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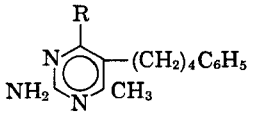
# Analogs of Tetrahydrofolic Acid XXVII

## Hydrophobic Bonding to Dihydrofolic Reductase by 2-Amino-4,6-disubstituted-5-alkylpyrimidines and 1-Alkyl-4,6-diamino-1,2-dihydro-*s*-triazines

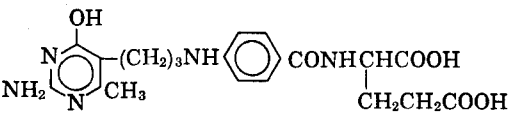
By B. R. BAKER, BENG-THONG HO, and DANIEL V. SANTI

**Strong hydrophobic bonding to dihydrofolic reductase has been observed by alkyl groups attached to the 5-position of pyrimidines or the 1-position of 1,2-dihydro-*s*-triazines. Studies were made with alkyl substituted 2,6-diamino-4-pyrimidinols (series *A*), 2-amino-6-methyl-4-pyrimidinols (series *B*), 2,4,6-triaminopyrimidines (series *C*), 2,4-diamino-6-methylpyrimidines (series *D*), and 4,6-diamino-1,2-dihydro-2,2-dimethyl-*s*-triazines (series *E*); 19 different alkyl, aryl, or aralkyl groups were investigated, but not in all series. Maximum hydrophobic bonding by an *n*-alkyl group was reached with *n*-butyl in all except series *B*; the *B* series required an *n*-amyl group. In all series, a further increment in binding over *n*-butyl was observed in the isoamyl group; the latter gave as good or better binding than the phenylpyrimidines or *s*-triazines of the pyrimethamine type. The butyl is probably complexed in a skew conformation. It is proposed that the 5-phenyl group of a pyrimidine or 1-phenyl group of 1,2-dihydro-*s*-triazine may also bind to dihydrofolic reductase by hydrophobic bonding. These observations on hydrophobic bonding can account for most of the discrepancies in increments of binding observed with the pyrimidines and dihydro-*s*-triazines in earlier papers. The possible uses of hydrophobic bonding to dihydrofolic reductase for species specificity are discussed.**

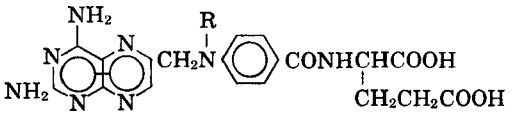
As a result of a previous study (1), it was reported that 2-amino-6-methyl-5-(4-phenylbutyl)-4-pyrimidinol (V*B*) was a threefold better inhibitor of dihydrofolic reductase than the pyrimidyl analog (I) (2, 3) containing the intact *p*-aminobenzoyl-L-glutamate moiety. With the assumption that the phenyl group of V*B* is binding at the same locus on dihydrofolic reductase as the *p*-aminobenzoyl moiety of I—and there was no *a priori* reason why this assumption should not hold—it was predicted (4) that 2,4-diamino-6-phenethylpteridine (IV) should be as good an inhbitor of dihydrofolic reductase as the potent aminopterin (II) or amethopterin (III). Synthesis and evaluation of the phenethylpteridine (IV) (4) did not support this prediction. In fact, 2,4-diamino-6-methyl-5-(4-phenylbutyl)-pyrimidine (V*D*), which was about one-thirtieth



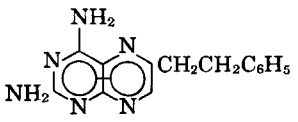
V*B*, R = OH
V*D*, R = $NH_2$



I



II, R = H
III, R = $CH_3$



IV

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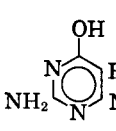
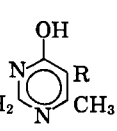
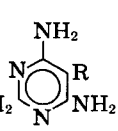
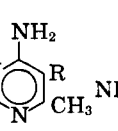
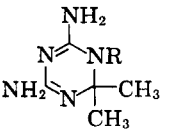
Previous paper: Baker, B. R., and Jordaan, J. H., *J. Heterocyclic Chem.*, **2**, 162(1965).

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE WITH 4,5,6-TRISUBSTITUTED-2-AMINOPYRIMIDINES AND 1-SUBSTITUTED-4,6-DIAMINO-1,2-DIHYDRO-*s*-TRIAZINES

| | | Series and $\mu M$ Concn. for 50% Inhibition | | | | |
|---|---|---|---|---|---|---|
| | |  |  |  |  |  |
| Compd. | R | *A* | *B* | *C* | *D* | *E* |
| V | $C_6H_5(CH_2)_4$— | 8.4[a] | 30[b] | 3.5[a] | 0.027[c] | 0.041[d] |
| VI | $C_6H_5(CH_2)_3$— | 60[a] | 160 | 7.1[a] | 0.18 | 0.028[d] |
| VII | $C_6H_5NH(CH_2)_3$— | | 800[e] | | 2.2[e] | |
| VIII | H— | 13,000[f] | 19,000[f] | 1200[f] | 1100[g] | |
| IX | $CH_3$— | | 7500[f] | | | 74 |
| X | $C_2H_5$— | 17,000 | 2200 | | | 220[d] |
| XI | $CH_2$=$CHCH_2$— | | 2000 | | | |
| XII | *n*-$C_3H_7$— | 450 | 1100 | 300 | | 11 |
| XIII | *n*-$C_4H_9$— | 40 | 260 | 23 | 2.0 | 0.36 |
| XIV | $(CH_3)_2C$=$CHCH_2$— | 17 | | | | |
| XV | *n*-$C_5H_{11}$— | | 30 | | 1.0 | |
| XVI | *i*-$C_5H_{11}$— | 4.0 | 8.5 | 2.1 | 0.24 | 0.058 |
| XVII | *n*-$C_6H_{13}$— | 30 | 35 | | | 0.32 |
| XVIII | *n*-$C_8H_{17}$— | 48 | 230 | | | 0.14 |
| XIX | $C_6H_5$— | 2.1 | | | | 0.11[h] |
| XX | 4-$ClC_6H_4$— | | | | 0.85[h] | 0.44[h] |
| XXI | 3-$CH_3C_6H_4$— | | | | | 0.078 |
| XXII | 3-$C_6H_5CH_2C_6H_4$— | | | | | 0.019[d] |
| XXIII | 3-$C_6H_5(CH_2)_2C_6H_4$— | | | | | 0.024[d] |
| XXIV | *n*-$C_4H_9\overset{+}{N}H_2(CH_2)_3$— | | >24,000[b] | | | |
| XXV | $C_6H_5CH_2\overset{+}{N}H_2(CH_2)_3$— | | >16,000[i] | | | |
| XXVI | $TsNH(CH_2)_3$—[j] | | 2400[k,l] | | | |
| XXVII | $TsN(CH_2)_3$—[j] $\lvert$ $C_4H_9$-*n* | | 190[k] | | | |

The dihydrofolic reductase was a 45–90% ammonium sulfate fraction prepared from pigeon liver acetone powder (General Biochemical) and assayed with 6 $\mu M$ dihydrofolate and 12 $\mu M$ TPNH in 0.05 *M* Tris buffer (pH 7.4) as previously described (34). Compounds in series *A*–*C* were assayed in the presence of 10% *N*,*N*-dimethylformamide to aid solubility, and master solutions were made in *N*,*N*-dimethylformamide unless otherwise indicated. Master solutions of compounds in series *D* were made in 0.01 *N* hydrochloric acid and in series *D* in water. The technical assistance of Miss Shirley Herrmann, Miss Karen Smith, and Miss Gail Westley is acknowledged. [a] Data from *Reference 35*. [b] Data from *Reference 1*. [c] Data from *Reference 4*. [d] Data from *Reference 16*. [e] Data from *Reference 34*. [f] Purchased from Aldrich Chemical Co. [g] Obtained through the courtesy of Dr. S. F. Zakrzewski *via* the Wellcome Research Laboratories; see *Reference 11*. [h] Data from *Reference 23*. [i] Data from *Reference 17*. [j] Ts = *p*-tolylsulfonyl. [k] Data from *Reference 18*. [l] Estimated from 20% observed inhibition at maximum solubility.

as effective an inhibitor of dihydrofolic reductase as aminopterin (II) (4), was 150-fold more potent than the phenethylpteridine (IV) (4).

## DISCUSSION

This enigma has now been partially resolved by the discovery that the *n*-butyl group of 5-*n*-butyl-2,4-diamino-6-methylpyrimidine (XIII*D*) (Table I) makes XIII*D* a 550-fold better inhibitor of dihydrofolic reductase than 2,4-diamino-6-methylpyrimidine (VIII*D*); a similar enhancement (320-fold) in binding by the 5-*n*-butyl group was also observed with 5-*n*-butyl-2,4-diamino-6-pyrimidinol (XIII*A*). A lesser, but significant enhancement in binding was observed in the 2-amino-6-methyl-4-pyrimidinol series (*B*) and the 2,4,6-triaminopyrimidine series (*C*).

An alkyl group such as a butyl can only bind to an enzyme by a combination of van der Waals forces (5) and hydrophobic bonding (6–9). The latter binding energy results from reassociation of water molecules previously separated by the alkyl group of the inhibitor and that part of the hydrophobic area of the enzyme ultimately involved in hydrophobic complex formation. Additional alkyl analogs were evaluated (Table I) as inhibitors of dihydrofolic reductase in the 2,6-diamino-4-pyrimidinol series (*A*), the 2-amino-6-methyl-4-pyrimidinol series (*B*), and the 4,6-diamino-1,2-dihydro-*s*-triazine series (*E*). Based on the inhibition data of the first three series, selected key alkyl derivatives of the 2,4,6-triaminopyrimidine series (*C*) and 2,4-diamino-6-methylpyrimidine series (*D*) were then synthesized and evaluated.

From Table I the following comparisons should be noted. (*a*) Maximum hydrophobic bonding is reached with a straight-chain hydrocarbon at *n*-butyl except for series *B*, which requires an *n*-amyl group; the amount of hydrophobic bonding is then not further increased significantly with *n*-hexyl or *n*-octyl. The maximum effect with an *n*-alkyl group on binding is 520-fold in series *A*, 630-fold in series *B*, and 1100-fold in series *D*. In series *E*, only the 1-methyl derivative could be compared with *n*-butyl, since R = H was not accessible; in series *E* a 200-fold increment in binding was observed between methyl and *n*-butyl. Only the triamino series *C*

differed in the magnitude of the maximum increment from hydrogen to butyl, being only 52.

These two discrepancies are of considerable theoretical interest. If it is assumed that in all five series, the heterocyclic ring binds to the same site on the enzyme, it is clear that the 2-amino-4-hydroxy-6-methylpyrimidine series *B* is not complexed with exactly the same stereochemistry as in the *A*, *D*, and *E* series. There are at least two ways in which the pyrimidine ring of series *B* could be complexed differently from series *A*, *D*, and *E*. When complexed with enzyme, the pyrimidine ring may be able to rotate in the plane of the complex to accommodate maximum binding, depending upon the interaction of the pyrimidine substituents with the enzyme as noted with the complexing of isoadenosine derivatives with enzymes (10). Such a rotation of the pyrimidine would be compatible if the pyrimidine is complexed *via* a charge-transfer complex, a mode of binding which is not likely (12). It is even less compatible with the four hydrogen bond formulation of Zakrzewski (11) or a van der Waals or hydrophobic interaction with the enzyme of such a hydrophilic moiety. Similarly, if there is one hydrogen bond interaction between the enzyme and the pyrimidine and one delocalized, but sometimes weak anionic–cationic interaction as recently proposed (12), then the pyrimidine might be able to rotate in the plane of the complex with the enzyme. Another possibility is that there are two hydrogen bonds between the enzyme and the pyrimidine ring, instead of four hydrogen bonds (11); in such a case, the angle of the plane of pyrimidine ring to the binding locus of the enzyme for the pyrimidine could vary, depending upon substitution at the 6-position and thus vary the distance required to reach the hydrophobic region.

The discrepancy in maximum hydrophobic bonding attainable (52-fold) with the 2,4,6-triaminopyrimidine series *C* is also of interest. First, there is no difference in binding when R = H (VIII) between series *C* and the 6-methyl series *D*. Therefore, it would appear that VIII*C* is more potent than it should be or that there is some opposing factor to hydrophobic bonding in series *C*. Since the compounds in series *C* are fairly strong bases (pKa of VIII*C* is 6.8) that are about 20% protonated at the assay pH of 7.4, part of this charge of these protonated species will be localized at the 6-amino group. If the 6-position must approach the hydrophobic region as the 5-alkyl group is complexing with the hydrophobic region, there would be a repulsion of the partially protonated 6-$NH_2$ which must be overcome at the expense of some of the energy of hydrophobic bonding of the 5-alkyl group. Such a 12-fold repulsion in XIII*C* compared to XIII*D* would not be out of the range of possibility. Thus, it is possible that the triaminopyrimidine (VIII*C*) can rotate so that the repulsion is minimal from any hydrophobic region, but that the binding by the *n*-butyl group of XIII*C* may force the partially protonated 6-amino group into the hydrophobic region with resultant loss in binding energy.

The relatively weak inhibition by the 2,4-diamino-6-hydroxy series *A*, compared to series *D*, might also be accounted for by the fact that either the hydroxyl or one of the amino groups, both of which are hydrophilic, would be in the 6-position near the supposed hydrophobic region. An alternate explanation has been offered by Zakrzewski (11) that one of the four hydrogen bonds between the enzyme and the pyrimidine is lost in the lowest member of this series (VIII*A*); however, the four hydrogen bond hypothesis is untenable with some recent results (12).

(*b*) The same increments per carbon atom in proceeding from H to butyl are not observed in all series. In series *E*, the ethyl group is less effective than methyl. Similarly, in series *A*, the ethyl group is less effective than hydrogen. In contrast, in series *B*, each added methylene group up to amyl gives an added increment in binding. Furthermore, in series *B*, the increment in binding between *n*-propyl (XII*E*) and *n*-butyl (XIII*E*) is thirtyfold, and the increment between *n*-propyl (XII*E*) and ethyl (X*E*) is twentyfold; the maximum increment in binding that could be expected for one methylene group from both hydrophobic bonding and van der Waals forces would be tenfold (8, 13). A possible explanation emerges if one considers that the total increment in binding between the methyl (IX*E*) and the *n*-butyl (XIII*E*) dihydro-triazines is 200-fold, whereas the maximum for the 3-methylene groups could be 1000-fold; if a conformational change in the enzyme or a rotational change of the pyrimidine must take place to get maximum hydrophobic binding, there may be some energy barrier to these changes that is only fully overcome when the *n*-butyl side chain (XIII*E*) is reached, and then the total free energy change of the four carbons for hydrophobic bonding is observed.

(*c*) Note that the *n*-butyl-triazine (XIII*E*) is almost as effective as the phenyl-triazine (XIX*E*); this threefold difference is about 600 cal. in binding energy.[1] Also note that in the *D* series the *n*-butyl derivative (XIII*D*) has the same magnitude of inhibition as the *p*-chlorophenyl analog (XX*D*), although the butyl is somewhat less effective. In the *A* series, the phenyl analog (XIX*A*) is about twentyfold more effective than the *n*-butyl analog (XIII*A*), a difference of about 1700 cal.; however, the isoamyl analog (XVI*A*) has the same order of activity as the phenyl analog (XIX*A*). Therefore, the possibility was considered that the *n*-butyl group and the phenyl group were both complexed to the same region of the enzyme by hydrophobic bonding.

In order for the *n*-butyl group to assume the conformation of a benzene ring, it would be necessary for the *n*-butyl group to assume an eclipsed con-

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[1] The calories of free energy change when an inhibitor complexes with an enzyme can be calculated from

$$\Delta F = -RT\ln\frac{I}{K_i} = -1300\log\frac{1}{K_i}\ \text{cal./mole} \quad \text{(Eq. 1)}$$

where $K_i$ = the inhibitor–enzyme dissociation constant. $K_i$ can be determined (3) from the $I/S$ ratio required for 50% inhibition if the concentration of inhibitor ($I$), the concentration of substrate ($S$), and the enzyme–substrate dissociation constant ($K_m$) are known and $S > 6\ K_m$ (as is the case in Table I) by use of

$$K_i = K_m\, I/S \quad \text{(Eq. 2)}$$

However, if the difference in free energy of binding between two inhibitors is to be calculated, then it is not necessary to know $K_m$ since:

$$\Delta\Delta F'' = \Delta F - \Delta F' = -RT\ln\frac{1}{K_i} + RT\ln\frac{1}{K_i'} = RT\ln\frac{K_i'}{K_i} \quad \text{(Eq. 3)}$$

Substituting Eq. 2 in Eq. 3 gives:

$$\Delta\Delta F'' = 1300\log\frac{I'}{I}\ \text{cal./mole}$$

where $I$ is the concentration necessary for 50% inhibition.

formation. The energy required to shift an *n*-butyl group from the most stable zigzag *trans* planar structure to the eclipsed conformation would require about 5000 cal. in energy (14). Since the total energy of hydrophobic bonding and van der Waals forces available for a butyl group complexing with an enzyme would be about 5600 cal. (8), there would be little net binding energy remaining if the butyl group had to assume an eclipsed conformation. However, there is no *a priori* reason why it should be assumed that the benzene ring has the optimum conformation for the most hydrophobic bonding. Therefore, the possibility should be considered that a skew conformation of the *n*-butyl group, which requires only 800 cal. in energy (14), could approach maximum hydrophobic bonding. The maximum increment in *n*-butyl binding of 550-fold was observed in the *D* series, thus giving a net free energy of binding of 3600 cal./mole. Since the *n*-butyl group could have a maximum free energy of binding of 5600 cal. (8, 13), and 800 cal. is necessary to skew the butyl group, a net maximum of 4800 cal. remains. This is well above the 3600 cal. noted for net binding of the *n*-butyl group of XIII*D*.

(*d*) The hydrophobic bonding of the isoamyl group (XVI) was then studied, although *a posteriori* the reasons for selecting this group may have been wrong. With the isoamyl group, the configuration giving the least interactions appears to give nearly a skew conformation of the *n*-butyl part of this moiety. Note that in series *A*, *C–E*, the isoamyl group gives a six- to elevenfold enhancement in binding over the *n*-butyl group; this enhancement is equivalent to a free energy change of 1000–1400 cal./mole, not much above the range of 800 cal. required to place the *n*-butyl group in a skew conformation. Thus, the results with the isoamyl group could be rationalized on the basis that the extra methyl in the isoamyl group does not necessarily give additional hydrophobic bonding, but allows a more skew-like conformation of the remaining group to be at the ground state.[2] It is then noteworthy that the isoamyl group gives the same order of binding as the phenyl in series *A*, *D*, and *E*.

A peculiarity in the isoamyl hydrophobic bonding still remains in the *B* series. The isoamyl group (XVI*B*) binds fourfold better than the *n*-amyl group (XV*B*) and thirtyfold better than the *n*-butyl group (XIII*B*); however, as discussed previously under (*a*), maximum straight-chain hydrophobic bonding is not reached in the *B* series until *n*-amyl. Furthermore, in the *B* series, the *n*-octyl group (XVIII*B*) causes an eightfold loss in the maximal straight-chain hydrophobic bonding, but little change in binding from *n*-butyl (XIII) to *n*-octyl (XVIII) occurs in the *A* or *E* series where maximal straight-chain hydrophobic bonding is reached with the *n*-butyl group.

The 3,3-dimethylalkyl side-chain (XIV) was selected as one fixed in a high-energy eclipsed conformation similar to benzene (XIX) and was synthesized in the 2,6-diamino-4-pyrimidinol series *A*; however, the angle between the $C_2$-$C_1$ and the $C_1$-pyrimidine bonds in the dimethylallyl group is about 25° different from the corresponding angle with the 5-phenylpyrimidine. The 3,3-dimethylallyl pyrimidine (XIV*A*) was intermediate in activity between the *n*-butyl pyrimidine (XIII*A*) and isoamyl pyrimidine (XVI*A*), but was somewhat less effective than the phenyl pyrimidine (XIX*A*). Remaining to be studied are predictions of less active conformations such as *trans*-crotyl, in order to verify further the conformation of binding of the alkyl groups.

(*e*) In all but series *D*, introduction of a phenyl group (V) on the terminal carbon of the *n*-butyl group (XIII) gives a five- to ninefold tightening in binding, a matter of 900–1200 cal. in free energy. In series *D*, 74-fold better binding was observed; since the *D* series contained the least synthetically accessible compounds in Table I, there were not sufficient data to rationalize why there was one magnitude greater binding with V*D* than with the other members of structure V.

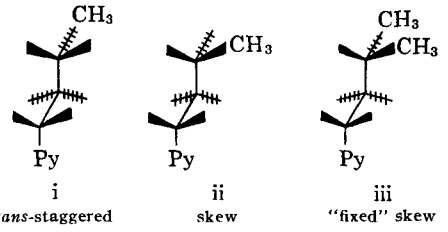
It is noteworthy that the *n*-octyl group (XVIII) in series *A*, *B*, and *D* gave little, if any, increase in binding to the enzyme; thus, these additional four carbons did not give the better binding seen when the phenyl group is attached to the butyl (V). If the phenyl group is binding to the enzyme by hydrophobic bonding, it is clear that there is insufficient binding to allow the second four carbon atoms of an *n*-octyl group to approach even a skew conformation in order to duplicate the conformation of benzene; of course, it is still possible that this benzene ring may complex with the enzyme in a charge–transfer complex (1, 15) which is not duplicatable by an alkyl group.

(*f*) The comparative increase in binding when the *n*-propyl side chain (XII) is terminally substituted by a benzyl group to give the 4-phenylbutyl group (V) is enlightening. In series *A*, *B*, and *C*, a 37–85-fold increase in binding was observed; this correlates with the 52-fold increase in binding when a benzyl group is placed on a 2,4-diamino-6-methylpteridine to give 2,4-diamino-6-phenethylpteridine (IV) (4) and helps explain why IV was a less effective inhibitor than anticipated before it was evaluated. As discussed at the beginning of this paper, this was one of the major discrepancies that led to the current hydrophobic bonding study.

In series *E*, compound V*E* gave an abnormally high increment of 270-fold in binding compared to the *n*-propyl derivative (XII*E*); most likely this high increment is due to the larger than expected increment between the *n*-butyl (XIII*E*) and *n*-propyl (XII*E*) groups as discussed under (*b*).

(*g*) In an earlier paper in this series, the 1-(*m*-benzylphenyl)-triazine (XXII*E*) and 1-(*m*-phenethylphenyl)-triazine (XXIII*E*) were synthesized and evaluated as inhibitors of dihydrofolic reductase (16). It had been anticipated that since the phenylbutyl side chain (V) in series *B* gave 27-fold better binding than the anilinopropyl side chain (VII*B*) that perhaps about 27-fold better binding would be

[2] The relationship of the *trans*-staggered butyl, skewed butyl, and fixed skewed isoamyl may be more easily seen as depicted in i, ii, and iii, respectively (14), where Py is the pyrimidine ring.




i *trans*-staggered ii skew iii "fixed" skew

observed when the phenethyl (XXIII*E*) or benzyl (XXII*E*) groups were placed on the 3-position of the 1-phenyl-triazine (XIX*E*). The actual observed increment in binding was about sixfold. With the concept that the *n*-butyl group of XIII*E* in a skewed conformation may bind to the same locus as the 1-phenyl group (XIX*E*), as discussed under (*c*), it would become clear why only a fivefold increment was observed. Note that placement of a phenyl group (V*E*) on the terminal carbon of the *n*-butyl-triazine (XIII*E*) gave a ninefold increment in binding, which is in agreement with the sixfold increment observed with XXII*E* and XIX*E*. Also note that the *m*-methyl group (XXI*E*) makes little contribution to binding when placed on the 1-phenyl-triazine (XIX*E*).

(*h*) An interesting discrepancy occurs when one compares the difference in binding of the phenylbutyl (V) and phenylpropyl groups (VI) as the heterocycle is varied from *A* to *E*. In the *C* and *E* series, the two moieties give about the same binding; in contrast, in series *A*, *B*, and *D*, the phenylbutyl side chain gives five- to eightfold better binding than the phenylpropyl side chain. These differences cannot be correlated with the basicity of the pyrimidine since the order of increasing basicity is *C* less than *D* less than *E*. It is clear that the conformational aspects of pyrimidine complexing with enzyme differ; whether this is due to different conformational changes in the enzyme or a rotation of the pyrimidine in its binding locus [see (*a*)] cannot yet be differentiated.

(*i*) It has been reported (1) that the *n*-butylaminopropyl side chain gives a compound (XXIV*B*) that is an extremely poor inhibitor. By comparison with other data in Table I on hydrophobic bonding, it is obvious that not only does hydrophobic bonding not occur with the propyl part of *n*-butylaminopropyl side chain of XXIV*B*, but that there is actually a repulsion to binding; since the *n*-butylamino group is protonated at the pH of the enzyme assay and is probably also solvated in water solution, possible repulsion of the compound from the hydrophobic area of the enzyme should by now not be too surprising. Furthermore, even the presence of a benzene ring on a protonated side chain such as the benzylaminopropyl side chain (XXV*B*) fails to give detectable inhibition; however, inhibitor properties return when the protonated amino group of XXV*B* is converted to an *N*-acetyl derivative (17).

(*j*) In view of the hydrophobic bonding of the *n*-butyl moiety of the 5-(4-phenylbutyl)pyrimidines (V) it is now possible to give a better rationalization of why the phenylbutyl pyrimidine (V*B*) is a 27-fold better inhibitor of dihydrofolic reductase than the anilinopropyl pyrimidine (VII*B*) (1). It was previously rationalized that the benzyl moiety of a phenylbutyl group would be a better electron acceptor than an anilino group in a charge–transfer complex (1). Although this mode of binding of the benzene may still be tenable, the effects of other substitutions of the benzene ring have so far been much smaller (17, 18) than the 27-fold increment in binding observed in the comparison of V*B* and VII*B*. Not only is one of the methylenes of V*B* missing in VII*B* that probably contributes to hydrophobic bonding, but in addition, there may be weak repulsion of the relatively polar NH group of the anilino moiety from the hydrophobic region of the enzyme. This repulsion is apparently weakened when the anilino NH group is acetylated since the resultant compound is about twice as effective (1). If it is assumed that the benzene ring of the anilinopropyl pyrimidine (VII*B*) gives a fivefold increment in binding, then the remaining aminopropyl moiety contributes only about as much hydrophobic bonding as an ethyl (X*B*) group attached to the pyrimidine.

(*k*) No rational reason for the increase in effectiveness of the tosylamidopropyl side chain (XXVI*B*), when an *N*-butyl group was inserted (XXVII*B*), was apparent (18) prior to the current study. That the hydrophobic bonding by the *N*-butyl group did not account for the better binding of XXVII*B* was readily eliminated (18). The acidic —$SO_2$NH— group, although not ionized at pH 7.4, can probably solvate with water in which case it could be repulsed from the hydrophobic region, thus accounting for the loss in binding.

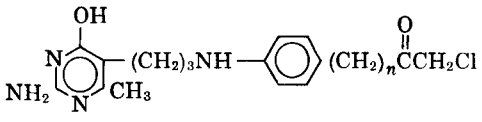
## DISCUSSION

Even though the discovery of the hydrophobic bonding of the alkylpyrimidines (*A*–*E*) to dihydrofolic reductase has led to firmer rationalizations on the mode of binding of a number of types of inhibitors to this enzyme, it has opened up a Pandora's box of new problems, the solutions to which are currently being pursued.

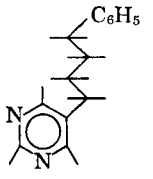
Probably the most serious problem from the standpoint of the design of an effective active-site-directed irreversible inhibitor (19) for dihydrofolic reductase is whether the phenylbutyl group of V and the anilinopropyl group of VII bind in such a way *via* the hydrophobic region that the aryl group is at the same locus as the *p*-aminobenzoyl moiety of folic acid; if the anilino group were complexed to a different locus, then an inhibitor of type XXVIII (15) would not have its alkylating function sufficiently neighboring to the binding points for the glutamate moiety of folic acid to alkylate such binding points irreversibly. Such a proposition is as yet almost impossible to prove unequivocally; therefore, only a massive amount of circumstantial evidence can be accumulated to make either one binding region for phenyl tenable or two different binding regions for phenyl tenable. Attempts are being made to accumulate some 18 lines of evidence, some of which have been presented here.

If only one binding region for phenyl of the phenylbutyl group is on the enzyme, then two possible skew conformations for hydrophobic bonding can be depicted (XXX, XXXI),[1] providing that an aryl group attached to a pyrimidine (XX*D*) or triazine (XIX*E*) is binding hydrophobically as assumed under (*c*); it should be possible not only to differentiate between these two modes of binding (XXX and XXXI) with properly constructed bicyclic derivatives, but also to obtain evidence against some other conformations of binding such as the most stable zigzag *trans*-planar conformation, XXIX.

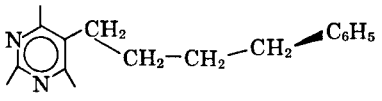
If only one locus for complexing the benzene ring of a phenylbutyl group is present on dihydrofolic reductase, then the phenyl group should be out of the near plane of the skewed butyl moiety, as depicted in XXX or XXXI. The aryl group of a 1-aryltriazine such as XIX*E* must be nearly coplanar with the triazine ring to have biological activity (20–23).



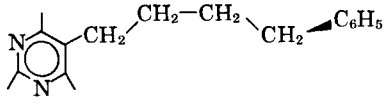
XXVIII



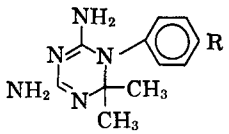
XXIX



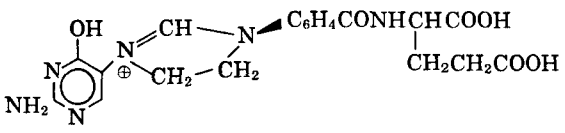
XXX



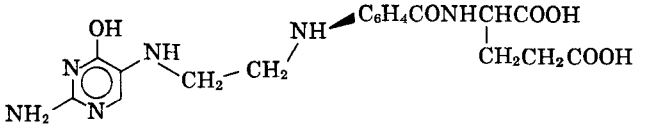
XXXI



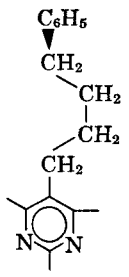
XIX*E*, R=H
XXXII, R=$C_6H_5$—



XXXIII



XXXIV



XXXV

Therefore, the imidazoline analog, recently reported by Tong *et al.* (24), would more likely have its double bond as indicated in XXXIII; this would place the *p*-benzoyl moiety out of the plane with the planar pyrimidyl-imidazoline moiety. Since XXXIII with its fixed angle of the benzene ring is as good an inhibitor of dihydrofolic reductase as the related compound without the anhydroformyl group (XXXIV) (24) (providing XXXIII was not converted to *N*-formyl XXXIV under the assay conditions) and since the phenyl moiety of XXXIII most probably binds to the enzyme, it is proposed that the enzyme has a contour that closely fits XXXIII in its two planes. Evidence that the enzyme has the contour proposed has been gained by measuring the 4-biphenyl triazine (XXXII) as an inhibitor. The introduction of this phenyl group in XXXII loosens binding by a factor of 1800-fold (25), indicating that the outside phenyl group does not even allow the inside phenyl group to bind to the enzyme due to the contact of the outside phenyl group with the bent contour of the enzyme as proposed. In contrast, note that the *m*-benzylphenyl triazine (XXII*E*) has an added increment in binding compared to the unsubstituted-phenyl triazine (XIX*E*), since the methylene group can allow the outside phenyl to assume a nonplanar conformation with respect to the inside phenyl group. Unfortunately, it cannot be ascertained at this time that the hydrophobic region is between the pyrimidyl and *p*-aminobenzoyl-L-glutamic moieties as depicted in XXX and XXXII.

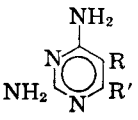
If additional evidence supports an inhibitor conformation of binding other than XXX or XXXI, such as XXIX or if the pyrimidine is rotated in its locus to give hydrophobic bonding as depicted in XXXV, then the question arises whether a pyrimidine derivative could be constructed that would bind to both the *p*-aminobenzoyl locus and the phenylbutyl locus. Thus, the conformation of either one aryl binding locus or two aryl binding loci for a phenylbutyl group and the *p*-aminobenzoyl moiety could have practical applications.

Further evidence for the proposed skew butane hydrophobic bonding should be obtained by evaluation of branched derivatives other than isoamyl and by evaluation of properly branched cycloalkane derivatives.

Some of the discrepancies in the mode of binding of 6-arylpyrimidines to folic reductase (26) have been previously enumerated (27). In view of the current observations on the hydrophobic bonding phenomenon, the mode of binding of the 6-phenylpyrimidines is being reinvestigated from the hydrophobic bonding viewpoint.

Another question to be answered is whether there is a similar type of hydrophobic bonding with folic cofactor enzymes (28, 29) other than folic reductase. Since the anilinopropyl (VII*B*) and phenylbutyl pyrimidines (V*B*) are inhibitors of thymidylate synthetase (1), the *n*-butyl pyrimidine (XIII*B*) was investigated as an inhibitor of this enzyme; in contrast to VII*B* and V*B*, which showed 50% inhibition at an inhibitor–substrate ratio of 50 and 35, respec-

TABLE II.—PHYSICAL CONSTANTS OF 5-ALKYL-2,4-DIAMINO-6-R′-PYRIMIDINES



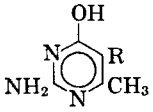
| Compd. | R′ | R | Yield,[a] % | M.p., °C. | Anal. Calcd. | Anal. Found |
|---|---|---|---|---|---|---|
| X*A* | OH | $C_2H_5$— | 43 | 289–291[b, c] | C, 46.7<br>H, 6.54<br>N, 36.3 | C, 46.5<br>H, 6.55<br>N, 36.4 |
| XII*A* | OH | *n*-$C_3H_7$— | 47 | 238–240[c] | C, 50.0<br>H, 7.19<br>N, 33.3 | C, 49.9<br>H, 7.37<br>N, 33.1 |
| XIII*A* | OH | *n*-$C_4H_9$— | 50 | 256–258[c] | C, 52.7<br>H, 7.74<br>N, 30.8 | C, 52.6<br>H, 7.77<br>N, 31.0 |
| XIV*A* | OH | $(CH_3)_2C{=}CHCH_2$— | 27 | 276–278 dec.[c] | C, 55.6<br>H, 7.27<br>N, 28.8 | C, 55.5<br>H, 7.30<br>N, 28.6 |
| XVI*A* | OH | *i*-$C_5H_{11}$— | 20[d] | 260–261[c] | C, 55.1<br>H, 8.22<br>N, 28.6 | C, 54.9<br>H, 8.13<br>N, 28.7 |
| XVII*A* | OH | *n*-$C_6H_{13}$— | 46 | 216–217[b, c] | C, 57.1<br>H, 8.63<br>N, 26.6 | C, 56.9<br>H, 8.61<br>N, 26.8 |
| XVIII*A* | OH | *n*-$C_8H_{17}$— | 52 | 184–185[b, c] | C, 60.5<br>H, 9.30<br>N, 23.5 | C, 60.4<br>H, 9.50<br>N, 23.4 |
| XIX*A* | OH | $C_6H_5$— | 16[e] | 283–285 dec.[i] | C, 58.3[i]<br>H, 6.18<br>N, 23.7 | C, 58.5<br>H, 6.24<br>N, 23.8 |
| XII*C* | $NH_2$ | *n*-$C_3H_7$— | 49 | 185–186[c] | C, 50.3<br>H, 7.84<br>N, 41.9 | C, 50.4<br>H, 7.90<br>N, 41.8 |
| XIII*C* | $NH_2$ | *n*-$C_4H_9$— | 46 | 205–206[c, f] | ... | ... |
| XVI*C* | $NH_2$ | *i*-$C_5H_{11}$— | 52 | 144–145[c] | C, 55.4<br>H, 8.78<br>N, 35.9 | C, 55.4<br>H, 8.74<br>N, 36.0 |
| VI*D* | $CH_3$ | $C_6H_5(CH_2)_3$— | 41 | 135–136[c] | C, 69.4<br>H, 7.49<br>N, 23.1 | C, 69.6<br>H, 7.60<br>N, 23.2 |
| XIII*D* | $CH_3$ | *n*-$C_4H_9$— | 35[g] | 150–152[h] | C, 60.0<br>H, 8.95<br>N, 31.1 | C, 60.1<br>H, 9.09<br>N, 31.3 |
| XV*D* | $CH_3$ | *n*-$C_5H_{11}$— | 47 | 142–144[c] | C, 61.8<br>H, 9.34<br>N, 28.8 | C, 62.1<br>H, 9.50<br>N, 29.0 |
| XVI*D* | $CH_3$ | *i*-$C_5H_{11}$— | 24 | 163–164[c] | C, 61.8<br>H, 9.34<br>N, 28.8 | C, 62.0<br>H, 9.32<br>N, 28.8 |

[a] Over-all yield of analytically pure material for two steps from ethyl cyanoacetate (series *A*) (35), malononitrile (series *C*) (35), or a 5-alkyl-2-amino-6-methyl-4-pyrimidinol (series *B* → series *D*) (4) by the procedures previously described. All compounds had ultraviolet and infrared spectra in agreement with their assigned structures; each compound in series *A* and *C* moved as a single spot on thin-layer chromatography on Brinkmann Silica Gel G in one of the following solvent systems: A, absolute ethanol; B, methyl ethyl ketone–*tert*-butyl alcohol (3:4). [b] Purified through the potassium salt which was insoluble in 1 *N* aqueous potassium hydroxide. [c] Recrystallized from aqueous ethanol. [d] Minimum yield due to mechanical loss. [e] Yield based on commercially available ethyl α-phenylcyanoacetate. [f] Lit. m.p. 199° (36). [g] The intermediate 2-amino-4-chloropyrimidine had m.p. 156–157° after recrystallization from aqueous ethanol. *Anal.*—Calcd. for $C_8H_{14}ClN_3$: C, 54.1; H, 7.07; N, 21.0. Found: C, 53.8; H, 6.86; N, 21.3. [h] Recrystallized from water. [i] Obtained as an ethanol solvate from 90% ethanol. Even after being dried at 100° in high vacuum for about 18 hr., the compound still had combustion values agreeing with 0.75 mole of ethanol. The presence of 0.75 mole of ethanol was verified by the NMR spectrum in $D_2O$ containing 1 *N* NaOH; the ratio of five phenyl protons at δ 7.37 to three methyl protons at δ 1.13 was in a ratio of 7:3.

tively (1), XIII*B* failed to show any inhibition at an inhibitor–substrate ratio of 100 (the maximum solubility) and thus was less than one-eleventh as effective as V*B*. Since it is therefore highly unlikely that thymidylate synthetase has any hydrophobic bonding capacity in the same region observed with dihydrofolic reductase, it now becomes relatively simple to design inhibitors that are more selective on dihydrofolic reductase than thymidylate synthetase; the converse, the probable hydrophobic repulsion from dihydrofolic reductase observed with the tosylamidopropyl side chain (XXVI*B*), can account for why XXVI*B* is considerably more effective on thymidylate synthetase than dihydrofolic reductase.

Appropriate for discussion at this point is whether the hydrophobic region is identical for the dihydrofolic reductases from different species. Considerable evidence is already available to support the idea that the hydrophobic bonding region can differ considerably among species. The outstanding success that Hitchings and his co-workers (30, 31) have had over the past 15 years with substituted 2,4-diamino-

TABLE III.—PHYSICAL CONSTANTS OF 5-ALKYL-2-AMINO-6-METHYL-4-PYRIMIDINOLS



| Compd. | R | Yield,[a] % | M.p., °C. | Anal. Calcd. | Anal. Found |
|---|---|---|---|---|---|
| X*B* | $C_2H_5$— | 42[b] | 284–286[c] | C, 54.9<br>H, 7.24<br>N, 27.4 | C, 54.6<br>H, 7.42<br>N, 27.1 |
| XI*B* | $CH_2$=$CHCH_2$— | | 275 dec.[b, d] | C, 58.2<br>H, 6.71<br>N, 25.4 | C, 58.0<br>H, 6.80<br>N, 25.3 |
| XII*B* | *n*-$C_3H_7$— | 52[e] | 271–274[f] | C, 57.5<br>H, 7.83<br>N, 25.1 | C, 57.6<br>H, 7.62<br>N, 25.1 |
| XIII*B* | *n*-$C_4H_9$— | 42[g] | 266–268[f] | C, 59.7<br>H, 8.34<br>N, 23.2 | C, 59.6<br>H, 8.31<br>N, 23.3 |
| XV*B* | *n*-$C_5H_{11}$— | 54 | 260–262[h] | C, 61.5<br>H, 8.78<br>N, 21.5 | C, 61.6<br>H, 8.96<br>N, 21.7 |
| XVI*B* | *i*-$C_5H_{11}$— | 53[i] | 270–271[h] | C, 61.5<br>H, 8.78<br>N, 21.5 | C, 61.3<br>H, 8.67<br>N, 21.3 |
| XVII*B* | *n*-$C_6H_{13}$— | 43 | 253–255[h] | C, 63.1<br>H, 9.15<br>N, 20.1 | C, 62.9<br>H, 9.01<br>N, 19.8 |
| XVIII*B* | *n*-$C_8H_{17}$— | 32[i] | 222–223[h] | C, 65.8<br>H, 9.76<br>N, 17.7 | C, 66.0<br>H, 9.90<br>N, 17.5 |

[a] Over-all yield of analytically pure material for two steps from ethyl acetoacetate, unless otherwise indicated. The alkylation of ethyl acetoacetate with excess bromoalkane was performed in dimethyl sulfoxide with one equivalent of sodium hydride (35) at ambient temperature until neutral; unchanged ethyl acetoacetate was removed by washing a chloroform solution with excess ice-cold aqueous sodium hydroxide, then the crude keto ester was condensed with guanidine hydrochloride and sodium methoxide in absolute ethanol. All the compounds had $\lambda_{max.}$ pH 1: 268 mμ, pH 7: 275 mμ, pH 13: 282 mμ and had infrared spectra in agreement with their assigned structures. [b] Yield from commercially available ethyl α-alkylacetoacetate. [c] Recrystallized from aqueous methanol. [d] Lit. (43) m.p. 266–267°. [e] Prepared by catalytic hydrogenation of X*IB* in 2-methoxyethanol with palladium–charcoal catalyst. [f] Recrystallized from methanol. [g] Alkylation of ethyl acetoacetate with *n*-butyl bromide performed in *tert*-butyl alcohol (1). [h] Recrystallized from aqueous ethanol. [i] Alkylation reaction in dimethyl sulfoxide run at 90° until neutral.

pyrimidines, 4,6-diamino-1,2-dihydro-*s*-triazines, and 2,4-diaminoquinazolines as useful chemotherapeutics for a number of infectious diseases is probably related to species differences in the mode of hydrophobic bonding by the respective dihydrofolic reductases.

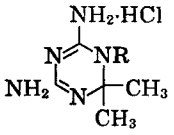
Regardless of whether the aryl group of a 1-aryltriazine such as XX*E* or a 5-arylpyrimidine such as XX*D* binds to dihydrofolic reductase by hydrophobic bonding [*cf.* (*c*)] or by some other means (32), species differences have already been observed (23, 31). It would therefore be worthwhile to investigate whether there are species differences in the mode of binding of alkyl pyrimidines, such as those in Table I, to the respective dihydrofolic reductases. These differences, if found, would have obvious chemotherapeutic applications. It is interesting to note that in 1952 Hitchings *et al.* (33) intuitively predicted the nonclassical antimetabolite concept (19) as applied to the present hydrophobic bonding study when they stated the following:

"Minor changes in their [2,4-diaminopyrimidines] fine chemical structure may adapt them to close approximations of the geometry and distribution of charge on protein surfaces of particular species and tissues. An analog more nearly related structurally to the metabolite [folic acid] would be less capable of species differentiation, because it would possess a greater number of features and centers, which are points of union common to the receptors of all species, and it would be these, rather than minor or incidental differences in chemical structure, which would primarily determine the dissociation of the cell receptor–analogue complex. In this view, therefore, the successful application of antimetabolites to problems of chemotherapy is dependent for success on a differential affinity of antimetabolite for the receptors of the parasite owing to details of structure in parasite and host which are largely indefinable and unpredictable. . . . The diaminopyrimidines are particularly suitable for these adaptions because of the high degree of specificity attainable through modifications of the substituents of the pyrimidine at the 5 and 6 positions."

Thirteen years later one need only to add that the "minor and incidental differences in chemical structure" can also change the dissociation of the enzyme–inhibitor complex if extra forces due to hydrophobic bonding or repulsion are present.

The concept of hydrophobic bonding to dihydrofolic reductase suggests another way in which species specificity may be achieved in addition to an increase or decrease in hydrophobic bonding *per se;* the reverse concept of hydrophobic repulsion would be worth exploring to see if a polar group on a side chain (such as XXIV*B* or XXV*B*) could be positioned in such a way that repulsion occurs from the hydrophobic region of the host dihydrofolic reductases, but not the dihydrofolic reductase of an invading cell, whether parasitic or cancerous.

TABLE IV.—PHYSICAL CONSTANTS OF 1-ALKYL-4,6-DIAMINO-1,2-DIHYDRO-2,2-DIMETHYL-*s*-TRIAZINE HYDROCHLORIDES



| Compd. | R | Yield,[a] % | $R_f$[b] | M.p., °C. | Anal. Calcd. | Anal. Found |
|---|---|---|---|---|---|---|
| IX*E* | $CH_3$ | 30[c] | 0.12 (A)<br>0.73 (E) | 209–211 | C, 37.6<br>H, 7.36<br>N, 36.5 | C, 37.6<br>H, 7.31<br>N, 36.7 |
| XII*E* | *n*-$C_3H_7$— | 19[d] | 0.27 (A)<br>0.84 (D) | 195–196 | C, 43.7<br>H, 8.25<br>N, 31.9 | C, 43.9<br>H, 8.35<br>N, 31.8 |
| XIII*E* | *n*-$C_4H_9$—[e] | 16[d, f] | 0.48 (A)<br>0.87 (C) | 196–198 | C, 46.2<br>H, 8.62<br>N, 30.0 | C, 46.4<br>H, 8.72<br>N, 30.2 |
| XVI*E* | *i*-$C_5H_{11}$— | 18[g] | 0.37 (A) | 193–195 | C, 48.5<br>H, 8.95<br>N, 28.3 | C, 48.5<br>H, 9.17<br>N, 28.1 |
| XVII*E* | *n*-$C_6H_{13}$— | 15[d] | 0.67 (A) | 186–187 | C, 50.5<br>H, 9.24<br>N, 26.8 | C, 50.5<br>H, 9.19<br>N, 26.9 |
| XVIII*E* | *n*-$C_8H_{17}$— | 15[h] | 0.70 (A) | 203–204 | C, 53.9<br>H, 9.74<br>N, 24.2 | C, 54.0<br>H, 9.82<br>N, 24.0 |
| XXI*E* | 3-$CH_3C_6H_5$— | 43[i] | | 202–204 | ... | ... |

[a] Yield of analytically pure, chromatographically homogeneous product, prepared as previously described (16). All compounds had $\lambda_{max.}$ 245 mμ in water and 252 mμ at pH 13; they had infrared spectra in agreement with their assigned structures. Paper chromatograms were run on Whatman No. 1 paper by the ascending technique in the following solvent systems (37): A, *tert*-butyl alcohol–methyl ethyl ketone–water–formic acid (44:44:11:0.27); B, *tert*-butyl alcohol–methyl ethyl ketone–water–formic acid (40:30:15:15); C, *n*-butyl alcohol–acetic acid–water (5:2:3); D, ethyl acetate–acetic acid–water (70:20:10); E, isopropyl alcohol–water–12 *N* HCl (130:37:33). All compounds were recrystallized from absolute alcohol–petroleum ether, b.p. 60–110°. [c] See *Reference 38* for intermediate alkyl biguanide (XXXVIII). [d] See *Reference 39* for intermediate alkyl biguanide (XXXVIII). [e] The isomeric 4-amino-6-(*n*-butylamino)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride (XXXIX, R = *n*-$C_4H_9$—) could be isolated from the filtrate of XIII*E* [see (40)]; recrystallization from absolute ethanol–petroleum ether (b.p. 60–110°) gave a 48% yield of white crystals, m.p. 145–146°; $R_f$ 0.74 (A), 0.92 (C); $\lambda_{max.}$ $H_2O$: 239, pH 13: 244 mμ. *Anal.*—Calcd. for $C_9H_{19}N_5 \cdot HCl$: C, 46.2; H, 8.62; N, 30.0. Found: C, 46.0; H, 8.59; N, 29.7. [f] See *Reference 41* for the intermediate alkyl biguanide (XXXVIII). [g] See *Reference 42* for the intermediate alkyl biguanide (XXXVIII). [h] The intermediate *n*-octyl biguanide (XXXVIII) was obtained in 36% yield, m.p. 130–133°, after recrystallization from isopropyl alcohol–ether when prepared as previously described. [i] Prepared by "three-component" method of Modest (20). [Lit. m.p. 212° (44).]

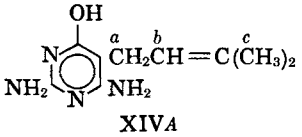
It is clearly within the realm of possibility that further new concepts on specificity will arise as additional observations are made on hydrophobic bonding with the dihydrofolic reductases.

## Chemistry

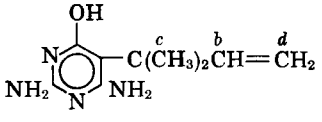
**Methods.**—The synthesis of V*A* (35), V*B* (1), V*C* (35), V*D* (4), and V*E* (16) has been described previously (Table I). All the compounds in Table I were made by these respective methods. However, comments on a few special cases should be made. Compound XIV*A* was synthesized by alkylation of

$(CH_3)_2C{=}CHCH_2Cl \rightarrow$

XXXVI



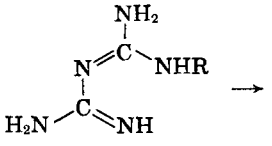
XIV*A*



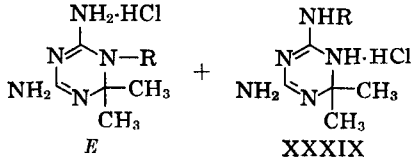
XXXVII

sodio ethyl cyanoacetate with 1-chloro-3-methyl-2-butene (XXXVI) in dimethyl sulfoxide, followed by condensation with alcoholic guanidine. Although it was considered highly unlikely that the rearranged 1,1-dimethylallylpyrimidine (XXXVII) had been obtained by an allylic rearrangement during the alkylation reaction with XXXVI, the structure of XIV*A* was checked by its NMR in 1 *N* NaOH in deuterium oxide; XIV*A* showed a triplet ($J_{ab}$ 6.5 c.p.s.) centered at $\delta$ = 5.06 for the methine protons, a doublet ($J_{ba}$ 6.5 c.p.s.) centered at $\delta$ = 3.0 for the methylene proton, and a singlet at $\delta$ = 1.67 for the methyl protons. The terminal methylene group of the isomeric XXXVII should appear at $\delta$ = 4.66. (See Tables II and III.)

The dihydro-*s*-triazines (series *E*) are synthesized



XXXVIII



*E* XXXIX

by condensative cyclization of an alkyl biguanidine (XXXVIII) with acetone (16). Since yields are usually low in this reaction (15–30%) and since the isomeric cyclization product bearing a 6-alkylamino group (XXXIX) can be formed (40), the products were carefully examined to see that they had structure *E*. This was accomplished by isolation of the isomeric 6-butylamino-*s*-triazine (XXXIX, R = *n*-$C_4H_9$) from the mother liquors of XIII*E*. The two isomeric *n*-butyl triazines were readily distinguishable by paper chromatography in solvent A (Table IV). Furthermore, XXXIX (R = *n*-butyl) had its ultraviolet maximum at 239 mμ in water and 244 mμ at pH 13, whereas XIII*E* had a maximum at 245 mμ in water and 252 mμ at pH 13; when XIII*E* was heated with about 1 *N* sodium hydroxide for 1 hr., the peaks shifted to those of XXXIX (R = *n*-$C_4H_9$), whereas the same treatment of XXXIX (R = *n*-$C_4H_9$) caused no spectral shift. All of the compounds in Table IV had an ultraviolet maximum at 245 mμ in water and moved as a single spot in solvent A and other paper chromatographic systems. The isomeric *n*-butylamino triazine (XXXIX, R = *n*-$C_4H_9$) gave 50% inhibition of dihydrofolic reductase at a concentration of 7.8 μ*M*; whether this 22-fold difference is due to the inherent activity of XXXIX (R = *n*-$C_4H_9$) or results from a 5% contamination with XIII*E* was not readily ascertained.

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