Analogs of Tetrahydrofolic Acid XV

Mode of Binding of 2,4-Diaminopyrimidines to Folic Reductase and Thymidylate Synthetase

By B. R. BAKER and BENG-THONG HO

A series of compounds related to 5-(3-anilinopropyl)-2,4-diamino 6-methyl-4-pyrimidine (XIII) was synthesized and evaluated as inhibitors of dihydrofolic reductase and thymidylate synthetase. Strong evidence was obtained that both amino groups were binding groups to both enzymes. Inhibition by 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (I*b*) was lost with both enzymes when the 2-amino group was replaced by 2-dimethylamino (XXII). When the 4-amino group of XIII was replaced by a 4-dimethylamino, a 26-fold decrease in binding to dihydrofolic reductase was observed. In contrast, this structural change caused little change in the extent of inhibition of thymidylate synthetase. The 2-amino group of I*b* probably binds to both enzymes (E) through an N—H ← E hydrogen bond. The 4-hydroxyl group of I*b* probably binds to dihydrofolic reductase by an O—H ← E hydrogen bond of the less favored 4-hydroxy tautomer but to thymidylate synthetase by a C=O → H—E hydrogen bond of the more stable 4-oxo tautomer. Since 2,4-diaminopteridines bind to folic reductase 10 to fiftyfold stronger than similarly substituted 2,4-diamino-5,6-dialkylpyrimidines, it is probable that N_5 or N_8 of the pteridine is an additional binding group. In contrast, 2-amino-4-hydroxy-5,6-dialkylpyrimidines bind to folic reductase about fivefold better than the corresponding 2-amino-4-hydroxypteridines, an indication that neither N_5 nor N_8 of the latter is a binding group. It is proposed that the imidazole ring of a histidine is present in the active site of folic reductase in order to transfer a proton to one side of a pyrazine double bond when hydride ion is transferred from TPNH to the other side of the double bond. It is also proposed that this imidazole ring forms a complex (*a*) with N_5 or N_8 of 2,4-diaminopteridines, (*b*) with a 6-phenyl or 6-benzyl group attached to a 2-amino-4-pyrimidinol, or (*c*) with a 5-phenyl group attached to a 2,4-diaminopyrimidine.

THE HIGH biological potency of 4-amino-4-deoxyfolic acid (aminopterin) (XXVIII) and the use of this compound as an antileukemic agent have been known for about 15 years (1). The lethal effect of aminopterin (XXVIII) on mammalian cells is most probably due to its strong inhibition of the important enzyme, folic reductase, which reduces folic acid (XXIX) to its cofactor form, tetrahydrofolic acid (1). Some years later, Werkheiser (2) showed that aminopterin (XXVIII) was bound to folic reductase 10,000 times stronger than the substrate, folic acid (XXIX), a remarkable increase in binding for such a simple structural change.

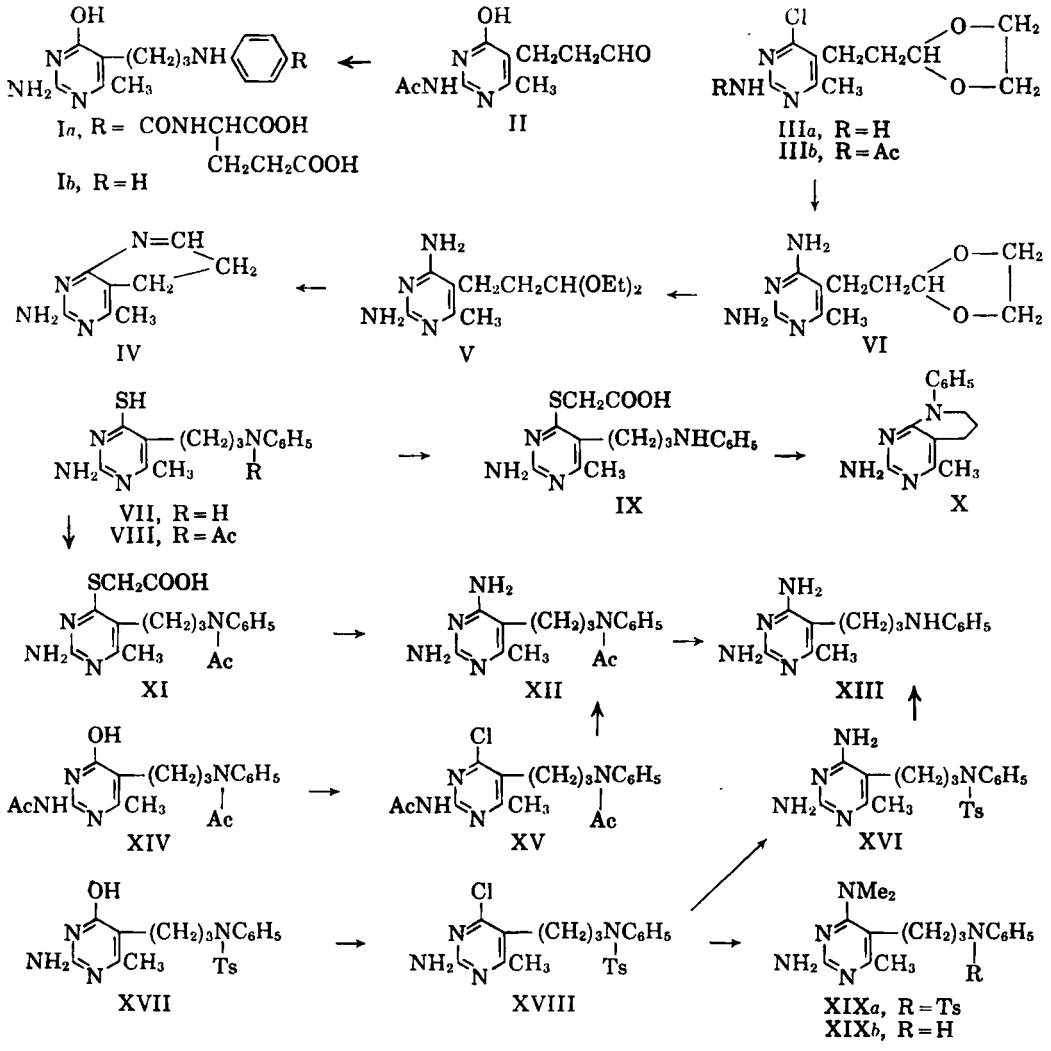
On the basis that the 2,4-diaminopteridine moiety of aminopterin was a much stronger base than the 2-amino-4-hydroxypteridine moiety of folic acid, Baker (3) proposed that the greater extent of protonation at the 2-amino group of aminopterin could lead to increased binding to an anionic site on the enzyme, and such an ionic bond could account for 6–7 Kcal. increase in free energy of binding. Zakrzewski (4) showed by thermodynamic studies that folate most probably had to be converted by the enzyme from its normal 3-H-4-oxo tautomer to the 4-hydroxy tautomer before hydrogen binding of this group to the enzyme could take place. The 4-amino group of aminopterin already exists mainly as the 4-amino tautomer and could therefore readily form two hydrogen bonds with the enzyme through the 4-NH and 3-N groups. Since hydrogen bonds have 2–5 Kcal. (5) of bonding energy, two additional hydrogen bonds in the 4-amino structure would give 4–10 Kcal. of free energy of binding, possibly within the range of the observed 6–7 Kcal. increase in free energy of binding with aminopterin. Furthermore, Zakrzewski (6) observed that there was no correlation between inhibition of folic reductase by a series of 2,4-diaminoheterocycles and their basicity, which made Baker's earlier hypothesis (3) seemingly untenable; however, these 2,4-diamino compounds (6) had other structural changes which might have influenced binding in a way to negate correlation with basicity. Therefore, the authors felt that a further study of binding to folic reductase with a more closely related series of compounds might shed additional light on the

Received April 16, 1964, from the Department of Medicinal Chemistry, School of Pharmacy, State University of New York at Buffalo, Buffalo.

Accepted for publication June 11, 1964.

This investigation was supported by grant CA-06624 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md. The authors thank Starks Associates, Inc., and the Cancer Chemotherapy National Service Center, National Cancer Institute, for large-scale preparation of certain intermediates, mediated by contract SA-43-ph4346.

Previous paper: Baker, B. R., Santi, D. V., and Shapiro, H. S., THIS JOURNAL, 53, 1317(1964).



remarkably strong binding of aminopterin (XXVIII).

A previous paper (7) from this laboratory showed that the tetrahydrofolate analog (I*a*) with $K_i = 2 \times 10^{-6}$ was bound to folic reductase stronger than the substrate, folic acid, with $K_m = 10 \times 10^{-6}$. Furthermore, the simplified structure I*b* without the carboxy-L-glutamate moiety was still a reasonably good inhibitor of folic reductase with $K_i = 63 \times 10^{-6}$ (8). A further study (9) showed that the 2-amino group of I*b* contributed more to binding to (dihydro)folic reductase than the 4-hydroxyl group did, but binding at the 4-position was increased with a 4-mercapto group. The synthesis and mode of binding of some pyrimidyl analogs, where the substituents at the 2- and 4-positions of I*b* have been varied with amino, dimethylamino, and hydrogen substituents, will now be reported. The effects of these groupings on binding of I*b* to thymidylate synthetase are also reported.

EXPERIMENTAL

Methods

Analogs of type I were originally synthesized by reductive condensation of the 5-pyrimidylpropionaldehyde (II) with the appropriate arylamine (7, 8). Therefore, the initial efforts toward synthesis of the 2,4-diamino analog (XIII) were aimed at synthesis of the key intermediate, 2,4-diacetamido-6-methyl-5-pyrimidylpropionaldehyde. Reaction of the 2-amino-4-chloropyrimidine bearing a dioxolane blocked aldehyde group (III*a*) (9) with methanolic ammonia for 24 hours in a sealed vessel at 150° gave the hydrochloride salt of VI in good yield. In the synthesis of II and related compounds it was necessary to exchange the dioxolane blocking group for the diethyl acetal blocking group (7, 10) before the aldehyde function could be regenerated; therefore, the 2,4-diaminopyrimidyl dioxolane (VI) was reacted with alcoholic sulfuric acid. Instead of the expected diethylacetal (V), an air-unstable product was obtained that showed a bathochromic shift in the ultraviolet from that expected for V. It appeared that cyclization to IV had taken place, a compound that would be expected to be unstable to air and would absorb at longer wavelength than V.

The availability of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinethiol (VII) (10) suggested that attempts be made to replace the mercapto group by amino. Since the carboxymethylthio group is a better leaving group than the methylthio group for nucleophilic displacement on a pyrimidine ring (11), VII was reacted with chloroacetic acid in water. Less than 2% of the desired 4-carboxymethylthiopyrimidine (IX) was formed; the major product was the cyclized piperidino [2,3-*d*] pyrimidine (X) formed by anchimeric displacement of the carboxymethylthio group of IX by the neighboring anilino group. The cyclic compound (X) had an ultraviolet spectrum almost identical to that of 2-amino-4-(*N*-methylanilino)-5,6-trimethylenepyrimidine (12), as would be expected. Thus, it was apparent that even if the yield of IX could be increased by conducting the reaction in acid solution to protonate the anilino group, it would not be possible to displace the carboxymethylthio group of IX with ammonia since the anchimeric cyclization to X would be more rapid.

To avoid cyclization of the anilino group, the *N*-acetylanilino pyrimidinethiol (VIII) (13) was reacted with chloroacetic acid in boiling methanol; the rate of reaction was followed by the shift of 350 mμ peak of VIII to 310 mμ. The reaction required 20 hours for completion. Reaction was faster in water, being complete in less than 2 hours. The yield of XI was better (91%) in the aqueous medium than in methanol (45%). Unfortunately, the carboxymethylthio group of XI was not a leaving group sufficiently strong to be displaced by ammonia below the decomposition temperature of XI. At 140° for 24 hours in concentrated ammonia water, conditions strong enough to convert a 2-amino-4-chloropyrimidine (III) to the diamine (VI), XI was recovered unchanged. At higher temperature (190°) conversion to XII apparently occurred along with some decomposition, as shown by the characteristic shift in ultraviolet spectrum in such a conversion (12). Although this route was probably successful, the length of the synthesis (15 steps) was considered impractical, and a shorter synthesis was sought.

Acetylation of I*b* with acetic anhydride in pyridine at about 90° gave an 80% yield of crystalline diacetyl derivative (XIV). Reaction of XIV with phosphorus oxychloride in benzene at 85° seemed rapid (10), but the resultant chloropyrimidine (XV) was difficult to purify and may have been contaminated with unchanged XIV. Treatment of crude XV with ammonia in methanol at 140° gave XII as a glass that had the proper ultraviolet spectrum; if the anilino *N*-acetyl group had not remained intact, then the product could have been expected to be piperidino-pyrimidine, X, which has a quite different ultraviolet spectrum. Hydrolysis of the *N*-acetyl group with boiling 6 *N* hydrochloric acid gave a mixture of the desired diaminopyrimidine (XIII) and the starting 2-amino-4-pyrimidinol (I*b*); these two compounds were separated without much difficulty by taking advantage of the stronger basicity of XIII; but the over-all yield was low. Whether the I*b* arose from contamination of the chloropyrimidine (XV) with unchanged XIV or whether the 2,4-diaminopyrimidine (XIII) was partially hydrolyzed to I*b* during the acid treatment (14) was not resolved.

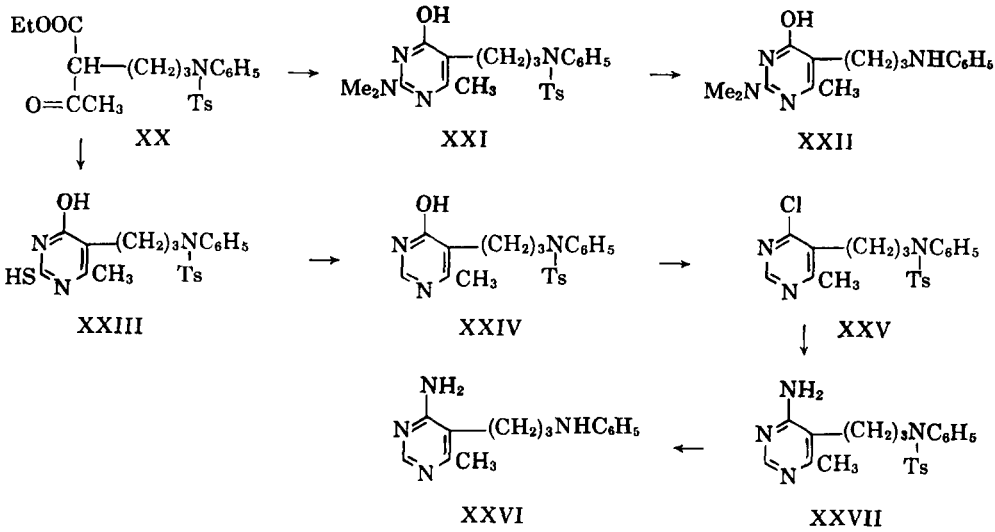
This route to the diaminopyrimidine (XIII) still involved 11 steps *via* 2-acetamido-4-hydroxy-6-methyl-5-pyrimidylpropionaldehyde (II) and I*b*, and the over-all yield was quite low. However, the high potency of XIII as an inhibitor of dihydrofolic reductase and the concomitant emergence of a facile three-step synthesis (15) of the *N*-tosyl derivative (XVII) of I*b* suggested that the conversion of XVII to the diaminopyrimidine (XIII) be investigated.

Reaction of pure XVII with boiling phosphorus oxychloride required 45 minutes for completion, and the crystalline chloropyrimidine (XVIII) was obtained in 80% yield. Treatment of XVIII with methanolic ammonia at 150° for 24 hours afforded the pure crystalline, blocked diaminopyrimidine (XVI) in 64% yield as its hydrochloride. The fusion of ammonium acetate with the chloropyrimidine (XVIII) at 165°—as described for the conversion of certain 2-methylthiopyrimidines to 2-aminopyrimidines (16)—mainly gave reversion to the pyrimidinol (XVII) in 71% yield, and only a 6% yield of the diaminopyrimidine (XVI) was obtained. The tosyl group of XVI was smoothly removed by reductive cleavage with sodium in liquid ammonia (15) to give the desired diaminopyrimidine (XIII) in 85% yield. Thus, this last synthesis of XIII required only six steps, each of which proceeded in 70–90% yield. The *N*-tosyl group of XVI could also be cleaved with 30% hydrogen bromide in acetic acid in the presence of phenol (15, 17) to give 82% of XIII.

Reaction of the chloropyrimidine (XVIII) with ethanolic dimethylamine at 140° for 24 hours—the conditions used for conversion of XVIII to XVI—led to a dark brown syrup which had the ultraviolet spectra expected for a 2-amino-4-dimethylaminopyrimidine (12); however, it could not be crystallized. The severity of the conditions was gradually reduced to the minimum, which surprisingly was only 1 hour at 120°. White crystalline XIX*a* was then readily isolated in 40% yield with the unexpectedly low melting point of 74°. Cleavage of the *N*-tosyl group of XIX*a* with hydrogen bromide in acetic acid containing phenol (15, 17) gave the dimethylamino pyrimidine (XIX*b*) in satisfactory yield.

When the keto ester (XX) (15) was reacted with *N,N*-dimethylguanidine, the crystalline 2-dimethylaminopyrimidine (XXI) was obtained in only fair yield. Cleavage of the *N*-tosyl group with sodium and liquid ammonia afforded the desired 2-dimethylamino - 5 - (anilinopropyl) - 6 - methyl - 4 - pyrimidinol (XXII) in 81% yield.

The final compound needed for enzymic comparison was the 4-aminopyrimidine (XXVI). The synthesis of XXVI started with condensation of the keto ester (XX) (15) with thiourea in methanolic sodium methoxide; the crystalline 2-mercaptopyrimidine (XXIII) was readily isolated in 71%. Desulfurization of XXIII with sponge nickel in boiling 2-methoxyethanol afforded 58% of the 4-pyrimidinol (XXIV). Treatment of XXIV with phosphorus oxychloride in boiling benzene gave a crystalline precipitate that was the phosphate salt of the chloropyrimidine (XXV). Conversion of the phosphate salt to the free base with aqueous sodium bicarbonate afforded an 84% over-all yield of crystalline XXV from XXIV. The chloro group of XXV was displaced with ammonia in methanol at 140° during 5 hours, giving a quantitative yield of the



blocked 4-aminopyrimidine (XXVII). Detosylation with sodium in liquid ammonia proceeded smoothly to afford the desired crystalline 4-aminopyrimidine (XXVI) in 76% yield.

Synthesis

Melting points were taken on a Fisher-Johns apparatus, and those below 230° are corrected. Infrared spectra were determined with a Perkin-Elmer 137 B spectrophotometer in KBr disk unless otherwise indicated; ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer.

2,4 - Diamino - 5 - [2 - (1,3 - dioxolan - 2 - yl)-ethyl]-6-methyl-4-pyrimidine (VI) Hydrochloride.—A mixture of 500 mg. of pure III*b* (10) and 10 ml. of ethanol saturated with ammonia at 0° was heated in a steel bomb at 150–155° for 24 hours. Evaporation to dryness *in vacuo* left 506 mg. of crude crystalline product, m.p. 207–210°. Recrystallization from methanol-acetone gave 234 mg. (51%) of white crystals, m.p. 214–215°; $\nu_{max.}$ 3300, 3150 (NH); 1660 (C=NH$^+$) 1625, 1520 (NH, pyrimidine); 1100, 1040, 1015 cm.$^{-1}$ (ether C—O—C); $\lambda_{max.}^{pH\ 1,7}$ 277 mμ (ε 7800); $\lambda_{max.}^{pH\ 13}$ 287 mμ (ε 8100).

Anal.—Calcd. for $C_{10}H_{17}ClN_4O_2$: C, 46.1; H, 6.57; N, 21.5. Found: C, 45.7; H, 6.40; N, 21.2

Similarly, III*a* (9) gave 65% of recrystallized product and was considered the method of choice because of the ease of obtaining pure III*a* and the large losses involved in obtaining pure III*b* (10).

2 - Amino - 4 - methyl - 8 - phenylpiperido[2,3-d]-pyrimidine (X).—A mixture of 100 mg. (0.364 mmole) of VII and 34 mg. (0.364 mmole) of chloroacetic acid in 2 ml. of water was refluxed with stirring for 45 minutes. After being cooled, the mixture was filtered, and the insoluble crystals were collected on a filter; yield, 2 mg. (1.6%). Its ultraviolet spectra indicated its structure was IX, since it was close to that described for 2-amino-4-methylthio-5,6-trimethylenepyrimidine (12); $\lambda_{max.}^{pH\ 1}$ 240, 274, 318, and $\lambda_{max.}^{pH\ 13}$ 310 mμ.

The filtrate was adjusted to pH 12 with 10% sodium hydroxide. The crystalline X was collected on a filter and washed with water; yield, 25 mg. (29%), m.p. 141°; $\nu_{max.}$ 3320, 3200 (NH); 1600, 1500 (NH, pyrimidine, C=C); 760, 695 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1,7}$ 290 (ε 13,100), inflection centering at 245 mμ (ε 9200); $\lambda_{max.}^{pH\ 13}$ 305 (ε 12,500), inflection centering at 252 mμ (ε 8200).

Anal.—Calcd. for $C_{14}H_{16}N_4$: C, 70.0; H, 6.71; N, 23.3. Found: C, 69.7; H, 6.70; N, 23.6.

2 - Amino - 4 - carboxymethylthio - 5 - (N - acetyl-3-anilinopropyl)-6-methylpyrimidine (XI).—A mixture of 100 mg. (0.32 mmole) of VIII (13), 4 ml. of water, and 41 mg. (0.43 mmole) of chloroacetic acid was refluxed for 2 hours, then chilled and neutralized to pH 5. The product was collected on a filter and washed with water; yield, 107 mg. (91%), m.p. 218–221°. Recrystallization from ethanol raised the melting point to 225–226°; $\nu_{max.}$ 3400, 3000, 2960, 2750, 2350, 1950–1860 (NH, NH$^+$); 1700 (C=NH$^+$); 1600 cm.$^{-1}$ (COO$^-$, pyrimidine), indicating a zwitterion structure; $\lambda_{max.}^{pH\ 1}$ 271 (ε 10,500), shoulder at 232 mμ (ε 20,600); $\lambda_{max.}^{pH\ 7,13}$ 233 (ε 20,000), 306 mμ (ε 9900).

Anal.—Calcd. for $C_{18}H_{22}N_4O_3S$: C, 57.7; H, 5.92; N, 15.0. Found: C, 57.7; H, 5.89; N, 14.8.

In boiling methanol, 22 hours were required for completion of the reaction, and XI was obtained in 45% yield. In boiling 0.1 *N* hydrochloric acid, reaction required 3 hours; XI was obtained in 68% yield.

2 - Acetamido - 5 - (N - acetyl - 3 - anilinopropyl)-6-methyl-4-pyrimidinol (XIV).—A solution of 520 mg. (2.01 mmoles) of I*b* (8) in 4 ml. of reagent pyridine and 2 ml. of acetic anhydride was heated in a bath at 90–92° for 1 hour, then spin-evaporated *in vacuo*. The gummy residue was spin-evaporated *in vacuo* with toluene (3 × 5 ml.). Trituration of the residue with ethyl acetate gave 543 mg. (80%) of product, m.p. 127–130°. Recrystallization from ethyl acetate afforded white crystals, m.p. 126–127°; $\nu_{max.}$ 3200, 2950 (NH, OH); 1650, 1600, 1570, 1530 (C=O, NH, pyrimidine, C=C); 697 cm.$^{-1}$ (phenyl CH); $\lambda_{max.}^{EtOH}$ 242 (ε 12,900), 294 mμ (ε 9100); $\lambda_{max.}^{pH\ 13}$ 278 (ε 8100), inflection centering at 247 mμ (ε 10,700).

Anal.—Calcd. for $C_{18}H_{22}N_4O_3$: C, 63.1; H, 6.48; N, 16.4. Found: C, 63.0; H, 6.49; N, 16.2.

2 - Amino - 4 - chloro - 6 - methyl - 5 - (N-tosyl-3-anilinopropyl)pyrimidine (XVIII).—A mixture of 1.075 Gm. (2.6 mmoles) of dry, recrystallized XVII (15) (m.p. 232–233°) and 5 ml. of phos-

phorus oxychloride was heated under a condenser protected from moisture in an oil bath preheated to 110°. After 45 minutes, the solution was cooled and poured into 50 Gm. of crushed ice and 15 ml. of petroleum ether with vigorous mechanical stirring. The gummy precipitate gradually solidified. The solid was ground with water, then filtered; this process was repeated until an aqueous washing was no longer acidic. After being dried over potassium hydroxide for 18 hours, the crude product weighed 1.166 Gm. (104%), m.p. 158–162°. Recrystallization from ethanol-water gave 0.892 Gm. (80%) of white crystals, m.p. 161–164°, that were suitable for further transformations. Several more recrystallizations from ethanol-water gave the analytical sample, m.p. 166–168°; $\nu_{max.}$ 3450, 3300, 3170 (NH); 1625, 1575, 1520 (NH, pyrimidine, C=C); 1340, 1150 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1}$ 232, 316 mμ; $\lambda_{max.}^{pH\ 7,13}$ 236, 305 mμ.

Anal.—Calcd. for $C_{21}H_{22}ClN_4O_2S$: C, 58.5; H, 5.38; N, 13.0. Found: C, 58.3; H, 5.55; N, 12.8.

2,4 - Diamino - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XVI) Hydrochloride.—A mixture of 240 mg. (0.557 mmole) of XVIII and 15 ml. of methanol saturated with ammonia at 0° was heated in a steel bomb in a bath at 150° for 24 hours. The solution was clarified by filtration, then spin-evaporated *in vacuo*. The residue was spin-evaporated *in vacuo* with methanol (3 × 5 ml.), leaving 260 mg. (104%) of crude product, m.p. 230–239° dec. Recrystallization from ethanol-petroleum ether (b.p. 60–110°) gave 160 mg. (64%) of white crystals, m.p. 254–257° dec.; $\nu_{max.}$ 3350, 3100 (NH); 1660 (C=NH$^+$); 1630, 1580 (C=C, C=N); 1350 1160 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{26}ClN_5O_2S$: C, 56.3; H, 5.85; N, 15.6. Found: C, 56.5; H, 5.86; N, 15.4.

5 - (3 - Anilinopropyl) - 2,4 - diamino - 6 - methylpyrimidine (XIII).—*Preparation A.*—To a magnetically stirred suspension of 500 mg. (1.12 mmoles) of XVI hydrochloride in 60 ml. of liquid ammonia was added sodium in small pieces until a permanent blue color was obtained that did not bleach in 15 minutes; a total of 167 mg. of sodium was required. After the cautious addition of 0.55 Gm. of ammonium chloride, the ammonia was allowed to evaporate, and the last traces were removed *in vacuo*. The residue was dissolved in 10 ml. of water plus 1.5 ml. of 6 *N* hydrochloric acid. The filtered solution was adjusted to pH 12 with 30% aqueous sodium hydroxide with cooling. The product was collected on a filter and washed with water; yield, 245 mg. (85%), m.p. 180–182°. Recrystallization from ethanol-water gave the analytical sample, m.p. 183–184°; $\nu_{max.}$ 3500, 3350, 3200 (NH); 1700, 1625, 1600, 1575 (NH, pyrimidine, C=C); 750, 690 (phenyl CH); no −SO_2N− near 1350 or 1160 cm.$^{-1}$; $\lambda_{max.}^{pH\ 1}$ 277 mμ (ε 7200); $\lambda_{max.}^{pH\ 7}$ 236 (ε 20,600), 282 mμ (ε 8200); $\lambda_{max.}^{pH\ 13}$ 237 (ε 20,500), 288 mμ (ε 10,100).

Anal.—Calcd. for $C_{14}H_{19}N_5$: C, 65.3; H, 7.44; N, 27.2. Found: C, 65.1; H, 7.51; N, 27.1.

Preparation B.—A suspension of 245 mg. (0.547 mmole) of XVI hydrochloride in 3.75 Gm. of 30% hydrogen bromide in acetic acid and 103 mg. of phenol (17) was stirred for 18 hours protected from moisture, then diluted with 15 ml. of anhydrous ether. The white hydrobromide salt was collected on a filter and washed with ether; yield, 247 mg., m.p. 253–255°. The hydrobromide salt was dissolved in 10 ml. of water; the solution, clarified by filtration, was basified to pH 12 with 30% aqueous sodium hydroxide. The product was collected on a filter and washed with water; yield, 115 mg. (82%), m.p. 183–184°, that was identical to *Preparation A*.

If the hydrogen bromide–acetic acid to XVI ratio was decreased threefold, most of the XVI was recovered unchanged due to its insolubility.

Preparation C.—A magnetically stirred mixture of 400 mg. (1.17 mmoles) of XIV, 4 ml. of benzene, and 0.4 ml. of phosphorus oxychloride was heated under a condenser in an oil bath preheated and maintained at 85°. Solution was completed in 1 minute, and the reaction was stopped after 3 minutes when the solution started to turn turbid; the mixture was immediately poured into a stirred solution of 2.6 Gm. of anhydrous sodium acetate in 10 ml. of water. After being stirred 10 minutes, the layers were separated, and the aqueous layer was extracted with dichloromethane (5 × 5 ml.). The combined organic solutions, dried with magnesium sulfate, were spin-evaporated *in vacuo*. Trituration of the residue with a mixture of ethyl acetate and ether gave 220 mg. (52%) of crude XV, m.p. 134–137°, which was difficult to purify further.

A mixture of 0.85 Gm. (2.36 mmoles) of XV and 8.5 ml. of ethanol saturated with ammonia at 0° was heated in a steel bomb at 140–145° for 24 hours. Spin-evaporation *in vacuo* left 0.87 Gm. of crude XII hydrochloride as a glassy solid; $\lambda_{max.}^{pH\ 1}$ 276 mμ, $\lambda_{max.}^{pH\ 7}$ 278 mμ; $\lambda_{max.}^{pH\ 13}$ 287 mμ; these spectra were in agreement with a 2,4-diamino-5,6-dialkylpyrimidine (12).

A solution of 700 mg. crude XII hydrochloride in 2 ml. of 6 *N* hydrochloric acid was refluxed for 2 hours, then spin-evaporated *in vacuo*. The residue was a mixture of *Ib* and XIII which were readily separated by fractional neutralization of an acid solution; *Ib* separated at pH 5, a mixture of the two at pH 7.5, and pure XIII at pH 13. Refractionation of the pH 7.5 precipitate gave additional XIII; yield, 26 mg. (9%), m.p. 182–184°, that was identical to *Preparation A*.

The yield of *Ib* was over 50%; since the ultraviolet spectrum of crude XII did not indicate that this much of the 2-amino-4-hydroxypyrimidine derivative was present, it would appear that *Ib* arose by acid cleavage of XIII (14).

Preparation D.—A solution of 19 mg. of XI in 4 ml. of concentrated ammonia water was heated in a steel bomb at 185–195° for 20 hours. The filtered solution was evaporated *in vacuo*, leaving 10 mg. of a gummy yellow solid whose ultraviolet spectra were in agreement with structure XII or XIII; $\lambda_{max.}^{pH\ 1}$ 275 mμ, $\lambda_{max.}^{pH\ 13}$ 288 mμ.

2 - Amino - 4 - dimethylamino - 6 - methyl - 5 - (N-tosyl-3-anilinopropyl)pyrimidine (XIXa).—A mixture of 280 mg. (0.65 mmole) of XVIII and 15 ml. of 20% ethanolic dimethylamine was heated in a steel bomb at 120° for 1 hour. The solution was spin-evaporated *in vacuo*. Crystallization of the residue from aqueous ethanol gave 115 mg. (40%) of white crystals, m.p. 70–74°, with softening at 65°. Recrystallization from the same solvent gave white crystals of unchanged melting point; $\nu_{max.}$ 3450, 3350, 3200 (NH); 1620, 1600, 1560–1540 (NH, pyrimidine, C=C); 1340, 1160 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1,7}$ 295 mμ with an inflection at 235 mμ; $\lambda_{max.}^{pH\ 13}$ 305 mμ.

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE AND THYMIDYLATE SYNTHETASE BY 2-R_1-4-R_2-5-(3-ANILINOPROPYL)-6-METHYLPYRIMIDINES

Compd.	R_1	R_2	Dihydrofolic Reductase[a,b] mM Concn. Inhibitor	% Inhibition	Inhibitor : Substrate[d]	Thymidylate Synthetase[b,c] mM Concn. Inhibitor	% Inhibition	Inhibitor : Substrate[e]	Synthetase : Reductase[f]
I*b*[g]	NH_2	OH	0.60[h]	43	130	0.62[i]	50	50	0.39
XIII[g]	NH_2	NH_2	0.0022	50	0.37	0.80	50	63	170
A[g]	NH_2	H	0.95	50[j]	80	0.70[i]	35	100	1.2
XXVII	H	NH_2	0.14	50	23	1.0	26	210	9.2
B[g]	H	OH	1.5	0	>1000	2.0[i]	26	420	<0.4
XXII	Me_2N	OH	6[h]	0	>4000	1.5[k]	0	>480	
XIX*b*	NH_2	NMe_2	0.058	50	9.7	1.3	50	100	10

[a] Dihydrofolic reductase from pigeon liver was prepared and assayed with 6 μm. dihydrofolate and 12 μm. TPNH in 0.05 *M* tris buffer at pH 7.4 as previously described (9). [b] The technical assistance of Mrs. Rita Zielinsky with these assays is acknowledged. [c] Thymidylate synthetase from *E. coli* B. was prepared and assayed with 80 μm. 2′-deoxyuridylate and 25.7 μm. *dl*-tetrahydrofolate, magnesium chloride, and formaldehyde in 0.05 *M* tris buffer at pH 7.4 as previously described (9). [d] Ratio of concentrations of inhibitor to dihydrofolate giving 50% inhibition. [e] Ratio of concentrations of inhibitor to the active 1-isomer of 5, 10-methylene-tetrahydrofolate giving 50% inhibition. [f] Ratio of inhibitor:substrate for 50% inhibition of thymidylate synthetase to inhibitor:substrate for 50% inhibition of dehydrofolic reductase. [g] Enzyme data from *Reference 9*. [h] Cell also contained 10% *N,N*-dimethylformamide. [i] Cell also contained 3% *N,N*-dimethylformamide. [j] In the presence of 12 μm. dihydrofolate. [k] Cell also contained 5% 2-methoxyethanol.

Anal.—Calcd. for $C_{23}H_{29}N_5O_2S$: C, 62.8; H, 6.65; N, 15.9. Found: C, 62.7; H, 6.59; N, 15.8.

2 - Amino - 5 - (3 - anilinopropyl) - 4 - dimethylamino-6-methylpyrimidine (XIXb) Dihydrochloride.—To 1.4 Gm. of 30% hydrogen bromide in acetic acid and 107 mg. of phenol (17) was added 250 mg. of XIX*a*. After being stirred for 16 hours, the mixture was processed as described for *XIII, Preparation B*. When the aqueous solution was basified to pH 12, a gum separated. The mixture was extracted with chloroform (5 × 4 ml.). The combined extracts were dried with magnesium sulfate and spin-evaporated *in vacuo*, leaving 145 mg. of crude XIX*b* as a gum. The gum was dissolved in 1 ml. of ethanol and the solution treated with 1 ml. of 12 *N* hydrochloric acid. Spin evaporation *in vacuo* left 150 mg. (74%) of crude XIX*b* dihydrochloride, m.p. 144–149°. Two recrystallizations from absolute ethanol–petroleum ether afforded 106 mg. (52%) of white crystals, m.p. 172–176°, which was unchanged after one more recrystallization. The analytical sample had $\nu_{max.}$ 3450, 3340, 3200 (NH); 2960–2900, 2740–2660 (NH^+); 1650 (C=NH^+); 1625, 1550 (C=C, C=N); 760, 690 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1}$ 291 mμ (ε 9800); $\lambda_{max.}^{pH\ 7}$ 242 (ε 19,700), 292 mμ (ε 11,100); $\lambda_{max.}^{pH\ 13}$ 302 (ε 8700), 240 mμ (shoulder, ε 18,300).

Anal.—Calcd. for $C_{16}H_{25}Cl_2N_5$: C, 53.6; H, 7.03 N, 19.5. Found: C, 53.9; H, 7.16; N, 19.3.

2 - Dimethylamino - 6 - methyl - 5 - (N - tosyl-3-anilinopropyl)-4-pyrimidinol (XXI).—A mixture of 2.75 Gm. of crude XX (73% pure by ultraviolet analysis) (15), 0.90 Gm. (3.29 mmoles) of 1,1-dimethylguanidine sulfate, 0.36 Gm. (6.59 mmoles) of sodium methoxide, and 15 ml. of methanol was refluxed with magnetic stirring for 20 hours protected from moisture. The mixture was spin-evaporated *in vacuo* and the residue triturated with 30 ml. of water; an oil separated that soon solidified. The crude product was collected on a filter and washed with water, then ethanol; yield, 1.36 Gm. (65%), m.p. 197–201°. Recrystallization from absolute ethanol gave 0.705 Gm. (34%) of pure product, m.p. 243–245°, that was unchanged on further recrystallization. The compound had $\nu_{max.}$ 1625, 1600, 1525 (pyrimidine, C=C); 1340, 1160 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1}$ 234, 265 mμ; $\lambda_{max.}^{pH\ 7}$ 230, plateau at 290–306 mμ; $\lambda_{max.}^{pH\ 13}$ 292, shoulder at 236 mμ.

Anal.—Calcd. for $C_{28}H_{28}N_4O_3S$: C, 62.7; H, 6.40; N, 12.7. Found: C, 62.9; H, 6.60; N, 12.6.

5 - (3 - Anilinopropyl) - 2 - dimethylamino - 6-methyl-4-pyrimidinol (XXII).—Treatment of 400 mg. (0.91 mmole) of XXI with sodium (0.15 Gm.) in liquid ammonia, as described for the conversion of *XVI* to *XIII*, gave upon neutralization of the hydrochloric acid solution, a solid that was collected on a filter, washed with water, and triturated with petroleum ether; yield, 218 mg. (81%), m.p. 120–121°. Recrystallization from petroleum ether (b.p. 60–110°) gave white crystals, m.p. 121–122°; $\nu_{max.}$ 3400 (NH or OH); 1630, 1575 (pyrimidine, C=C); 750, 690 cm.$^{-1}$ (aromatic CH); $\lambda_{max.}^{pH\ 1}$ 231 (ε 14,700), 271 mμ (ε 7700); $\lambda_{max.}^{pH\ 7}$ 234 (ε 26,800), inflection centering at 285 mμ (ε 5400); $\lambda_{max.}^{pH\ 13}$ 242 (ε 23,700), 290 mμ (ε 8100).

Anal.—Calcd. for $C_{16}H_{22}N_4O$: C, 67.1; H, 7.74; N, 19.6. Found: C, 67.3; H, 7.92; N, 19.5.

2 - Mercapto - 6 - methyl - 5 - (N - tosyl - 3-anilinopropyl)-4-pyrimidinol (XXIII).—To a solution of 5.6 Gm. of crude XX (73% pure by ultraviolet analysis) (15) in 30 ml. of reagent methanol was added 1.58 Gm. (29.2 mmoles) of sodium methoxide and 1.00 Gm. (13.1 mmoles) of thiourea. After being refluxed for 22 hours with magnetic stirring and protected from moisture, the mixture was spin-evaporated *in vacuo*. The residue was dissolved in 60 ml. of water and filtered from some insoluble by-product; acidification to about pH 4 with acetic acid gave 2.95 Gm. (71%) of product, m.p. 265–269°. Recrystallization from 2-methoxyethanol by addition of water gave 2.66 Gm. (64%) of pure product, m.p. 250–252°; $\nu_{max.}$ 3050 (NH or OH); 2980–2880 (acidic hydrogen); 1630, 1550 (pyrimidine, C=C); 1330, 1155 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 1.7}$ 221, 278 mμ; $\lambda_{max.}^{pH\ 13}$ 313, plateau at 253–263, inflection centering at 235 mμ.

Anal.—Calcd. for $C_{21}H_{23}N_3O_3S_2$: C, 58.7; H, 5.40; N, 9.78. Found: C, 58.5; H, 5.35; N, 9.53.

6 - Methyl - 5 - (N - tosyl - 3 - anilinopropyl) - 4-pyrimidinol (XXIV).—To a solution of 1.00 Gm. (2.33 mmoles) of XXIII in 25 ml. of 2-methoxyethanol was added 4.5 Gm. of sponge nickel catalyst (Davison Co.). After being refluxed with stirring for 3 hours, the mixture was filtered through a Celite pad, then the insolubles were washed with hot 2-methoxyethanol (2 × 10 ml.). The combined filtrate and washings were spin-evaporated *in vacuo*,

leaving 0.54 Gm. (58%) of white solid m.p. 214–215°. Recrystallization from ethyl acetate gave 0.35 Gm. of white crystals, m.p. 214–215°; $\nu_{max.}$ 2950–2850 (acidic hydrogen); 1640, 1600 (C=O, pyrimidine, C=C); 1340, 1150 cm.$^{-1}$ (−SO_2N−); $\lambda_{max.}^{pH\ 13}$ 265, with a shoulder at 233 mμ.

Anal.—Calcd. for $C_{21}H_{23}N_3O_3S$: C, 63.5; H, 5.83; N, 10.6. Found: C, 63.7; H, 5.91; N, 10.4.

4 - Chloro - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XXV).—A mixture of 200 mg. (0.50 mmole) of XXIV, 2 ml. of benzene, and 0.2 ml. of phosphorus oxychloride was heated under reflux for 30 minutes protected from moisture in an oil bath preheated to 85°. Within 10 minutes, the phosphate salt of XXV began to separate. The white crystals were collected on a filter and washed with benzene; yield, 0.252 Gm., m.p. 155–157°, which showed NH^+ bands at 2600–2350 and 2100–1900 cm.$^{-1}$ and a P → O band 1290 cm.$^{-1}$.

A mixture of the phosphate salt, chloroform (5 ml.), and 5% aqueous sodium bicarbonate (5 ml.) was shaken until solution was complete. The separated chloroform solution was washed with water, dried with magnesium sulfate, then spin-evaporated *in vacuo;* yield, 0.175 Gm. (84%) of white crystals, m.p. 124–125°. Recrystallization from ethanol-water gave white crystals of unchanged melting point; $\nu_{max.}$ 1580, 1540–1520 (C=N, C=C); 1320, 1150 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{22}ClN_3O_2S$: C, 60.6; H, 5.33; N, 10.1. Found: C, 60.5; H, 5.30; N, 10.2.

4 - Amino - 6 - methyl - 5 - (N - tosyl - 3 - anilinopropyl)pyrimidine (XXVII).—A mixture of 465 mg. (1.12 mmoles) of XXV and 10 ml. of methanol saturated with ammonia at 0° was heated in a steel bomb at 140° for 5 hours. The cooled contents were clarified by filtration, and the solution was spin-evaporated *in vacuo.* The residual hydrochloride was dissolved in 30 ml. of 25% aqueous acetic acid, then the solution was adjusted to pH 12 with 30% sodium hydroxide. The product was collected on a filter and washed with water; yield, 470 mg. (100%), m.p. 169–172°. Recrystallization from ethanol-water gave 375 mg. (85%) of product, m.p. 170–172°. Further recrystallization raised the melting point to 173–175°; $\nu_{max.}$ 3450, 3300, 3180–3100 (NH); 1630, 1560 (NH, C=C, C=N); 1330, 1150 cm.$^{-1}$ (−SO_2N−).

Anal.—Calcd. for $C_{21}H_{24}N_4O_2S$: C, 63.6; H, 6.10; N, 14.1. Found: C, 63.8; H, 6.28; N, 13.9.

4 - Amino - 5 - (3 - anilinopropyl - 6 - methylpyrimidine (XXVI).—Reduction of 368 mg. (0.93 mmole) of XXVII with 177 mg. of sodium in liquid ammonia, as described for the conversion of XVI to XII, gave 170 mg. (76%) of product, m.p. 144–146°. Recrystallization from ethyl acetate–petroleum ether afforded 130 mg. (58%) of pure product as white crystals, m.p. 147–148°; $\nu_{max.}$ 3350, 3150 (NH); 1640, 1570, 1540 (NH, C=C, C=N); 790, 680 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}^{pH\ 1}$ 257 mμ (ε 12,300); $\lambda_{max.}^{pH\ 7}$ 242 (ε 17,600), 272 mμ (inflection, ε 8400); $\lambda_{max.}^{pH\ 13}$ 238 (ε 19,800), 275 mμ (ε 7600, shoulder).

Anal.—Calcd. for $C_{14}H_{18}N_4$: C, 69.4; H, 7.49; N, 23.1. Found C, 69.2; H, 7.32; N, 23.0.

RESULTS AND DISCUSSION

As stated in the introduction, the objective of this study was to shed further light on the remarkable increase in binding by aminopterin (XXVIII) and other 2,4-diaminoheterocycles to the enzyme, (dihydro)folic reductase.

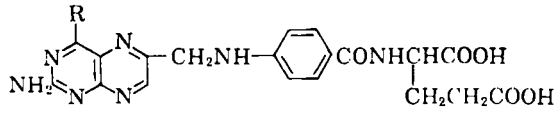
The early proposal by Baker (3) that greater protonation of the 2-amino group of the strongly basic aminopterin (XXVIII) (compared to folic acid, XXIX) could lead to increased binding to an anionic site was untenable with the later studies by Zakrzewski (4), who proposed that stronger binding could be accounted for by binding of the 4-amino group and the N-3 nitrogen.

That the 4-amino group does give increased binding is demonstrated clearly by comparing the binding of the 4-pyrimidinol (B) with the 4-aminopyrimidine (XXVII) (Table I); there is a greater than fiftyfold increase in binding to dihydrofolic reductase by this structural change, an indication that the 4-amino group binds to the enzyme. That the 2-amino group also contributes to binding to dihydrofolic reductase is seen clearly by comparing the 2-amino-4-pyrimidinol (I*b*) to the 4-pyrimidinol (B) (Table I). Removal of the 2-amino group led to a greater than eightfold loss in binding. The 2,4-diaminopyrimidine (XIII) binds 350-fold stronger than the 2-amino-4-pyrimidinol (I*b*), thus indicating that both the 2-amino and 4-amino are involved in binding to the enzyme, probably by hydrogen bonds as proposed by Zakrzewski (4). To this point, Zakrzewski's explanation is satisfactory that (*a*) four hydrogen bonds exist between the 2,4-diaminopyrimidine moiety and the enzyme (XXX) and (*b*) the energy change necessary to convert the normal 4-oxo tautomer (XXXII) of folic acid to the 4-hydroxy tautomer (XXXI) leads to a net decrease in binding compared to XXX.

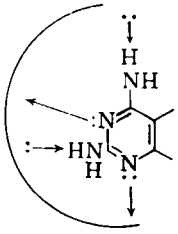
The first discrepancy appears when one considers the extent of binding of the 2-aminopyrimidine (A) compared to the 2,4-diaminopyrimidine (XIII) and the 2-amino-4-pyrimidinol (I*b*) (Table I). The 2-aminopyrimidine clearly has its N-3 nitrogen available for binding to the enzyme without a tautomeric shift. Thus, if Zakrzewski's explanation of N-3 and 4-amino binding (XXX) were correct, then the 2-aminopyrimidine (A) with its available N-3 should have been bound to the enzyme considerably stronger than the 2-amino-4-pyrimidinol (I*b*), but not so strong as the 2,4-diaminopyrimidine (XIII). At least a tenfold tighter binding should have been seen. These results indicate that the N-3 of the 2,4-diaminopyrimidyl moiety does not bind to folic reductase as previously indicated in XXX. There are a number of other discrepancies which are not completely compatible with Zakrzewski's formulation of XXX as the mode of binding of the 2,4-diaminopyrimidyl moiety of aminopterin (XXVIII) and other 2,4-diaminoheterocycles.

To make comparisons more valid between the compounds in Table I and the binding of aminopterin to rat liver folic reductase, the 2,4-diaminopyrimidine (XIII) was measured as an inhibitor of this enzyme system using folic acid as substrate; XIII had[1] $K_i = 2.4 \times 10^{-8}$. Compared to I*b*, which has $K_i = 6.3 \times 10^{-5}$ in this system (8), the 2,4-diaminopyrimidine (XIII) binds to rat liver folic reductase 2600-fold more tightly than I*b;* in contrast, aminopterine (XXVIII) binds to rat liver

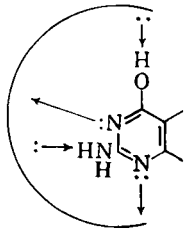
[1] The authors thank Dr. W. C. Werkheiser, Roswell Park Memorial Institute, for this determination on rat liver folic reductase.



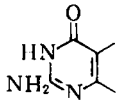
XXVIII, R = NH_2
XXIX, R = OH



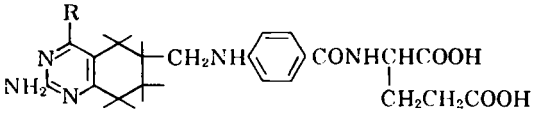
XXX



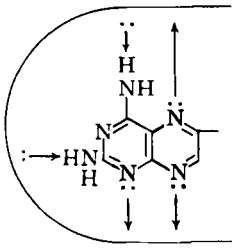
XXXI



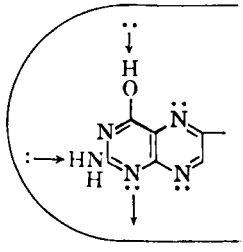
XXXII



XXXIII, R = NH_2
XXXIV, R = OH



XXXV



XXXVI

folic reductase in the same assay system 100,000-fold more tightly than folic acid (XXIX) (2). Thus, a discrepancy of 100,000/2600 = 38-fold remains when one compares the pair XIII–I*b* with the pair XXVIII–XXIX. Furthermore, comparison of folic acid (XXIX, $K_m = 1 \times 10^{-5}$) to the corresponding pyrimidyl analog (I*a*, $K_i = 2 \times 10^{-6}$) (8) shows that the pyrimidyl portion of folic acid accounts for all of the binding of its pteridine moiety. These discrepancies can be accounted for by some binding of the pyrazine portion of the 2,4-aminopteridine system in XXVIII but no binding by the pyrazine portion of the 2-amino-4-hydroxypteridine system of folic acid (XXIX). Support for this additional binding point of 2,4-diaminopteridines can be gleaned from the literature.

(*a*) 2,4-Diamino-5-methylpteridine ($K_i = 4.7 \times 10^{-7}$) binds sixtyfold tighter than 2,4-diamino-5,6-dimethylpyrimidine ($K_i = 2.8 \times 10^{-5}$) (4).

(*b*) Aminopterin (XXVIII) binds to folic reductase 10-to 100-fold stronger than its tetrahydroquinazoline analog (XXXIII) (18), whereas the tetrahydroquinazoline analog (XXXIV) (19) of folic acid binds five times stronger than folic acid (XXIX) (20).

(*c*) 5-(*p*-Chlorophenyl)-2,4-diamino-6-ethylpyrimidine ($K_i = 7 \times 10^{-9}$)[1] binds much stronger than 2,4-diamino-6-methylpyrimidine ($K_i = 2.6 \times 10^{-5}$) (4), an indication that the chlorophenyl group is an additional binding point (8).

(*d*) 2-Amino-5-(3-anilinopropyl)-4-pyrimidinol with a 6-phenyl or 6-benzyl substituent has $K_i = 1.8 \times 10^{-6}$ and 4.0×10^{-6}, respectively (15), compared to the 6-methyl substituent (I*b*) with $K_i = 6.3 \times 10^{-5}$ (8). These data indicate that the 6-phenyl or 6-benzyl group have an additional point of binding to folic reductase.

It is probable that the extra binding point needed for (*a*)–(*d*) is the same, providing this binding point is a locus of fairly broad dimensions (21). A logical possibility for this locus can be surmised when one considers what is known about the mechanism of some dehydrogenases (or hydrogenases if the reaction is a reduction).

In lactic dehydrogenase, where pyruvate is reduced by DPNH to lactate, it has been reasonably well established that the imidazole ring of a histidine is present at the active site (22). The imidazole in its protonated form not only acts as a proton donor during the reduction, but also is probably a binding point for the ketone oxygen atom (23). This has been represented diagrammatically by XXXVII (24), and the mechanism of reduction proceeds through the transition state, XXXVIII, to the products XXXIX. If the reaction is reversible, the electron flow shown in XXXIX will return the components to the transition state, XXXVIII. It is logical to assume that all enzymic hydrogenases and dehydrogenases would have an imidazole group at the active site as a proton donor or acceptor, thus resulting in a more facile reaction than if the substrate had to pick up a proton from the solvent.

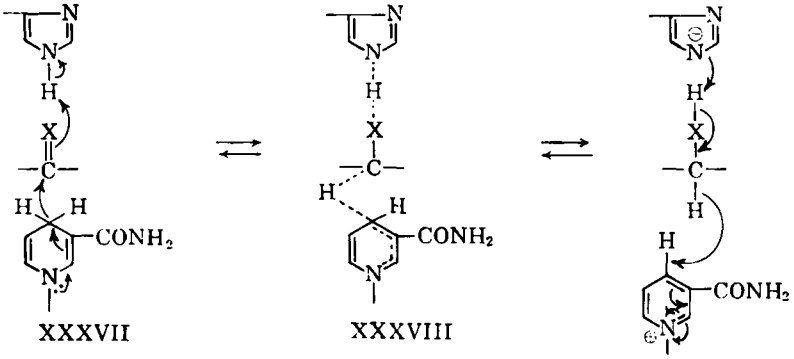
Therefore, it is logical to invoke the presence of an imidazole ring in the active site of folic reductase to aid in the reduction of the double bonds of the pyrazine ring. Molecular models show that the imidazole can be juxtapositioned more or less face-to-face with the pyrazine ring so that in one form (XL) the imidazole proton could easily be transferred to the 7- or 8-position of the pteridine; in the other form (XLII) a proton could easily be transferred to the 6- or 5-position of the pteridine, thus accounting for any possible structure for the intermediate dihydrofolate. The TPNH for hydride transfer can be juxtapositioned on the opposite side of the pteridine from the imidazole so that it can readily transfer a hydride anion to either C_6 or C_7 of the pteridine.

The supposed presence of the imidazole ring at the active site of folic reductase juxtapositioned face-to-face with the pyrazine moiety will now allow explanation of the binding discrepancies (*a*)–(*d*) cited earlier. Aminopterin, which is about 1000 times more basic than folic acid (6), could bind at either N-8 (XLI) or N-5 (XLIII) with the acidic NH group of the imidazole to give a quasi-ionic bond. Even though N-5 or N-8 may not be the first point of protonation in free solution (6), when aminopterin is complexed with the enzyme the juxtapositioning of the imidazole NH to N-5 or N-8 could certainly protonate one of these two groups first. Hydroxylation of the pteridine, as in folic acid (XXVIII), decreases basicity of the ring system by 1000-fold, which would be sufficient to negate appreciable binding to the imidazole of the type indicated in XL and XLII.

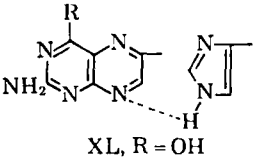
The extra point of binding of certain 6-phenyl and 6-benzyl pyrimidines (15) can be depicted as a charge-transfer complex between the imidazole and the benzene ring where the latter is an electron donor (XLIV). Studies on the mode of binding of the 6-phenyl group are so far in agreement with this concept (24). The binding of the phenyl group of the 5-phenylpyrimidines can also be depicted as a charge-transfer complex of either the π-type (XLV) or the n-type.

Whether an imidazole is in the juxtaposition at the active site of folic reductase as depicted (XL–XLV) is being vigorously pursued in this laboratory by construction of the proper type of active-site-directed irreversible inhibitor that could label the imidazole (25, 26).

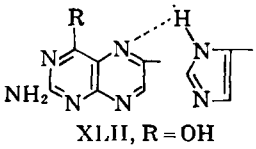
The relatively tight fit of the 2,4-diaminopyrimidyl moiety to folic reductase, as depicted by Zakrzewski in structure XXX (4) but modified as in structure XXXV, was studied by synthesis and enzymic



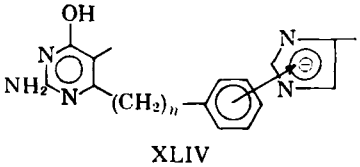
XXXVII XXXVIII XXXIX



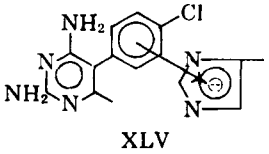
XL, R = OH
XLI, R = NH_2



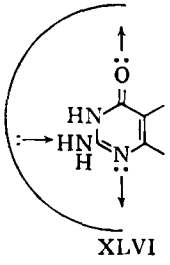
XLII, R = OH
XLIII, R = NH_2



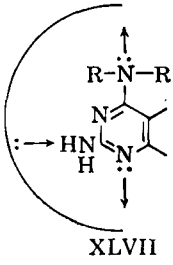
XLIV



XLV



XLVI



XLVII

evaluation of the 2-dimethylaminopyrimidine (XXII) and the 4-dimethylaminopyrimidine (XIX*b*). A greater than thirtyfold loss in binding occurred with the 2-dimethylamino-4-pyrimidinol (XXII) compared to the 2-amino-4-pyrimidinol (I*b*) (Table I). This loss in binding can be attributed either to steric hindrance to binding by the two bulky methyl groups or the loss of the necessary NH for hydrogen bonding or both.

The 2-amino-4-dimethylaminopyrimidine (XIX*b*) suffered a 26-fold loss in binding compared to the 2,4-diaminopyrimidine (XXVII) (Table I). Actually, one could have expected a greater loss in binding since XIX*b* still binds eightfold stronger than the 2-aminopyrimidine (A), which does not have a substituent at the 4-position. These results indicate that there is no steric hindrance to binding created by the two bulky methyl groups, but that the 4-NH group necessary for binding is no longer present. That the 2-amino-4-dimethylaminopyrimidine (XIX*b*) binds eightfold stronger than the 2-aminopyrimidine (A) suggests that the dimethylamino group causes the N-1 and C_2-NH to bind more tightly due to the increased basicity in the dimethylamino system.

Although the compounds in Table I were synthesized to study the mode of binding of 2,4-diaminopyrimidines to dihydrofolic reductase, the fact that I*b* was also an inhibitor of thymidylate synthetase suggested that these compounds also be investigated as inhibitors of thymidylate synthetase for what information could be gleaned on the mode of binding of the 2-amino-4-pyrimidinol moiety to this enzyme.

In a previous paper (9) it was observed that both the 2-amino group and 4-hydroxyl (or 4-oxo) group contributed to binding to thymidylate synthetase and that the 4-hydroxyl contributed to binding to the latter enzyme relatively more than to dihydrofolic reductase. The results shown in Table I are compatible with the binding depicted in XLVI. The 2,4-diaminopyrimidine (XIII) has about the same amount of binding as the 2-amino-4-pyrimidinol (I*b*). This indicates that the 4-oxo group of I*b* (depicted in XLVI) binds equally as well as the N: → H-E bond depicted in XLVII. Thus, the 3-NH of XLVI and the 3-N of XLVII are not likely to be binding points. Furthermore, the 2-amino-4-dimethylaminopyrimidine (XIX*b*) binds almost as well as the 2,4-diaminopyrimidine (XIII). Therefore, (*a*) the 4-NH of XIII cannot be a binding point, (*b*) the two bulky methyl groups are tolerated, and (*c*) the mode of binding of XIII and XIX*b* at the 4-position is most probably *via* an N: → H-E bond, as depicted in XLVII.

In contrast, the 2-dimethylamino-4-pyrimidinol (XXII) shows a greater than ninefold loss in binding compared to the 2-amino-4-pyrimidinol (I*b*). These results indicate that either the loss of the 2-N-H destroys the N-H ← E type of binding depicted in XLVI or there is no room for the two bulky methyl group or both.

Although 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (I*b*) can inhibit both thymidylate synthetase and dihydrofolic reductase, being slightly more effective on the former enzyme, the mode of binding of I*b* to the two enzymes is different; the differences and similarities are summarized as follows.

(*a*) The 2-amino group apparently binds to both enzymes by an N-H ← E bond, and binding is considerably decreased if the 2-amino group is converted to a 2-dimethylamino group.

(*b*) The 4-hydroxyl group binds to dihydrofolic reductase by an O-H ← E bond but to thymidylate synthetase by an = O: → H-E bond of the 4-oxo tautomer. As a result, conversion of the 4-hydroxyl group to a 4-amino group (XIII) causes a 350-fold increase in binding to dihydrofolic reductase, but no increase in binding to thymidylate synthetase.

(*c*) A 6-phenyl group, instead of the 6-methyl group on I*b*, causes a thirtyfold increase in binding to dihydrofolic reductase—presumably by binding to an imidazole on the protein surface—but gives no change in binding to thymidylate synthetase (15).

(*d*) The binding of the anilino moiety to dihydrofolic reductase is presumably through a charge-transfer complex and is readily influenced by change in the electronegativity of the benzene ring; this influence on binding to thymidylate synthetase is much less pronounced (13).

(*e*) The bridge length between the pyrimidyl and anilino moieties of I*b* is more restrictive on dihydrofolic reductase than on thymidylate synthetase, *i.e.*, the locus on thymidylate synthetase binding the anilino group seems to have larger dimensions than the locus on dihydrofolic reductase (27). As a result, compounds more selective on thymidylate synthetase have been obtained (27).

Studies to obtain greater selectivity of inhibition of thymidylate synthetase than dihydrofolic reductase and vice versa, dependent upon the differences cited above, are continuing.

REFERENCES

(1) Jukes, T. H., and Broquist, H. P., in "Metabolic Inhibitors," Hochster, R. M., and Quastel, J. H., eds., Academic Press Inc., New York, N. Y., 1963, pp. 481–534.
(2) Werkheiser, W. C., *J. Biol. Chem.*, **236**, 888(1961).
(3) Baker, B. R., *Cancer Chemotherapy Rep.*, **4**, 1(1959).
(4) Zakrzewski, S. F., *J. Biol. Chem.*, **238**, 1485(1963).
(5) Pauling, L., "Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1948.
(6) Zakrzewski, S. F., *J. Biol. Chem.*, **238**, 4002(1963).
(7) Baker, B. R., and Morreal, C. E., THIS JOURNAL, **52**, 840(1963).
(8) Baker, B. R., *et al.*, *J. Med. Chem.*, **7**, 24(1964).
(9) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(10) Baker, B. R., Morreal, C. E., and Ho, B.-T., *J. Med. Chem.*, **6**, 658(1963).
(11) Hitchings, G. H., and Russell, P. B., *J. Chem. Soc.*, **1949**, 2454.
(12) Ross, L. O., Goodman, L., and Baker, B. R., *J. Am. Chem. Soc.*, **81**, 3108(1959).
(13) Baker, B. R., Ho, B.-T., and Chheda, G. B., *J. Heterocyclic Chem.*, **1**, 88(1964).
(14) Hitchings, G. H., Russell, P. B., and Whittaker, N., *J. Chem. Soc.*, **1956**, 1019.
(15) Baker, B. R., Santi, D. V., and Shapiro, H. S., THIS JOURNAL, **53**, 1317(1964).
(16) Curran, W. V., and Angier, R. B., *J. Org. Chem.*, **28**, 2672(1963).
(17) Weisblat, D. I., Magerlein, B. J., and Myers, D. R., *J. Am. Chem. Soc.*, **75**, 3630(1953).
(18) DeGraw, J., *et al.*, *J. Org. Chem.*, **27**, 576(1962).
(19) DeGraw, J. I., Goodman, L., and Baker, B. R., *ibid.*, **26**, 1156(1961).
(20) Baker, B. R., "Analogs of Tetrahydrofolic Acid," Preprint C-I, presented to the Scientific Section, A.PH.A., Las Vegas meeting, March 1962.
(21) Wallace, R. A., Kurtz, A. N., and Niemann, C., *Biochemistry*, **2**, 824(1963).
(22) Winer, A. D., and Schwert, G. W., *J. Biol. Chem.*, **231**, 1065(1958).
(23) Baker, B. R., *et. al.*, *J. Med. Pharm. Chem.*, **2**, 633 (1960).
(24) Baker, B. R., Shapiro, H. S., and Werkheiser, W. C., unpublished data.
(25) Baker, B. R., THIS JOURNAL, **53**, 347(1964).
(26) Schoellman, G., and Shaw, E., *Biochemistry*, **2**, 252 (1963).
(27) Baker, B. R., and Ho, B.-T., unpublished data.