foam. This material was dissolved in 8 ml of hot H_2O, treated with Norit, filtered, and cooled at 2° for several days. The colorless crystals of **10** (0.6 g, 47%) which separated were collected by filtration. A second crop (0.3 g, 23%) raised the yield to 70%. A small sample was recrystallized from H_2O for analysis: mp 180.5–181.5°; uv max (pH 1) 256 mμ (ε 14,700), (pH 11) 259 mμ (ε 14,700); ir (KBr) 1704 cm^{-1} ($NHCO_2Et$); nmr (DMSO-d_6) δ 1.16 (t, 3, J = 7 Hz, $-COOCH_2CH_3$), 4.04 (q, 2, J = 7 Hz, $-CO_2CH_2CH_3$), 7.54 (m, 1, 5′-CH_2NHCO_2Et), 5.44 (m, 1, 3′-OH plus remainder of β-2′-deoxynucleoside spectrum; nmr (DMSO-d_6, D_2O) peaks at δ 7.54 and 5.44 missing. *Anal.* ($C_{14}H_{17}N_5O_4$) C, H, N.

Dihydrofolate Reductase from *Trypanosoma equiperdum*.[1] II. Inhibition by 2,4-Diaminopyrimidines and Related Heterocycles

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A number of 2,4-diaminopyrimidines and related heterocyclic compounds have been evaluated as inhibitors of dihydrofolate reductase obtained from *Trypanosoma equiperdum*, chicken liver, and rat liver. 2,4-Diaminopyrimidine itself (at 10^{-4} M) was not effective as an inhibitor of dihydrofolate reduction in all three systems studied but 5-aryl derivatives of 2,4-diaminopyrimidine were good inhibitors (ID_{50} = 10^{-8} to 10^{-6} M) of this enzymatic reaction. 2,4-Diamino-5-benzylpyrimidines and 2,4-diamino-5-aryloxypyrimidines were considerably more effective as inhibitors of the trypanosomal enzyme system than of the mammalian and avian systems. Although 2,4-diamino-6-phenyl-*s*-triazine was not active as an inhibitor of the enzymes studied, related 4,6-diamino-1,2-dihydro-*s*-triazines were potent inhibitors of the reductases. 2,4-Diamino-6,7-diphenylpteridine was found to be approximately tenfold more effective as an inhibitor of the three reductase systems than was 2,4-diamino-6,7-dimethylpteridine; 2-amino-6,7-diphenylpteridine and 4-amino-6,7-diphenylpteridine were not effective as inhibitors of these enzymes. 2,4,7-Triamino-6-arylpteridines bearing an *ortho* substituent in the 6-aryl moiety were found to be 10–100-fold more potent as inhibitors of the reductase systems than were the corresponding *para*-substituted derivatives. The 2-amino-4-hydroxypteridine derivatives biopterin, xanthopterin, and isoxanthopterin were found to be effective neither as substrates nor as inhibitors of the trypanosomal reductase.

We have described recently[2] the isolation of dihydrofolate reductase from the protozoan *Trypanosoma equiperdum* and have shown that the pattern of susceptibility of this trypanosomal enzyme system to inhibition by several diamino heterocycles is different from those observed for reductases isolated from bacterial and mammalian sources. The extensive studies carried out by Burchall and Hitchings[3] and by Baker and his colleagues[4] on the structural requirements for inhibition of dihydrofolate reductase from different sources have established that seemingly small changes in chemical structure can produce marked alterations in the ability of an agent to inhibit a particular reductase. We have initiated a similar comparative study of the relationship between the chemical structure of various 2,4-diaminopyrimidines and related heterocyclic systems, and their ability to inhibit dihydrofolate reductases from *T. equiperdum*, chicken liver, and rat liver. It was hoped that such a study would provide information which might prove useful in the design of new agents for use in the chemotherapy of trypanosomiases and other protozoal diseases.

Experimental Section

Initially dihydrofolate reductase from *T. equiperdum* was prepared exactly as outlined in ref 1; in later studies acetone powders, prepared from 3–6 × 10^{10} trypanosomes, were extracted with 7 ml of pH 7.0 phosphate buffer (0.1 M) and the extracts were centrifuged for 40° at 100,000g and 4° in a Beckman Model L refrigerated centrifuge; the resulting supernatant solutions served as the source of enzyme. This modified procedure for obtaining trypanosomal enzyme was introduced because of the high loss of enzyme activity encountered during the dialysis step employed in our original procedure. It yielded an enzyme preparation which did not differ significantly, with respect to sensitivity to various inhibitors, from the enzyme preparation made by the previous procedure. In this connection, it is worth pointing out that Schrecker and Huennekens[5] found that dihydrofolate reductase in crude extracts of chicken liver and this enzyme after partial purification showed similar sensitivity to the diaminopteridine inhibitor, aminopterin. Acetone powders[6] were prepared from sections of chicken liver, the powders were extracted as for the trypanosomal preparation, the extracts were dialyzed overnight against 100 vol of pH 5.5 buffer (0.01 M), and the dialyzed material was used for enzymatic assays. Acetone powders were prepared also from sections of rat liver and were extracted and dialyzed as described for the chicken liver preparations. A similar preparation[7] of (dihydro) folate reductase, obtained by high-speed centrifugation of a rat liver homogenate, was used by Hampshire and her colleagues[8] for evaluating the inhibitory potency of a series of 2,4-diamino-5-arylazopyrimidines.

Reduced nicotinamide–adenine dinucleotide phosphate (NADPH) was purchased from P. L. Biochemicals, Inc., Milwaukee, Wis.; folic acid was purchased from Calbiochem, Los Angeles, Calif. Dihydrofolate was prepared by the method of Futterman[9] and also by the procedure of Friedkin and his colleagues.[10] No differences were observed between the rates of enzymatic reduction of dihydrofolate prepared by the different methods. References to syntheses of pteridines carried out in this laboratory are given in the tables.

(1) A preliminary account of a portion of this work was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Washington, D. C., Aug 1967; J. J. McCormack and J. J. Jaffe, *Pharmacologist*, **9**, 193 (1968).

(2) J. J. Jaffe and J. J. McCormack, *Mol. Pharmacol.*, **3**, 359 (1967).

(3) J. J. Burchall and G. H. Hitchings, *ibid.*, **1**, 126 (1965).

(4) See, for example, (a) B. R. Baker and B-T. Ho, *J. Pharm. Sci.*, **53**, 1137 (1964); (b) B. R. Baker and B-T. Ho, *ibid.*, **55**, 470 (1966); (c) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967); (d) B. R. Baker, *ibid.*, **11**, 483 (1968).

(5) A. W. Schrecker and F. M. Huennekens, *Biochem. Pharmacol.*, **13**, 731 (1964).

(6) S. F. Zakrzewski, *J. Biol. Chem.*, **235**, 1776 (1960).

(7) S. F. Zakrsewski and C. A. Nichol, *J. Pharmacol. Exptl. Therap.*, **137**, 162 (1962).

(8) J. Hampshire, P. Hebborn, A. M. Triggle, D. J. Triggle, and S. Vickers, *J. Med. Chem.*, **8**, 745 (1965).

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

(10) M. Friedkin, E. S. Crawford, and D. Misra, *Fed. Proc.*, **21**, 176 (1962).

Assay of dihydrofolate reductase activity was carried out spectrophotometrically by following the decrease in optical density at 340 mμ which occurs on reduction of dihydrofolate and concomitant oxidation of NADPH. The assay solution contained 0.16 μmole of dihydrofolate, 0.24 μmole of NADPH, 33 μmoles of mercaptoethanol, the appropriate enzyme extract (sufficient extract to give a change in absorption of at least 0.01 OD unit/min), and pH 7.0 phosphate buffer (0.1 *M*) in a total volume of 3 ml. Spectrophotometric measurements were made using a Beckman model DU spectrophotometer equipped with thermospacers to maintain the temperature of the cell compartment at 37°.

All drugs were preincubated at 37° for 10 min in the presence of enzyme, mercaptoethanol, and buffer, then NADPH and dihydrofolate were added to the cuvette, and reductase activity was measured at 1-min intervals over a 10-min period. The level of drug required to produce 50% inhibition was estimated graphically from a plot of V_0/V_1 *vs.* increasing inhibitor concentration where V_0 = reaction rate in absence of inhibitor and V_1 = reaction rate in presence of inhibitor.[11] Stock solutions of diaminopteridine derivatives were made up in DMSO and appropriate amounts of DMSO were added to control cuvettes where required.

Results and Discussion[12]

2,4-Diaminopyrimidines.—The effects of various derivatives of 2,4-diaminopyrimidine on dihydrofolate reductase activity in extracts prepared from *T. equiperdum*, chicken liver, and rat liver are summarized in Table I. Compounds **1**–**4** appear to possess no useful degree of inhibitory potency against the reductases studied; the lack of efficacy of these compounds as inhibitors is consistent with previous observations by others.[8,11,13,14] Introduction of a suitably substituted aromatic function into position 5 of the pyrimidine nucleus can greatly enhance the ability of the 2,4-diaminopyrimidine system to inhibit enzymatic reduction of dihydrofolate,[14] and **5** with a 4-chlorophenyl substituent in the 5 position was an effective inhibitor of dihydrofolate reductase in the trypanosomal system as well as in the chicken liver and rat liver systems. The ID_{50} value for **5** is approximately tenfold lower for the trypanosomal reductase than for the avian and mammalian enzymes, but it should be stressed that this difference may simply reflect the order of magnitude difference in K_m values for dihydrofolate which exists between the trypanosomal reductase ($K_m = 4.3 \times 10^{-6}$ *M*)[2] and those from chicken liver (1.7×10^{-7} *M*)[15] and rat liver (2×10^{-7} *M*).[16] Introduction of CH_3 into the 6 position of **5** results in increased inhibitory potency against the rat liver and chicken liver enzymes, but no significant alteration in activity was observed in the trypanosomal system. 2,4-Diamino-5-(2-bromophenyl)pyrimidine (**7**) was a much poorer inhibitor of dihydrofolate reductase from all three sources than was the 4-chlorophenyl analog; it may be that the poor inhibition observed with the 2-bromophenyl derivative is due, at least in part, to steric complications[17–19] arising from the proximity of the bromo function to the pyrimidine ring. In this regard it should be noted that *o*-halophenyl derivatives of 2,4-diaminopyrimidine have been shown to be considerably less effective than the corresponding *p*-halophenyl derivatives as antimalarial agents.[20]

2,4-Diamino-6-ethyl-5-(4-chlorophenyl)pyrimidine (**8**, pyrimethamine), a clinically useful antiplasmodial agent, has been found very recently[21] to be a highly selective inhibitor ($ID_{50} \cong 10^{-10}$ *M*) of dihydrofolate reductase isolated from *Plasmodium berghei;* this pyrimidine derivative is also a potent inhibitor of dihydrofolate reductase isolated from Erlich ascites carcinoma cells.[22] Pyrimethamine was also found to be an effective inhibitor of dihydrofolate reductase from *T. equiperdum*[2] and analogous reductases from other trypanosomal species of African origin.[23] As can be seen in Table I, the rat liver enzyme system and the chicken liver system exhibited sensitivities to inhibition by pyrimethamine which were not very different from that of the trypanosomal enzyme. These comparative data are of considerable interest because they indicate that the reductase obtained from the protozoan, *T. equiperdum*, is more akin to the mammalian and avian enzymes used in this study, with respect to sensitivity to pyrimethamine, than to the reductase isolated by Ferone, *et al.*,[21] from the protozoan *P. berghei*. The ID_{50} values obtained for pyrimethamine in the rat liver and chicken liver systems are considerably (ten- to twentyfold) higher than those reported[24] for pyrimethamine as an inhibitor of dihydrofolate reduction by reductases obtained from guinea pig liver and small intestine.

The introduction of a second Cl in the 3′ position of pyrimethamine resulted in little alteration of inhibitory potency against the trypanosomal reductase, compared with that of pyrimethamine. On the other hand, the 3′,4′-dichloro derivative (**9**) proved to be considerably more effective than pyrimethamine as an inhibitor of reductases from chicken liver and rat liver. Replacement of 6-Et of pyrimethamine and its 3′-Cl analog (**9**) by CH_3 was found to produce a tenfold decrease in the ability of the resulting compounds (**6**, **10**) to inhibit the trypanosomal reductase, but against the avian and mammalian enzymes the decrease in activity was not so marked. Baker and Ho[4a] have shown, using dihydrofolate reductase systems from both pigeon liver and *Escherichia coli* B, that there was relatively little difference between the inhibitory potencies of **9** and its 6-Me analog **10**. It is noteworthy that the decrease in ability to inhibit the trypanosomal reductase, which is a consequence of the replacement of the ethyl group of pyrimethamine by a methyl group, correlates roughly with the decrease in antiplasmodial activity observed[20] for the 6-Me analog relative to pyrimethamine. Compound **6** was considerably less effective than **10** as an inhibitor of dihydrofolate reductase from pigeon liver[4a] and guinea pig tissues[24] and, as seen in

(11) B. R. Baker and H. S. Sachdev, *J. Pharm. Sci.*, **52**, 933 (1963).

(12) Because many of the compounds used in this study were at or near solubility limits at 10^{-4} *M*, we chose this concentration as a "cut-off point," and compounds which did not produce appreciable inhibition at this level were classified as "inert."

(13) S. F. Zakrzewski, *J. Biol. Chem.*, **238**, 1489 (1963).

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

(15) B. T. Kaufman and R. G. Gardiner, *J. Biol. Chem.*, **241**, 1319 (1966).

(16) D. Wang and W. C. Werkheiser, *Fed. Proc.*, **23**, 324 (1964).

(17) I. M. Rollo, *Trans. Roy. Soc. Trop. Med. Hyg.*, **46**, 474 (1952).

(18) P. B. Russell, *J. Chem. Soc.*, 2951 (1954).

(19) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967).

(20) E. A. Falco, L. G. Goodwin, G. H. Hitchings, I. M. Rollo, and P. B. Russell, *Brit. J. Pharmacol.*, **6**, 185 (1951).

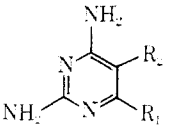
(21) R. Ferone, J. J. Burchall, and G. H. Hitchings, *Mol. Pharmacol.*, **5**, 49 (1969).

(22) J. R. Bertino, J. P. Perkins, and D. G. Johns, *Biochemistry*, **4**, 839 (1965).

(23) J. J. Jaffe, J. J. McCormack, and W. E. Gutteridge, *Exptl. Parasitol.*, in press.

(24) J. R. Bertino, A. T. Iannotti, J. P. Perkins, and D. G. Johns, *Biochem. Pharmacol.*, **15**, 563 (1966).

TABLE I
INHIBITION OF DIHYDROFOLATE REDUCTION BY 2,4-DIAMINOPYRIMIDINES



Compd	R_1	R_2	ID_{50} (10^{-8} M) *T. equiperdum*	Chicken liver	Rat liver	Source[a]
1	H	H	Inert	Inert	Inert	Aldrich
2	NH_2	H	Inert	Inert	Inert	Aldrich
3	H	NH_2	Inert	Inert	Inert	Aldrich
4	NH_2	NH_2	Inert	Inert	Inert	C
5	H	4-ClC_6H_4	150	1,000	900	BW
6	Me	4-ClC_6H_4	180	120	270	BW
7	H	2-BrC_6H_4	14,000	18,000	11,000	BW
8	Et	4-ClC_6H_4	20	55	80[b]	BW
9		3,4-$Cl_2C_6H_3$	14	2	0.8	BW, NSC
10	Me	3,4-$Cl_2C_6H_3$	60	1	1.6	BW
11	Et	3-NO_2-4-ClC_6H_3	10	0.65	1.5	BW
12	Et	3-NH_2-4-ClC_6H_3	160	140	250	BW
13	Me	4-MeC_6H_4	1,000	16	100	BW
14	Me	3,4-$(MeO)_2C_6H_3$	30,000	280	700	BW
15	NH_2	Phenylazo	750	1,500	1,000	Ref 8
16	H	3,4,5-$(MeO)_3C_6H_2CH_2$	100	40,000	13,000[c]	BW
17	H	2,4,5-$(MeO)_3C_6H_2CH_2$	200	35,000	6,500	BW
18	H	3,4-$(MeO)_2C_6H_4CH_2$	70	15,000	1,800	BW
19	CH_3	3,4-$(MeO)_2C_6H_4CH_2$	270	25,000	25,000	BW
20	H	2-Br-4,5-$(MeO)_2C_6H_2CH_2$	80	12,000	7,800	BW
21	H	2-Br-3,4,5-$(MeO)_3C_6H_2CH_2$	90	35,000	23,000	BW
22	H	3-Br-4,5-$(MeO)_2C_6H_2CH_2$	18	25,000	5,000	BW
23	H	4-$ClC_6H_4CH_2$	130	7,300	20,000	BW
24	CH_3	4-$ClC_6H_4CH_2$	250	2,700	5,000	BW
25	CH_3	4-$O_2NC_6H_4CH_2$	100	5,300	1,200	BW
26	H	4-ClC_6H_4O	240	16,000	8,000	BW
27	CH_3	4-ClC_6H_4O	200	4,800[d]	2,200	BW
28	H	4-$MeOC_6H_4O$	150	2,400	6,000	BW
29	CH_3	4-$O_2NC_6H_4O$	650	7,500	1,500	BW

[a] Sources of inhibitors are identified by the following designations: SKF, supplied by Dr. A. Maass of Smith Kline and French Laboratories, Philadelphia, Pa.; WY, supplied by Dr. P. B. Russell of Wyeth Laboratories, Radnor, Pa.; BW, supplied by Dr. G. H. Hitchings of Burroughs Wellcome Laboratories, Tuckahoe, N. Y.; NSC, supplied by Dr. H. Wood of the Cancer Chemotherapy National Service Center, Bethesda, Md.; A, purchased from Aldrich Chemical Co., Milwaukee, Wis.; C, purchased from Cyclo Chemical Co., Los Angeles, Calif. [b] Lit.[2] 80. [c] Lit.[2] 26,000. [d] Lit.[4a] (pigeon liver) 4800.

Table I, similar observations have been made in the systems studied by us.

The 3′-NO_2 analog **11** of pyrimethamine was approximately 100-fold more effective than pyrimethamine as an inhibitor of dihydrofolate reductase from rat liver and chicken liver, but it was several-fold less potent than pyrimethamine as an inhibitor of the reductase obtained from *T. equiperdum*. Replacement of NO_2 of **11** by NH_2 resulted in a tenfold decrease in inhibitory potency against the protozoal reductase and a 150–250-fold decrease in activity against both the avian and the mammalian enzymes. The effect of inserting NO_2 in the 3′ position of pyrimethamine was closely similar to that observed when Cl was substituted in the 3′ position of pyrimethamine. The marked activity of the 3′-NO_2 compound against a mammalian reductase suggests potential value for the compound as an inhibitor of neoplasms which are resistant to methotrexate. In this regard it should be noted that **10** has been found[25] to be an effective inhibitor of Walker carcinoma 256, a tumor which is rather insensitive to methotrexate. The increased efficacy of **10** over methotrexate in this tumor system may be due to differences in the permeability characteristics of these dihydrofolate reductase inhibitors.[26] The differential ability to penetrate various cellular membranes between methotrexate and "small-molecule" dihydrofolate reductase inhibitors such as pyrimethamine has been clearly demonstrated.[27] The observation that the 3′-NH_2 and 3′-NO_2 derivatives of pyrimethamine are roughly equiactive as inhibitors of the trypanosomal reductase but differ considerably in their inhibitory potency against the rat liver and chicken liver enzymes may indicate a considerable difference in the electronic requirements for binding of inhibitors to the protozoal enzyme on the one hand, and to the avian and mammalian enzymes on the other.

2,4,6-Triamino-5-phenylazopyrimidine (**15**) was found[8] capable of inhibiting rat liver folic acid reductase, and the suggestion has been made that the 5-aryl group contributes heavily to the "binding" of this agent to the reductase enzymes. We found it to be an inhibitor of trypanosomal dihydrofolate reductase as well as reductase from chicken and rat liver; the K_i value for inhibition of rat liver dihydrofolate reductase

(25) L. C. Mishra, F. Rosen, and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.*, **8**, 47 (1967).

(26) L. C. Mishra, F. Rosen, and C. A. Nichol, *ibid.*, **9**, 49 (1968).

(27) R. C. Wood, R. Ferone, and G. H. Hitchings, *Biochem. Pharmacol.*, **6**, 113 (1961).

by **15**, estimated from the relationship,[8] $K_I = K_M (I_{50}/S_{50})$, where I_{50} and S_{50} represent, respectively, the inhibitor and substrate concentrations present at 50% inhibition was found to be 2×10^{-8} *M*, a value which is essentially the same as that reported for this substance as an inhibitor of folic acid reduction by rat liver reductase.

The trimethoxybenzylpyrimidine, trimethoprim (**16**), was found to be several-thousand-fold more effective as an inhibitor of dihydrofolate reductase from bacterial sources than as an inhibitor of reductase preparations from mammalian liver.[3] Recently Baker[19] made the interesting observation that trimethoprim was much less active as an inhibitor of dihydrofolate reductase from T_2 bacteriophage than of bacterial reductases *per se*. We have found that dihydrofolate reductase in extracts prepared from chicken liver was only poorly inhibited by trimethoprim, a concentration in the order of 10^{-4} *M* being required for 50% inhibition. The low susceptibility of the chicken liver enzyme to inhibition by trimethoprim parallels the low sensitivities of pigeon liver reductase[17] and reductase from guinea pig tissues[24] to this inhibitor.

We found earlier[2] that dihydrofolate reductase from *T. equiperdum* is intermediate, in its sensitivity to inhibition by trimethoprim, between the high sensitivity of bacterial reductases and the rather extreme insensitivity of mammalian liver reductases. As can be seen in Table I, several analogs of trimethoprim (**17**–**25**) have also been found to be considerably more effective as inhibitors of dihydrofolate reductase from *T. equiperdum* than of the analogous enzymes extracted from mammalian or avian liver. It is possible that the difference in susceptibility of trypanosomal and mammalian reductases to inhibition by pyrimidines of the trimethoprim type might be used to advantage in the strategy of chemotherapy of certain trypanosomiases.

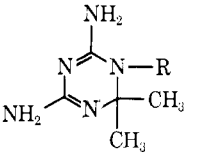
Although certain 5-benzylpyrimidines and 5-phenylpyrimidines were found to exhibit similar potencies as inhibitors of the protozoal reductase obtained from *T equiperdum*, these classes of compounds differed markedly with respect to antimalarial activity against *Plasmodium gallinaceum* and *P. berghei*.[20] Thus, the ID_{50} values for **6** and the related 5-benzylpyrimidine (**24**) were essentially the same with regard to the trypanosomal reductase, but in antiplasmodial tests, the 5-phenyl analog proved to be approximately 50 times more potent than the 5-benzyl derivative.

A large series of 5-aryloxy derivatives of 2,4-diaminopyrimidine was synthesized at the Wellcome Research Laboratories during the course of an intensive research program aimed at developing new antimalarial agents. In general, these aryloxy derivatives showed relatively poor antiplasmodial activity.[20] The efficacies of the several 5-aryloxy derivatives (**26**–**29**) of 2,4-diaminopyrimidine which we have investigated as inhibitors of the trypanosomal, rat liver, and chicken liver systems rather closely resembled those observed for analogous 5-benzyl derivatives since in both the 5-benzyl and the 5-aryloxy series inhibitory potency was higher against the trypanosomal reductase than against the other two systems.

4,6-Diamino-1,2-dihydrotriazines.—A number of 4,6-diamino-1,2-dihydrotriazines show high antiprotozoal activity,[28–31] and such substances have been found also to be potent antagonists of the growth-promoting action of folic acid for certain microorganisms[32] and to possess potentially useful anthelmintic activity.[33] Compounds of this class are very effective inhibitors of dihydrofolate reductases from mammalian[3] and avian sources.[34] The dihydrotriazine derivatives (Table II)

TABLE II

INHIBITION OF DIHYDROFOLATE REDUCTION BY 4,6-DIAMINO-2,2-DIMETHYL-1-ARYL-1,2-DIHYDRO-*s*-TRIAZINES



		ID_{50} (10^{-8} *M*)			
Compd	R	*T. equiperdum*	Chicken liver	Rat liver	Source[a]
30	3,4-$Cl_2C_6H_3$	350	2.5	0.4	NSC
31	4-BuC_6H_4	2000	30	7.2[b]	BW
32	3-ClC_6H_4	210	5.5	10	NSC
33	3-BrC_6H_4	340	4	3	NSC
34	4-ClC_6H_4	100	50	120	NSC
35	2-Naphthyl	400	100	65	Aldrich

[a] See footnote *a*, Table I. [b] Lit.[2] 14.

were, with the exception of **34**, less active as inhibitors of trypanosomal dihydrofolate reductase than of reductases from chicken liver or rat liver. For example, the 3′,4′-dichlorophenyl derivative **30** was approximately 200-fold more effective as an inhibitor of the avian reductase than of the trypanosomal reductase, and this observation is consistent with the finding, previously reported,[2] that the related 4′-butylphenyl derivative **31** was nearly 200 times less potent an inhibitor of trypanosomal reductase than of rat liver reductase. The increased efficacies, as inhibitors of chicken liver reductase, of the 3′-chlorophenyl- and 3′,4′-dichlorophenyldihydrotriazines over that of the 4′-butylphenyl analog parallel observations[4a,17] on the relative effectiveness of these compounds as inhibitors of pigeon liver dihydrofolate reductase. Furthermore, Baker and Ho[4a] found that replacement of the 3′-Cl of **32** by Br resulted in little change in inhibitory activity against dihydrofolate reductases from *E. coli* and pigeon liver, and we also found a similar degree of activity for these halogenated derivatives in the systems studied by us. The 3′-chlorophenyldihydrotriazine **32** and the 3′,4′-dichloro analog **30** showed comparable inhibitory potencies against the chicken liver enzyme, but we found that the 3′,4′-dichloro derivative was about 20-fold more effective than the 3′-monochloro compound in the rat liver system. Similar observations

(28) R. D. Powell, R. L. DeGowin, and R. B. Eppes, *Am. J. Trop. Med. Hyg.*, **14**, 913 (1965).

(29) B. C. Walton, D. A. Person, M. H. Ellman, and R. Bernstein, *ibid.*, **17**, 814 (1968).

(30) C. M. Johnson, *ibid.*, **17**, 819 (1968).

(31) R. I. Hewitt, W. S. Wallace, A. Gumble, E. White, and J. H. Williams, *ibid.*, **3**, 225 (1954).

(32) E. J. Modest, G. E. Foley, M. M. Rechet, and S. Farber, *J. Am. Chem. Soc.*, **74**, 855 (1952).

(33) B. Roth, R. B. Burrows, and G. H. Hitchings, *J. Med. Chem.*, **6**, 370 (1963).

(34) For a summary of a series of studies on dihydrofolate reductase inhibitors of the dihydrotriazine class see B. R. Baker, "Design of Active-Site-Directed Irreversible Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967.

concerning the relative activities of these compounds have been made[4a] for mouse liver and pigeon liver reductases but Baker[4b] has recently reported that the 3′,4′-dichlorophenyl derivative is only threefold more potent than the 3′-chlorophenyl derivative as an inhibitor of rat liver reductase. The 4′-butylphenyl derivative was found to be less effective than the corresponding naphthyl (**35**) derivative as an inhibitor of trypanosomal reductase although the reverse was seen in the case of reductases from rat and chicken liver.

It has been noted[4a] that the ability of several 4,6-diamino-1,2-dihydrotriazines to inhibit dihydrofolate reductases, obtained from mouse liver, pigeon liver, and *E. coli*, did not correlate well with the antimalarial activity of these compounds. We also found a lack of correlation between the ability of such triazines to inhibit trypanosomal dihydrofolate reductase and the reported antimalarial activity of these triazines. For example, the 3′,4′-dichlorophenyl analog **30** was 20-fold more effective than the 3′-chlorophenyl (**32**) derivative when evaluated against *P. berghei* in mice, whereas little, if any, difference was detected between the ability of these compounds to inhibit the reductase of *T. equiperdum*. This lack of correlation between *in vitro* reductase inhibition and *in vivo* antimalarial activity could, of course, be attributed to differences in absorption, distribution, metabolism, or excretion of the drugs *in vivo*, but it may also be due to a pronounced difference between the plasmodial reductase and, not only the avian and mammalian reductases, but also the phylogenetically more closely related trypanosomal reductase. In this regard, Ferone and his colleagues[21] have found recently that dihydrofolate reductase obtained from *P. berghei* shows a different pattern of sensitivity to certain small-molecule inhibitors than does the enzyme from *T. equiperdum*.

In contrast to the activity of the 4,6-diaminodihydrotriazines, 2,4-diamino-6-phenyl-*s*-triazine showed virtually no inhibitory potency against the reductases studied by us. Baker[35] has also reported that the fully aromatic triazine has little efficacy as an inhibitor of reductase prepared from pigeon liver.

2,4-Diaminopteridines.—2,4-Diaminopteridines have long been known[36] to possess antifolic activity in bacterial systems. Such substances are capable of inhibiting folate reduction by a reductase obtained from chicken liver,[13] and an attempt has been made recently[37] to correlate the ability of diaminopteridines to inhibit chicken liver folate reductase with their calculated electronic parameters. We have investigated several 2,4-diaminopteridines as inhibitors of dihydrofolate reductase prepared from *T. equiperdum* as well as from chicken liver and rat liver; the results obtained are summarized in Table III. 2,4-Diaminopteridine (**36**) itself was found to be essentially inactive as an inhibitor of the three enzyme systems. On the other hand, the 6-Me (**37**) derivative and the 6,7-Me_2 (**38**) derivatives showed somewhat higher inhibitor activity. Insertion of phenyl groups into the pyrazine ring of 2,4-diaminopteridines resulted in a marked increase in inhibitory potency which may reflect differences in the ability of these compounds to undergo hydrophobic interactions with the various reductases.[14,34] It should be noted that Greenberg[38] reported in 1948 that 2,4-diamino-6,7-diphenylpteridine (**39**) was an effective antimalarial agent, against *P. gallinaceum*, while the 6,7-Me_2 analog exhibited negligible activity. The observation that unsubstituted 2,4-diaminopteridine had a very low order of efficiency as a reductase inhibitor indicates that use[37] of this compound as a model for quantum mechanical estimations of efficiency of inhibitor binding to dihydrofolate reductase is of extremely limited value. Of particular relevance in this regard is the observation that folic acid itself was a better inhibitor[2] of trypanosomal dihydrofolate reductase than was 2,4-diaminopteridine.

Neither 4-amino-6,7-diphenylpteridine (**40**) nor its 2-amino analog (**41**) showed appreciable activity as inhibitors of reductases from *T. equiperdum*, chicken liver, or rat liver. Replacement of the 4-NH_2 of 2,4-diamino-6,7-diphenylpteridine by NHMe resulted in a loss of inibitory potency; methylation of exocyclic amino groups of other diaminopteridine dihydrofolate reductase inhibitors was reported previously by Johns, *et al.*,[24,39] to produce a profound decrease in inhibitory activity. The lack of efficacy of the 2- and 4-NH_2 and 2-amino-4-methylamino derivatives of 6,7-diphenylpteridine finds a parallel in studies, by Baker and his colleagues,[40,41] of structure–activity relationships among related 2,4-diaminopyrimidine reductase inhibitors.

The recent report[42] by Osdene, *et al.*, on the effectiveness of certain 2,4,7-triaminopteridines in suppressing experimental malarial infections, coupled with our earlier finding[2] that the clinically useful diuretic agent, triamterene (**42**, 2,4,7-triamino-6-phenylpteridine), was a good inhibitor of trypanosomal dihydrofolate reductase, prompted a study of the abilities of members of a series of 2,4,7-triaminopteridines to act as inhibitors of the trypanosomal reductase. It can be seen in Table IV that 2,4,7-triaminopteridines which bear an *ortho*-substituted phenyl group in the 6 position were not greatly different with respect to ability to inhibit reductase preparations from all three sources employed than were those compounds bearing a *meta* substituent in the 6-phenyl moiety. There was a much larger difference in the abilities of the *ortho*-substituted compounds and of the corresponding *para*-substituted analogs to inhibit these enzymes. These observations would suggest rather stringent steric requirements for effective interaction between the triaminopteridine derivatives and the reductases from various sources. It is noteworthy that the higher inhibitory potency of the *ortho*-substituted 6-phenylpteridines, compared with that of the *para*-substituted derivatives, correlates well with the finding that the *ortho*-substituted derivatives were effective antimalarial agents, when evaluated against *P. gallinaceum* in chicks and *P. berghei* in mice, while the *para*-substituted derivatives were essentially inactive in suppressing these plasmodial infections. On the other hand, comparison

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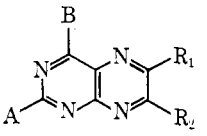
(36) (a) L. J. Daniel and L. C. Norris, *J. Biol. Chem.*, **170**, 747 (1949); (b) G. B. Elion and G. H. Hitchings, *ibid.*, **188**, 611 (1951).
(37) W. B. Neely, *Mol. Pharmacol.*, **3**, 108 (1967).
(38) J. Greenberg, *J. Pharmacol. Exptl. Therap.*, **97**, 484 (1949).
(39) D. G. Johns, A. C. Sartorelli, A. T. Ianotti, and J. R. Bertino, *Proc. Am. Assoc. Cancer Res.*, **5**, 32 (1964).
(40) B. R. Baker and B-T. Ho, *J. Pharm. Sci.*, **53**, 1457 (1964).
(41) B. R. Baker and H. S. Shapiro, *ibid.*, **55**, 308 (1966).
(42) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, **10**, 431 (1967).

TABLE III

INHIBITION OF DIHYDROFOLATE REDUCTION BY SELECTED AMINOPTERIDINES

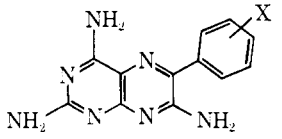


Compd	A	B	R_1	R_2	ID_{50} (10^{-8} M) *T. equiperdum*	ID_{50} (10^{-8} M) Chicken liver	ID_{50} (10^{-8} M) Rat liver	Source[a]
36	NH_2	NH_2	H	H	Inert	Inert	25,000	*b*
37	NH_2	NH_2	Me	H	4000	25,000[d]	7,000	C
38	NH_2	NH_2	Me	Me	800	7,500	17,000[e]	*b*
39	NH_2	NH_2	Ph	Ph	40	1,000	400	*b*
40	H	NH_2	Ph	Ph	Inert	Inert	Inert	*c*
41	NH_2	H	Ph	Ph	Inert	Inert	Inert	*c*

[a] See footnote *a*, Table I. [b] M. F. Mallette, E. C. Taylor, and C. K. Cain, *J. Am. Chem. Soc.*, **69**, 1814 (1947). [c] M. D. Potter and T. Henshall, *J. Chem. Soc.*, 2000 (1956); for the 2-amino derivative the literature procedure was modified by dissolving the pyrimidine at pH 7 rather than in dilute acetic acid before the condensation with benzil. [d] K_I, calculated[8] from $ID_{50} = 5 \times 10^{-7}$ *M*; lit.[13] 4.7×10^{-7} *M*. [e] K_I, calculated from $ID_{50} = 1 \times 10^{-6}$ *M*; lit.[47] 2×10^{-6} *M*.

TABLE IV

INHIBITION OF DIHYDROFOLATE REDUCTION BY 2,4,7-TRIAMINO-6-ARYLPTERIDINES



Compd	X	ID_{50} (10^{-8} M) *T. equiperdum*	ID_{50} (10^{-8} M) Chicken liver	ID_{50} (10^{-8} M) Rat liver	Source[a]
42	H	250	150	160[e]	Aldrich
43	2-Me	400	300	130	SKF
44	3-Me	800	330	190	WY
45	4-Me	10,000	12,000	1,700	WY
46	2-Cl	400	250	150	SKF
47	3-Cl	130	100	25	WY
48	4-Cl	15,000	10,000	1,800	WY
49	2,6-Cl_2	180	300	60	WY
50	2,4-Cl_2	5,000	5,700	740	WY
51	3,4-Cl_2	50,000	2,200	200	WY
52	2-Br	90	260	100	WY
53	4-Br	8,200	29,000	7,200	WY
54	2-I	180	330	120	WY
55	4-I	12,000	11,000	5,000	WY
56	2-F	500	400	210	WY
57	4-F	3,300	4,500	1,400	WY
58	2-Ph	110	9,000	1,400	WY
59	1-Naphthyl[b]	120	790	100	WY
60	4-Ph	20,000	8,500	23,000	WY
61	4,5-Me_2	9,000	3,000	8,500	WY
62	Ph[c]	Inert	Inert	Inert	SKF
63	Ph[d]	Inert	Inert	Inert	SKF

[a] See footnote *a*, Table I. [b] Naphthyl function in place of the phenyl group of **42**. [c] 4-Amino group replaced by a *p*-chlorophenylamino group. [d] 2-Amino group replaced by a phenylamino group. [e] K_I, calculated from $ID_{50} = 6 \times 10^{-9}$ *M*; lit.[47] 1×10^{-8} *M*.

of ID_{50} values obtained in the trypanosomal system for 2,4,7-triamino-6-*o*-chlorophenylpteridine (**46**), which has marked antimalarial activity, and the related 6-*m*-chlorophenyl compound (**47**), which shows little antimalarial potency, indicates quite clearly that no necessary correlation can be demonstrated between *in vitro* inhibition of trypanosomal reductase and *in vivo* antimalarial activity of these pteridines.

The report[42] that 2,4,7-triamino-6-*o*-tolylpteridine (**43**) has activity against leishmania *in vitro* as well as the recent demonstration by Corbascio[43] of the ability of this compound to antagonize arrhythmias induced by cardiac glycosides further extend the range of pharmacological activity shown by pteridines of this type; it would be of considerable interest to determine what effect alteration of the position of substituents in the 6-aryl function exerts on the antileishmanial and antiarrhythmic activity of such triamterene derivatives. When the primary amino group in either the 2 or the 4 position of triamterene was replaced by an aromatic amino function, ability to inhibit the reductase systems studied was found to decrease substantially. Comparable decreases in ability to inhibit dihydrofolate reductase were obtained[24] when the amino group in position 2 or 4 of analogous pteridine derivatives was replaced by an alkylamino function.

The potency of triamterene as an inhibitor of rat liver reductase is comparable to its potency as an inhibitor of reductase preparations from Ehrlich ascites tumor cells[22] and mouse leukemia cells.[44] Doctor, in 1958, reported that 2,4,7-triamino-6-phenylpteridines were able to inhibit conversion of folic acid to 5-formyltetrahydrofolic acid by an extract prepared from chicken liver;[45] the order of inhibitory activity which Doctor found for certain 6-arylpteridines was similar to that found for inhibition of dihydrofolate reduction by the crude extract from chicken liver which we have used. Thus in Doctor's experiments the inhibitory potencies of 2,4,7-triamino-6-phenylpteridine and its *o*-methyl and *m*-methyl derivatives differed by a factor of only three- to fourfold, whereas the *p*-methyl analog was about 150-fold less potent than the *o*-methyl derivative. As summarized in Table IV, the same qualitative relationship exists among these compounds as inhibitors of dihydrofolate reductases. It should be noted that addition of *p*-Cl to either the *o*- or the *m*-chlorophenyl derivatives of 2,4,7-triaminopteridine resulted in a reduction of inhibitory potency, indicating that the simple presence of *ortho* or *meta* substituents *per se* is not sufficient to produce optimal inhibitory activity of such pteridine derivatives against the reductase systems.

(43) A. N. Corbascio, *Fed. Proc.*, **27**, 348 (1968).

(44) A. R. Maass, V. D. Wiebelhaus, G. Sosnowski, B. Jenkins, and G. Gessner, *Toxicol. Appl. Pharmacol.*, **10**, 413 (1967).

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Since 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)-pteridine (biopterin) is a growth-promoting agent for the trypanosomatid flagellate, *Crithidia fasciculata*,[46] we decided to determine whether this agent was capable of serving as a substrate for the reductase obtained from *T. equiperdum;* biopterin (at 10^{-4} *M*) was not a substrate for the trypanosomal reductase, nor was it capable (again at 10^{-4} *M*) of inhibiting the reduction of dihydrofolate by this preparation. Biopterin has also been found by Roberts and Hall[47] to be ineffective as an inhibitor of rat liver dihydrofolate reductase. The related 2-amino-4-hydroxypteridines, xanthopterin (2-amino-4,6-dihydroxypteridine) and isoxanthopterin (2-amino-4,7-dihydroxypteridine), also proved ineffective both as substrates and inhibitors of the trypanosomal reductase. A number of 4-substituted 2,6-diaminopyridines can inhibit the growth of such protozoa as *Tetrahymena pyriformis* and *C. fasciculata*.[48] In the case of 4-alkoxy-2,6-diaminopyridines, inhibitory activity has been ascribed to interference with biopterin function in *C. fasciculata*.[49] We found that 4-butoxy-2,6-diaminopyridine[50] had no appreciable activity as an inhibitor of trypanosomal dihydrofolate reductase, and this observation seems in accord with the lack of activity of biopterin itself in the protozoal reductase system. Recent experiments[51] with dihydrofolate reductase obtained from *C. fasciculata* indicated that biopterin shows no activity in this system. Folic acid was found previously[2] to be essentially inactive as a substrate for trypanosomal dihydrofolate reductase, but it was found to be a reasonably effective inhibitor of the enzyme (ID_{50} *ca.* 10^{-5} *M*); similar results were obtained with the triglutamate derivative[52] of folic acid which proved to be ineffective as a substrate while possessing inhibitory potency comparable to that of folic acid itself.

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(46) H. N. Guttman and F. G. Wallace in "Biochemistry and Physiology of Protozoa," Vol. III, S. H. Hutner, Ed., Academic Press, New York, N. Y., 1964, p 460.

(47) D. Roberts and T. C. Hall, *J. Clin. Pharmacol.*, **8**, 217 (1968).

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(50) Provided by Dr. A. Maass of Smith Kline and French Laboratories, Philadelphia, Pa.

(51) W. E. Gutteridge, J. J. McCormack, and J. J. Jaffe, *Biochim. Biophys. Acta*, in press.

(52) Kindly supplied by Dr. E. Patterson, Lederle Laboratories, Pearl River, N. Y.

Irreversible Enzyme Inhibitors. CLIV.[1,2] Some Factors in Cell Wall Transport of Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase[3] Derived from 5-Substituted 2,4-Diaminopyrimidines

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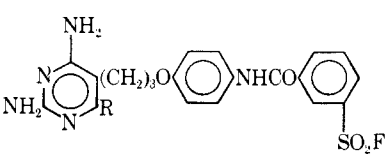
5-[*p*-(*m*-Fluorosulfonylbenzamido)phenoxypropyl]-2,4,6-triaminopyrimidine (**1a**) was previously observed to be an isozyme-specific active-site-directed irreversible inhibitor of dihydrofolic reductase from L1210 mouse leukemia that showed no irreversible inhibition of the enzyme from normal mouse liver; however, **1a** was ineffective *in vivo* due to poor penetration of the L1210 cell wall. Replacement of the ether bridge of **1a** by thioether (**2**) or methylene (**3**) gave irreversible inhibitors of similar isozyme specificity that could operate at much lower concentration due to their better reversible binding than **1a**: cell wall transport was not improved with **2** or **3**. This difficulty in cell wall transport of **1a**, **2**, and **3** compared to 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (**5**) was traced to two factors: (a) replacement of the 3,4-dichlorophenyl group of **5** by phenylbutyl (**7**) or *p*-aminophenoxypropyl (**6**) was extremely detrimental to transport, and (b) further smaller losses in transport occurred when **6** or **7** were converted to the *m*-fluorosulfonylbenzamido type of irreversible inhibitor (**1b**, **8**). In contrast to **7**, 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-phenylbutyl-2-triazine (**16**) was transported as well as **5**.

5-Phenoxypropylpyrimidines of type **1**[4,5] were found to be excellent active-site-directed inhibitors[6] of the dihydrofolic reductase from L1210 mouse leukemia, but showed no inactivation of this enzyme from normal mouse liver, intestine, or spleen.[7] However, **1a** and **1b**



1a, R = NH_2
b, R = CH_3

(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 J. A. Hurlbut, *J. Med. Chem.*, **12**, 415 (1969).

(3) For the previous paper on this enzyme see B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 224 (1969), paper CLI of this series.

(4) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **11**, 489 (1968), paper CXIX of this series.

(5) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 108 (1969), paper CXLIII of this series.

(6) 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.

(7) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 67 (1969), paper CXXXIII of this series.