Analogs of Tetrahydrofolic Acid. X.[1,2] Synthetic and Enzymic Studies on the Contribution of the *p*-Aminobenzoyl-L-glutamate Moiety of Pyrimidyl Analogs to Binding to Some Folic Cofactor Area Enzymes

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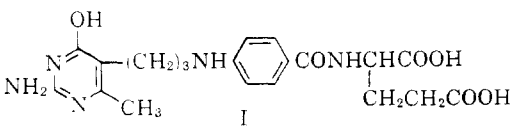
Received July 12, 1963

The pyrimidyl analog of tetrahydrofolic acid, N-(2-amino-4-hydroxy-6-methyl-5-pyrimidylpropyl)-*p*-aminobenzoyl-L-glutamic acid (I), is an inhibitor of both folic reductase and 5,10-methylenetetrahydrofolate dehydrogenase. Six compounds related to I with changes in the *p*-aminobenzoyl-L-glutamate moiety were synthesized, namely, by removal of the carboxy-L-glutamate fragment (XII), removal of the L-glutamate fragment (XIII), removal of the γ-carboxyl and the adjoining two carbons (XIV), removal of the α-carboxyl (XV), by conversion of the γ-carboxyl to an amide (XVI), and by changing the bridge between the *p*-amino group and the pyrimidine moiety from an amine to an amide (IX). The seven compounds were compared as inhibitors of folic reductase and 5,10-methylenetetrahydrofolate dehydrogenase in order to determine which groups of the *p*-aminobenzoyl-L-glutamate moiety were required for enzymic binding. The groups necessary for complexing were remarkably different for the two enzymes; the main contribution by the moiety to binding to the reductase was the *p*-amino group with lesser contribution by the carboxy-L-glutamate fragment; the opposite was true for the dehydrogenase.

Since some fifteen or more enzymes that use folic acid, tetrahydrofolic, or derivatives of tetrahydrofolic used for "one-carbon" transfer reaction are known,[3-5] a program was initiated on synthesis and evaluation of analogs of tetrahydrofolic acid. A rationale for,[6] and a synthesis of,[7,8] a pyrimidyl analog (I) of tetrahydrofolic acid has been described. Since I could inhibit some of these enzymes, we embarked on the synthesis and enzymic evaluation of additional analogs that might shed light on the mode of binding of the *p*-aminobenzoyl-L-glutamate moiety of I to these enzymes; the resultant findings are the subject of this paper.



I

The analog of I containing an amide bridge (IX) between the *p*-aminobenzoyl-L-glutamate and pyrimidyl moieties was synthesized for two reasons. First, IX would give insight on whether or not the basic *p*-amino group of the *p*-aminobenzoyl-L-glutamate was necessary for binding of I to a given enzyme. Second, if this basic amino group was not necessary for binding, the sequence envisioned for the synthesis of IX would have operational advantages over the sequence for I for synthesis of certain compounds related to I.

In order to evaluate the contribution to enzymic binding by the carboxy-L-glutamate portion of I, analogs XII–XVI were prepared by use of the appropriate aniline derivative (XI) in a reductive condensation with 2-acetamido-4-hydroxy-6-methyl-5-pyrimidylpropionaldehyde.[7,8]

Chemistry.—Ethyl α-(2-cyanoethyl)acetoacetate (III), obtained by Michael addition of ethyl acetoacetate to acrylonitrile,[9] smoothly condensed with guanidine hydrochloride in ethanolic sodium ethoxide to give a 67% yield of crystalline 2-amino-4-hydroxy-6-methyl-5-pyrimidylpropionitrile (V). Hydrolysis of the nitrile group with boiling 6 *N* hydrochloric acid afforded the corresponding propionic acid (IV), in 86% yield.

Two possible routes were envisioned for activation of the carboxyl group of IV in order to form the final amide linkage in IX. Treatment of IV with acetic anhydride would acetylate the amino group[7,8]; the resultant compound was then expected to cyclize to lactone VI, an activated form of the carboxyl which could react with aromatic amines. The product obtained from IV and acetic anhydride had carbonyl absorption at 1780 cm.$^{-1}$ corresponding to that for a lactone, but also showed the presence of a second carbonyl at 1725 cm.$^{-1}$ suggesting a mixed anhydride structure with acetic acid. The latter structure could explain the failure of the mixed anhydride to give the N^2-acetyl derivative of IX; apparently the acetyl portion of the mixed anhydride reacts preferentially with the *p*-aminobenzoyl-L-glutamic acid. Successful activation of the carboxyl was accomplished by refluxing the acid (IV) with thionyl chloride containing a small amount of pyridine.[10] Although solution did not occur, in 0.5

(1) This work was supported in part by the U. S. Public Health Service through Grants CY-5867, CY-06624, 2G-555, and CA-5298.

(2) For the previous paper of this series see B. R. Baker and H. S. Shapiro, *J. Med. Chem.*, **6**, 664 (1963).

(3) M. Friedkin, *Ann. Rev. Biochem.*, **32**, 185 (1963).

(4) J. F. Holland, *Clin. Pharm. Therap.*, **2**, 374 (1961).

(5) T. H. Jukes and H. P. Broquist, in "Metabolic Inhibitors," edited by R. M. Hochster and J. H. Quastel, Academic Press, Inc., New York, N. Y., 1963, pp. 481–534.

(6) B. R. Baker, Preprints of the Scientific Session of the American Pharmaceutical Association, Las Vegas, Nevada, 1962; paper V of this series.

(7) B. R. Baker and C. E. Morreal, *J. Pharm. Sci.*, **51**, 596 (1962); paper VI of this series.

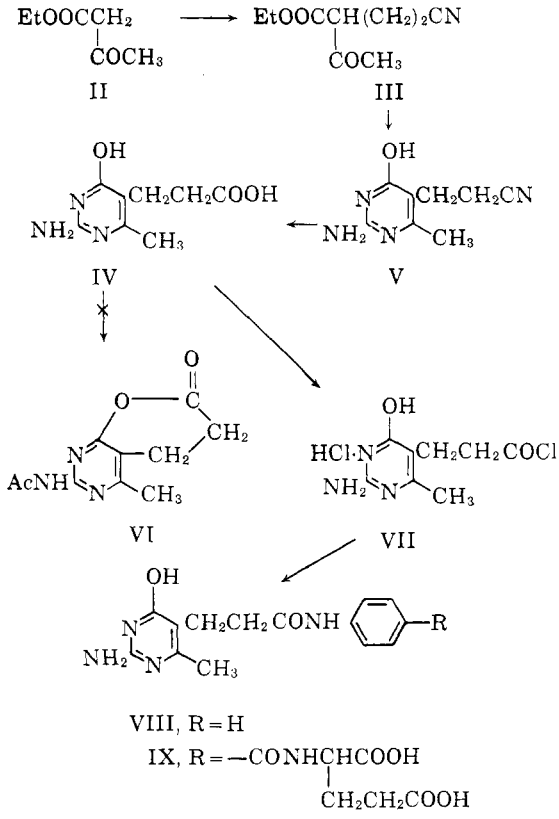
(8) B. R. Baker and C. E. Morreal, *ibid.*, **52**, 840 (1963); paper VII of this series.

(9) N. F. Albertson, *J. Am. Chem. Soc.*, **72**, 2594 (1950).

(10) A similar procedure for conversion of 2-amino-4-hydroxy-5,6,7,8-tetrahydroquinazoline-6-carboxylic acid to the acid chloride has been described by R. Koehler, L. Goodman, J. DeGraw, and B. R. Baker, *ibid.*, **80**, 5779 (1958); paper I of this series.

hr. IV was converted to 71% of the acid chloride hydrochloride (VII) as shown by the carbonyl absorption at 1790 cm.$^{-1}$ and C=NH$^+$ absorption at 1660 cm.$^{-1}$. The moisture-sensitive VII was immediately treated with aniline or *p*-aminobenzoyl-L-glutamic acid in pyridine to give VIII and IX in 81 and 36% yields, respectively.

The prototype pyrimidyl analog (I) of tetrahydrofolic acid was originally synthesized by the condensation of



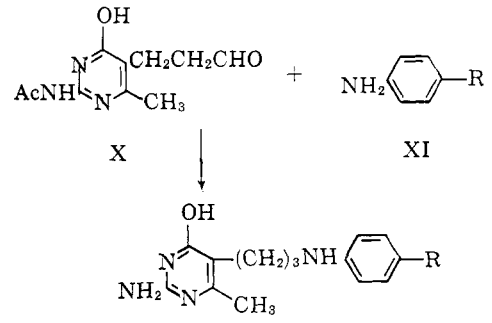
VIII, R = H

IX, R = —CONHCHCOOH
|
CH_2CH_2COOH

2-acetamido-4-hydroxy-6-methyl-5-pyrimidylpropionaldehyde (X) with *p*-aminobenzoyl-L-glutamic acid in dimethylformamide, followed by reduction of the intermediate anil with methanolic sodium borohydride[7,8]; the product (I) was difficult to purify since it was insoluble in all solvents except aqueous acid or base, or hot dimethylformamide, giving only 20–25% yields of pure product. Since the enzymic study required the synthesis of four analogs (XIII–XVI) derived from *p*-aminobenzoic acid, a detailed study of the variables in this two-step sequence was performed with *p*-aminohippuric acid to give XIV. The results of some 25 experiments can be summarized as follows.

(1) Increasing the quantities of, or time for, the borohydride reduction did not change the yield or purity; therefore reduction at room temperature with methanolic sodium borohydride for 16–20 hr. were used in all the reactions.

(2) Increasing the time of the anil condensation at 25° from 15 min. to 18 hr. or increasing the temperature to 80° for 2–4 hr. gave nearly double the crude yield; however, this crude product contained 23–25% of Bratton–Marshall positive material (diazotizable amine), calculated as the "anil", when treated with acid.[8] Actually this by-product hydrolyzable to *p*-aminobenzoyl-L-glutamic acid was not the anil since the anil appears to be readily reducible by borohydride; the nature of this nonreducible, acid hydrolyzable by-product is not known, and the relative amount was not decreased on reprecipitation of the crude product. Treatment of a crude product (25% "anil") with 3 *N* hydrochloric acid at 80° showed that 19% of the diazotizable amine was released immediately, then 5% more over the next 8 min. Neutralization gave an



I, R = CONHCHCOOH
|
$(CH_2)_2COOH$

XII, R = H

XIII, R = COOH

XIV, R = $CONHCH_2COOH$

XV, R = $CONH(CH_2)_3COOH$

XVI, R = CONHCHCOOH
|
$(CH_2)_2CONH_2$

analytically pure product, but the over-all yield was not improved. In addition, *p*-aminohippuric acid suffered a small amount of hydrolysis to glycine, as detected quantitatively by ninhydrin[11]; thus, a compound with an even more labile amide group such as XVI would not be compatible with the hot acid purification method.

(3) The use of dimethyl sulfoxide or dimethylformamide–acetic acid (10–1) as solvent for the anil formation gave decreased yields with an increased amount of Bratton–Marshall positive material.

(4) The optimum conditions for ease of purification and overall yield were formation of the anil in dimethylformamide for 15 min. at room temperature, followed by the addition of methanol and sodium borohydride, then allowing reduction to proceed for 16–20 hr.

These optimum conditions were used for synthesis of XIII–XVI in 20–30% yield of analytically pure material (Table II). Since these amorphous compounds failed to have definite melting points and gave poor infrared spectra, the extent of purification was based on the molecular extinction coefficient of the ultraviolet peaks, the Bratton–Marshall test, and combustion analyses.

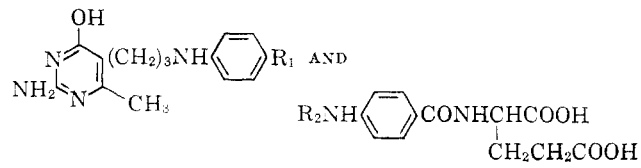
Since it was necessary to synthesize two of the starting *p*-aminobenzoyl derivatives, their description is warranted. γ-(*p*-Nitrobenzamido)butyric acid was obtained in 67% yield by reaction of γ-aminobutyric acid with *p*-nitrobenzoyl chloride in aqueous base. Catalytic reduction of the nitro group with a palladium catalyst afforded the desired γ-(*p*-aminobenzamido)-butyric acid in 50% yield; reduction of the nitro group with ferrous ammonium sulfate gave the pure compound in 60% yield.

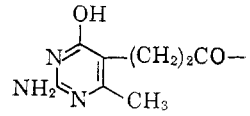
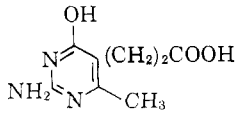
The preparation of *p*-nitrobenzoyl-L-glutamine from *p*-nitrobenzoyl-L-glutamic acid γ-ethyl ester and ammonia[12] proceeded poorly in this Laboratory. However, treatment of an aqueous solution of the sodium salt of L-glutamine in the presence of magnesium oxide with a chloroform solution of *p*-nitrobenzoyl chloride

(11) W. Troll and R. K. Cannan, *J. Biol. Chem.*, **200**, 803 (1953).
(12) F. E. King and P. C. Spencely, *J. Chem. Soc.*, 3159 (1950).

TABLE I

INHIBITION OF FOLIC REDUCTASE AND 5,10-METHYLENETETRAHYDROFOLIC DEHYDROGENASE BY



No.	R_1	R_2	Folic reductase[a] $K_i \times 10^6$	5,10-Methylenetetrahydrofolic dehydrogenase[b] mM concn. for 50% inhibition[c]	5,10-Methylenetetrahydrofolic dehydrogenase[b] Estimated[d] $K_i \times 10^4$
I	—CONHCHCOOH $(CH_2)_2COOH$		2.0	1.1	10
IX			160	0.47	4.4[e]
XII	H		63	[f]	
XIII	—COOH		13	8.5	80
XIV	—$CONHCH_2COOH$		13	4.5	42
XV	—$CONH(CH_2)_3COOH$		4.8	11	100
XVI	—CONHCHCOOH $(CH_2)_2CONH_2$		1.4	1.7	16
XXII		CH_3CO—	>2000	3.1	29
IV			>2000		

[a] Folic reductase from the liver of mature Holtzman rats was prepared and assayed as previously described[16]; K_i values were determined by the reciprocal plot method and all the compounds showed "competitive" kinetics. Folic acid had $K_m = 10 \times 10^{-6}$. [b] Pigeon liver acetone powder (Sigma) was stirred with 12 volumes of 0.05 *M* Tris buffer (pH 7.4)/g. and 1 g./g. of analytical grade Celite (Johns-Manville Co.) for 1 hr. in an ice bath; suction filtration through a pad of Celite into an ice-cooled receiver was rapid and gave 7 volumes of clear filtrate/g. Less preferable, the mixture containing no Celite could be clarified by centrifugation at 100,000*g*. The desired enzyme was stable for at least 1 month when the solution was kept frozen at −20°; usually 1 ml. aliquots were frozen in order to avoid repeated thawing and freezing. In the upper and lower compartments of a Cary 11 recording spectrophotometer were placed cuvettes containing 2.70 ml. of 0.05 *M* Tris buffer (pH 7.4), 100 λ of enzyme solution, and 0.20 ml. of a solution containing 0.48 m*M* tetrahydrofolate, 0.3 *M* mercaptoethanol, 5 m*M* Versene, 75 m*M* magnesium chloride, and 45 m*M* formaldehyde in 0.05 *M* Tris buffer (pH 7.4). The enzymic reaction was started by addition of 100 λ of 3.1 m*M* TPN to the upper cuvette, then the change in optical density at 340 mμ was recorded by use of a 0–0.1 optical density slide wire. The enzyme solution, prepared as above, showed an initial rate of about 0.03 optical density unit change/min. [c] 5,10-Methylenetetrahydrofolate had $K_m = 3.0 \times 10^{-5}$ *M* (average of 7 runs), when determined by the reciprocal plot method. The 50% inhibition values were determined by plotting V_0/V against I where V_0 = velocity without inhibitor, V = velocity at I concentration of inhibitor in the presence of 31 μ*M* 5,10-methylenetetrahydrofolate. [d] The estimated K_i values were calculated by the equation[26] $K_i = K_m \times I/S$, where I = inhibitor concentration necessary to give 50% inhibition ($V_0/V = 2$) and S was 31 μ*M* 5,10-methylenetetrahydrofolate. The estimated K_i values for IX and other compounds agreed with K_i values determined by the reciprocal plot method within a factor of 1.25 or less. [e] By the reciprocal plot method using 100 μ*M* TPN, 31, 15.5, and 10.3 μ*M* 5,10-methylenetetrahydrofolate, and 0.60 and 0.30 m*M* IX, K_i was found to be 3.6×10^{-4} and 3.4×10^{-4}, respectively; the kinetics were "competitive." [f] No inhibition was obtained at a cuvette concentration of 0.60 m*M* ($20 \times S$), the maximum solubility of XII at pH 7.4.

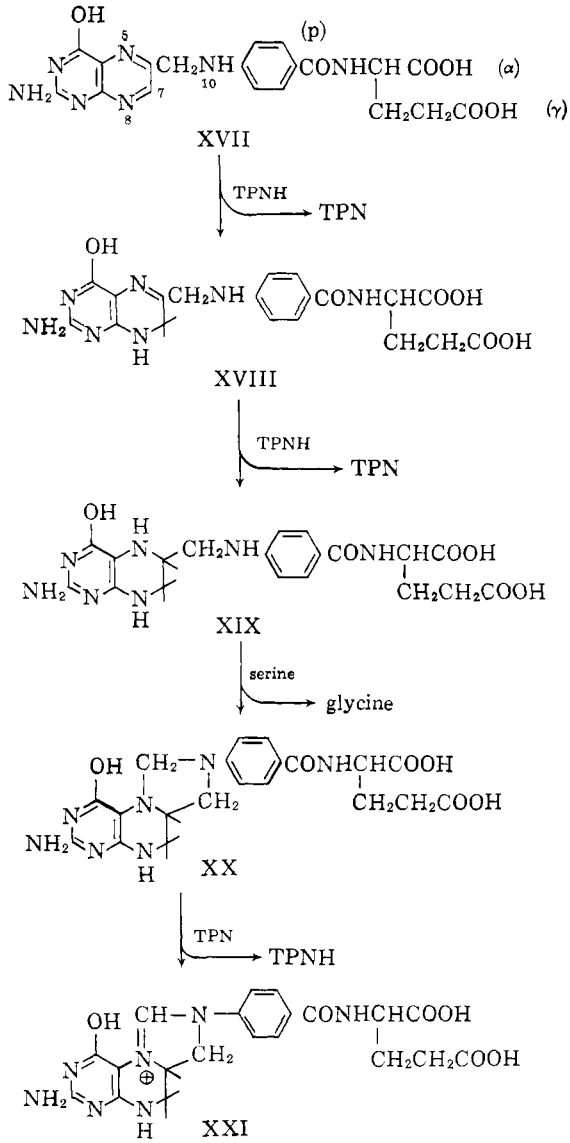
gave a 62% yield of pure *p*-nitrobenzoyl-L-glutamine. Catalytic reduction of the nitro group with a palladium catalyst[12] proceeded smoothly to give 87% of recrystallized *p*-aminobenzoyl-L-glutamine.

The last compound needed for enzymic studies, namely, the 5-(anilino-propyl)pyrimidine, XII, was readily prepared by reductive condensation of the pyrimidylpropionaldehyde (X) with excess aniline in 98% yield. This compound was the only member of the series that could be recrystallized from an organic solvent and then had a definite melting point (218°) presumably because of the lack of the *p*-carboxyl function.

Enzymic Evaluation.—Compounds I, XII–XVI were evaluated as inhibitors of folic reductase (XVII → XIX) from rat liver and 5,10-methylenetetrahydrofolate dehydrogenase (XX → XXI) from pigeon liver; the results are recorded in Table I. From the inhibition values in the Table, considerable insight into the mode of binding of these inhibitors to the two enzymes can be gained; presumably, the individual substrates have a similar mode of binding to their respective enzymes.

The 5- and 8-nitrogens of folic acid (XVII) do not appear to contribute to binding to the enzyme, since the K_i of I (devoid of these nitrogens) is less than K_m; this result was anticipated in view of the proposition that positions on a substrate or cofactor involved in the enzymic-catalyzed transfer of a group from cofactor to substrate, or *vice versa*, are most probably not positions of binding to the enzyme.[6,13] The possibility that loss in binding from the lack of 5- and 8-nitrogens in I is compensated by increase in binding associated with the pyrimidine ring of I cannot be excluded at this

(13) B. R. Baker, *Cancer Chemotherapy Rept.*, **4**, 1 (1959).



time. However, a more drastic change in binding was noted with compound IX; the K_i value increased about 80-fold indicating that the basic amino group (N^{10} of XVII) of the *p*-aminobenzoyl-L-glutamate moiety was necessary for good binding. In fact, the 30-fold decrease in the binding of XII compared to I, indicated that the *p*-amino group of the *p*-aminobenzoyl-L-glutamate contributed more to binding to folic reductase than did the entire carboxy-L-glutamate group. The lesser contribution by the latter group was due primarily to the carbonyl groups of the *p*- and γ-carboxyls, as shown by comparison to the binding of I with that of XIII–XVI. Since both of these carbonyl groups are in the amide form in XVI, which binds to folic reductase as well as I, it is most probable that these carbonyls bind through hydrogen bonding since the possible ionic binding of the γ-carboxyl is blocked in XVI. The α-carboxyl probably contributes little to binding as can be seen by comparison of I with XIII–XVI.

That both the pyrimidyl and benzoyl-L-glutamate moieties joined together were necessary for the binding of IX to folic reductase was shown by the lack of inhibition of the fragmented moieties, IV and XXII.

The preceding results with compounds I and XII–XVI offer an explanation of why pteroic acid, pteroyl-L-glutamic acid (XVII), pteroyl-D-glutamic acid (the unnatural isomer), pteroyltriglutamic acid, and their dihydro derivatives can be substrates for folic reductase (XVII → XIX) or dihydrofolic reductase (XVIII → XIX)[14]; the contribution to binding by the carboxy-L-glutamate moiety is minimal. Since a γ-amide (XVI) can still bind as well as I, it is likely that the extra glutamate residues in the triglutamate are sufficiently removed from the enzyme surface in the enzyme–substrate complex so not to interfere with binding. In addition, that the α-carboxyl of I and XVI contributes little if any to binding offers an explanation of why the unnatural isomer—pteroyl-D-glutamate—can still be a substrate; apparently the α-carboxyl of pteroyl-D-glutamate neither binds nor interferes with binding in either the D or L form, that is, this group is not in the area of contact with the enzyme.

Previous reports from this laboratory have indicated that the contribution of the pyrimidyl moiety of I to binding to folic reductase can be increased by replacement of the 6-methyl with 6-phenyl[2] or the replacement of the 4-hydroxyl with 4-mercapto.[15] In fact, the loss in binding to folic reductase by the removal of the carboxy-L-glutamate moiety in I can be fully compensated for by increasing the contribution of the pyrimidyl moiety when the 6-methyl group of XII is replaced by a 6-phenyl group.[2] The 10^5 increase in binding[16] of folic acid (XVII) to folic reductase when the 4-hydroxyl group is replaced by 4-amino to give aminopterin has been long known.[5] In fact, the increase in binding by replacing the 4-hydroxyl group by 4-amino more than compensates for the total binding of the entire *p*-aminobenzoyl-L-glutamate moiety, as shown[17] by the $K_i = 4.7 \times 10^{-7}$ for 2,4-diamino-6-methylpteridine; even 2,4-diamino-6-methylpyrimidine ($K_i = 2.6 \times 10^{-5}$)[17] binds about one-fourth as well as folate. Compared to 2,4-diamino-6-methyl-pyrimidine, the increase in binding to folic reductase by the antimalarial, 5-chlorophenyl-2,4-diamino-6-ethylpyrimidine ($K_i = 7 \times 10^{-9}$)[18] indicates the chlorophenyl group most probably is an additional binding group.

The relatively small contribution of the carboxy-L-glutamate moiety to binding to folic reductase was completely unanticipated, but is a result that should have considerable utility in chemotherapy. 4-Amino-4-deoxyfolic acid (aminopterin) and folic acid (XVII) are concentrated in several types of bacterial cells by active transport,[19,20] whereas the folic reductase inhibitor, 5-*p*-chlorophenyl-2,4-diamino-6-ethylpyrimidine (pyrimethamine) enters some of the same bacterial cell walls by passive diffusion[19,21]; this suggests that the carboxy-L-glutamate moiety is directly involved in active transport. Thus an investigation of the synthesis and biological evaluation of aminopterin analogs without the carboxy-L-glutamate moiety has been initiated, particularly since one tumor (Walker 256) is known to be naturally resistant to aminopterin ap-

(14) R. Nath and D. Greenberg, *Biochemistry*, **1**, 435 (1962); S. F. Zakrzewski, *J. Biol. Chem.*, **235**, 1780 (1960); R. L. Blakely and B. M. McDougall, *ibid.*, **236**, 1163 (1961).

(15) B. R. Baker, C. E. Morreal, and B. T. Ho, *J. Med. Chem.*, **6**, 658 (1963); paper VIII of this series.

(16) W. C. Werkheiser, *J. Biol. Chem.*, **235**, 1776 (1960).

(17) S. F. Zakrzewski, *ibid.*, **238**, 1485 (1963).

(18) W. C. Werkheiser, Roswell Park Memorial Institute, unpublished data.

(19) R. C. Wood and G. H. Hitchings, *J. Biol. Chem.*, **234**, 2381 (1959).

(20) M. J. Pine, *J. Bact.*, **79**, 827, 835 (1960).

(21) R. C. Wood and G. H. Hitchings, *J. Biol. Chem.*, **234**, 2377 (1959).

parently due to the failure of the tumor cell line to take up the drug[22] (presumably by active transport); other susceptible cell lines became resistant presumably by loss of the active transport system.[20,23,24] Therefore, tumor cells having a natural or acquired resistance resulting from a defective active transport system for folate, should be susceptible to inhibition by inhibitors of type XII containing a 4-amino-4-deoxy group; any normal tissue that has an adequate active transport system could presumably be protected from the effect of such a drug by use of large doses of folic acid or 5-formyltetrahydrofolic acid (leucovorin). In fact, the mechanism of selectivity of action of pyrimethamine on the malaria parasite has been attributed to the lack of an active transport system in the parasite for folic acid and leucovorin, whereas the host can incorporate leucovorin from the blood stream into host cells *via* active transport, thus protecting the host against the effect of passive entrance of the drug into host cells[25]; furthermore, *Escherichia coli* and the malaria parasite are naturally resistant to aminopterin since neither cell contains an active transport system for folate or aminopterin.[25]

The same compounds (Table I) showed that the mode of binding of the *p*-aminobenzoyl-L-glutamate moiety to 5,10-methylenetetrahydrofolate dehydrogenase (XX → XXI) contrasted sharply with the mode of binding of this moiety to folic reductase. Based on the hypothesis that a group involved in a transfer reaction from substrate to cofactor cannot be a binding group to the enzyme,[6,13] it could be predicted that N^5 and N^{10} of XX or the *p*-amino group of the *p*-aminobenzoyl-L-glutamate moiety on an inhibitor such as I should not be binding points on the enzyme. That this assumption has experimental support is clearly indicated by the fact that compounds I and IX bound about equally well to the dehydrogenase; since it is likely that an amide and an amino would have different binding properties, the *p*-amino group is presumably not involved in binding to this enzyme.

Complete removal of the carboxy-L-glutamate moiety, as in XII, caused loss of inhibition properties. The only change in the carboxy-L-glutamate residue (XIII–XVI) that did not significantly reduce the inhibition was conversion of the γ-carboxyl to an amide (XVI); therefore the γ-carboxyl group presumably binds through a hydrogen bond rather than through an anion. That both glutamate carboxyls are involved in binding is shown by the considerable decrease in binding if either of these carboxyl groups is removed, as is seen by comparing I with XIV and XV. That the carbonyl of the *p*-carboxyl group contributes to binding is indicated by the better binding of XIII than XII, although XIII binds poorly compared to I. Thus, all three carbonylated functional groups of the carboxy-L-glutamate moiety appear to be involved in enzymic binding.

The fact that *p*-acetamidobenzoyl-L-glutamic acid (XXII) binds about one-third as well as I indicates that the main contributions to the enzymic binding of I are through the carboxy-L-glutamate moiety; it is notable that removal of a single carboxyl (XIV, XV) from I causes greater loss in binding than removal of the entire pyrimidyl moiety (XXII). Previous reports[3] from this Laboratory have indicated that the weak binding by the pyrimidyl moiety can be increased by conversion of the 4-hydroxyl to 4-mercapto[15] or the 6-methyl to 6-phenyl.[2]

There are eleven polar atoms in I capable of relatively strong binding to an enzyme in addition to the binding forces present in the π-electrons of the two aromatic rings and the smaller contribution by CH van der Waals forces; the substrates for the two enzymes (XVII and XX) contain two additional polar atoms, both involved in the respective transfer reaction. Binding by at least three and probably no more than six of the polar groups to either enzyme, depending upon the relative number of hydrogen bonds and anionic–cationic interactions should be sufficient to account for the magnitudes of the observed K_i and K_m values. Therefore it would be unlikely that the identical 3–6 polar atoms would be involved in binding of an inhibitor such as I, or of the required substrate, to both enzymes; although differences in the mode of binding of I to the two enzymes could thus be anticipated, the specific differences other than transfer points on the substrate would not be predictable. Comparisons among the other 14 or more enzymes in the folic cofactor area[5,6] are continuing with our spectrum of potential inhibitors; searches for differences in inhibitor binding to a substrate-identical enzyme such as folic reductase in different tissues or species is also warranted. Such differences in mode of binding of inhibitors could have considerable utility in chemotherapy and could also indicate differences in the nature of the active sites of these enzymes.

By studying the mode of binding of selected pyrimidyl inhibitors (Table I) to folic reductase (XVII → XIX) and 5,10-methylenetetrahydrofolic dehydrogenase (XX → XXI), we hoped to established the main groups for binding and to find one or more areas of the inhibitor not in contact with the enzyme surface when complexed with the enzyme; such noncontact areas could then be used for placement of groups which have the potential ability to form a covalent linkage with the enzyme, thus giving irreversible inhibition.[6,13]

There are at least two such apparent noncontact areas in compound I when it is complexed with folic reductase, (a) the area at the 6-position of the pyrimidyl moiety[2] and (b) part of the area of the carboxy-L-glutamate moiety; in the former case it should be possible to find an active-site-directed irreversible inhibitor of the "*exo*-alkylation" type and in the latter case an irreversible inhibitor of either the "*exo*- or *endo*-alkylation" type.[13]

In the case of 5,10-methylenetetrahydrofolate dehydrogenase there is so far only one clear noncontact area when I is complexed, that near the 6-position of the pyrimidyl moiety[2]; thus, it should be possible to construct an irreversible inhibitor of this enzyme of the "*exo*-alkylation" type by proper substitution on the 6-phenyl group of a 6-phenylpyrimidyl analog of IX; it is clear that IX would be more suitable for these studies than I, since IX is a better reversible inhibitor of the dehydrogenase than of folic reductase, whereas in the case of I the reverse is true. Irreversible in-

(22) W. C. Werkheiser, *Proc. Am. Assoc. Cancer Res.*, **3**, 371 (1962).
(23) G. A. Fischer, *Biochem. Pharmacol.*, **11**, 1233 (1962).
(24) W. C. Werkheiser, L. W. Law, R. A. Roosa, and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.*, **4**, 71 (1963).
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hibitors of the "*endo*-alkylation" type should be possible with the dehydrogenase if either the α- or γ-carboxyl were converted to a small group with covalent bond forming properties, although some loss of reversible binding might occur.

An extra dimension of specificity with active-site-directed irreversible inhibitors has previously been noted with other closely substrate-related enzymes or even a substrate-identical enzyme from two different tissues.[26] Synthetic and enzymic studies to find such specificity with folic cofactor enzymes are continuing in this laboratory.

Experimental[27]

2-Amino-4-hydroxy-6-methyl-5-pyrimidinepropionitrile (V).—To a solution of 4.30 g. (0.80 mole) of sodium methoxide in 200 ml. of absolute ethanol was added 7.60 g. (0.080 mole) of guanidine hydrochloride followed by 14.6 g. (0.080 mole) of ethyl α-(2-cyanoethyl)acetoacetate (III).[9] After being refluxed with magnetic stirring for 19 hr., the solvent was removed by spin evaporation *in vacuo*. Trituration of the residue with 20 ml. of water gave 9.5 g. (67%) of product, m.p. 265–270°, that was suitable for further transformations. Recrystallization from water gave white crystals, m.p. 266–267°; ν_{max}^{KBr} 3500, 3400 (OH, NH); 2250 (C≡N); 1640, 1600, 1540 cm.$^{-1}$ (NH, pyrimidine).

Anal. Calcd. for $C_8H_{10}N_4O$: C, 54.0; H, 5.60; N, 30.5. Found: C, 54.0; H, 5.38; N, 30.7.

When the reaction was run only 3 hr., the yield was considerably less.

2-Amino-4-hydroxy-6-methyl-5-pyrimidylpropionic Acid (IV).—A mixture of 6.4 g. (0.036 mole) of V and 70 ml. of 6 *N* hydrochloric acid was refluxed for 2 hr., then cooled and neutralized to about pH 5 with *N* potassium hydroxide. The product was collected on a filter and washed with water; yield 6.00 g. (86%), m.p. 291–301° dec., that was suitable for further transformations. For analysis, a sample was dissolved in 5% aqueous sodium bicarbonate. The filtered solution was acidified with dilute acetic acid; the product was collected and washed well with water. This reprecipitation was repeated twice more to give a white powder, m.p. 301–303° dec.; $\lambda_{max}^{pH\ 1}$ 227 (ϵ 8600), 265 mμ (ϵ 7500); $\lambda_{max}^{pH\ 8.4}$ 275 mμ (ϵ 5000); $\lambda_{max}^{pH\ 13}$ 279 mμ (ϵ 6700); ν_{max}^{KBr} 3500, 3400 (NH, OH); 1710 (carboxyl C=O); 1650, 1520–1490 cm.$^{-1}$ (NH, pyrimidine).

Anal. Calcd. for $C_8H_{11}N_3O_3$: C, 48.6; H, 5.61; N, 21.2. Found: C, 48.5; H, 5.86; N, 21.0.

2-Amino-4-hydroxy-6-methyl-5-pyrimidylpropionyl Chloride Hydrochloride (VII).—To a mixture of 591 mg. (3 mmoles) of IV and 19.3 g. of thionyl chloride was added 0.10 ml. of reagent pyridine.[10] After being refluxed for 45 min., the suspension was cooled and diluted with 20 ml. of reagent ether. The solid was collected on a filter and washed with 10 ml. of reagent ether; this product was protected from moisture and used immediately for the subsequent reactions described below. The yield was 470 mg. (71%), m.p. 150–200° dec.; ν_{max}^{KBr} 3400 (NH); 1790 (acid chloride C=O); 1660 (C=NH$^+$); 1625, 1600, 1525 cm.$^{-1}$ (NH and pyrimidine ring). The compound was characterized as the anilide, **2-amino-4-hydroxy-6-methyl-5-pyrimidylpropionanilide** (VIII). To a magnetically stirred solution of 93 mg. (1 mmole) of aniline in 1 ml. of reagent pyridine was added 112 mg. (0.5 mmole) of freshly prepared VII. The solution was stirred for 30 min. protected from moisture, then poured into 10 g. of ice. The product was collected on a filter and washed with water; yield, 100 mg. (81%), m.p. 290–300° dec. Recrystallization from 2-methoxyethanol by addition of water gave white crystals, m.p. 305–307° dec.; ν_{max}^{KBr} 3400, 3100 (NH); 1650 (NH, amide C=O); 1580, 1540 (NH, pyrimidine ring); 760, 690 cm.$^{-1}$ (monosubstituted phenyl).

Anal. Calcd. for $C_{14}H_{16}N_4O_2$: C, 61.5; H, 5.91; N, 20.5. Found: C, 61.3; H, 5.99; N, 20.3.

N-(2-Amino-4-hydroxy-6-methyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic Acid (IX).—To a stirred solution of 266 mg. (1 mmole) of *p*-aminobenzoyl-L-glutamic acid in 1 ml. of reagent pyridine was added 233 mg. (1 mmole) of freshly prepared VII in one portion. After being stirred for 18 hr. at room temperature protected from moisture, the solution was added to 10 g. of iced water. The resultant solution was acidified to about pH 4 with acetic acid, then left at 5° for 48 hr. to complete crystallization of the product. The white powder was collected on a filter, then washed successively with water, ethanol, and ether; yield, 160 mg. (36%), m.p. 180–225° dec. The material gave a negative Bratton–Marshall test for primary aromatic amine.[8] Two recrystallizations from methanol gave the analytical sample as white crystals, m.p. 180–230° dec.; $\lambda_{max}^{pH\ 1}$ 266 mμ (ϵ 27,000); $\lambda_{max}^{pH\ 8.4}$ 268 mμ (ϵ 25,000); $\lambda_{max}^{pH\ 13}$ 270 mμ (ϵ 25,600).

Anal. Calcd. for $C_{20}H_{23}N_5O_7$: C, 54.0; H, 5.16; N, 15.7. Found: C, 53.9; H, 5.25; N, 15.6.

***p*-Acetamidobenzoyl-L-glutamic Acid (XXII).**—Although this compound is mentioned in the biological literature several times, neither its method of preparation nor its physical properties could be found.

To a solution of 1.00 g. (3.76 mmoles) of *p*-aminobenzoyl-L-glutamic acid in 10 ml. of 50% aqueous acetic acid was added 1.5 ml. (16 mmoles) of acetic anhydride. After standing for 24 hr. the mixture was filtered and the product washed with water; yield, 0.82 g. (72%), m.p. 210–215°. Recrystallization from water gave golden leaflets, m.p. 213–215°; ν_{max}^{KBr} 3500, 3380 (NH); 1740 (carboxyl C=O); 1670 (amide C=O); 1630, 1600 cm.$^{-1}$ (amide NH).

Anal. Calcd. for $C_{14}H_{16}N_2O_6$: C, 54.5; H, 5.22; N, 9.10. Found: C, 54.3; H, 5.39; N, 9.05.

γ-(*p*-Nitrobenzoyl)butyric Acid.—To a magnetically stirred solution of 1.03 g. (0.01 mole) of γ-aminobutyric acid in 10 ml. of 10% sodium hydroxide cooled in an ice bath was added 2.4 g. (0.13 mole) of *p*-nitrobenzoyl chloride in portions over 10 min. The mixture was stirred in an ice bath until the acid chloride dissolved, then acidified to about pH 2 with hydrochloric acid. The solid was collected on a filter and washed with cold water; yield 2.6 g. (67%), m.p. 155–157°. Two recrystallizations from water gave nearly white crystals, m.p. 164–166°; ν_{max}^{KBr} 3400 (NH); 1690 (carboxyl C=O); 1620 (amide C=O); 1340 cm.$^{-1}$ (NO_2).

Anal. Calcd. for $C_{11}H_{12}N_2O_5$: C, 52.4; H, 4.80; N, 11.1. Found: C, 52.2; H, 4.86; N, 11.0.

γ-(*p*-Aminobenzoyl)butyric Acid.—A suspension of 1.26 g. (5 mmoles) of γ-(*p*-nitrobenzoyl)butyric acid in 4 ml. of water was dissolved by the addition of just sufficient concentrated ammonium hydroxide, then added to a hot solution of 9.04 g. (0.032 mole) of $FeSO_4.7H_2O$ in 20 ml. of water. The stirred, hot mixture was treated with 8 ml. of concentrated ammonium hydroxide in five portions over 10 min. After being heated on a steam bath for an additional 20 min., the mixture was filtered through a Celite pad and the filtrate spin evaporated *in vacuo*. The residue was extracted with several portions of boiling ethanol until a white inorganic residue remained. The combined extracts were spin evaporated *in vacuo*. Crystallization of the resultant sirup from 5 ml. of water gave 0.86 g. (68%) of product, m.p. 144–145°. Recrystallization from water gave buff-colored crystals, m.p. 146–148°; ν_{max}^{KBr} 3500, 3400 (NH); 1720 (carboxyl C=O); 1640 (amide C=O); no NO_2 band at 1340 cm.$^{-1}$.

Anal. Calcd. for $C_{11}H_{14}N_2O_5$: C, 59.4; H, 6.35; N, 12.6. Found: C, 59.3; H, 6.50; N, 12.4.

***p*-Nitrobenzoyl-L-glutamine.**—To a solution of 5.85 g. (0.04 mole) of L-glutamine and 1.6 g. (0.04 mole) of sodium hydroxide in 80 ml. of water was added 4.84 g. (0.12 mole) of magnesium oxide. To the stirred mixture, cooled in an ice bath, was added dropwise a solution of 11.2 g. (0.06 mole) of *p*-nitrobenzoyl chloride in 75 ml. of chloroform over a period of 90 min. After being stirred an additional 18 hr. at 0–3°, the aqueous layer was separated, acidified rapidly to pH 1 with 3 *N* HCl and immediately filtered from *p*-nitrobenzoic acid. The product slowly separated from the filtrate; it was recrystallized from water to give 7.23 g. (62%) of product, m.p. 190–191°; ν_{max}^{KBr} 3450, 3250 (NH); 1729 (carboxyl C=O); 1655, 1635 (amide C=O); 1340 cm.$^{-1}$ (NO_2). King and Spencer[12] have recorded m.p. 175–176° for this material prepared by ammonolysis of *p*-nitrobenzoyl-L-glutamic acid γ-ethyl ester, a route which worked poorly in this laboratory.

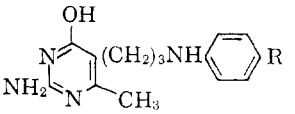
Reduction of the nitro group with hydrogen–palladium[12] afforded an 87% yield of recrystallized *p*-aminobenzoyl-L-glutamine, m.p. 195–196°, lit.[12] m.p. 197°.

(26) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962); **12**, 293 (1963); B. R. Baker and R. P. Patel, *Biochem. Biophys. Res. Comm.*, **9**, 199 (1962).

(27) Melting points were taken in capillary tubes in a Mel-Temp block; all melting points below 230° are corrected. Infrared and ultraviolet spectra were determined on Perkin–Elmer Models 137B and 202 spectrophotometers, respectively.

TABLE II

PHYSICAL CONSTANTS OF PYRIMIDYL ANALOGS



No.	R	% calcd. C	H	N	% found C	H	N	Ultraviolet spectra, λ_{max} (mμ) (ε × 10³) pH 1	pH 13
XII	H	65.1	7.02	21.7	65.2	7.05	21.5	225 (11.1) 264 (10.1)	239 (17.2) 280 (9.36)
XIII	—COOH	59.6	6.00	18.5	59.4	6.75	18.6[a]	227 (21.4) 267 (11.0) 293–308 (plateau)	282 (24.4)
XIV	—CONHCH₂COOH	56.8	5.89	19.5	56.7	5.70	19.5	222 (20.5) 271 (13.2) 292–308 (plateau)	283 (21.3)
XV	—CONH(CH₂)₃COOH	58.9	6.50	18.1	58.7	6.40	17.9	225 (19.6) 269 (10.5) 305 (inflection)	282 (26.5)
XVI	—CONHCHCOOH (CH₂)₂CONH₂	55.7	6.09	19.1	55.7	6.28	19.0[b]	222 (26.4) 268 (10.0) 302 (inflection)	285 (26.0)

[a] Oxygen: calcd., 15.9; found, 15.8. [b] Oxygen: calcd., 18.6; found, 19.0.

Preparation of Compounds XII–XVI (Table II).—A solution of 112 mg. (0.5 mmole) of X and 0.5 mmole of XI in 2 ml. of dimethylformamide was allowed to react for 15 min., then diluted with 10 ml. of ethanol. With magnetic stirring, 0.80 g. of sodium borohydride was added in portions over a period of 30 min. After being stirred overnight, the mixture was diluted with 5 ml. of 0.1 *N* sodium hydroxide, then spin evaporated to dryness *in vacuo*. The residue was dissolved in 3 ml. of water, clarified by centrifugation, then adjusted to pH 4.5 with 3 *N* hydrochloric acid. The product was collected by centrifugation, then washed successively with two 5-ml. portions of water, one 5-ml. portion of hot water to remove unreacted XI, two 5-ml. portions of ethanol, one 5-ml. portion of hot ethanol, and finally ether or dichloromethane. For further purification, the compound was dissolved in 1–5% aqueous sodium bicarbonate, the solution was filtered, then acidified to pH 4.5. The product was collected and washed as above; yield 20–30%; the Bratton–Marshall assay at this point was 0–3%, calculated as "anil." The reprecipitation was repeated, if necessary, to obtain constant ultraviolet molecular extinction coefficients. The samples retain moisture tenaciously and are obtained anhydrous only after long drying under high vacuum at 100°.

The following exceptions should be noted in the general procedure:

(1) Compound XV is somewhat soluble in hot ethanol; this washing step was omitted.

(2) Compound XIII dissolves in 5% sodium bicarbonate only on heating, since the sodium salt is insoluble in cold water.

(3) Solution of the crude product in dimethylformamide and reprecipitation with water is an alternate method of purification used on occasion for XV and XVI.

(4) Compound XII, was prepared using a 5:1 ratio of aniline to X. The lack of a carboxyl group required, of course, a different isolation procedure. After evaporation of the sodium hydroxide solution, the residue from 1 mmole of X was dissolved in 5 ml. of 3 *N* hydrochloric acid. The filtered solution was adjusted to pH 8–9 with 1.5 *N* sodium hydroxide. The crystalline product was collected on a filter and washed with water; yield, 250 mg. (97%), m.p. 213–215°. Recrystallization from absolute ethanol gave 175 mg. (68%) of white crystals, m.p. 218°; ν_{max}^{KBr} 3400, 3200 (NH); 1640, 1610, 1530, 1500 (C=C, C=N, NH); 745, 692 cm.$^{-1}$ (monosubstituted phenyl).