degree of butyrylation and biological activity on thyroid function. Evidently other factors besides penetration, e.g., binding requirements of various enzyme systems involved in the mechanism of action of cAMP, will have to be considered to explain our results.

Several groups[26,27] have reported variations in the PDE isoenzyme composition of different tissues and we might speculate that there are also differences in the protein kinase system of various tissues.

Comparing the effect of the various cyclic nucleotides on the thyroidal function with results previously obtained on the release of growth hormone[28,29] or prolactin,[30] we find some degree of tissue specificity. For example, Bt_2-8HS-cAMP, which was one of the most active compounds on growth hormone release, shows only a marginal effect on thyroid function. On the other hand, $8H_2N$-cAMP, which is a very strong stimulator of the thyroid, is one of the least active cAMP analogues with respect to growth hormone release. This observation of relative specificity of certain cAMP derivatives could contribute to a better understanding of the mechanism of action of cAMP and lead to further study of possible therapeutic applications of cyclic nucleotides.

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Synthetic Inhibitors of *Escherichia coli*, Calf Thymus, and Ehrlich Ascites Tumor Thymidylate Synthetase

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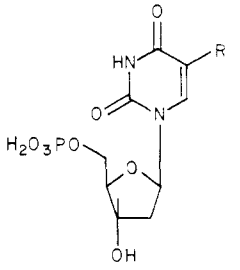
In a study of active site binding the inhibition of thymidylate synthetase derived from *Escherichia coli*, calf thymus, and Ehrlich ascites tumor was examined using eight inhibitors. 5-Substituted 2′-deoxyuridine 5′-phosphate analogues used in this study are the hydroxymethyl, methoxymethyl, benzyloxymethyl, formyl, acetyl, allyl, and two potential active site alkylating substituents: 2,3-oxypropyl and the azidomethyl analogues. All compounds were competitive with the substrate, 2′-deoxyuridine 5′-phosphate; the most potent inhibitor was 5-formyl-dUMP (K_i = 0.1, 0.09, and 0.08 μM for the respective enzyme). The 5-hydroxymethyl, 5-benzyloxymethyl, and 5-azidomethyl derivatives of dUMP showed some differential inhibition; these compounds were two to three times more active against the ascites tumor enzyme than against the thymus enzyme.

Thymidylate synthetase catalyzes the conversion of dUMP[1] to dTMP, a reductive methylation reaction utilizing formaldehyde as the carbon source and tetrahydrofolic acid as the reducing agent.[1b] The fluoro analogues of both the substrate (5-fluoro-dUMP) and product (5-trifluoromethyl-dUMP), potent inhibitors of the enzyme, are effective in the treatment of cancer.[2] The primary effect, inhibition of DNA synthesis, is caused by a depletion of dTTP.

An approach to the treatment of cancer that offers greater selectivity is the design of inhibitors that have different affinities for isoenzymes. The key features in

Table I. Structures of the Substrate, Product, and Inhibitors of Thymidylate Synthetase[a]



No.	Compd	R	Source
1	dUMP	H	
2	dTMP	CH_3	
3	5-Hydroxymethyl-dUMP	CH_2OH	From 5[b]
4	5-Methoxymethyl-dUMP	CH_2OCH_3	$POCl_3$ method[c]
5	5-Benzyloxymethyl-dUMP	$CH_2OCH_2C_6H_5$	$POCl_3$ method[b]
6	5-Formyl-dUMP	CHO	Thymidine kinase[d]
7	5-Acetyl-dUMP	$COCH_3$	$POCl_3$ method[e]
8	5-Allyl-dUMP	$CH_2CH=CH_2$	$POCl_3$ method[f]
9	5-(2,3-Oxypropyl)-dUMP	$CH_2CH(O)CH_2$	From 8
10	5-Azidomethyl-dUMP	CH_2N_3	$POCl_3$ method[e]

[a] Nucleotides prepared chemically used the procedure of Yoshikawa et al.[19] which is selective for 5′-phosphorylation. Purification was by either paper or DEAE cellulose chromatography. All nucleotides synthesized by chemical means were purified and examined for impurities (3′-phosphate) by reaction with 5′-ribonucleotide phosphohydrolase (E.C. 3.1.3.5). Electrophoresis showed essentially complete hydrolysis. [b] Nucleoside synthesis was reported by Mertes and Shipchandler[7] and Brossmer and Rohm.[6] Compound 3 can be prepared from the corresponding cytosine derivative: A. H. Algeria, *Biochim. Biophys. Acta*, 149, 317 (1967). [c] See ref 5. [d] Nucleoside synthesis was reported by Mertes and Shipchandler.[10] Conversion to 6 using carrot phosphotransferase was reported by Santi and Sakai.[11] Synthesis of 6 is described in the Experimental Section. [e] The synthesis of the nucleoside precursors will be published elsewhere. [f] Nucleoside synthesis was reported by Montgomery and Hewson.[9]

such an approach are the tertiary structural differences that may exist between isoenzymes in the proximity of the active site.[3] One example of isoenzymic thymidylate synthetases has been described; the host and phage-infected host enzymes are distinct proteins.[4]

In this preliminary study of structural features important for active site binding, a series of eight 5-substituted substrate analogues was examined for inhibition of thymidylate synthetase from *Escherichia coli*, calf thymus, and Ehrlich ascites tumor.

The synthesis of the nucleotides (Table I) was done using chemical synthesis or enzyme-catalyzed phosphorylation. The synthesis of some of the nucleosides of the compounds in Table I has been reported. 5-Methoxymethyl-2′-deoxyuridine,[5] 5-benzyloxymethyl-2′-deoxyuridine,[6,7] 5-acetyl-2′-deoxyuridine,[8] 5-allyl-2′-deoxyuridine,[9] and 5-azidomethyl-2′-deoxyuridine[8] were converted selectively to the 5′-phosphate using phosphorus oxychloride in trimethyl phosphate. These, with the exception of the 5-acetyl, 7, were purified in 45–60% yield by preparative paper chromatography. Compound 7 was found to decompose on elution from paper with 2% ammonia; it was purified on DEAE cellulose column

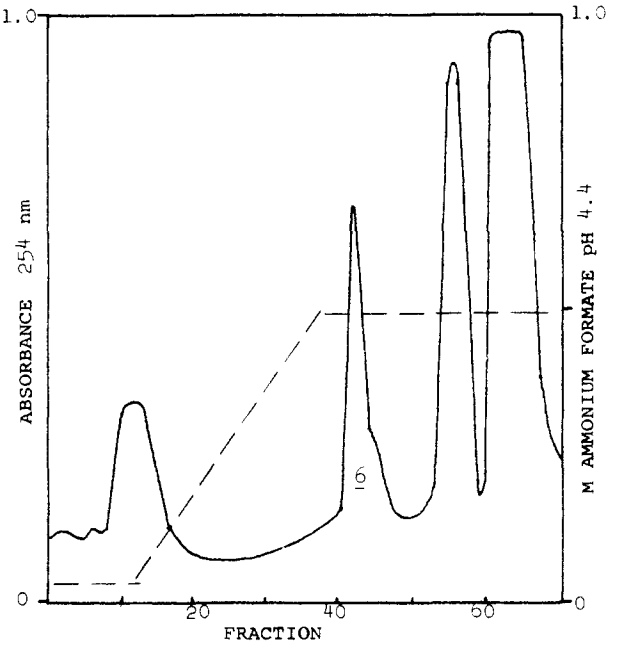


Figure 1. The synthesis of 6 catalyzed by the action of thymidine kinase is described in the Experimental Section. The product 6 (4.5 μmol) was resolved from the starting nucleoside, ADP, and ATP using a wash of 100 ml of 0.005 M buffer followed by a linear gradient of 125 ml of 0.005 M and 125 ml of 0.5 M ammonium formate, pH 4.4; a final wash of 0.5 M buffer eluded ATP. Fractions (10 ml) were collected; the nucleoside (1.45 μmol) was found in fractions 11–15, and the product 6 (2.77 μmol) was collected in fractions 40–42.

chromatography. The nucleotides prepared by chemical synthesis were examined for impurities by treatment with 5′-ribonucleotide phosphohydrolase (E.C. 3.1.3.5). In all cases the enzyme completely hydrolyzed the nucleotides **4**, **5**, **7**, **8**, and **10** whereas there was no hydrolysis of uridine 2′(3′)-phosphate. Compound **6**, the 5-formyl derivative, could not be prepared using the phosphorus oxychloride method starting with the nucleoside.[7,10,11] Conversion of the 5-formyl nucleoside to **6** was accomplished in 65% yield using thymidine kinase.[12] The product was resolved using DEAE cellulose (Figure 1).

Compound **3** was prepared by hydrogenolysis of the benzyloxy nucleotide **5**. Attempts to prepare the nucleoside precursor leading to **9** were unsuccessful; **9** which was not isolated in crystalline form could, however, be prepared by epoxidation of the allyl derivative **8** by treatment with *m*-chloroperbenzoic acid.

The ultraviolet absorption characteristics of the nucleotides are listed in Table II.

All of the nucleotides in this study except the formyl, **6**, were competitive inhibitors of thymidylate synthetase from the three sources (Table III). The general pattern of activity within the series (compounds **3–10**) was similar for all three enzyme preparations. 5-Hydroxymethyl-dUMP (**3**) was a potent inhibitor approximately ten times as active as either the 5-methoxy derivative **4** or 5-benzyloxymethyl-dUMP (**5**).

5-Formyl-dUMP (**6**) was found to be the most potent inhibitor of the three enzyme preparations. The bacterial enzyme showed competitive inhibition with respect to substrate and a K_i of 10×10^{-8} M. Compound **6** was a noncompetitive inhibitor of the thymus enzyme; a K_i of 9×10^{-8} M was observed (Figure 2). The results found on inhibition of ascites tumor enzyme were a K_i of 8×10^{-8} M, competitive with substrate (Figure 3). In contrast to the formyl compound **6**, 5-acetyl-dUMP (**7**) was a weak

Table II. Ultraviolet Absorption Nucleotides

Compound	pH[a]	λ_{max}, nm	ϵ	λ_{min}, nm	ϵ
5-Hydroxymethyl-dUMP (3)	1	264	10700	233	2300
	7	264	10700	233	2300
	13	264	8200	239	4500
5-Methoxymethyl-dUMP (4)	1	261	10700	232	2600
	7	207	10600		
		261	11100	232	2600
	13	261	7700	242	5800
5-Benzyloxy-methyl-dUMP (5)	1	264	9700	235	2700
	7	264	9800	235	3000
	13	263	7600	240	4000
5-Formyl-dUMP (6)	2[b]	279	13100	252	5300
	4.4[b]	279	14000	250	5300
	13	281	10000	258	6800
5-Acetyl-dUMP (7)	1	228	10300	211	7600
		281	12400	250	3900
	7	228	12200	208	7700
		281	13000	250	4000
	13	284	10000	257	5100
5-Allyl-dUMP (8)	1	266	9800	234	3800
	7	266	9800	234	3900
	13	264	6800	244	5400
5-(2,3-Oxypropyl)-dUMP (9)	1	268	10100	237	4200
	7	268	10200	237	4300
	13	266	7500	245	6500
5-Azidomethyl-dUMP (10)	1	263	9700	233	4500
	7	263	9700	233	4400
	13	261	7000	243	5800

[a] Solutions at pH 1 were 1 M HCl and at pH 13 were 1 M NaOH. [b] These solutions also contained 0.01 M ammonium formate.

Table III. Inhibition of Thymidylate Synthetase Purified from Three Sources[a]

Inhibitor	K_m (no inhibn) and K_i values, μM		
	E. coli	Calf thymus	Ehrlich ascites
None	25	9	4
5-Hydroxymethyl-dUMP (3)	4[b]	4	0.6
5-Methoxymethyl-dUMP (4)	42	32	19
5-Benzyloxymethyl-dUMP (5)	25	42	9
5-Formyl-dUMP (6)	0.1[b]	0.09	0.08
5-Acetyl-dUMP (7)	64	73	22
5-Allyl-dUMP (8)	135	130	40
5-(2,3-Oxypropyl)-dUMP (9)	25	33	14
5-Azidomethyl-dUMP (10)	18	21	5

[a] Assays were run without preincubation as described in the Experimental Section. K_m and K_i values were determined by reciprocal plotting of velocity vs. dUMP concentration. Concentrations of substrate used were 2.5, 3.25, 5, 7.5, and 10 × 10^{-6} M. All inhibitors were competitive with substrate in these assays, with the exception of **6** against the thymus enzyme. [b] Santi and Sakai[11] reported K_i's of 10.5 μM for 3 and 0.013 μM for **6** when tested against the bacterial enzyme.

inhibitor of all three enzymes; a K_i of 6.4 × 10^{-5} M was observed for the bacterial enzyme.

5-Allyl-dUMP (8) was found to be an exceptionally weak inhibitor. Conversion of the allyl analogue 8 to the epoxide **9** increased potency and is a chemically reactive analogue potentially capable of alkylating a nucleophilic site on the enzyme. Compound **9** is approximately four times more potent than 8 as an inhibitor of the enzyme from all three sources. Against the ascites tumor enzyme the calculated K_i was 1.5 × 10^{-5} M.

5-Azidomethyl-dUMP (**10**) is another chemically reactive analogue potentially capable of alkylation of the enzyme. Against the bacterial and thymus enzyme it is a competitive inhibitor, slightly more effective than **9**; the K_i

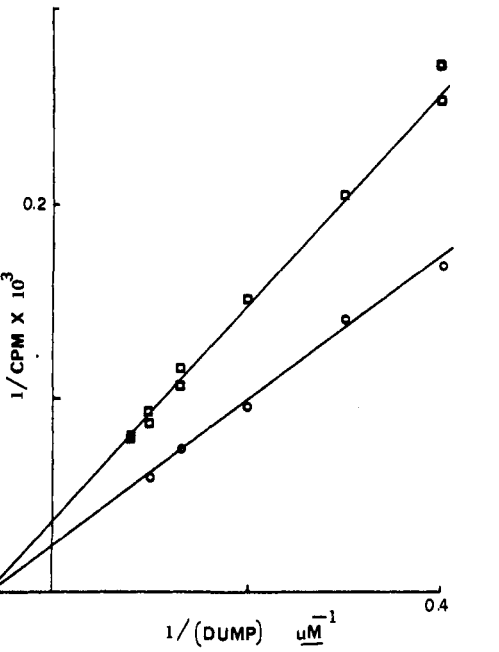


Figure 2. Reciprocal plot of inhibition of calf thymus thymidylate synthetase by 5-formyl-dUMP (**6**): no inhibitor (○); **6**, 4.6 × 10^{-8} M (□).

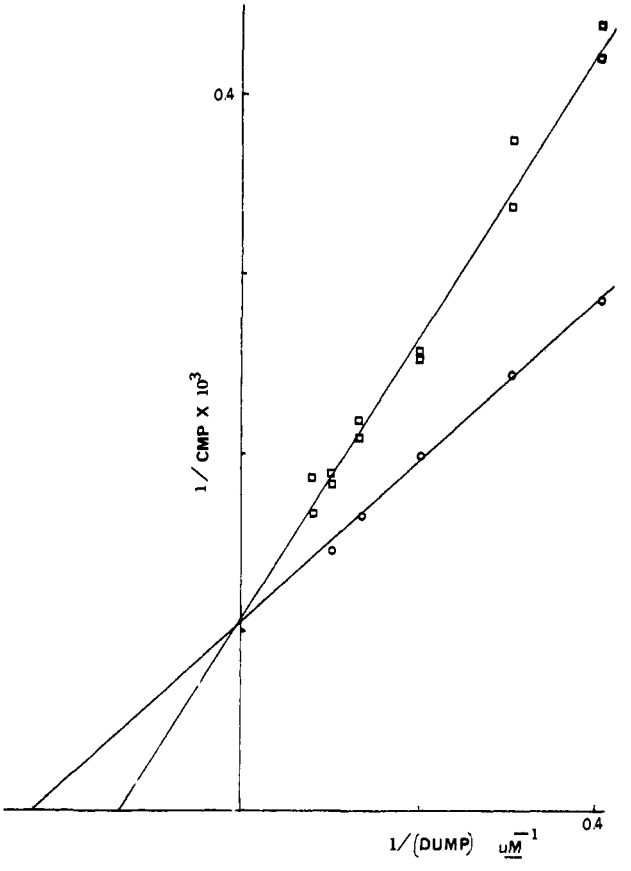


Figure 3. Reciprocal plot of inhibition of Ehrlich ascites tumor thymidylate synthetase by 5-formyl-dUMP (**6**): no inhibitor (○); **6**, 5.8 × 10^{-8} M (□).

against thymus thymidylate synthetase was 2.1 × 10^{-5} M.

Preliminary testing for irreversible inhibition was done by preincubation of the chemically reactive inhibitors **9** and **10** for 3 h in the absence of substrate. Studies were also done to compare the inactivation of the enzyme when preincubated with and without cofactor. After the preincubation the reaction mixture was dialyzed to remove

inhibitor and the activity of the enzymes was compared to controls using 1×10^{-5} M substrate concentration. Incubation for 3 h did inactivate the enzymes; the calf thymus enzyme was 40% of the activity of the control (not preincubated), whereas the ascites tumor enzyme was 15% as active as the fresh enzyme.

5-(2,3-Oxypropyl)-dUMP (**9**) incubated for 3 h with the thymus and ascites tumor enzyme, with or without added cofactor, failed to show any appreciable inactivation (Table V). The activity of the thymus enzyme did show a slight decrease; however, the ascites tumor enzyme was more active, 120% of control when **9** was incubated in the presence of cofactor.

5-Azidomethyl-dUMP (**10**) under the same preincubation conditions showed about 20% inactivation of thymus enzyme and 40% inactivation of the ascites tumor enzyme (Table V). When the preincubation with inhibitor was done in the presence of cofactor only the tumor enzyme showed any significant protection by the cofactor against inactivation. In this case only 10% inactivation was observed.

Discussion

The inhibitors examined in this study (Table I) are of three general types of substitution patterns. In the oxymethyl series, compounds **3**, **4**, and **5** were prepared to examine the inhibitory effect of a hydroxyl substituent (**3**) compared to the product of the reaction dTMP (**2**). Replacement of the hydrogen in **3** by two substituents of different size led us to prepare the methoxymethyl (**4**) and the benzyloxymethyl (**5**) analogues.

The results (Table II) show that the hydroxymethyl derivative **3** is a potent inhibitor of all three enzymes. With *E. coli* thymidylate synthetase we observed a K_i of 4×10^{-6} M compared to the reported value of 1.05×10^{-5} M.[11] Compound **3** had the same K_i (4×10^{-6} M) against the thymus enzyme; however, greatest activity was observed against the ascites tumor enzyme ($K_i = 6 \times 10^{7}$ M). The product of the reaction, dTMP, is a weak inhibitor of ascites tumor thymidylate synthetase ($K_i = 4.1 \times 10^{-4}$ M);[2] the substitution of a hydroxyl for a hydrogen substantially increases the affinity of **3** for thymidylate synthetase. Compound **4**, the methoxy derivative, cannot act as a proton donor in hydrogen bonding to the enzyme as can **3**; a tenfold reduction in affinity was observed. It should be pointed out that the difference in activity of **3** and **4** could be a steric effect caused by replacing a hydrogen with a methyl group. However, compound **5** with a benzyl group has essentially the same affinity as the methyl compound **4**. The comparable inhibitory effect of **4** and **5** also suggests that the enzyme can tolerate reasonably large substituents on the 5 position of the pyrimidine ring; this observation suggests that placement of steric bulk in this position could be a useful probe for exploring isoenzyme differences.[3]

The second group of inhibitors, the formyl **6** and the acetyl **7**, having a carbonyl α to the ring, potentially could react with a nucleophilic group at the active site and reversibly inhibit the enzyme. 5-Formyl-dUMP (**6**) is a potent inhibitor of thymidylate synthetase from the three sources. Compound **6** has been reported to be a competitive inhibitor with respect to substrate of the bacterial enzyme with a K_i of 1.3×10^{-8} M;[11] we found a K_i of 1×10^{-7} M, competitive with substrate. Similar findings with the ascites tumor indicated little differential inhibition; however, the thymus enzyme showed noncompetitive inhibition kinetics (Figure 2).

In contrast to **6**, the acetyl derivative **7** was a weak inhibitor. Although both **6** and **7**, having carbonyl groups

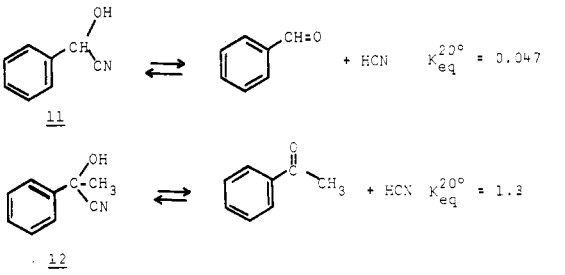


Figure 4. Dissociation constants at 20° of the cyanohydrin addition complexes of benzaldehyde (**11**) and acetophenone (**12**); data were taken from Lapworth and Manske.[14,15]

α to the ring, should have similar reactivity it is recognized that the inductive effect of the methyl group in **7** decreases the electrophilic character of the carbonyl carbon.

The fact that **6** is almost 1000 times as potent as **7** (thymus) clearly suggests a unique binding mode, perhaps an additional binding site for **6** that, due to the inductive effect of the methyl group, is not feasible with **7**. Two modes of binding to the carbonyl are possible. A nucleophile could form a reversible covalent bond by addition to the carbonyl carbon. Comparison of the inductive effect of aldehydes and ketones clearly shows that the carbonyl carbon of an aromatic aldehyde (**6**) is more electropositive than the ketone **7**. For example, both 3- and 4-hydroxybenzaldehyde are stronger acids (pK_a = 9.02 and 7.62) than the corresponding ketones, 3- and 4-hydroxyacetophenone (pK_a = 9.19 and 8.05); the difference in the substituent electronic effects is due to the increased electron attraction exerted by the aldehyde group compared to the acetyl.[13] This difference in electropositive character is a real effect that greatly influences nucleophilic additions to the carbonyl carbon. A study of the addition of cyanide to benzaldehyde and acetophenone showed that the cyanohydrin addition complex of benzaldehyde (**11**), with a dissociation constant of 0.047, was more stable than that of acetophenone cyanohydrin (**12**) (K_{eq} = 1.3, Figure 4). At equilibrium at 20° 99% of the aldehyde complex **11** is present. In contrast, only 46% of the ketone complex **12** is present at equilibrium.[14,15]

A second mode of binding is possible for **6**; the carbonyl oxygen could act as an electron donor for hydrogen bond formation. Enzyme–inhibitor binding of this type is less feasible since the carbonyl of the acetyl **7** through the methyl inductive effect should have a higher electron density and form a stronger hydrogen bond than the aldehyde group in **6**. The finding that the aldehyde **6** is more potent than the methyl ketone **7** discounts this possibility.

The third group of compounds in this study are the allyl **8**, the epoxypropyl **9**, and the azidomethyl **10** derivatives. All represent a three- or four-atom chain with enhanced electron density. The allyl analogue **8** was a very weak inhibitor, comparable to the product dTMP. Apparently the electronic character of the double bond does not contribute to binding to the active site. Compounds **9** and **10**, approximately the same size as **8**, are five times more inhibitory.

The results of this study also can be examined for differential effects that suggest mammalian isoenzyme specificity. In any considerations of this type the relative K_m's must be taken into account since these adjust for the absolute K_i differences observed and the only realistic comparison that is applicable for detecting isozyme specificity that is extendable to the in vivo case is the K_i/K_m ratio as shown in Table IV. The results show that 5-hydroxymethyl-dUMP (**3**) is three times more effective

Table IV. Isoenzyme Specific Inhibition of Calf Thymus and Ehrlich Ascites Tumor Thymidylate Synthetase

No.	Inhibitor	K_i/K_m, calf thymus	K_i/K_m, Ehrlich ascites	K_i/K_m, ascites / K_i/K_m, thymus
3	5-Hydroxymethyl-dUMP	0.44	0.15	3
4	5-Methoxymethyl-dUMP	3.5	4.7	0.7
5	5-Benzyloxymethyl-dUMP	4.6	2.2	2
6	5-Formyl-dUMP	0.01	0.02	0.5
7	5-Acetyl-dUMP	8.1	5.5	1.5
8	5-Allyl-dUMP	14	10	1.4
9	5-(2,3-Oxypropyl)-dUMP	3.6	3.5	1
10	5-Azidomethyl-dUMP	2.3	1.2	1.9

against the ascites tumor enzyme. The same pattern, to a lesser extent, is seen with the benzyloxymethyl (**5**) and the azidomethyl (**10**) derivatives. Only in the case of compound **5** would this difference be useful for detecting tertiary structural differences due to bulk tolerance effects as defined by Baker.

Compound **9**, an epoxide, is susceptible to nucleophilic attack at either position 2 or 3 of the chain. Incubation of **9** with the enzyme should give irreversible inactivation if an appropriate nucleophile (mercaptan, hydroxyl, or amine) is so positioned on the enzyme. Such nucleophiles have been implicated to add to carbon 6 of the substrate pyrimidine ring in the enzyme-catalyzed reaction.[16,17] Apparently this group is not in a position to attack the epoxide ring since preincubation of **9** with the tumor enzyme for 20 min and analysis of the reciprocal plots failed to show any irreversible inhibition.

5-Azidomethyl-dUMP (**10**) should also be susceptible to nucleophilic attack at the CH_2 group or C_6 of the uracil ring with subsequent displacement of azide anion, a good leaving group. Incubation of **10** with the thymus enzyme for 20 min showed noncompetitive inhibition which suggests that some irreversible inhibition may be occurring. Incubation of **10** for longer periods in the absence of added cofactor followed by dialysis gave 40% inactivation of the ascites enzyme again suggesting an irreversible binding of **10** to the active site (Table V). Essentially complete protection from inactivation was afforded by the cofactor tetrahydrofolate.

Experimental Section

5-[^{3}H]-dUMP was obtained from Schwartz-Mann and a stock solution at 5×10^{-5} M was prepared using dUMP at 400 μCi/μM. The cofactor *dl*-L-tetrahydrofolic acid (Sigma Chemicals) was dissolved in 1 M 2-mercaptoethanol, the pH adjusted to 7.4 with 1 M potassium hydroxide, and the solution stored in small aliquots at −20° at a concentration of 0.032 M.

Preparation of Thymidylate Synthetase. All operations were carried out at 2–4°. *E. coli* frozen cells were thawed and suspended in three volumes of buffer A consisting of 0.05 M Tris HCl, pH 7.4, 0.01 M 2-mercaptoethanol, and 0.001 M EDTA. This suspension was homogenized with a Polytron homogenizer and 0.1 μg of deoxyribonuclease per gram of packed cells was added. After 20 min the suspension was clarified by centrifugation at 17 000g for 15 min. Ammonium sulfate was added to the solution to 45%; the solution was stirred for 30 min and centrifuged at 17 000g for 15 min. Ammonium sulfate was added to the supernatant to 85%; the solution was stirred 30 min and centrifuged again. The pellet was redissolved in a minimum volume of buffer A and dialyzed overnight in the same buffer and the solution concentrated by covering the dialysis tube with sucrose for 18 h.

Table V. Irreversible Inactivation of Thymidylate Synthetase by Preincubation with 5-(2,3-Oxypropyl)-dUMP (9) and 5-Azidomethyl-dUMP (10)[a]

Compd	Additions	% control reaction: Calf thymus	% control reaction: Ehrlich ascites
9	None	104	94
9	Tetrahydrofolate	85	120
10	None	77	60
10	Tetrahydrofolate	83	87

[a] Tetrahydrofolate concentrations during preincubation were 0.00027 M. Assay of the enzyme after 3 h of preincubation was done as described in the Experimental Section using saturating substrate concentrations: dUMP, 1×10^{-5} M. Inhibitor concentrations during preincubation were 3.3×10^{-4} M.

Aliquots were stored at −70°. The K_m of this preparation for dUMP was 2.5×10^{-5} M.

Enzyme was extracted from fresh calf thymus by homogenizing minced thymus in an equal volume of buffer A containing 0.1 M sodium chloride. After centrifugation at 28 000g for 30 min the supernatant was passed through cheesecloth to remove fat droplets; protamine sulfate was added to give a 0.35% solution. After stirring for 20 min the solution was centrifuged at 28 000g for 30 min and the supernatnat saturated to 45% with ammonium sulfate. After 30 min the suspension was centrifuged again to give the supernatant which was made 85% in ammonium sulfate. The pellet was dissolved in the minimum volume of buffer A containing 0.1 M sodium chloride and 5% sucrose, dialyzed against the same buffer, concentrated in sucrose, and stored in small aliquots at −70°. The calf thymus enzyme had a K_m value of 9×10^{-6} M for dUMP.

Thymidylate synthetase from Ehrlich ascites tumor cells was purified from cells harvested 8 days after inoculation of male BDF-1 mice. Cells stored at −70° were thawed and packed by centrifugation at 7000g. The pellet was suspended in an equal volume of buffer containing 0.005 M potassium phosphate at pH 7.1 and 0.005 M 2-mercaptoethanol. The cells were disrupted with a Polytron homogenizer and the solution was centrifuged at 105 000g for 1 h. The supernatant was stored at −70°. This preparation had a K_m of 4×10^{-6} M for the substrate.

Enzyme Assay. The assay reported by Roberts[18] was modified. The reaction mixture contained 0.02 M formaldehyde, 0.02 M magnesium chloride, 0.02 M Tris HCl, pH 7.4, 0.05 M 2-mercaptoethanol, 0.0005 M tetrahydrofolic acid, 5-[^{3}H]-dUMP ($2.5–10 \times 10^{-6}$ M, 400 μCi/μM) enzyme, and inhibitor when required. Controls lacked the cofactor, tetrahydrofolic acid. Assays were run in a total volume of 0.1 ml at 37° for 7 min using the ascites tumor and *E. coli* enzyme and for 10 min using the thymus enzyme. The reaction was stopped by the addition of 50 μl of 20% trichloroacetic acid and the unreacted substrate was removed by adding 0.25 ml of activated charcoal in a 20% aqueous suspension. After standing 15 min the mixture was filtered through a glass wool plugged Pasteur pipet and 0.1 ml of the filtrate was counted in a scintillation fluid containing 0.5% 2,-5-diphenyloxazole and 10% Beckman BBS-3 solubilizer in toluene.

Preincubation studies were carried out using the ascites and thymus derived enzyme. The reaction mixture for preincubation contained 0.005 M formaldehyde, 0.05 M 2-mercaptoethanol, 0.13 M potassium phosphate buffer, pH 6.7, 0.00027 M tetrahydrofolic acid (when needed), enzyme, and inhibitor. Tetrahydrofolic acid was not added to the control assays or to those mixtures preincubated in the absence of cofactor. After preincubation an equal volume of water containing substrate, 5-[^{3}H]-dUMP, and, when needed, tetrahydrofolic acid was added to initiate the reaction.

Measurements of irreversible inhibition of the enzyme by chemically reactive inhibitors were done by preincubation with the inhibitor followed by dialysis of the ascites enzyme mixture against two changes of 10 000 vol of the same buffer (excluding tetrahydrofolic acid and inhibitor) during a 24-h period. Dialysis of the thymus enzyme used the same buffer and, in addition, contained 0.033 M sodium chloride and 0.0003 M EDTA. After

dialysis the assays were run by adding an equal volume of solution containing 0.0033 M formaldehyde, 0.033 M 2-mercaptoethanol, 0.1 M potassium phosphate buffer, pH 6.1, the required amount of substrate, and 0.0002 M tetrahydrofolic acid.

Synthesis of 5-Formyl-2′-deoxyuridine 5′-Phosphate (6). Thymidine kinase was isolated from *E. coli* B.[12] The enzyme from 30 g of packed cells was added to a 15-ml solution containing 0.07 M Tris HCl, pH 7.8, 0.0075 M magnesium chloride, 0.0075 M ATP, 4.5 mg of bovine serum albumin (D. V. Santi, personal communication), and 15 μmol of 5-formyl-2′-deoxyuridine. After incubation for 3 h at 37°, the mixture was heated to 95° for 5 min, cooled, and centrifuged at 6500g for 10 min. The resulting supernatant was brought to pH 4.4 with formic acid and a portion (one-third) of the mixture diluted with 2 vol of water and applied to a 2.6 × 35 cm column of DEAE cellulose (Whatman DE81) equilibrated in 0.005 M ammonium formate, pH 4.4.[20] After washing with 100 ml of the same buffer a linear gradient was applied using 125 ml of 0.005 M and 125 ml of 0.5 M ammonium formate, pH 4.4. The product, **6**, was formed in 65% yield. The uv in ammonium formate buffered at pH 4.4 had a λ_{max} at 279 nm; the reported λ_{max} (0.1 N HCl) for the nucleoside is 281 nm (ϵ 13 200).[7]

Chemical Phosphorylation of Nucleosides. The procedure of Yoshikawa et al.[19] for the selective phosphorylation of nucleosides was used. The nucleoside (0.5 mmol) was dissolved in 1.45 ml of a trimethyl phosphate solution containing 0.14 ml (0.5 mmol) of phosphorus oxychloride and 0.01 ml of water at 2° and the mixture stirred at that temperature for 4 h. Diethyl ether (50 ml) was added followed by water (1–2 ml); after shaking the mixture to extract organic materials the ether was decanted and the aqueous phase applied to two sheets of Whatman 3MM paper. Purification of nucleotides **4**, **5**, **8**, and **10** was done using 2-propanol–ammonia–water (7:1:2) as the solvent. The acetyl derivative **7** was purified on a DEAE cellulose column using a linear gradient of 0.005–0.5 M ammonium formate, pH 4.4.[20] Yields in the synthesis of **4**, **5**, **7**, **8**, and **10** ranged from 45 to 60%.

Compounds **4**, **5**, **7**, **8**, and **10** were confirmed as the 5′-phosphates by hydrolysis by 5′-ribonucleotide phosphohydrolase (E.C. 3.1.3.5). The nucleotide (1 mg) was incubated with 2.5 units of the enzyme in 1 ml of a 0.1 M glycine buffer and 0.01 M magnesium chloride solution at a pH of 9 for 30 min at 37°. The reaction mixture was resolved by electrophoresis in 0.05 sodium phosphate buffer at a pH of 8.5. Uridine 2′(3′)-phosphate was not hydrolyzed by this enzyme whereas dTMP was converted completely to thymidine. Compounds **4**, **5**, **7**, **8**, and **10** were completely hydrolyzed by this enzyme.

Synthesis of 5-Hydroxymethyl-2′-deoxyuridine 5′-Phosphate (3). 5-Benzyloxymethyl-2′-deoxyuridine 5′-phosphate diammonium salt (**4**, 100 mg, 0.22 mmol) was dissolved 2 ml of water and this solution added to a suspension of 100 mg of prereduced 5% palladium-on-charcoal catalyst in 25 ml of water. Hydrogenolysis at 25° and atmospheric pressure was done for 90 min, the suspension filtered, and the filtrate lyophilized. The product was purified on Whatman 3MM paper using 2-propanol–ammonia–water (7:1:2) to give a quantitative yield of **3**. Analysis for starting material (**4**), product (**3**), or dTMP (**2**), a side product, can be done on TLC using silica developed in 1-butanol–acetic acid–water (5:2:3).

Synthesis of 5-(2,3-Oxypropyl)-2′-deoxyuridine 5′-Phosphate (9). 5-Allyl-2′-deoxyuridine 5′-phosphate diammonium salt (**8**, 525 mg, 1.38 mmol) in absolute methanol (30 ml) was treated with a solution of *m*-chloroperbenzoic acid (2 g, 11.6 mmol) in absolute methanol (20 ml) at room temperature. The mixture was stirred at the same temperature for 17 h. TLC at this stage reveals only trace amounts of starting material (silica plate, developed with 1-butanol–acetic acid–water, 5:2:3). The solution was evaporated to dryness (bath temperature was kept at 25°) and the white solid residue was partitioned between water and ether (50 ml each). The aqueous layer was washed four times with ether and lyophilized to yield a colorless syrup (520 mg). Chromatographic analysis showed the optimum separation of starting material (R_f ~0.55) and product (R_f ~0.45) using isobutyric acid–ammonium–water (66:1:30). The product was purified in 50% yield on Whatman 3 chroma paper.

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References and Notes

(1) (a) Abbreviations used are dTMP, 2′-deoxythymidine 5′-phosphate; dUMP, 2′-deoxyuridine 5′-phosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; EDTA, ethylenediaminetetracetic acid; ATP, adenosine 5′-triphosphate. (b) M. Friendkin, *Adv. Enzymol.*, **38**, 235 (1973).
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