evaporation from absolute ethanol afforded 1 g of viscous product, 1-(2,3,5-tri-O-acetyl-β-D-arabinofuranosyl)-5-hydroxyuracil, which was used directly in the next step.

The acetylated 5-hydroxyuracil derivative (1 g) was dissolved in CH_3OH, and the solution was saturated at 0 °C with NH_3 gas. After overnight reaction, the solution was evaporated to dryness and acetamide was sublimed off at 60 °C (0.1 mmHg). Evaporation of the residue from absolute EtOH produced crystals of **6**. Recrystallization from EtOH–H_2O (1:1) yielded 517 mg (50%) of **6**: mp 252–254 °C.

1-(β-D-Arabinofuranosyl)-5-(propynyloxy)uracil (5). 1-(β-D-Arabinofuranosyl)-5-hydroxyuracil (**6**, 650 mg, 2.5 mmol) was dissolved in methanol–water (1:1) containing KOH (2.5 mmol). Propargyl bromide (439 mg, 3.7 mmol) was added to this stirred solution. After overnight reaction at room temperature, the solvent was evaporated in vacuo. Absolute ethanol was added to the viscous residue, and the resulting solution was stored at 4 °C for several days. The colorless crystals (432 mg, 54%) that formed were collected by filtration: mp 163–166 °C; UV λ_{max} (H_2O) 277 nm (ε 8940); CIMS (NH_3) *m/e* 299 (P + 1, 100%); ^1H NMR (Me_2SO-d_6) 11.46 (br s, 1, N_3-H), 7.52 (s, 1, H-6), 5.97 (d, *J* = 4-Hz, 1, H-1′), 5.54 (d, *J* = 5 Hz, 1, 2′- or 3′-OH), 5.40 (d, *J* = 4 Hz, 1′-, 2′- or 3′-OH), 5.07 (t, *J* = 4 Hz, 1, 5′-OH), 4.56 (d, *J* = 2.5 Hz, 2, propynyl CH_2), 3.98 (m, 2, H-2′ and H-3′), 3.64 (m, 3, H-4′ and H-5′ H's), 3.51 (t, *J* = 2.5 Hz, 1, acetylene H). Anal. C, H, N.

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Enzyme Affinity of the 5,6-Dihydro Derivatives of the Substrate and Product of Thymidylate Synthetase Catalysis

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The 5,6-dihydro derivatives of 2′-deoxyuridine 5′-phosphate (**2**) and 2′-deoxythymidine 5′-phosphate (**3**) were synthesized and characterized. The affinities of **2** and **3** were compared to those of the substrate (2′-deoxyuridine 5′-phosphate) and product (2′-deoxythymidine 5′-phosphate) of the reaction catalyzed by thymidylate synthetase. In both cases, the enzyme affinity of the 5,6-dihydro derivatives was 50 times less than that of the substrate or product. The conclusions from this study are that a noncovalent complex of enzyme and a dihydro substrate or dihydro product is improbable in thymidylate synthetase catalysis and the covalent enzyme–substrate complex is more reasonable.

Thymidylate synthetase catalyzes the conversion of 2′-deoxyuridine 5′-phosphate (dUMP) to 2′-deoxythymidine 5′-phosphate (dTMP).[1] The cofactor, tetrahydrofolic acid, partakes in the reaction initially as the carbon-transferring agent by the activation of formaldehyde or the equivalent biological source of formaldehyde. Subsequent cofactor involvement is through alkylation of the 5-carbon of the substrate, followed by reduction of the intermediate complex and release of the oxidized cofactor 7,8-dihydrofolate.

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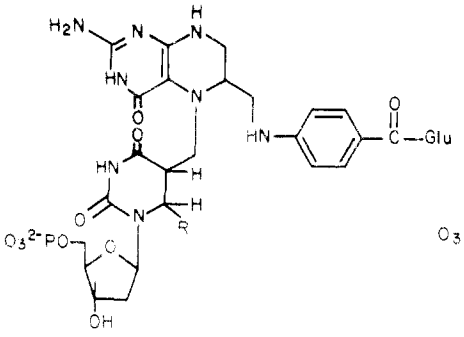
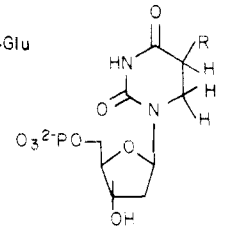
Recent studies by several research groups have elucidated details of this conversion. Santi and McHenry[2] found that 5-fluoro-2′-deoxyuridine 5′-phosphate, in the presence of cofactor, formed a covalent complex with the enzyme from a resistant strain of *Lactobacillus casei*. Similar studies by Langenbach and co-workers,[3] using the Ehrlich ascites tumor enzyme, confirmed these findings. After the initial binding of substrate and cofactor to the enzyme, it would appear that the first reaction in the catalysis is the formation of the intermediate 1a as the



1a, R = enzyme
b, R = nucleophile



2, R = H
3, R = CH_3

product of the reaction of the substrate, the activated cofactor, and the enzyme.[4]

Substrate and product analogues that are strongly bound to the enzyme[4] are 5-halo, 5-trifluoromethyl, 5-mercapto, 5-formyl and, more recently, the 5-nitro derivative[5] of 2′-deoxyuridine 5′-phosphate. This report examines the effect of reduction of the 5,6 double bond on the affinity of the substrate (dUMP) and product (dTMP) for the enzyme.

Reduction of the 5,6 double bond in pyrimidine nucleotides has been reported by Cohen and Doherty[6] using rhodium on alumina. Using their procedure 2′-deoxyuridine 5′-phosphate was reduced in aqueous acid, and the product, 5,6-dihydro-2′-deoxyuridine 5′-phosphate (2), was isolated in 55% yield by gradient elution on a DEAE-cellulose column. The same procedure applied to 2′-deoxythymidine 5′-phosphate gave an identical yield of 5,6-dihydro-2′-deoxythymidine 5′-phosphate (3). The reduced derivatives showed a 208-nm ultraviolet absorption peak in water, as reported in the studies of Janion and Shugar;[7] the characteristic change in the NMR was the loss of the aromatic resonance signals in both 2 and 3.

These dihydro derivatives were examined for their affinity to thymidylate synthetase purified from *Lactobacillus casei*. Figure 1 is a double-reciprocal plot of the velocity of the enzymatic reaction vs. substrate (dUMP) concentration. This assay method, a measure of ^3H_2O released from 2′-deoxy[5-^{3}H]uridine 5′-phosphate, gave a K_m for substrate affinity of 1.7×10^{-6} M, which compares favorably with the reported K_m range of 0.7 to 5.2×10^{-6} M using the spectrophotometric assay.[8-10] 5,6-Dihydro-2′-deoxyuridine 5′-phosphate (2) was a weak competitive (substrate) inhibitor with a K_i of 1.0×10^{-4} M. The product of the enzymatic conversion (dTMP) was found to be a competitive substrate inhibitor with a K_i of 2.8×10^{-6} M; Daron and Aull[10] noted a value of 2.37×10^{-6} M. The dihydro product analogue, 5,6-dihydro-2′-deoxythymidine 5′-phosphate (3), as seen in Figure 1 is also a weak competitive (substrate) inhibitor with a calculated K_i of 1.5×10^{-4} M. Comparing the affinities of the substrate and product to their respective 5,6-dihydro

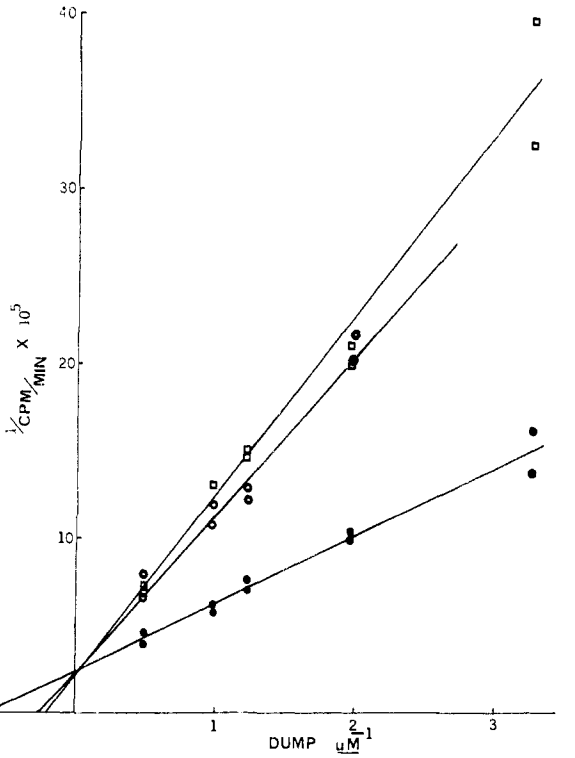


Figure 1. Double-reciprocal plot of the velocity of formation of ^3H_2O vs. concentration of substrate, 2′-deoxyuridine 5′-phosphate (DUMP), in the absence and presence of 5,6-dihydro-2′-deoxyuridine 5′-phosphate (H_2dUMP) or 5,6-dihydro-2′-deoxythymidine 5′-phosphate (H_2dTMP): (●) no inhibitor; (□) 200 μM H_2dUMP; (○) 200 μM H_2dTMP.

derivatives 2 and 3 shows, in both cases, that the dihydro analogues bind approximately 50 times less effectively.

Analysis of the chemical reactivity of uracil shows that the 5-carbon undergoes aromatic electrophilic substitution reactions. Similarly, uracil reacts with the formaldehyde and tetrahydroquinoline[11] to give the Mannich product; base-catalyzed reaction of uracil with formaldehyde leads to 5-hydroxymethyluracil.[12] Halogenation,[13] nitration,[14] and hydroxymethylation[15] have been reported for the uracil nucleotides. At some stage in the reaction profile the transient formation of a substituted sp^3 carbon at position 5 would be expected.

Although the mechanism of thymidylate synthetase catalysis is reasonably assumed to proceed through the covalent ternary complex 1, there is no direct evidence that this is the case, since the model on which this is based is the isolated complex from the reaction of the enzyme, cofactor, and 5-fluoro-2′-deoxyuridine 5′-phosphate.

An alternate mechanism is the initial addition of a nonenzymatic nucleophilic group to carbon-6 with subsequent nucleophilic attack of the anion at carbon-5 on the formyliminium derivative of tetrahydrofolic acid in either a stepwise or concerted reaction to give the enzyme complex **1b**.

If the enzyme complex similar to **1b** represented a transition state in some stage of the reaction leading to product and that transition state has an sp^3 carbon at the reactive 5 position, then a simple comparison of the enzyme affinity of the substrate or product to the respective 5,6-dihydro derivatives should address the question.

From transition-state theory and studies on transition-state inhibitors,[16] if a dihydro derivative, not covalently bound to the enzyme, were involved at any stage in the catalysis then greatly enhanced affinity should be

observed. The fact that the dihydro derivatives bind 50 times less effectively than the substrate or product does not support any transient formation of a noncovalently bound dihydro intermediate in thymidylate synthetase catalysis. Furthermore, these results suggest that the dihydro forms of the substrate or product do not occur as free intermediates in the reaction.

Experimental Section

IR spectra were measured with a Beckmann IR-33, UV spectra with a Cary 219 recording spectrophotometer, and NMR spectra with a Varian Model EM-360 or T-60. Microanalyses (±0.4% of theory) were obtained from a Hewlett-Packard 185B at the Department of Medicinal Chemistry, University of Kansas. The starting nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo.; DEAE-cellulose was the product of Whatman Biochemicals, Ltd.

Thymidylate synthetase purified from methotrexate-resistant *Lactobacillus casei* was purchased from the New England Enzyme Center, Tufts University, at a specific activity of 0.91 μmol of dTMP formed min^{-1} (mg of protein)$^{-1}$. The substrate 2′-deoxy[5-^{3}H]uridine 5′-monophosphate at a specific activity above 15 Ci/mmol was purchased from Moravek Biochemicals, Industry, Calif., and diluted with cold substrate purchased from Sigma Chemical Co. The cofactor *dl*-tetrahydrofolic acid was also purchased from Sigma Chemical Co.

5,6-Dihydro-2′-deoxyuridine 5′-[Bis(triethylammonium) Phosphate] (2). 2′-Deoxyuridine 5′-(disodium phosphate)·2.5H_2O (200 mg, 0.39 mmol) was dissolved in 20 mL of water, and the pH of the solution was adjusted to 2 with 1 N HCl. Rhodium catalyst (5% rhodium on alumina, 50 mg) was added and the flask flushed with nitrogen for 10 min. The hydrogenation was done at atmospheric pressure for 5 h; within 1 h, almost all of the calculated hydrogen was consumed. After removal of catalyst by centrifugation, the solution was lyophilized to a glassy solid residue. The solid material was dissolved in 3 mL of water and applied on a 2.5 × 50 cm column of DEAE-cellulose. Gradient elution was performed using 0.01 and 0.3 M of triethylammonium bicarbonate (pH 7.5). The corresponding fractions (retention volume 530 mL) were collected and lyophilized to give a white solid, which was dried at 60 °C for 24 h to give 150 mg (55%) of the product **2** as a hygroscopic powder: UV λ_{max} (H_2O) 208 nm; NMR (D_2O) δ 6.2 (t, 1 C_1-H), 4.6–3.4 (m, 6), 3.2 (q, 12, NCH_2), 2.9–1.9 (m, 4), 1.25 (t, 18, CH_2CH_3). Anal. Calcd for $C_{21}H_{45}N_4O_8P$·1.5H_2O: C, 46.74; H, 8.97; N, 10.38. Found: C, 46.65; H, 8.95; N, 10.20.

5,6-Dihydro-2′-deoxythymidine 5′-[Bis(triethylammonium) Phosphate] (3). Using the same procedure as described in the synthesis of **2**, 2′-deoxythymidine 5′-(disodium phosphate)·2H_2O (150 mg) was hydrogenated with 80 mg of 5% rhodium on alumina at pH 2.0 for 15 h. The same treatment as described for **2** gave 70 mg (56%) of the product **3** as a hygroscopic powder: UV λ_{max}(H_2O) 208 nm; NMR (Me_2SO-d_6) δ 6.15 (t, 1, C_1-H), 3.8–3.3 (m, 6), 3.0 (q, 12, NCH_2), 2.4–1.6 (m, 3), 1.2 (apparent t, 21, NCH_2CH_3 and $CHCH_3$). Anal. Calcd for $C_{22}H_{47}N_4O_8P$·2.5H_2O: C, 46.22; H, 9.17; N, 9.80. Found: C, 46.05; H, 9.18; N, 9.62.

Enzyme Assay. The enzyme was assayed by a modification of the radioisotope assays described by Roberts[17] and Lomax and Greenberg.[18] The solution, 0.1 mL, contained 25 mM mercaptoethanol; 0.22 mM *dl*-tetrahydrofolic acid; 6.75 mM formaldehyde; 5 mM sodium bicarbonate; 3 mM magnesium chloride; 0.12 mM EDTA; 6 mM Tris–acetate buffer (pH 6.8); 5 μL of the diluted enzyme solution (0.1 μg); substrate; and, when indicated, inhibitor. Control reactions lacked the cofactor, tetrahydrofolic acid. The substrate 2′-deoxy[5-^{3}H]uridine 5′-monophosphate was used at a specific activity of 500 μCi/μmol. The assays were started by the addition of the enzyme to the complete mixture and then incubated at 30 °C. Incubation was stopped at 30 s by the addition of 50 μL of 20% trichloroacetic acid. A 20% aqueous suspension of charcoal (0.25 mL) was added, and the solution was vortexed and allowed to stand for 15 min. The suspension was filtered through a glass-wool plugged Pasteur pipette, 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. Counting efficiency was 33%; control samples lacking the cofactor were found to have less than 5% of the respective sample counts.

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Preparation and Antidiabetic Activity of Some Sulfonylurea Derivatives of 3,5-Disubstituted Pyrazoles

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Four series of *p*-[3,5-dimethyl- (and 5-methyl-3-carboxy-) pyrazole-1]benzenesulfonylureas, thioureas, 2-thiohydantions, and 5,6-dihydro-4(3*H*)-oxo-2(1*H*)-pyrimidinethiones were prepared for evaluation as hypoglycemic agents. Biological testing of these compounds showed that some possessed antidiabetic activity.

Dulin, Gerritsen, and co-workers showed that 3,5-dimethylpyrazole and its active metabolite 5-methylpyrazole-3-carboxylic acid had a potent hypoglycemic activity.[1-5] Later, they found that several 3,5-dimethylpyrazoles possess hypoglycemic activities as great as 100 times that of tolbutamide in glucose-primed intact rats.

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