values calculated by each method. It was shown that ΔKOH at any pH was equal to a function containing the hydrogen and hydroxide ion concentrations, dissociation constants, the stoichiometric concentrations of ligand and acid, and the concentration of potassium hydroxide in the ligand-metal titration. Comparison of $\bar{n}$ values calculated by the two methods gives a check on the validity of the data and for the concentration of ligand.

It was pointed out that calculations for tetracycline analogs at a high pH would be inaccurate since a dissociable hydrogen on a complexed molecule, but remote from the site of coordination, will have a larger dissociation constant than the same hydrogen on a free ligand.

The thermodynamic dissociation and stoichiometric stability constants were calculated for five tetracycline analogs with cupric ion and these values were compared with the data of previous workers. Contrary to the conclusions of Doluisio and Martin, the biologically inactive analogs, 4-epi-chlortetracycline and 4-epi-anhydrotetracycline, were found to form 2:1 complexes, which were quite insoluble and which precipitated at $\bar{n}$ values just above 1. Methods for the calculations of the biligand stability constants were discussed.

REFERENCES

(1) Bjerrum, J., "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, Denmark, 1941.
(2) Calvin, M., and Wilson, K. W., *J. Am. Chem. Soc.*, **67**, 2003(1945).
(3) Albert, A., *Biochem. J.*, **50**, 690(1952).
(4) Albert, A., *Nature*, **172**, 201(1953).
(5) Albert, A., and Reese, C. W., *ibid.*, **177**, 433(1956).
(6) Doluisio, J. T., and Martin, A. N., *J. Med. Chem.*, **6**, 16(1963).
(7) Green, R. W., Hallman, P. S., and Lions, F., *Inorg. Chem.*, **3**, 376(1964).
(8) Albert, A., and Serjeant, E. P., "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962, p. 24.
(9) Noyes, A. A., *Z. Physik. Chem.*, **11**, 495(1893); *cf.* Britton, H. T. S., "Hydrogen Ions," vol. I, 4th ed., Chapman and Hall Ltd., London, England, 1955.
(10) Kielland, J., *J. Am. Chem. Soc.*, **59**, 1675(1937).
(11) Speakman, J. C., *J. Chem. Soc.*, **1940**, 855.
(12) Stephens, C. R., *et al.*, *J. Am. Chem. Soc.*, **78**, 4155 (1956).
(13) Rossotti, F. J. C., and Rossotti, H. S., "The Determination of Stability Constants," McGraw-Hill Book Co., Inc., New York, N. Y., 1961, Chap. 5.
(14) Irving, H., and Rossotti, H. S., *J. Chem. Soc.*, **1953**, 3397.
(15) Leeson, L. J., Krueger, J. E., and Nash, R. A., *Tetrahedron Letters*, **1963**, 1155.

Nonclassical Antimetabolites XX

Simulation of 5′-Phosphoribosyl Binding IV. Attempted Simulation with Nucleoside-5′-carbamates

By B. R. BAKER, PRAFULLCHANDRA M. TANNA, and GRAHAM D. F. JACKSON

Twenty-two different ways can be envisioned for the mode of binding of the phosphate moiety of a nucleotide to an enzyme depending upon whether one, two, or three phosphate oxygens are involved and the specific groups involved on the enzyme are disregarded. A nucleoside-5′-carbamate should be able to simulate 13 of these possibilities, that is, all the modes of binding involving hydrogen bonds, but not anionic–cationic interactions. 5′-*O*-Carbamoylthioinosine (XII*a*) was synthesized, but failed to simulate the binding of thioinosinic acid (IV) to succinoadenylate kinosynthetase. Similarly, 5′-*O*-carbamoyl-2′-deoxy-5-fluorouridine (XXIV) was synthesized and failed to simulate the binding of 2′-deoxy-5-fluoro-5′-uridylic acid (VII) to thymidylate synthetase. In conjunction with some previous data, it has been concluded that the most likely mode of binding of nucleotides to these two enzymes involves one hydrogen bond and one anionic–cationic interaction of which there are four possible combinations.

IN THE FIRST paper of this series (1) on simulation of phosphate binding, the underlying biochemical reasons for the utility of this simulation were discussed; in a relatively simple assay system, that is, simulation of the inhibitory properties of 5′-adenylate on lactic dehydrogenase and glutamic dehydrogenase by 9H-adenine-9-yl alkanoic acids, the results were considered to be successful. However, later attempts to obtain phosphate simulation in binding to thymidylate synthetase with uracil-1-alkanoic acids and their derivatives were unsuccessful (2). It was concluded (2) that a more systematic study on the groups of a 5′-phosphoribosyl moiety necessary for binding to the enzyme would be required before relatively simple side chains could be used for binding in place of the 5′-phosphoribosyl binding; such a study with succinoadenylate kinosynthetase has been reported recently (3). Another approach to the simulation of phosphate binding of a nucleotide would be the replacement of the phosphate group on a known nucleotide

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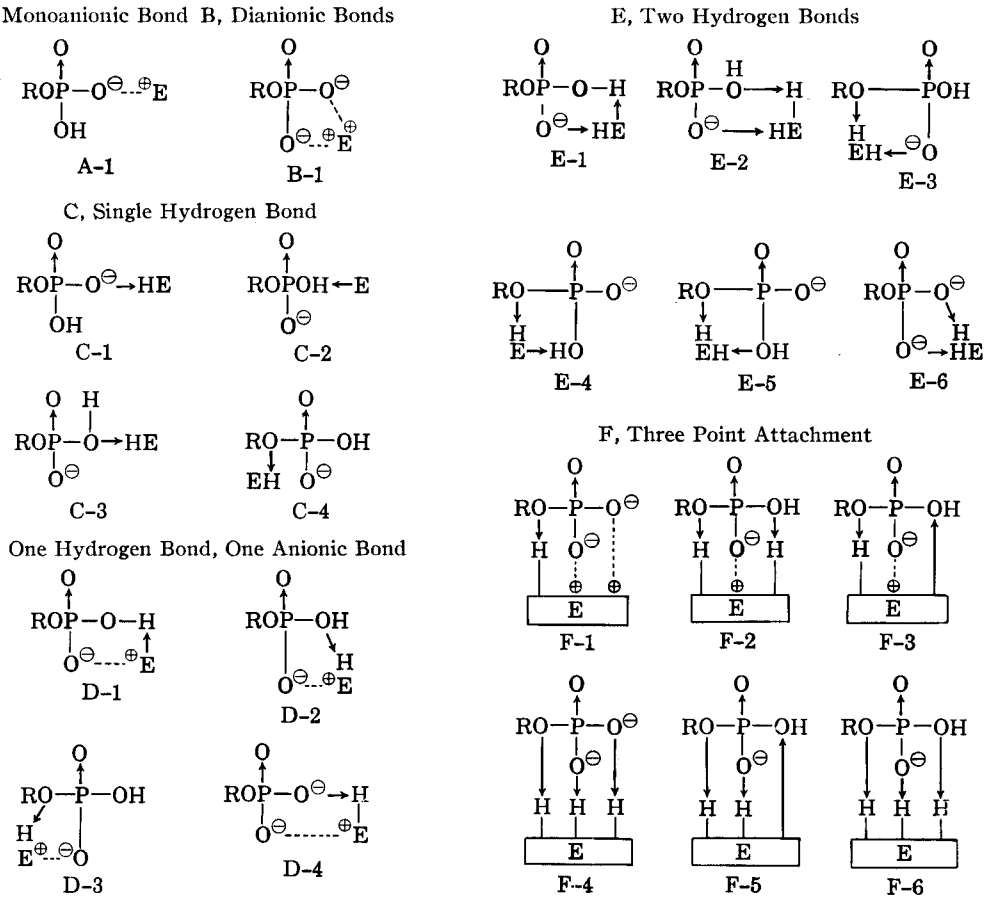
Previous paper: Baker, B. R., and Tanna, P. M., *J. Pharm. Sci.*, **54**, 845(1965).

Possible Modes of Binding of a Phosphate Ester to an Enzyme, E



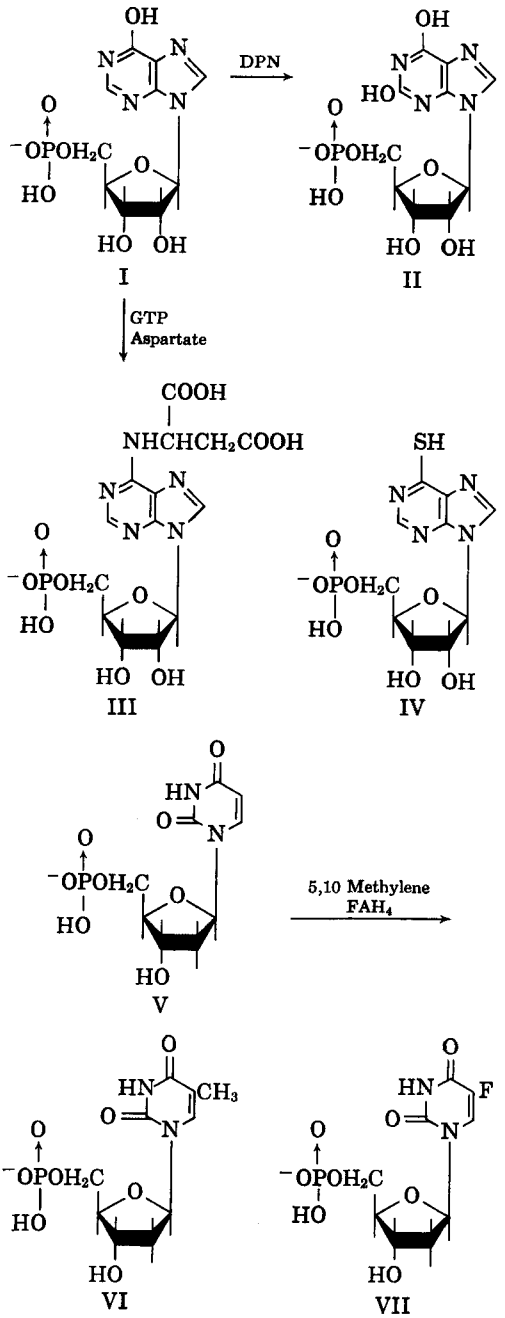
Scheme I

inhibitor by some candidate binding group; such a candidate group is the 5′-carbamate group. The synthesis and enzymic evaluation of the 5′-*O*-carbamoyl derivatives of thioinosine (XII*a*) and 2′-deoxy-5-fluorouridine (XXIV) are the subjects of this paper.

DISCUSSION

Over 20 different ways can be envisioned (Scheme I) for the mode of binding of the phosphate group of a phosphate ester-containing substrate, depending upon whether one, two, or three oxygen functions of the phosphate are involved in binding and whether the mono-anionic form or di-anionic form of the phosphate binds by hydrogen bonds or by anionic interaction with a cationic group on the enzyme surface. This cationic group can be either directly on the enzyme, or the anionic group can be bridged to an anionic group on the enzyme by the divalent metal ion frequently needed with phosphate ester substrates. Furthermore, there is no *a priori* reason to believe that all phosphate esters would bind to different enzymes in an identical fashion. If there are indeed different modes of binding of phosphate esters to their respective enzymes, then molecules properly designed to simulate one mode of phosphate binding might not bind to a different enzyme requiring the same substrate or the same enzyme from a different species; for example, if the mode of binding of the phosphate group of inosinate (I) to inosinic dehydrogenase (I→II, Scheme II) is different than the mode of binding of inosinate (I) to succinoadenylate kinosynthetase (I→III), then it should be ultimately possible to design an inhibitor that will block one enzyme and not the other or vice versa, thus achieving specificity. Replacement of the phosphate group of a nucleotide by a carbamate group, as discussed in this paper, serves to sort out some of the likely *versus* unlikely modes of phosphate binding to thymidylate synthetase (V→VI) and succinoadenylate kinosynthetase (I→III).

Of the 22 possible modes of phosphate binding listed in Scheme I, a carbamate ester such as XII*a* should be able to simulate 13 of these modes, that is, all of the modes of binding except those involving an anionic interaction of phosphate with a cationic site on the enzyme. Since thioinosinate (IV) has been reported to inhibit succinoadenylate kinosynthetase



Scheme II

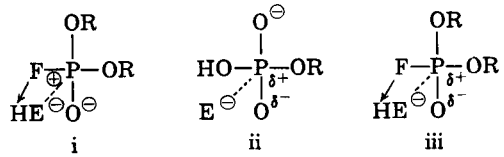
(I→III) (4–7), the 5′-carbamate of thioinosine (XII*a*) was selected as a candidate inhibitor for this enzyme. Similarly, since 2′-deoxy-5-fluorouridylic acid (VII) is an excellent inhibitor of thymidylate synthetase (V→VI) (8, 9) the 5′-carbamate of 2′-deoxy-5-fluorouridine (XXIV) was also selected for study.

That thioinosinic acid (IV) was a good inhibitor of succinoadenylate kinosynthetase (I→III) can be seen in Table I. However, thioinosine 5′-carbamate (XII*a*) was less than one-thirtieth as effective as IV.

2′-Deoxy-5-fluorouridine-5′-carbamate (XXIV) did inhibit thymidylate synthetase, but was less than 10^{-5} as effective as 2′-deoxy-5-fluorouridylate (VII) (Table I) (8, 9); in fact, the carbamate (XXIV) was even less effective than 2′-deoxy-5-fluorouridine (XIX) by a factor of 4.5. Similarly, thymidine-5′-carbamate (XXIII) was less effective than thymidylate (VI), a known inhibitor of thymidylate synthetase (10).

The fact that the 5′-carbamate group does not bind in place of the phosphate of these two nucleotides (IV, VII) in two enzyme systems is quite enlightening; thus, of the 22 possible modes of phosphate binding, those modes involving one, two, or three hydrogen bonds (C-1–C-4, E-1–E-6, F-4–F-6, respectively) (Scheme I) are rendered unlikely. Furthermore, since uracil-1-valeric acid fails to inhibit thymidylate synthetase (2), and 6-mercapto-9H-purine-9-ylvaleric acid (1) is a poor inhibitor of succinoadenylate kinosynthetase (11), a simple mono-anionic–cationic interaction (A-1) is also unlikely. Of the remaining eight possible modes of binding, one involves a di-anionic–cationic interaction (B-1), four involve a mono-anionic–cationic interaction plus one hydrogen bond (D-1–D-4), and three involve a mono-anionic–cationic interaction plus two hydrogen bonds (F-1–F-3). At this point, one or more of the members of the D class seem to be the most likely candidates since the F class and B-1 would appear to give more free energy of binding than required for the observed K_i's of IV and VII. Therefore, current attention is being focused on synthesis of candidate inhibitors that could simulate phosphate binding of type D, that is one anionic–cationic interaction and one hydrogen bond.[1]

[1] A polarized P → O form such as $\overset{\delta+}{\text{P}}\text{—}\overset{\delta-}{\text{O}}$ has been suggested (25) as a possible mode of binding of diisopropylphosphofluoridate to esterases by a structure such as i or ii. Since the phosphate monoester of a nucleotide already has one hydroxyl group fully ionized and a second hydroxyl group partially ionized, the polarization of the P → O group of a phosphate monoester, as in iii could be expected to be considerably repressed. Therefore, enzyme-substrate binding having one of the phosphate binding points being of type iii is not yet seriously being considered for the design of inhibitors for phosphate simulation.



Binding of the type $E^{\oplus} \ldots \overset{\delta-}{\text{O}}\text{—}\overset{\delta+}{\text{P}}\text{—}O^{\ominus}$ could be expected to shift to $E^{+} \ldots {}^{\ominus}\text{O—}\overset{\delta+}{\text{P}}\text{—}\overset{\delta-}{\text{O}}$, which would be a polarized form of A-1 and therefore equivalent to A-1. Hydrogen bonding to the P → O linkage as in iv would be equivalent to C-1 or C-3.

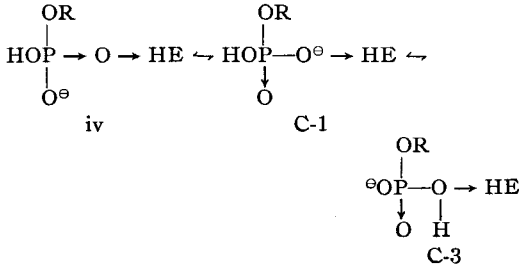
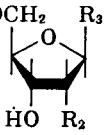


TABLE I.—INHIBITION OF SUCCINOADENYLATE KINOSYNTHETASE AND THYMIDYLATE SYNTHETASE BY



Compd.	R_1	R_2	R_3	Enzyme[a]	mM Concn. Inhibitor	% Inhibition	I_{50}[b]
IV[c]	$-PO(OH)_2$	OH	MP[d]	A	0.070	50	2.3
XII*a*	$-CONH_2$	OH	MP[d]	A	0.90	0	>85[e]
VII	$-PO(OH)_2$	H	FU[f]	B			0.001[g]
XIX	H	H	FU[f]	B	3.6	50	90
XXIV	$-CONH_2$	H	FU[f]	B	20	50	500
VI	$-PO(OH)_2$	H	T[h]	B			0.67[i]
XXIII	$-CONH_2$	H	T[h]	B	5	0	>500[e]

[a] Succinoadenylate kinosynthetase (enzyme A) from *Escherichia coli* B was isolated and assayed with 30.6 μM IMP, 100 μM GTP, 3.75 mM L-aspartate, and 10 mM $MgCl_2$ in glycine buffer (pH 8.0) as previously described (3). Thymidylate synthetase (enzyme B) from *E. coli* B was isolated and assayed with 214 μM *dl*-tetrahydrofolate, 40 μM 2′-deoxyuridylate, 20 mM formaldehyde, and 12 mM $MgCl_2$ in Tris buffer (pH 7.4) as previously described (15). [b] The ratio of concentration of inhibitor to IMP (enzyme A) or dUMP (enzyme B) giving 50% inhibition. [c] Data previously reported (3). [d] MP = 6-mercapto-9H-purine-9-yl. [e] Since 20% inhibition is readily observed, the concentration for 50% inhibition is estimated to be greater than four times the concentration measured. [f] FU = 5-fluoro-1-uracilyl. [g] Data taken from *References 8, 9,* and *24*. [h] T = 1-thyminyl. [i] Data taken from *Reference 10;* calculated from ratio of K_I:K_m.

EXPERIMENTAL

Enzyme Measurements[2]

Reagents.—*Escherichia coli* B, sodium guanosine 5′-triphosphate (GTP), and *dl*-tetrahydrofolic acid (FAH_4) were purchased from General Biochemicals. Sodium 5′-inosinate (IMP) and sodium 2′-deoxy-5′-uridylate (dUMP) were purchased from the Sigma Chemical Co. Thymidine and streptomycin sulfate were purchased from Nutritional Biochemicals.

Succinoadenylate Kinosynthetase.—This enzyme was isolated from *E. coli* B by a modification of the procedure of Lieberman (12) and assayed by the method of Wyngaarden and Greenland (13), as previously described (3). Cuvette concentrations of 30.6 μM IMP, 100 μM GTP, 10 mM $MgCl_2$, and 3.75 mM L-aspartate in glycine buffer (pH 8.0) in 1 ml. of total solution were employed. The reaction was initiated by the final addition of L-aspartate and the increase in absorbance at 280 mμ was followed with a Cary model 15 recording spectrophotometer on a 0–0.1 absorbance slide wire; the initial slopes without inhibitor were about 0.004 absorbance units per minute.

Thymidylate Synthetase.—This enzyme was isolated from *E. coli* B by a modification of the procedure of Wahba and Friedkin (14) and assayed by a modification of their method as previously described (15). Cuvette concentrations of 214 μM *dl*-FAH_4, 40 μM dUMP, 12.1 mM formaldehyde, and 20 mM $MgCl_2$ in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM tetrasodium ethylenediaminetetraacetate[3] in 3.1 ml. of total solution were employed. The reaction was initiated by final addition of dUMP after the system had balanced, and the increase in absorbance at 338 mμ was followed with a Cary model 11 recording spectrophotometer on a 0–0.1 absorbance slide wire; the initial slopes in the absence of inhibitor were about 0.004 absorbance units per minute.

For assay of both enzymes, the inhibitors were dissolved in the respective buffer solutions and sufficient was added to give 30–70% inhibition. By plotting V_0/V_I against I, the 50% inhibition concentration (I_{50}) when $V_0/V_I = 2$ was readily determined, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = concentration of inhibitor (1, 16); the I_{50} values are recorded in Table I.

Chemistry

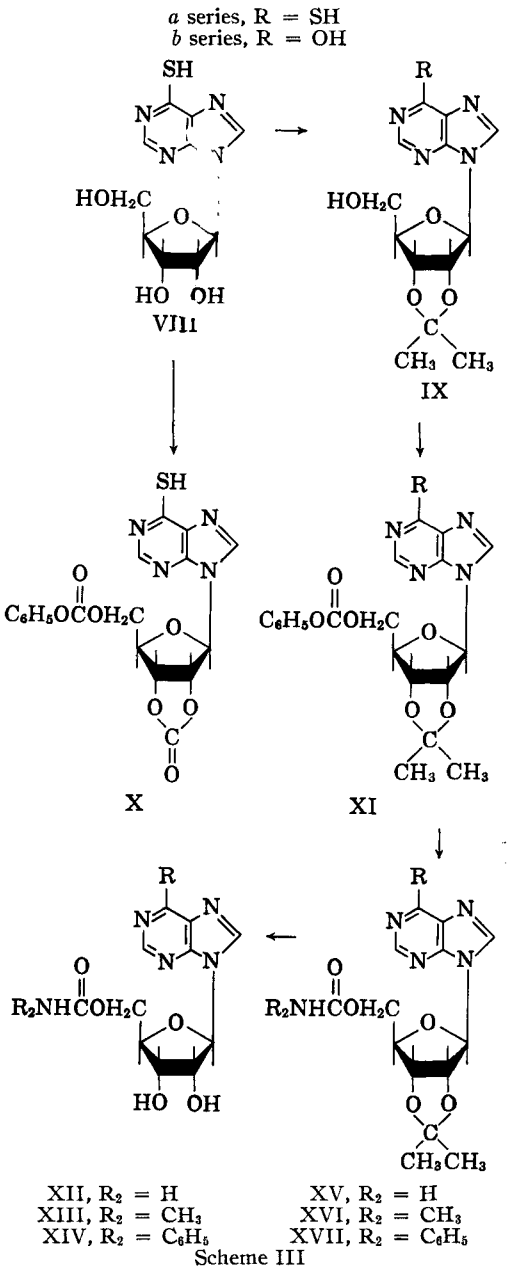
Methods.—There are two major routes to the preparation of carbamates. The first method involves conversion of the alcohol to a mixed carbonate with phenyl chloroformate in pyridine, followed by treatment with ammonia (17, 18). Both steps were considered compatible with a nucleoside, since basic conditions were involved. The second method involves reaction of an alcohol with sodium cyanate and trifluoroacetic acid in concentrated benzene solution (19); the solubility of a nucleoside derivative such as IX was not considered compatible with their stringent requirements for solvent.

Reaction of 2′,3′-*O*-isopropylideneinosine (IX*b*) with phenyl chloroformate in pyridine afforded the mixed carbonate (XI*b*) as an amorphous solid in 63% yield. With concentrated ammonia water at room temperature, XI*b* was smoothly converted to the crystalline carbamate (XV*b*) in 64% yield. Attempted removal of the isopropylidene group from XV*b* with aqueous acetic acid was followed by thin-layer chromatography; with conditions sufficient for complete removal of the isopropylidene group, considerable cleavage to hypoxanthine had already occurred. Attempts to separate hypoxanthine from the desired carbamate (XII*b*) appeared unpromising due to the poor crystallizing properties of inosine derivatives. Therefore, attention was turned to synthesis of the thioinosine-5′-carbamate (XII*a*) since previous experience in this laboratory had indicated that 9-alkyl-6-mercaptopurines had better crystallizing powers than the corresponding hypoxanthine derivatives.

A number of attempts to acylate selectively the 5′-hydroxyl group of thioinosine (VIII) with phenyl chloroformate proved unpromising since a mixture

[2] The technical assistance of Miss Shirley Herrmann, Miss Karen Smith, and Miss Gail Westley is acknowledged.

[3] Marketed as Versene.



Scheme III

of products was always obtained; this mixture contained a cyclic carbonate such as X, as could readily be seen by twin carbonyl bands at 1760 cm.$^{-1}$ for the carbophenoxy carbonyl group and 1810 cm.$^{-1}$ for the cyclic carbonate. Even treatment of VIII with one equivalent of phenyl chloroformate led to products with 1810 cm.$^{-1}$ absorption, indicating that there was insufficient selectivity in acylation of the 5′-hydroxyl over the 3′- or 2′-hydroxyl groups; considerable nucleoside was recovered unchanged. Therefore, it was necessary to block the nucleoside as its 2′,3′-*O*-isopropylidene derivative (IX*a*). Since trace amounts of cupric ion in the final inhibitor preparation have been shown to change the inhibitor properties of thioinosinate (IV) (20) for succinoadenylate lyase, the enzyme that converts succinoadenylate (III) to adenylate, a modification of the method of Hampton (21), which does not use cupric sulfate, was utilized for preparation of IX*a*. Thioinosine (VIII) was smoothly converted to its isopropylidene derivative (IX*a*) in 84% yield when a 2:1 ratio of methanesulfonic acid to VIII was employed in acetone containing 2,2-dimethoxypropane as a water scavenger.

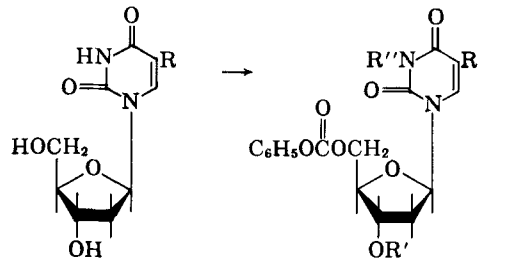
The isopropylidene nucleoside (IX*a*) reacted smoothly with excess phenyl chloroformate in pyridine to give an 87% yield of the mixed carbonate (XI*a*) which showed a single carbonyl band at 1760 cm.$^{-1}$. When reacted with aqueous ammonia at room temperature, XI*a* was readily converted to the crystalline 5′-carbamate (XV*a*) in 91% yield. Considerable study was devoted to the best conditions for removal of the isopropylidene group of XV*a* since concurrent nucleoside cleavage to 6-mercaptopurine was a serious side reaction; the reactions were followed by thin-layer chromatography. The best conditions found were refluxing in 20% acetic acid for 45 min.; at this time some XV*a* was still present, but 33% cleavage to the water-insoluble 6-mercaptopurine had already occurred. The desired carbamate (XII*a*) was readily separated from the water-insoluble 6-mercaptopurine since XII*a* is readily soluble in hot water, but insoluble in cold; XII*a* was isolated as a beautifully crystalline material in 33% yield.

Although the isopropylidene derivative (IX*a*) could be converted directly to the *N*-phenylcarbamate (XVII*a*) in *N*,*N*-dimethylformamide with phenylisocyanate, attempted selective removal of the isopropylidene group of XVII*a* to form XIV*a* gave a complex mixture which was unpromising. However, the *N*-methylcarbamate (XVI*a*), prepared from XI*a* with aqueous methylamine in 78% yield, could be converted to crystalline XIII*a* in 37% yield with a 29% concomitant cleavage to 6-mercaptopurine. (Scheme III.)

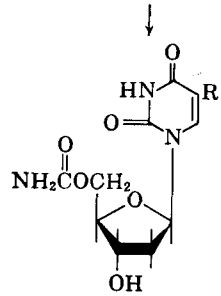
Since thymidylate is an inhibitor of thymidylate synthetase (10) and since thymidine (XVIII) is considerably more accessible than 5-fluoro-2′-deoxyuridine (XIX), the synthesis of thymidine 5′-carbamate (XXIV) was investigated first. Even though the properly blocked derivative of thymidine—namely, its 3′-acetate—can be prepared in three steps *via* 5′-tritylthymidine in a reported overall yield of 61% (22, 23), the selective acylation of thymidine (XVIII) with phenyl chloroformate to XX was explored; the direct acylation proceeded to the crystalline 5′-*O*-carbophenoxy derivative (XX) in 24% yield, and some unchanged thymidine could be recovered. Formation of the 5′-carbamate (XXIII) from XX with aqueous ammonia, as described for the preparation of XV*a*, proceeded in 90% yield or 22% over-all from thymidine (XVIII).

Since the remote possibility existed that the product isolated by selective acylation of thymidine (XVIII) was the isomeric 3′-*O*-carbophenoxythymidine, which in turn would give a 3′-carbamate, 3′-*O*-acetylthymidine (23) was reacted with excess carbophenoxy chloride in pyridine to give XXI. Treatment of XXI with methanolic ammonia resulted in cleavage of the phenoxy ester and *O*-acetyl groups to give authentic thymidine 5′-carbamate (XXIII) in about the same over-all yield from thymidine (XVIII); this was identical with XXIII prepared *via* XX.

In a similar manner, 5-fluoro-2′-deoxyuridine (XIX) was selectively acylated to crystalline XXII*a* in 27% yield. Conversion of the carbophenoxy derivative (XXII*a*) to the 5′-carbamate (XXIV) with aqueous ammonia, as described for preparation of the analogous thymidine derivative (XXIII), gave an oily mixture from which the desired XXIV could only be isolated with difficulty. In contrast, XXII*a* was readily converted with methanolic ammonia to the crystalline 5′-carbamate (XXI) in near quantitative yield. That the product was indeed a 5′-carbamate (XXI) and not a 3′-carbamate was demonstrated by an alternate unequivocal synthesis of the 5′-carbamate *via* the 3′-acetate (XXII*b*); the over-all yield from XIX was considerably poorer by this alternate route. (Scheme IV.)



XVIII, R = CH_3
XIX, R = F
XX, R = CH_3, R′ = H, R″ = H
XXI, R = CH_3, R′ = CH_3CO, R″ = H
XXII*a*, R = F, R′ = H, R″ = H
XXII*b*, R = F, R′ = CH_3CO, R″ = C_6H_5OCO—



XXIII, R = CH_3
XXIV, R = F

Scheme IV

Synthesis

Melting points were taken in capillary tubes on a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer in aqueous solution. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel G with benzene–methanol (3:1) (solvent A), benzene–ethanol (5:1) (solvent B), water-saturated *n*-butanol (solvent C), or benzene–ethanol (20:3) (solvent D). Spots were detected by iodine vapor or inspection under ultraviolet light; in each case, a solvent system was chosen that differentiated starting material from product.

2′,3′-O-Isopropylidenethioinosine (IXa).—To a magnetically stirred mixture of 2.50 Gm. (8.8 mmoles) of thioinosine (VIII) in 60 ml. of reagent acetone and 3.8 ml. of 2,2-dimethoxypropane was added 1.25 ml. (17.5 mmoles) of methanesulfonic acid. After being stirred in a stoppered flask for 6 hr., the reaction mixture was added to a mixture of 100 ml. of iced water and 63 ml. of 1 *N* ammonium hydroxide with stirring. The resulting solution was spin-evaporated *in vacuo* to about 75 ml. when the product separated; yield, 2.39 Gm. (84%), m.p 225–226°, that was suitable for further transformations; $\nu_{max.}$ 3500 (OH); 1600, 1570, 1530 (C=C, C=N); 1100 cm.$^{-1}$ (C—O—C). [Lit. m.p. 235° (21).]

5′ - O - Carbophenoxy - 2′,3′-O-isopropylidenethioinosine (XIa).—To a magnetically stirred solution of 0.972 Gm. (3 mmoles) of IX*a* in 15 ml. of reagent pyridine cooled in an ice bath was added 0.45 ml. (3.3 mmoles) of phenyl chloroformate over a period of about 5 min. After an additional 15 min. in the ice bath, the mixture was magnetically stirred at ambient temperature in a stoppered flask for 3.75 hr. The mixture was spin-evaporated *in vacuo* and the residue was triturated well with 20 ml. of water, then collected on a filter and washed with water (3 × 15 ml.). After being dried on a steam bath, the solid was washed with benzene (3 × 10 ml.) to remove traces of diphenyl carbonate whose presence was indicated by carbonyl absorption at 1770 cm.$^{-1}$; yield, 1.165 Gm. (87%), m.p. 235–238° dec., that was suitable for further transformation. Recrystallization of a sample from 50% aqueous acetone gave buff-colored crystals, m.p. 240–242° dec.; $\nu_{max.}$ 1760 (ester C=O); 1590, 1560, 1530 (C=C, C=N); 1240, 1200 (ester C—O—C); 1100 (ether C—O—C); 730, 685 (C_6H_5); no OH near 3500 cm.$^{-1}$. The compound moved as a single spot on TLC in solvent A.

Anal.—Calcd. for $C_{20}H_{20}N_4O_6S$: C, 54.1; H, 4.54; N, 12.6. Found: C, 54.2; H, 4.54; N, 12.6.

Similarly, IX*b* was converted to XI*b*, except that the benzene washings were omitted. XI*b* was obtained as an amorphous solid in 63% yield that could not be crystallized; $\nu_{max.}$ 1760 (ester C=O); 1690, 1650, 1580, 1540 (C=O, C=N, C=C); 1260, 1230 (ester C—O—C); 1060 (ether C—O—C); 750, 685 cm.$^{-1}$ (C_6H_5). Although a trace of diphenyl carbonate was present, as shown by a small carbonyl peak at 1770 cm.$^{-1}$, TLC in solvent A showed a single major spot and diphenyl carbonate moved with the solvent front.

5′ - O - Carbamoyl - 2′,3′ - O - isopropylidenethioinosine (XVa).—A solution of 889 mg. (2 mmoles) of XI*a* in 40 ml. of concentrated ammonia water was allowed to stand 5 hr. at room temperature, then spin-evaporated *in vacuo*. Trituration with ether to remove phenol gave 671 mg. (91%) of product, m.p. 240–250°. Recrystallization from 75 ml. of 50% aqueous acetone gave 224 mg. (34%) of near colorless needles of analytically pure material that moved as a single spot on TLC in solvent A and had m.p. 243–247° dec.; $\nu_{max.}$ 3500, 3350, 3200 (NH); 1730–1700 (broad carbamate C=O); 1600 1560, 1530 (NH, C=C, C=N); 1200 (ester C—O—C); 1100 (ether C—O—C); no phenoxy near 1760, 730, and 685 cm.$^{-1}$.

Anal.—Calcd. for $C_{14}H_{17}N_5O_5S$: C, 45.8; H, 4.67; N, 19.1. Found: C, 45.6; H, 4.80; N, 19.1.

From the mother liquor of the 224 mg. there was isolated 210 mg. (total 62%) of a second crop, m.p. 240–242° dec., that was also suitable for further transformations.

In the same manner, reaction of XI*a* with 40% aqueous methylamine gave XVI*a* in 78% yield as an amorphous powder that could not be crystallized but was uniform on TLC in solvent A and had $\nu_{max.}$ 1730, 1710 (C=O); 1600, 1575, 1540 (C=C, C=N, NH); 1200 (carbamate C—O—C); 1100 (ether C—O—C); no phenoxy ester near 1760, 760, or 690 cm.$^{-1}$.

5′ - O - Carbamoyl-2′,3′ - O - isopropylideneinosine (XVb).—XI*b* was converted to this in 64% yield, m.p. 149–151°; no suitable solvent for recrystallization could be found. TLC in solvent C showed a single spot. The compound had $\nu_{max.}$ 1710 (carbamate C=O); 1690, 1650, 1590, 1550 (NH, C=C, C=N, C=O); 1220 (ester C—O—C); 1080 (ether C—O—C); no phenoxy bands near 1760, 750, or 685 cm.$^{-1}$.

Anal.—Calcd. for $C_{14}H_{17}N_5O_6$: C, 47.9; H, 4.88; N, 19.9. Found: C, 47.6; H, 5.01; N, 20.1.

2′,3′ - O - Isopropylidene-5′ - O - (N - phenylcarbamoyl)thioninosine (XVIIa).—A mixture of 324 mg. (1 mmole) of IX*a*, 0.8 ml. of *N,N*-dimethylformamide, and 0.12 ml. (1.1 mmoles) of phenylisocyanate was heated on a steam bath for 45 min. The solution was spin-evaporated *in vacuo*. The residue was triturated with 10 ml. of ether, then collected on a filter and washed with two 10-ml. portions of ether. Recrystallization from 70% aqueous acetone gave 279 mg. (63%) of nearly colorless crystals, m.p. 249–250°; $\nu_{max.}$ 3400 (NH), 1750, 1725 (C=O); 1600, 1550, 1530 (NH, C=C, C=N); 1220 (carbamate C—O—C); 1100 (ether C—O—C); 760, 690 cm.$^{-1}$ (C_6H_5). The compound showed one spot on TLC in solvent A.

Anal.—Calcd. for $C_{20}H_{21}N_5O_5S$: C, 54.2; H, 4.77; N, 15.8. Found: C, 54.0; H, 4.91; N, 15.6.

5′-O-Carbamoylthioinosine (XIIa).—A solution of 200 mg. (0.54 mmole) of XV*a* in 10 ml. of 20% acetic acid was refluxed for 45 min., then spin evaporated *in vacuo* in a 30° bath. The residue was heated with 8 ml. of water, and the insoluble 6-mercaptopurine (30 mg., 33%) was removed by filtration. On being chilled, the filtrate deposited 59 mg. (33%) of XII*a*, m.p. 225–227° dec.; the filtrate contained a mixture of XII*a* and XV*a* as shown by TLC in solvent A. Recrystallization from water gave buff-colored crystals, m.p. 227–228° dec.; $\nu_{max.}$ 3500, 3400 (OH, NH); 1700 (carbamate C=O); 1600, 1575, 1560, 1530 (NH, C=C, C=N); 1200 (carbamate C—O—C); 1080 cm.$^{-1}$ (C—OH); $\lambda_{max.}$ (pH 1) 324 mμ (ε 22,000); $\lambda_{max.}$ (H_2O) 321 mμ (ε 20,400); $\lambda_{max.}$ (pH 13) 312 mμ (ε 22,200); TLC showed a single spot in solvent A.

Anal.—Calcd. for $C_{11}H_{13}N_5O_5S$: C, 40.4; H, 4.00; N, 21.4. Found: C, 40.5; H, 4.20; N, 21.2.

When the reflux time was increased to 3.5 hr., 80% cleavage to 6-mercaptopurine occurred. A 1.5-hr. reflux gave 54% cleavage and 14% of XII*a*. The reaction was not more selective when performed at 60°.

5′-O-(N-Methylcarbamoyl)thioinosine (XIIIa).—A solution of 333 mg. (0.875 mmole) of XVI*a* in 10 ml. of 20% acetic acid was refluxed for 45 min., then spin-evaporated *in vacuo* to about 5 ml. The 6-mercaptopurine (38 mg., 29%) was removed by filtration. The filtrate was spin-evaporated to dryness *in vacuo*. Crystallization from 50% ethanol gave 70 mg. (23%) of nearly colorless needles, m.p. 212–216° dec., which moved as a single spot on TLC in solvent A and had $\nu_{max.}$ 3500, 3400 (NH, OH); 1700 (carbamate C=O); 1600, 1575, 1550, 1530 (NH, C=C, C=N); 1200 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (pH 1) 324 mμ (ε 22,400), $\lambda_{max.}$ (pH 7), 321 mμ (ε 20,600); $\lambda_{max.}$ (pH 13) 312 mμ (ε 20,600).

Anal.—Calcd. for $C_{12}H_{15}N_5O_5S$: C, 42.4; H, 4.44; N, 20.6. Found: C, 42.5; H, 4.40; N, 20.4.

From the mother liquor of the 70 mg. was isolated an additional 40 mg. (total 37%) of product, m.p. 206–209° dec.

5′-O-Carbophenoxythymidine (XX).—To a magnetically stirred and ice-cooled solution of 968 mg. (4 mmoles) of thymidine in 15 ml. of reagent pyridine was added dropwise 782 mg. (5 mmoles) of phenyl chloroformate over a period of 30 min. protected from moisture. After being stirred at ambient temperature for an additional 5 hr., the mixture was spin-evaporated *in vacuo*. Trituration of the residue with water gave 695 mg. of white solid; from the filtrate thymidine could be isolated. The white solid was extracted with two 30-ml. portions of boiling petroleum ether (b.p. 60–110°) to remove diphenyl carbonate (133 mg., C=O at 1780 cm.$^{-1}$). The insoluble material was leached with two 15-ml. portions of boiling benzene, leaving the insoluble product, m.p. 167–168°; yield, 345 mg. (24%) which showed one spot on TLC in solvent A. Recrystallization from ethanol gave white crystals, m.p. 168–169°; $\nu_{max.}$ 3430 (OH); 1770 (ester C=O); 1725, 1690, 1660 (C=O, C=C, C=N); 725, 688 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{17}H_{18}N_2O_7$: C, 56.4; H, 5.01; N, 7.73. Found: C, 56.6; H, 5.14; N, 7.82.

TLC of the benzene mother liquor showed three components.

3′-O-Acetyl-5′-O-carbophenoxythymidine (XXI).—To a magnetically stirred solution of 705 mg. (2.5 mmoles) of 3′-*O*-acetylthymidine (23) in 10 ml. of reagent pyridine cooled in an ice bath and protected from moisture was added 626 mg. (4 mmoles) of phenyl chloroformate over a period of 20 min. After being stirred at ambient temperature for 3 hr., the mixture was spin-evaporated *in vacuo*, and the residual oil was washed with water (2 × 10 ml.). The oil was leached with boiling petroleum ether (b.p. 60–110°) (2 × 25 ml.) to remove diphenyl carbonate. Crystallization from benzene–petroleum ether (b.p. 30–60°) gave 870 mg. (87%) of crude product, m.p. 68–78°. Recrystallization from benzene gave 725 mg. (73%) of white crystals, m.p. 84–87°; $\nu_{max.}$ 1760 (carbophenoxy C=O); 1725 (acetyl C=O); 1700, 1670 (C=O, C=N, C=C); 768, 682 (C_6H_5); no OH near 3500. The compound moved as one spot on TLC in solvent D, but analyzed for a benzene solvate.

Anal.—Calcd. for $C_{19}H_{20}N_2O_8 \cdot {}^1/_2C_6H_6$: C, 59.6; H, 5.23; N, 6.32. Found: C, 59.8; H, 5.30; N, 6.68.

Recrystallization from ethanol gave solvate-free white crystals, m.p. 138–139°.

Anal.—Calcd. for $C_{19}H_{20}N_2O_8$: C, 56.4; H, 4.99; N, 6.93. Found: C, 56.5; H, 5.01; N, 7.04.

5′-O-Carbamoylthymidine (XXIII).—*Preparation A.*—A solution of 121 mg. (0.33 mmole) of XX in 7 ml. of concentrated ammonia water was allowed to stand for 4 hr., then spin-evaporated *in vacuo*. Trituration of the residue with ether to remove phenol gave 85 mg. (90%) of product, m.p. 219–220°. Recrystallization from ethanol afforded white needles, m.p. 220–221°, that were uniform on TLC in solvent B; $\nu_{max.}$ 3500 (NH, OH); 1710 (amide and ring C=O); 1660, 1630 (C=C, C=N, NH); no phenoxy group near 1770, 725, or 688 cm.$^{-1}$; $\lambda_{max.}$ (H_2O) 268 mμ (ε 9100).

Anal.—Calcd. for $C_{11}H_{15}N_3O_6$: C, 46.3; H, 5.30; N, 14.7. Found: C, 46.4; H, 5.30; N, 14.6.

Preparation B.—Reaction of 186 mg. (0.46 mmole) of XXI with concentrated ammonia water was conducted as described under *Preparation A*, except that reaction proceeded for 18 hr., gave 91 mg. (71%) of recrystallized product, m.p. 219–221°, that was identical with preparation *A* by TLC, mixed melting point, and spectral data.

5′-O-Carbophenoxy-2′-deoxy-5-fluorouridine (XXIIa).—Reaction of 782 mg. (5 mmoles) of phenyl chloroformate with 984 mg. (4 mmoles) of XIX, as described for the preparation of XX gave 395 mg. of benzene insoluble material, m.p. 131–132°. Recrystallization from ethanol gave 385 mg. (27%) of white crystals, m.p. 135–136°, that were uniform on TLC in solvent B; $\nu_{max.}$ 3450 (OH); 1765 (ester C=O); 1700, 1660 (uracil); 770, 685 cm.$^{-1}$ (C_6H_5).

Anal.—Calcd. for $C_{16}H_{15}FN_2O_7$: C, 52.4; H, 4.10; N, 7.64. Found: C, 52.3; H, 4.06; N, 7.61.

3′-O-Acetyl-3,5′-N,O-dicarbophenoxy-2′-deoxy-5-fluorouridine (XXIIb).—Reaction of 158 mg. (0.55 mmole) of 3′-*O*-acetyl-2′-deoxy-5-fluorouridine (26) with 234 mg. (1.5 mmoles) of phenyl chloroformate was performed as described for the preparation of XXI. Crystallization from benzene, then recrystallization from ethanol gave 138 mg. (48%) of white needles, m.p. 153–154°; $\lambda_{max.}$ (EtOH) 271 mμ (ε 6300); $\nu_{max.}$ 1800, 1770 (carbophenoxy C=O); 1735 (acetyl C=O); 1690, 1590 (C=O, C=C, C=N); 745, 688 (C_6H_5); no OH near 3500 cm.$^{-1}$.

Anal.—Calcd. for $C_{25}H_{21}FN_2O_{10}$: C, 56.8; H, 3.98; N, 5.30. Found: C, 57.0; H, 4.02; N, 5.50.

Impure mono-5′-*O*-carbophenoxy derivative, m.p. 133–136°, could be isolated from the filtrate.

5′-O-Carbamoyl-2′-deoxy-5-fluorouridine (XXIV).—*Preparation A.*—To 10 ml. of methanol saturated with ammonia at 0° was added 182 mg. (0.5 mmole) of XXII*a*. After 30 min. at ambient temperature, the solution was spin-evaporated *in vacuo*. Trituration with ether, then recrystallization from ethyl acetate gave 140 mg. (99%) of product, m.p. 90–100°. Recrystallization from ethyl acetate afforded 98 mg. (68%) of white crystals, m.p. 101–102°, that were uniform on TLC in solvent B and had $\nu_{max.}$ 3500–3400 (OH, NH); 1710, 1660, 1600 (C=C, C=N, C=O, NH); no phenoxy bands near 1765, 770, 685 cm.$^{-1}$; $\lambda_{max.}$ (pH 7) 268 mμ (ε 7700).

Anal.—Calcd. for $C_{10}H_{12}FN_3O_6$: C, 41.5, H, 4.18; N, 14.5. Found: C, 41.8; H, 4.29; N, 14.2.

Preparation B.—A mixture of 90 mg. (0.22 mmole) of XXII*b* and 7 ml. of concentrated ammonia water was stirred for 72 hr., then spin-evaporated *in vacuo*. The residue was leached with ether (3 × 10 ml.) to remove phenol and acetamide, then crystallized from ethyl acetate–petroleum ether (b.p. 30–60°); yield of crude product, 28 mg. (57%), m.p. 80–89°. Recrystallization from ethyl acetate gave 20 mg. (41%) of white crystals, m.p. 100–101°, that had ultraviolet and infrared spectra identical with preparation *A*; there was no depression on mixed melting point, and both preparations moved as a single spot with the same mobility on TLC in solvent B. The reaction conditions in preparation *A* failed to remove the *N*-carbophenoxy group completely; the *N*-carbamate was still present even after 24 hr., as shown by carbonyl absorption at 1800 cm.$^{-1}$.

REFERENCES

(1) Baker, B. R., and Sachdev, H. S., *J. Pharm. Sci.*, **52**, 933(1963).
(2) Baker, B. R., and Chheda, G. B., *ibid.*, **54**, 25(1965).
(3) Baker, B. R., and Tanna, P. M., *ibid.*, **54**, 845(1965).
(4) Salser, J. S., Hutchison, D. J., and Balis, M. E., *J. Biol. Chem.*, **235**, 429(1960).
(5) Davidson, J. D., *Cancer Res.*, **20**, 225(1960).
(6) Atkinson, M. R., Morton, R. K., and Murray, A. W., *Biochem. J.*, **89**, 167(1963).
(7) Hampton, A., *Federation Proc.*, **21**, 370(1962).
(8) Cohen, S. S., *et al.*, *Proc. Natl. Acad. Sci. U.S.*, **44**, 1004(1958).
(9) Hartmann, K.-U., and Heidelberger, C., *J. Biol. Chem.*, **236**, 3006(1961).
(10) Blakely, R. L., *ibid.*, **238**, 2113(1963).
(11) Baker, B. R., and Tanna, P. M., unpublished data.
(12) Liebermann, I., *J. Biol. Chem.*, **223**, 327(1956).
(13) Wyngaarden, J. B., and Greenland, R. A., *ibid.*, **238**, 1054(1963).
(14) Wahba, A. J., and Friedkin, M., *ibid.*, **237**, 3794 (1962).
(15) Baker, B. R., Ho, B.-T., and Neilson, T., *J. Heterocyclic Chem.*, **1**, 79(1964).
(16) Baker, B. R., *et al.*, *J. Med. Pharm. Chem.*, **2**, 633 (1960).
(17) McLamore, W. M., P'An, S. Y., and Bavley, A., *J. Org. Chem.*, **20**, 1379(1955).
(18) Melander, B., *J. Med. Pharm. Chem.*, **1**, 443(1959).
(19) Loev, B., and Kormendy, M. F., *J. Org. Chem.*, **28** 3421(1963).
(20) Bridger, W. A., and Cohen, L. H., *Biochim. Biophys. Acta*, **73**, 514(1963).
(21) Hampton, A., *J. Am. Chem. Soc.*, **83**, 3640(1961).
(22) Michelson, A. M., and Todd, A. R., *J. Chem. Soc.*, **1955**, 816.
(23) Horwitz, J. P., Urbanski, J. A., and Chua, J., *J. Org. Chem.*, **27**, 3300(1962).
(24) Mathews, C. K., and Cohen, S. S., *J. Biol. Chem.*, **238**, 367(1963).
(25) Saunders, B. C., "Some Aspects of the Chemistry and Toxic Action of Organic Compounds Containing Phosphorous and Fluorine," Cambridge University Press, Cambridge, England, 1957, pp. 188–191.
(26) Thomas, H. J., and Montgomery, J. A., *J. Med. Pharm. Chem.*, **5**, 24(1962).