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Analogs of Tetrahydrofolic Acid. XII. On the Relative Contribution by the 2-Amino and 4-Hydroxy Groups of 2-Amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol to Inhibition of Folic Reductase and Thymidylate Synthetase (1,2)

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The structure of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol (II), an inhibitor of both dihydrofolic reductase and thymidylate synthetase, has been modified by replacement of the 2-amino group by hydrogen or the 4-hydroxyl group by hydrogen, mercapto or amino. Enzymic evaluation showed that 5-(3-anilinopropyl)-2,4-diamino-6-methylpyrimidine was a 170-fold more effective inhibitor of dihydrofolic reductase than thymidylate synthetase. This specificity is due to the fact that replacement of the 4-hydroxyl group of II with 4-amino enhances binding to dihydrofolic reductase by 350-fold, but binding to thymidylate synthetase is unchanged. No significant cross-over specificity was noted with the six compounds studied.

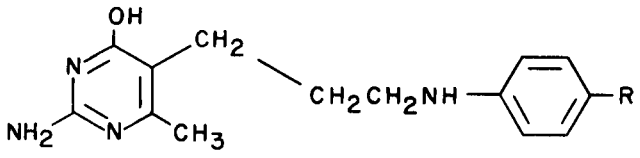
Replacement of the 4-hydroxyl group of II by hydrogen gave a compound (XVII) that was a slightly more effective inhibitor of dihydrofolic reductase than II, but was slightly less effective on thymidylate synthetase. Binding to dihydrofolic reductase and to thymidylate synthetase could be increased by 17-fold and 5-fold, respectively, by replacement of the 4-hydroxyl group of II by 4-mercapto (XVI). It was concluded that the mode of binding in the N-3 and 4-hydroxyl region of II was not the same for the two enzymes.

Synthesis of the 2-amino-, the 4-hydroxy- and the 4-mercaptopyrimidyl analogs of II are described. Also described are the partial purification of and assays for dihydrofolic reductase and thymidylate synthetase by procedures more familiar to the organic chemist.

Previous studies from this laboratory have shown that the tetrahydrofolate analog (I) was a good inhibitor of folic reductase, being bound to the enzyme somewhat stronger than the substrate, folic acid (3, 4). Further studies on the contribution of the aminobenzoyl-L-glutamate moiety of I to binding to folic reductase showed that the *p*-aminophenyl group contributed more to binding than the carboxy-L-glutamate residue; furthermore, when the carboxy-L-glutamate group was removed from I, the resultant compound (II) had a dissociation constant (K_i) about only six times the K_m of folic acid (5). In order to determine the relative contribution of the 2-amino and 4-hydroxyl groups to enzyme binding by II, the 2-deamino, the 4-deoxy, and two 4-mercapto analogs were synthesized and evaluated as inhibitors of folic reductase and thymidylate synthetase; the results are the subject of this paper.

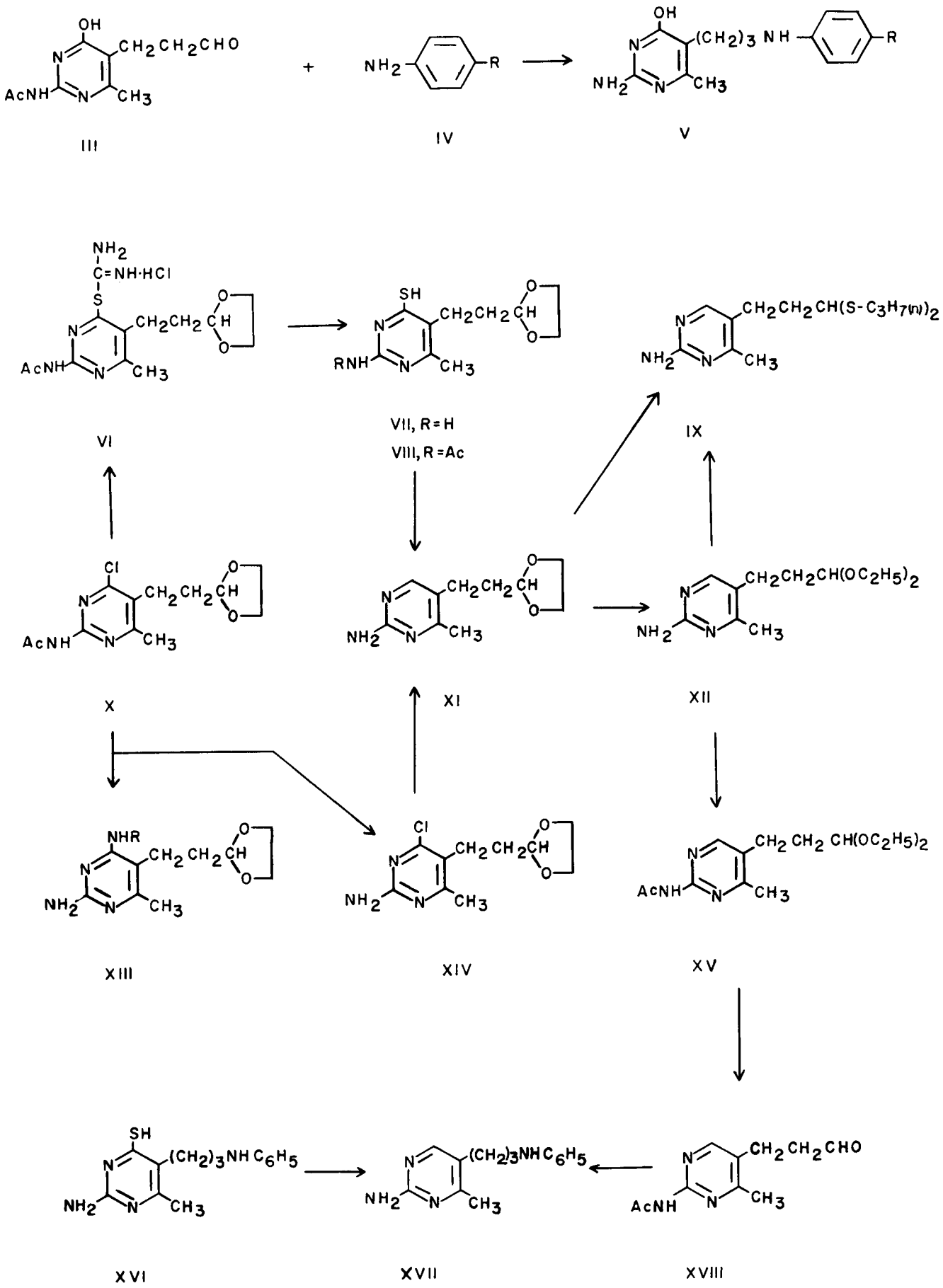
Compounds of type V related to I and II have been previously synthesized by a general method (3-5) which involved reductive condensation of the pyrimidine-5-propionaldehyde (III) with the proper aromatic amine (IV); the sodium borohydride used in the reduction generated sufficient base to cleave the N^2-acetyl group. In order to utilize this method for the synthesis of 4-deoxy derivatives of I and II, an intermediate such as XVIII would be required; XVIII should be synthesizable from the previously described 2-acetamido-4-chloropyrimidine (X) (6).

Two routes were envisioned for the replacement of the 4-chloro group of X by hydrogen, namely, by hydrogenolysis of the chloro group of X or by desulfurization of a 4-thiol function such as that in VI-VIII; the hydrogenolysis reaction was investigated first. There were two disadvantages in trying to hydrogenolyze the chloro group of X; firstly, the N-acetyl group of X is quite labile and might be partially removed by solvent during the reaction, and secondly, purification losses of X were high (6) and fairly pure



I, R = CONHCHCOOH
CH2CH2COOH

II, R = H


III
IV
V
VI
VII, R = H
VIII, R = Ac
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII

X would be needed for catalytic hydrogenolysis. Attempts to replace the chloro group of X by ammonia in ethanol for another study (7) led to the formation of 55% the 2-amino-4-chloropyrimidine (XIV), rather than the corresponding 2,4-diaminopyrimidine, if the conditions were too mild; XIV was found to be a nicely crystalline compound that was readily purified. Therefore efforts were directed towards finding simpler optimum conditions for the conversion of crude X to pure 2-amino-4-chloropyrimidine (XIV).

When *n*-butylamine in boiling methanol, a reagent previously used for removal of an N^6-benzoyl and sugar acyl groups from a blocked N^6-benzoyl adenine nucleoside (8, 9), was used for de-N-acetylation of crude X, a 50% yield of XIV of excellent purity was obtained after five hours of reflux. The mother liquor from XIV contained an oily material, the ultra-violet spectrum of which was in agreement with that of a 2-amino-4-alkylamino-5,6-dialkylpyrimidine (10), presumably the 4-butylaminopyrimidine (XIII, R = H). Since the 4-chloro group was being displaced by the amine during the deacetylation, a time study was made by examining the ultraviolet spectrum at *p*H 1 of aliquots; *p*H 1 was chosen since XIV has a peak at 314 mμ, a wave length where X and the presumed XIII have no significant absorption. The extinction at 314 mμ rose to a maximum after three hours reflux in methanol, then was constant for the next five hours. These data showed that the desired 2-amino-4-chloropyrimidine (XIV) was stable to the reaction conditions after it had formed; it was therefore logical to assume that XIII most probably formed by displacement of the 4-chloro group of X before deacetylation occurred. That the 4-chloro group of the 2-acetamidopyrimidine (X) was considerably more reactive than the 4-chloro group of the 2-aminopyrimidine (XIV) was later shown to be the case with thiourea as the nucleophile; X was rapidly converted to the thiouronium salt (VI) in boiling *t*-butyl alcohol, conditions where XIV was recovered unchanged (6).

Further variations in conditions for increasing the conversion of X to XIV were dependent upon whether *n*-butylamine removed the acetyl group by direct aminolysis or by base-catalyzed methanolysis. That the reaction could proceed by both mechanisms, but predominately by methanolysis, was indicated by replacing the methanol by acetonitrile; in a comparable time the yield of XIV was 18% and must have formed by aminolysis in this aprotic solvent. Since the major route of N-deacetylation of X was methanolysis, a sterically hindered amine was used in order to slow down displacement of the chloro group to form XIII. With methanolic diisopropylamine, twenty hours at the b.p. was necessary to complete the reaction and the yield of XIV of good purity was 75% starting with crude 2-acetamidopyrimidine (X). No reaction occurred in acetonitrile, indicating that this sterically hindered amine in methanol removed the N-acetyl group only by methanolysis.

Hydrogenolysis of the 4-chloropyrimidine (XIV) to XI with palladium-charcoal in the presence of triethylamine as an acid acceptor proceeded smoothly to XI, m.p. 117°, in 85% yield. The subsequent synthesis (6) of the 4-pyrimidinthiol derivatives, VI, VII and VIII, gave an alternate method for conversion of the crude 2-acetamido-4-chloropyrimidine (X) to the 2-aminopyrimidine (XI). Desulfurization of VI, VII or VIII in aqueous ammonia with sponge nickel showed that VII gave the best yield (62%) of XI; the overall yield from crude X was 42%. Thus, the best method for conversion of the crude 2-acetamido-4-chloropyrimidine (X) to the 2-aminopyrimidine (XI) was by hydrogenolysis of XIV, the overall yield being 64%.

As could be anticipated from previous work from this laboratory (6), the dioxolane group of XI was stable to boiling water. Therefore the dioxolane blocking group of XI was converted in 94% yield to the ethyl acetal blocking group (XII) by reflux with ethanolic sulfuric acid. The diethyl acetal (XII), m.p. 115°, had an infrared spectrum quite similar to that of XI, as well as an identical ultraviolet spectrum; however XII was readily differentiated from XI by N.M.R. since XII showed its two methyls of the ethyl groups at δ 1.22, a signal not present in XI. Acetylation of XII in acetic anhydride at room temperature gave an N-acetyl derivative which could not be crystallized, but showed carbonyl absorption of an N^2-acetyl group at 1680 cm^{-1}, a band not present in XII. In view of the (a) difficulty of purification of this N^2-acetyl derivative, (b) the general lability of N^2-acetyl groups and (c) the necessity for the presence of a 4-hydroxy for the anchimeric assistance in the water hydrolysis of the ethyl acetal group (11) - which was not known when this problem was started - an alternate blocking group for the aldehyde function was needed that could theoretically be removed in the presence of the labile N^2-acetyl group; the mercaptal blocking group is commonly used in the carbohydrate area since it can be removed with mercuric chloride in the presence of an insoluble acid acceptor at room temperature even with other sensitive groups present (12-14).

Treatment of either the dioxolane (XI) or the ethyl acetal (XII) with 1-propanethiol and concentrated aqueous hydrochloric acid afforded the crystalline mercaptal (IX) as its hydrochloride. Although acetylation, then selective removal of the mercaptal group with mercuric chloride to give the desired 2-acetamido-5-pyrimidylpropionaldehyde (XVIII) should be feasible, the problem was not investigated further when the synthesis of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinethiol (XVI) was completed (6); desulfurization of XVI with sponge nickel gave, in 77% yield, sufficient of the 4-deoxy inhibitor (XVII) for enzymic investigation. If XVII had been a sufficiently more potent enzyme inhibitor than II, the alternate synthesis of XVII *via* the mercaptal (IX) might have been worthy of further pursuit.

The synthesis of the desamino derivative (XXV) of the folic reductase inhibitor, II, started with the dioxolanyl-keto ester (XIX) (6). Condensation of XIX with thiourea in methanolic sodium methoxide gave the 2-mercaptopyrimidine (XX) in 60% yield. Desulfurization of XX in aqueous ammonia with sponge nickel afforded the water soluble pyrimidinol (XXI) in 86% yield. The dioxolane blocking group of XXI was converted to a diethyl acetal blocking group with boiling alcoholic sulfuric acid; although the acetal (XXIII) was relatively low melting, it readily formed beautiful crystals, m.p. 77°, in 66% yield. Treatment of the

acetal (XXIII) with boiling water gave the aldehyde (XXII) as a glass that could not be crystallized but showed an aldehyde carbonyl band at 1720 cm^{-1}. Reductive condensation of the crude aldehyde (XXII) and aniline with sodium borohydride (4, 5) afforded crystalline XXV, the 2-deamino derivative of the folic reductase inhibitor, II, in 20% overall yield for the two steps from XXIII. The 2-deamino analog of I, prepared by reductive condensation of XXII with *p*-aminobenzoyl-L-glutamic acid could not be purified.

Although replacement of the 4-hydroxyl group of I with mercapto increased binding to folic reductase only two-fold, the increase in binding to 5, 10-methylenetetrahydrofolate dehydrogenase was five-fold (5). Thus it could be anticipated that replacement of the 4-hydroxy group of XXV with 4-mercapto (XXX) might increase binding to other enzymes in the folic cofactor area (15, 16) than folic reductase; that such was the case has now been noted in the Results and Discussion Section with thymidylate synthetase. The 4-mercapto analog (XXX) was synthesized as follows: short reaction of the 4-pyrimidinol (XXI) with phosphorous oxychloride in warm benzene gave a 74% yield of the 4-chloropyrimidine (XXIV) as a mobile, somewhat volatile oil with the expected ultraviolet peaks (10); the chloro pyrimidine (XXIV) was characterized by conversion to the crystalline 4-amino pyrimidine (XXVII) in 71% yield. Reaction of the 4-chloropyrimidine (XXIV) with thiourea in boiling ethanol did not stop at the thiouronium salt stage (XXVI), but spontaneously eliminated cyanamide and hydrogen chloride - a reaction previously noted with 9-alkyl-6-chloropurines and thiourea (17); however, the ultraviolet spectra of the product were characteristic of a 4-alkylthiopyrimidine rather than a 4-mercaptopyrimidine (20). As anticipated from the ultraviolet spectra, combustion analyses confirmed the structure of the product (83% overall yield) as the cyclic thioacetal, XXVIII; apparently the one equivalent of hydrogen chloride generated by breakdown of the thiouronium salt (XXVI) is sufficient to cause a neighboring-group facilitated exchange of the blocking functions of the latent aldehyde group.

Hydrolysis of the ethoxy group of XXVIII with aqueous sulfuric acid, followed by basification with ammonia did not give the expected 7-hydroxythiopyran (XXXI), but further reaction with ammonia took place to give the imino bis-thiapyran (XXXII). The evidence for this unusual structure, XXXII, consisted of (a) combustion analyses, (b) the high melting point (216°) compared to the more than 100° lower m.p. for its precursors, thus indicating a dimeric product and (c) its ultraviolet maximum in 0.1 N base at 290 mμ rather than at 299-302 mμ if it had formed the mercaptide ion (see u.v. of XXX) at this *p*H (10) as previously noted with the corresponding 2-amino derivative of XXXI (6); XXXII could be expected to require stronger basic conditions for conversion to the mercaptide ion than would XXXI.

Reaction of XXXII with a large excess of aniline in boiling ethanol evolved ammonia; the 7-anilinothiapyran (XXIX) presumably formed was not purified, but was directly reduced with sodium borohydride in methanolic sodium methoxide (6) to give a 41% overall yield of the beautifully crystalline mercapto analog (XXX) with the expected spectral characteristics.

EXPERIMENTAL

Melting points were taken on a Fisher-Johns apparatus or in capillary tubes on a Mel-temp block; those below 230° are corrected. Infrared and ultraviolet spectra were determined on Perkin-Elmer recording spectrophotometers 137B and 202, respectively.

2-Amino-4-chloro-5-[2-(1, 3-dioxolan-2-yl)ethyl]-6-methylpyrimidine (XIV).

A solution of 400 mg. (1.4 mmoles) of crude X (6) and 0.4 ml. of diisopropylamine in 2.8 ml. of reagent methanol was refluxed with magnetic stirring for 22 hrs. protected from moisture; during this time part of the product crystallized from the hot solution. The mixture was cooled several hrs. at 3-5°, then filtered and the product washed with ice cold methanol; yield, 238 mg., m.p. 156-157°, that was suitable for hydrogenolysis. By concentration of the combined mother liquor and washings, an additional 22 mg. (total 76%) was obtained, m.p. 156-157°.

With *n*-butylamine, the reaction time was complete in six hours and a yield of 170 mg. (50%), m.p. 157-158°, was obtained. Recrystallization from methanol gave nearly white crystals, m.p. 159-160°; ν max (KBr) 3420, 3350, 3200 (NH); 1650, 1580, 1520 (NH, pyrimidine); 1020 cm^{-1} (ether C-O-C); λ max (*p*H 1) 229 (ε, 17,300), 314 mμ (ε, 6,100); λ max (*p*H 7) 233 (ε, 16,800); 302 mμ (ε, 4,700); λ max (*p*H 13) 302 (ε, 4,800); inflection centering at 228 mμ.

Anal. Calcd. for $C_{10}H_{14}ClN_3O_2$: C, 49.3; H, 5.79; N, 17.2. Found: C, 49.1; H, 5.81; N, 17.2.

Evaporation of the mother liquor from the *n*-butylamine reaction gave 224 mg. of a yellow oil with λ max (*p*H 1) 277; λ max (*p*H 7) 278, and λ max (*p*H 13) 287 mμ in agreement (10) with structure (XIII (R = *n*-C_4H_9).

2-Amino-5-[2-(1, 3-dioxolan-2-yl)ethyl]-6-methylpyrimidine (XI).

(A). A mixture of 200 mg. (0.82 mmole) of XIV, 200 mg. of 5% palladium charcoal, and 83 mg. (0.82 mmole) of triethylamine in absolute ethanol was shaken with hydrogen at 2-3 atm. for 2 hrs. when hydrogen uptake was complete. The mixture was clarified by filtration through a pad of Celite (Johns-Manville Co.). The residue remaining after spin-evaporation of the filtrate *in vacuo* was extracted with boiling benzene (5 x 5 ml.), decanting each time from the residual triethylamine hydrochloride. Spin-evaporation *in vacuo* gave 145 mg. (85%) of product, m.p. 111-113°. Recrystallization from benzene afforded 130 mg. (76%) of pure product, m.p. 115-116°; ν max (KBr) 3350, 3180 (NH); 1650, 1590, 1550 (NH, pyrimidine); 1050, 1030, 1015 cm^{-1} (ether C-O-C); λ max (*p*H 1) 227 (ε, 16,500); 310 mμ (ε, 4,000); λ max (*p*H 7) 230 (ε, 15,000); 300 mμ (ε, 3,400); λ max (*p*H 13) 300 mμ (ε, 3,300).

Anal. Calcd. for $C_{10}H_{15}N_3O_2$: C, 57.4; H, 7.23; N, 20.1. Found: C, 57.2; H, 7.12; N, 20.1.

(B). A mixture of 400 mg. (1.66 mmoles) of the pyrimidinethiol (VII), 20 ml. of water and 0.5 g. of sponge nickel catalyst (Davison Chemical Co.) was refluxed with stirring for 6 hrs., then filtered through a pad of Celite. The filter cake was washed with 10 ml. of hot water. The combined filtrate and washings were spin-evaporated *in vacuo*. The residue was extracted with boiling benzene (2 x 5 ml.), then boiling chloroform (2 x 5 ml.). The combined organic extracts were spin-evaporated *in vacuo*; yield, 216 mg. (62%) of nearly white crystals, m.p. 115-116°, that were identical with preparation A.

2-Amino-6-methyl-5-pyrimidylpropionaldehyde Diethyl Acetal (XII).

A solution of 1.00 g. (4.78 mmoles) of XI in 55 ml. or absolute ethanol containing 0.29 ml. of 96% sulfuric acid was refluxed for 6 hours, then neutralized with alcoholic potassium hydroxide. The solvent was removed by spin-evaporation *in vacuo* and the residue was extracted with boiling dichloromethane (4 x 15 ml.). Spin-evaporation *in vacuo* of the combined extracts and recrystallization of the residue from carbon tetrachloride gave 1.08 g. (94%) of crystals, m.p. 112-113°. Further recrystallization of a similar preparation from benzene afforded white crystals, m.p. 114-115°; a mixture with XI melted at 104-110°. The product had ν max (KBr) 3340, 3180 (NH); 1670, 1600, 1560 (NH, pyrimidine); 1060, 1040, 1010 cm^{-1} (ether C-O-C); λ max (*p*H 1) 227 (ε, 17,800); 310 mμ (ε, 3,700).

Anal. Calcd. for $C_{12}H_{21}N_3O_2$: C, 60.2; H, 8.85; N, 17.6. Found: C, 60.5; H, 9.06; N, 17.8.

2-Amino-6-methyl-5-pyrimidylpropionaldehyde Di-*n*-propyl Mercaptal Hydrochloride (IX).

To a solution of 200 mg. (0.95 mmole) of XI in 0.5 ml. of concentrated hydrochloric acid at 0° was added 0.5 ml. of 1-propanethiol. The mixture was magnetically stirred for 22 hrs. in a lightly stoppered flask, then spin-evaporated *in vacuo*. The spin-evaporation was repeated with ethanol (2 x 2 ml.) leaving 283 mg. (88%) of white solid, m.p. 149-151°. Recrystallization of the hydrochloride from either benzene or ethyl acetate gave 262 mg. (72%) of white crystals, m.p. 151-153°, which was unchanged on further recrystallization. The analytical sample had ν max (KBr) 3400, 3150 (NH); 2500, 1850 (broad NH^+); 1675 ($C{=}NH^+$); 1625, 1550 cm^{-1} (NH, pyrimidine); λ max (*p*H 1) 228 (ε, 20,700); 313 mμ (ε, 5,600); λ max (*p*H 7) 230 (ε, 18,200); 300 mμ (ε, 4,100); λ max (*p*H 13) 300 mμ (ε, 4,100).

Anal. Calcd. for $C_{14}H_{28}N_3S_2 \cdot HCl$: C, 50.1; H, 7.80; N, 12.5. Found: C, 50.3; H, 7.85; N, 12.7.

2-Amino-5-(3-anilinopropyl)-6-methylpyrimidine (XVII).

A mixture of 823 mg. (3 mmoles) of XVI (6), 55 ml. of absolute ethanol and 1.6 g. of sponge nickel catalyst (Davison Chem. Co.) was refluxed with stirring for 6 hrs., then filtered through a pad of Celite. The cake was washed with 4-50 ml. portions of hot ethanol. Spin-evaporation *in vacuo* of the combined filtrate and washings left 550 mg. (77%) of product, m.p. 147-148°. Recrystallization from carbon tetrachloride gave the analytical sample, m.p. 148-149°; ν max (KBr) 3300, 3150 (NH); 1650, 1600, 1545 (NH, C=C, C=N); 745, 690 cm^{-1} (C_6H_5-); λ max (*p*H 1) 228 (ε, 16,600); 312 mμ (ε, 4,700); λ max (*p*H 7) 235 (ε, 22,400); 298 mμ (ε, 4,700); λ max (*p*H 13) 297 (ε, 5,400); inflection at 240 mμ.

In an earlier preparation, a less stable dimorph, m.p. 120-122°, was obtained with spectra identical with the higher melting dimorph.

Anal. Calcd. for $C_{14}H_{18}N_4$: C, 69.4; H, 7.49; N, 23.1. Found: (high melting dimorph) C, 69.2; H, 7.24; N, 23.1; (low melting dimorph) C, 69.1; H, 7.46; N, 23.2.

5-[2-(1,3-Dioxolan-2-yl)ethyl]-2-mercapto-6-methyl-4-pyrimidinol (XX).

To a solution of 15.5 g. of sodium methoxide in 200 ml. of methanol was added 30 g. of XIX (6) and 10 g. of thiourea. After being refluxed with stirring for 20 hrs., the orange solution was spin-evaporated *in vacuo* and the residue dissolved in 200 ml. of warm water. Acidification with 16 ml. of glacial acetic acid gave 20 g. of product. Recrystallization from water afforded 19 g. (60%) of yellow needles, m.p. 234-235°; ν max (Nujol) 3200-3080 (broad NH, OH); 1690 (C=O); 1560 cm^{-1} (pyrimidine); λ max (*p*H 1, 7) 280 mμ (ϵ, 18,300); λ max (*p*H 13) 263 (ϵ, 14,500); 309 mμ (ϵ, 8,000).

Anal. Calcd. for $C_{10}H_{14}N_2O_3S$: C, 49.6; H, 5.80; N, 11.5; S, 13.2. Found: C, 49.4; H, 5.72; N, 11.3; S, 13.4.

5-[2-(1,3-Dioxolan-2-yl)ethyl]-6-methyl-4-pyrimidinol (XXI).

A mixture of 10 g. (0.041 mole) of XX, 20 ml. of concentrated ammonia water, 250 ml. of water and 45 g. of sponge nickel catalyst was refluxed with stirring for 90 min. The catalyst was removed by filtration through a pad of Celite and the filtrate was spin-evaporated *in vacuo*. Recrystallization of the residue from ethyl acetate by addition of petroleum ether (b.p. 30-60°) gave 7.5 g. (86%) of white needles, m.p. 159-160°; ν max (Nujol) 3150 (NH, OH); 2850-2700 (broad acidic hydrogen); 1660, 1610, 1550 cm^{-1} (C=O, C=C, C=N); λ max (*p*H 1) 239 mμ (ϵ, 10,200); λ max (*p*H 7) 237 (ϵ, 6,600); 261 mμ (ϵ, 5,900); λ max (*p*H 13) 268 (ϵ, 5,400); inflection at 230 mμ (ϵ, 10,800).

Anal. Calcd. for $C_{10}H_{14}N_2O_3$: C, 57.1; H, 6.69; N, 13.3. Found: C, 57.3; H, 6.77; N, 13.3.

4-Hydroxy-6-methyl-5-pyrimidylpropionaldehyde Diethyl Acetal (XXIII).

A solution of 4.0 g. (19 mmoles) of XXI in 160 ml. of absolute ethanol containing 0.5 ml. of 96% sulfuric acid was refluxed for 20 hrs., then neutralized with methanolic ammonia. The solvent was removed by spin-evaporation *in vacuo* and the residue was partitioned between 50 ml. of dichloromethane and 20 ml. of water. The separated organic layer was further washed with water (2 x 20 ml.), dried with magnesium sulfate, then spin-evaporated *in vacuo*. Recrystallization of the residue (3.5 g.) from petroleum ether (b.p. 60-110°) gave 3.0 g. (66%) of white needles, m.p. 76-77°; ν max (Nujol) 3190, 3080 (NH, OH); 2700 (broad acidic hydrogen); 1670, 1610, 1560 cm^{-1} (C=O, C=N, C=C); λ max (*p*H 1) 238 mμ (ϵ, 8,950); λ max (*p*H 7) 236 (ϵ, 6,200); 263 mμ (ϵ, 5,600); λ max (*p*H 13) 231 (ϵ, 9,250); 271 mμ (ϵ, 5,300).

Anal. Calcd. for $C_{12}H_{20}N_2O_3$: C, 57.1; H, 6.69; N, 13.3. Found: C, 57.3; H, 6.77; N, 13.3.

4-Hydroxy-6-methyl-5-pyrimidylpropionaldehyde (XXII).

A solution of 400 mg. (1.67 mmoles) of XXIII in 10 ml. of water was refluxed for 1 hr., then spin-evaporated *in vacuo*. The glassy residue could not be crystallized but showed ν max (Nujol) 3250, 3180 (NH, OH); 2720 (acidic hydrogen); 1720 (aldehyde C=O); 1660, 1610, 1540 cm^{-1} (C=O, C=C, C=N). The compound was impure since it would not give satisfactory combustion values, but was probably partially hydrated.

Anal. Calcd. for $C_8H_{10}N_2O_2$: C, 57.8; H, 6.06; N, 16.8. Found: C, 54.5; H, 6.62; N, 15.2.

5-(3-Anilinopropyl)-6-methyl-4-pyrimidinol (XXV).

A solution of 280 mg. of crude XXII and 0.5 ml. of aniline in 20 ml. of methanol was allowed to stand for 1 hr., then 500 mg. of sodium borohydride was added portionwise with stirring over a period of 30 min. After being stirred for about 18 hrs. more, the mixture was spin-evaporated *in vacuo*. The residue was partitioned between 20 ml. of 0.1 N sodium hydroxide and 20 ml. of chloroform. The separated aqueous layer was neutralized with acetic acid, then extracted with chloroform (3 x 20 ml.). The combined chloroform extracts were washed with water, dried with magnesium sulfate, and spin-evaporated *in vacuo*. Crystallization from ethyl acetate by addition of petroleum ether (b.p. 30-60°) gave 80 mg. (20%) of white needles, m.p. 141-142°; ν max (Nujol) 3330 (NH, OH); 2690 (acidic hydrogen); 1650, 1600, 1550, 1520, 1490, (C=O, C=C, C=N); 745, 690 cm^{-1} (C_6H_5-); λ max (*p*H 1) 238 mμ (ϵ, 9,950); λ max (*p*H 7) 240 (ϵ, 16,300); inflection at 273 mμ; λ max (*p*H 13) 238 (ϵ, 18,800); inflection at 273 mμ.

Anal. Calcd. for $C_{14}H_{17}N_3O$: C, 69.1; H, 7.06; N, 17.3. Found: C, 69.1; H, 6.99; N, 17.1.

4-Chloro-5-[2-(1,3-dioxolan-2-yl)ethyl]-6-methylpyrimidine (XXIV).

A mixture of 1.00 g. (5.23 mmoles) of XXI, 10 ml. of benzene and 1.0 ml. of phosphorus oxychloride was refluxed with stirring for 10 min. during which time oil droplets separated. The mixture was quickly cooled, then diluted with 50 ml. of chloroform and washed with ice water (5 x 20 ml.). Dried with magnesium sulfate, the chloroform layer was spin-evaporated *in vacuo* (bath 40-50°). The residual product (0.8 g., 74%) was a mobile, nearly colorless oil that could not be crystallized but had ν max (film) 1550, 1525 (C=C, C=N), and no OH, NH near 3000 cm^{-1}; λ max (*p*H 1-13) 258 mμ.

The compound was characterized by conversion to XXVII as follows:

4-Amino-5-[2-(1,3-dioxolan-2-yl)ethyl]-6-methylpyrimidine (XXVII).

A mixture of 0.80 g. (3.53 mmoles) of XXIV and 20 ml. of methanol saturated with ammonia at 0° was heated in a steel bomb at 140° for 19 hrs. Solvent was removed by spin-evaporation *in vacuo* and the residual crystalline hydrochloride was dissolved 25 ml. of water. The aqueous solution was basified to *p*H 12, then extracted with chloroform (4 x 25 ml.). The combined extracts, dried with magnesium sulfate, were spin-evaporated *in vacuo*. Crystallization from ethyl acetate by addition of petroleum ether (b.p. 30-60°) gave 0.52 g. (71%) of white needles, m.p. 107-108°; ν max (Nujol) 3390, 3180 (NH); 1650, 1590, 1560 cm^{-1} (NH, pyrimidine); λ max (*p*H 1) 260 mμ (ϵ, 12,900); λ max (*p*H 7) 240 (ϵ, 8,870); 268 mμ (ϵ, 7,150); λ max (*p*H 14) 235 (ϵ, 10,700); 273 mμ (ϵ, 5,920).

Anal. Calcd. for $C_{10}H_{15}N_3O_2$: C, 57.4; H, 7.24; N, 20.1. Found: C, 57.1; H, 7.17; N, 20.3.

5,6-Dihydro-7-ethoxy-4-methyl-7H-thiopyrano[2,3-d]pyrimidine (XXVIII).

A mixture of 800 mg. (3.53 mmoles) of XXIV and 1.5 g. of thiourea in 20 ml. of absolute ethanol was refluxed for 3 hrs., then diluted with 25 ml. of 1 N aqueous sodium hydroxide. The mixture was spin-evaporated *in vacuo* to about one-third volume, then cooled. The product (430 mg.) was collected by filtration and the filtrate was extracted several times with chloroform. The combined extracts were dried with magnesium sulfate and spin-evaporated *in vacuo* leaving an additional 230 mg. of crystalline residue. Recrystallization of the combined solids from ethyl acetate by addition of petroleum ether (b.p. 30-60°) gave 610 mg. (83%) of white needles, m.p. 87-88°; ν max (Nujol) 1540, 1515 (C=C, C=N); 1085 (ether C-O-C); no NH or OH near 3000 cm^{-1}; λ max (*p*H 1) 228 (ϵ, 8,450); 301 mμ (ϵ, 16,600); λ max (*p*H 7-13) 249 (ϵ, 6,680); 282 mμ (ϵ, 9,400).

Anal. Calcd. for $C_{10}H_{14}N_2OS$: C, 57.1; H, 6.69; N, 13.3; S, 15.2. Found: C, 57.0; H, 6.89; N, 13.1; S, 15.4.

Bis-(5,6-dihydro-4-methyl-7H-thiopyrano[2,3-d]pyrimidine-7-yl)imine (XXXII).

A solution of 330 mg. (1.56 mmoles) of XXVIII in 15 ml. of 2 N sulfuric acid was refluxed for 1 hr., then cooled and basified with ammonia. The product (150 mg.) was collected by filtration and washed with water. Concentration of the combined filtrate and washings *in vacuo* gave 110 mg. (total 93%) of a second crop. The combined crops were recrystallized from water to give 220 mg. (80%) of white needles, m.p. 215-216°; ν max (Nujol) 3480, 3380, 3250, 3220 (H_2O, NH); 1540, 1510, 1490, (C=C, C=N); no C-O near 1080 cm^{-1}; λ max (*p*H 1) 227 (ϵ, 7,800); 298 mμ (ϵ, 15,300); λ max (*p*H 7) 253 (ϵ, 5,600); 284 mμ (ϵ, 9,000); λ max (*p*H 13) 290 mμ (ϵ, 9,500).

Anal. Calcd. for $C_{16}H_{18}N_6S_2 \cdot 1/2H_2O$: C, 54.4; H, 5.72; N, 19.8; S, 18.1. Found: C, 54.0, 54.2; H, 5.57, 5.59; N, 19.4, 19.6; S, 18.3, 18.2.

5-(3-Anilinopropyl)-6-methyl-4-pyrimidinethiol (XXX).

A mixture of 300 mg. (0.85 mmoles) of XXXI, 1.5 ml. of aniline and 30 ml. of absolute ethanol was refluxed for 1 hr., then the solvent was removed by spin-evaporation *in vacuo*. The excess aniline was removed by leaching with petroleum ether. The crude, gummy XXIX was dissolved in 50 ml. of methanol containing 60 mg. of sodium methoxide. After 30 min. the solution was treated portionwise with 1.0 g. of sodium borohydride with stirring. When addition was complete, the mixture was refluxed with stirring for 90 min., then spin-evaporated *in vacuo*. The residue was dissolved in water and the solution neutralized to *p*H 7 with hydrochloric acid. The crystalline product (220 mg., 53%) was collected and recrystallized from ethanol; yield, 170 mg. (41%) of yellow plates, m.p. 177-178°; ν max (Nujol) 3370 (NH); 1600, 1560, 1500 (C=C, C=N); 755, 695 cm^{-1} (C_6H_5-); λ max (*p*H 1) 322 mμ (ϵ, 13,800); λ max (*p*H 7) 240 (shoulder, ϵ, 13,100); 288 (ϵ, 10,300); 333 mμ (ϵ, 11,400); λ max (*p*H 13) 240 (inflection, 17,000); 302 mμ (ϵ, 14,300).

Anal. Calcd. for $C_{14}H_{17}N_3S$: C, 64.8; H, 6.62; N, 16.2; S, 12.3. Found: C, 64.8; H, 6.66; N, 16.0; S, 12.2.

ENZYME MEASUREMENTS (37) REAGENTS.

Tris (tris-hydroxymethyl aminomethane), tris hydrochloride, folic acid and TPNH were highest quality purchasable from Sigma Chemical Co. Pigeon liver acetone powder was purchased from General Biochemicals. Dihydrofolic acid was prepared by the sodium dithionite reduction of folic acid as described by Futterman (18) and stored as a 1.86 mM homogenized suspension in 0.005 N hydrochloric acid containing 0.1 M mercaptoethanol; the dihydrofolate in the suspension was stable at 3-4° for at least five months. Tetrahydrofolic acid (sealed under nitrogen) and frozen *E. coli* B cells (30-35% solids) were purchased from General Biochemicals. Sodium 2'-deoxyuridylate was purchased from Sigma Chemical Co.

Buffers.

Buffer A was 0.05 M Tris hydrochloride, *p*H 7.4. Buffer B was the same as A, but with the addition of 10 mM mercaptoethanol and 1 mM Versene.

Dihydrofolic Reductase (19).

In the small head (prechilled to -15°) of a Waring blendor was placed 5 g. of pigeon liver acetone powder, 5 g. of analytical grade Celite (Johns-Manville Co.) and 50 ml. of ice cold Buffer A. The mixture was blended for 2 min., then a spatula-tip of General Electric 60 Antifoam was added. The mixture was suction filtered through a thick Celite pad on a 90 mm. Buchner funnel into an ice-cooled receiver; volume, 30 ml. To the magnetically stirred filtrate cooled in an ice-bath was added 8.3 g. of ammonium sulfate (45% of saturation) over a period of 2 min. After being stirred for an additional 10 min., 1 g. of Celite was added and the mixture was filtered through a Celite pad on a 75 mm. Buchner funnel into an ice-cooled receiver; volume = 27 ml. To the stirred filtrate cooled in an ice-bath was added 9.2 g. of ammonium sulfate (90% of saturation). After being stirred for 10 min., 1 g. of Celite was added and the mixture was filtered through a 2 g. Celite pad on a 50 mm. Buchner funnel. The filter cake was immediately transferred to 20 ml. of ice-cold Buffer A, the lumps were crushed, then the mixture was stirred in an ice-bath for 10 min. Filtration through a 50 mm. Buchner funnel into an ice-cooled receiver gave 20 ml. of clear yellow filtrate. When assayed for dihydrofolic reductase as described below, 50 λ of solution gave an optical change of 0.010-0.013 units per min. The enzyme was stable for at least two months when stored at 2-5°.

Dihydrofolic Reductase Assay (19).

An 0.20 ml. aliquot of the homogenized dihydrofolic acid suspension was added to 1.80 ml. of Buffer B; this 0.186 mM solution was protected from light and was made up fresh for each day's use.

A 3.72 mM solution of TPNH in 0.01 M sodium hydroxide could be used for five days if stored at 3-5°; 0.20 ml. was diluted with 1.80 ml. of Buffer B to give an 0.372 mM solution and was used the same day.

In the upper and lower cuvettes of a Cary 11 spectrophotometer were placed 2.80 ml. of Buffer B, 50 λ of enzyme solution and 100 λ of 0.372 mM TPNH. After the system had balanced the enzyme reaction was started by addition of 100 λ of 0.186 mM dihydrofolate to the upper cuvette, then the decrease in optical density at 340 mμ with time was recorded (19) with a 0-0.1 optical density slide wire; the cuvette concentration of TPNH and dihydrofolate were 12 and 6 μM, respectively. No reaction occurred if either the enzyme or TPNH were omitted from both cuvettes, or dihydrofolate was omitted from the upper cuvette. In some inhibitor runs (Table I), the dihydrofolate concentration was 3 or 12 μM. The content of the bottom cuvette could be used for several hrs. without change.

Thymidylate Synthetase.

The literature procedure (20) was modified as follows: To 135 g. of neutral alumina (anhydrous, 80-200 mesh, Brockman activity I) in a mortar was added 30 ml. of water; the mixture was ground until uniform, then chilled in the mortar to -20°. After addition of 75 g. of frozen *E. coli* B cells (30-35% solids), the mixture was ground until it became stiff paste (5-10 min.). The grinding was continued after addition of 75-ml. portions of Buffer B, each portion being poured off until a total or 350 ml. had been used. The mixture was blended in a Waring blendor (head pre-chilled to -20°) for 30 seconds at high speed. After addition of a spatula-tip of General Electric 60 antifoam, the mixture was blended 10 seconds more. Insolubles were removed by centrifugation at less than 5° at 4000 r.p.m. in a No. 21 rotor of a Spinco L centrifuge for 30 min.

The viscous, slightly cloudy supernatant liquid (240 ml.) was cooled in an ice-bath and with magnetic stirring was treated with 14 ml. of 5% aqueous streptomycin sulfate solution; a stringy precipitate of nucleic acids separated. After being stirred 10 min., the mixture was further treated with 10 g. of analytical grade Celite (Johns-Manville) and stirred 5 min. more. The mixture was filtered by suction through a thick Celite pad on a 130 mm. Buchner funnel into an ice-cooled receiver. To the clear yellow mobile filtrate (195 ml.) was added with stirring and ice-cooling 54 g. of ammonium sulfate (45% of saturation). After being stirred for 7 min., the mixture was treated with 3 g. of Celite, stirred 3 min. more, then filtered through a Celite pad on a 90 mm. Buchner funnel into an ice-cooled receiver. To the filtrate (195 ml.) was added with magnetic stirring and ice-cooling 65 g. of ammonium sulfate (90% of saturation). After being stirred 7 min., the mixture was treated with 5 g. of Celite and stirred 3 min. more. The mixture was filtered through a 5 g. Celite pad on a 90 mm. Buchner funnel with suction and the original flask rinsed with some of the filtrate.

The Celite cake was quickly transferred to a flask containing 50 ml. of ice-cooled Buffer B. A trace of General Electric 60 antifoam was added, the lumps were crushed with a spatula, then the mixture was magnetically stirred in an ice-bath for 15 min. The Celite was removed by suction filtration through a 65 mm. Buchner funnel into an ice-cooled receiver, and the filter cake washed with 15 ml. of ice-cold Buffer B. The light yellow enzyme solution (65 ml.) was stored at -20° in 1-2 ml. aliquots and was stable for at least one month. When assayed as described below with "assay mix A", 50 λ gave an optical density change of 0.003 units per min., equivalent to 2.85 μmoles of thymidylate synthesized per hour or 56 Wahba-Friedkin (20) units per ml.

Thymidylate Synthetase Assay.

The literature procedure (20) was modified slightly. A 100 mg. sealed ampoule of tetrahydrofolic acid diacetate salt was opened and added to 20 ml. of 1 M mercaptoethanol previously adjusted to *p*H 7.4. The solution was divided into 0.90 ml. aliquots and frozen at -20°; in the frozen state the solution is stable at least several months.

An "assay mix A" was made up and used on the same day by adding to the vial containing 0.90 ml. of tetrahydrofolate solution, the following solution: 3.00 ml. of 1 M mercaptoethanol (*p*H 7.4), 1.50 ml. of 0.3 M formaldehyde (*p*H 7.4), 1.50 ml. of 0.5 M magnesium chloride and 2.10 ml. of Buffer B.

In the upper and lower cuvettes of a Cary 11 spectrophotometer were placed 50 λ of enzyme, 0.75 ml. of "assay mix A" and 2.20 ml. of Buffer B. When the system had balanced to no further optical density change (2-4 min.), the enzyme reaction was started by addition of 100 λ of 2.48 mM 2'-deoxyuridylate in Buffer A, then the increase in optical density at 338 mμ with time recorded (20) with a 0-0.1 optical density slide-wire; the cuvette concentrations of *dl*-tetrahydrofolate and 2'-deoxyuridylate were 0.214 mM and 0.080 mM, respectively. No reaction occurred if the enzyme, the tetrahydrofolate "mix" or the 2'-deoxyuridylate were singly omitted.

In some assays, a cuvette concentration of 25.7 μM tetrahydrofolate was employed by using "assay mix B" prepared as follows. To 0.30 ml. of the tetrahydrofolate master solution was added 0.54 ml. of 0.3 M formaldehyde (*p*H 7.4), 4.40 ml. of 0.5 M magnesium chloride, 5.00 ml. of 1 M mercaptoethanol (*p*H 7.4) and 7.66 ml. of Buffer B; 0.5 ml. of "assay mix B" and 100 λ of enzyme was used in each cuvette.

Inhibitors.

Master solutions were prepared in N,N-dimethylformamide at 31-62 mM or in 0.01 N hydrochloric acid at 3.1 mM. Up to 1 ml. of the latter could be added to the thymidylate synthetase assay in place of 1 ml. of Buffer B without changing the *p*H; similarly, up to 1.5 ml. could be used in the dihydrofolic reductase assay.

RESULTS AND DISCUSSION

A plot of V_o/V_I against I for several concentrations of I was made in order to determine the 50% inhibition point ($V_o/V_I = 2$), where V_o = velocity of the enzyme reaction without inhibitor, V_I = velocity of the reaction in the presence of inhibitor, and I = concentration of the inhibitor. In some cases the insolubility of the compound precluded the concentration necessary to obtain 50% inhibition; a V_o/V_I plot was made up to a concentration of I approaching the limit of solubility and the concentration for the maximum inhibition obtainable is recorded in Table I.

When 10% N,N-dimethylformamide was used in the dihydrofolic reductase assay the V_o was decreased about 20%. Similarly, 3% N,N-dimethylformamide in the thymidylate synthetase assay decreased the V_o to about one-half. In the latter cases, the enzyme concentration was therefore doubled.

The apparent K_m of *dl*-5,10-methylene-tetrahydrofolate with thymidylate synthetase was 3×10^{-5} molar when determined by the reciprocal plot method; a K_m of 4.5×10^{-6} for this compound has been previously recorded (20). The apparent K_m of dihydrofolate was difficult to determine. The K_m of dihydrofolate was so small that it was difficult to get below rate saturation and still have an accurate rate of optical density change. For example, 1.5 μM dihydrofolate appeared to be somewhat below rate saturation, but the total optical density change was only about 0.01 units when all the dihydrofolate was reduced. It appeared that the K_m of dihydrofolate was in the range of 10^{-6} to 10^{-7} molar. Reported values of the K_m of dihydrofolate have varied from 5×10^{-7} for the chicken liver enzyme (22) and 4×10^{-6} for the *S. faecalis* R enzyme (23) to 2×10^{-5} for the embryonic chick liver enzyme (24) and the leucocyte enzyme (24) and 1×10^{-4} for the calf thymus enzyme (25); such wide divergence may be due partially to the above mentioned technical difficulties.

Since in our hands the determination of the K_m of

TABLE I

Inhibition of Dihydrofolic Reductase and Thymidylate Synthetase by 2-R_1-4-R_2-5-(3-Anilinopropyl)-6-methylpyrimidines

Compound No.	R_1	R_2	Dihydrofolic Reductase: μM Conc. FAH_2 (a)	mM Conc. Inhibitor	Percent Inhibition	Inhibitor: Substrate (b)	Thymidylate Synthetase: μM Conc. M-FAH_4 (c)	mM Conc. Inhibitor	Percent Inhibition	Inhibitor: Substrate (d)	Synthetase: Reductase
II	NH_2	OH	6	0.60 (e)	43	130	25.7	0.62 (f)	50	50	0.39
XVII	NH_2	H	12	0.95	50	80	25.7	0.70 (f)	35	100	1.2
XXV	H	OH	6	1.5	0	>1000	25.7	2.0 (f)	26	420	< 0.4
XVI (g)	NH_2	SH	3	0.022	50	7.3	25.7	0.080	38	11	1.5
XXX	H	SH	3	0.070	0	> 90	25.7	0.20	15	90	< 1
A (h)	NH_2	NH_2	6	0.0022	50	0.37	25.7	0.80	50	63	170

(a) FAH_2 = dihydrofolic acid. (b) Ratio of inhibitor to FAH_2 giving 50% inhibition. (c) M-FAH_4 = *dl*-5,10-methylene-tetrahydrofolic acid. (d) Ratio of inhibitor to 1-M-FAH_4 giving 50% inhibition. (e) Cuvette also contained 10% N,N-dimethylformamide. (f) Cuvette also contained 3% N,N-dimethylformamide. (g) See reference (6) for preparation. (h) B. R. Baker and B. T. Ho, to be published.

dihydrofolate was not accurate, it follows that the K_i of inhibitors determined by the reciprocal plot would be just as inaccurate. Furthermore, comparison of the effect of a single inhibitor on two different enzymes by comparing the respective K_i values fails to consider the relative K_m values of the substrates for the two enzymes, that is, if a single inhibitor had the same K_i value with two different enzymes with differing substrate K_m values it is obvious that the inhibitor will be more effective on the enzyme using a substrate with the larger dissociation constant. In order to compare the relative effect of an inhibitor against two different enzymes, the chemotherapeutic situation is closely approached by comparing the inhibitor: substrate ratios that give 50% inhibitions; even this comparison fails to consider the relative concentration of the two enzymes, but should be one-step closer in approximation of specificity than comparing K_i values of a single inhibitor on two different enzymes. Therefore, the inhibitor: substrate ratios necessary to give 50% inhibition are recorded in Table I; for thymidylate synthetase the concentration of the natural *l*-substrate was used rather than the concentration of *dl*-isomer.

When the 50% inhibition point could not be reached, due to the insolubility of the compound, the V_o/V_I against I curve was extended to the 50% inhibition point; these inhibitor: substrate ratios obtained by this procedure may be up to 50% less accurate than those 50% inhibition values obtained by actual experimental observation. Therefore differences in inhibitor: substrate ratios less than two-fold are probably not significant.

In two cases - the effect of XXV and XXX on dihydrofolic reductase - no inhibition could be detected at maximum solubility or light transmission. Since 13% inhibition is readily detectable, the 50% inhibition were estimated to be greater than four-times the maximum concentration reached; for example, the inhibitor: substrate ratio is greater than 1000 and greater than 90, respectively, for XXV and XXX on dihydrofolic reductase.

The contribution by the carboxy-L-glutamate side chain of XXXIII (6) to binding to dihydrofolic reductase and thymidylate synthetase is relatively minimal when compared to the binding of XVI (Table I). For 50% inhibition of thymidylate synthetase a inhibitor: substrate (XXXIII) ratio of 2.1 was required, compared to 11 for XVI. Similarly for inhibition of the dihydrofolic reductase from pigeon liver, a inhibitor: substrate (XXXIII) ratio of 0.50 was required compared to 7.3 for XVI. This 14-fold spread between XXXIII and XVI for 50% inhibition of pigeon liver dihydrofolic reductase does not agree with a spread of 4-fold previously recorded (5) for rat liver folic reductase. This disagreement could be the result of either a species difference or the difference in assay method. That this difference was not due to the former was shown by rechecking the inhibition of rat liver (dihydro) folic reductase using dihydrofolate as substrate; values were obtained identical with the dihydrofolic reductase from pigeon liver. Several other differences in the results obtained by the two different assay methods have been noted and will be subsequently reported; a rational reason why different substrates should give different K_i values has still not become apparent.

The approximate K_i values for some of these compounds with thymidylate synthetase can be approximated from equation (a) (26).

(a) $K_i = I\ K_m/S$ where I = inhibitor concentration required to give 50% inhibition in the presence of an S concentration of *dl*-substrate, and $K_m = 3 \times 10^{-5}$ molar.

Thus, II, XVI, XXX (Table I), and XXXIII have approximate K_i values of 75, 14, and 7×10^{-5} molar, respectively, for thymidylate synthetase. Apparent K_i values of 1 and 4 μM previously had been observed for XXXIII and XVI (6), respectively, with rat liver folic reductase when assayed with folic acid as substrate.

The relative contribution to binding to the two enzymes by the 2-amino and 4-hydroxyl groups of II can be gleaned by comparison of the inhibition by II, its 2-deamino analog (XXV), and its 4-deoxy analog (XVII). Removal of the hydroxyl group as in XVII, gave essentially no changes in the capacity of II to bind to either enzyme. The most obvious explanation is that the 4-hydroxyl group (or its oxo tautomer) makes no contribution to binding; that this obvious explanation is wrong can be seen by noting that replacement of the 4-hydroxyl group of II with a 4-mercapto group (XVI) gives a 17-fold increase in binding with dihydrofolic reductase and a 5-fold increase in binding with thymidylate synthetase. Since the mercapto group and hydroxyl group most probably would bind in the same manner, it would indicate that the 4-hydroxyl does bind.

An alternate explanation for the fact that the deoxy analog (XVII) binds as well as II brings into play the possibility for an increase in binding by a second group when a particular group is removed; *since only a net change in binding is observed experimentally*, the loss of binding by one group being made up by a corresponding increase in binding by a second group would give no net change in binding. The 2-amino group of dihydrofolic reductase inhibitors most probably binds to the enzymes by a hydrogen bond (27). Removal of the 4-hydroxyl group of a 2-amino-4-hydroxypyrimidine makes the resultant 2-aminopyrimidine a slightly weaker base (28). Even though the 2-aminopyrimidine is a weaker base a logical explanation for the nearly equal binding of II and its 4-deoxy analog (XVII) is that there is a gain in binding by the 2-amino group of XVII which compensates for loss of binding by removal of the 4-hydroxyl group.

The contribution of the 2-amino group of II to binding to both enzymes can be seen by comparing the inhibition of II and its 2-deamino analog (XXV) (Table I). The resultant XXV could not be detected as an inhibitor of dihydrofolic reductase; a greater than eight-fold decrease in binding occurred. The effect of removing the 2-amino group was less on binding to thymidylate synthetase; about a four-fold decrease in binding occurred, indicating that the 4-hydroxyl group of II contributes more to binding to thymidylate synthetase than to dihydrofolic reductase.

This difference in the binding to the two enzymes by the 2-amino group is also apparent when one compares the 2-amino-4-mercapto analog (XVI) with its 2-deamino derivative (XXX) (Table I). Removal of the 2-amino group (XXX) causes a greater than twelve-fold decrease in binding of XVI to dihydrofolic re-

ductase; only about an eight-fold decrease in binding to thymidylate synthetase occurs when the 2-amino group is removed from XVI to give XXX.

That 5-(3-anilinopropyl)-2,4-diamino-6-methylpyrimidine (compound A, Table I) is an excellent inhibitor of folic reductase, binding about 350 times tighter than the corresponding 2-amino-4-hydroxy derivative (II), should come as no great surprise; the strong inhibition of folic reductase by 2,4-diaminopyrimidines and pteridines, and 2,6-diaminopurines - such as aminopterin and pyrimethamine has been known for about 15 years (29). Compound A is not as good inhibitor of dihydrofolic reductase as aminopterin by a factor of at least 100 (25); part but not all of this difference can be accounted for by the lack of the carboxy-L-glutamate side chain. However, compound A inhibits dihydrofolic reductase better than other 2,4-diaminopyrimidines not containing the anilinopropyl side chain (27).

In contrast, there was little *a priori* information to indicate whether or not Compound A (Table I) should be a good inhibitor of thymidylate synthetase; in fact, little significant change in binding of Compound A - compared to II - to thymidylate synthetase takes place. The effects of only six tetrahydrofolate analogs as inhibitors of thymidylate synthetase could be found in the literature. Tetrahydro-aminopterin and tetrahydro-amethopterin were bound to the enzyme stronger than the substrate (30), and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine about one-sixth as well as substrate (31); aminopterin (30), amethopterin (30), and 9-methyl-tetrahydrofolate (29) failed to inhibit the enzyme at the concentration tested. The best inhibitor of thymidylate synthetase is still 5-fluoro-2'-deoxyuridylate; since this is an analog of the other substrate, 2'-deoxyuridylate, its effects (32-34) are not germane to this paper.

In a previous paper (5) on the relative mode of binding of tetrahydrofolate analogs to folic reductase and 5,10-methylene-tetrahydrofolate dehydrogenase, it was pointed out that the tetrahydrofolate analog (I) contained eleven polar atoms capable of strong binding to an enzyme in addition of the binding forces present in the π-electrons of the two aromatic rings. It was also pointed out that binding by at least three and probably no more than six of these thirteen groups in I should be sufficient to account for the magnitude of the K_i value observed; therefore it would be unlikely that the identical 3-6 groups would be involved in binding to any two enzymes in the folic cofactor area. Note that thymidylate synthetase and 5,10-methylene-tetrahydrofolate dehydrogenase - using the identical substrate - have different modes of binding. The carboxy-L-glutamate moiety of inhibitors such as I and XXXIII make a relatively minor contribution to binding to thymidylate synthetase and folic reductase, but account for a major portion of binding to the dehydrogenase (5,6).

Even with a structure such as II without the carboxy-L-glutamate residue, there are five polar groups and two aromatic rings which can bind; therefore it would not be surprising if the mode of binding of II to dihydrofolic reductase and thymidylate synthetase were dissimilar. There is only a 2-3 fold difference in inhibition of the two enzymes by II (Table I).

By replacement of the 4-hydroxyl group of II by amino (Compound A, Table I), a 170-fold specificity in favor of dihydrofolic reductase is obtained, since the 4-amino group gives tremendous enhancement to binding to dihydrofolic reductase, but not thymidylate synthetase. None of the compounds in Table I have a significant cross-over in specificity in favor of thymidylate synthetase. Whether or not greater specificity can be obtained by use of the bulk principle of specificity (21) or the bridge principle of specificity (26) remains to be determined. However, it is apparent that the mode of binding of II in the region of its N-3 and 4-OH groups is different for the two enzymes. Light on which one of the several tautomeric forms of the 2-amino-4-hydroxypyrimidine system is actually complexed to thymidylate synthetase might be shed by a thermodynamic study of the type done by Zakrezewski (27) on the mode of binding of 2,4-diaminopyrimidines to folic reductase.

A difference in the mode of binding of the anilino moiety of II has also been noted and is reported in an accompanying paper. Such difference in the mode of binding to the various enzymes in the folic cofactor area are of obvious importance in chemotherapy in order to gain specificity (15).

Finally, it has been noted that the 2-amino-4-mercapto analog (XXXIII) is an effective inhibitor of *in vitro* growth of Sarcoma 180 cells, giving 50% inhibition at 5×10^{-5} molar; furthermore the growth of a cell line 174-fold resistant to amethopterin by virtue of a 100-fold increase in folic reductase level is still inhibited 50% by XXXIII at 8×10^{-5} molar (35). It follows that XXXIII does not inhibit cell growth of these cells by a block of folic reductase, but by a blockade of some other enzyme - perhaps thymidylate synthetase. None of the compounds in Table I have as yet been evaluated in the Sarcoma 180 tissue culture system.

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