2-proton singlet at 3.38 ($NCH_2C_6H_5$), 8-proton series of multiplets at 2.2–3.0 (CH_2 adjacent to aromatic nucleus or N), and 6-proton series of multiplets at 1.2–2.0 (CH_3).

Anal. Calcd for $C_{22}H_{29}O_2N$: C, 77.8; H, 8.6; N, 4.1. Found: C, 76.9; H, 8.8; N, 3.9.

B.—The benzazecine **3** (R = H) (0.12 g) was refluxed for 16 hr with triethylamine (0.3 g) and 3,4-dimethoxybenzyl chloride (0.3 g) in $CHCl_3$ (5 ml). Distillation of the product at 170° (bath, 0.1 mm) gave the benzazecine **3** [R = 3,4-$(CH_3O)_2$-$C_6H_3CH_2$], mp 53–56°, undepressed by the sample prepared as in A.

Reductive Fission of Salt 6 (R = $C_6H_5CH_2$; X = Br). Lithium (56 mg) was added piecemeal with stirring to the salt (1.5 g) in liquid NH_3 (200 ml) containing 1-methoxy-2-propanol (0.45 g). After 5 min the blue color was discharged with a few drops of water, and ether and water were added. The oily product in ether was treated with isopropyl alcohol previously saturated with HCl at 0°, and the resulting suspension was filtered to give the **hydrochloride** of base **12**, identical in melting point infrared absorption spectrum, and thin layer chromatographic behavior (on silica gel using NH_3-saturated benzene) with an authentic sample.

Acknowledgments.—We thank Messrs. C. Koo and T. Pattison for the preparation of key intermediates and Drs. M. Gluckman and M. E. Rosenthale and Mr. A. Begany, Pharmacological Evaluation Section, Wyeth Laboratories Inc., for the biological test data.

Synthesis of Methylenetetrahydrofolic Acid Analogs[1]

MATHIAS P. MERTES AND NATUBHAI R. PATEL

Department of Medicinal Chemistry, School of Pharmacy, University of Kansas, Lawrence, Kansas

Received April 28, 1966

The synthesis of analogs of N^5,N^{10}-methylenetetrahydrofolic acid is described. These compounds are 2-substituted 1-*p*-carbethoxyphenylimidazolidines and 3-substituted 2-*p*-carbethoxyoctahydroimidazo[1,5-*a*]-pyrazines, structural modifications of the cofactor involved in the synthesis of thymidylic acid. The 5′-uracil derivatives are analogs of the proposed intermediate in the biosynthesis of thymidylic acid. The inhibitory effects of these compounds on dihydrofolate reductase and thymidylate synthetase are described.

The role of folate coenzymes in biochemical pathways has been amply reviewed by many investigators.[2] Several aspects of folate utilization have been of particular interest in the design of agents effective in the treatment of cancer, *e.g.*, aminopterin and amethopterin. Tetrahydrofolic acid was recognized in 1957 as an essential metabolite in the synthesis of thymidine 5′-monophosphate.[3] The unique character of the one-carbon transfer, a reductive methylation, stimulated further interest in this pathway.

If the limiting factor in deoxyribonucleic acid synthesis and ultimately cell division is the availability of thymidine 5′-triphosphate,[4] then an obvious approach to potential anticancer agents is through the inhibition of thymidine 5′-triphosphate formation. One such step in the sequence is the synthesis of thymidine 5′-monophosphate. Several studies on the mechanism of the one-carbon transfer to deoxyuridine 5′-monophosphate (dUMP) have demonstrated that tetrahydrofolic acid, the reducing agent, is converted to dihydrofolic acid[5] *via* a hydrogen (hydride?) transfer in the intermediate complex to give the product, thymidine 5′-monophosphate. The enzyme thymidylate synthetase, isolated from microbial and mammalian sources,[5,6] catalyzes the reductive methylation of deoxyuridine 5′-monophosphate by transfer of a methyl group from N^5,N^{10}-methylenetetrahydrofolic acid. The kinetics of the reaction support the view that a binary complex is formed between the enzyme and the cofactor with subsequent formation of a ternary intermediate with the substrate (dUMP).[7] After transfer of the methyl, the binary enzyme–dihydrofolic acid complex dissociates. Regeneration of tetrahydrofolic acid is mediated by nicotinamide–adenine dinucleotide phosphate (NADPH) reduction of dihydrofolic acid in the presence of dihydrofolic acid reductase.

Inhibition of the latter step in the sequence by aminopterin and amethopterin has been demonstrated as the site of action for these drugs.[8] Direct inhibition of thymidylate synthetase has been reported for 5-fluoro- and 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate[6b,7c] which can be termed, respectively, "substrate" and "product" inhibitors. The proposed formation of a binary complex and the kinetics of the enzymatic reaction suggest the feasibility of inhibition by analogs of the cofactor, N^5,N^{10}-methylenetetrahydrofolic acid.

The design of cofactor inhibitors of thymidylate synthetase is not new. Kisliuk[9] reported growth

(1) This work was generously supported by Grant CA-7522 of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Md., and by the Graduate School, University of Kansas.

(2) (a) T. H. Jukes and H. P. Boroquist in "Metabolic Inhibitors," Vol. I, R. M. Hochster and J. M. Quastel, Ed., Academic Press Inc., New York, N. Y., 1963, p 481; (b) M. Friedkin, *Ann. Rev. Biochem.*, **32**, 185 (1963); (c) F. M. Huennekens, *Biochemistry*, **2**, 151 (1963); (d) J. S. O'Brien, *Cancer Res.*, **22**, 267 (1962).

(3) (a) M. Friedkin and A. Kornberg in "The Chemical Basis of Heredity," W. D. McElroy and H. B. Glass, Ed., The Johns Hopkins Press, Baltimore, Md., 1957, p 609; (b) M. Friedkin and D. Roberts, *Federation Proc.*, **14**, 215 (1955); (c) P. Reichard, *Acta Chem. Scand.*, **9**, 1275 (1955); (d) E. A. Phear and D. M. Greenberg, *J. Am. Chem. Soc.*, **79**, 3737 (1957).

(4) For a discussion of this point see K. G. Lark in "Molecular Genetics, Part I," J. H. Taylor, Ed., Academic Press Inc., New York, N. Y., 1963, p 153.

(5) (a) G. K. Humphreys and D. M. Greenberg, *Arch. Biochem. Biophys.*, **78**, 275 (1958); (b) B. M. McDougall and R. L. Blakley, *Biochem. Biophys. Acta*, **39**, 176 (1960); (c) M. Friedkin, *Federation Proc.*, **18**, 230 (1959); M. Friedkin in "The Kinetics of Cellular Proliferation," F. Stohlman, Ed., Grune and Stratton, New York, N. Y., 1959, p 99; (d) R. L. Blakley, B. V. Ramasastri, and B. M. McDougall, *J. Biol. Chem.*, **238**, 3075 (1963).

(6) (a) R. Nath and D. M. Greenberg, *Federation Proc.*, **20**, 227 (1961); (b) K. U. Hartmann and C. Heidelberger, *J. Biol. Chem.*, **236**, 3006 (1961); (c) R. Silber, B. W. Grabrio, and F. M. Huennekens, *Federation Proc.*, **21**, 241 (1962); (d) V. K. Whittaker and R. L. Blakley, *J. Biol. Chem.*, **236**, 838 (1961).

(7) (a) A. J. Wahba and M. Friedkin, *ibid.*, **237**, 3794 (1962); (b) R. L. Blakley, *ibid.*, **238**, 2113 (1963); (c) P. Reyes and C. Heidelberger, *Mol. Pharmacol.*, **1**, 14 (1965).

(8) M. J. Osborn, M. Freeman, and F. M. Huennekens, *Proc. Soc. Exptl. Biol. Med.*, **97**, 429 (1958).

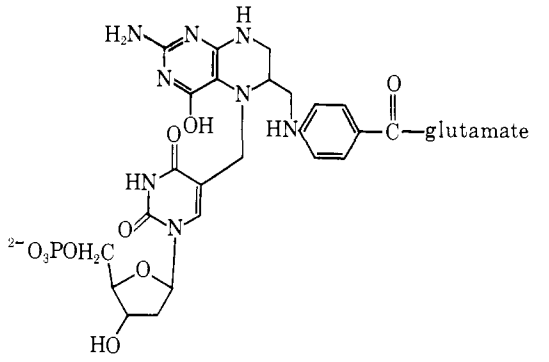
(9) R. L. Kisliuk, *Nature*, **188**, 781 (1960).

inhibition in microorganisms with tetrahydroaminopterin; further investigation revealed that the completely reduced forms of aminopterin and amethopterin were effective inhibitors of thymidylate synthetase.[10] A remarkable change in enzyme specificity is noted in comparing aminopterin, which is a potent inhibitor of dihydrofolate reductase but only weakly inhibits thymidylate synthetase, to the reduced forms. Dihydroaminopterin is as potent as aminopterin against the reductase enzyme but is twenty times more inhibitory against thymidylate synthetase. Tetrahydroaminopterin, a weak inhibitor of the reductase, is ten times as effective as aminopterin against the synthetase enzyme. Although Kisliuk did not determine whether the 5,10-methylene derivative was the actual inhibitor, N^5,N^{10}-methylenetetrahydroaminopterin was reported by Slavik and Slavikova[11] to be more effective than either dihydro- or tetrahydroaminopterin.

Further, Goodman, *et al.*,[12] reported that a methylene homolog of dihydrofolic acid was reduced by the reductase enzyme to tetrahydrohomofolic acid, a potent thymidylate synthetase inhibitor. Tong, Lee, and Goodman[13] reported the synthesis of pyrimidine analogs of tetrahydrofolic acid active against thymidylate synthetase. Baker, *et al.*,[14] also have studied substituted pyrimidines and the relative effects of substituents in the 2, 4, and 5 positions of 6-methylpyrimidines on binding to dihydrofolic acid reductase and thymidylate synthetase. The most effective inhibitors of the latter enzyme were 2-amino-4-mercapto-6-methyl-5-(3-anilinopropyl)pyrimidines with various groups either on the anilino nitrogen (hydrogen, acetyl, benzoyl, carbobenzoxy, tosyl) and *para* substitution on the anilino ring. The most effective inhibitor was reported to be 2-amino-4-mercapto-5-(3-*p*-chloroanilinopropyl)-6-methylpyrimidine.[14b]

The findings of Kisliuk,[10] that thymidylate synthetase binding was enhanced in the reduced pyrazine ring derivatives of folate analogs, prompted a study of the necessary binding sites for thymidylate synthetase inhibition in cofactor analogs that lack the pyrimidine portion of the molecule. In the series examined, compounds **1a** and **b** and **2a** and **b** were synthesized as structural analogs of N^5,N^{10}-methylenetetrahydrofolic acid. For simplicity, the glutamate portion of the molecule was omitted since Baker[14b] reported that in his series of compounds this plays a minor role in binding to thymidylate synthetase. More recently Friedkin,[15] *et al.*, have found that the amino acid portion of tetrahydrohomofolic acid derivatives is a critical factor in inhibition.

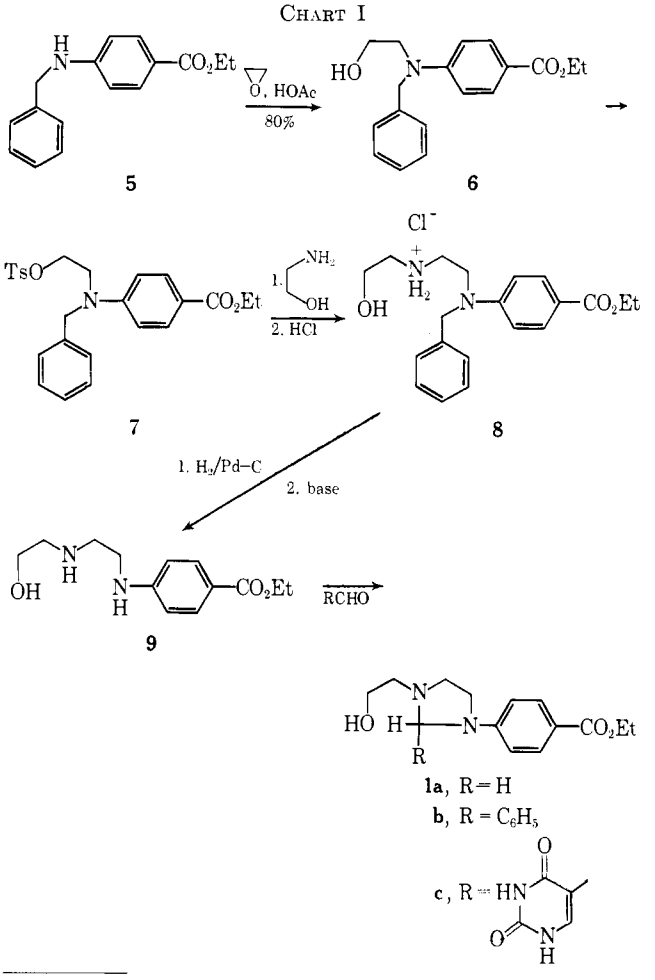
Furthermore, since Friedkin[5c] proposed a mechanism for the biosynthesis of thymidine 5′-monophosphate involving an intermediate complex (**3**) bridging both the cofactor and the substrate, compounds **1c**, **2c**, and **4**, substituting uracil on the methylene carbon, were synthesized in an effort to bridge binding sites on both the cofactor and the substrate and thereby study the inhibitory effect on the enzyme. A series of compounds resembling the proposed intermediate **3** have recently been reported by Gupta and Huennekens.[16]



3

The synthesis (Chart I) of the imidazoline series (**1**) was accomplished by condensation of the diamine **9** with the appropriate aldehyde. Ethyl *p*-benzyl-

CHART I



(10) (a) M. Friedkin, E. J. Crawford, and D. Misra, *Federation Proc.*, **21**, 176 (1962); (b) A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **236**, PC11 (1961); (c) R. L. Kisliuk and M. D. Levine, *ibid.*, **239**, 1901 (1964).

(11) K. Slavik and V. Slavikova, Proceedings of the 5th International Congress of Biochemistry, Moscow, Aug 10–16, 1961, Vol. 9, The Macmillan Co., New York, N. Y., 1963, p 139.

(12) (a) L. Goodman, J. I. DeGraw, R. L. Kisliuk, M. Friedkin, E. J. Pastore, E. J. Crawford, L. T. Plante, A. Al-Nahas, J. F. Morningstar, Jr., G. Kwok, L. Wilson, E. F. Donovan, and J. Ratzan, *J. Am. Chem. Soc.*, **86**, 308 (1964); (b) J. I. DeGraw, J. P. Marsh, E. M. Action, O. P. Crews, C. W. Mosher, A. N. Fujiwara, and L. Goodman, *J. Org. Chem.*, **30**, 3404 (1965).

(13) G. L. Tong, W. W. Lee, and L. Goodman, *J. Am. Chem. Soc.*, **86**, 5664 (1964).

(14) (a) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964); (b) B. R. Baker, B.-T. Ho, and G. B. Cldeda, *ibid.*, **1**, 88 (1964); (c) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **54**, 1187 (1965); (d) B. R. Baker and J. K. Coward, *ibid.*, **54**, 714 (1965).

(15) M. Friedkin, L. T. Plante, and E. J. Crawford, *Federation Proc.*, **24**, 541 (1965).

(16) V. S. Gupta and F. M. Huennekens, *ibid.*, **24**, 541 (1965).

aminobenzoate (**5**), prepared by reduction of the Schiff base and esterification,[17] was treated with ethylene oxide to give ethyl *p*-N-benzyl-N-(2-hydroxyethyl)-aminobenzoate (**6**). Treatment with *p*-toluenesulfonylchloride gave the tosylate **7** isolated in two different crystalline forms.

The second two-carbon fragment was introduced by displacement of the tosylate group with ethanolamine. Treatment of the tosylate **7** with ethanolamine,[18] followed by the addition of ether saturated with dry hydrogen chloride yielded N-benzyl-N-*p*-carbethoxyphenyl-N′-(2-hydroxyethyl)ethylenediamine hydrochloride (**8**). The removal of the protective benzyl group was accomplished by hydrogenolysis of the diamine hydrochloride **8** to the desired product, N-*p*-carbethoxyphenyl-N′-(2-hydroxyethyl)ethylenediamine (**9**).

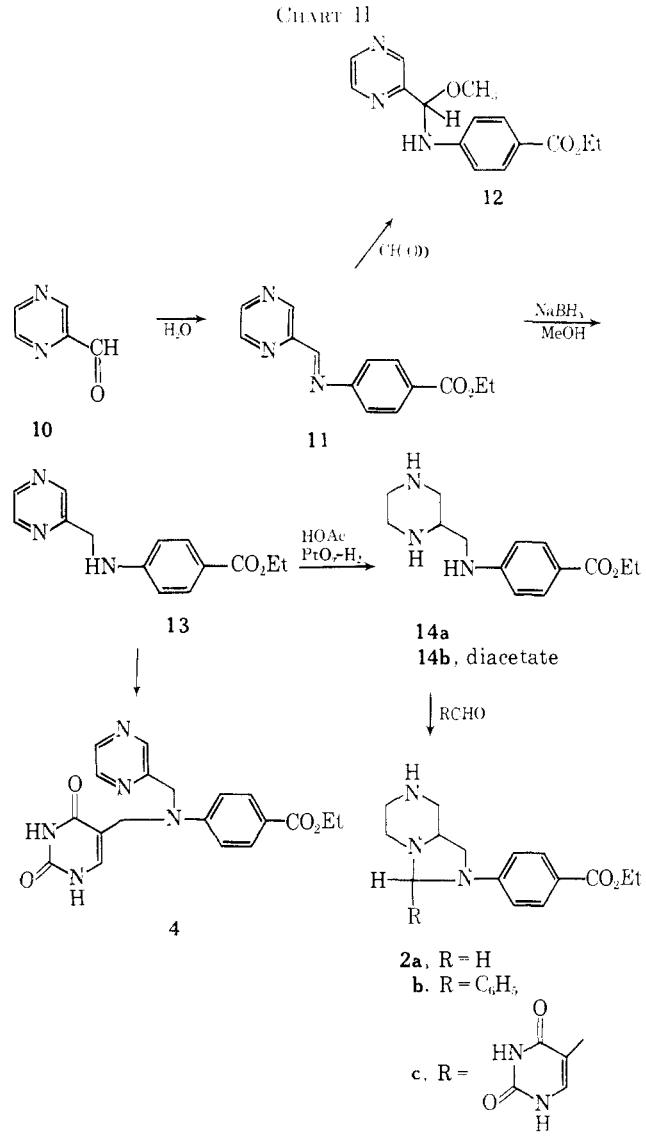
The diamine **9** was condensed with formaldehyde and benzaldehyde yielding 1-*p*-carbethoxyphenyl-3-(2′-hydroxyethyl)imidazolidine (**1a**) and 1-*p*-carbethoxyphenyl-2-phenyl-3-(2′-hydroxyethyl)imidazolidine (**1b**), respectively. However, this procedure did not afford the desired product with 5-formyluracil possibly because of the insolubility of the aldehyde in benzene. The diamine **9** and 5-formyluracil[19] were refluxed in ethanol, giving 1-*p*-carbethoxyphenyl-2-(5′-uracil)-3-(2′-hydroxyethyl)imidazolidine (**1c**).

The octahydroimidazo[1,5-*a*]pyrazine compounds (**2**) were synthesized by the route shown in Chart II. Methyl 2-pyrazinoate, prepared from pyrazine-2-carboxylic acid and dry methanol saturated with hydrogen chloride, was selectively reduced to the corresponding aldehyde **10** with lithium aluminum hydride, using the procedure of Rutner and Spoerri.[20] Ethyl *p*-N-(2-pyrazal)aminobenzoate (**11**) characterized by nmr was obtained by condensing the pyrazinealdehyde (**10**) with ethyl *p*-aminobenzoate. Recrystallization of **11** from absolute methanol gave the adduct of methanol, compound **12**. Alcohol addition products of Schiff bases have been reported.[21] The reduction of the Schiff base **11** to ethyl *p*-N-(2-piperazinylmethyl)-aminobenzoate (**14**) was attempted with hydrogen and platinum oxide in acetic acid, but the only product identified from the reaction mixture was ethyl *p*-aminobenzoate. Thus, it was necessary to reduce **11** with sodium borohydride in methanol to ethyl *p*-(2-pyrazinylmethyl)aminobenzoate (**13**) which was separated from ethyl *p*-aminobenzoate by column chromatography.

The reduction of **13** to **14** was tried in several ways (tin and hydrochloric acid, platinum oxide in ethanol) and eventually was accomplished by platinum oxide in acetic acid to give **14b**.

The desired compounds, 2-*p*-carbethoxyphenyloctahydroimidazo[1,5-*a*]pyrazine (**2a**), 2-*p*-carbethoxyphenyl-3-phenyloctahydroimidazo[1,5-*a*]pyrazine (**2b**), and 2-*p*-carbethoxyphenyl-3-(5′-uracil)octahydroimidazo-

CHART II



[1,5-*a*]pyrazine (**2c**) were prepared by condensing the amine **14a** with formaldehyde, benzaldehyde, and 5-formyluracil, respectively.

Ethyl *p*-N-(2-pyrazinylmethyl)-N-thyminylaminobenzoate (**4**) was obtained by treating the amine (**13**) with 5-bromomethyluracil.[22]

It is possible that the condensation of **14** with the aldehydes may proceed to give the following types of compounds: the desired product **2**, compound **15** involving condensation with N^4 of the piperazine ring, and a bridged compound (**16**).

The nmr spectrum of the amine **14a** gives the aromatic amine proton signal at δ 4.87 (1 proton) and aliphatic amine proton signal at δ 1.9 (2 protons). However, in the nmr spectrum of the condensation product **2b** the aromatic amine proton signal disappears and the signal at δ 1.62 reduces to one proton. This indicates that the condensation involves the aromatic amine and one of the aliphatic amines, eliminating **16** as the product. Although structure **15** has not been disproved, from inspection of Dreiding models it appears sterically unfavorable since it requires formation of a 1,3-diaxial system.

(17) (a) W. Manchot and J. R. Furlong, *Ber.*, **42**, 4389 (1909); (b) G. Reddelien and H. Dannilof, *ibid.*, **54**, 3132 (1921); A. Roe and J. A. Montgomery, *J. Am. Chem. Soc.*, **75**, 910 (1953); (c) A. Skita and W. Stuhmer, German Patent 716,668 (Dec 24, 1941); *Chem. Abstr.*, **38**, 2345 (1944).

(18) D. Triggle and B. Belleau, *Can. J. Chem.*, **40**, 1201 (1962).

(19) R. H. Wiley and Y. Yamamoto, *J. Org. Chem.*, **25**, 1906 (1960).

(20) (a) S. A. Hall and P. E. Spoerri, *J. Am. Chem. Soc.*, **62**, 664 (1940); (b) H. Rutner and P. E. Spoerri, *J. Org. Chem.*, **28**, 1898 (1963).

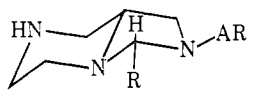
(21) (a) G. Cavallani, E. Massarani, and D. Nardi, *J. Med. Pharm. Chem.*, **2**, 99 (1960); (b) E. L. Anderson, J. E. Casey, Jr., M. Emas, E. E. Force, E. M. Jensen, R. S. Matz, and D. E. Rivard, *ibid.*, **6**, 787 (1963).

(22) J. A. Carbon, *J. Org. Chem.*, **25**, 1531 (1960).

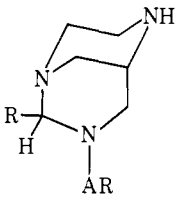
TABLE I
ENZYME STUDIES

Compd[a]	Solvent	Ratio of inhibitor/substrate concn for 50% inhibition: Thymidylate synthetase [I]/[THFA]	Thymidylate synthetase [I]/[dUMP]	Dihydrofolate reductase [I]/[DHFA]	Dihydrofolate reductase [I]/[NADPH]
Ethyl *p*-aminobenzoate	95% EtOH	No inhib at 1.7×10^{-3} *M*		No inhib at 3.3×10^{-3} *M*	
1a	95% EtOH	0.6	2.6	15.5	7.7
1b	95% EtOH	0.4	1.9	23.	11.
1c	0.02 *M* NaOH	0.4	1.7	5.2	2.5
13	0.02 *M* NaOH	2.5	10.5	14.0	6.9
4	95% EtOH	0.4	1.8	1.9	0.9
14b	H_2O	0.4	1.7	17.	8.5
2a	95% EtOH	0.4	1.8	4.9	2.4
2b	95% EtOH	0.5	2.3	19.	9.4
2c	0.02 *M* NaOH	0.6	2.8	6.0	3.0
Aminopterin	0.02 *M* NaOH	0.2 (0.33[b])	0.8 (2.5[b])	0.0009 (0.0008[c])	0.0004 (0.0008[c])

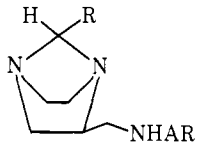
[a] A study of the rate of saponification of ethyl *p*-aminobenzoate in 0.02 *M* NaOH showed a half-life of approximately 160 min. The inhibitors **1c**, **13**, and **2c** were dissolved in 0.02 *M* NaOH and assayed within 60 min after solution. The results, therefore, are considered to indicate inhibition by the ester, not the saponification product. [b] Reported in ref 10c. [c] Reported in ref 24.



2



15



16

Enzyme Inhibition Studies.—Inhibitors were studied at several concentrations that gave values ranging from 80 to 20% inhibition of the rate. By plotting the ratio of the velocity of the uninhibited reaction to the velocity of the inhibited reaction (V_0/V_i) *vs.* inhibitor concentration, the concentration of inhibitor necessary for 50% inhibition was estimated. Baker[14] has used this method to relate the ratio of inhibitor to substrate necessary for 50% inhibition in an evaluation of the inhibitor strength. The inhibitor–substrate or cofactor ratios for 50% inhibition are given in Table I for both thymidylate synthetase and dihydrofolate reductase.

The results of inhibition of dihydrofolate reductase demonstrate that the pyrimidine portion of the molecule is necessary in these compounds for binding to the enzyme. In the imidazoline series (**1a** or **1b**), relatively weak inhibition is noted, 10^{-4} *M* being required for 50% inhibition. The pyrazine analog **13**, the piperazine analog **14**, and the benzaldehyde derivative (**2b**) of **14** also are weak inhibitors of the enzyme. The most effective inhibitors, the thymidyl analog **4** and the 5-formyluracil derivatives of the amines (**1c** and **2c**), have as additional binding sites the pyrimidine ring which probably accounts for the weakly enhanced binding to the reductase enzyme. The activity of **2a** cannot be justified on the basis that the precursor diamine **14** has one-third the activity of the formaldehyde derivative **2a**.

The thymidylate synthetase preparation appears to be slightly more sensitive to inhibition than that reported by Friedkin.[7a] For comparison, the effect of aminopterin on the enzyme was studied. Kisliuk and Levine[10c] reported that aminopterin gave 50% inhibition of Friedkin's enzyme preparation at a concentration of 1×10^{-4} *M*. The calculated ratio for 50% inhibition was 0.33 for aminopterin/tetrahydrofolic acid (THFA). In Table I it can be noted that 50% inhibition of thymidylate synthetase was achieved with a ratio of 0.2 for aminopterin/THFA. This slight difference in sensitivity could be due to the fact that Kisliuk and Levine used purified aminopterin whereas the commercially available material was used in these studies.

None of the inhibitors (Table I) are as effective as aminopterin. The slight variation between the inhibitors suggests that little change has been made in the binding affinity of the inhibitors by substituting phenyl or uracil on the methylene carbon. Thus, bridging of the binding sites of the enzyme for the substrate and cofactor was not achieved. The weak inhibition noted for the pyrazine **13** compared to **14** suggests the necessity of a basic nitrogen corresponding to the N^5 of tetrahydrofolic acid which is the common feature of all of the inhibitors in this study except **13** and **4**, the thymidyl analog of the pyrazine derivative. The most obvious finding from the enzyme studies is that the pyrimidine ring moiety of the pteridine system is not essential for binding to thymidylate synthetase and in fact may contribute little to the binding. The common features of the inhibitory compounds in this series are the *p*-aminobenzoate ester and the basic nitrogen joined to the anilino group by a two-carbon chain. Compound **4**, having comparable inhibition and lacking the basic nitrogen, presumably has an additional binding site, the uracil ring. If bridging of the two binding sites occurs in **4**, it was not observed in the other series and hence requires further study.

Experimental Section

Melting points were obtained on a calibrated Thomas–Hoover Unimelt and are corrected. Infrared data were recorded on Beckman IR5, IR8, and IR10 spectrophotometers. Nmr data were recorded on a Varian Associates Model A-60 spectrophotometer. Ultraviolet data were recorded on a Cary 14 and Beckman DB spectrophotometers. Microanalyses were carried out by Drs. G. Weiler and F. B. Strauss, Oxford, England.

Ethyl *p*-N-Benzyl-N-(2-hydroxyethyl)aminobenzoate (6).—A solution of 240 ml of 80% acetic acid and 5.1 g (0.02 mole) of ethyl *p*-benzylaminobenzoate[13] (**5**) was chilled to 0°, and 26 ml of ethylene oxide was added slowly with vigorous stirring at 0°. The mixture was stirred for 3 hr at 0° and allowed to stand at room temperature for 24 hr. The clear solution was added with continuous stirring to 600 ml of ice-cold water. The white substance which precipitated was filtered, washed with cold water, and dried to give, after drying *in vacuo* at 25° for 6 hr, 5.8 g (100%), mp 50–51°.

Ethyl *p*-(N-Benzyl-N-2-tosylethyl)aminobenzoate (7).—Dry pyridine (60 ml) and 7.0 g (0.022 mole) of **6** were mixed and cooled to −4° in an ice–salt bath. *p*-Toluenesulfonyl chloride (8.9 g, 0.45 mole) was added and mixed thoroughly. After 15 hr at room temperature, the reaction mixture was added with stirring to 1000 ml of ice-cold water. The white precipitate (9.9 g) was filtered, dried, and recrystallized from absolute ethanol as white flakes: 7.9 g (75%); mp 91°; $\lambda_{max}^{CHCl_3}$ 305 mμ (ε 17,900), 290 mμ (ε 16,440).

Anal. Calcd for $C_{25}H_{25}NO_5S$: C, 66.20; H, 6.00; N, 3.08; S, 7.06. Found: C, 66.15; H, 6.04; N, 3.08; S, 7.42.

N-Benzyl-N-*p*-carbethoxyphenyl-N′-(2-hydroxyethyl)ethylenediamine Hydrochloride (8).—2-Aminoethanol (2.44 g, 0.04 mole) in 100 ml of dry benzene was treated with 4.53 g (0.01 mole) of the tosylate **7**, and the mixture refluxed for 40 hr. After cooling, the benzene layer was separated and washed three times with 25-ml portions of water until the washings were neutral. The benzene solution was dried ($MgSO_4$) and evaporated to dryness yielding a thick syrup. Absolute ethanol (10 ml) was added and the solution was cooled overnight in a refrigerator, whereby unreacted **7** separated as a white powder. It was removed by filtration, and ethereal HCl was added to the filtrate. The hydrochloride thus obtained was recrystallized from ethanol–ether: white flakes, 2.5 g (65%), mp 143–145°.

Anal. Calcd for $C_{20}H_{26}N_2O_3$: C, 63.40; H, 7.18; N, 7.39; Cl, 9.35. Found: C, 63.32; H, 7.18; N, 7.41; Cl, 9.38.

N-*p*-Carbethoxyphenyl-N′-(2-hydroxyethyl)ethylenediamine (9).—The hydrochloride **8** (2.2 g, 0.006 mole) was dissolved in 125 ml of absolute ethanol with heating. The solution was cooled and 0.5 g of 5% palladium on carbon was added. The mixture was hydrogenated at 2 atm for 25 min in a Parr apparatus, whereby the theoretical amount of hydrogen was absorbed. The alcoholic solution was filtered and evaporated to dryness giving a residue that was dissolved in 5 ml of absolute ethanol and precipitated with ether. Recrystallization from alcohol-ether gave the hydrochloride as white flakes, 1.58 g (95%), mp 112–113°.

A solution of 1.25 g (0.004 mole) of the hydrochloride in 25 ml of water was made alkaline with saturated $NaHCO_3$ and saturated with NaCl. The resulting solution was extracted six times with 50-ml portions of ether. The ether extracts were combined and washed with 15 ml of water. Drying ($MgSO_4$) and concentrating to a small volume gave a white solid which was recrystallized from ether–Skelly B (bp 60–70°) to give **9** as white flakes, 0.9 g (82%), mp 96–96.5°, λ_{max}^{EtOH} 304 mμ (ε 22,130).

Anal. Calcd for $C_{13}H_{20}N_2O_3$: C, 61.88; H, 7.98; N, 11.10. Found: C, 61.51; H, 8.15; N, 11.38.

1-*p*-Carbethoxyphenyl-3-(2′-hydroxyethyl)imidazolidine (1a).—The diamine **9** (0.504 g, 0.002 mole) was dissolved in 25 ml of dry benzene, and 0.21 ml (0.002 moles) of 37% formaldehyde solution was added. The mixture was refluxed, for 2 hr, and water was collected in a Dean–Stark trap. After the reaction mixture was evaporated to dryness the white solid (0.52 g, quantitative yield) melted at 76.5°, λ_{max}^{EtOH} 307 mμ (ε 14,800).

Anal. Calcd for $C_{14}H_{20}N_2O_3$: C, 63.31; H, 7.62; N, 10.59. Found: C, 63.52; H, 7.45; N, 10.61.

1-*p*-Carbethoxyphenyl-2-phenyl-3-(2′-hydroxyethyl)imidazolidine (1b).—Benzaldehyde (0.106 g, 0.001 mole) and 0.236 g (0.0009 mole) of **9** were refluxed in 30 ml of dry benzene for 2 hr; water was collected in a Dean–Stark trap. The reaction mixture was evaporated to dryness, giving a yellow, syrupy residue. The residue was taken up in absolute ethanol and treated with activated charcoal, and the solvent was removed. The residue was crystallized from anhydrous ether–skelly B, as white crystals, 0.175 g (55%), mp 92.5°, λ_{max}^{EtOH} 308 mμ (ε 22,930).

Anal. Calcd for $C_{20}H_{24}N_2O_3$: C, 70.56; H, 7.10; N, 8.22. Found: C, 70.64; H, 7.01; N, 8.13.

1-*p*-Carbethoxyphenyl-2-(5′-uracil)-3-(2′-hydroxyethyl)imidazolidine (1c).—5-Formyluracil[19] (0.117 g, 0.0008 mole) was dissolved in 90% ethanol by refluxing for 6 hr. To this solution, 0.228 g (0.0009 mole) of **9** was added and the reaction mixture refluxed for 24 hr. The yellowish mixture was treated with activated charcoal and evaporated to dryness. The unreacted diamine was removed by extracting with ether, and the residue was recrystallized from ethanol, affording 0.1 g (32%) of yellowish white crystals, mp 220–221°, λ_{max}^{EtOH} 303 mμ (ε 14,640).

Anal. Calcd for $C_{18}H_{21}N_4O_5$: C, 57.74; H, 5.92; N, 14.96. Found: C, 57.79; H, 5.96; N, 14.72.

Ethyl *p*-(2-Pyrazal)aminobenzoate (11).—Dry benzene (35 ml), 1.08 g (0.01 mole) of aldehyde (**10**),[20] and 1.65 g (0.01 mole) of ethyl *p*-aminobenzoate were refluxed overnight to give the theoretical amount of water collected in a Dean–Stark trap. The solution was evaporated to dryness *in vacuo* to give a yellow residue. The oil on silica gel 5 (No. GF 254, E. Merck AG., Darmstadt) showed two spots under ultraviolet light after developing in 4% methanol in $CHCl_3$. One of the spots was found to be ethyl *p*-aminobenzoate by comparison with the authentic sample. Fractional crystallization from ether, ether–Skelly B, benzene–Skelly B, or ethanol was not successful. The compound **11** was used in the next step without further purification.

Ethyl *p*-(2-Pyrazinyl)methoxymethylaminobenzoate (12).—The Schiff base **11**, 0.5 g, was dissolved in 10 ml of absolute methanol at room temperature and kept at 0° for 2 days. The white solid that separated in the methanolic solution was filtered, recrystallized from absolute methanol, and dried under vacuum at room temperature: mp 100.5–101.5°.

Anal. Calcd for $C_{15}H_{17}N_3O_3$: C, 62.70; H, 5.96; N, 14.62. Found: C, 62.37; H, 6.18; N, 14.92.

Ethyl *p*-[(2-Pyrazinylmethyl)aminobenzoate (13).—The Schiff base **11** (2.55 g, 0.01 mole) was dissolved in 35 ml of methanol and cooled to 0°, and 0.8 g of sodium borohydride was added in portions during 10 min. The temperature of the mixture was raised to 60–70° for 45 min with continuous stirring. The reaction mixture was cooled to room temperature, acidified with acetic acid, diluted with 60 ml of water, made alkaline with NaOH solution, and extracted four times with 50-ml portions of ether. The ether extracts were combined, dried over $MgSO_4$, and evaporated to dryness giving a yellow powder, 2.4 g. The substance was found by tlc on silica gel 10 to be a mixture of the desired **13** and ethyl *p*-aminobenzoate. The mixture was separated on a silica gel column (E. Merck AG., Darmstadt, 0.05–0.20 mm, 130 g). The substances were eluted with ethyl acetate, and 15-ml fractions were collected. Ethyl *p*-aminobenzoate (0.03 g) was found in fractions 1–10; the product (1.8 g) was collected in fractions 11–25 with an additional 0.2 g found in tubes 26–40. The yellowish white compound, 2.0 g (78%), was recrystallized from ether as white needles: mp 117–118.5°; λ_{max}^{EtOH} 299 mμ (ε 22,380), shoulder at 275 mμ (ε 11,990).

Anal. Calcd for $C_{14}H_{17}N_3O_2$: C, 65.35; H, 5.87; N, 16.33. Found: C, 64.96; H, 5.65; N, 16.21.

Ethyl *p*-(2-Piperazinylmethyl)aminobenzoate (14a).—Platinum oxide (1.0 g) was suspended in 5 ml of glacial acetic acid and reduced for 50 min at atmospheric pressure in a micro-hydrogenation unit. To this suspension, a solution of 1.0 g (0.004 mole) of ethyl *p*-(2-pyrazinylmethyl)aminobenzoate (**13**) in 5 ml of glacial acetic acid was added and the solution was hydrogenated for 65 min. The theoretical amount of hydrogen was absorbed, and the reduction was stopped. After removal of the catalyst, evaporation of the filtrate gave a thick syrup which was taken up in 15 ml of water and extracted five times with 30 ml of ether. The ether extracts were combined, washed with 10 ml of water, dried over $MgSO_4$, and evaporated to dryness, giving 0.13 g of ethyl *p*-aminobenzoate. The aqueous solution was adjusted to pH 10 with 5% NaOH, saturated with NaCl, and extracted with eight 30-ml portions of $CHCl_3$. The $CHCl_3$ extracts were combined, washed with 10 ml of water, filtered, dried over $MgSO_4$, and evaporated to dryness, giving a yellow, syrupy liquid, 0.78 g (76%). The diacetate (**14b**) was a white powder: mp 94.5–95.5°; mol wt, 371, 382, and 389 (calcd 383.4); λ_{max}^{EtOH} 303 mμ (ε 22,190).

2-*p*-Carbethoxyphenyloctahydroimidazo[1,5-*a*]pyrazine (2a).—The amine **14a** (0.311 g, 0.001 mole) was dissolved in 5 ml of absolute methanol and a solution of 0.10 g (0.001 mole) of 37% formaldehyde in 10 ml of absolute methanol was added. The reaction mixture was refluxed for 6 hr, cooled, and evaporated to dryness. The yellow, semisolid residue was dissolved in 5 ml of dry benzene, evaporated to dryness, and dried under vacuum overnight. The yellow powder was extracted four times with 30 ml of hot ether. The ether solution, upon cooling, gave a white powder, 0.187 g (58%), which was dissolved in methanol,

treated with activated charcoal, and evaporated to dryness. Recrystallization from ether gave white crystals, mp 174–175.5°, λ_{max}^{EtOH} 307 mμ (ε 30,830).

Anal. Calcd for $C_{15}H_{21}N_3O_2$: C, 65.48; H, 7.68; N, 15.26. Found: C, 65.74; H, 7.50; N, 15.45.

2-*p*-Carbethoxyphenyl-3-phenyloctahydroimidazo[1,5-*a*]pyrazine (2b).—In a round-bottom flask attached to a Dean–Stark trap and a reflux condensor, 0.65 g (0.002 mole) of **14a** was dissolved in 35 ml of dry benzene. To this solution, 0.26 g (0.002 mole) of benzaldehyde was added and the mixture refluxed for 24 hr. The mixture was cooled and evaporated to dryness to give a brown syrup. The product was purified on a neutral alumina column (Woelm, grade III, 75 g), using ethyl acetate to give, after a forerun containing benzaldehyde, a white compound, 0.34 g (39%), which was dissolved in ethyl acetate, treated with activated charcoal and recrystallized from ethyl acetate; white crystals, mp 165–166.5°, λ_{max}^{EtOH} 307 mμ (ε 31,300).

Anal. Calcd for $C_{21}H_{25}N_3O_3$: C, 71.76; H, 7.17; N, 11.95. Found: C, 71.44; H, 6.99; N, 12.22.

2-*p*-Carbethoxyphenyl-3-(5′-uracil)octahydroimidazo[1,5-*a*]pyrazine (2c).—The amine **14a** (0.35 g, 0.001 mole) and 0.19 g (0.001 mole) of 5-formyluracil[19] were added to 40 ml of anhydrous methanol. The reaction mixture was refluxed for 27 hr, cooled to room temperature, concentrated to a small volume under vacuum, and stored at 10° overnight. The yellow, crystalline product was collected, 0.24 g (51%), dissolved in hot ethanol, treated with activated charcoal, and recrystallized from ethanol–methanol to give white crystals, mp 219–220°, λ_{max}^{EtOH} 304 mμ (ε 29,300).

The analytical sample prepared by drying over P_2O_5 under vacuum at room temperature showed the following analysis.

Anal. Calcd for $C_{19}H_{23}N_5O_5 \cdot C_2H_5OH$: C, 58.45; H, 6.77; N, 16.23. Found: C, 58.21; H, 6.73; N, 16.49.

After drying the above crystals at 100° under vacuum the analysis showed loss of the molecule of ethanol.

Anal. Calcd for $C_{19}H_{23}N_5O_4$: C, 59.20; H, 6.01. Found: C, 59.54; H, 6.07.

Ethyl *p*-N-(2-Pyrazinylmethyl)-N-thyminylaminobenzoate (4).—To a solution of 0.257 g (0.001 mole) of **13** in 20 ml of dry tetrahydrofuran was added 0.228 g (0.001 mole) of 5-bromomethyluracil,[22] 0.053 g of Na_2CO_3, and a catalytic amount of NaI. The mixture was cooled and filtered, and the residue was washed with a few milliliters of tetrahydrofuran (solution A). The gummy residue was washed with two 15-ml portions of water to remove water-soluble, inorganic materials leaving 0.2 g of powder B. The solution A was evaporated to dryness to give 0.257 g of yellow powder A. Tlc with alumina (GF 254, E. Merck AG., Darmsdt) using $CHCl_3$–methanol (9:1) showed A to be a mixture of the product **4**, the amine **13**, and unidentified material, and B to be a mixture of the product **4** and unidentified material. A and B were chromatographed separately as follows and as shown in Table II to obtain the product. Fractions

TABLE II

A, ON NEUTRAL ALUMINA (WOELM, GRADE I, 20 g)

Fraction	Effluent, vol (ml)	Description of material
1 and 2	Chloroform, 25	Amine **13**
3	Chloroform, 50	Amine **13**
4 and 4	Chloroform, 60	...
6 and 7	Chloroform–methanol (9:1), 100	Product **4**
8 and 9	Chloroform–methanol (8.5:1.5), 200	Product **4**

6–9 gave 0.109 g of the desired product. Similarly B was purified on an alumina column with $CHCl_3$–methanol to give 0.097 g of the desired product **4**. The products from A and B were combined to give 0.22 g (58%). Recrystallization from anhydrous methanol–ether gave white flakes: mp 193.5–194.5°; λ_{max}^{EtOH} 303 mμ (ε 25,000), 271 mμ (ε 18,000).

Anal. Calcd for $C_{19}H_{19}N_5O_4$: C, 59.83; H, 5.02; N, 18.36. Found: C, 59.52; H, 5.04; N, 18.41.

Enzyme Purification Studies. Thymidylate Synthetase.—Thymidylate synthetase was purified according to the procedure of Wahba and Friedkin[7a] from *E. coli* B.[23] The assay solution contained 0.05 μmole of deoxyuridine 5′-monophosphate (Sigma Chemical Corp.), 0.22 μmole of tetrahydrofolic acid (General Biochemicals Corp.), 15 μmoles of formaldehyde, 25 μmoles of $MgCl_2$, 130 μmoles of mercaptoethanol, 0.9 μmoles of ethylenediaminetetraacetic acid, 45 μmoles of Tris, pH 7.4, and enzyme in a total volume of 1.2 ml. Tetrahydrofolic acid was added to 1 *M* mercaptoethanol and adjusted to pH 7.4. This solution, containing approximately 20 μmoles/ml, was divided into small fractions and frozen. Deoxyuridine 5′-monophosphate was dissolved in water to give a stock solution containing approximately 1 μmole/ml and stored frozen. The assay stock solution (minus mix) contained 12 mmoles of mercaptoethanol, 27 μmoles of tetrahydrofolic acid, 1.8 mmoles of formaldehyde, and 3 mmoles of $MgCl_2$ in a total volume of 30 ml. The sample cell contained 0.25 ml of the minus mix; 0.05 ml of the deoxuridine 5′-monophosphate stock solution; enzyme, inhibitor solution, or inhibitor solvent; and buffer A to a total of 1.2 ml in the assay mixture. Deoxyuridine 5′-monophosphate was replaced by water in the reference cell.

The change in absorbance at 340 mμ was read at 30° in a Beckman DB spectrophotometer, recorded with 10 × expansion of the transmission scale, and converted to absorbance. Under these conditions the change in absorbance was linear for the first 10 min. Due to occasionally inconsistent rate changes with thymidylate synthetase, several of the compounds were preincubated at 30° with the minus mixture and buffer until the absorbance at 340 mμ stabilized. The addition of the enzyme and substrate then gave linear rates.

Dihydrofolates Reductase.—Dihydrofolic acid reductase was purified by a slight modification of the method of Mathews and Huennekens[24] from chicken livers. The enzyme preparation had a specific activity of 0.36 μmole/hr per mg of protein. This was divided into small fractions and frozen.

The stock solution of NADPH was prepared by dissolving NADPH (Sigma Chemical Corp.) in 0.02 *M* Tris buffer at a pH of 8.5 to give a final concentration of 0.5 μmole/ml. Dihydrofolic acid, prepared by dithionite reduction of folic acid according to Futterman,[25] was dissolved in 0.005 *M* acetate buffer containing 0.01 *M* mercaptoethanol at a pH of 4.5 to give a final concentration of 0.5 μmole/ml. The assay was that of Friedkin and co-workers[26] and contained 0.2 ml of the NADPH stock solution, 0.1 ml of the dihydrofolic acid stock solution, 0.75 ml of 0.02 *M* mercaptoethanol in 0.1 *M* phosphate buffer, pH 7.5, and enzyme, inhibitor, inhibitor solvent, or water to a total of 1.5 ml. Dihydrofolic acid was replaced by 0.005 *M* acetate buffer containing 0.01 *M* mercaptoethanol at pH 4.5 in the reference cell.

The change in absorbance at 340 mμ was recorded at 32° in a Beckman DB with a 10 × expansion of the transmission scale and was linear for 10 min. After conversion to absorbance units, 52% of the change in absorbance represents the utilization of dihydrofolic acid, and this change was used in determining the specific activity of the enzyme.

Inhibitors were dissolved in water, alcohol, or 0.02 *M* NaOH to give stock solutions containing 1 μmole/0.1 ml. When base or alcohol was used as the solvent, the effect of the solvent on the uninhibited reaction was examined and these rates compared to the inhibited reaction.

A study of the rate of saponification of ethyl *p*-aminobenzoate in 0.02 *M* NaOH showed a half-life of approximately 160 min. The inhibitors **1c**, **13**, and **2c** were dissolved in 0.02 *M* NaOH and assayed within 60 min after solution. The results, therefore, are considered to indicate inhibition by the ester, not the saponification product.

Acknowledgment.—The authors wish to acknowledge the assistance of Mrs. William Riggs for the enzyme studies, Mr. Larry Hare for the synthesis of starting materials, and Professor Morris Friedkin and associates for helpful discussions.

(23) Grain Processing Corp., Muscatine, Iowa.

(24) C. K. Mathews and F. M. Huennekens, *J. Biol. Chem.*, **238**, 3436 (1963).

(25) S. Futterman, *ibid.*, **228**, 1031 (1957).

(26) M. Friedkin, E. J. Crawford, S. R. Humphreys, and A. Goldin, *Cancer Res.*, **22**, 600 (1962).