(6) W. Muller and D. Crothers, *J. Mol. Biol.*, **35**, 251 (1968).
(7) D. M. Hayes and P. A. Kollman, *J. Am. Chem. Soc.*, **98**, 3335 (1976).
(8) H. Weinstein, D. Chou, S. Kang, C. L. Johnson, and J. P. Green, *Int. J. Quantum Chem.*, **QBS3**, 135 (1976).
(9) H. Weinstein, S. Masyani, S. Srebrenik, S. Cohen, and M. Sokolvosky, *Mol. Pharmacol.*, **9**, 820 (1973).
(10) G. Loew, D. S. Berkowitz, H. Weinstein, and S. Srebrenik in "Molecular and Quantum Pharmacology", B. Pullman and E. Bergmann, Ed., Dordrecht, Holland, 1975, p 355.
(11) P. Kollman, *J. Am. Chem. Soc.*, **99**, 4875 (1977).
(12) W. J. Hehre, R. F. Stewart, and J. A. Pople, *J. Chem. Phys.*, 51, 2651 (1969).
(13) V. Bloomfield, D. Crothers, and I. Tinoco, "Physical Chemistry of Nucleic Acids", Harper and Row, New York, 1974.
(14) R. Ditchfield, W. J. Hehre, and J. A. Pople, *J. Chem. Phys.*, **54**, 724 (1971).
(15) J. A. Pople, D. P. Santry, and G. A. Segal, *J. Chem. Phys.*, **43**(S), 129 (1965).
(16) F. A. Momamy, L. M. Carruthers, R. F. McGuire, and H. A. Scheraga, *J. Phys. Chem.*, **78**, 1595 (1974).
(17) J. Meisenhofer, *Cancer Chem. Rep.*, **58**, 21 (1974).
(18) F. Quadofoglio and V. Crescenzi, *Biophys. Chem.*, **2**, 64 (1974).
(19) D. Perahia, A. Kollman, and B. Pullman, *Theor. Chim. Acta*, 51, 349 (1971).

Design of Species- or Isozyme-Specific Enzyme Inhibitors. 2.[1] Differences between a Bacterial and a Mammalian Thymidine Kinase in the Effect of Thymidine Substituents on Affinity for the Thymidine Site

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Derivatives obtained by monosubstitution at six positions of thymidine, 5′-amino-5′-deoxythymidine, or 5-bromo-5,6-dihydrothymidine have been studied as inhibitors of *Escherichia coli* and hamster thymidine kinases (TK). Affinity for the enzymatic thymidine binding sites was assessed from apparent enzyme–inhibitor dissociation constants (K_i values; for inhibitions competitive with respect to thymidine at near-saturating ATP levels) or I_{50} values (for noncompetitive inhibitions). To provide indices of relative affinity for each enzyme, the K_i and I_{50} values were divided by the appropriate K_M value (33 or 3.3 μM) of thymidine with the *E. coli* and hamster enzymes, respectively. 3-Amylthymidine gave $I_{50}/K_M = 20$ with *E. coli* and $K_i/K_M = 21$ with hamster TK; 5-amino-2′-deoxyuridine gave $I_{50}/K_M = 840$ with *E. coli* TK and $K_i/K_M = 135$ with hamster TK; *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine diastereoisomers at 16 mM showed almost no inhibition of *E. coli* TK and gave $K_i = 0.2$–0.3 mM with hamster TK; 3′-acetamido- and 3′-(ethylthio)-3′-deoxythymidines gave $I_{50}/K_M = 183$ and 9.6, respectively, with *E. coli* TK and $K_i/K_M = 750$ and 3.6, respectively, with hamster TK; 5′-*C*-(acetamidomethyl)- and 5′-*C*-(propionamidomethyl)thymidine epimers inhibited both enzymes competitively ($K_i/K_M = 26$–198 for *E. coli* and 20–330 for hamster), and the extra methyl present in the propionamido derivatives produced 7.5- and 9-fold differential effects on binding; 5′-amino-5′-deoxythymidine also inhibited competitively ($K_i/K_M = 9.6$ for *E. coli* and 1.8 for hamster), and addition of a 5′-*N*-hexyl group reduced the differential affinity ($K_i/K_M = 78$ for *E. coli* and 54 for hamster); some 5′-(alkylthio)-5′-deoxythymidines inhibited hamster TK competitively but activated *E. coli* TK, possibly by interacting at its dCDP–dCTP activator site. The evidence indicates that thymidine derivatives suitably substituted at any one of the above six positions can bind to the thymidine sites of the *E. coli* and hamster thymidine kinases in a species-selective manner.

Increasing evidence indicates that enzymes from different species which catalyze an identical reaction are frequently structurally dissimilar from each other at regions that are situated outside the enzymatic active sites. In view of this, B. R. Baker[2] proposed that one approach to the design of species-selective enzyme inhibitors could be to determine a position of a substrate or a substrate analogue at which a substituent could be attached without preventing binding to the substrate site of the target enzyme and to vary the nature of the substituent with the object of bringing about differential reversible enzyme inhibition. This type of approach to species-specific inhibitors has been successfully utilized in studies with dihydrofolate reductases,[3-5] thymidine phosphorylases,[6] and adenylate kinases.[7] Baker[2] also suggested that the design of species-selective enzyme inhibitors might be aided by incorporation of a leaving group into the substituent, because species-specific neighboring group effects could come into play during displacement of such a group by a nucleophilic group of the target enzyme. This approach has subsequently given rise to several well-documented examples of substrate-site-directed reagents which bring about species-selective irreversible enzyme inhibition.[8-10]

Another potentially useful approach in the design of species-selective enzyme inhibitors would comprise attaching relatively small substituents to a substrate with the object of permitting or possibly promoting binding to the substrate site of the target enzyme while hindering or possibly preventing binding to the substrate site of the same enzyme from another species. The present report, which describes the first of several studies of this approach, is concerned with the interaction of *E. coli* and hamster thymidine kinases with a series of compounds obtained by monosubstitution at six positions of thymidine, 5′-amino-5′-deoxythymidine, or 5-bromo-5,6-dihydrothymidine. Positions of substitution were selected on the basis of synthetic accessibility but were representative of most areas of the thymidine molecule. For the most part, the substituents selected were short, relatively flexible, and nonbulky in order to increase the probability that they could permit binding to thymidine sites. The affinity of the compounds for the thymidine binding site of the *E. coli* enzyme has been evaluated by kinetic analysis of their inhibition of the enzyme-catalyzed reaction in the presence of near-saturating levels of the second substrate, ATP. The results have been compared with the previously reported

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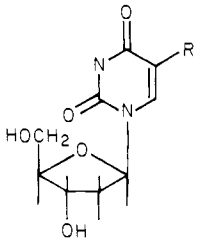
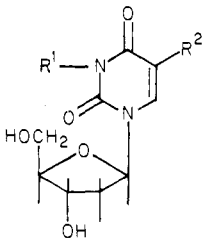
inhibitor properties of these compounds with the cytoplasmic isozyme of hamster thymidine kinase.[1] The study has revealed that marked species-selective effects on affinity for the thymidine sites are produced by substituents located at most of the positions studied.

Chemical Syntheses. 5-(3-Acetamidopropionamido)-2′-deoxyuridine (**1d**) was prepared from 5-amino-2′-deoxy-

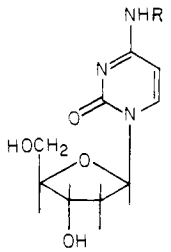


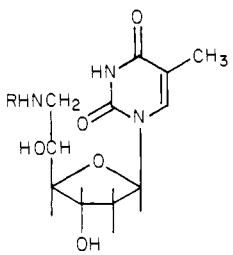


R

1a NH_2
b NHC_2H_5
c $NHCOCH_3$
d $NHCO(CH_2)_2NHCOCH_3$
e CN
f CH_2NH_2
g $CH_2NHCOCH_3$

	R^1	R^2
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



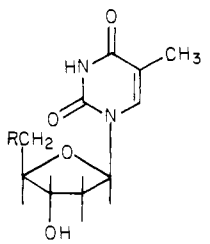


R

2a H
b C_2H_5

R

4a $COCH_3$ (more polar 5′ epimer)
b $COCH_3$ (less polar 5′ epimer)
c COC_2H_5 (more polar 5′ epimer)
d COC_2H_5 (less polar 5′ epimer)



R

5a NH_2
b $NHCOCH_3$
c $NHCO(CH_2)_2CH_3$
d NHC_2H_5
e $NH(CH_2)_3CH_3$
f $NH(CH_2)_5CH_3$
g $N(CH_3)(CH_2)_2N(CH_3)COCH_3$
h $N(CH_3)(CH_2)_4N(CH_3)COCH_3$
i $N(CH_3)(CH_2)_6N(CH_3)COCH_3$

R

5j SC_2H_5
k SCH_2CH_2OH
m $S(CH_2)_2CO_2H$
n $S(CH_2)_3CO_2H$
o $S(O)C_2H_5$
p $S(O)(CH_2)_2CO_2H$

uridine (**1a**) by procedures described previously[1] for other 5′-[[ω-(acylamino)acyl]amino]-2′-deoxyuridines. The 5′-[(ω-acetamidoalkyl)amino]-5′-deoxythymidines **5h** and **5i** were obtained from 5′-*O*-tosylthymidine by a method described previously for the preparation of **5g**.[1] The synthesis of the remaining compounds in this study has been reported.[1] In the case of compounds **4a–d**, the terms "more polar" and "less polar" refer to compounds synthesized from one or other of the two 5′ epimers of 3′-*O*-acetyl-5′-*C*-(nitromethyl)thymidine which had been separated by partition chromatography.[11]

Studies with *E. coli* Thymidine Kinase. Inhibition of the enzyme-catalyzed conversion of thymidine to TMP

Table I. Inhibition of Thymidine Kinase of *E. coli*[a]

compd	inhibns, %	inhibitor concns, mM
1b	0, 0	0.5, 2.0
1c	15, 15	0.5, 2.0
1d	0, 0	0.5, 2.0
1e	23, 40	0.5, 2.0
1f	14, 25	0.5, 1.0
2a	0, 0	0.5, 2.0
2b	0, 0	0.5, 2.0
3a	12, 22	0.5, 1.0
3b	18, 35	0.5, 1.0
3c	30, 44	0.5, 1.0
3d	28, 39	0.5, 1.0
3e	45, 59	0.5, 1.0
5b	0, 0	0.5, 1.0
5c	0, 0	0.5, 1.0
5d	28, 36	4.0, 8.0
5e	9, 12	0.5, 2.0
5g	0, 0	1.0, 2.0
5h	14, 14	1.0, 2.0
5i	14, 14	1.0, 2.0
7a	11, 17	2.0, 16.0
7b	0	16.0

[a] The thymidine level was 82 μM; see the Experimental Section for the remaining conditions of the inhibition determinations.

Table II. Inhibition Constants of Derivatives of 2′-Deoxyuridine and Thymidine with Thymidine Kinase of *E. coli*

compd	type of inhibn[a]	K_i,[b] mM	I_{50},[c] mM	inhibitor concns, mM
1a	NC, L		28	8, 16
3c	NC, L		1.25	0.5, 1.0
3e	NC, L		0.65	0.5, 1.0
4a	C, L	1.25		1.1, 2.2
4b	C, L	2.0		1.6, 3.2
4c	C, L	6.6		1.2, 2.4
4d	C, L	0.85		1.0, 2.0
5a	C, L	0.32		1.0, 2.0
5f	C, L	2.6		2.3, 4.6
6a	NC, L		2.75	2.0, 4.0
6b	NC, L		6.1	2.0, 4.0
6c	NC, L		0.32	1.0, 2.0, 4.0
2′-dUrd (1, R = H)	C, L	0.29		0.15, 0.30
Urd	NC, L		47.5	20, 40

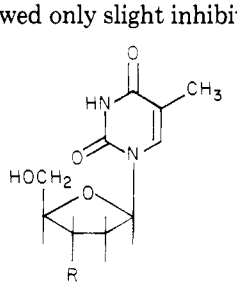
[a] L = linear plot of inhibitor level vs. slope of the Lineweaver-Burk plot; C = competitive, NC = noncompetitive (both with respect to thymidine). [b] Enzyme-inhibitor dissociation constant. [c] Inhibitor level giving 50% inhibition.

brought about by some of the present series of thymidine derivatives is given in Table I; the level of thymidine in these studies was approximately three times higher than its Michaelis constant. Inhibition by the remaining derivatives was studied in the presence of variable thymidine levels, and from these data a series of inhibition constants (Table II) was derived.

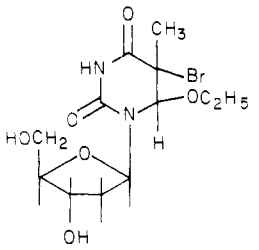
a. Substituents at C-5 of Thymidine. 5′-Deoxyuridine (1, R = H), which is a substrate of *E. coli* thymidine kinase,[12] was a competitive inhibitor with respect to thymidine with a K_i value of 290 μM (Table II). The K_M of thymidine under the same conditions was 33 μM, and, on the assumption that the K_i of thymidine (apparently unreported) is of similar magnitude, it would follow that the methyl group of thymidine enhances adsorption to the thymidine site by a factor of about 9. Substitution of an amino group for that methyl group (compound **1a**) produced a weak noncompetitive inhibitor, showing that the amino group greatly hinders adsorption to the form of the enzyme which binds thymidine and suggesting that the methyl group of thymidine adsorbs to a nonpolar region.

When small alkyl or acyl groups were attached to the amino group of **1a** (compounds **1b–d**), the feeble inhibition persisted (Table I). Attachment of an amino group to the methyl of thymidine (**1f**) also reduced binding to the enzyme very considerably, a finding again consistent with the view of a nonpolar acceptor region for the thymidine methyl. In addition, attachment of the relatively nonpolar $-CH_2OCH_3$ group to C-5 of 2′-deoxyuridine permits substrate and inhibitor activity with this enzyme,[13] in contrast to the feeble inhibitory properties resulting from attachment of the comparably large but more polar $-NHCOCH_3$ group of **1c**. Similarly, 5-(ethoxymethyl)-2′-deoxyuridine is a weak substrate,[14] whereas the isosteric 5-(acetamidomethyl)-2′-deoxyuridine (**1g**) had no detectable substrate activity (in this instance, less than 0.021% that of thymidine under comparable conditions).

b. Substituents at N-3, C-4, and C-6 of Thymidine. Neither 2′-deoxycytidine (**2a**), which is not a substrate of the enzyme,[12] nor its N^4-ethyl derivative **2b** inhibited the enzyme at a level of 2 mM (Table I). Of the two diastereoisomers of *trans*-5-bromo-6-ethoxy-5,6-dihydrothymidine, **7a** showed only slight inhibition at 16 mM and



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



7a, more polar diastereoisomer
b, less polar diastereoisomer

7b showed no inhibition at that level. 5,6-Dihydrothymidine itself has been reported to be noninhibitory at 1 mM,[14] and the inactivity of **7a** and **7b** is, hence, attributable, at least in part, to their 5,6-dihydro structure. Replacement of H-3 of thymidine by ethyl or *n*-amyl groups (**3c**, **3e**) produced inhibitors which were noncompetitive with respect to thymidine (Table II). Removal of the 5-methyl of the 3-ethyl- and 3-*n*-propylthymidines **3c** and **3d** to give the 3-alkyl-2′-deoxyuridines **3a** and **3b**, respectively, resulted in both cases in an approximately twofold increase in the I_{50} value.

c. Substituents at C-3′ of 3′-Deoxythymidine and C-5′ of Thymidine. 3′-Amino-, 3′-acetamido-, and 3′-(ethylthio)-3′-deoxythymidine (**6a–c**) showed noncompetitive inhibition. Baker and co-workers[14] reported that replacement of the 3′-OH of thymidine by H produced an 80-fold loss in binding, and the presence in the thymidine–enzyme complex of enzymatic atoms within bonding distance of the 3′-OH might account for the lack of room in that complex for the 3′ substituents of compounds **6a–c**. Uridine, which is not a substrate,[12] is a weak noncompetitive inhibitor (Table II), indicating the possibility that an area of limited bulk tolerance may also be located near the adjacent C-2′ of thymidine. Another possibility is that introduction of a 2′-OH into 2′-deoxyuridine may cause conformational changes which are unfavorable for recognition of the molecule by the thymidine site.

Table III. Activation of *E. coli* Thymidine Kinase by 5′-Substituted 5′-Deoxythymidines[a]

	activation, %, at various concns, mM				
compd	0.1	0.5	1.0	2.0	8.0
5j	70	63		46	
5k		15	28	25	
5m	120	126		125	106
5n	61	60		62	35
5o	−5			−4	22
5p	−2	−1		42	65

[a] The assay system (see Experimental Section) contained 82 μM thymidine.

That the 5′-OH of thymidine also participates in binding to the enzyme is indicated by the 30-fold loss of binding which accompanies substitution of H for the 5′-OH.[14] The nearby 5′-*C*-[(acylamino)methyl] substituents of compounds **4a–d** do not, however, prevent adsorption to the thymidine site, as evidenced by the competitive character of the inhibitions exerted by those compounds (Table II). No substrate activity was exhibited by the 5′ epimers **4a** or **4b** labeled with ^{14}C in their acetyl groups. Addition of an additional methylene to **4a** to give **4c** decreased the affinity fivefold, whereas the same modification of **4b** doubled the affinity.

d. Substituents as C-5′ of 5′-Deoxythymidine. 5′-Amino-5′-deoxythymidine (**5a**), which is not a substrate of *E. coli* thymidine kinase,[13] was found to be a competitive inhibitor with about one-tenth as much affinity for the enzyme as thymidine itself. The 5′-*N*-acetyl and 5′-*N*-butyryl derivatives **5b** and **5c** were not inhibitory at the levels tested (Table I), possibly because of limited bulk tolerance near the reaction center at O-5′ or of decreased capacity of N-5′ to form a hydrogen bond with an enzymatic group. The 5′-*N*-hexyl derivative **5f** was a competitive inhibitor with an affinity for the enzyme about eightfold less than that shown by **5a**. The 5′-*N*-ethyl and 5′-*N*-butyl derivatives **5d** and **5e** (Table I) were significantly weaker inhibitors than **5f**. Some 5′-amino-5′-*N*-(ω-aminoalkyl)-5′-deoxythymidines have been reported to be inhibitors (no quantitative data furnished) but not substrates of the *E. coli* enzyme.[13]

The 5′-(alkylthio)-5′-deoxythymidines **5j–n** and, to a lesser extent, the related sulfoxides **5o** and **5p** activated the thymidine kinase catalyzed reaction (Table III). The most effective compound was 5′-[(2-carboxyethyl)thio]-5′-deoxythymidine (**5m**). The carboxyl of **5m** is situated the same number of bond distances from C-5′ as is the β-phosphate of a nucleoside 5′-diphosphate. *E. coli* thymidine kinase is activated by certain 2′-deoxynucleoside 5′-di- and triphosphates,[15] most markedly by dCDP and dCTP, and it is thus possible that **5j–p** could be acting at the same locus.

Comparison of *E. coli* and Hamster Cytoplasmic Thymidine Kinases. All of the present series of compounds, with the exception of **1d**, **5h**, and **5i**, had previously been examined as inhibitors of the cytoplasmic isozyme of hamster thymidine kinase.[1] The studies with the hamster enzyme were carried out at pH 8, which is only 0.2 pH unit higher than the pH of the present studies, and thus both enzymes were exposed to essentially the same ratio of ionic species in the case of partially ionized compounds. Additionally, the levels of MgATP employed in the inhibition studies (5 mM for the hamster enzyme, 5.7

Table IV. Inhibition of *E. coli* and Hamster Cytoplasmic Thymidine Kinases by Derivatives of Thymidine and 2'-Deoxyuridine

	E. coli enzyme[a]			hamster cytoplasmic enzyme[b]	
compd	type[c]	K_i, mM	I_{50}, mM	type[c]	K_i, mM
Thd		0.033[g]			0.003[g]
3e	NC		0.65	C	0.07
1, R = H	C	0.29		C[d]	0.1
1a	NC		28.0	C	0.45
7a			*e*	C	0.31
7b			*e*	C	0.18
6b	NC		6.1	C	2.5
6c	NC		0.32	C	0.012
4a	C	1.25		C	0.27
4b	C	2.0		C	1.10
4c	C	6.6		C	0.16
4d	C	0.85		C	0.065
5a	C	0.32		C[d]	0.006
5f	C	2.6		C	0.18
5j	activator[f]			NC	21.5
5m	activator[f]			C	0.5
5n	activator[f]			C	1.2

[a] Values from Table II. [b] Values from ref 1. [c] C = competitive, NC = noncompetitive (both with respect to thymidine). [d] Assumed to be competitive by analogy with other mammalian thymidine kinases (ref 1). [e] Inhibition too weak to allow measurement of an inhibition constant; see Table I. [f] Not inhibitory at 0.1–8.0 mM; see Table III. [g] K_M.

mM for the *E. coli* enzyme) were in both cases near-saturating.

Representative inhibition constants for the six types of thymidine and 2'-deoxyuridine derivatives studied are compiled in Table IV. The two enzymes show many contrasting properties. Thus, 3-*n*-amylthymidine (**3e**) exhibited noncompetitive inhibition with the *E. coli* enzyme but competitive inhibition with the hamster enzyme. In both cases, as discussed above and previously,[1] the inhibitory potency of the corresponding 3-alkyl-2'-deoxyuridines supported the most probable interpretation of the kinetic results; namely, that the K_i value of **3e** with the hamster enzyme is a measure of its affinity for the thymidine site, whereas the noncompetitive inhibition with the *E. coli* enzyme indicates preferential adsorption of **3e** to a form of the enzyme which is not the same as the enzyme form with which thymidine combines.

The two enzymes showed pronounced differences in respect to their 5-methyl binding regions. Thus, the 5-methyl group of thymidine appears to enhance binding ca. 9-fold to the *E. coli* enzyme but ca. 33-fold to the hamster enzyme. In addition, 5-amino-2'-deoxyuridine (**1a**) was a weak noncompetitive inhibitor of the *E. coli* enzyme but a more powerful competitive inhibitor of the hamster enzyme. Very large differences were also shown by the two enzymes in their interactions with the 5-bromo-6-ethoxy-5,6-dehydrothymidines (**7a** and **7b**), which had virtually no inhibitory effect on the *E. coli* enzyme at 16 mM, yet had K_i values of 0.2–0.3 mM with the hamster enzyme. It appears from this that the hamster enzyme is better able than the *E. coli* enzyme[14] to bind the 5,6-dihydrothymine ring. The 3'-substituted 3'-deoxythymidines **6b** and **6c** produced noncompetitive inhibition with the *E. coli* enzyme but competitive inhibition with the hamster enzyme.

The 5'-*C*-substituted thymidines **4a–d** inhibited both enzymes competitively and served as probes which revealed considerable structural differences between the enzymes near C-5' in the enzyme–thymidine complex. Thus, addition of an extra methyl to **4a** to give **4c** decreased binding to the thymidine site 5.3-fold with the *E. coli* enzyme but increased binding 1.7-fold with the hamster enzyme; moreover, addition of a methyl to **4b** produced a 2.3-fold increase in binding with the *E. coli* enzyme but a 17-fold increase with the hamster enzyme.

The K_i value for inhibition of the hamster enzyme by 5'-amino-5'-deoxythymidine (**5a**) was only twice the K_M of thymidine, whereas with the *E. coli* enzyme a factor of 10 was observed. The 5'-(alkythio)-5'-deoxythymidines **5j**,**m**,**n** activated the *E. coli* enzyme but inhibited the hamster enzyme (Table IV). It has been reported that dCTP inhibits human cytoplasmic thymidine kinase,[16] whereas it activates *E. coli* thymidine kinase by interacting at a 2'-deoxynucleoside di- or triphosphate effector site.[15] On this basis, it can be suggested, tentatively, that the above 5'-(alkylthio) derivatives might activate the *E. coli* enzyme by complexing at its positive effector site. On the other hand, the hamster enzyme, like other mammalian thymidine kinases,[17] presumably lacks such a deoxynucleoside di- or triphosphate activator site, and the 5'-(alkylthio)-thymidines **5m** and **5n**, at higher levels than those at which they activate the *E. coli* enzyme, appear to adsorb instead to the thymidine site of the hamster enzyme, as indicated to the linear competitive nature of their inhibition.[1]

The present series of inhibitors have thus proved effective in revealing many structural differences between the *E. coli* and hamster cytoplasmic thymidine kinases in regions near their respective thymidine binding sites. With respect to the particular substituents and positions of their attachment used in this study, the enzyme–thymidine complex of the *E. coli* enzyme shows, in general (Table IV), less substituent tolerance than the enzyme–thymidine complex of the hamster cytoplasmic enzyme. This is particularly clear in the case of alkyl substituents at N-3, an amino group at C-5, or an acetamido or ethylthio group at C-3', all of which markedly hinder adsorption of the corresponding thymidine derivatives to the thymidine site of the *E. coli* enzyme as evidenced by the weak noncompetitive inhibition which they produce.

Experimental Section

Chemical Synthesis. General. Thin-layer chromatograms were obtained with Merck F-254 silica gel plates in (A) chloroform–methanol, 9:1, or (B) chloroform–methanol, 4:1. Preparative layer chromatography was conducted with 2-mm layers of silica gel on glass. Spots on chromatograms were detected by their ultraviolet absorption and 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 15 spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and unless otherwise indicated are within ±0.4% of the theoretical values.

5-(3-Acetamidopropionamido)-2'-deoxyuridine (1d). A solution of **1a** (242 mg, 1 mmol), *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (494 mg, 2 mmol), and 3-[*N*-(carbobenzyloxy)-amino]propionic acid (446 mg, 2 mmol) in ethanol–H_2O (4:1, 10 mL) was stored at room temperature for 3 h. After removal of the solvents in vacuo, the residue was purified by preparative layer chromatography in solvent A, and the material at R_f 0.22 was extracted with ethyl acetate. The solubles were dissolved in ethanol (100 mL) containing 5% Pd/charcoal catalyst (100 mg) and hydrogenolyzed at 25 psi for 20 min. The catalyst was removed by filtration and the filtrate concentrated. Addition of acetone yielded 170 mg (54%) of the intermediate 5-[(3-aminopropionyl)amino]-2'-deoxyuridine as a white powder, which migrated as a monocation upon electrophoresis at pH 7.6 and reacted positively to the ninhydrin reagent. A solution of this intermediate (0.5 mmol) in ethanol–H_2O (2:1, 10 mL) was treated with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (247 mg, 1 mmol) and acetic acid (60 mg, 1 mmol) and stored at room temperature for 3 h. After removal of the solvents in vacuo, the residue was

purified by preparative layer chromatography in solvent B, and material at R_f 0.22 was eluted with ethanol and crystallized from ethanol to yield 125 mg (70%) of 1d as white plates: mp 195–196 °C; UV λ_{max} 281 nm (ϵ 8200). Anal. ($C_{14}H_{20}N_4O_7$) C, H, N.

5′-[[4-(*N*-Methylacetamido)butyl]methylamino]-5′-deoxythymidine (5h). This was prepared by a previously described procedure for **5g**[1] and obtained in 32% yield as a hygroscopic solid. Anal. ($C_{18}H_{30}N_4O_5 \cdot H_2O$) C, H; N: calcd, 14.00; found, 13.55. The analyzed material was converted to a hydrochloride salt[1] for enzyme work: mp 115–117 °C; UV λ_{max} 267 nm (ϵ 9500).

5′-[[6-(*N*-Methylacetamido)hexyl]methylamino]-5′-deoxythymidine (5i). This was prepared by the same method as **5h** and obtained in 48% yield as its hydrochloride: mp 95–97 °C; UV λ_{max} 267 nm (ϵ 9700). Anal. ($C_{20}H_{35}N_4O_5Cl \cdot 1.75H_2O$) C, H, N.

Enzyme Kinetic Studies. *E. coli* B cells (harvested in late log phase) were obtained from Miles Laboratories, Inc. [2-^{14}C]-Thymidine (57 mCi/mmol) and [1-^{14}C]acetic acid (4 mCi/mmol) were obtained from New England Nuclear, Inc. Thymidine kinase was isolated from 100-g batches of frozen cells by the method of Okazaki and Kornberg.[12] Purification to fraction V gave an enzyme preparation with a specific activity of 0.006 μmol min^{-1} (mg of protein)$^{-1}$. Fraction V was stable at −15 °C for the duration of this work. The assay mixture and volume was that of Okazaki and Kornberg and it contained 0.1 μL (7.2×10^{-6} units) of fraction V, Tris-HCl buffer (71 mM), $MgCl_2$ (2.85 mM), $MnCl_2$ (0.71 mM), ATP (5.7 mM), thymidine (0.86 mM), and bovine serum albumin (0.285 μg/μL). 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 20 μL of the supernatant was applied, together with 0.1 μmol of carrier TMP (thymidine 5′-phosphate), on Whatman No. 3MM paper. Electrophoresis was carried out in 0.05 M triethylammonium bicarbonate buffer, pH 7.6, at a gradient of 27 V/cm for 1 h. After the paper was dried, the TMP spot was cut out, 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 present in the 20-μL aliquot generated no less than 1000 cpm. Reaction rates remained linear for 20 min and were proportional to the enzyme concentration. The K_M value for thymidine was 33 ± 3 μM (reported[15] 33 μM).

In inhibition studies, four or more levels of thymidine in the range 20–160 μM were used for each of two levels of inhibitor and also for a control which lacked inhibitor, and all the enzyme-catalyzed reaction mixtures were made up in duplicate. Apparent 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 of velocity vs. substrate level (all the latter were linear). The K_i or I_{50} values were reproducible to within ±7%.

Compounds **1g**, **4a**, and **4b** were labeled with [1-^{14}C]acetic acid as previously described.[1] These compounds were tested as substrates by incubating with 5 μL of fraction V for 1 h at 37 °C in the above assay mixture lacking thymidine. The compounds were tested at the following levels: **1g**, 775 μM; **4a**, 450 μM; **4b**, 500 μM. In no case was radioactivity detected in the TMP region.

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References and Notes

(1) For Part 1, see A. Hampton, F. Kappler, and R. R. Chawla, *J. Med. Chem.*, **22**, 621 (1979).
(2) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, 1967.
(3) J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, 1, 126 (1965).
(4) J. J. Burchall, *J. Infect. Dis.*, **128**, S437 (1973).
(5) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967).
(6) B. R. Baker and J. L. Kelley, *J. Med. Chem.*, **14**, 812 (1971).
(7) A. Hampton, L. A. Slotin, F. Kappler, T. Sasaki, and F. Perini, *J. Med. Chem.*, **19**, 1371 (1976).
(8) B. R. Baker and R. P. Patel, *J. Pharm. Sci.*, **53**, 714 (1964).
(9) A. Hampton, L. A. Slotin, and R. R. Chawla, *J. Med. Chem.*, **19**, 1279 (1976).
(10) R. L. Barfknecht, R. A. Huet-Rose, A. Kampf, and M. P. Mertes, *J. Am. Chem. Soc.*, **98**, 5041 (1976).
(11) F. Kappler and A. Hampton, *J. Carbohydr., Nucleosides, Nucleotides*, **2**, 109 (1975).
(12) R. Okazaki and A. Kornberg, *J. Biol. Chem.*, **239**, 269 (1964).
(13) W. Rohde and A. G. Lezius, *Z. Physiol. Chem.*, **352**, 1507 (1971).
(14) B. R. Baker, T. J. Schwan, and D. V. Santi, *J. Med. Chem.*, **9**, 66 (1966).
(15) R. Okazaki and A. Kornberg, *J. Biol. Chem.*, **239**, 275 (1964).
(16) L.-S. Lee and Y.-C. Cheng, *Biochemistry*, **15**, 3686 (1976).
(17) E. P. Anderson, *Enzymes*, **9**, 69–74 (1973).