tometer thermostated at 30 °C. The regular assay mixture contained the following: Tris–acetate, 70 mM, pH 7.4; mercaptoethanol, 80 mM; $MgCl_2$, 20 mM; d,l-L-tetrahydrofolate, 0.3 mM; CH_2O, 12 mM; EDTA, 0.3 mM; 2′-deoxyuridylate, 1.0 mM; and sufficient enzyme to produce an absorbance change of 0.015–0.025 OD unit/min at 340 nm in the absence of inhibitors.

Inhibitory constants (apparent K_i values) were calculated from the data represented in Figure 2, using an apparent K_m value of 5×10^{-6} M, independently determined for the substrate 2′-deoxyuridylate.

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References and Notes

(1) D. L. Klayman and W. H. H. Gunther, in "Organic Selenium Componds: Their Chemistry and Biology", Wiley, New York, 1973.
(2) H. G. Mautner and J. J. Jaffe, *Cancer Res.*, **18**, 294 (1958).
(3) H. G. Mautner, S. H. Chu, J. J. Jaffe, and A. C. Sartorelli, *J. Med. Chem.*, **6**, 36 (1963).
(4) S. Chu and D. D. Davidson, *J. Med. Chem.*, **15**, 1088 (1972).
(5) L. B. Townsend and G. H. Milne, *Ann. N.Y. Acad. Sci.*, **255**, 91 (1975).
(6) T. J. Bardos, J Aradi, Y. K. Ho, and T. I. Kalman, *Ann. N.Y. Acad. Sci.*, **255**, 522 (1975).
(7) S. H. Schwartz, T. J. Bardos, G. H. Burgess, and E. Klein, *J. Med. (Basel)*, **1**, 174 (1970).
(8) T. J. Bardos, *Top. Curr. Chem.*, **52**, 63–98 (1974).
(9) P. Chandra and T. J. Bardos, *Res. Commun. Pathol. Pharmacol.*, **4**, 615 (1972).
(10) T. J. Bardos, R. R. Herr, and T. Enkoji, *J. Am. Chem. Soc.*, **77**, 960 (1955).
(11) L. B. Agenas, *Acta Chim. Scand.*, **17**, 268 (1963).
(12) L. Szabo, T. I. Kalman, and T. J. Bardos, *J. Org. Chem.*, **35**, 1434 (1970).
(13) D. L. Klayman and T. S. Griffin, *J. Am. Chem. Soc.*, **95**, 197 (1973).
(14) D. V. Santi and C. F. Brewer, *Biochemistry*, **12**, 2416 (1973).
(15) T. J. Bardos and T. I. Kalman, *J. Pharm. Sci.*, **55**, 606 (1966).
(16) T. I. Kalman and T. J. Bardos, *J. Am. Chem. Soc.*, **89**, 1171 (1967).
(17) K. Baranski, T. J. Bardos, A. Bloch, and T. I. Kalman, *Biochem. Pharmacol.*, **18**, 347 (1969).
(18) U. Svankolm, in ref 1, p 903.
(19) R. P. Leary and R. L. Kisliuk, *Prep. Biochem.*, **1**, 47 (1971).
(20) T. I. Kalman and T. J. Bardos, *Mol. Pharmacol.*, **6**, 621 (1970).
(21) E. S. Gould, *Anal. Chem.*, **23**, 1502 (1951).
(22) J. F. Alicino, in ref 1, p 1059.
(23) R. Duschinsky, T. Gabriel, W. Tautz, A. Nussbaum, M. Hoffer, E. Grunberg, J. H. Burchenal, and J. J. Fox, *J. Med. Chem.*, **10**, 47 (1967).
(24) G. E. Hilbert and T. B. Johnson, *J. Am. Chem. Soc.*, **52**, 2001 (1930).
(25) A. J. Wahba and M. Friedkin, *J. Biol. Chem.*, **236**, PC 11 (1961).
(26) T. I. Kalman, *Biochem. Biophys. Res. Commun.*, **49**, 1007 (1972).

Design of Species- or Isozyme-Specific Enzyme Inhibitors. 1. Effect of Thymidine Substituents on Affinity for the Thymidine Site of Hamster Cytoplasmic Thymidine Kinase

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5-(Ethylamino)- and 5-acetamido-2′-deoxyuridine 5′-triphosphates were synthesized; the extent and concentration dependence of their inhibitory action on the title enzyme resembled that of the feedback inhibitor TTP. This and other findings provide a tentative indication that bulk tolerance near C-5 of the thymine ring may be more extensive at the TTP site than at the thymidine site. Enzyme–inhibitor dissociation constants (K_i values) were determined for thymidine derivatives monosubstituted at various positions. Competitive inhibition with respect to thymidine (indicative of substituent tolerance in the enzyme–thymidine complex) was produced by 3-amylthymidine (K_i = 65 μM), *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine diastereoisomers (K_i = 180 and 310 μM), 5′-*C*-(acetamidomethyl)- and 5-*C*-(propionamidomethyl)thymidine epimers (K_i range 65–1100 μM), 3′-acetamido- and 3′-(ethylthio)-3′-deoxythymidines (K_i = 2.5 mM and 12 μM, respectively), and certain 5′-(alkylamino)- and 5′-(alkylthio)-5′-deoxythymidines (K_i range 180–1200 μM). Evidence indicates that bulk tolerance at some, if not most, of the above atoms of thymidine is found in the enzyme–thymidine complexes of human and other mammalian thymidine kinases; attachment of suitable substituents to such atoms could, in principle, lead to thymidine site directed isozyme-specific inhibitors of human cytoplasmic thymidine kinase, which is a candidate target in the design of antineoplastic drugs.

The TMP[1] required for cell multiplication can be furnished either from dUMP via the de novo pathway of biosynthesis or from exogenous thymidine. Several drugs, e.g., methotrexate, are effective in blocking the de novo pathway. The alternate route to TMP involves transfer of phosphate from a nucleoside 5′-triphosphate to thymidine, a reaction catalyzed by thymidine kinase. In neoplastic tissue and rapidly proliferating cells, the activity of thymidine kinase is elevated to a level which is sufficiently high to permit this enzyme to play a major role in TMP production in vivo.[2,3] Two isozymes of thymidine kinase, associated with the mitochondrial and cytoplasmic fractions, respectively, of mammalian cells, have been recognized.[4–11] The mitochondrial isozyme is the preponderant form in human adult spleen, liver, and fibroblasts,[5,6] whereas the cytoplasmic isozyme is reported to be the sole or predominant form in KB and HeLa human tumor cell lines,[6] in human fibroblasts transformed by SV40 virus,[5,6] in human fetal liver,[5,6] and in the following tumor tissues of clinical origin: Wilm's tumor,[5] rhabdomyosarcoma,[5] bladder adenocarcinoma,[5] and cervical carcinoma.[12] A nonmalignant uterine fibroma, on the other hand, contained principally the mitochondrial isozyme.[5] These data suggest that effective antineoplastic chemotherapy might be obtainable by the coadministration of a drug which can block de novo TMP biosynthesis with a drug which can inhibit cytoplasmic thymidine kinase without appreciably inhibiting mitochondrial thymidine kinase. In this context, the present work has been aimed at the attempted design of thymidine site directed selective inhibitors of the cytoplasmic isozyme. Baby hamster kidney cells grown in culture served as a convenient source

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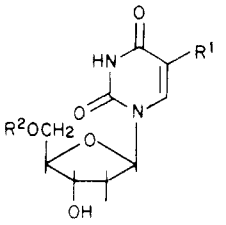
of a mammalian cytoplasmic thymidine kinase. This paper describes a survey of possible positions of the thymidine molecule at which substituents might be attached without preventing adsorption to the enzymatic thymidine binding site. For this purpose, the various thymidine derivatives were studied as inhibitors of the thymidine kinase, and their affinity for the thymidine site was evaluated kinetically. Substituents which permit binding to the site were sought because they have the potential to selectively enhance thymidine site directed adsorption to the cytoplasmic isozyme. This could ensue, for instance, from specific binding of the substituents to regions adjacent to the thymidine site of the cytoplasmic isozyme which differ structurally from corresponding regions of the mitochondrial isozyme.

Chemical Syntheses. 5-(Ethylamino)-2′-deoxyuridine (**1b**) was reported to be obtained in 20% yield by heating

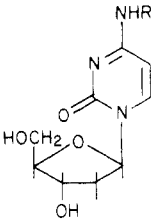


	R¹	R²
1a	NH_2	H
b	NHC_2H_5	H
c	$NHCOCH_3$	H
d	$NHCO(CH_2)_2NHCOCH_2I$	H
e	$NHCO(CH_2)_3NHCOCH_2I$	H
f	$NHCO(CH_2)_4NHCOCH_2I$	H
g	$NHCO(CH_2)_5NHCOCH_2I$	H
h	$NHCO(CH_2)_3NHCOCH_3$	H
i	CN	H
j	CH_2NH_2	H
k	$CH_2NHCOCH_3$	H
m	NHC_2H_5	PO_3H_2
n	$NHCOCH_3$	PO_3H_2
o	NHC_2H_5	$P_3O_9H_4$
p	$NHCOCH_3$	$P_3O_9H_4$

5-bromo-2′-deoxyuridine with ethylamine.[13] In this laboratory, the method gave 23% of **1b** with 5-iodo-2′-deoxyuridine as starting material. In the presence of triethylamine and *N*,*N*-dimethylformamide, the reaction proceeded at room temperature and gave **1b** in 65% yield. These conditions had previously been reported to promote the reaction of amines with 6-chloropurine ribonucleoside.[14] 5-Acetamido-2′-deoxyuridine (**1c**) was obtained in 70% yield by selective N-acetylation of **1a**[13] with ethoxycarbonic acetic anhydride formed in situ from acetic acid and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), a reagent shown to be useful for N-acylation of cytidine.[15] The 5′-phosphate derivatives, **1m–n**, of **1b** and **1c** were synthesized from 5-iodo-2′-deoxyuridine 5′-phosphate[16] by similar methods to those used for **1b** and **1c** and were found to be readily convertible to the corresponding triphosphates **1o** and **1p** by the general procedures of Michelson[17] and Hoard and Ott,[18] respectively. For preparation of the 5-[[(ω-(iodoacetamido)acyl]-amino]-2′-deoxyuridines **1d–h**, compound **1a** was 5-N-acylated by treatment with the appropriate *N*-[(benzyloxy)carbonyl]-ω-substituted amino acid and EEDQ, following which the blocking group was removed by catalytic hydrogenolysis and the resulting amine was acylated with EEDQ–iodoacetic acid to furnish compounds **1d–h** in 25–35% overall yield.

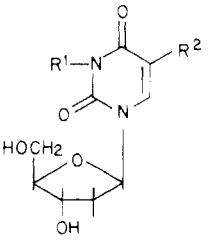
5-Cyano-2′-deoxyuridine (**1i**) was obtained in 75% yield by treatment of 5-iodo-2′-deoxyuridine at room temperature with KCN and dicyclohexyl-18-crown-6 in *N*,*N*-dimethylformamide. Compound **1i** had previously been obtained in 5% yield from the reaction of silylated 5-iodo-2′-deoxyuridine and cuprous cyanide in hot pyridine[19] and in 45% yield from the reaction of 3′,5′-di-*O*-acetyl-5-bromo-2′-deoxyuridine with KCN in Me_2SO.[20] 5-(Aminomethyl)-2′-deoxyuridine (**1i**) was isolated in crystalline form in 70% yield following hydrogenation of **1i** with rhodium–alumina.[21] Selective N-acetylation of **1j** by the EEDQ method described above furnished homogeneous **1k** in 73% yield.

The synthesis of N^4-ethyl-2′-deoxycytidine (**2b**) was



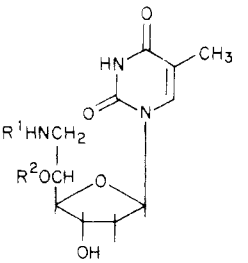
2a, R = H
b, R = C_2H_5

initially approached by silylation–amination[22] of 2′-deoxyuridine. This produced numerous products, and purification of **2b** was tedious. Compound **2b** was obtained in 70% yield by the reaction of 3′,5′-di-*O*-benzoyl-4-thio-2′-deoxyuridine[23] with ethylamine under the conditions of Fox and co-workers[23] for the synthesis of N^4-methyl-2′-deoxycytidine. The 3-alkylthymidines and 3-alkyl-2′-deoxyuridines **3a–e** were obtained in 70–75%



	R¹	R²
3a	C_2H_5	H
b	n-C_3H_7	H
c	C_2H_5	CH_3
d	n-C_3H_7	CH_3
e	n-C_5H_{11}	CH_3

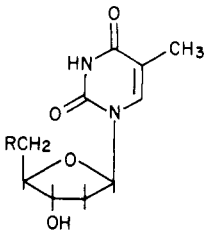
yield by treatment of the parent nucleosides with the appropriate alkyl iodide and potassium carbonate in dimethyl sulfoxide by the method of Baker et al.;[24] **3c**[25] and **3e**[24] were described previously by other workers. The method of synthesis of compounds **4a–f** has been de-



	R¹	R²
4a	$COCH_3$ (more polar epimer)	H
b	$COCH_3$ (less polar epimer)	H
c	COC_2H_5 (more polar epimer)	H
d	COC_2H_5 (less polar epimer)	H
e	COC_2H_5 (more polar epimer)	$P_3O_9H_4$
f	COC_2H_5 (less polar epimer)	$P_3O_9H_4$

scribed;[26] the designations "more polar" and "less polar" refer to derivatives of one or other of the two 5′ epimers of 3′-*O*-acetyl-5′-*C*-(nitromethyl)thymidine which had been separated by partition chromatography.[26]

5′-Amido-5′-deoxythymidines **5a–c** were obtained in

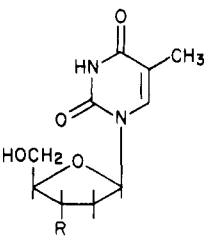


	R		R
5a	$NHCOCH_3$	**5j**	SC_2H_5
b	$NHCO(CH_2)_2CH_3$	**k**	SCH_2CH_2OH
c	$NHCO(CH_2)_4CH_3$	**m**	SCH_2CO_2H
d	NHC_2H_5	**n**	$S(CH_2)_2CO_2H$
e	$NH(CH_2)_3CH_3$	**o**	$S(CH_2)_3CO_2H$
f	$NH(CH_2)_5CH_3$	**p**	$S(O)C_2H_5$
g	$N(COCH_3)(CH_2)_5CH_3$	**q**	$S(O)(CH_2)_2CO_2H$
h	$NH(CH_2)_2NHCOCH_3$	**r**	$S(O)(CH_2)_3CO_2H$
i	$N(CH_3)(CH_2)_2N(CH_3)COCH_3$		

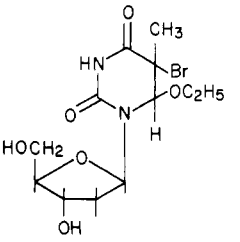
58–68% yields by acylation of 5′-amino-5′-deoxythymidine[27] with EEDQ and the appropriate carboxylic acid. The 5′-(alkylamino)-5′-deoxythymidines **5d–f** were isolated in 60–75% yield following treatment of 5′-*O*-*p*-toluenesulfonylthymidine[28] at room temperature with alkylamines. Compound **5g** was made by acetylation of **5f** by the EEDQ method. For the synthesis of compounds **5h** and **5i**, 5′-*O*-tosylthymidine was converted by reaction with ethylenediamine or N^1,N^2-dimethylethylenediamine to the respective intermediate 5′-[(ω-aminoalkyl)-amino]thymidines. From the appropriate one of these intermediates, **5i** was obtained in good yield by acetylation by means of the EEDQ method. Treatment of 5′-[(aminoethyl)amino]-5′-deoxythymidine with 1 molecular equiv each of EEDQ and acetic acid led to a mixture of the starting nucleoside (ca. 20%) with the desired **5h** (ca. 35%), together with an approximately equal amount of a less polar, ninhydrin-negative component which had the same UV absorption spectrum as **5h** and which behaved as a neutral species on paper electrophoretograms run at pH 7.5 and was concluded to be the 5′-*N*-acetyl derivative of **5h**. More selective synthesis of **5h** was obtained by acetylation with 1.2 equiv of *N*-acetoxysuccinimide, which converted the diamine to a mixture of **5h** (85–90%) and its 5′-*N*-acetyl derivative (10–15%).

Reaction of thymidine with diethyl disulfide and tri-*n*-butylphosphine, according to a general method for the conversion of nucleosides to their 5′-(alkylthio)-5′-deoxy derivatives,[29] gave 5′-deoxy-5′-(ethylthio)thymidine (**5j**) in 27% yield. The 5′-(alkylthio)-5′-deoxythymidines **5k–o** were conveniently prepared from the reaction of the appropriate thiols with 5′-*O*-tosylthymidine. Oxidation of the thioethers **5j**, **5n**, and **5o** by the sodium periodate method for synthesis of sulfoxides[30] furnished the 5′-(alkylsulfinyl)-5′-deoxythymidines **5p–r** in high yield.

3′-Acetamido-3′-deoxythymidine (**6b**)[31] was obtained in 80% yield by acetylation of **6a**[32] by the EEDQ procedure. 3′-(Ethylthio)-3′-deoxythymidine (**6c**) was synthesized by treatment of 5′-*O*-trityl-2,3′-anhydro-2′-deoxy-1-(β-D-xylofuranosyl)thymine[33] with ethyl mercaptan followed by removal of the 5′-*O*-trityl group with 80% acetic acid. The two diastereoisomers, **7a** and **7b**, of *trans*-5-bromo-6-



6a, R = NH_2
b, R = $NHCOCH_3$
c, R = SC_2H_5



7a, more polar diastereoisomer
b, less polar diastereoisomer

ethoxy-5,6-dihydrothymidine were prepared and separated by silica gel chromatography by previously described methods.[34] Physical properties of the compounds are listed in Tables I and II.

Effects on BHK21 Thymidine Kinase of Phosphate Donors and Inhibitors Diagnostic for the Cytoplasmic and Mitochondrial Isozymes. Useful properties by which to distinguish the two isozymes are the effectiveness of CTP as a phosphate donor for the mitochondrial form[6,10,11] and the effectiveness of dATP as a phosphate donor for the cytoplasmic form[10] (see Table III); in addition, dCTP[6,10] and 5-iodo-2′-deoxycytidine[10] inhibit the mitochondrial form more strongly than the cytoplasmic form, whereas 5-iodo-2′-deoxyuridine inhibits the cytoplasmic form more strongly than the mitochondrial form.[10] The effects of these phosphate donors and inhibitors on the thymidine kinase preparation from BHK21 cells were determined under conditions similar to those employed with purified preparations of human mitochondrial and cytoplasmic isozymes.[10] Reference to Table III shows that the properties of the BHK21 preparation closely resemble those of the cytoplasmic isozyme and indicate that the preparation contains minor amounts, at most, of a mitochondrial form.

Inhibition of Thymidine Kinase by Derivatives of TTP and dUTP. Thymidine kinase from almost all sources is subject to feedback inhibition by low concentrations of TTP.[35] The effect is thought to be allosteric and with mammalian thymidine kinases can be either competitive or noncompetitive with respect to thymidine, according to the source of the enzyme (for a summary see ref 10). Two dUTP derivatives, **1o,p**, and two TTP derivatives, **4e,f**, were compared with TTP for inhibitory effects on the BHK21 thymidine kinase in the presence of near-saturating levels of thymidine (3.5 times the K_M level) and Mg-ATP (7.5–35 times the K_M range of 70–340 μM[2,3,10,36] reported for various mammalian thymidine kinases). With all compounds, excepting **4f**, the inhibition tended to approach a limit value of 40–50%. The same type of inhibition by TTP of BHK21 thymidine kinase has been described previously.[37] Inhibition by 100–200 μM **4e** and **4f** (Figure 1) was of similar magnitude to the inhibition (competitive with respect to thymidine) exerted under the same conditions by the corresponding nucleosides **4c** and **4d** (Table V). The inhibitions of **4e** and **4f** might,

Table I. Physical Properties of Compounds Obtained by General Methods Given Under the Experimental Section

compd	yield, %	mp (solvent), °C	UV λ_{max} (H_2O), nm ($\epsilon \times 10^{-3}$)	formula	anal.
1d	30	166-168 (CH_3OH-Et_2O)	278 (8.2)	$C_{14}H_{19}N_4O_7I$	C, H, N, I
1e	26	220-222[d] (EtOH)	278 (8.4)	$C_{15}H_{21}N_4O_7I \cdot 0.5EtOH$	C, H, N; I[a]
1f	35	140-142 (CH_3OH-Et_2O)	278 (8.1)	$C_{16}H_{23}N_4O_7I \cdot CH_3OH$	C, H, N, I
1g	35	115-116 (CH_3OH-Et_2O)	278 (8.0)	$C_{17}H_{25}N_4O_7I$	C, H, N, I
3a	74		263 (8.2)	$C_{11}H_{16}N_2O_5$	C, H, N
3b	70		263 (8.4)	$C_{12}H_{18}N_2O_5$	C, H, N
3d	71	89-91 (C_6H_6-ligroin)	267 (9.2)	$C_{13}H_{20}N_2O_5$	C, H, N
4a	63	102-104 (MeOH-Et_2O)	267 (9.5)	$C_{13}H_{19}N_3O_6 \cdot 0.5CH_3OH$	C, H, N
4b	68	144-145 (EtOH-Et_2O)	267 (9.6)	$C_{13}H_{19}N_3O_6$	C, H, N
4c	58	153-155 (EtOH-Et_2O)	267 (9.6)	$C_{14}H_{21}N_3O_6$	C, H, N
4d	67	165-167 (EtOH)	267 (9.4)	$C_{14}H_{21}N_3O_6$	C, H, N
4e	55		266 (9.5)	$C_{14}H_{20}N_3O_{15}P_3Na_4 \cdot 4H_2O$	P
5a	63	221-223 (EtOH)	267 (9.3)	$C_{12}H_{16}N_3O_5$	C, H, N
5b	71	238-240 (EtOH)	267 (9.2)	$C_{14}H_{20}N_3O_5$	H, N; C[b]
5c	68	225-227 (EtOH)	267 (9.4) (EtOH)	$C_{16}H_{24}N_3O_5$	C, H, N
5m	20	240-242[d] (EtOH-ether)	267 (9.5)	$C_{12}H_{16}N_2O_6S \cdot H_2O$	C, H[c]
5n	58	155-156 (EtOH-ether)	267 (9.6)	$C_{13}H_{18}N_2O_6S$	C, H, N, S
5o	53	116-117 (EtOAc)	267 (9.7)	$C_{14}H_{20}N_2O_6S$	C, H, N, S
5q	76	199-201[d] (EtOH-H_2O)	267 (9.6)	$C_{13}H_{18}N_2O_7S \cdot H_2O$	C, H
5r	79	175-176 (EtOH-H_2O)	267 (9.7)	$C_{14}H_{20}N_2O_7S$	C, H

[a] I: calcd, 24.45; found, 24.90. [b] C: calcd, 54.02; found, 54.58. [c] C: calcd, 43.11; found, 42.52. H: calcd, 5.38; found 4.91. [d] Decomposition.

Table II. Paper Chromatography and Electrophoresis

compd	R_f in system										electrophoretic mobility, cm[a]	
	A	B	C	D	E	F	G	H	I	J	pH 7.6	pH 3.5
1a		0.22		0.25						0.60		
1b		0.52		0.58						0.77		
1c		0.44		0.41						0.66		
1d	0.09	0.38		0.29								
1e		0.42		0.30						0.64		
1f	0.11	0.50		0.36								
1g	0.14	0.54		0.44								
5-I-dUMP					0.11	0.27			0.25		−14.9	−8.2
5-NH_2-dUMP					0.10				0.21		−11.2	
1m					0.15	0.34			0.37		−14.0	−6.1
1n					0.12	0.22			0.36		−15.3	−8.7
TTP							0.40	0.22	0.30			−13.9
1o									0.29			−12.1
1p								0.20	0.33			−14.4
4e							0.45	0.44				−11.3
5d			0.10		0.75					0.54	+12.3	+14.8
5e			0.21		0.84					0.73	+11.0	+13.5
5f			0.32		0.87					0.82	+10.2	+12.8
5g		0.42	0.89		0.90					0.91	+2.5	+1.2
5h			0.12								+9.8	+12.4
5i		0.20									+9.5	+12.2
5j	0.38	0.75		0.74								
5k	0.22	0.64		0.58								
5m		0.09		0.06								−8.1
5n		0.16	0.40									−7.5
5o		0.25	0.51	0.26								−7.1
5p	0.18	0.57		0.21								
5q			0.05									−6.6
5r			0.09									−6.4
6c	0.44			0.79								

[a] Distance moved toward the cathode in 30 min at a gradient of 47 V/cm.

therefore, equally well be due to binding to the thymidine site, to the TTP site, or to both; binding to the ATP site is also possible but appears less likely in view of the high level of Mg-ATP present.

5-(Ethylamino)- (**1o**) and 5-acetamido-2′-deoxyuridine 5′-triphosphate (**1p**) at 200 μM gave approximately the same degree of inhibition as TTP itself at 50 μM (Figure 1). The feeble inhibitory properties of **1b** and **1c** (Table IV) indicate that the enzyme–thymidine complex does not readily accommodate an ethylamino or acetamido substituent at C-5 of thymidine. It follows that the thymine portion of the triphosphates **1o** and **1p**, at the levels tested, likewise can not bind significantly to the thymine portion of the thymidine site. In addition, it appears unlikely that **1o** and **1p** bind significantly to the ATP site because of the high level (5 mM) of Mg-ATP present, and their inhibitory action is, therefore, most likely exerted principally at the regulatory TTP site. This would imply, tentatively, that the thymine rings of TTP and thymidine bind to separate sites of the enzyme, and that the TTP site differs from the thymidine site in possessing more bulk tolerance at C-5 of the thymine moiety.

Table III. Effects of Various Inhibitors and Phosphate Donors on Thymidine Kinase of BHK21(C13) Cells

enzyme	potency of phosphate donors, rel %				effect of inhibitors,[a] % inhibn		
	ATP	dATP	GTP	CTP	dCTP	5-I-UdR	5-I-CdR
BHK21(C13) prep	100[b]	107[b]	6[b]	15[b]	27[c]	37[d]	0[d]
cytoplasmic isozyme (human)[e]	100[f]	108[f]	36[f]	21[f]	15[g]	56[d]	0[d]
mitochondrial isozyme (human)[e]	100[f]	56[f]	46[f]	79[f]	82[g]	31[d]	64[d]

[a] The assay systems contained 0.19 mM [^{14}C]thymidine. [b] The nucleoside triphosphates (5 mM) were employed in the assay system given under the Experimental Section, which thereby initially contained 2.5 mM of the corresponding Mg^{2+} triphosphates; the [^{14}C]thymidine level was 14.4 μM. [c] The assay system included 5 mM dCTP, 5 mM ATP, and 7.5 mM $MgCl_2$. [d] 5-Iodo-2'-deoxyuridine and 5-iodo-2'-deoxycytidine were each 0.19 mM in the assay system and Mg^{2+}-ATP was at a saturating level. [e] The values for these enzymes were reported in ref 10. [f] The assays were performed with 2 mM Mg^{2+} triphosphates and saturating thymidine levels. [g] The assay included 2 mM dCTP, 2 mM ATP, and 4 mM $MgCl_2$.

Table IV. Inhibition of Thymidine Kinase[a]

compd	inhibitions, %	inhibitor concns, mM
1b	13, 25	3.4, 6.8
1c	8, 13	2.0, 4.0
1d	22, 36	2.0, 4.0
1k	0, 0	6.0, 12.0
2a (2'-deoxycytidine)	10, 24	0.37, 0.74
2b	24, 38	5.0, 10.0
2'-deoxyuridine (1, $R^1 = R^2 = H$)	38, 59	0.1, 0.2
3a	24, 45	7.2, 14.4
3b	18, 36	7.8, 15.5
3c	13, 26	0.1, 0.2
3d	16, 34	0.1, 0.2
5'-amino-5'-deoxythymidine	46, 63	0.012, 0.024
5b	38, 77	6.6, 13.2
5c[b]		
5p	13, 30	1.4, 5.6
6c	31, 60	0.28, 1.4

[a] The thymidine level was 3.6 μM; the remaining assay conditions are given under the Experimental Section. [b] Not tested because of poor solubility in the assay buffer.

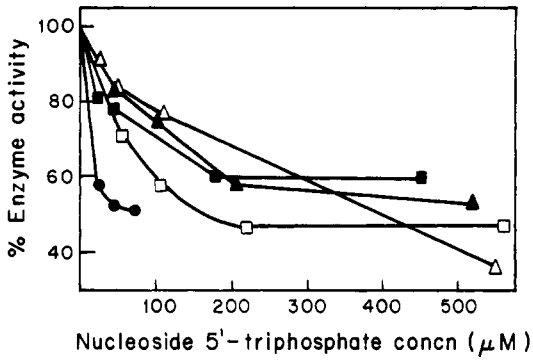


Figure 1. Inhibition of BHK21(C13) thymidine kinase by TTP and dUTP derivatives with 9.0 μM [^{14}C]thymidine present in the standard assay system: (●) TTP; (△) 4f; (▲) 4e; (□) 1o; (■) 1p.

Inhibition of Thymidine Kinase by Thymidine Derivatives. Table IV lists the inhibition of enzyme-catalyzed formation of TMP effected by some of the present series of thymidine derivatives in the presence of a level of thymidine close to its Michaelis constant. Inhibition by the remaining thymidine derivatives was studied in the presence of variable levels of thymidine; the enzyme–inhibitor dissociation constants (K_i values) thereby obtained are given in Table V.

(a) Substituents at C-5. 2'-Deoxyuridine (1, $R^1 = R^2 = H$) is a substrate and a competitive inhibitor of mammalian thymidine kinases.[38] In the cases examined, the ratio of the K_i of 2'-deoxyuridine to the K_M of thymidine (taken as a rough index of the K_i of thymidine) has indicated that the 5-methyl of thymidine enhances adsorption to the enzymatic thymidine site by a factor which varies between 30 and 100.[2,10,36] If one assumes that the inhibition of the BHK thymidine kinase by 2'-deoxyuridine is likewise competitive in nature with respect to thymidine, the observed inhibitions (Table IV) correspond to a K_i value of ca. 100 μM and again point to a substantial (30-fold) contribution to binding by the 5-methyl group of thymidine. In contrast, the 5-amino group of **1a** is seen (Table V) to cause a 4.5-fold loss in binding to the thymidine site, suggesting that the 5-methyl may interact with a hydrophobic region of the enzyme. Consistent with this view, the present studies (Table V) indicate that 5-(aminomethyl)-2'-deoxyuridine (**1j**) binds ca. 17 times less strongly to the thymidine site than does 2'-deoxyuridine itself. Attachment of an ethyl (**1b**) or an acetyl group (**1c**) to N^5 of **1a** (Table IV) reduced binding to the thymidine site by an amount which was at least 90-fold if the in-

Table V. Inhibition Constants of Derivatives of 2'-Deoxyuridine and Thymidine with Thymidine Kinase

compd	types of inhibn[a]	K_i,[b] mM	I_{50},[b] mM	inhibitor concns, mM
1a	C, L	0.45		0.44, 0.88
1e	NC, L		3.1	2.0, 4.0
1g	NC, L		3.0	2.0, 4.0
1i	C, L	0.22		1.0, 2.0
1j	C, L	1.70		1.9, 3.8
3e	C, L	0.072		0.2, 0.4
4a	C, L	0.27		0.2, 0.4
4b	C, L	1.10		0.22, 0.44
4c	C, L	0.16		0.095, 0.19
4d	C, L	0.065		0.135, 0.27
5a	C, L	6.5		8.0, 16.0
5d	C, L	0.19		0.55, 1.1
5e	C, L	0.27		0.45, 0.9
5f	C, L	0.18		0.26, 0.52
5g	C	4.4		4.4
5h	C, L	0.25		0.76, 1.52
5i	C, L	0.90		1.8, 3.6
5j	NC, L		21.5	3.0, 6.0
5k	C, L	0.8		2.0, 4.0
5m	C-NC, L[c]		1.2	0.8, 1.6
5n	C, L	0.5		1.4, 2.8
5o	C, L	1.2		1.0, 2.0
5q	NC, L		0.13	0.125, 0.25
5r	NC, L		2.5	1.6, 3.2
6b	C, L	2.5		1.4, 2.8
6c	C, L	0.012		0.04, 0.08
7a	C, L	0.31		0.28, 0.56
7b	C, L	0.18		0.28, 0.56

[a] L = linear, NL = nonlinear plot of inhibitor level vs. slope of the Lineweaver-Burk plot; C = competitive, NC = noncompetitive (both with respect to thymidine). [b] K_i = enzyme-inhibitor dissociation constant; I_{50} = inhibitor level causing 50% inhibition. The procedures used to derive the K_i and I_{50} values are detailed under the Experimental Section. [c] I_{50} determined by plotting inhibitor level against intercept on the $1/V$ axis of the Lineweaver-Burk plot was 2.7 mM.

hibitions were noncompetitive and 16-fold if they were competitive. When a methylene was interposed between the uracil ring and the **1c** acetamido group (compound **1k**), an additional marked diminution in inhibition resulted (Table IV), suggesting that there may be insufficient room in the thymidine–enzyme complex to accommodate the 5 substituents of **1b**, **1c**, and **1k**. Compounds **1c**, **1h**, and **1k** labeled with ^{14}C in their acetyl groups had no detectable substrate activity (i.e., less than 0.25% that of thymidine under similar conditions). Interestingly, **1c** and **1h** exhibited substrate activity after the enzyme preparation had been frozen and thawed, a process associated with changes in the tertiary structure of the enzyme, as evidenced by a threefold increase in the K_M value of thymidine.

An homologous series of four 5-[[(iodoacetamido)-acyl]amino] derivatives of 2′-deoxyuridine, **1d**–**1g**, was prepared; **1e** and **1g** were noncompetitive inhibitors (Table V), indicating that they combine with an enzyme–substrate complex rather than with free enzyme and providing further evidence for lack of room in the enzyme–thymidine complex to accommodate most of the N^5 substituents of the present study. Treatment of the enzyme with ca. 2 mM levels of **1d**–**g** in the assay buffer for 2 h, 0 °C, did not affect its activity. TLC indicated that the compounds were stable under these conditions.

(b) Substituents at N-3 and C-4. In exploratory studies of substituent tolerance at C-4, it was found that attachment of an ethyl group (**2b**) to N^4 of 2′-deoxycytidine (**2a**) decreased inhibitory potency by a factor of 7. An *n*-amyl group attached to N-3 of thymidine gave rise to a strong competitive inhibitor (**3e**), K_i = 65 μM. On the assumption that attachment of shorter substituents to N-3 would also produce competitive type inhibition, the inhibitions of 3-ethyl- and 3-*n*-propylthymidines (**3c**,**d**) shown in Table IV correspond to a K_i value for each compound of 200–250 μM, thus suggesting that the distal methylenes of the *n*-amyl group of **3e** may interact with a nonpolar region of the enzyme to produce the enhanced binding. The 3-alkyl-2′-deoxyuridine derivatives **3a** and **3b** produced inhibitions (Table IV) which, if competitive in nature, correspond to a K_i value of ca. 11 mM for **3a** and ca. 14 mM for **3b**. Introduction into **3a** and **3b** of a 5-methyl group, to give **3c** and **3d**, respectively, increased affinity for the thymidine site (as judged from K_i values) 45-fold for **3a** and 70-fold for **3b**. These factors are similar in magnitude to the approximately 30-fold difference, discussed above, in the affinity of 2′-deoxyuridine and thymidine for the thymidine site, and the parallelism constitutes additional evidence that the K_i value of the competitive inhibitor **3e** does, as expected, represent the affinity of **3e** for the thymidine site rather than its affinity for a disparate site.

(c) Substitution at C-5′ of Thymidine. All four of the 5′-*C*-[(acylamino)methyl]thymidines **4a**–**d** were competitive inhibitors, indicating that these substituents do not prevent binding to the thymidine site despite their proximity to the potential reaction center at *O*-5′. The epimeric compounds **4a** and **4b** labeled with ^{14}C showed no substrate activity. The propionyl derivative **4d** bound 17-fold more strongly than its acetyl counterpart **4b**, and **4c** bound approximately twice as well as **4a**, suggesting that the terminal methyl groups of the 5′-substituents of **4c** and **4d** are binding to nonpolar regions of the enzyme.

(d) Substitution at C-5′ of 5′-Deoxythymidine. 5′-Amino-5′-deoxythymidine is a strong inhibitor, competitive with respect to thymidine, of Walker 256 rat carcinoma[39] and mouse ascites sarcoma 180[40] thymidine kinases, the K_i values being 2.4 and 3 μM, respectively. With the BHK enzyme, the inhibition observed (Table IV), if assumed to be competitive, corresponds to a K_i value of ca. 6 μM. The 5′-*N*-acetyl derivative **5a** (Table V) is a competitive inhibitor with a K_i value of 6.5 mM, and the 5′-*N*-butyryl derivative **5b** behaves similarly with a K_i value of 6 mM calculated from data in Table IV assuming competitive-type inhibition. The 1000-fold reduced affinity of **5a** and **5b** can be ascribed, at least in part, to decreased availability of potential bonding electrons on N(5′) coupled with an enlargement of the 109.5° bond angle at N(5′) of 5′-amino-5′-deoxythymidine to the 123° angle in the C(5′)–N(5′)–CO system of **5a** and **5b**. Thus, attachment to **5a** of a 5′-*N*-hexyl group (**5g**), although adding to molecular crowding near the reaction site at O(5′), nevertheless, decreased the K_i from 6.5 to 4.4 mM, possibly as a result of the increase in electron density at N(5′) and/or the much closer approximation of the C(5′)–N(5′)–CO bond angle to that at N(5′) in 5′-amino-5′-deoxythymidine. The affinity of the 5′-*N*-alkyl derivatives **5d**–**f** for the thymidine site, as indicated by their K_i values as competitive inhibitors, was 30–40 times less than that of 5′-amino-5′-deoxythymidine, conceivably indicative of repulsion between polar regions of the enzyme and the alkyl chains.

5′-(Ethylthio)-5′-deoxythymidine (**5j**), in contrast to its 5′-(ethylamino) analogue **5d**, was a noncompetitive inhibitor of the enzyme. This is not ascribable to the absence in **5j** of a hydrogen at the heteroatom attached to C(5′) because **5g** and **5i** also lack such a hydrogen, yet are competitive inhibitors. The poor affinity of **5j** for the thymidine site could be associated with the reduced bond angle (C–O–H, 110°; C–N–C, 109.5°; C–S–C, 104°) and the increased bond distances (covalent radii: O, 0.66 Å; N, 0.70 Å; S, 1.04 Å) occasioned by replacement of the 5′-oxygen by sulfur. Introduction of hydrophilic groups at two or three bond distances from the 5′-sulfur of **5j** and its higher homologue (to give **5k**,**n**,**o**) increased affinity for the thymidine site and produced competitive inhibitors; this effect was less pronounced when a hydrophilic group was only one bond distance from the 5′-sulfur (compound **5m**). On the other hand, sequential introduction of hydrophobic groups at the same and greater distances from the 5′-nitrogen of **5d** (to give **5e**,**f**) at first slightly decreased the binding (**5e**) and then restored it (**5f**), suggesting little or no interaction of the substituents with the enzyme. The inhibitions by **5d**–**f** and **5j**–**o** tentatively suggest the existence of a polar region of the enzyme adjacent to the 5′-oxygen of thymidine in the enzyme–thymidine complex. The sulfoxide derivatives, **5q** and **5r**, corresponding to **5n** and **5o**, respectively, were noncompetitive inhibitors (Table V), possibly because the increased bulk near the 5′-sulfur offset the tendency of the carboxyl groups to promote binding to the thymidine site.

(e) Substitution at C-3′ of 3′-Deoxythymidine and C-6 of 5,6-Dihydrothymidine. 3′-Acetamido-3′-deoxythymidine (**6b**) was a weak competitive inhibitor; 3′-deoxy-3′-(ethylthio)thymidine (**6c**) also inhibited in competitive fashion and was the most powerful inhibitor of the present series with a K_i value of 12 μM. The two diastereoisomers **7a** and **7b** of *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine were both moderately effective competitive inhibitors (Table V).

Substituent Tolerance at the Thymidine Site of Mammalian Thymidine Kinases in General. Lee and Cheng[10] have reported that 5-propyl-2′-deoxyuridine is a strong competitive inhibitor ($K_i \approx 20$ μM) of the cytoplasmic and mitochondrial isozymes of human thymidine kinase; the present studies showed that the isosteric compound 5-(ethylamino)-2′-deoxyuridine is a very weak

inhibitor of the hamster enzyme and that longer-chain 5-(acylamino) derivatives of 2′-deoxyuridine are weak noncompetitive inhibitors. It is not clear to what extent these differences in affinity may be due to differences in tolerance to the various types of 5 substituents (alkyl and alkyl- or acylamino) and to what extent they may be related to structural differences between the human and hamster enzymes. With regard to substituent tolerance at N(3) of thymidine, Baker and Neenan[41] found that the *n*-amyl derivative **3e** inhibited thymidine kinase of Walker 256 rat carcinoma slightly more effectively than did 2′-deoxyuridine; similar relative inhibitions are reported here for the hamster enzyme. Data from enzyme inhibition studies,[40] together with affinity column chromatography of thymidine kinase preparations from mouse gut,[42] mouse sarcoma 180,[40] and human leukocytes,[9] has indicated that tolerance to 3′-*O*-phenyl phosphate substituents exists and that the overall bulk tolerance may be more marked at the 3′ than at the 5′ position in the case of the mouse[40] and human[9] thymidine kinases. It has been reported that *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine (mixed diastereoisomers) (**7a** and **7b**) shows pronounced competitive inhibition (K_i = 24 μM) of thymidine kinase of mouse Ehrlich's ascites tumor cells,[43] to indicate that both mouse and hamster thymidine kinases can tolerate short alkoxy groups at the 6 position.

The present studies have furnished evidence that certain substituents can be attached to the 3, 6, 3′, and 5′ positions of thymidine, as well as the 5′ position of 5′-deoxythymidine, without preventing adsorption to the thymidine site of hamster cytoplasmic thymidine kinase. Evidence from other laboratories, summarized above, suggests that substituent tolerance at most of the above positions, and also at the 5 position,[10] may be a common feature of many mammalian thymidine kinases. Therefore, if substituents with affinity for regions of the enzyme exo to the thymidine site are introduced at one or more of these positions, it may prove possible to obtain strong, selective inhibitors of human cytoplasmic thymidine kinase. The feasibility of obtaining an isozyme-selective inhibitor of thymidine kinase was demonstrated recently by Lee and Cheng,[10] who reported that two 5-halo-2′-deoxycytidines are strong inhibitors of human thymidine kinase and are selective for the mitochondrial isozyme, and, in addition, that the K_i of 5-vinylthymidine is 20-fold lower with the human mitochondrial isozyme than with the cytoplasmic isozyme.

Experimental Section

Chemical Synthesis. General. Tri-*n*-butylammonium pyrophosphate was prepared at room temperature.[44] Pyridine and *N*,*N*-dimethylformamide were distilled from calcium hydride. Petroleum ether employed in purifications boiled at 30–60 °C. Thin-layer chromatograms were obtained with Merck F-254 silica gel plates in (A) chloroform–methanol, 9:1; (B) chloroform–methanol, 4:1; (C) chloroform–methanol, 2:1; or (D) ethyl acetate–ethanol, 4:1. Preparative layer chromatography was conducted with 2-mm layers of silica gel on glass. Paper chromatography (descending) employed Whatman no. 1, 3MM, or 17 papers in (E) 2-propanol–concentrated NH_4OH–water, 7:1:2; (F) 1-butanol–acetic acid–water, 5:2:3; (G) 1-propanol–concentrated NH_4OH–water, 55:10:35; (H) isobutyric acid–1 M NH_4OH–0.1 M EDTA, 100:60:1.5; (I) 2-propanol–concentrated NH_4OH–water, 55:10:35; or (J) 2-propanol–H_2O, 4:1. Electrophoresis was performed on Whatman no. 1 paper at 40–80 V/cm for 30–60 min at pH 7.6 [0.05 M $(Et)_3NHCO_3$] or pH 3.5 (0.05 M citrate). Spots on chromatograms were detected by their ultraviolet absorption and (in the case of silica gel chromatograms) by spraying with the Molisch reagent. Melting points (uncorrected) were determined on a Fisher-Johns melting point apparatus. Ultraviolet spectra were determined with a Cary Model 15 spectrophotometer. Infrared spectra were determined in KBr disks with a Perkin-Elmer spectrophotometer Model 137, and ^1H NMR spectra were obtained with a Varian XL-100-15 spectrometer and are recorded as parts per million downfield from an external standard (concentric capillary) of $SiMe_4$; use of the external standard caused a downfield shift of 0.4–0.5 ppm for all protons. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and unless otherwise indicated are within ±0.4% of the theoretical values. Phosphate analyses of nucleoside triphosphates were performed by the method of Lowry and Lopez[45] after treatment of ca. 1 μmol of these compounds for 60 min at 22 °C in 1 mL of Tris buffer, pH 10.4, containing 0.02 mg of alkaline phosphatase of calf intestinal mucosa (type VII, Sigma Chemical Co.).

5-Amino-2′-deoxyuridine (1a) was prepared from 5-iodo-2′-deoxyuridine as described by Shen et al.[13] The product was obtained in 68% yield: mp 170–171 °C dec; UV λ_{max} 265 nm at pH 1 (ε 7900; reported[13] 7950), 289 nm at pH 12 (ε 5500; reported 5400). It was homogeneous on chromatograms run in solvents B, D, and J.

5-(Ethylamino)-2′-deoxyuridine (1b). To a solution of 5-iodo-2′-deoxyuridine (710 mg, 2 mmol) and triethylamine (200 μL) in dimethylformamide (2 mL) was added anhydrous ethylamine (5 mL). The solution was stored in a stainless-steel bomb at room temperature for 72 h. The residue obtained upon removal of volatiles in vacuo was dissolved in methanol and purified by chromatography in solvent B on silica gel. The band of R_f 0.52 was eluted with ethanol–chloroform (1:4) to yield 350 mg (65%) of **1b** as an off-white powder: mp 178–180 °C (reported[13] 179–181 °C); UV λ_{max} 267 nm at pH 1 (ε 9400; reported[13] ε 9500), 292 nm at pH 12 (ε 5500; reported 5600).

5-(Acetylamino)-2′-deoxyuridine (1c). Compound **1a** (60 mg, 0.25 mmol) in ethanol–H_2O (4:1, 5 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (125 mg, 0.5 mmol) and acetic acid (30 mg, 0.5 mmol). The solution was maintained at 37 °C for 30 min and then evaporated to a gum, which was triturated with benzene. The resulting solid was crystallized from methanol to yield **1c** (50 mg, 71%): mp 260–262 °C dec; UV λ_{max} 277 nm at pH 1 (ε 7500), 273 nm at pH 12 (ε 6000). Anal. ($C_{11}H_{15}O_6N_3$) C, H, N.

General Method for the Synthesis of 5-[[ω-(Iodoacetamido)acyl]amino]-2′-deoxyuridines 1d–g. **1a** (242 mg, 1 mmol) was dissolved in methanol–H_2O (4:1, 10 mL) and treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (494 mg, 2 mmol) and the appropriate *N*-[(benzyloxy)carbonyl]-ω-substituted amino acid (2 mmol). The solution was stored at room temperature for 3 h and then evaporated. The residue was leached with petroleum ether and chromatographed in solvent B over silica gel. The major band was extracted with ethyl acetate, which was then evaporated. The residue was dissolved in ethanol (100 mL) containing 5% Pd/charcoal catalyst (100 mg) and was hydrogenolyzed at 25 psi for 20 min. The catalyst was removed by filtration and the filtrate concentrated to small volume. Addition of acetone precipitated the intermediate 5-[[ω-(amino)acyl]-amino]-2′-deoxyuridines as white powders which migrated as monocations on electrophoresis at pH 7.6 and reacted positively to ninhydrin reagent. Solutions of these intermediates (0.25 mmol) in ethanol–H_2O (2:1, 6 mL) were treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (125 mg, 0.5 mmol) and iodoacetic acid (94 mg, 0.5 mmol) at room temperature for 16 h. After evaporation of the solvents, the residue was triturated with benzene and dissolved in MeOH–H_2O (2:1, 2 mL) and chromatographed in solvent B over silica gel. The major band was eluted with EtOH–$CHCl_3$ (1:4). The eluate was concentrated and diethyl ether was added to precipitate **1d–1g**. Physical properties are listed in Tables I and II.

5-Cyano-2′-deoxyuridine (1i). A solution of 5-iodo-2′-deoxyuridine (354 mg, 1 mmol), potassium cyanide (260 mg, 4 mmol), and dicyclohexyl-18-crown-6 (1.54 g, 4 mmol) in *N*,*N*-dimethylformamide (10 mL) was stored at room temperature for 72 h. The dimethyl formamide was evaporated, the residual yellow oil was dissolved in EtOH, and the nucleoside was precipitated by the addition of ether. Three additional precipitations removed the crown ether. The solid was chromatographed in solvent B on preparative silica gel plates. Extraction of the major band (R_f 0.3) with hot ethanol (5 × 50 mL) gave 190 mg (75%) of **1i** which was recrystallized from ethanol: mp 170–172 °C (reported[19] 161

°C); TLC R_f 0.12 (solvent A), 0.39 (solvent B); NMR (100 MHz, Me_2SO-d_6) δ 8.84 (s, 1, H-6) (reported 8.9;[19] 8.68 for 5-cyanouridine,[46] 6.29 for H-5 of 6-cyanouridine[46]); IR 2250 cm^{-1} (CN); UV λ_{max} 278 nm at pH 1 (ϵ 12700), 278 nm at pH 12 (9900). Anal. ($C_{10}H_{11}N_3O_5$) C, H, N.

5-(Aminomethyl)-2′-deoxyuridine (1j). Compound **1i** (50 mg, 0.2 mmol) in 15% aqueous NH_4OH (25 mL) was hydrogenated over 5% rhodium–alumina (25 mg) at 40 psi for 1 h. The mixture was filtered and then evaporated. The residue was dissolved in H_2O (100 mL) and percolated through a column of Dowex 50 (NH_4) ion-exchange resin (1.25 × 12.5 cm). The column was washed with H_2O until the absorbancy of the eluent at 267 nm was less than 0.1, after which **1j** was eluted with 1% NH_4OH. Evaporation of the eluent gave a white solid, which crystallized from ethanol to yield **1j** (36 mg, 70%): mp 184–186 °C dec; it migrated as a monocation when subjected to paper electrophoresis at pH 4.5; UV λ_{max} 266 nm at pH 1 (ϵ 9200), 266 nm at pH 12 (ϵ 6700). Anal. ($C_{10}H_{15}N_3O_5$) C, H, N.

5-(Acetamidomethyl)-2′-deoxyuridine (1k). Compound **1j** (26 mg, 0.1 mmol) in ethanol (4 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (48 mg, 0.2 mmol) and acetic acid (0.25 mmol). The solution was stored at room temperature for 2 h and then evaporated. The residue was leached with light petroleum and the resulting oil was chromatographed in solvent B over silica gel. Extraction of the major zone (R_f 0.22) with hot ethanol (4 × 50 mL) gave 22 mg (73%) of **1k** as a white crystalline solid, which was recrystallized from ethanol: mp 186–188 °C; TLC R_f 0.10 (solvent A), 0.30 (solvent B); UV λ_{max} 267 nm at pH 1 (ϵ 10000), 267 nm at pH 12 (ϵ 7400). Anal. ($C_{12}H_{17}N_3O_6$) C, H, N.

5-(Ethylamino)-2′-deoxyuridine 5′-Phosphate (1m). 5-Iodo-2′-deoxyuridine 5′-phosphate (100 mg of the free acid form, 0.325 mmol) (obtained from dUMP by the method used by Michelson[16] for UMP) and ethylamine (5 mL) were heated in a stainless-steel bomb at 60–65 °C for 21 h. The residue obtained upon evaporation was dissolved in water (1 mL) and purified by chromatography in solvent E on Whatman 3MM paper (eight sheets, 23 × 56 cm). The band at R_f 0.25 was eluted with water, and **1m** was obtained as its white disodium salt (30% yield) by addition of NaI in acetone to a solution of **1m** in methanol, followed by precipitation with acetone: UV λ_{max} 267 nm at pH 1 (ϵ 9300), 293 nm at pH 12 (ϵ 5600); ^1H NMR (D_2O, 100 MHz) δ 7.44 (s, 1, H-6), 6.82 (t, 1, J = 6.5 Hz, H-1′), 4.58 (m, 4, H-3′, -4′, -5′), 3.46 (q, 2, J = 7 Hz, $-CH_2CH_3$), 2.70 (dd, 2, J = 6 and 7 Hz, H-2′, -2″), 1.66 (t, 3, J = 7 Hz, $-CH_2CH_3$). Anal. ($C_{11}H_{16}N_3O_8PNa_2 \cdot 0.5H_2O$) C, H, N, P.

5-Acetamido-2′-deoxyuridine 5′-Phosphate (1n). 5-Iodo-2′-deoxyuridine 5′-phosphate (434 mg of the free acid form, 1 mmol) and liquid ammonia were heated at 50 °C in a stainless-steel bomb for 48 h. The residue obtained upon evaporation was purified by chromatography in solvent E on Whatman no. 17 paper (eight sheets, 23 × 56 cm). The major band was eluted with water and lyophilized to give 205 mg (62%) of 5-amino-2′-deoxyuridine 5′-phosphate as a white powder: UV λ_{max} 265 nm at pH 1, 289 nm at pH 12; homogeneous in solvents E and I. This nucleotide (100 mg, 0.3 mmol) was dissolved in ethanol–water (1:1, 5 mL), and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (150 mg, 0.6 mmol) and acetic acid (36 mg, 0.6 mmol) were added. The solution was maintained at 37 °C for 1 h and then evaporated in vacuo. The residue was triturated with benzene, then dissolved in water, and purified by chromatography in solvent F on Whatman 3MM paper (eight sheets, 23 × 56 cm). The band of R_f 0.22 was eluted with water, and **1n** was obtained as its white disodium salt (102 mg, 82%) by the procedure described for **1m**. The product was homogeneous in solvents E, F and I: UV λ_{max} 273 nm at pH 1 (ϵ 8400), 270 nm at pH 12 (ϵ 6900); ^1H NMR (D_2O, 100 MHz) δ 8.66 (s, 1, H-6), 6.75 (t, 1, J = 7 Hz, H-1′), 4.54 (m, 4, H-3′, -4′, -5′), 2.85 (dd, 2, J = 6 and 7 Hz, H-2′, -2″), 2.65 (s, 3, $-CH_3$), 2.37 (s, 3, 0.5 acetone). Anal. ($C_{11}H_{14}N_3O_9PNa_2 \cdot 0.5Me_2CO \cdot 1.25H_2O$) C, H, N, P.

5-(Ethylamino)-2′-deoxyuridine 5′-Triphosphate (1o). Compound **1m** (80 mg, 0.2 mmol) was converted to **1o** by the method of Michelson.[17] The crude **1o** (95 mg) was purified by chromatography in solvent I on Whatman 3MM paper (eight sheets, 23 × 56 cm). The band at R_f 0.3 was eluted with water and converted to its white tetrasodium salt (65 mg, 55% yield) as described for **1m**. The product was homogeneous in solvent I and upon electrophoresis at pH 3.5 and pH 7.5: UV λ_{max} 267 nm at pH 1 (ϵ 9300), 293 nm at pH 12 (ϵ 5500). Anal. ($C_{11}H_{16}N_3O_{14}P_3Na_4 \cdot 3H_2O$) P. The ratio of the inorganic phosphate released by alkaline phosphatase to 5-(ethylamino)-2′-deoxyuridine was 2.99:1.

5-Acetamido-2′-deoxyuridine 5′-Triphosphate (1p). Compound **1n** (65 mg, 0.16 mmol) was converted to **1p** by the method of Hoard and Ott;[18] **1p** was purified by chromatography in solvent I on Whatman 3MM paper (six sheets, 23 × 56 cm). The band at R_f 0.32 was eluted with water and converted to its white tetrasodium salt (72 mg, 68% yield) by the procedure described for **1m**. The product was homogeneous in solvents H and I and upon electrophoresis at pH 3.5 and pH 7.5: UV λ_{max} 272 nm at pH 1 (ϵ 8400), 269 nm at pH 12 (ϵ 7000). Anal. ($C_{11}H_{14}N_3O_{15}P_3Na_4 \cdot 6H_2O$) P. The ratio of the inorganic phosphate released by alkaline phosphatase to 5-acetamido-2′-deoxyuridine was 2.92:1.

***N*^{4}-Ethyl-2′-deoxycytidine (2b).** A mixture of 3′,5′-dibenzoyl-4-thio-2′-deoxyuridine[23] (1 g, 2.2 mmol), ethylamine (3 mL), and ethanol (20 mL) was heated at 100 °C in a stainless-steel bomb for 12 h. The solution was evaporated to a syrup, which was dissolved in water (50 mL) and extracted with methylene chloride (4 × 25 mL). The aqueous solution was decolorized by treatment with Norite and then percolated through Dowex 50 (H^+) ion-exchange resin (1.25 × 11.5 cm). Elution was carried out with a linear gradient of water to 0.5 M NH_4OH (1 L). The fractions containing **2b** were lyophilized to a white powder. After drying at 78 °C (0.01 mm) for 72 h, **2b** was obtained as a white powder (410 mg, 73%): mp 79–82 °C; UV λ_{max} 278 nm at pH 1 (ϵ 15900), 272 nm at pH 12 (13900). Anal. ($C_{11}H_{16}N_3O_4 \cdot 0.5H_2O$) C, H, N.

General Synthesis of 3-Alkylthymidines and 3-Alkyl-2′-deoxyuridines 3a–e. A solution of 2′-deoxyuridine or thymidine (1 mmol) in dimethyl sulfoxide (2 mL) was treated with potassium carbonate (50 mg) and the appropriate alkyl halide (4 mmol). The suspension was stirred for 18 h and then filtered, and the filtrate was evaporated to a thin syrup, which was chromatographed in solvent A over silica gel. Ethanol extraction of the main bands gave heavy syrups which resisted crystallization in the case of **3a** and **3b**; **3c**[25] was obtained as a syrup which set to a crystalline mass over several weeks: TLC (solvent A) for **3a** and **3c**, R_f 0.31; **3b** and **3d**, 0.32; **3e**, 0.37; TLC (solvent B) for **3a** and **3b**, R_f 0.66. Other physical properties are listed in Table I.

5′-*C*-[(Acylamino)methyl]thymidines 4a–d were prepared from their 3′-*O*-acetyl derivatives by a previously described method:[26] TLC (solvent A) for **4a,b**, R_f 0.11; **4c,d**, 0.15; TLC (solvent B) for **4a,b**, R_f 0.34; **4c,d**, 0.41. Other physical properties are listed in Table I.

5′-*C*-(Propionamidomethyl)thymidine 5′-triphosphate (4e) was prepared by the procedures described for its 5′ epimer **4f**.[26] See Tables I and II for properties.

General Synthesis of 5′-(Acylamino)-5′-deoxythymidines 5a–c. 5′-Amino-5′-deoxythymidine[27] (35 mg, 0.145 mmol) in methanol (2 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (72 mg, 0.3 mmol) and the required carboxylic acid (0.3 mmol). The solution was stored at room temperature for 1 h and then evaporated. The residue was leached with light petroleum and then dissolved in methanol and chromatographed in solvent B over silica gel. The major band was extracted with hot ethanol (5 × 50 mL). Upon concentration of the ethanol, crystallization occurred. Compounds **5a–c** crystallized from ethanol as colorless needles: TLC (solvents A and B, respectively) for **5a**, R_f 0.16, 0.49; **5b**, 0.20, 0.54; **5c**, 0.25, 0.58. Other physical properties are listed in Table I.

5′-(Ethylamino)-5′-deoxythymidine (5d). A solution of 5′-*O*-tosylthymidine[28] (198 mg, 0.5 mmol) in ethylamine (2 mL) was stored in a stainless-steel bomb at room temperature for 16 h. The residue from evaporation of ethylamine was dissolved in methanol and purified on silica gel with solvent C. The band of R_f 0.1 was eluted with ethanol. Evaporation of the ethanol gave a white solid which was crystallized from ethanol–ether (1:1) to give 97 mg (75%) of **5d**: mp 161–163 °C; UV λ_{max} 267 nm at pH 1 (ϵ 9400). Anal. ($C_{12}H_{19}N_3O_4 \cdot 0.5H_2O$) C, H, N.

5′-(*n*-Butylamino)-5′-deoxythymidine (5e). A mixture of 5′-*O*-tosylthymidine (396 mg, 1 mmol) and *n*-butylamine (2.5 mL) was stirred at room temperature for 16 h. The residue upon

removal of volatiles in vacuo was dissolved in water (10 mL) and the pH was adjusted to 6 with 1 N HCl. The solution was percolated through a column of carboxymethylcellulose (H^+ form; 5 × 10 cm). The column was washed with water (100 mL) and then with 0.4 N NH_4OH (100 mL). The ammoniacal eluant was evaporated, and the residue was dissolved in methanol and purified by chromatography on silica gel with solvent C. The band of R_f 0.2 was eluted with ethanol to give **5e**, 180 mg (61%), as a white powder which became a gum upon exposure to air. The gum was dissolved in water (2 mL) and the pH adjusted to 2 with 2 N HCl. The water was removed by azeotropic distillation with ethanol–benzene. The residue was dissolved in ethanol and precipitated by addition of chloroform to give **5e** (190 mg, 57% yield) as its white monohydrochloride: mp 152–154 °C; UV λ_{max} 267 nm at pH 1 (ϵ 9600). Anal. ($C_{14}H_{23}O_4N_3$·HCl·0.25H_2O) C, H, N.

5′-(*n*-Hexylamino)-5′-deoxythymidine (5f). This was prepared according to the procedure used for **5e**. From 5′-*O*-tosylthymidine (792 mg, 2 mmol) was obtained, after purification on silica gel, 400 mg (62%) of **5f** as a gum; 200 mg of this product was converted to its white monohydrochloride (225 mg) in the manner described for **5e**: mp 175–176 °C; UV λ_{max} 267 nm at pH 1 (ϵ 9600). Anal. ($C_{16}H_{27}N_3O_4$·HCl·0.75H_2O) C, H, N; Cl: calcd, 9.45; found, 10.67.

5′-*N*-Acetyl-5′-(*n*-hexylamino)-5′-deoxythymidine (5g). To a solution of **5f** (65 mg, 0.2 mmol) in ethanol (2 mL) was added *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (100 mg, 0.4 mmol) and acetic acid (25 mg, 0.4 mmol). This mixture was stirred for 3 h at room temperature and then evaporated to dryness, and the residue was dissolved in methanol (1 mL). The solution was applied to two 20 × 20 cm silica gel plates (2-mm thickness), which were developed successively in chloroform and then solvent B. The band of R_f 0.42 (thymidine had R_f 0.17) was eluted with chloroform–ethanol (1:1) and the eluant evaporated to dryness. The residue was dissolved in chloroform (2 mL) and precipitated with light petroleum (100 mL) to yield 45 mg (63%) of **5g**: mp 129–130 °C; UV λ_{max} 267 at pH 1 (ϵ 9500). Anal. ($C_{18}H_{29}N_3O_5$) C, H, N.

5′-[(2-Acetamidoethyl)amino]-5′-deoxythymidine (5h). To a solution of 5′-*O*-tosylthymidine (396 mg, 1 mmol) in *N*,*N*-dimethylformamide (2 mL) was added ethylenediamine (2 mL). The solution was stored at room temperature for 24 h. After removal of the volatiles in vacuo, the residue was dissolved in water (4 mL) and the solution was applied to a column of carboxymethylcellulose (5 × 7 cm). The column was washed with water (50 mL), after which the product was eluted with 1 M NH_4OH. The eluate was concentrated in vacuo to 10 mL and centrifuged to remove a finely divided precipitate. The supernatant was evaporated to yield 170 mg (60%) of the nucleoside diamine as a gum. This migrated as a ninhydrin-positive dication on electrophoresis at pH 3.5. To a solution of 0.25 mmol of the nucleoside diamine in methoxyethanol (2 mL) was added *N*-acetoxysuccinimide (55 mg, 0.3 mmol). The mixture was stored at 22 °C for 16 h and then evaporated to dryness. The residue was chromatographed on silica gel in solvent C. The major band was eluted with ethanol to give a gum (42 mg, 52% yield). When a solution of this in methanol (0.5 mL) was added to an excess of ether, **5h** was obtained as a white hygroscopic powder, which was homogeneous in solvents C and D and on HVE at pH 3.5 and pH 7.6 (Table II); UV λ_{max} (H_2O) 267 nm (ϵ 9500).

5′-[(2-(*N*-methylacetamido)ethyl]methylamino]-5′-deoxythymidine (5i). A mixture of 5′-*O*-tosylthymidine (2.0 g, 5.05 mmol) and N^1,N^2-dimethylethylenediamine (5 mL) was stirred at room temperature for 24 h. The solution was diluted with chloroform (10 mL) and precipitated by slow addition to light petroleum (400 mL). The gum obtained was dissolved in chloroform (20 mL) and precipitated by dropwise addition to anhydrous ether (400 mL). The precipitate was obtained by centrifugation, and a solution of it in water (20 mL) was percolated through Dowex 50 (NH_4^+) ion-exchange resin (5 × 7 cm). The column was washed with water until the absorbancy of the eluant at 267 nm was less than 0.1. The desired material was eluted with 0.2 M NH_4OH. The eluant was concentrated to ca. 10 mL and diluted with ethanol (100 mL). The resulting precipitate was removed by centrifugation and discarded. The supernatant was evaporated. To a solution of the residue in methanol (5 mL) was added anhydrous ether, giving 5′-[2-(methylamino)ethyl]-methylamino]-5′-deoxythymidine (1.0 g, 63%) as an off-white powder which migrated as a dication on electrophoresis at pH 7.5. This intermediate (0.5 mmol) in ethanol–H_2O (1:1, 5 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (250 mg, 1 mmol) and acetic acid (60 mg, 1 mmol). The solution was stored at room temperature for 3 h and then evaporated. The residue was dissolved in methanol and chromatographed in solvent B over silica gel. The major band was eluted with ethanol–$CHCl_3$ (1:4). The solvents were evaporated, and a solution of the residue in chloroform was added to an excess of light petroleum. The solid so obtained became a gum upon exposure to air. The gum was dissolved in water and the pH was adjusted to 6 by addition of 4 N HCl. The solution was diluted with ethanol (4 volumes) and evaporated to dryness. A solution of the residue in methanol (2 mL) was added to anhydrous ether to yield 220 mg (56%) of the hydrochloride of **5i** as a white powder: mp 130–131 °C; UV λ_{max} (H_2O) 267 nm (ϵ 9600). Anal. ($C_{16}H_{27}N_4O_5Cl$·0.75H_2O) C, H, N, Cl.

5′-Deoxy-5′-(ethylthio)thymidine (5j). This was prepared by a procedure used for other 5′-(alkylthio)-5′-deoxynucleosides: white plates (27% yield); mp 188–190 °C (EtOH–ether); UV λ_{max} (pH 2) 267 nm (9700). Anal. ($C_{12}H_{18}N_2O_4S$) C, H, N, S.

5′-Deoxy-5′-[(2-hydroxyethyl)thio]thymidine (5k). To a solution of 5′-*O*-tosylthymidine (1 g, 2.5 mmol) in dry *N*,*N*-dimethylformamide (5 mL) was added β-mercaptoethanol (1 mL) and triethylamine (0.25 mL). The solution was stirred at 40–45 °C for 16 h. The residue obtained upon removal of volatiles in vacuo was dissolved in methanol (2 mL) and chromatographed in solvent A over silica gel. The major product was eluted with ethanol and crystallized from ethanol–ether (1:1) to yield 480 mg (63%) of white plates: mp 120–121 °C; UV λ_{max} (H_2O) 267 nm (9800). Anal. ($C_{12}H_{18}N_2O_5S$) C, H, N, S.

General Synthesis of 5′-[(ω-Carboxylalkyl)thio]-5′-deoxythymidines 5m–o. To a solution of sodium ethoxide (20 mmol) in ethanol (10 mL) was added *N*,*N*-dimethylformamide (30 mL) and the appropriate ω-mercaptocarboxylic acid (10 mmol). The solution was stirred for 10 min. 5′-*O*-Tosylthymidine (792 mg, 2 mmol) was added and the solution was stirred at room temperature for 16 h. The residue obtained upon removal of volatiles in vacuo was dissolved in water (25 mL) and the solution was percolated through a column (5 × 10 cm) of Dowex 50 (H^+) ion-exchange resin. The column was washed with water (100 mL) and the combined eluants were concentrated to ca. 10 mL and poured into ethanol (100 mL). The solution was clarified by centrifugation and then concentrated and chromatographed in solvent B over silica gel. The major product was eluted with ethanol and chromatographed in ethyl acetate–CH_3OH–$CHCl_3$ (2:1:2) over silica gel. The major product was eluted and crystallized. Physical properties are given in Tables I and II.

5′-(Ethylsulfinyl)-5′-deoxythymidine (5p). To a solution of **5j** (86 mg, 0.3 mmol) in DMF–H_2O (1:1, 5 mL) was added dropwise 0.25 M aqueous $NaIO_4$ (1.2 mL). After 30 min, TLC in solvent A indicated that the reaction was complete. The residue obtained upon evaporation of volatiles under reduced pressure was extracted with ethanol (2 × 10 mL). The solubles were chromatographed in solvent A over silica gel. The major product was eluted with ethanol and crystallized from ethanol–ether (1:1) to yield 70 mg (77%) of white plates: mp 208–210 °C; UV λ_{max} (H_2O) 267 nm (ϵ 9600); IR 9.23 μm (S→O). Anal. ($C_{12}H_{18}N_2O_5S$) C, H, N, S.

General Synthesis of 5′-[(ω-Carboxyalkyl)sulfinyl]-5′-deoxythymidines 5q,r. To a solution of **5n** or **5o** (0.3 mmol) in ethanol–water (1:1, 2 mL) was added dropwise 0.25 M $NaIO_4$ (1.2 mL); TLC in solvent C showed that the conversion was complete. The volatiles were removed in vacuo and the residue was extracted at 22 °C with DMF (2 × 10 mL). The DMF was evaporated and the residue was crystallized from ethanol–water (4:1). For physical properties, see Tables I and II. Both **5q** and **5r** showed strong IR absorption at 9.25 μm (S→O), which was absent in **5n** and **5o**.

3′-Amino-3′-deoxythymidine (6a). Compound **6a** was prepared in 51% yield by catalytic hydrogenation (10% Pd/charcoal in 1:1 EtOH–H_2O) of the corresponding azide. A portion was converted to the hydrochloride, which was crystallized from methanol–2-propanol: mp 250–251 °C dec (lit.[32] 249–250 °C dec).

3′-(Acetylamino)-3′-deoxythymidine (6b). A solution of **6a** (241 mg, 1 mmol of the crude free base form) in ethanol–water (1:1, 5 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (494 mg, 2 mmol) and acetic acid (120 mg, 2 mmol). The solution was stored at room temperature for 1 h and evaporated to dryness. The residue was chromatographed on silica gel in solvent B. The major product was eluted with ethanol and then dissolved in methanol (1 mL), and this solution was added to ether to yield 225 mg (80%) of a white powder, mp 184–186 °C (lit.[31] 183–184 °C).

3′-(Ethylthio)-3′-deoxythymidine (6c). A solution of sodium ethoxide (10 mmol) in EtOH (5 mL) was added to a mixture of ethanethiol (1 mL) and *N*,*N*-dimethylformamide (5 mL). 5′-*O*-Trityl-2,3′-anhydro-2′-deoxy-1-(β-D-xylofuranosyl)thymine (466 mg, 1 mmol) was added and the solution was maintained at 75 °C for 16 h; TLC in $CHCl_3$–MeOH (19:1) showed complete conversion to a major UV-absorbing product (R_f 0.58) and a minor product (R_f 0.33). The residue from evaporation of solvents was dissolved in methanol (5 mL) and the solution was poured into water (100 mL). The solid obtained was dissolved in 80% acetic acid (10 mL) and heated on a steam bath for 1 h. The acetic acid was removed by azeotropic distillation with benzene under reduced pressure. The residue was chromatographed in solvent A over silica gel. The major product (R_f 0.44) was eluted with ethanol–$CHCl_3$ (1:1), then dissolved in $CHCl_3$ (2 mL), and precipitated by addition to petroleum ether to yield 135 mg (47%) of a white powder: mp 95–96 °C; UV λ_{max} (H_2O) 267 nm (ε 9700). Anal. ($C_{12}H_{18}N_2O_4S$) C, H, N, S.

Enzyme Kinetic Studies. Baby hamster kidney cells BHK21(C13) grown to confluency in roller bottles were obtained as a suspension at 0–1 °C in complete medium from Grand Island Biological Co. and maintained at 37 °C for 0.5 h prior to collecting by centrifugation. [2-^{14}C]Thymidine (57 mCi/mmol) and [1-^{14}C]acetic acid (4 mCi/mmol) were obtained from New England Nuclear, Inc. Diethylaminoethylcellulose sheets were purchased from Whatman. Thymidine kinase preparations were obtained from 1-g batches of wet packed cells, which were washed twice at 2 °C with aqueous 0.05 M NaCl and disrupted at 2 °C in 0.05 M NaCl by ultrasonic vibration (60 W for 2 × 10 s). The supernatant (2 mL) obtained after centrifugation at 100000*g* was diluted with an equal volume of glycerol for storage at 4 °C. As judged by the Michaelis constant of thymidine and the maximal velocity, the thymidine kinase activity remained stable for 1–3 weeks under these conditions and for lesser periods when glycerol was omitted. A single freezing of the high-speed supernatant increased the Michaelis constant of thymidine from the normal range of 2.5–4.0 μM (reported, 2.5[37] and 2.3[47] μM) to 8–9 μM. The assay mixture contained in a 0.1 mL final volume Tris-HCl buffer (0.05 M) at pH 8.0, ATP (5 mM), $MgCl_2$ (2.5 mM), [^{14}C]thymidine (3.6 to 18 μM, 57 mCi/mmol), and thymidine kinase (5 or 10 μL of the enzyme preparation). The mixture was rocked in a water bath at 37 °C for 10 min and then immersed in boiling water for 2 min and cooled in ice. Denatured protein was removed by centrifugation and 0.02 mL of the supernatant was applied to a 16-mm DEAE-cellulose disk. Each disk was washed three times with 1 mM ammonium formate and once with ethanol, dried under an IR lamp, immersed in a toluene–phosphor mixture, and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 2425. The radioactivity was corrected for the blank value found for unincubated reaction mixtures; in all reactions, the [^{14}C]TMP (thymidine 5′-phosphate) present in the 20-μL aliquot generated more than 500 cpm. Reaction rates remained linear for 20 min and were proportional to the enzyme concentration. When AMP (5 mM) was substituted for ATP in the thymidine kinase assay mixture, no TMP was detected, showing the absence of phosphotransferase activity, which is sometimes present in thymidine kinase preparations, e.g., in extracts of chick embryo cells.[48]

In inhibition studies, four or more levels of thymidine in the range 3.6–18 μM were used for each of the two levels of inhibitor given in Table V and also for a control which lacked inhibitor, and all the enzyme-catalyzed reaction mixtures were made up in duplicate. Enzyme–inhibitor dissociation constants (K_i values; for competitive inhibitors) and I_{50} values (for noncompetitive inhibitors) were obtained from replots of inhibitor concentrations vs. slopes of Lineweaver–Burk double-reciprocal plots (all the latter were linear). The K_i and I_{50} values represent the inhibitor level which doubles the slope; they were reproducible to within 8%. For noncompetitive inhibitors, the same I_{50} values are given by a replot of inhibitor level vs. intercepts on the $1/V$ axis of Lineweaver–Burk plots. For mixed competitive–noncompetitive inhibitors, on the other hand, the two types of replots give nonidentical I_{50} values, and the magnitude of the difference between the values provides a measure of the competitive-type contribution to the overall inhibition; this is illustrated by compound **5m** (Table V).

Attempted alkylation of the enzyme by **1d–g** and iodoacetamide (respective concentrations 1.7, 1.9, 1.9, 1.4, and 1.6 mM) was carried out in the assay buffer for 2 h at 0 °C. Aliquots of the mixtures were assayed at 0.5-h intervals by a twofold dilution to give the standard assay conditions in which the thymidine level was 36 μM. Control mixtures lacking inhibitor lost 12–15% enzyme activity during the 2-h test period. TLC showed that **1d–g** remained unchanged during 16 h under the above test conditions.

^{14}C-labeled derivatives of thymidine or 2′-deoxyuridine were tested as substrates by incubating with 40 μL of enzyme preparation for 30 min at 37 °C in the above assay mixture lacking thymidine; 20 μL of the mixture was subjected to electrophoresis on Whatman 3MM paper in 0.05 M triethylammonium bicarbonate, pH 7.6, after which the radioactivity in the TMP region (14 cm toward the anode) was determined. This electrophoretic procedure was employed in preference to the DEAE-cellulose disk procedure because it could detect smaller amounts of TMP. The compounds were tested at the following levels: **1c**, 350 μM; **1h**, 62 μM; **1k**, 54, 271, and 542 μM; **4a**, 400 μM; **4b**, 400 μM. In no case was significant radioactivity present in the TMP region. Under the same conditions, 14.4 μM thymidine of the same specific activity gave rise to 2200 cpm of TMP. The once-frozen high-speed supernatant enzyme preparation (20 μL) converted 2% of **1c** (70 μM) and 1.3% of **1h** (62 μM) to their 5′-phosphates under these conditions.

Compounds **4a** and **4b** were labeled for substrate studies by reacting an excess of the corresponding 3′-*O*-acetyl-5′-*C*-(aminomethyl)thymidine epimers with [1-^{14}C]acetic acid and EEDQ by a method previously used for an unlabeled compound of this type.[26] The ^{14}C-labeled **1h** was prepared by substituting [1-^{14}C]acetic acid for iodoacetic acid in the described preparation of **1e**.

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References and Notes

(1) Abbreviations used are: AMP, TMP, and dUMP, adenosine, thymidine, and 2′-deoxyuridine 5′-phosphates, respectively; dATP, CTP, GTP, TTP and dUTP, 2′-deoxyadenosine, cytidine, guanosine, thymidine, and 2′-deoxyuridine 5′-triphosphates, respectively; BHK, baby hamster kidney; DEAE, diethylaminoethyl; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.
(2) E. Bresnick and U. B. Thompson, *J. Biol. Chem.*, **240**, 3967 (1965).
(3) T. Hashimoto, T. Arima, H. Okuda, and S. Fujii, *Cancer Res.*, **32**, 67 (1972).
(4) B. Attardi and G. Attardi, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2874 (1972).
(5) M. A. Stafford and O. W. Jones, *Biochim. Biophys. Acta*, **277**, 439 (1972).
(6) A. T. Taylor, M. A. Stafford, and O. W. Jones, *J. Biol. Chem.*, **247**, 1930 (1972).
(7) A. J. Berk and D. A. Clayton, *J. Biol. Chem.*, **248**, 2722 (1973).
(8) S. Kit and W.-C. Leung, *J. Cell Biol.*, **61**, 35 (1974).
(9) L.-S. Lee and Y.-C. Cheng, *J. Biol. Chem.*, **251**, 2600 (1976).
(10) L.-S. Lee and Y.-C. Cheng, *Biochemistry*, **15**, 3686 (1976).

(11) S. Kit, W.-C. Leung, D. Trkula, and G. Jorgensen, *Int. J. Cancer*, **13**, 203 (1974).
(12) W. E. Rawls, G. Cashon, E. Adam, T. Ogino, R. Duff, and F. Rapp, *Cancer Res.*, **34**, 362 (1974).
(13) T. Y. Shen, J. F. McPhenson, and B. O. Linn, *J. Med. Chem.*, **9**, 366 (1966).
(14) J. Zemlicka and F. Sorm, *Collect. Czech. Chem. Commun.*, **30**, 1880 (1965).
(15) J. F. B. Mercer and R. H. Symons, *Biochim. Biophys. Acta*, **238**, 27 (1971).
(16) A. M. Michelson, *Synth. Proced. Nucleic Acid Chem. 1968–1973*, **1**, 491 (1968).
(17) A. M. Michelson, *Biochim. Biophys. Acta*, **91**, 1 (1964).
(18) D. E. Hoard and D. G. Ott, *J. Am. Chem. Soc.*, **87**, 1785 (1965).
(19) R. C. Bleackley, A. S. Jones, and R. T. Walker, *Nucleic Acids Res.*, **2**, 683 (1975).
(20) P. F. Torrence, B. Bhooshan, J. Descamps, and E. DeClercq, *J. Med. Chem.*, **20**, 974 (1977).
(21) M. Freifelder, *J. Am. Chem. Soc.*, **82**, 2386 (1960).
(22) H. Vorbruggen, K. Krolikiewicz, and U. Niedballa, *Ann. Chem.*, **988** (1975).
(23) I. Wempen, R. Duschinsky, L. Kaplan, and J. J. Fox, *J. Am. Chem. Soc.*, **83**, 4755 (1961).
(24) B. R. Baker, T. J. Schwan, and D. V. Santi, *J. Med. Chem.*, **9**, 66 (1966).
(25) R. T. Markiw and E. S. Canellakis, *J. Org. Chem.*, **34**, 3707 (1969).
(26) F. Kappler and A. Hampton, *J. Carbohydr., Nucleosides, Nucleotides*, **2**, 109 (1975).
(27) J. P. Horwitz, A. J. Tomson, J. A. Urbanski, and J. Chua, *J. Org. Chem.*, **27**, 3045 (1962).
(28) E. J. Reist, A. Benitez, and L. Goodman, *J. Org. Chem.*, **29**, 554 (1964).
(29) I. Nakagawa and T. Hata, *Tetrahedron Lett.*, 1409 (1975).
(30) N. J. Leonard and C. R. Johnson, *J. Org. Chem.*, **27**, 282 (1962).
(31) N. Miller and J. J. Fox, *J. Org. Chem.*, **29**, 1772 (1964).
(32) J. P. Horwitz, J. Chua, and M. Noel, *J. Org. Chem.*, **29**, 2076 (1964).
(33) R. P. Glinski, M. S. Khan, and R. L. Kalamas, *J. Org. Chem.*, **38**, 4299 (1973).
(34) R. Teoule, B. Fouque, and J. Cadet, *Nucleic Acids Res.*, **2**, 487 (1975).
(35) J. S. Roth in "Protein Metabolism and Biological Function", C. P. Bianchi and R. Hilf, Eds., Rutgers University Press, New Brunswick, N.J., 1970, p 141.
(36) E. Bresnick, U. B. Thompson, and K. Lyman, *Arch. Biochem. Biophys.*, **114**, 352 (1966).
(37) H. G. Klemperer, G. R. Haynes, W. I. H. Shedden, and D. H. Watson, *Virology*, **31**, 120 (1967).
(38) E. P. Anderson, *Enzymes, 3rd Ed.*, **9**, 69–74 (1973).
(39) J. P. Neenan and W. Rohde, *J. Med. Chem.*, **16**, 580 (1973).
(40) Y.-C. Cheng and W. H. Prusoff, *Biochemistry*, **13**, 1179 (1974).
(41) B. R. Baker and J. P. Neenan, *J. Med. Chem.*, **15**, 940 (1972).
(42) E. P. Kowal and G. Markus, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **34**, 700 (1975).
(43) B. Fouque and R. Teoule, *Chemotherapy*, **20**, 221 (1974).
(44) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).
(45) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).
(46) H. Inoue and T. Ueda, *Chem. Pharm. Bull.*, **19**, 1743 (1971).
(47) A. T. Jamieson and J. H. Subak-Sharpe, *J. Gen. Virol.*, **24**, 481 (1974).
(48) S. Kit, W.-C. Leung, D. Trkula, and D. R. Dubbs, *Arch. Biochem. Biophys.*, **169**, 66 (1975).

S-2,ω-Diaminoalkyl Dihydrogen Phosphorothioates as Antiradiation Agents

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To enable further structure–activity comparisons among radioprotective phosphorothioates, *S*-2,ω-diaminoalkyl dihydrogen phosphorothioates were synthesized from L-2,4-diaminobutyric acid, L-ornithine, L-lysine, and DL-2,7-diaminoheptanoic acid as homologues of *S*-2,3-diaminopropyl dihydrogen phosphorothioate (**4**) and as isomeric analogues of *S*-2-[(ω-aminoalkyl)amino]ethyl dihydrogen phosphorothioates (e.g., **1**). The preferred route that evolved from exploratory trials retained optical activity and involved the reduction of methyl 2,ω-bis(benzoylamino)alkanoates with lithium borohydride, debenzoylation–bromodehydroxylation, and reaction of the resulting 1-(bromomethyl)-1,ω-alkanediamine dihydrobromides with trisodium phosphorothioate. The products of an alternative route that involved the reduction of phthaloylated intermediates with sodium borohydride were racemic. Exploratory conversions of *N*-(ω-alkenyl)phthalimides failed to provide suitable precursors of the target compounds. In terms of a protective index, these homologues were significantly more radioprotective than the parent phosphorothioate **4** when administered intraperitoneally to mice prior to whole-body γ irradiation. The homologues derived from L-lysine also showed good peroral activity. No apparent difference was observed in the protection afforded by optically active homologues and the corresponding racemates.

In the search for effective structural modifications of radioprotective phosphorothioates, *S*-2,3-diaminopropyl dihydrogen phosphorothioate (**4**) was prepared[1] and its structure proved by desulfurization of the derived thiol (Scheme I). The observed radioprotective properties of **4** suggested the synthesis of a homologous series, which would be isomeric with a group of radioprotective *S*-2-[(ω-aminoalkyl)amino]ethyl dihydrogen phosphorothioates.[2] One of these, *S*-2-[(3-aminopropyl)amino]ethyl dihydrogen phosphorothioate (**1**), is being considered for

$$\underset{\mathbf{1}}{H_2N(CH_2)_3NH(CH_2)_2SPO_3H_2}$$

use as a possible adjuvant to the radiotherapy of solid tumors because of observed selective protection of normal tissues and favorable toxicity.[3]

Chemistry. The structure assigned to the intermediate bromide **3** resulting from a possibly ambiguous opening

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