J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. I, Academic Press Inc., New York, N. Y., 1963, p. 106.

TABLE I

2,4-DIAMINO-6-SUBSTITUTED 5-ARYLAZOPYRIMIDINES



No	R	R'	M.p., °C.	Recrystn. solvent	Yield, %	Formula	Calcd., %				Found, %			
							C	H	Cl	N	C	H	Cl	N
II	NH_2	H	265	EtOH	85	*a*								
III	Cl	$COOC_2H_5$	268–270	$C_2H_5OCH_2CH_2OH$	63	$C_{13}H_{13}ClN_6O_2$	48.65	4.08	11.06	26.22	48.2	4.09	10.81	26.2
IV	Cl	$CONHCHCO_2H$ $(CH_2)_2CO_2H$	195–200 dec.	EtOH–H_2O	44	$C_{16}H_{16}ClN_7O_5$	45.5	3.8	8.4	19.0	45.3	3.87	8.23	18.45
V	NH_2	$COOC_2H_5$	256–258	$C_2H_5OCH_2CH_2OH$	83	$C_{13}H_{15}N_7O_2$	51.8	5.02	...	32.55	51.7	5.01	...	35.2
VI	NH_2	SO_2NH_2	331–334	$C_2H_5OCH_2CH_2OH$	47	*b*								
VII	NH_2	$CONHCHCO_2H$ $(CH_2)_2CO_2H$	235–237	EtOH–H_2O	39	$C_{16}H_{18}N_8O$	47.75	4.51	...	28.03	47.6	4.8	...	27.81
VIII	NH_2	$COONa$	...	EtOH–H_2O	91	$C_{11}H_{10}N_7NaO_2$	44.75	3.41	...	...	44.37	3.64	...	...
IX	NEt_2	$COOC_2H_5$	202–204	EtOH	85	$C_{17}H_{23}N_7O_2$	57.2	6.5	...	...	57.25	6.55	...	...
X	NEt_2	$CONHCHCO_2H$ $(CH_2)_2CO_2H$	260–265 dec.	*i*-PrOH–H_2O	73	$C_{20}H_{26}N_8O_5$	52.35	5.75	...	...	52.6	5.41	...	...
XI	NH_2	NEt_2	248–250	MeOH–H_2O	46	$C_{14}H_{20}N_8$	56.0	6.67	...	37.28	56.1	6.75	...	37.6
XII	NH_2	$N(CH_2CH_2Cl)_2$	245–247	MeOH–H_2O	83	$C_{14}H_{18}Cl_2N_8 \cdot H_2O$	43.4	4.67	...	28.87	43.18	4.82	...	28.63
XIII	NH_2	$N(CH_2CH_2Br)_2$	250	MeOH	80	$C_{14}H_{18}Br_2N_8$	36.7	3.93	...	24.42	36.9	4.06	...	24.2
XIV	NH_2	$N(Et)CH_2CH_2Cl$	210–214	MeOH	65	$C_{14}H_{19}ClN_8$	50.23	5.67	...	...	49.84	5.62	...	...
XV		$COOEt$	224–226	EtOH	95	$C_{19}H_{30}N_8O_3$	55.2	6.28	...	27.1	55.14	6.29	...	27.26
XVI		$COOEt$	230–233	EtOH–Et_2O	83	$C_{19}H_{30}Cl_2N_8O_2 \cdot 2H_2O$	45.65	5.75	14.13	22.19	45.32	5.61	14.09	22.7

[a] Known compound, see ref. 2. [b] Known compound, see ref. 4b.

TABLE II

ANTITUMOR AND FOLIC ACID REDUCTASE DATA

No.	Folic acid reductase inhibition[a] $([I]/[S])_{50}$	Mouse toxicity,[b] mg./kg.	Rat toxicity,[c] mg./kg. q.d. 5–9	Antitumor activity[d] Dose, mg./kg. q.d. 5–9	Antitumor activity[d] % body wt. change[e]	Antitumor activity[d] T/C[f]
II	0.001	170	80	50	−1	0.6
IV	4.0	>1000				
V	0.011	>1000	>400	400	+1	0.3
VI	1.1	500				
VII	1.8	>1000	>100	100	+18	1.05
VIII	1.6[g]	80	220	200	−13	0.24
				100	+16	0.9
IX	3.75[g]	400	>200	200	+20	0.95
X	0.125	>1000	>200	200	+18	1.1
XI	0.1	240				
XII	0.17	290				
XIII	0.15					
XIV	0.125	170				
XV	9.0[g]	>1200	>400	400	+10	0.35
XVI	1.4	180	140	100	−13	0.18
TAP[h]	5.0	600				

[a] Ratio of concentration of inhibitor required for 50% inhibition of enzyme activity to concentration of substrate (see text). [b] Approximate acute LD_{50} (see Experimental Section). [c] Approximate LD_{50}, 5 daily doses on days 5–9 (see Experimental Section). [d] Against established Murphy–Sturm lymphosarcoma. [e] Weight of rats on day 5 taken as 100%. [f] Ratio of volume of treated and control tumors, estimated on day 12. [g] Values obtained by extrapolation. Low solubility of the compounds prevented determination of $[I]_{50}$ directly. [h] TAP = 2,4,5,6-tetraaminopyrimidine.

ing the $([I]/[S])_{50}$ ratio as a measure of relative change in K_i. The K_i for 2,4,6-triamino-5-phenylazopyrimidine (II) obtained from this expression was 6.2×10^{-9} *M*. Analysis of data for this compound using the procedure of Lineweaver and Burk[10] (Figure 1) gave a K_i of 1.4×10^{-8} *M*, in reasonable agreement with the value obtained by the more approximate technique. Furthermore, the data of Figure 1 indicate that II is a competitive inhibitor of folic acid reductase. From this study and the previous studies of Timmis, *et al.*,[2] we conclude that the compounds described in this paper are (with the possible exception of XVI, Tables I and III) competitive reversible antagonists of folic acid reductase.

TABLE III

EFFECT OF PREINCUBATING FOLIC ACID REDUCTASE WITH 5-ARYLAZOPYRIMIDINES[a]

No.[b]	2 hr.[c] A	2 hr.[c] B	4 hr.[c] A	4 hr.[c] B	8 hr.[c] A	8 hr.[c] B
XI	94/95	93/95	86/91	85/88	78/80	70/75
XII	95/95	94/95	90/91	88/88	78/80	74/75
XIII			82/80		65/68	
XIV	95/95				79/80	
XV	93/94	92/91	89/89	82/80	75/75	68/65
XVI	90/94	88/91	83/89	78/80	62/75	49/65

[a] Activity of enzyme after preincubation with inhibitor (numerator) or without inhibitor (denominator) expressed as per cent of activity at 0 hr. [b] See Table I for formulas. [c] Time of preincubation before adding substrate. A and B are separate experiments. [S] = 8×10^{-5} *M*, [I] is sufficient to produce 20% inhibition at zero time.

Comparison of the structures of 5-arylazopyrimidines and folic acid reveals that the benzene ring of the inhibitor molecules is two atoms closer to the pyrimidine ring than in folic acid. The benzene ring of the inhibitor and substrate molecules cannot, therefore, bind to the same site on the enzyme. In view of the

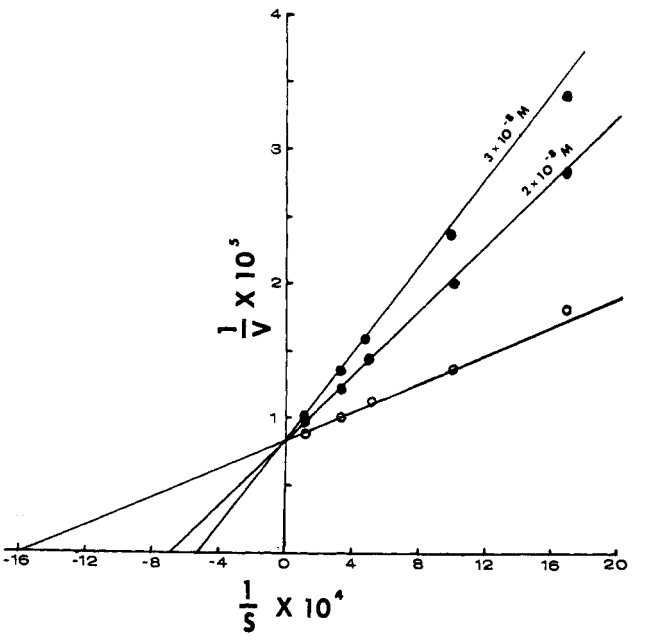


Figure 1.—Inhibition of the reduction of folate by compound II: open circles, absence of inhibitor; closed circles, presence of inhibitor at concentrations stated.

significant structural difference it is probably not surprising that the introduction of *para* substituents into the benzene ring of the parent member of this series of antagonists, 2,4,6-triamino-5-phenylazopyrimidine (II), produces compounds which are significantly less potent as inhibitors. Thus the introduction of the *p*-sulfonamido (VI), *p*-carbethoxy (V), *p*-carboxy-L-glutamyl (VIII), or *p*-diethylamino (XI) substituents produces relative increases of approximately 1100-, 10-, 1800-, and 100-fold, respectively, in the $([I]/[S])_{50}$ ratios. Baker[11] has shown that the *p*-carboxy and *p*-

(10) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

(11) (a) B. R. Baker, D. V. Santi, P. L. Almaula, and W. C. Werkheiser, *J. Med. Chem.*, **7**, 24 (1964); (b) B. R. Baker, B-T. Ho, and G. B. Chheda, *J. Heterocyclic Chem.*, **1**, 88 (1964).

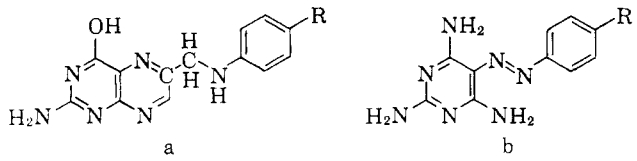


Figure 2.

carboxy-L-glutamyl substituents actually increase the affinity of 5-(3-anilinopropyl)pyrimidines for folic reductase. However, Baker's compounds actually conform to the same steric and structural pattern as folic acid and the divergent effects of substituents in these two types of antagonists are not surprising.

Despite the structural differences between folic acid and 2,4,6-triamino-5-arylazopyrimidines, it is apparent, from the high affinity of some of the compounds of the latter type, that the 5-aryl group is probably making a significant binding contribution. A number of studies have shown that several 5-substituted pyrimidines possess folic acid inhibitory properties and that the binding requirements for the 5-substituent at folic acid reductase are relatively flexible. Thus, 5-aryl-2,4-diaminopyrimidines and 1-aryl-4,6-diamino-1,2-dihydro-5-triazines include powerful inhibitors of folic acid.[12] The aryl ring in these compounds contributes significantly to the binding energy but cannot bind to the same site as the benzene ring of folic acid.

In one of the planar conformations of the 5-arylazopyrimidines the azo linkage corresponds to that portion of the enzyme surface normally complementary to the N-5–C-6 double bond of folic acid (Figure 2). The high inhibitory potency of some 5-arylazopyrimidines and the isosterism of the azo and azomethine linkages suggests that Figure 2b does represent the configuration of enzyme-bound inhibitor. Speculations concerning the binding site of the 5-aryl group of the 5-arylazopyrimidines depend, however, upon a knowledge (currently unavailable) of the conformation of enzyme-bound folic acid. A planar conformation (*i.e.*, Figure 2a) necessitates that the aryl group of the inhibitor molecule binds to the enzyme surface normally, complementary (in part at least) to the 9–10 (CH_2NH) bond of folic acid. Evidence in favor of this assumption is equivocal[13] and it is equally probable that folic acid is bound to folic reductase in a nonplanar conformation. The 5-aryl group of the inhibitors may then bind to an area of the enzyme adjacent to that normally required for binding the folic acid molecule.

Since the nature of this proposed additional binding site is unknown, it is difficult to rationalize the effects of substituents in the aryl ring of 5-arylazopyrimidines. They may sterically hinder binding of the aryl ring, although the results of Table II are not entirely consistent with this assumption; thus V is 100 times more effective than VI as a folic reductase inhibitor and VII is equipotent with VIII. More probably the substituents alter the electron density of the aromatic ring and thus influence its ability to bind either through charge-transfer complex formation or through hydrophobic bonding.

The effects of 6-N-substitution on the inhibitory activities of these compounds are of some interest: introduction of the 6-(N,N-diethylamino) (IX), 6-N-(N′-2-hydroxyethyl)piperazino (XV), and 6-N-(N′-2-chloroethyl)piperazino (XVI) into 2,4,6-triamino-5-(4-carbethoxy)phenylazopyrimidine (V) leads to substantial decreases in inhibitory activity. In contrast, the introduction of the 6-(N,N-diethylamino) (X) substituent into 2,4,6-triamino-5-(4-carboxy-L-glutamyl)-phenylazopyrimidine (VII) leads to an increase in inhibitory activity. The reasons for the differing effects of the 6-substituents are not clear at the present time. It is interesting to note that Timmis, *et al.*,[2] also found that the introduction of 6-(N,N-dialkyl) substituents into the parent 2,4,6-triamino-5-arylazopyrimidine reduced growth inhibitory potency to *S. faecalis*.

It was anticipated that the introduction of appropriately located alkylating groups into the 5-arylazopyrimidine molecule might result in irreversible antagonists of folic acid reductase. The data presented in Table III indicate that an alkylating function in the 4′-position of the 5-arylazo group (XII, XIII, and XIV) has no effect on the extent of enzyme inactivation with increasing incubation time. The rate of enzyme inactivation is essentially the same with the potential alkylating compounds (XII, XIII, and XIV), with the nonalkylating analog (XI), and in the absence of inhibitor. This is not surprising, since on the basis of the interaction model discussed these alkylating substituents are in the wrong position and, in any event, the effect of the *p*-azo linkage will be to reduce their reactivity drastically.[14] However, N-2-chloroethyl-N′-[2,4-diamino-5-(4-carbethoxyphenylazo)-6-pyrimidyl]piperazine (XVI), where the alkylating function is attached through the 6-position of the pyrimidine ring and which will be more reactive than the previously discussed compounds, binds approximately 6-fold more tightly than its nonalkylating analog (XV) and shows evidence of irreversible inhibition of the enzyme after 8 hr. of preincubation. Further work is now in progress on related compounds where the 6-substituents will be less conformationally rigid.

Tumor Studies.— While there is an indication of a structure–activity relationship when inhibitory effects upon folic acid reductase are considered, there is no direct correlation between enzyme-inhibitory activity and toxicity or antitumor activity. The most efficient enzyme-inhibitory compound (II) was relatively toxic to both mice and rats. However, V, which was also a potent enzyme inhibitor, was nontoxic at relatively high doses. Both compounds showed only moderate growth inhibitory effects on the Murphy–Sturm tumor which is extremely sensitive to other folic acid antagonists such as amethopterin.[15] Compounds XV and XVI both showed moderate tumor growth inhibitory activity but were relatively inefficient inhibitors of folic acid reductase. Antitumor activity

(12) T. H. Jukes and H. P. Broquist, "Metabolic Inhibitors," Vol. I, R. M. Hochster and J. H. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, p. 481.

(13) It is interesting to note, however, that Baker[11b] has shown that replacement of the anilino NH group of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol by CH_2 gives a compound that is a 26-fold more effective inhibitor against dihydrofolic reductase. One explanation of this finding is that the NH group does not contribute to binding *per se*, but acts indirectly by altering the binding capacity of the adjacent aromatic ring. It is possible, therefore, that the enzyme area complementary to the CH_2NH– of folic acid may be relatively nonpolar in character.

(14) W. C. J. Ross and G. P. Warwick, *J. Chem. Soc.*, 1364, 1719, 1724 (1956).

(15) W. C. Werkheiser, *Cancer Res.*, **23**, 1277 (1963).

of VII was seen only at the maximum tolerated dose and was associated with severe body weight loss. Differences in toxicity values between members of this series of compounds are probably due to differences in solubility which affect the rate of absorption from the injection site and distribution in the body. It is possible that some of these compounds are inhibiting folate metabolizing enzymes other than folic acid reductase. This possibility is being investigated.

Experimental Section[16]

A. New Syntheses.—2,4,6-Triamino-5-arylazopyrimidines (II, V–VIII, and XI–XIV of Table I) were prepared by the general method described by Timmis and co-workers.[2] The diazotized amine (0.1 mole), free from nitrous acid, was added to a solution of 2,4,6-triaminopyrimidine (0.1 mole) in water (350 ml.) containing sufficient sodium acetate to maintain the pH at 6–7 and cooled to 0–5°. When the addition was complete, the mixture was stirred at 0–5° for 5 hr. and then at 10° for 2–3 days. The product was collected, washed well with water, and recrystallized.

N,N-Di(2-bromoethyl)-*p*-phenylenediamine Dihydrochloride.—N,N-Di(2-bromoethyl)aniline[17] was converted to the 4-nitroso derivative by the method of Ross[17] in 73% yield. It had m.p. 96–98° (Et_2O–EtOH).

Anal. Calcd. for $C_{10}H_{12}Br_2N_2O$: C, 35.7; H, 3.6; Br, 47.18. Found: C, 35.2; H, 3.59, Br, 47.55.

4-Nitroso-N,N-di(2-bromoethyl)aniline (6.8 g., 0.02 mole) was dissolved in 50 ml. of concentrated HCl and cooled to 0°. Stannous chloride (8.0 g., 0.042 mole) was added with stirring: after 30 min. the mixture was added to an excess of ice-cooled 2 *N* NaOH (300 ml.) covered with ether (150 ml.). The mixture was shaken, the ether layer was removed, and the aqueous portion was extracted twice with 100-ml. portions of ether. The ether extract was dried ($MgSO_4$) and HCl was passed through until precipitation was complete. The oily precipitate was triturated with 2-propanol and recrystallized from EtOH–Et_2O to give the dihydrochloride, m.p. >280°, yield 3.5 g. (44%).

Anal. Calcd. for $C_{10}H_{16}Br_2Cl_2N_2$: C, 30.4; H, 4.08; Br, 40.5; Cl, 17.95; N, 7.1. Found: C, 30.72; H, 4.08; Br, 40.91; Cl, 17.84; N, 7.1.

N-2-Chloroethyl-N-ethyl-*p*-phenylenediamine Dihydrochloride.—N-Ethyl-N-2-hydroxyethylaniline (33 g., 0.2 mole) in $CHCl_3$ (100 ml.) was added to a stirred solution of PCl_5 (36 g., 0.2 mole) in $CHCl_3$ (100 ml.) at 0°. When the addition was complete the mixture was refluxed for 3 hr. and then poured into vigorously stirred aqueous $NaHCO_3$. The $CHCl_3$ layer was separated, washed with water, dried ($MgSO_4$), and distilled to give N-2-chloroethyl-N-ethylaniline, b.p. 150–155° (18 mm.), yield 72%.

Anal. Calcd. for $C_{10}H_{14}ClN$: Cl, 19.3. Found: Cl, 19.15.

N-2-Chloroethyl-N-ethylaniline was converted to the 4-nitroso derivative according to the method of Ross[17] in 69% yield. It had m.p. 52–54° (Et_2O–EtOH).

Anal. Calcd. for $C_{10}H_{13}ClN_2O$: C, 56.5; H, 6.16; Cl, 16.66; N, 13.17. Found: C, 56.2; H, 6.4; Cl, 16.91; N, 13.35.

The nitroso compound was reduced to N-2-chloroethyl-N-ethyl-*p*-phenylenediamine dihydrochloride by the method described above for 4-nitroso-N,N-di(2-bromoethyl)aniline. The yield was 65%, m.p. 175–180°.

Anal. Calcd. for $C_{10}H_{17}Cl_3N_2$: C, 44.2; H, 6.31; Cl, 39.2; N, 10.31. Found: C, 43.8; H, 6.58; Cl, 39.5; N, 10.21.

2,4-Diamino-5-arylazo-6-chloropyrimidines (III and IV, Table I) were prepared by the general method described by Timmis and co-workers.[2] The diazotized amine (0.1 mole), free from nitrous acid, was added to a stirred solution of 2,4-diamino-6-chloropyrimidine (0.1 mole) in acetic acid (300 ml., 3 *N*) at 0°. After 10 min. crystalline sodium acetate was added to bring the pH to 6–7 and the solution was maintained at 10° for 2–3 days; the precipitate was filtered, washed with water, and recrystallized.

2,4-Diamino-6-diethylamino-5-(4-carbethoxyphenylazo)pyrimidine (IX).—2,4-Diamino-6-chloro-5-(4-carbethoxyphenylazo)pyrimidine (I, 3.2 g., 0.01 mole) in ethanol (75 ml.) and diethylamine (2 ml.) were heated at 100° for 12 hr. The solution was cooled and filtered to give IX in 78% yield. One recrystallization from ethanol gave analytically pure material, m.p. 202–204°. Compounds X and XV were prepared in a similar manner from IV and diethylamine and II and N-2-hydroxyethylpiperazine, respectively.

N-2-Chloroethyl-N′-[2,4-dimaino-5-(4-carbethoxyphenylazo)-6-pyrimidyl]piperazine (XVI) Hydrochloride.—N-2-Hydroxyethyl-N-[2,4-diamino-5-(4-carbethoxyphenylazo)-6-pyrimidyl]-piperazine (XV) (2.1 g., 0.005 mole) was finely powdered and suspended in anhydrous ether (30 ml.) at 0°. Thionyl chloride (0.7 g., 0.006 mole) was added and the mixture was maintained at 35° for 1 hr. Excess $SOCl_2$ was destroyed by the addition of ethanol (2 ml.) and the product was filtered, washed with ether, and recrystallized (EtOH–Et_2O) to give a slightly hygroscopic product, m.p. 230–233°, whose analysis agrees well with a dihydrate.

B. Inhibition of Folic Acid Reductase.—A high-speed supernatant fraction of rat liver homogenate was used as the source of folic acid reductase.[18] Rat livers were homogenized with 3 vol. of ice-cold 0.25 *M* sucrose containing 2 m*M* trishydroxymethylaminomethane buffer, pH 7.8. The homogenate was centrifuged at 13,000*g* for 4 hr. at 2°, and the supernatant was stored at −20° until used.

The assay procedure for folic acid reductase activity was similar to that described by Werkheiser.[19] The incubation mixture contained 10 mμmoles of NADPH, 5 μmoles of sodium citrate, 5 μmoles of $MgCl_2$, 50 μmoles of dimethyl glutarate buffer (pH 6.1), 40 μmoles of folate, and rat liver supernatant in a total volume of 0.5 ml. Dimethyl sulfoxide or a solution of compound dissolved in dimethyl sulfoxide was also included. After incubating at 37° for 20 min., 100 μl. of 5 *N* HCl and 400 μl. of acetone were added. The amount of *p*-aminobenzoyl-L-glutamic acid formed (from tetrahydrofolic acid in the acidified solution) was determined spectrophotometrically at 560 mμ after diazotization using the Bratton–Marshall reagents.[20] After applying corrections for the optical density values of incubation mixtures completely inactivated by the prior addition of excess amethopterin and for inhibition of enzyme activity due to the solvent, the ratio of the amount of amine produced in control tubes to the amount produced in tubes containing inhibitor was plotted against the concentration of the inhibitor. From this plot, the concentration of inhibitor required to produce a 50% inhibition of enzyme activity was estimated.

C. Biological Test Methods.—Toxicity determinations were performed using male Swiss mice (22–26 g). The compound dissolved in saline or suspended in 10% gum acacia was administered by intraperitoneal injection to groups of 3–6 mice/dose level. Deaths within a 21-day period were recorded and approximate LD_{50} values were estimated graphically from per cent mortality/log dose plots.

Antitumor activities of the compounds against the Murphy–Sturm lymphosarcoma were assessed as follows. The tumor was implanted subcutaneously into male Holtzman rats using a trochar and cannula. Five days later, when the tumor had reached a size of about 5 g., the compound was injected intraperitoneally daily for 5 days. Control animals received the vehicle only. On day 12, the volumes of the tumors were calculated from measurements taken by a caliper,[21] and the mean tumor volume of treated rats was compared with the mean tumor volume of control rats (T/C in Table II). Rats were subsequently observed to determine whether complete regression of the tumors occurred.

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(16) Melting points were recorded on a Thomas–Kofler hot stage and are corrected. Analyses are by Galbraith Laboratories Inc., Knoxville, Tenn., and Dr. A. E. Bernhardt, Mülheim, Ruhr, West Germany.

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