hydrophobic interactions taking place. This conclusion does not appear applicable to CoA–ChA interactions. Elution of ChA from Blue Dextran columns can be accomplished readily with 0.5 M KCl, while 60% ethylene glycol is required for elution to take place in the absence of salt. This observation, coupled with the fact that the 3′-phospho group of CoA and acetyl-CoA is essential for tight binding to ChA,[12,13] suggests the importance of coulombic interactions. We had shown previously that ChA binds increasingly tightly to alkylagarose columns as the length of the alkyl group is extended.[13] These findings have been cited as supporting the importance of hydrophobic interactions in ChA binding. The CoA-binding and dye-binding studies suggest that coulombic interactions are at least as important as hydrophobic interactions when CoA or aromatic dyes are attached to this enzyme.

A recent X-ray diffraction study of the binding of the para isomer of Reactive Blue 2 to horse liver alcohol dehydrogenase[4] showed an interaction of a sulfonate group in the para position of ring A with an arginine guanidino group, while anthraquinone was attached to the adenine binding site.[4] We are assuming that, in the case of ChA, the anthraquinone portion of the dye also interacts with the adenosine binding site, while the sulfonate group interacts with an active-site arginine residue. We have already shown that "specific" arginine reagents, such as phenylglyoxal,[24] inhibit ChA, with CoA being more effective than 3′-dephospho-CoA in protecting against inhibition.[25a,b]

The present studies suggest that aromatic dyes should be useful as probes of the CoA binding site of ChA. Since tetraiodofluorescein is highly fluorescent while Reactive Blue 2 and Congo Red show induced CD spectra when introduced into the asymmetric environment of enzyme binding sites,[26,27] these dyes should be useful not only for studying the topography of the CoA binding site but also for studying conformational changes induced by choline, the other substrate of the enzyme.

Experimental Section

Aromatic Dyes. Generous samples of Reactive Blue 2 (isomer mixture), as well as of the meta and para isomers, were obtained from Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland. Purification was carried out by the procedure of Beissner and Rudolph.[19] The hydroxy derivative of Cibacron Blue was prepared from the chlorotriazine by the procedure of Moe and Piszkiewics.[18] Completion of the reaction was ascertained by showing that the C–Cl stretching band in the infrared spectrum had disappeared.[18] Thin-layer chromatography was used to establish lack of contaminants in the purified compounds. 1,8-ANS and its 1,2, 1,5, 2,6, and 2,8 isomers were purchased from Molecular Probes, Roseville, MN, as was the dimer of 1,8-ANS 4,4′-bis[1-(phenylamino)naphthalene 8-sulfonate]. All compounds were recrystallized. Procion Red HE3B (Reactive Red 120) was a gift from the Amicon Corp., Lexington, MA. The cyclic analogue of 1,8-ANS was synthesized by the procedure of Cory et al.[21] The content of Reactive Blue 2 in Blue Dextran (Pharmacia) was determined by the procedure of Easterday and Easterday.[28]

Choline Acetyltransferase (ChA). ChA derived from squid head ganglia was partially purified by the procedure of Husain and Mautner[29] using the following modifications: The 40–30% $(NH_4)_2SO_4$ extraction step and the chromatography on mercurial Sepharose were omitted. Chromatography on phosphocellulose resin was followed by chromatography on hydroxylapatite, yielding activity ranging from 3 to 7 $\mu M\ min^{-1}$ (mg of protein)$^{-1}$. The enzyme was stabilized by the addition of ethylene glycol to a concentration of 10% (v/v) to all buffers. Recovery was ~40% of starting enzyme activity. The enzyme assay procedure of Fonnum[30] was used; inhibition assays, in the presence of 10 mM choline, were carried out as described previously,[12] except that lysozyme, previously used by us to stabilize ChA, was omitted because of its ability to bind aromatic dyes. [1-^{14}C]Acetyl-CoA (or [2-^{3}H]acetyl-CoA) for enzyme assays was purchased from New England Nuclear Corp. Inhibition data were analyzed by the procedure of Cleland,[31] utilizing the computer programs described to obtain K_i and standard error values. A DEC System 10 computer was used.

Acknowledgment. We are indebted to the National Science Foundation for a grant (BNS-80-40348) for support of this work and to Andrew A. Pakula for his assistance in the determination of kinetic parameters.

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Interaction of 5-Ethynyl-2′-deoxyuridylate with Thymidylate Synthetase

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The interaction of 5-ethynyl-2′-deoxyuridylate (5-ethynyl-dUMP; 1) with thymidylate (dTMP) synthetase has been investigated. The compound was an inhibitor of the enzyme, competitive with 2′-deoxyuridylate (dUMP) when the reaction was initiated by addition of enzyme ($K_i = 2.7 \times 10^{-6}$ M). However, upon preincubation of 1 with dTMP synthetase, the inhibition pattern became noncompetitive. The time course of the enzyme reaction in the presence of 1 was nonlinear, indicating an increase in binding with time. Irreversible inactivation of the enzyme did not occur. The compound did not appear to become altered structurally as a result of interaction with the enzyme. A ternary complex was formed among dTMP synthetase, compound 1, and 5,10-methylenetetrahydrofolate, which was stable enough to survive Sephadex G-25 filtration but dissociated upon denaturation of the enzyme.

A number of compounds containing an acetylenic moiety have been shown to be mechanism-based inhibitors of various enzymes.[1] For example, the mechanism postulated for the irreversible inactivation of lactate dehydrogenase

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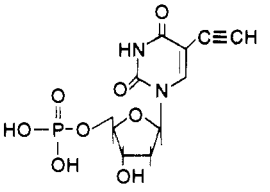
by 2-hydroxy-3-butynoic acid involves the initial abstraction of a β proton to give rise to an anion at the allylic position.[2] This species then is thought to rearrange to a highly reactive allene group, which then reacts with the enzyme. It was first suggested by Rando[1] that 5-ethynyluracil (5-ethynyl-Ura) might be a potential mechanism-based inhibitor of thymidylate (dTMP) synthetase. Subsequently, it was shown that the nucleoside, 5-ethynyl-2′-deoxyuridine (5-ethynyl-dUrd), inhibits dTMP synthetase in intact cells.[3] The mechanism of dTMP synthetase, as it is currently accepted, provides a pathway for the conversion of 5-ethynyl-dUMP (1) to a reactive



1

allenic compound. Experiments with model compounds[4] and studies of the structure of the covalent complex formed with dTMP synthetase, 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate), and the tight-binding inhibitor, 5-fluoro-2′-deoxyuridylate (FdUMP),[5] have established that the first step of the mechanism of catalysis involves nucleophilic attack of a thiolate anion at the C-6 position of the pyrimidine ring, resulting in the formation of a covalent bond from sulfur to the C-6 position, and an anion at the C-5 position. Subsequent protonation of this species would complete the saturation of the 5,6 double bond and form a 5,6-dihydropyrimidine intermediate similar to the product of bisulfite addition to pyrimidines.[6] This reaction is thought to facilitate the subsequent methylene-transfer step by providing activation of the C-5 position toward electrophilic attack.[4] The above mechanism operating on 1 would generate an anion adjacent to the acetylenic group, thereby providing the possibility for rearrangement to the 5-allene. An irreversible inactivation of the enzyme would then depend on the presence of a proximal nucleophilic group in the active site. dTMP synthetase, in fact, has been postulated to contain an active-site nucleophile which functions in the role of a general acid–base catalyst.[7] In this regard, an enzyme-generated affinity label would aid in identifying such a putative catalytic residue. Accordingly, we prepared 1 and investigated its interaction with dTMP synthetase from *Lactobacillus casei*.

Chemistry. 5-Ethynyl-Ura was converted to 5-ethynyl-dUrd essentially by the produre reported by Barr et al.[8] The 5′-hydroxyl group of the nucleoside was selectively phosphorylated using the procedure of Sowa et al.[9] to give 1, which was purified on a DEAE-cellulose column using a LiCl gradient.

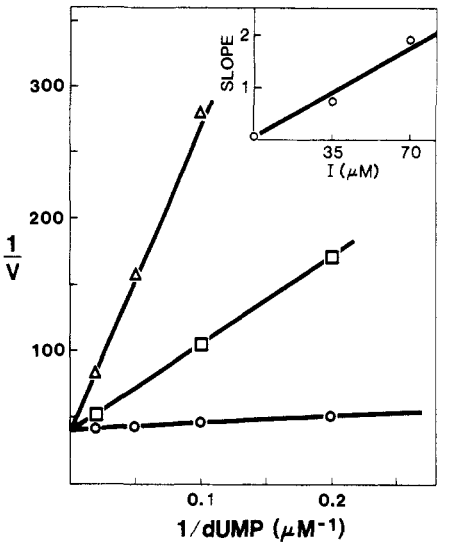


Figure 1. Double-reciprocal plot of the inhibition of dTMP synthetase in the presence of 5-ethynyl-dUMP (1), with dUMP as the variable substrate: (○) no inhibitor; (□) 35 μM inhibitor; (△) 70 μM inhibitor. The reactions contained 0.1 mM 5,10-CH_2-H_4folate, variable concentrations of dUMP, and 10 mM β-mercaptoethanol in 0.1 M Tris, pH 7.5.

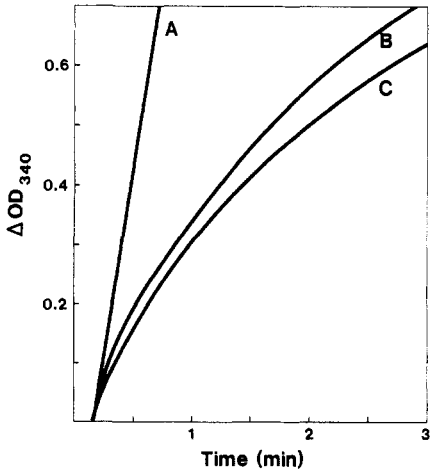


Figure 2. The time course of the dTMP synthetase reaction in the absence of the inhibitor (curve A) and in the presence of 35 μM 5-ethynyl-dUMP (curve B) and 70 μM 5-ethynyl-dUMP (1) (curve C). Conditions were as described under Figure 1, except that dUMP was held constant at 50 μM.

Results and Discussion

Interaction of 5-Ethynyl-dUMP (1) with dTMP Synthetase. A consideration of the currently accepted mechanism of dTMP synthetase suggested the possibility that the pyrimidine ring of 5-ethynyl-dUMP (1) might interact with the sulfhydryl group in the active site of the enzyme so as to generate an allenic moiety at the C-5 position. This reactive species would then be available to react with an enzymatic nucleophile and thus produce an irreversible covalent bond. We could not test this hypothesis directly because of the lack of a radiolabeled analogue of 1, but instead we measured time-dependent effects of the inhibitor on enzyme activity. Compound 1 was competitive with dUMP when the enzyme reaction was initiated by addition of enzyme (Figure 1) with a K_i

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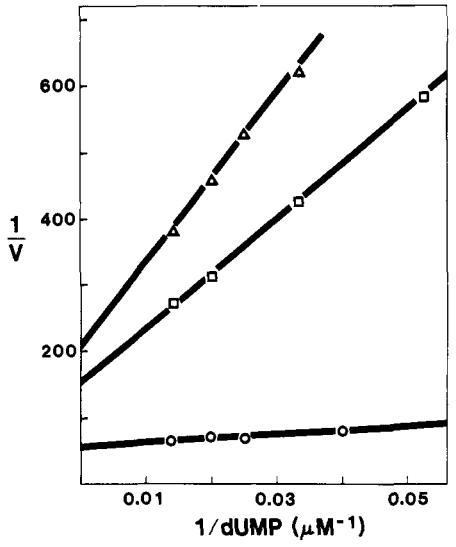

Figure 3. Double-reciprocal plot of the inhibition of dTMP synthetase activity when 5-ethynyl-dUMP (1) was preincubated with the enzyme for 15 min before adding dUMP: (○) no inhibitor; (□) 35 μM inhibitor; (△) 70 μM inhibitor. Other components were as described in the legend to Figure 1.

of 2.7×10^{-6} M calculated from the resulting Lineweaver–Burk plot. It was apparent, however, from the nonlinear time course of these reactions (Figure 2) that the inhibition did not remain at the initial value but rather increased with time. This type of behavior has been described by Cha[11] as being characteristic of "tight-binding" (stoichiometric) inhibitors. The apparent "tightening up" of the inhibition was confirmed by preincubating the enzyme with compound 1 and initiating the reaction by addition of the substrate 2′-deoxyuridylate (dUMP). This procedure resulted in a noncompetitive type of pattern (Figure 3) with a nonlinear slope replot (not shown). A 30-min incubation of 1 at a concentration of 0.1 mM with dTMP synthetase did not, however, lead to irreversible inactivation, because we were able to recover full enzyme activity after dialysis in the presence of dUMP. Therefore, if the 5-allenic species is indeed formed in the active site, covalent reaction with the enzyme occurs very slowly, if at all.

There are several possible mechanisms that could account for the tightening-up behavior that is observed with 1. The compound may be a quasi-substrate or "mechanism-based" inhibitor which is converted by the enzyme to a more inhibitory or reactive form, as suggested previously.[1] In order to test this possibility, we incubated 1 with dTMP synthetase for periods of time up to 24 h but observed no time-dependent changes in the UV spectrum of the nucleotide, strongly suggesting that the pyrimidine moiety had not undergone any modification by the enzyme. Alternately, the inhibitor may induce a conformational change in the enzyme that leads to a tightly bound binary or ternary complex, without itself undergoing any structural alterations (e.g., as in the inhibition of dihydrofolate reductase by methotrexate),[12] or the compound may be modified while on the enzyme surface but released unchanged when the bonds linking it to the enzyme are broken. The formation of 5,6-dihydropyrimidine derivatives via sulfhydryl addition to the 5,6 double bond is thought to be the basis for the tight-binding inhibition of dTMP synthetase by a number of pyrimdine nucleotides,

Table I. Binding of [^{14}C]5,10-CH_2-H_4folate to dTMP Synthetase in the Presence of 5-Ethynyl-dUMP (1) as Measured by Sephadex Filtration

reaction mixture[a] contained	dpm bound to protein
no nucleotide	75
5-ethynyl-dUMP (1)	1 106
FdUMP	15 500

[a] The reaction contained 0.1 mM [^{14}C]CH_2-H_4-folate, 10 μM nucleotide, 2.5 nmol of dTMP synthetase from *L. casei* in 1.0 mL of 0.1 M Tris buffer, pH 7.5, containing 10 mM β-mercaptoethanol.

including 5-(trifluoromethyl)-2′-deoxyuridylate (CF_3dUMP)[13] and 5-nitro-2′-deoxyuridylate (NO_2dUMP).[14] Such adducts are generally freely reversible, so the nucleotides are found to be unchanged when released from the complex.[13,14] The presence of 5,10-CH_2-H_4folate is not required for the tight binding of CF_3dUMP and NO_2dUMP.[13,14] In contrast, the very potent inhibitor FdUMP undergoes further alteration by the enzyme: a methylene bridge is formed from H_4folate to the C-5 position of FdUMP, resulting in the covalent binding of both ligands to the enzyme.[4] This complex is stable when the enzyme is denatured, and dissociates only via enzymatic cleavage of the covalent bonds. It was of interest, therefore, to determine whether 1 was capable of forming a ternary covalent complex with enzyme and 5,10-CH_2-H_4folate in a manner analogous to FdUMP. The compound was incubated with dTMP synthetase and [^{14}C]-5,10-CH_2-H_4folate, and the mixture subjected to Sephadex filtration. Table I shows that a ternary complex is indeed isolated, but with only about 7% of the amount of cofactor that is bound when the stoichiometric inhibitor FdUMP is used. This very appreciable dissociation during chromatography indicates that a substantially weaker ternary complex is formed with 1 than with FdUMP. We estimate the K_d value for this complex to be on the order of 10^{-9} M,[15] which represents about a 1000-fold increase in binding compared to the complex existing, initially, before the tightening-up reaction occurs. Treatment of the complex isolated from the Sephadex column with trichloroacetic acid did not result in any precipitated radioactivity, demonstrating that stable covalent bonds to the cofactor are not formed as is the case with the FdUMP–5,10-CH_2-H_4folate–dTMP synthetase complex.

To obtain an indication of whether 1 is modified while in the active site by nucleophilic saturation of the 5,6 double bond would require detailed spectral studies, which in any case are often difficult to interpret. However, in analogy to other 5-substitued dUMP analogues,[16] it can probably be assumed that this reaction does occur to some extent and may be responsible for the increase in binding that we observe. If so, the C-5 anion initially generated by the nucleophilic attack at C-6 must be assumed to be too rapidly protonated to allow the ethynyl to allenyl rearrangement that is observed with other acetylenic

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(15) This K_d value is an estimate based on a similar degree of dissoication upon Sephadex filtration of a dTMP synthetase–dUMP–pteroyltriglutamate ternary complex for which the K_d was determined by Scatchard analysis to be 5×10^{-9} M. See A. Lockshin and P. V. Danenberg, *J. Biol. Chem.*, **254**, 12 285 (1979).
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mechanism-based inhibitors.

Experimental Section

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. The UV spectra were recorded on a Beckman Model 25 spectrophotometer. All chemicals were reagent grade.

2′-Deoxy-5-ethynyluridine. This compound was synthesized according to the procedure outlined by Barr et al.,[8] except that $SnCl_4$ was used for the condensation of silylated 5-ethynyl-Ura with 1-chloro-2-deoxy-3,5-di-*o*-*p*-toluoyl-α-D-*erythro*-pentafuranose. Deblocking of the intermediate and separation of the α and β anomers as described[8] gave 5-ethynyl-dUrd in 50% yield, mp 196–197 °C (lit.[8] mp 195–197 °C). The anomeric purity of the β isomer was established by NMR.

2′-Deoxy-5-ethynyluridylate (1). To a mixture of freshly distilled phosphoryl chloride (0.041 mL, 0.44 mmol), water (0.005 mL, 0.28 mmol), and pyridine (0.039 mL, 0.48 mmol) in acetonitrile (2 mL) was added 5-ethynyl-dUrd (25.2 mg, 0.1 mmol). After stirring for 4 h at 0 °C, the reaction mixture was poured into ice–water (2 mL), which contained pyridine (1 mL). The reaction mixture was placed on a DEAE-cellulose column (2 × 30 cm), which was washed with water and then eluted with a 0–0.5 M LiCl gradient with 500 mL in each reservoir. The single UV-absorbing peak was collected, and the solvent was evaporated. The white residue was taken up in MeOH, and then acetone was added to precipitate the lithium salt of 1. The compound was homogeneous on two cellulose TLC systems: *i*-PrOH–NH_4OH–H_2O, 7:1:2 (R_f 0.2); *n*-BuOH–AcOH–H_2O, 5:2:3 (R_f 0.6). The UV spectrum was identical with that of the nucleoside [λ_{max} (H_2O) 286 nm]. NMR (D_2O) δ 3.9 (s, C≡CH). Anal. Calcd base/phosphorus ratio, 1:1; found, 1:1.05.

dTMP Synthetase Assay. The activity of dTMP synthetase purified to homogeneity from *L. casei* was assayed according to the procedure of Wahba and Friedkin.[10]

Incubation of 1 with dTMP Synthetase. Compound 1 (0.1 mM) and dTMP synthetase (2.5 nM) were incubated at 32 °C in 0.1 M Tris buffer, pH 7.5, containing 10 mM β-mercaptoethanol. The UV spectrum was scanned at intervals.

Acknowledgment. This work was supported by American Cancer Society Grant CH1-D, the Lillian L. Lyman Memorial Grant, and NCI Contract NO1-CM-67084. P.V.D. is the recipient of American Cancer Society Faculty Research Award FRA-197.

Additions and Corrections

1980, Volume 23

Daniel F. Heiman, Stephen G. Senderoff, John A. Katzenellenbogen,* and Richard J. Neeley: Estrogen Receptor Based Imaging Agents. 1. Synthesis and Receptor Binding Affinity of Some Aromatic and D-Ring Halogenated Estrogens.

Page 1001. The nuclear magnetic resonance spectra for **13a** and **13b** should read as follows. 16α-Bromoestradiol-17β (**13a**): ^1H NMR (Me_2SO-d_6) δ 0.67 (s, 3, 18-methyl), 3.79 (t, 1, J = 5 Hz, 17α-H), 4.17 (m, 1, 16β-H), 5.36 (d, 1, J = 5 Hz, 17β-OH), 6.43, 6.52, 7.04 (3), 9.01 (s, 1, 3-OH). 16α-Bromoestradiol-17α (**13b**): ^1H NMR (Me_2SO-d_6) δ 0.72 (s, 3, 18-methyl), 3.50 (t, 1, J = 5 Hz, 17β-H), 4.72 (m, 1, 16β-H), 5.13 (d, 1, J = 6 Hz, 17α-OH), 6.43, 6.52, 7.03 (3), 9.05 (s, 1, 3-OH).

M. H. Fleysher,* R. J. Bernacki, and G. A. Bullard: Some Short-Chain N^6-Substituted Adenosine Analogues with Antitumor Properties.

Page 1449. The head for columns 3–10 of Table I and for columns 2 and 3 of Table II should read molar concentration for 50% growth inhibition (ID_{50}), M × 10^{-6}.

Page 1450. In Table IV, the mean life span of mice treated with N^6-propargyladenosine (compound 3) should read 55.0 days.

1981, Volume 24

Peter Boehm, Kelvin Cooper, Alan T. Hudson, Jane P. Elphick, and Nicholas McHardy: In Vitro Activity of 2-Alkyl-3-hydroxy-1,4-naphthoquinones against *Theileria parva*.

Page 296. In line 23, 2-chloro-3-(3-cyclopropyl)-1,4-naphthoquinone (6) should read 2-chloro-3-(3-cyclohexylpropyl)-1,4-naphthoquinone.