0.08 mL of 95% ethanol, ground together with 50 mg of dry gum acacia with a mortar and pestle, and brought to a volume of 5 mL with distilled water. A dose of 4 mg/kg of arachidonic acid produced a diarrhea graded 3 to 4+ intensity in all mice. The diarrhea was graded on paper towels as follows: 0 = solid pellet or no bowel movement; 1 = slightly soft pellet with little or no wet ring formation; 2 = moderately soft pellet with definite wet ring formation; 3 = soft pellet with large ring formation; 4 = amorphous pellet with very large wet ring formation. The ED_{50} was the dose which reduced the expected diarrhea score of six pretreated mice by 50% compared to the total diarrhea score of six control mice 30 min after arachidonic acid administration.

Gastric Ulcer Induction. This test is a modification of that described.[23,24] Male rats were deprived of food for 18 h prior to testing, while tap water was permitted ad libitum. The test compounds were administered orally 4 h prior to autopsy, at which time the stomachs were removed. The stomachs were divided along the lesser curvature, everted, rinsed in saline, and examined for the presence of focal petechiae. Ulcers were rated on an all or none basis and, in addition, each stomach was graded for the severity of ulcers formed using the following ratings: 0 = none; 1 = trace; 2 = mild; 3 = moderate; 4 = severe. The results of the ulcer scores were subjected to statistical analysis by the student's *t* test.

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5-Fluoro-2'-deoxyuridine 5'-(*p*-Azidophenyl phosphate), a Potential Photoaffinity Label of Thymidylate Synthetase

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5-Fluoro-2'-deoxyuridine 5'-(*p*-azidophenyl phosphate) (1), a potential photoaffinity labeling reagent for thymidylate synthetase from a methotrexate-resistant strain of *Lactobacillus casei*, has been synthesized and characterized. UV_{254} irradiation of mixtures of thymidylate synthetase with 1, containing ^{14}C-labeled phenyl and ^{3}H-labeled pyrimidine rings, in the presence of excess 5,10-methylenetetrahydrofolate, the cofactor for the reaction, produced two complexes, separable from the native enzyme by polyacrylamide gel electrophoresis, in which only the ^{3}H-containing moiety was bound to the protein. When mixtures of enzyme and 1 were irradiated in the absence of cofactor, complexes separable from the native enzyme were not observed. However, the ^{14}C-containing component of 1 was now bound to the protein in the absence of the ^{3}H-containing portion. The results are discussed in terms of the topography of the enzyme active site.

Thymidylate synthetase, which is essential for the replication of both mammalian and bacterial cells, has been a tempting target for investigation during the past 2 decades because control of its function may have potential utility in cancer chemotherapy. The system is also of interest because of the unique mechanistic role played by 5,10-methylenetetrahydrofolate, which acts both as methylene group donor and reductant in the enzymatic synthesis of thymidylate from 2'-deoxyuridylate. Recently, elegant proteolytic degradation studies of the complex formed between the enzyme and 5-fluoro-2'-deoxyuridylate have culminated in the isolation of active-site peptides bound to the pyrimidine moiety of this substrate analogue.[1,2] However, few investigations have been directed toward the phosphate-binding portion of the receptor site since the initial observation that a phosphate group is essential for substrate or inhibitor activity,[3,4] although a recent report has appeared indicating that arginine is important for enzyme activity and the authors suggest it may form an ionic bond to the phosphate dianion.[5] In

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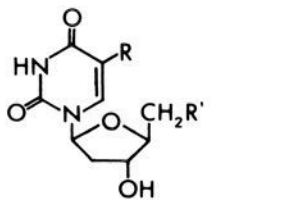
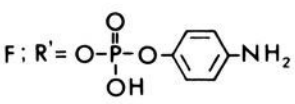
order to probe this portion of the active site of thymidylate synthetase, a potential photolabile inhibitor, 5-fluoro-2′-deoxyuridine 5′-(*p*-azidophenyl phosphate) (**1**) has been

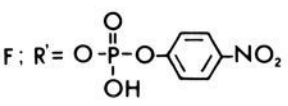


1, R = F; R′ = O-P(=O)(OH)-O-C6H4-N3



2, R = F; R′ = O-P(=O)(OH)-O-C6H4-NH2

3, R = F; R′ = OH



4, R = F; R′ = O-P(=O)(OH)-O-C6H4-NO2

5, R = H; R′ = N3

synthesized from its *p*-aminophenyl analogue **2** by diazotization and azide substitution.

Chemistry. To obtain the 5′-*p*-aminophenyl phosphate precursor **2** of the azide **1**, a previously reported synthesis[6,7] which employed the carbodiimide-promoted condensation of *p*-nitrophenyl phosphate and 5-fluoro-2′-deoxyuridine (**3**), followed by catalytic reduction, was modified because wide variations in yields of the intermediate *p*-nitrophenyl ester **4** were obtained. In the new procedure, *p*-nitrophenyl phosphorodichloridate[8] was reacted with **3** in anhydrous dioxane/pyridine to give the *p*-nitrophenyl ester **4**. Because thin-layer chromatography of the reaction mixture showed **4** to be the primary product, catalytic hydrogenation over 5% palladium/charcoal was performed without isolation of the nitro intermediate to give the desired aminophenyl derivative **2** with an overall 45% recovery. The amine was then converted to the azido derivative **1** in 75% yield by the usual diazotization and azide-substitution sequence.[9] The efficiency of this preparative route additionally allowed the synthesis of a ^{14}C and ^{3}H doubly labeled sample of the azide starting from *p*-nitro[2,6-^{14}C]phenol and 5-fluoro-2′-deoxy[6-^{3}H]uridine. Although it had initially been expected that the strong acid used in the diazotization and azide-substitution reaction might cause degradation of the glycosidic bond, the aminophenyl ester **2** was surprisingly stable in cold concentrated HCl, being unchanged after 24 h. The ^{1}H NMR spectrum of the azide product was essentially the same as that of its precursor,[6] except for a 0.2-ppm downfield shift of the aromatic AB quartet and an increase in J of 1.5 Hz. Catalytic hydrogenation of a methanolic solution of **1** over 10% palladium/charcoal caused reversion to **2**. An aqueous solution of the barium salt of the azide was stable for at least 2 days at room temperature in a Pyrex container exposed to external light, and for at least 7 days at 4 °C in the dark. When subjected to low-intensity UV irradiation (254 nm) it decomposed with $t_{1/2}$ of 2.5 min when measured by LC on a C_{18} μBondapak column. The $t_{1/2}$ did not alter in water, 90% aqueous acetonitrile, or 30% aqueous acetonitrile containing 5 mM tetrabutylammonium phosphate; this observation is consistent with decomposition via a nitrene intermediate.[10] Furthermore, **2** was not detected as a product of photolysis of **1**, which indicated that decomposition occurred via a singlet nitrene.[11]

Biochemistry. **1** was, as expected, a competitive inhibitor (K_i = 1 μM) of thymidylate synthetase, whereas a second analogue, 5′-azido-2′,5′-dideoxyuridine, prepared by the nucleoside substitution method of Hata et al.,[12] was devoid of activity as an inhibitor or as a substrate. These results emphasize the reported[3,4] requirement for a 5′-phosphate monoester but indicate that the single ionic charge of the phosphate diester is sufficient to induce strong binding to the active site and suggests that the phosphate-binding region is not sterically constrained, since the azidophenyl substituent is readily accommodated. Despite **1** being a good inhibitor of thymidylate synthetase, light-protected mixtures of enzyme and **1**, with or without *dl*-5,10-methylenetetrahydrofolate, the cofactor for the enzymatic reaction, showed only one band when subjected to polyacrylamide gel electrophoresis,[13,14] which corresponded to the migration of free enzyme. In addition, full enzymatic activity could be recovered by dialysis or Sephadex G-50 filtration. Similarly, a 5-min irradiation of an enzyme solution, with or without the cofactor, did not affect the catalytic activity or the electrophoretic pattern of the enzyme. However, irradiation of a mixture of enzyme, **1**, and cofactor resulted in inactivation of the enzyme and formation of complexes separable on polyacrylamide gels which were stable to dialysis and gel filtration.

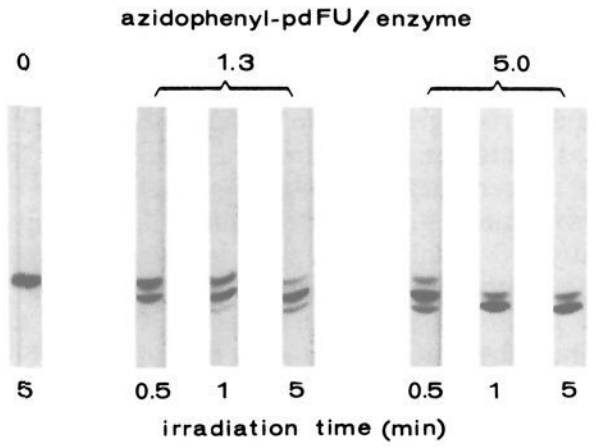


Figure 1. Polyacrylamide gel electrophoresis of thymidylate synthetase from a methotrexate-resistant stain of *Lactobacillus casei* and the products which formed when mixtures of the enzyme, *dl*-5,10-methylenetetrahydrofolate, and 5-fluoro-2′-deoxyuridine 5′-(*p*-azidophenyl phosphate) (**1**) were irradiated at 254 nm for 0.5, 1, or 5 min at ambient temperature. Electrophoretic samples (50 μL), which contained 4–6 μM protein, a tenfold excess of the folate cofactor, and either 0, 1.3, or 5.0 mol of inhibitor/mol of enzyme in 0.1 M potassium phosphate buffer, pH 6.8, 0.02 M 2-mercaptoethanol, were applied to 7.5-cm polyacrylamide gels and subjected to electrophoresis as described under the Experimental Section.

Figure 1 shows that up to three resolvable forms of thymidylate synthetase were present in these irradiated reaction mixtures. The uppermost band (R_f 0.56), designated form A, corresponds to native enzyme. The second band (R_f 0.60) and lowermost band (R_f 0.63) have been designated form B and form C, respectively. The electrophoretic behavior of forms B and C is consistent with the increasing anionic character of the ternary protein complexes which would be expected if complexation occurred with, in the case of form B, 1 equiv each of cofactor and inhibitor and, in the case of form C, 2 equiv each of cofactor and inhibitor. Forms B and C were enzymatically inactive, since the activity of irradiated solutions could be accounted for by the content of form A. Separation of unbound ligands could be accomplished by gel filtration or by dialysis overnight, although on prolonged dialysis enzyme activity was slowly recovered.

In the presence of excess 5,10-methylenetetrahydrofolate, the relative amounts of forms A, B, and C depend on the time of irradiation of the mixtures and on the molar ratio of inhibitor **1** to thymidylate synthetase.

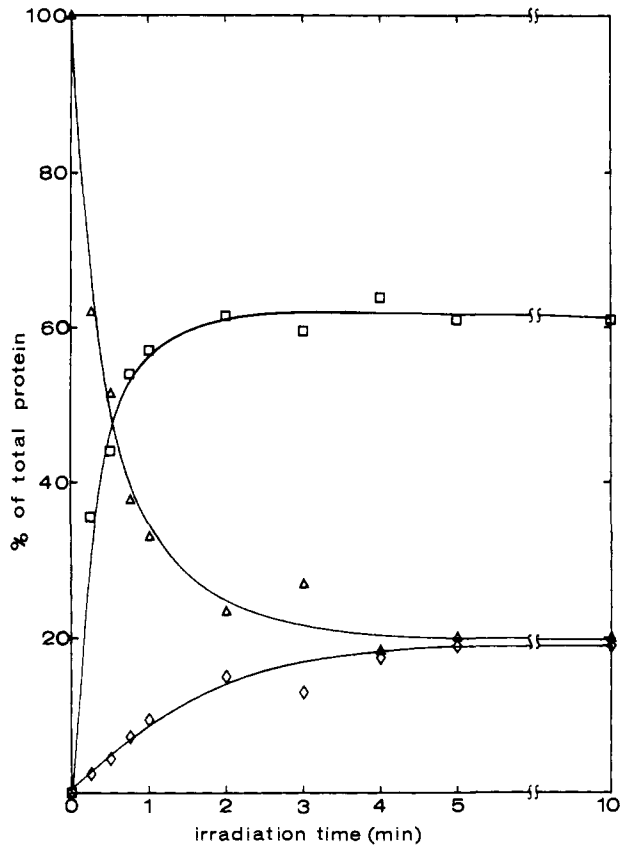


Figure 2. The effect of irradiation time on the formation of complexes between 5-fluoro-2'-deoxyuridine 5-(*p*-azidophenyl phosphate) (1) and thymidylate synthetase. Solutions containing a 1.33:1 molar ratio of the nucleotide inhibitor and enzyme, in the presence of a tenfold excess of *dl*-5,10-methylenetetrahydrofolate, were irradiated and samples were withdrawn at intervals and subjected to polyacrylamide gel electrophoresis. Percentages of total protein contained in bands A (Δ–Δ), B (□–□), and C (◇–◇) were determined, after staining, with a scanning densitometer. Each point represents the mean of five determinations (standard errors ≤2.6%).

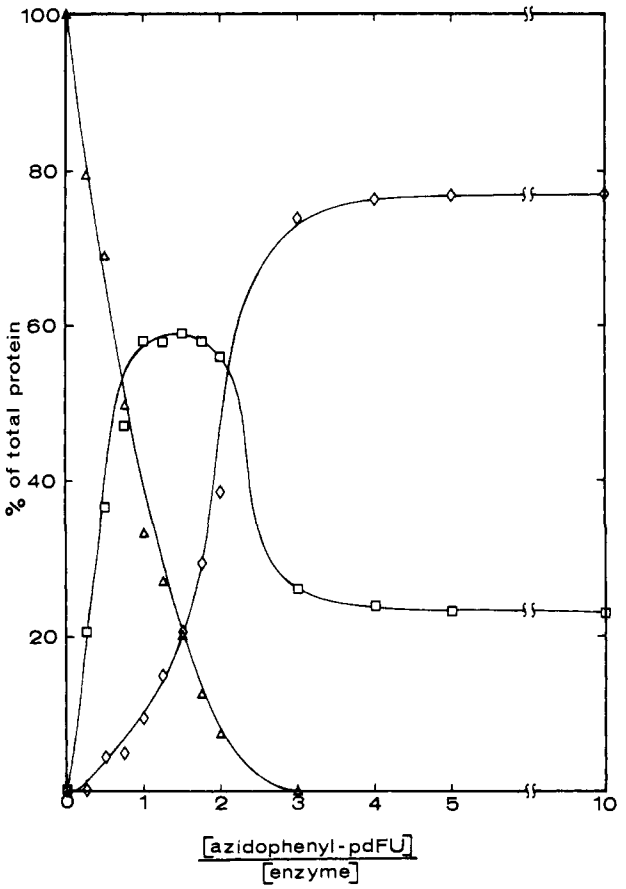


Figure 3. The effect of changing the molar ratio of 5-fluoro-2'-deoxyuridine 5'-(*p*-azidophenyl phosphate) (1) and thymidylate synthetase in the presence of a tenfold excess of *dl*-5,10-methylenetetrahydrofolate on the formation of the ternary complexes. Each mixture of inhibitor and enzyme was irradiated for 5 min, and 50-μL aliquots were extracted for electrophoresis. Protein percentages contained in bands A (Δ–Δ), B (□–□), and C (◇–◇) were determined, after staining, with a scanning densitometer. Each point represents the mean of five determinations (standard errors ≤3.4%).

Figure 2 indicates that for a constant ratio of inhibitor 1 to enzyme, the amount of native enzyme decreases with time while the amount of forms B and C increase until a limiting value is reached at $t = 5$ min. Similar results were obtained for a range of inhibitor/enzyme molar ratios. Although the half life of 1 is 2.5 min in solutions not containing protein, it is possible that a change in conformation when bound to the synthetase, or a transfer of energy from the protein, make 1 more susceptible to photolysis when bound to the enzyme.

When mixtures containing increasing molar ratios of inhibitor 1 to enzyme were irradiated for 5 min, in the presence of excess 5,10-methylenetetrahydrofolate, there was a rapid decrease in the amount of free enzyme, accompanied by an initial increase in the proportion of forms B and C (Figure 3). Form B then decreased to a value of 23%, while form C continued to increase until it represented 77% of the protein present. These values appear to be limiting states; even with very large excesses of inhibitor and prolonged irradiation times, the product ratio did not vary. This suggests that up to 20–25% of the enzyme has only one catalytically competent active site, although previous physicochemical studies have demonstrated that the enzyme is composed of two identical subunits, each containing an active site.[15] These results are similar to those obtained by Donato et al.[16] in studies of the interaction of 5-fluoro-2'-deoxyuridylate (FdUMP) with thymidylate synthetase. The complexes which formed by irradiating inhibitor 1 and thymidylate synthetase plus cofactor could not be distinguished electrophoretically from those formed between 5-fluoro-2'-deoxyuridylate and thymidylate synthetase in the presence of cofactor. Additionally, the UV spectrum of a limiting mixture of forms B and C, freed from excess ligands by Sephadex G-10 chromatography, was identical with that reported[16] for a limiting mixture of complexes formed between thymidylate synthetase, FdUMP, and cofactor.

Irradiation of 5-fluoro-2'-deoxy[6-^{3}H]uridine 5'-(*p*-azido[2,6-^{14}C]phenyl phosphate) in the presence of the synthetase and cofactor also gave rise to the same electrophoretic patterns. In addition, the pattern was unaltered whether 0.02 M 2-mercaptoethanol was present or absent from the pH 6.8, 0.1 M potassium phosphate buffer solution. Attempts to quantitate the bound radioactivity by dialysis, counting of gel slices, or by nitrocellulose adsorption of the complexes[17] indicated that tritium (i.e., the pyrimidine portion of the nucleotide ester) was indeed bound to the enzyme, but the lack of ^{14}C radioactivity indicated that the 5'-phenyl group had been lost. Changes of buffer ions and pH (0.1 M potassium phosphate buffer, pH 5.4–7.6; 0.1 M Tris-HCl buffer, pH 7–9; 0.1 M Tris–maleate buffer, pH 4.7–6.3) in the irradiation mixtures did not alter this result. When a mixture of forms B and C containing radiolabeled inhibitor was allowed to equilibrate

for 24 h at room temperature with a 10 M excess of unlabeled FdUMP, 90% of the radioactivity was released from the protein. Similar results were obtained when the radiolabeled protein was incubated with a 10 M excess of dUMP. Thin-layer chromatography in systems A and C indicated that 70–80% of the released radioactivity was present as FdUMP and about 20% was identified as the nucleoside, results consistent with those previously reported for complexes formed between enzyme, FdUMP, and cofactor.[18] The amount of ^3H bound per mole of synthetase in form C was twice that bound in form B. Heat denaturation of the enzyme complexes prevented release of radioactivity from the protein. The above data demonstrate that irradiation of thymidylate synthetase, inhibitor 1, and methylenetetrahydrofolate results in the decomposition of the inhibitor to form FdUMP, which reacts to form ternary complexes. Addition of preirradiated inhibitor 1 to a mixture of thymidylate synthetase and methylenetetrahydrofolate also produced a mixture of the ternary complexes, indicating that the formation of FdUMP is not enzyme mediated, although it may be enzyme assisted since the $t_{1/2}$ for decomposition is reduced in the presence of the enzyme.

Since 2-mercaptoethanol is known to reduce aromatic azides in aqueous solution,[19] the photolysis of thymidylate synthetase, inhibitor 1 and CH_2FH_4 was repeated in the absence of exogenous thiol. (A solution of thymidylate synthetase previously activated by dialysis against 0.02 M mercaptoethanol in 0.1 M potassium phosphate buffer and then dialyzed against scrupulously argon-deaerated phosphate buffer retained a specific activity over 3.0 units/mg.) The results of the photolysis in the absence of mercaptoethanol were the same as those performed in the presence of mercaptoethanol.

In contrast to the above data, when inhibitor 1 was photolyzed with thymidylate synthetase at a molar ratio of 3:1 in the absence of the cofactor, 1 mol of a ^{14}C-containing component was bound per mole of protein, in the absence of a tritium-containing component. A previous study has reported one binding site for FdUMP or dUMP per mole of enzyme in phosphate buffer when the cofactor was excluded from the reaction mixture.[15] Complexes separable from the native enzyme by polyacrylamide gel electrophoresis were not formed in the photolysis reaction; however, if cofactor was added complexes were observed to form, suggesting FdUMP had been liberated during the photolysis; moreover, the labeled enzyme could still complex with authentic FdUMP in the presence of the cofactor. The specific activity of the enzyme–inhibitor complex was the same as that of the control, indicating that the phosphate-binding site is still free to interact with the nucleotide. Because of the structure of inhibitor 1, labeling would be expected to occur at a residue near the phosphate-binding site rather than directly to that site. The relationship between reactants included in the photolysis mixture and the subsequent radioactivity which becomes bound to the protein is shown in Figure 4.

The results of the dual-labeling experiments suggest that in the absence of cofactor the 5′-(*p*-azidophenyl) ester 1 is positioned so that the nitrene formed by irradiation can react with a nearby portion of the enzyme. This is followed by a reaction-dependent decomposition, which results in the loss of (at least) the pyrimidine portion of the inhibitor molecule. In the presence of the cofactor, it appears the azidophenyl moiety of the reversibly bound inhibitor now protrudes into the solvent surrounding the enzyme, and thus nitrene production leads to preferential reaction with the solvent. The resulting unstable intermediate then

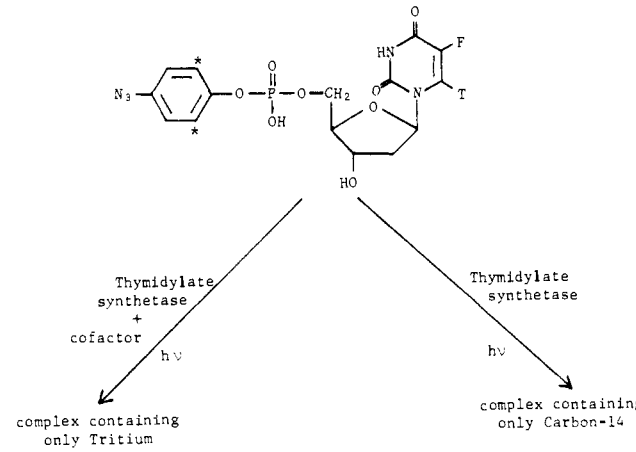


Figure 4. The radioactivity that is observed bound to thymidylate synthetase after irradiating: (A) the ternary complex of enzyme, 5,10-methylenetetrahydrofolate, and 5-fluoro-2′-deoxy[6-^3H]-uridine 5′-(*p*-azido[2,6-^{14}C]phenyl phosphate) or (B) the binary complex, which excludes the folate derivative. An asterisk indicates carbon-14 and T indicates tritium atoms.

eliminates a phenoxide ion, leaving 5-fluoro-2′-deoxyuridylate bound to the enzyme.[15,16] Further studies of the interaction of inhibitor 1 with thymidylate synthetase in the absence of the cofactor, including attempts to isolate and characterize the radioactively labeled peptide components of the proteins which are formed during irradiation, are currently in progress.

Experimental Section

Absorbance, IR and NMR spectra were recorded with Beckman DB-G and Cary Model 14, Perkin-Elmer Model 337, and Varian T60-A instruments, respectively. The NMR spectra expressed in parts per million were obtained in D_2O employing sodium trimethylsilyl propionate as internal standard (Merck and Co., Inc.) or in Me_2SO-d_6 with tetramethylsilane as internal standard (Aldrich Chem. Co.). Radioactivity of 0.1-mL samples was measured in Aquasol (10 mL, New England Nuclear) using a Beckman LS-233 scintillation counter. TLC systems used were as follows: (A) PEI-F cellulose (E.M. Laboratories) with development by 0.25 M LiCl; (B) cellulose (Eastman no. 13254) with development by *tert*-amyl alcohol–formic acid–water (3:2:1); (C) cellulose with development by acetonitrile–0.1 M NH_4Cl (3:2). Column chromatograph fractions (20 mL) were collected automatically with an LKB Ultrorac 7000 fraction collector and were monitored continuously for absorbance at 280 nm with an LKB Uvicord II 8300 detector unit. Elemental analyses were performed by Spang Microanalytical Laboratories (Eagle Harbor, Michigan). 5-Fluoro-2′-deoxyuridine was a gift from Hoffman-La Roche Research Laboratories.

5-Fluoro-2′-deoxyuridine 5′-(*p*-Aminophenyl phosphate) (2). A solution of dry 5-fluoro-2′-deoxyuridine (49.2 mg, 0.20 mmol) in anhydrous pyridine (1 mL) was added to *p*-nitrophenyl phosphorodichloridate (51.2 mg, 0.20 mmol) dissolved in an anhydrous mixture of dioxane (2 mL) and pyridine (0.25 mL), and the reactants were stirred for 15 min at room temperature. After dilution with H_2O (5 mL), the reaction products were chromatographed on a Dowex 50(H^+) column (3 × 28 cm). The pooled effluent (150 mL) obtained by aqueous elution was adjusted to pH 3.5 with 1 M NH_4OH, and the solution was extracted with ether until the extract showed no yellow coloration when mixed with concentrated NH_4OH. The aqueous solvent was removed by rotary evaporation, maintaining an external bath temperature of <40 °C, and the residue, dissolved in MeOH (50 mL), was hydrogenated over 5% Pd/C (100 mg) at 35 psi for 1 h. Filtration and evaporation of the filtrate gave an oily product, which was dissolved in H_2O (5 mL) and applied to a similar Dowex 50(H^+) column. Aqueous elution of the column afforded **2** as the principal component. After neutralization with a saturated solution of $Ba(OH)_2$, lyophilization, and precipitation from aqueous EtOH, this product (45.8 mg, 0.089 mmol, 45%) was identical with an

authentic sample of barium 5-fluoro-2'-deoxyuridine 5'-(p-aminophenyl phosphate).[6]

5-Fluoro-2'-deoxyuridine 5'-(p-Azidophenyl phosphate) (1). Concentrated HCl (0.15 mL) was added with stirring to an ice-cooled suspension of barium 5-fluoro-2'-deoxyuridine 5'-(p-aminophenyl phosphate) (39.1 mg, 0.075 mmol) in H_2O (0.4 mL). An aqueous solution of 0.3 M $NaNO_2$ (0.4 mL) was then added with stirring, followed by a cold solution of 0.8 M NaN_3 (0.15 mL), and the mixture was stirred at 4 °C for 20 h. The products were purified by successive column chromatography on (i) Dowex 50(H^+) (2.5 × 32 cm) with aqueous elution; (ii) AE-cellulose (3 × 35 cm) with elution by a 2-L linear gradient of 0–0.5 M ammonium acetate, and (iii) a second passage through Dowex 50(H^+). In each case, the azido derivative was the principal component. Neutralization of the final effluent with saturated aqueous $Ba(OH)_2$, lyophilization, and reprecipitation from 90% aqueous ethanol by ether addition gave the barium salt of 1 as a white powder: yield 35.0 mg, 0.056 mmol (75%); UV λ_{max} (0.1 M HCl) 252 nm (ϵ 19 105); λ_{max} (0.1 M NaOH) 251 nm (ϵ 18 695); IR ν_{max} (Nujol) 2100, 2120 cm^{-1} ($-N_3$); TLC R_f (systems A and B) 0.66; NMR (D_2O) 7.85 (d, 1 H, J_{HF} = 6 Hz, 6-H), 7.18 and 6.93 ppm (AB q, 4 H, J_{AB} = 8 Hz, aromatic). Anal. ($C_{15}H_{14}FN_5O_8P$-$Ba_{0.5}$·$6H_2O$) N; P: calcd, 5.00; found, 4.47.

5-Fluoro-2'-deoxy[6-^{3}H]uridine 5'-(p-Azido[2,6-^{14}C]phenyl phosphate). An aqueous solution of p-nitro[2,6-^{14}C]phenol (50 μCi, 32.8 mCi/mmol; 3 mL) was added to sodium p-nitrophenoxide[8] (65.0 mg, 0.40 mmol) in a 1 × 10 cm test tube. After lyophilization and drying at 80 °C (0.03 mm), the red powdery residue was slowly added in a closed system to stirred, freshly distilled, ice-cooled $POCl_3$ (2 mL) contained in a second 1 × 10 cm vessel. After warming to room temperature, the resulting mixture was filtered and washed with $POCl_3$ (1 mL), and the filtrate was evaporated to yield a yellow oil which was maintained at 0.03 mm for 2 h before being dissolved in an anhydrous mixture of dioxane (2 mL) and pyridine (0.25 mL). The solution was then combined with a solution of 5-fluoro-2'-deoxy[6-^{3}H]uridine (50.0 mg, 0.20 mmol, 250 μCi, 1.23 mCi/mmol) in anhydrous pyridine (1 mL). The synthetic sequence then followed the procedure described above for the nonradioactive materials and afforded the barium salt of the desired product in 11% overall yield with the following specific activities: ^{3}H = 1.06 mCi/mmol; ^{14}C = 0.125 mCi/mmol.

Photolysis Experiments. Photolyses of 1 were performed in dilute (10^{-4} M) aqueous solutions contained in 0.6 × 9.4 cm quartz tubes revolving at a distance of 4 cm around a quartz-enclosed mercury lamp (Westinghouse Lifeguard; peak energy 254 nm). The lamp and sample tubes were immersed in a room-temperature water bath. Samples were withdrawn at intervals, and the amount of 1 remaining in solution was determined by paired ion reverse-phase LC on a C_{18} μBondapak column (0.4 × 30 cm), using 5 mM tetrabutylammonium phosphate in 30% acetonitrile/water as the eluant.

Biochemistry. Thymidylate synthetase was isolated from a methotrexate-resistant strain of *Lactobacillus casei* (kindly supplied by Dr. Roy Kisliuk, Tufts University) by the procedure of Galivan et al.[20] and was assayed by the method of Wahba and Friedkin.[21] Enzyme used in these experiments had a specific activity of 3.0–3.5 units/mg of protein. Inhibition constants (K_i) were determined in 0.1 M potassium phosphate buffer, pH 6.8, in the usual manner[22] from the reciprocal relationship between rate and inhibitor concentration. The enzyme concentration was 4.5 μM; substrate concentrations were 25, 50, 75, and 100 μM; and inhibitor concentrations were 0, 0.25, 0.5, 1.0, 1.5, and 2.0 μM.

Enzyme photolyses were carried out as described above on solutions of thymidylate synthetase (4–6 μM) in 0.02 M 2-mercaptoethanol, 0.1 M potassium phosphate buffer, pH 6.8. The enzyme was irradiated with varying molar ratios of inhibitor 1, either in the presence or absence of a tenfold excess of *dl*-5,10-methylenetetrahydrofolate. Solutions were assayed for enzymatic activity and 50-μL aliquots were subjected to polyacrylamide gel electrophoresis, according to the procedures of Ornstein[13] and Davis,[14] to detect the formation of complexes. Quantitation of the complexes was achieved with a Clifford Model 443 Densicomp scanning densitometer. To achieve the increased resolution necessary for gel scanning, protein solutions were applied to 10-cm gels, which were electrophoresed at 2.5 mA/gel until 1 h after the Bromophenol blue marker dye reached the bottom of the gel. Protein was visualized by staining with 2% Aniline black in 7% acetic acid, followed by electrophoretic destaining.

Determination of bound radioactivity was carried out essentially by the method of Santi et al.,[17] in which aliquots (500 μL) of irradiated mixtures were adsorbed onto 24-mm nitrocellulose membranes (Schleicher and Schuell, BA-85) and washed with 7 × 0.5 mL portions of buffer, and the membranes were dissolved in Aquasol (10 mL). After 24 h, samples were counted with a Beckman LS-233 scintillation counter.

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