were evaporated and a sample of each residue was chromatographed on paper using solvent system 1 (Table II). A compound which absorbed ultraviolet light and had the same mobility as acetophenetidine (R_f 0.80) was obtained from fraction 1. After spraying the chromatogram with the Pauly reagent, a spot with the same mobility (R_f 0.50) and orange color as I was obtained from both fractions, and a second spot (R_f 0.33) with a red color was also obtained from fraction 2. Fraction 2 was evaporated and recrystallized twice from aqueous ethanol and once from ethyl acetate–cyclohexane. Crystals were obtained, m.p. 169–171°, alone or in mixture with an authentic sample of I. The infrared spectra of the metabolite and of I were identical. The ultraviolet spectra of the metabolite and the reference compound were similar in 0.5 *N* HCl (λ_{max} 281 mμ) and in 0.5 *N* NaOH (λ_{max} 297 mμ).

Acknowledgments. The authors wish to acknowledge the excellent assistance of Miss L. Beauchamp and Miss M. Bordun.

Nonclassical Antimetabolites. XXV.[1] Inhibitors of Thymidine Kinase. I. A New Spectrophotometric Assay. Inhibition by Nucleoside Derivatives

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A spectrophotometric assay for thymidine kinase has been devised that employs 2′-deoxy-5-fluorouridine (FUDR) as the substrate; the concentration of the resultant 2′-deoxy-5-fluorouridylate (FUDRP) was assayed by inhibition of thymidylate synthetase. The assay has a magnification of 1000-fold since FUDRP is known to bind to thymidylate synthetase 1000-fold better than the substrate, 2′-deoxyuridylate; the assay has then the same order of sensitivity as the usual type of radioactive assay. The mode of binding of the 2′-deoxyribosyl moiety of thymidine to thymidine kinase was investigated with deoxy derivatives of thymidine. Both the 5′- and 3′-hydroxyls contribute to binding, the latter more strongly. Whether or not the furanose oxygen contributed to binding could not be ascertained with any certainty. Bulk-tolerance studies indicated that larger groups, placed on the 5′-hydroxyl or 5-methyl of thymidine, caused a greater than 600-fold loss in binding. In contrast, an *n*-amyl group could be introduced on the N^3-position of thymidine with retention of enzyme binding, but the binding was decreased about 50-fold compared to thymidine.

One of the major endeavors in this laboratory is the design of suitable, active-site-directed, irreversible inhibitors[2] of dihydrofolic reductase and thymidylate synthetase.[3] An efficient blockade of either enzyme would result in "thymine-less death" of cells[4] from lack of the thymidylate necessary for DNA synthesis. Even if one did achieve the hoped-for species or tissue specificity predicted from the bridge principle of specificity with irreversible inhibitors,[2] the target cells still may be able to obtain sufficient thymidylate by converting thymidine, scavenged from the host blood stream, to thymidylate with the enzyme, thymidine kinase. Therefore, a nontissue-specific blockage of thymidine kinase might be a necessary adjunct to achieve "thymine-less death," the tissue specificity being achieved during blockage of dihydrofolic reductase or thymidylate synthetase. For this reason, a study on the mode of binding of inhibitors to thymidine kinase has been initiated.

Enzyme Studies.—The isolation of thymidine kinase from *E. coli* B and its assay with radioactive thymidine has been described by Okazaki and Kornberg.[5] These workers found that 2′-deoxyuridine and its 5-halogen derivatives were as effective as thymidine as substrates. Although their radioactive assay is quite satisfactory for evaluation of possible substrates, this assay is more laborious for quantitative evaluation of potential inhibitors; therefore, three possible methods for spectrophotometric assay of the enzyme were investigated, the third being considered the best because of the large magnification built into the assay.

(a) The measurement of ATP conversion to ADP as thymidine is converted to thymidylate (dTMP)[6] by measuring the generated ADP in a coupled system with pyruvate kinase and lactic dehydrogenase was investigated.[7] Although this coupled system worked reasonably well with the measurement of the ADP generated by the hexokinase-catalyzed phosphorylation of D-glucose, our preparation of thymidine kinase gave erratic results.

(b) The measurement of 2′-deoxyuridine conversion to 2′-deoxyuridylate (dUMP)[6] by coupling the latter as a substrate to thymidylate synthetase was investigated. This method was the simplest for detection of the presence of thymidine kinase in an extract, but the levels of thymidylate synthetase needed to make the rate of the coupled system dependent upon the thymidine kinase were uneconomically high.

(1) (a) This work was supported by Grant No. CA-05845 and CA-05867 from the National Cancer Institute, U. S Public Health Service. (b) For the previous paper of this series see B. R. Baker and G. D. F. Jackson, *J. Pharm. Sci.*, in press. (c) Address inquiries to Department of Chemistry, University of California, Santa Barbara, Calif. 93106.

(2) For a review on the factors in the design of active-site-directed irreversible inhibitors see B. R. Baker, *ibid.*, **53**, 347 (1964).

(3) (a) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **2**, 21 (1965); (b) B. R. Baker and J. H. Jordaan, *ibid.*, **2**, 162 (1965); (c) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965); (d) B. R. Baker and J. K. Coward, *ibid.*, **54**, 714 (1965); (e) B. R. Baker and J. H. Jordaan, *J. Med. Chem.*, **8**, 35 (1965); (f) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964); (g) B. R. Baker and H. S. Shapiro, *J. Med. Chem.*, **6**, 664 (1963).

(4) S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lechtenstein, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1004 (1958).

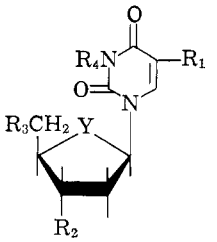
(5) R. Okazaki and A. Kornberg, *J. Biol. Chem.*, **239**, 269, 275 (1964).

(6) The following abbreviations are used: FUDR, 5-fluoro-2′-deoxyuridine; FUDRP, 5-fluoro-2′-deoxyuridylate; dUMP, 2′-deoxyuridylate; dTMP, thymidylate; M-FAH_4, 5,10-methylene-*dl*-tetrahydrofolate; FAH_2, dihydrofolate; dCTP, 2′-deoxycytidine triphosphate.

(7) This type of system has been used for the spectrophotometric assay of uridine kinase; see P. Reichard and O. Sköld, *Methods Enzymol.*, **6**, 194 (1963).

TABLE I

FUDR CONVERSION TO FUDRP BY THYMIDINE KINASE.[a] INHIBITION BY

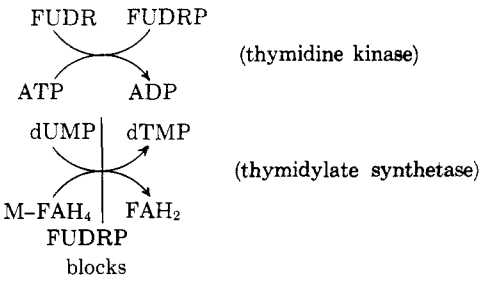


Compd.	R_1	R_2	R_3	R_4	Y	Concn., mM	% inhib.
II	CH_3	OH	OH	H	O	0.1	85 ± 7[b]
						0.04	68 ± 10[c]
III	H	OH	OH	H	O	0.2	53 ± 8[b]
IV	CH_3	H	OH	H	O	3	64
V	CH_3	OH	H	H	O	3	84
						1	16
VI	CH_3	OH	OH	H	CH_2	2	44
VII	CH_3	OH	C_2H_5OOCO	H	O	3	0[d]
VIII[e]	CH_3	OH	C_6H_5OOCO	H	O	3	0[d]
IX	CH_3	OH	CH_3NHCO	H	O	3	0[d]
X	CH_3	OH	$C_6H_5(CH_2)_2NHCO$	H	O	3	0[d]
XII	$EtOCH_2$	OH	OH	H	O	3	21[d]
XIV	CH_3	OH	OH	n-C_5H_{11}	O	3	70[d]
XIII	4-Thiothymidine					3	74[d]
XV	5,6-Dihydrothymidine					1	0

[a] The thymidine kinase assay was performed with 0.1 mM FUDR (I) as described in the Experimental Section. The technical assistance of Gail Westley is acknowledged. [b] Average and deviation in six determinations. [c] Average and deviation in four determinations. [d] See ref. 18. [e] For preparation see ref. 23.

(c) The measurement of 2′-deoxy-5-fluorouridine (FUDR) conversion to 2′-deoxy-5-fluorouridylate (FUDRP) was investigated by the ability of the generated FUDRP to inhibit thymidylate synthetase (see Chart I). FUDRP has previously been observed to bind

CHART I



to thymidylate synthetase 1000 times stronger than the substrate, dUMP[4]; therefore, the assay has a built-in magnification of 1000-fold, giving a sensitivity approaching that of a radioactive assay[5] but not requiring the separation of substrate from product before assay.

Although activity could be detected by either method b or c, the assays were still erratic. It was reasoned that the major difficulties were due to the further enzymatic conversion of FUDRP or dUMP to the triphosphate level and to the phosphorolytic cleavage of the nucleoside, nucleotide, and nucleoside triphosphate to the respective pyrimidines. Since fluoride ion is known to inhibit all of these side reactions, the possible inhibitory effect of fluoride on thymidine kinase and thymidylate synthetase was examined and found to be absent at the concentration (5 mM) used to inhibit these side reactions. In the presence of 5 mM fluoride, assay c became readily duplicable and the yield of FUDRP, as expected, was greatly increased. Furthermore, duplicable results could be obtained with an enzyme solution prepared by a simple three-step purification procedure. In addition, the thymidylate synthetase fraction was readily separated from the thymidine kinase fraction and could be used for the second step of the assay. The thymidine kinase was then shown to be linear over a period of at least 40 min., that is, the amount of FUDRP solution necessary for 50% inhibition of thymidylate synthetase was inversely proportional to the incubation time of the thymidine kinase reaction.

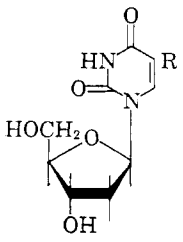
Since both thymidine and FUDR are substrates for the thymidine kinase, thymidine should act as a competitive substrate of FUDR. When equal concentrations of thymidine and FUDR were incubated with thymidine kinase, only about one-fifth as much FUDRP was formed, that is, five times as much of the FUDRP solution was then needed to give 50% inhibition of the thymidylate synthetase reaction (Table I). Since deoxythymidine monophosphate (dTMP) is bound to the thymidylate synthetase about as effectively as dUMP[8] and since FUDRP binds 1000-fold better than dUMP,[4] the dTMP generated by the thymidine kinase does not interfere with the determination of the FUDRP concentration. Therefore thymidine has the over-all effect of inhibiting the formation of FUDRP from FUDR catalyzed by thymidine kinase.

2′-Deoxyuridine (III), a known substrate for thymidine kinase,[5] was also a competitive substrate (Table I), showing 53% inhibition of FUDRP formation at a concentration of 0.2 mM; in contrast, thymidine II

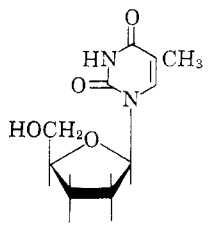
(8) R. L. Blakely, *J. Biol. Chem.*, **238**, 2113 (1963).

showed 68% inhibition at 0.04 m*M*. Thus, the order of binding to thymidine kinase is thymidine > FUDR > 2′-deoxyuridine since FUDR (I) is used at a concentration of 0.1 m*M* in the assay system. Okazaki and Kornberg[5] have noted that FUDR and 2′-deoxyuridine are about equally effective substrates, but that thymidine is a better substrate.

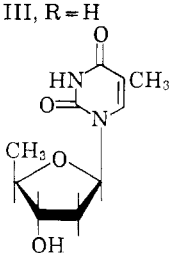
Several classes of studies were performed on the mode of binding of thymidine and related nucleosides to thymidine kinase. The first study was on the relative contribution of the three oxygen functions of the 2′-deoxyribosyl moiety. 3′-Deoxythymidine (IV)[9] at 3 m*M* showed 64% inhibition of FUDRP formation (Table I); thus, about an 80-fold loss in binding occurred when the 3′-hydroxyl of thymidine (II) was replaced by hydrogen (IV). 5′-Deoxythymidine (V)[10] at 3 m*M* showed 84% inhibition of FUDRP formation; the loss in binding when the 5′-hydroxyl of II was replaced by hydrogen (V) was thus about 30-fold. The cyclopentane analog VI of II at 2 m*M* showed 44% inhibition of FUDRP formation. Thus replacement of the furanose oxygen of thymidine (II) by a methylene group (VI) led to an estimated 120-fold loss in binding.



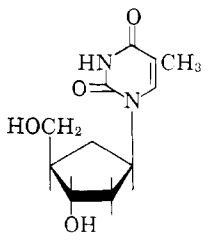
I, R = F
II, R = Me
III, R = H



IV



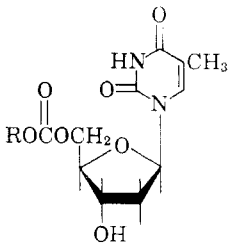
V



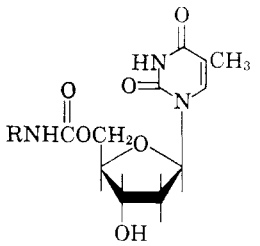
VI

It is clear that both the 3′- and 5′-hydroxyls of thymidine contribute to binding to thymidine kinase. Since a hydrogen atom is smaller than a hydroxyl group, there could not have been any steric effects on binding. The situation with the cyclopentane analog VI is less clear since the methylene group is larger than the furanose oxygen. Therefore, such a structural change leading to a 120-fold decrease in binding could be due to either a steric interaction of the methylene group with the enzyme that decreases binding, or the furanose oxygen is one of the binding points of thymidine to the enzyme, or both.

The fact that the 5′-hydroxyl of thymidine (II) contributes to binding to thymidine kinase is somewhat surprising. The 5′-hydroxyl is the position on the substrate where transfer of phosphate from ATP occurs. Usually a transfer point is not involved in binding to an enzyme using a cofactor.[11–14] An exception can occur when the enzyme aids in breaking an O–H bond. For example, the H of the O–H in lactate presumably complexes with an imidazole of lactic dehydrogenase thus aiding in the breaking of the O–H bond.[15–17] It could be, therefore, that the 5′-hydroxyl of thymidine (II) is complexed with thymidine kinase in order to aid bond breaking of the O–H group as phosphorylation by ATP takes place. Such a possibility would call for the hydrogen of the 5′-hydroxyl to complex with the enzyme rather than the oxygen atom.



VII, R = C_2H_5
VIII, R = C_6H_5



IX, R = CH_3
X, R = $C_6H_5CH_2CH_2$

To devise an unequivocal experiment that can distinguish between H and O bonding of the 5′-hydroxyl to thymidine is difficult. Replacement of the hydroxyl hydrogen by a carbon might lead to a bulk intolerance to complexing with the enzyme which could also be mistakenly interpreted to mean that a binding point (the hydroxyl hydrogen) had been lost. For example, four compounds (VII–X), that were made for a different purpose discussed below, failed to inhibit thymidine kinase at a concentration of 3 m*M*.[18a] The fact that even 5′-deoxythymidine (V) gives 84% inhibition at 3 m*M* shows that the enzyme does not have a bulk tolerance for the relatively large carbamate and carbonate groups of VII–X, else VII–X should have been at least as effective as 5′-deoxythymidine (V). Therefore, these experiments cannot be used as evidence either to support or to refute the concept that the hydrogen of the 5′-hydroxyl of thymidine is a binding point to the enzyme.

Compounds VII–X were actually synthesized to determine if the enzyme did have a bulk tolerance for the carbonate and carbamate groups at the 5′-position of thymidine (II). If such had been the case, this posi-

(9) Synthesized by the method of A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 816 (1955), as modified by J. P. Horwitz, J. Chua, and M. Noel, *J. Org. Chem.*, **29**, 2007 (1964), for introduction of the 3′-iodo group.

(10) Synthesized by the method of J. P. Horwitz, J. A. Urbanski, and J. Chua, *ibid.*, **27**, 3300 (1962).

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

(12) B. R. Baker, Presented to the Scientific Section of the American Pharmaceutical Association, Las Vegas, Nev., March 1962, Preprint C-I.

(13) B. R. Baker and H. S. Shapiro, *J. Med. Chem.*, **6**, 664 (1963).

(14) B. R. Baker, D. V. Santi, P. I. Almaula, and W. C. Werkheiser, *ibid.*, **7**, 24 (1964).

(15) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *ibid.*, **2**, 633 (1960).

(16) D. Dennis and N. O. Kaplan, *J. Biol. Chem.*, **235**, 810 (1960).

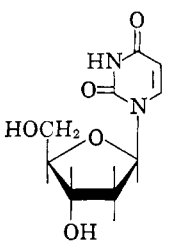
(17) A. D. Winer and G. W. Schwert, *ibid.*, **234**, 1155 (1959).

(18) (a) Samples of VII, IX, and X were employed for enzyme assay that were "analytically pure" by combustion analyses and moved as single spots on t.l.c. when run in the standard manner; these samples showed some inhibition of thymidine kinase at 3 m*M*. Since the compounds were made from thymidine, it was possible that the amount of inhibition observed was actually due to undetected thymidine. That such was the case was determined by the quantitative t.l.c. procedure described in the Experimental Section. When quantitated in this way, using 3:1 benzene–methanol, VII, IX, and X had 2–5% thymidine present which accounted for the inhibition observed at 3 m*M*. Two other compounds, VIII and XIV, prepared from thymidine, had less than 0.4% thymidine present. Similarly, using 4:1 $CHCl_3$–ethanol, "4-thiothymidine" (XIII)[18b] contained less than 0.4% thymidine; XII (= XIXc), using 5:3 $CHCl_3$–ethanol, contained less than 0.8% of 2′-deoxyuridine. (b) J. J. Fox, D. V. Pragg, I. Wempen, I. L. Doerr, L. Cheong, J. E. Knoll, M. L. Eidenoff, A. Bendich, and G. B. Brown, *J. Am. Chem. Soc.*, **81**, 178 (1959).

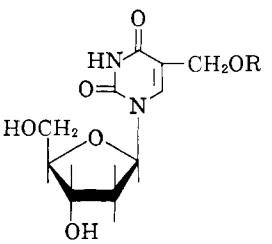
tion would have been a prime area on the inhibitor to make further structural changes in the search for possible, active-site-directed, irreversible inhibitors.[2]

Few other enzymes utilizing nucleosides or nucleotides have been studied from the standpoint of the mode of binding of the sugar moiety. For example, Schaeffer and his co-workers[19] have studied the mode of binding of the sugar moiety of adenosine to adenosine deaminase. They observed[19c] that 9-hydroxyethyladenine was bound to adenosine deaminase somewhat better than the substrate, adenosine, but that 9-cyclopentyladenine[19a] was bound less effectively. These results could mean that all of the binding of the ribosyl moiety of the adenosine is due to the 2′-hydroxyl group. Baker and Tanna[20] observed that when the 2′-hydroxyl group of adenylic acid was absent, as in 2′-deoxyadenylic acid, the latter was complexed to succinoadenylate kinosynthetase one sixth as effectively as adenylic acid; further removal of the 3′-hydroxyl and furanose oxygen led to little change in inhibitory properties. Thus the mode of binding of the (deoxy)ribosyl moiety of nucleosides and nucleotides has so far varied considerably from enzyme to enzyme. The mode of binding of inhibitors to additional enzymes utilizing nucleosides or nucleotides as substrates will have to be studied before it can be stated that definitive classes of binding exist.

The third type of study involved the bulk tolerance by thymidine kinase to substituents on the 5-position of the pyrimidine ring. Okazaki and Kornberg[5] have reported that 2′-deoxyuridines with a 5-methyl, 5-fluoro, 5-chloro, 5-bromo, or 5-iodo group are still substrates. We therefore investigated whether or not 5-ethoxymethyl-2′-deoxyuridine (XII) could inhibit



III



XI, R = H
XII, R = C_2H_5

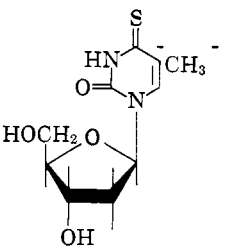
the conversion of FUDR to FUDRP by thymidine kinase. Compound XII, readily prepared by hydroxymethylation of 2′-deoxyuridine (III) to XI followed by acid-catalyzed etherification,[21] showed only about 20% inhibition of the enzyme reaction at a concentration of 3 mM,[18] about a 600-fold loss in binding. Thus, there is probably not sufficient bulk tolerance at the 5-position of the pyrimidine to warrant consideration of compounds of type XII with larger ether groups, as active-site-directed, irreversible inhibitors.[2]

(19) (a) H. J. Schaeffer, S. Marathe, and V. Alks, *J. Pharm. Sci.*, **53**, 1368 (1964); (b) R. H. Shah, H. J. Schaeffer, and D. H. Murray, *ibid.*, **54**, 15 (1965); (c) H. J. Schaeffer and P. S. Bhargava, *Biochemistry*, **4**, 71 (1965), and references therein.

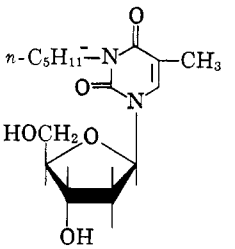
(20) B. R. Baker and P. M. Tanna, *J. Pharm. Sci.*, **54**, 845 (1965); paper XIX of this series.

(21) Although XII (= XIXc) had not heretofore been synthesized, 5-ethoxymethyluridine (XIXb), 5-ethoxymethyluracil (XIXa), and other 5-alkoxymethyluracils have presumably been synthesized by R. E. Cline, R. M. Fink, and K. Fink, *J. Am. Chem. Soc.*, **81**, 2521 (1959), by acid-catalyzed etherification of the corresponding hydroxymethylpyrimidines, XVIIb and XVIIa; other than mobility on paper chromatography, the compounds were not further characterized.

The lack of bulk tolerance at the 5-position was also indicated by the lack of inhibition of the enzyme by 5,6-dihydrothymidine at a concentration of 1 mM. In dihydrothymidine, the 5-methyl group is out of plane with the pyrimidine and thus might be less tolerated by the enzyme. Of course, it is also possible that the pseudo-aromatic pyrimidine system of thymidine is necessary for good binding. If the pseudo-aromatic system with a 5,6 double bond were the main mode of binding of the pyrimidine ring system, such as in a charge-transfer complex, then "4-thiothymidine" (XIII)[18b] ought to inhibit thymidine kinase conversion of FUDR to FUDRP in the same order as thymidine. In fact, XIII at a concentration of 3 mM[18] showed 74%



XIII



XIV

inhibition of the enzyme, thus being complexed to the enzyme about one-seventieth as well as thymidine. This result would indicate that the 4-oxo group of thymidine is hydrogen bonded to the enzyme, since the thione group forms hydrogen bonds poorly. Furthermore, this result with XIII indicates that the 3-hydrogen contributes weakly, if at all, to binding to the enzyme, since the thione at the 4-position makes the 3-H more acidic and therefore more capable of bonding to some anionic site on the enzyme.[3b] However, it is also possible that the 3-NH does contribute to binding, but that the loss of 4-oxo binding is more deleterious than the increased 3-NH binding.

Further evidence that the 3-NH probably does not complex with a cationic site on the enzyme can be gleaned by again comparing the binding of FUDR and 2′-deoxyuridine, but with the additional consideration of the relative pK_a values or the 3-NH group. The 3-NH for FUDR has pK_a = 7.66, and 2′-deoxyuridine has pK_a = 9.3[22a]; thus FUDR is 35% ionized at the pH 7.4 used in the assays, whereas 2′-deoxyuridine is only 1.2% ionized. Therefore, the 3-NH group does not bond with a cationic site on the enzyme, or else FUDR should have been complexed far stronger than twofold greater than 2′-deoxyuridine as previously observed with the 1000-fold greater binding of FUDRP than dUMP to thymidylate synthetase.[4,22b] However, if the 3-NH group is hydrogen bonded to the enzyme, then a similar strength of 3-NH binding of FUDR and 2′-deoxyuridine might be possible, depending upon the group on the enzyme that is the electron donor for this presumed hydrogen bond. That there is little difference in binding between FUDR and 2′-deoxyuridine can also be rationalized on the basis that the 3-NH is not a binding point.

Since it appeared possible that the 3-H may not have been binding to the enzyme, thymidine was converted to its 3-*n*-amyl derivative XIV. At a concentration of 3

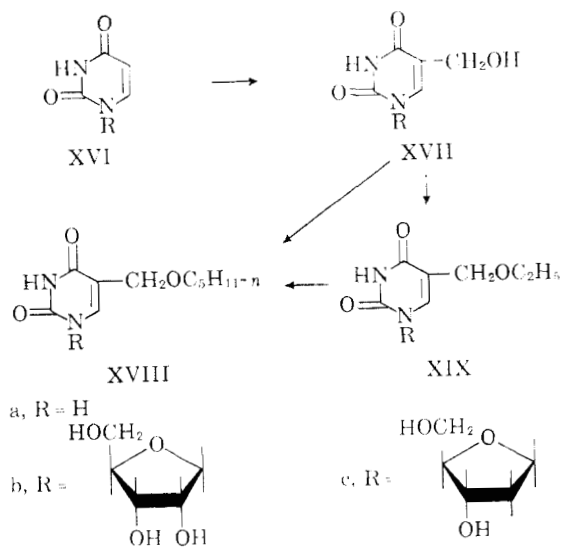
(22) (a) I. Wempen, R. Duschinsky, L. Kaplan, and J. J. Fox, *ibid.*, **83**, 4755 (1961); (b) B. R. Baker, *Cancer Chemotherapy Rept.*, **4**, 1 (1959).

mM,[18] XIV showed 74% inhibition of thymidine kinase, about a 50-fold loss in binding compared to thymidine. To determine whether this loss is due to steric interaction of the *n*-amyl group with the enzyme in the enzyme–inhibitor complex (low bulk tolerance) or is due to the loss of the possible 3-NH binding point or both will require further study.

Chemistry.—The synthesis of 5′-O-carbophenoxythymidine (VIII) by selective acylation of thymidine with phenyl chloroformate has been previously described.[23] Similarly, thymidine could be selectively acylated with ethyl chloroformate to crystalline 5′-O-carbethoxythymidine (VII). That the carbethoxy was on the 5′-hydroxyl was verified by conversion to the 3′-O-acetyl derivative which was identical with a sample of 3′-O-acetyl-5′-O-carbethoxythymidine prepared by reaction of 3′-O-acetylthymidine[24] with excess ethyl chloroformate in pyridine. Treatment of 5′-O-carbophenoxythymidine (VIII) with methylamine or with phenethylamine gave the carbamates IX and X, respectively; a similar reaction with ammonia has been described.[23]

Two routes to 5-hydroxymethyl-2′-deoxyuridine (XVIIc), the key intermediate to the desired XIXc, are (a) the acid-catalyzed hydroxymethylation of 2′-deoxyuridine (XVIc) in 19% yield to XVIIc, ion-exchange chromatography being necessary for isolation, and (b) a nucleoside coupling between 2′-deoxy-3,5-di-*O*-(*p*-tolyl)-D-ribofuranosyl chloride and 5-benzyloxymethyluracil followed by a two-step removal of the blocking groups which gave XIXc in 39% over-all yield.[25] As route b is fairly lengthy, we decided to reinvestigate the hydroxymethylation of 2′-deoxyuridine.

The initial studies of hydroxymethylation were performed on uridine (XVIb). The acid-catalyzed conditions previously described[21] gave, on paper chro-



matography, a spectrum of at least five spots; the yield of XVIIb was about 10%. The conditions described for hydroxymethylation of XVIc gave even less of the desired product XVIIc and such a myriad of spots that the paper chromatogram was almost a continuous streak. Among the spots was one that corresponded to a considerable amount of uracil (XVIa), a result of acid-catalyzed cleavage of XVIc. Since (a) the 2′-deoxyuridine is partially hydrolyzed under these conditions, (b) XVIa has been hydroxymethylated by base catalysis,[21] and (c) nucleosides are base stable, the base-catalyzed hydroxymethylation of uridine (XVIb) and XVIc were investigated.

Reaction of XVIb with formaldehyde and potassium hydroxide in a ratio of 1:2:2 in water at 50° gave a mixture of four products, as shown by paper chromatography. It was estimated that 30% of XVIIb was present. Since the reaction mixture became neutral after 1 day, no further reaction took place. It was reasoned that the formaldehyde was undergoing a slow Cannizarro reaction under these conditions and the generated formic acid was neutralizing the basic catalyst. Therefore, the reaction was run at 50° in a ratio of 1:10:2. 2 parts of potassium hydroxide was added each day for 8 days when the paper chromatogram showed a constancy. At this point the yield of 5-hydroxymethyluridine (XVIIb) was about 50%. At 65°, the reaction was complete in 5 days and the yield of XVIIb was over 81%.

When these last conditions were applied to XVIc, the chromatographic pattern became constant after 6 days, and the yield of 5-hydroxymethyl derivative was about 80%; this major spot also gave a positive Dische test for 2′-deoxynucleosides.[26] Deionization of the solution and evaporation gave an oil that was primarily the desired 5-hydroxymethyl-2′-deoxyuridine (XVIIc), which was difficult to crystallize. It was noted that XVIIc was readily converted to the ether[21] XIXc with ethanol and a small amount of hydrochloric acid and that XIXc was more readily crystallized. However, it was still difficult to separate more than about 15% over-all yield of good material. Since thin layer chromatography (t.l.c.) showed that the filtrates still contained mainly the desired XIXc, preparative t.l.c. easily removed the by-products and a 56% over-all yield of pure XIXc from XVIc was obtained.

Direct preparation of a higher ether such as XVIIIc from XVIIc proceeded poorly due to the insolubility of the nucleoside. However, an acid-catalyzed ether exchange reaction to convert XIXc to XVIIIc proceeded smoothly, as shown by t.l.c., but the product was not further characterized.

Depending upon the ratio of alkyl halide to uracil, uracil can be converted to either a 1-alkyluracil or a 1,3-dialkyluracil[27] in dimethyl sulfoxide in the presence of potassium carbonate. Treatment of thymidine with 1-bromopentane under these conditions gave analytically pure, crystalline 3-*n*-amylthymidine (XIV), in 41% yield.

Enzyme Assays

Materials.—The FUDR (I) was a generous gift from Dr. Harry B. Wood, Jr., of the Cancer Chemotherapy National Service Center. Dr. R. B. Angier, Lederle Laboratories, generously provided a sample of the cyclopentane analog (VI) of thymidine. We are indebted to Dr. J. J. Fox, Sloan Kettering Institute for the "4-thiothymidine" (XIII).[22a] Recrystallized and lyophilized bovine serumal bumin, 2′-deoxyuridine, and

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thymidine were purchased from Nutritional Biochemicals Co.; ATP, 2-deoxycytidine triphosphate (dCTP), and dUMP[6] were purchased from Sigma Chemical Co.; *Escherichia coli* B and tetrahydrofolate (FAH_4) were purchased from General Biochemicals. Professor Arthur Kornberg graciously sent us 0.3 ml. of his fraction IV of thymidine kinase[5] having 12 units/ml., used for the early work on spectrophotometric assays. Buffer A was 0.05 *M* Tris hydrochloride, pH 7.4. Buffer B was the same as A, but with the addition of 10 m*M* of mercaptoethanol and 1 m*M* Versene. Buffer C was 0.05 *M* glycylglycine, pH 7.0.[5]

Isolation of Thymidine Kinase.—A suspension of 40 g. of *E. coli* B cells in 40 ml. of buffer B was passed through a French pressure cell (American Instrument Co.) at 4000–6000 p.s.i. and the exudate was caught in a beaker immersed in an ice bath; the pressure cell had been precooled overnight at 5°. The exudate was passed once more through the pressure cell and was caught in the small head of a Waring Blendor, cooled in an ice bath. After the addition of 80 ml. of buffer B and 1 drop of GE-60 antifoam, the mixture was blended at high speed for 30 sec. Cell walls were removed by centrifugation at 5000 r.p.m. (Spinco Rotor 21) at 3° for 45 min. The supernatant (117 ml.) was placed in an ice bath and 7.7 ml. of 5% streptomycin sulfate was added with magnetic stirring. After 10 min., 10 g. of analytical grade Celite (Johns-Manville Co.) was added, and stirring was continued for an additional 10 min. The precipitated nucleic acid was removed by filtration through a Celite pad on a 90-mm. Büchner funnel into an ice-cooled receiver, and the cake was washed with 25 ml. of buffer B. To the clear, mobile filtrate (114 ml.) was added portionwise with magnetic stirring 25.9 g. of $(NH_4)_2SO_4$ (45% of saturation). After 10 min., 5 g. of Celite was added, and the mixture was stirred in the ice bath an additional 10 min. The mixture was filtered through a Celite pad on a 65-ml. Büchner funnel into an ice-cooled receiver. The filtrate (103 ml.) was brought to 90% of saturation with 34.9 g. of $(NH_4)_2SO_4$, and this fraction containing thymidylate synthetase was isolated as previously described.[3f] The filter cake of the 0–45% $(NH_4)_2SO_4$ fraction was immediately suspended in 35 ml. of ice-cold buffer A and kept at 5° until it could be further processed. The lumps were dispersed, and the mixture was magnetically stirred for 5 min. in an ice bath. The Celite was removed by filtration and the filtrate was caught in an ice-cooled receiver; total volume, 32 ml. Even fresh preparations needed dCTP as an activator.[5] For reasons unknown a 70° treated crude extract of subsequent fractions[5] failed to show any thymidine kinase activity even with dCTP present.

Assay of Thymidine Kinase.—Assay mix A was composed of 10 ml. of 0.72 *M* buffer A, 2 ml. of 0.275 *M* $MgCl_2$, and 0.08 *M* $MnCl_2$, 35 ml. of 31 m*M* LiF, and 10 ml. of 2.1 m*M* FUDR. This solution could be stored indefinitely at room temperature. Assay mix B was prepared on the day to be used as follows: 0.86 ml. of assay mix A, 0.15 ml. of bovine serum albumin (14 mg./ml., stored at 5°), 0.15 ml. of 57 m*M* ATP (can be stored 5 days at −15°), and 0.10 ml. of 9.3 m*M* dCTP (can be stored 5 days at −15°); total volume, 1.76 ml. Inhibitors were dissolved in water to give a 5.4 m*M* solution or multiple thereof; thus 0.10 ml. of 5.4 m*M* inhibitor solution would give a 1 m*M* solution of inhibitor in the incubation mixture. The incubation was performed as follows: in a series of five 12-ml., heavy-walled centrifuge tubes were placed 0.30 ml. of assay mix B, 0.22 ml. of water plus inhibitor solution, and 20 μl. of enzyme preparation; total volume, 0.54 ml. The following solutions were incubated 20 min. at 37°: (a) control with no inhibitor; (b) standard of 0.2 m*M* 2′-deoxyuridine (III) or 0.04 m*M* or 0.1 m*M* thymidine (II); (c), (d), and (e) three inhibitors or an inhibitor at three concentrations or combinations thereof. The incubation concentrations were 0.03 *M* Tris buffer, 2.6 m*M* $MgCl_2$, 0.6 m*M* $MnCl_2$, 5 m*M* LiF, 0.1 m*M* FUDR, 0.67 mg./ml. of bovine serum albumin, 2.7 m*M* ATP, and 1.7 m*M* dCTP.[5] After the 20-min. incubation period, the tubes were immediately placed in a boiling-water bath for 5 min. Then 3.63 ml. of buffer B was added, and the mixture was centrifuged in a clinical centrifuge for 2 min. at highest speed. The supernatant solution was removed with an eye-dropper and stored at 5° until ready for assay of the FUDRP content. The thymidylate synthetase from *E. coli* B (45–90% saturated $(NH_4)_2SO_4$ fraction) was assayed with 214 μ*M* *dl*-FAH_4 and 80 μ*M* dUMP as previously described[3f] by noting the optical density change/min. at 338 mμ; this was usually about 0.006 O.D. unit/min. when 200 μl. of enzyme preparation was used in 3.1 ml. of total solution. The amount of diluted incubation solution (a) necessary to give 50% inhibition was determined by using varying amounts that would give 30–70% inhibition. A plot of V_0/V_I against volume of dilute incubation solution gave the volume for 50% inhibition by extrapolating the point of V_0/V_I = 2 to the volume axis; V_0 = velocity without inhibitor and V_I = velocity with FUDRP solution added. Between 0.1 and 0.2 ml. of incubation solution should be necessary for 50% inhibition. With more active enzyme preparations, less enzyme was used; with less active enzyme preparations, the time of incubation could be increased to 40 min. or more enzyme used. Similarly, the volumes of solution of b–e were determined that would give 50% inhibition of thymidylate synthetase. The larger the volume necessary to give 50% inhibition of thymidylate synthetase, the more effective was the block of the enzymic conversion of FUDR to FUDRP. The per cent inhibition of thymidine kinase is readily calculated from the expression

$$\% \text{ inhibition} = \left(1 - \frac{V_a}{V_x}\right)100$$

where V_a = volume of diluted solution of the control incubation necessary to give 50% inhibition of thymidylate synthetase and V_x = volume of dilute incubation mixture b–d to give 50% inhibition of thymidylate synthetase. Since 0.1 ml. of dilute incubation solution can give 50% inhibition of thymidylate synthetase in the presence of 80 μ*M* dUMP and since only 1/1000 as much FUDRP as dUMP will give 50% inhibition,[4] it can be calculated that the cuvette concentration of FUDRP was 0.080 μ*M*. It can then be back-calculated that the total FUDRP generated in the incubation mixture was 10 μmoles in 20 min. or 30 μmoles in 60 min. The Okazaki–Kornberg radioactive assay[5] uses 1 hr. to generate 21 μmoles of FUDRP or 26 μmoles of dTMP; thus this spectrophotometric assay is as sensitive as the radioactive assay in the normal operating range, although about eight times as much solution is used in our assay. It can also be back-calculated that our enzyme preparation contained 1.5 enzyme units/ml. where one unit is defined as the amount catalyzing the conversion of 1 μmole of FUDR to FUDRP in 60 min. under the assay conditions; yield, 1.2 units/g. of wet *E. coli* B. Okazaki and Kornberg[5] report a yield of 3 units/g. of wet *E. coli* cells after $(NH_4)_2SO_4$ precipitation (fraction IV) where their units are corrected for the slower rate of FUDR reaction compared to thymidine as substrate.

Experimental Section

Melting points were determined in capillary tubes on a Mel-Temp block; those below 230° are corrected. Infrared spectra were determined in Nujol mull with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer in 10% alcohol unless otherwise indicated. Thin layer chromatograms were run on Brinkmann silica gel HF_{254} with 3:1 benzene–methanol, unless otherwise indicated; spots were detected by visual examination under ultraviolet light.

5′-O-Carbethoxythymidine (VII).—To a magnetically stirred solution of 969 mg. (4 mmoles) of thymidine (II) in 15 ml. of reagent pyridine, cooled in ice, was added dropwise during 10 min. 548 mg. (5 mmoles) of ethyl chloroformate. After being stirred at ambient temperature for 4 more hr., the mixture was spin evaporated *in vacuo* and the residue crystallized from 20 ml. of water at 0°. The crude product (440 mg.) was collected on a filter, washed with cold water, dried, and leached with two 15-ml. portions of boiling benzene; yield 411 mg., m.p. 155–157°. Recrystallization from ethyl acetate gave 227 mg. (18%) of analytical sample as white crystals: m.p. 159–161°; λ_{max} (pH 1, 7, 13) 268 mμ; λ_{max} 2.93, 3.14 (OH, NH), 5.70 (ester C=O), 5.85–6.10 (uracil), 7.75–8.00 (ester C–O–C), 9.33, 9.65 μ (COH).

Anal. Calcd. for $C_{13}H_{18}N_2O_7$: C, 49.7; H, 5.77; N, 8.91. Found: C, 49.9; H, 5.97; N, 8.87.

T.l.c. in the usual fashion showed only a single spot. Quantitative t.l.c., described below showed 2–5% thymidine.

Quantitative T.l.c. for Determination of Thymidine Content.—A 10-μl. aliquot of a thymidine solution (207 μg./ml.) was spotted on a t.l.c. plate. After development with 3:1 benzene–methanol, a single spot was detected under ultraviolet light. Thus, thymidine is detectable under these conditions in amounts as small as 2.07 μg.

A 10-μl. aliquot of a solution of the "analytically pure" 5-O-carbethoxythymidine (VII) (20 mg./ml.) and 25- and 10-μl.

aliquots of a solution of VII containing 4 mg./ml. were individually spotted on a t.l.c. plate. After development, a spot corresponding to the thymidine was detected in the case of the first and second samples, but not the third sample. Since the third sample (40 μg.) showed no thymidine, and since 2 μg. of thymidine is detectable, VII contained less than 5% thymidine. The second sample (100 μg.) showed thymidine; thus there was greater than 2 μg. of thymidine present or greater than 2%. For the results of similar quantitative determinations see, reference 18.

3′-O-Acetyl-5′-O-carbethoxythymidine. A.—A solution of 62 mg. (0.2 mmole) of VII in 3 ml. of reagent pyridine and 0.1 ml. (1 mmole) of acetic anhydride was left at room temperature, protected from moisture, for 20 hr. The solution was poured into 15 g. of ice water, then extracted with $CHCl_3$ (five 15-ml. portions). The combined, dried extracts were spin evaporated *in vacuo*. Recrystallization from toluene gave 38 mg. (53%) of white crystals, m.p. 145–147°, which were uniform on t.l.c. and had λ_{max} (pH 1, 7, 13) 268 mμ; λ_{max} 3.20 (NH), 5.73 (carbonate C=O), 5.82 (sh) (acetate C=O), 5.90, 6.10 (uracil), 7.92–8.06 μ (carbonate and acetate C–O–C).

Anal. Calcd. for $C_{15}H_{20}N_2O_8$: C, 50.6; H, 5.66; N, 7.86. Found: C, 50.6; H, 5.88; N, 7.98.

B.—To a magnetically stirred solution of 188 mg. (0.67 mmole) of 3′-O-acetylthymidine[24] in 3 ml. of pyridine cooled in ice and protected from moisture was added, in one portion, 148 mg. (1.4 mmoles) of ethyl chloroformate. After being stirred at ambient temperature for 24 hr., the mixture was processed as in A; yield 82 mg. (34%), m.p. 144–147°; identical with preparation A based on mixture melting point and comparative infrared spectra.

5′-O-(N-Methylcarbamoyl)thymidine (IX).—A solution of 121 mg. (0.33 mmole) of VIII[23] in 7 ml. of 40% aqueous methylamine was allowed to stand for 15 hr., then spin evaporated *in vacuo*. The residue was triturated with 15 ml. of ether to remove phenol; yield 82 mg., m.p. 220–225°. Recrystallization from absolute ethanol gave 62 mg. (62%) of white crystals, m.p. 223–225°, homogeneous on t.l.c.[18]

Anal. Calcd. for $C_{12}H_{17}N_3O_6$: C, 48.2; H, 5.73; N, 14.0. Found: C, 48.1; H, 5.67; N, 13.8.

5′-O-(N-Phenethylcarbamoyl)thymidine (X) was prepared in the same fashion from VIII with phenethylamine in methanol; white crystals from absolute ethanol, m.p. 179–180°, homogeneous on t.l.c.[18] and had λ_{max} (pH 1, 7, 13) 269 mμ; λ_{max} 2.96, 3.15 (OH, NH), 5.92, 6.05, 6.57 (amide, uracil), 9.38, 9.60 μ (COH).

Anal. Calcd. for $C_{19}H_{23}N_3O_6$: C, 58.6; H, 5.95; N, 10.8. Found: C, 58.5; H, 5.86; N, 10.6.

5′-O-(N,N-Dimethylcarbamoyl)thymidine was prepared from VIII and 20% ethanolic dimethylamine as described for the preparation of IX. Recrystallization from absolute ethanol gave a 66% yield of the analytical sample as white crystals: m.p. 158°, resolidified, then remelted at 179–180°; λ_{max} (pH 1, 7, 13) 269 mμ; λ_{max} 3.00, 3.13 (OH, NH), 5.81–6.00 (amide, uracil), 9.65 μ (COH).

Anal. Calcd. for $C_{13}H_{19}N_3O_6$: C, 49.8; H, 6.11; N, 13.4. Found: C, 49.7; H, 6.17; N, 13.6.

3-(*n*-Amyl)thymidine (XIV).—A mixture of 484 mg. (2 mmoles) of II, 307 mg. (2 mmoles) of 1-bromopentane, 276 mg. (2 mmoles) of anhydrous K_2CO_3, 300 mg. (2 mmoles) of NaI, and 25 ml. of dimethyl sulfoxide was magnetically stirred at 80–90° for 15 hr. The mixture was spin evaporated in high vacuum. The residue was dissolved in 35 ml. of water and the solution was extracted with four 30-ml. portions of $CHCl_3$. The combined, dried extracts were spin evaporated *in vacuo*. The residue (670 mg.) was recrystallized from ethyl acetate–petroleum ether (b.p. 60–110°); yield 258 mg. (41%); m.p. 115–117°; homogeneous on t.l.c.[18]; λ_{max} (pH 1, 7, 13) 270 mμ; λ_{max} 2.82, 2.89 (OH), 5.93, 6.00, 6.15 (C=O, C=C), 9.10, 9.21, 9.40, 9.60 μ (COH).

Anal. Calcd. for $C_{15}H_{22}N_2O_6$: C, 57.7; H, 7.74; N, 8.97. Found: C, 57.7; H, 7.80; N, 8.99.

5-Ethoxymethyl-2′-deoxyuridine (XII).—A solution of 1.141 g. (5 mmoles) of III and 5 ml. of 37% aqueous formaldehyde in 5 ml. of 1 *N* aqueous KOH was heated at 65°. Four 5-ml. portions of 1 *N* KOH were added at 24-hr. intervals, then the mixture was heated 48 hr. longer; some III was still present after 4 days which did not seem to decrease appreciably after the sixth day. To the cooled reaction mixture was added 30 ml. (wet volume) of a strong acid ion-exchange resin (Dowex 50W-X8-H* form) and sufficient water to bring the total volume to 100 ml. After being stirred at room temperature for 1 hr., the resin was removed by filtration and washed with 100 ml. of water in portions. The combined filtrate and washings were spin evaporated *in vacuo* (without heating) to a syrup; paper chromatography showed this syrup to be mainly XI with a small amount of unchanged III present. To a solution of the syrup in 50 ml. of absolute ethanol was added 75 μl. of 12 *N* aqueous HCl. After being refluxed for 4 hr., the solution was spin evaporated *in vacuo* (without heating) to a syrup; paper chromatography showed that all of the XI had been converted to XII. A solution of the syrup in 10 ml. of ethanol was streaked across twelve 20 × 20 cm. thin layer plates coated to a thickness of 1.25 mm. with silica gel HF; the solution was applied with a Brinkmann adjustable applicator Model S-11. The plates were developed with $CHCl_3$–ethanol (10:7). The ultraviolet-absorbing zones corresponding to XII were marked, then scraped from the plates. The combined zones were extracted with hot 50-ml. portions of ethanol until no appreciable ultraviolet-absorbing material was eluted (4–12 portions). The combined ethanol extracts were spin evaporated *in vacuo* (bath 25°). The crystalline residue was recrystallized from ethyl acetate to give 0.84 g. (56% based on III) of pure product, m.p. 135–136°, that was uniform on t.l.c.[18] A pilot run, recrystallized to constant melting point, had m.p. 135–136°; λ_{max}^{EtOH} 266 mμ; λ_{min}^{EtOH} 233 mμ; O.D. ratios: 250/260 = 0.73, 280/260 = 0.57; λ_{max} (KBr) 3.12 (OH, NH), 6.3 (broad C=C, C=O, C=N), 9.10 μ (ether C–O–C).

Anal. Calcd. for $C_{12}H_{18}N_2O_6$: C, 50.4; H, 6.34; N, 9.79. Found: C, 50.1; H, 6.29; N, 9.73.

The product could also be isolated by direct ethyl acetate extraction of the syrup remaining prior to preparative t.l.c.; considerable resinification occurred, and the yield was only about 15%.