Thymidylate Synthetase from Amethopterin-Resistant *Lactobacillus casei*. Purification by Affinity Chromatography†

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ABSTRACT: 5-Fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (III) has been obtained by catalytic hydrogenation of the *p*-nitrophenyl ester (II) resulting from the condensation of a molar equivalent of *p*-nitrophenyl phosphate and 5-fluoro-2′-deoxyuridine (I). 5-Fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII) was prepared in a similar manner by reduction of the 3′-(*p*-nitrophenyl phosphate) 5′-phosphate derivative (VI) which, in turn, was obtained by treating the 3′,5′-bis(*p*-nitrophenyl phosphate) ester (V) with snake venom phosphodiesterase. The latter diester was synthesized by reacting: 5-fluoro-2′-deoxyuridine (I) with excess *p*-nitrophenyl phosphate; and *p*-nitrophenol with the 3′,5′-diphosphate (IV) obtained when the 3′,5′-bis(cyanoethyl phosphate) derivative of (I) is hydrolyzed. Affinity column materials were prepared by coupling: (A) (III) and (VII) directly to cyanogen bromide activated Sepharose; (B) (III) to Sepharose through a succinic anhydride–1,6-diaminohexane linkage; (C) (III) as in (B), but blocking excess succinyl carboxyl groups with glycinamide. Each column material was tested for its ability to adsorb thymidylate synthetase from cell-free extracts of an amethopterin-resistant strain of *Lactobacillus casei*. Protein and enzymatic activity were monitored following stepwise elution of each column with increasing concentrations (0.01–0.3 M) of phosphate buffer (pH 7). Consistent with the weak inhibitory properties of (VII) ($K_i \sim 10^{-4}$ M), column materials containing this compound were unable to retain the enzyme. In contrast, (III) ($K_i \sim 10^{-6}$ M) attached to Sepharose (particularly in mode (C)) showed a pronounced ability to absorb the enzyme from the cell-free extract (2.32 units/ml of sedimented absorbent). Elution of the enzyme was accomplished with 0.2–0.3 M buffer. After two cycles through this column, pure enzyme (3.3 units/mg; single band at R_F 0.67 upon polyacrylamide electrophoresis) was obtained in a >50% overall yield.

Thymidylate synthetase, which catalyzes reaction (eq 1), has been studied extensively because of its importance

2′-deoxyuridylate + 5,10-methylene-5,6,7,8-tetrahydrofolate ⟶ 5-methyl 2′-deoxyuridylate (thymidylate) + 7,8-dihydrofolate (1)

in providing a DNA precursor and its unusual mechanism in which a methylene group is both transferred from the folate coenzyme and reduced (Humphreys and Greenberg, 1958; Pastore and Friedkin, 1962; Blakley, 1963; Reyes and Heidelberger, 1965).

5-Fluoro-2′-deoxyuridine 5′-phosphate is a potent inhibitor of thymidylate synthetase (Cohen *et al.*, 1958; Bosch *et al.*, 1958). In this laboratory we have been studying the nature of the nucleotide site on the enzyme through the use of substrate and inhibitor analogs. During the course of this investigation, it became apparent that the 5′-(*p*-aminophenyl phosphate) ester of 5-fluoro-2′-deoxyuridine (III) and the 3′-(*p*-aminophenyl phosphate) ester of 5-fluoro-2′-deoxyuridine 5′-phosphate (VII) interact reversibly with the synthetase. Moreover, these analogs contain a reactive substituent, the amine, which could be linked to an inert, *insoluble*, or *soluble* macromolecular support. In the former case, the material could be used for the purification *via* affinity chromatography of thymidylate synthetase, and in the latter instance the material might serve as a high molecular weight, site-directed antimetabolite (Robinson *et al.*, 1973). This paper describes the synthesis and properties of 5-fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (III) and 5-fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII) (Figure 1), their coupling to Sepharose, and the use of the resulting products as affinity column materials for the purification of thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*.

Experimental Section

Materials. Chemicals were obtained from the following sources: 5-fluoro-2′-deoxyuridine (Hoffmann-La Roche Research Laboratories), barium 2-(cyanoethyl phosphate) (Sigma Chemical Co.), *p*-nitrophenol, *p*-nitrophenyl phosphate, dicyclohexylcarbodiimide, and 10% palladium on charcoal (British Drug Houses), Dowex 50 (H^+ form, 200–400 mesh × 8) (Bio-Rad Laboratories), DEAE-cellulose (Whatman DE-22) and cellulose (Whatman CF-11) (W & R Balston Ltd.), Sepharose 4B (Pharmacia Fine Chemicals). A molar solution of 2-cyanoethyl phosphate was prepared using the procedure described by Tener (1961). Pyridine, dimethylformamide, and acetonitrile were redistilled and dried prior to use by standing over a molecular sieve, Linde Type 4A (Matheson Coleman and Bell). 5-Fluoro-2′-deoxyuridine 5′-phosphate was prepared by the carbodiimide-mediated action of a molar equivalent of 2-(cyanoethyl phosphate) on the free nucleoside (Tener, 1961). The product after hydrolysis was routinely purified by passage through DEAE-cellulose, in the bicarbonate form, and isolated as the barium salt after fractional crystallization from an ethanol–water mixture. Snake venom phosphodiesterase was obtained from the Worthington Biochemical Corp.

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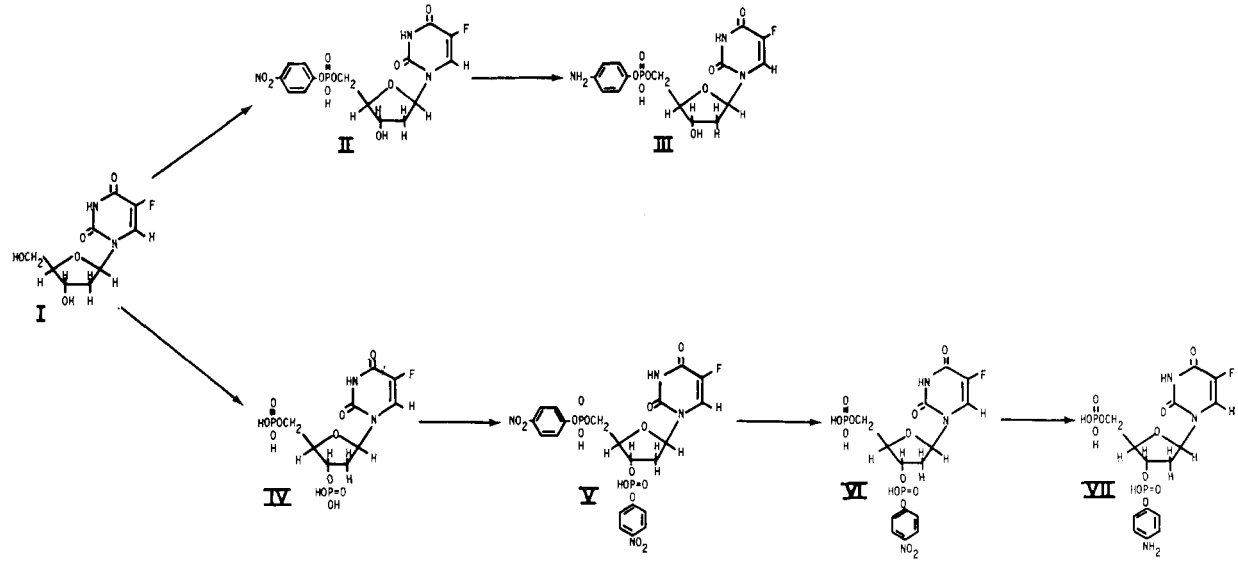

FIGURE 1: Synthetic routes for the preparation of 5-fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (III) and 5-fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII).

Methods. Ultraviolet and infrared absorption spectra were obtained with Cary, Model 14, and Perkin-Elmer, Model 337, recording spectrophotometers. Proton magnetic resonance (pmr) spectra were determined on a Jeol Instrument, Model JNM-PS-100 (courtesy of Dr. J. Rivier, Salk Institute for Biological Studies); hexamethyldisiloxane (K & K Laboratories, Inc.) was used as the internal standard.

Solvent evaporation was achieved with either a Virtis lyophilizer or with a Buchi rotary evaporator. Hydrogenation was accomplished in a Parr hydrogenator. Thin-layer chromatography was carried out using the following systems: (A) 0.5 M LiCl with Macherey-Nagel PEI-cellulose-UV_{254}; (B) 2-propanol–ammonia–water (7:1:2 v/v); and (C) ethanol–1-propanol–water (4:1:2 v/v) with cellulose-UV_{254} (Brinkman Instrument Co.). Compounds were visualized as quenching spots under ultraviolet light in a Chromatovue, or by spraying with a solution of ammonium molybdate–perchloric acid (Hanes and Isherwood, 1949). Microanalyses were performed by Dr. F. B. Strauss (Microanalytical Laboratory, Oxford, England).

Cell-free extracts of amethopterin-resistant *L. casei* (Dunlap *et al.*, 1971) were prepared in the following manner. Frozen cells (33 g) were suspended in 400 ml of a pH 7.2 buffer containing Tris (as the free base), 50 mM; KCl, 50 mM; mercaptoethanol, 10 mM; and EDTA, 1 mM. Aliquots of this suspension were placed in a 400-ml rosette flask immersed in ice–water, and the cells were lysed with a Branson Sonifier, Model 5125 (Danbury, Conn.) at a setting of 8 for one 5-min and seven 3-min periods, with intervals of 7 min to allow for cooling to 2°. Centrifugation at 20,000*g* for 50 min yielded a pale yellow supernatant (400 ml) which was dialyzed against 12 l. of 0.01 M potassium phosphate (pH 7) containing 0.02 M mercaptoethanol. The solution (380 ml) was concentrated threefold by ultrafiltration through a Diaflo pressure cell (Amicon PM10 membrane at 40 psi argon pressure).

Thymidylate synthetase was assayed spectrophotometrically by the procedure of Wahba and Friedkin (1961). Specific activity is defined as μmoles of deoxyuridylate converted to thymidylate per min per mg of protein. Protein was determined by the method of Lowry *et al.* (1951). Inhibition constants were obtained from plots of $1/v$ against i at varying substrate concentrations, where v is the initial rate of reaction and i is the inhibitor concentration. Regression analyses of the experimental results were carried out using a Hewlett-Packard calculator (Model 2810A).

Disc electrophoresis on 7.5% (w/v) polyacrylamide gels was carried out in 0.6 × 6 cm tubes. Protein was visualized by staining with Amido Black (Davis, 1964).

5-Fluoro-2′-deoxyuridine 5′-(Cyanoethyl phosphate). 5-Fluoro-2′-deoxyuridine (I) (0.500 g) in dry pyridine (10 ml) was combined with a molar solution of 2-(cyanoethyl phosphate) (8.4 ml) and the mixture was evaporated to dryness *in vacuo*. A further quantity of dry pyridine (10 ml) was added and evaporation was repeated. The residue, dissolved in a 1:1 v/v mixture of dry pyridine and acetonitrile (30 ml) containing dicyclohexylcarbodiimide (1.50 g), was protected from moisture by a $CaCl_2$ tube and stirred for 5 days at 20°. The reaction was terminated by the addition of water (2 ml). After the solution was stirred for an additional 2 hr, the solvent was evaporated. The residue was suspended in water (30 ml) and insoluble material was removed by filtration. The filtrate was chromatographed on a 3 × 20 cm column of Dowex 50, by elution with water. Peak fractions determined by absorption at 280 nm were combined, neutralized with a suspension of calcium hydroxide, and lyophilized. The white product was taken up in water (10 ml), and the precipitate, resulting from the addition of methanol (195 ml), was discarded. The filtrate was again evaporated to give an oily product which solidified after drying in a desiccator over $CaCl_2$ for 12 hr at a pressure of 1.0 mm. The solid was dissolved in 95% methanol (150 ml), and any further insoluble material was removed by filtration. The filtrate was evaporated to yield the calcium salt of 5-fluoro-2′-deoxyuridine 5′-(cyanoethyl phosphate) as a white, microcrystalline solid (0.720 g): λ_{max} (0.1 N HCl) 268 nm (ϵ 9300 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 268 nm (ϵ 7400); ν_{max} (KBr) 2250 cm^{-1}; pmr spectrum (D_2O) showed δ 2.8 (m, 2 H, methylene of the cyanoethyl), 4.0 (m, 3 H, second methylene of the cyanoethyl overlapping the 4′ proton). R_F 0.70 in system A. *Anal.* Calcd for $C_{12}H_{13}N_3O_8PFCa_{0.5}\cdot 6H_2O$: N, 8.20; P, 6.12. Found: N, 8.30; P, 6.11.

5-Fluoro-2′-deoxyuridine 3′,5′-Bis(cyanoethyl phosphate). 5-Fluoro-2′-deoxyuridine (I) (0.500 g), a molar solution of 2-(cyanoethyl phosphate) (8.4 ml) and dicyclohexylcarbodiimide (1.50 g) were reacted in a 1:1 v/v mixture of dry pyridine–dimethylformamide (50 ml) by stirring at 37° for 5 days. Water (4 ml) was added to terminate the reaction. After 1 hr

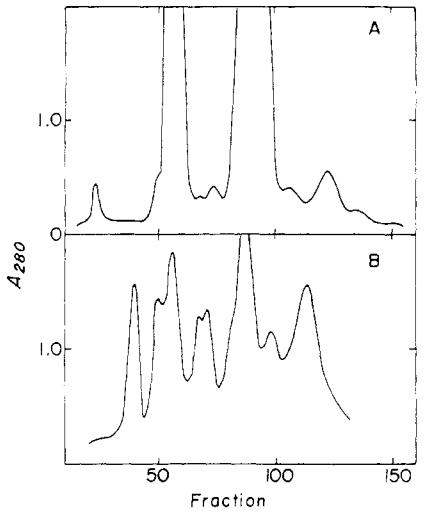

FIGURE 2: Chromatography of 5-fluoro-2′-deoxyuridine 3′,5′-bis-(*p*-nitrophenyl phosphate) on DEAE-cellulose. Reaction between: (A) 5-fluoro-2′-deoxyuridine (I) and *p*-nitrophenyl phosphate; and (B) 5-fluoro-2′-deoxyuridine-3′,5′-diphosphate (IV) and *p*-nitrophenol. Elution was obtained with a linear gradient of 0 → 0.5 M ammonium acetate (1 l. of each) followed by 0.5 M ammonium acetate. Fractions (20 ml) were collected automatically and monitored for absorbance at 280 nm.

a further addition of water (20 ml) was made and the urea precipitate was removed by filtration. The filtrate was evaporated to dryness *in vacuo* at 37°. The residue was taken up in water (10 ml) and neutralized with a suspension of calcium hydroxide. Methyl alcohol (20 ml) was added and the precipitate was discarded. The filtrate was evaporated to dryness at a temperature no greater than 37°. The residue was resuspended in 95% ethyl alcohol and then filtered to give a gummy, hygroscopic solid (0.853 g). This material was redissolved in 80% acetone (55 ml) and any insoluble components were removed by filtration. The filtrate was evaporated and the residue dissolved in water (1 ml). Fractional crystallization with the addition of increasing quantities of methanol gave the calcium salt of 5-fluoro-2′-deoxyuridine 3′,5′-bis-(cyanoethyl phosphate) (0.413 g): λ_{max} (0.1 N HCl) 268 nm (ϵ 8100 (based on the anhydrous calcium salt)); ν_{max} (KBr) 2250 cm^{-1}; R_F 0.66 in system B; pmr spectrum (D_2O) 2.70 (m, 4 H, two cyanoethylmethylene groups), 3.96 (m, 6 H, the remaining two cyanoethylmethylene groups overlapping the 5′-methylene).

5-Fluoro-2′-deoxyuridine 3′,5′-Diphosphate (IV). Calcium 5-fluoro-2′-deoxyuridine 3′,5′-bis(cyanoethyl phosphate) (0.335 g) was dissolved in 0.1 N NaOH and heated under reflux for 30 min. The solution was cooled and allowed to stand at 0° for 12 hr; the precipitate was removed by filtration. The filtrate was chromatographed on a 3 × 20 cm column of Dowex 50, by elution with water. Peak fractions were combined, adjusted to pH 7 with a suspension of calcium hydroxide, and lyophilized to dryness. The residue was taken up in water (20 ml), filtered to remove insoluble material, and 95% ethyl alcohol (30 ml) was added to the filtrate. Calcium 5′-fluoro-2′-deoxyuridine 3′,5′-diphosphate was obtained as a white microcrystalline precipitate (0.215 g): λ_{max} (0.1 N HCl) 268 nm (ϵ_{268} 8800 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 268 nm (ϵ 7000 M^{-1} cm^{-1}); pmr spectrum (D_2O) showed δ 2.25 (m, 2 H, 2′ methylene), 4.00 (m, 2 H, 5′ methylene), 4.35 (m, 1 H, 4′ proton), 6.10 (m, 1 H, 1′ proton), 7.85 (d, 1 H, J_{HF} = 7.5 Hz, 6 proton). The 3′ proton was obscured by the solvent peak. *Anal.* Calcd for $C_9H_9N_2O_{11}P_2FCa_2 \cdot H_2O$: N, 5.60; P, 12.4. Found: N, 5.57; P, 12.5.

5-Fluoro-2′-deoxyuridine 3′,5′-Bis(p-nitrophenyl phosphate) (V). (a) *From 5-Fluoro-2′-deoxyuridine (I) and p-Nitrophenyl Phosphate.* Disodium *p*-nitrophenyl phosphate tetrahydrate (8.00 g) was converted to the acid form by passage through a 3 × 20 cm column of Dowex 50; the aqueous effluent was lyophilized. The white solid was twice taken up in dry pyridine (40 ml) and evaporated to dryness. 5-Fluoro-2′-deoxyuridine (I) (2.00 g) was treated in the same manner. After the second evaporation, the nucleoside was taken up again in dry pyridine (40 ml) and added to the *p*-nitrophenyl phosphate; the mixture was then evaporated to dryness. Dicyclohexylcarbodiimide (6.18 g) in pyridine (10 ml) was added and the volume was increased to 75 ml with a 1:1 (v/v) mixture of dry pyridine and dimethylformamide. The suspension was stirred for 3 days at 37° in a flask protected from moisture by a $CaCl_2$ drying tube. Water (20 ml) was added and the mixture was stirred for an additional 2 hr. The precipitate was removed by filtration and the filtrate was evaporated to dryness. Water (20 ml) was again added to the residue and insoluble material was removed by filtration. The filtrate was passed through a Dowex 50 column and the aqueous eluent was adjusted to pH 3.5 with NH_4OH. The solution was extracted repeatedly with ether until the extracts showed no yellow color when tested with NH_4OH. The aqueous phase was concentrated by rotary evaporation to 20 ml, at a temperature not exceeding 30°. The resulting solution was chromatographed on a 4 × 50 cm column of DEAE-cellulose and eluted with a linear gradient of 0–0.5 M ammonium acetate (Figure 2A). Fractions 80–100 were combined, concentrated, and passed successively through a 4 × 50 cm column of cellulose CF11 and a 3 × 20 cm column of Dowex 50. Effluent from the latter column was neutralized with saturated $Ba(OH)_2$ and concentrated. Fractional crystallization with ethanol led to barium 5-fluoro-2′-deoxyuridine 3′,5′-bis(*p*-nitrophenyl phosphate) as a white microcrystalline solid (3.05 g, 75%): λ_{max} (0.1 N HCl) 278 nm (ϵ 21,750 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 280 nm (ϵ 19,700 M^{-1} cm^{-1}); pmr spectrum (D_2O) δ 7.08 (m, 4 H, aromatic), 7.52 (d, 1 H, 6 proton, J_{HF} = 6 Hz), 7.98 (m, 4 H, aromatic); R_F 0.27, 0.52, and 0.43 in systems A, B, and C, respectively. *Anal.* Calcd for $C_{21}H_{17}N_4O_{15}P_2FBa \cdot 3H_2O$: N, 6.67; P, 7.36. Found: N, 6.22; P, 7.36. For the free acid, $C_{21}H_{19}N_4O_{15}P_2F \cdot 2H_2O$: C, 36.7; H, 3.36; N, 8.18; P, 9.05. Found: C, 36.4; H, 3.63; N, 7.80; P, 8.83.

(b) *From 5-Fluoro-2′-deoxyuridine 3′,5′-Diphosphate (IV) and p-Nitrophenol.* Calcium 5-fluoro-2′-deoxyuridine 3′,5′-diphosphate (0.190 g) was converted to the acid (IV) by passage through a 4 × 20 cm column of Dowex 50. The aqueous eluate was evaporated to dryness, and then converted to the dry pyridinium salt, as described above. *p*-Nitrophenol (0.127 g) in dry pyridine (10 ml) was added and the mixture was again evaporated to dryness. The residue was taken up in a 1:1 (v/v) mixture of dry pyridine and dimethylformamide (5 ml) containing dicyclohexylcarbodiimide (0.300 g). This mixture was stirred for 3 days at 37°. The product was isolated by the sequence described above. The DEAE-cellulose profile is illustrated in Figure 2B. Fractions 82–92 were combined and barium 5-fluoro-2′-deoxyuridine 3′,5′-bis(*p*-nitrophenyl phosphate) (0.059 g, 19%) was isolated as described previously.

5-Fluoro-2′-deoxyuridine 5′-(p-Nitrophenyl phosphate) (II). By procedures similar to those described above, hydrated sodium *p*-nitrophenyl phosphate (3.00 g) was reacted at ambient temperature with 5-fluoro-2′-deoxyuridine (I) (2.00 g) in a 1:1 (v/v) mixture of dry pyridine and dimethylform-

amide (40 ml) containing dicyclohexylcarbodiimide (2.50 g) to give 5-fluoro-2′-deoxyuridine 5′-(*p*-nitrophenyl phosphate) (II) (1.14 g). The white microcrystalline product was isolated as the monohydrated barium salt after the chromatographic and fractional crystallization procedures described above: R_F 0.60 in system A; λ_{max} (0.1 N HCl) 272 nm (ϵ 14,700 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 272 nm (ϵ 13,100 M^{-1} cm^{-1}); pmr spectrum (D_2O) δ 7.10 (d, 2 H, aromatic), 7.55 (d, 1 H, pyrimidine, 6 proton, J_{HF} = 6 Hz), 7.95 (d, 2 H, aromatic). *Anal.* Calcd for $C_{15}H_{14}N_3O_{10}PFBa_{0.5}\cdot H_2O$: N, 7.88; P, 5.82. Found: N, 7.61; P, 6.05.

5-Fluoro-2′-deoxyuridine 3′-(p-Nitrophenyl phosphate) 5′-Phosphate (VI). Barium 5-fluoro-2′-deoxyuridine 3′,5′-bis(*p*-nitrophenyl phosphate) (1.11 g) was converted to the acid (V) by passage through a 3 × 20 cm column of Dowex 50. The aqueous eluate was concentrated to 40 ml and then diluted to 150 ml with 0.2 M ammonium bicarbonate containing 2 mM $MnCl_2$. After adjusting the pH to 8.2 with NH_4OH, snake venom phosphodiesterase (5 mg) was added. The solution was allowed to stand until there was no further release of *p*-nitrophenol (40 hr). The pH was adjusted to 4 with 1 M HCl and the solution was extracted repeatedly with ether until the extracts produced no yellow color upon addition of NH_4OH. The aqueous phase was concentrated to 20 ml and passed through an 8 × 28 cm column of Sephadex G-15. Nucleotide-containing fractions were combined, concentrated, and applied to a Dowex-50 column. The eluate was neutralized with saturated barium hydroxide and this solution was concentrated prior to fractional crystallization with ethanol. Barium 5-fluoro-2′-deoxyuridine 3′-(*p*-nitrophenyl phosphate) 5′-phosphate was obtained as a white microcrystalline solid (0.770 g): R_F 0.12 in system A; λ_{max} (0.1 N HCl) 272 nm (ϵ 15,500 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 272 nm (ϵ 13,800 M^{-1} cm^{-1}); pmr spectrum (D_2O) showed δ 7.65 (d, 2 H, aromatic), 8.50 (m, 3 H, the two additional aromatic proton resonances overlapping the pyrimidine 6 single proton doublet). *Anal.* Calcd for $C_{15}H_{13}N_3O_{13}P_2FBa_{1.5}\cdot H_2O$: N, 5.62; P, 8.30. Found: N, 5.61; P, 8.58.

5-Fluoro-2′-deoxyuridine 3′-(p-Aminophenyl phosphate) 5′-Phosphate) (VII). Barium 5-fluoro-2′-deoxyuridine 3′-(*p*-nitrophenyl phosphate) 5′-phosphate (0.310 g) was converted to the acid VI by passage through Dowex 50. The aqueous effluent was evaporated; the residue was taken up in methanol and hydrogenated over 10% palladium charcoal at 35 psi and room temperature for 60 min. The catalyst was removed by filtration. Ether was added to the filtrate to give 5-fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII) (0.158 g) as a white precipitate which was isolated by centrifugation: R_F 0.15 in system A; λ_{max} (0.1 N HCl) 268 nm, (ϵ 8950 M^{-1} cm^{-1}); λ_{max} (0.1 N NaOH) 279 nm (ϵ 7400 M^{-1} cm^{-1}); pmr spectrum (D_2O) δ 7.65 (m, 4 H aromatic), 8.25 (d, 2 H, J_{HF} = 7 Hz, 6 proton). *Anal.* Calcd for $C_{15}H_{18}N_3O_{11}P_2F\cdot H_2O$: C, 35.0; H, 3.93; N, 8.14; P, 12.0. Found: C, 35.5; H, 4.38; N, 7.61; P, 11.9.

5-Fluoro-2′-deoxyuridine 5′-(p-Aminophenyl phosphate) (III). Barium 5-fluoro-2′-deoxyuridine 5′-(*p*-nitrophenyl phosphate) (0.440 g) was converted to the acid II by passage through Dowex 50. The water effluent was evaporated and the gummy residue was taken up in methanol (100 ml); 10% palladium on charcoal (0.10 g) was added and the mixture was hydrogenated at 35 psi for 1 hr. The catalyst was removed by filtration, the filtrate was concentrated to 10 ml, and the solution was chromatographed on Dowex 50. Elution with water afforded initially any unchanged starting material, followed by the product. The latter fractions were combined and neutralized with saturated barium hydroxide. Concentration, followed by the addition of ethanol, gave barium 5-fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) as a white precipitate (0.200 g): R_F 0.72 in system A; λ_{max} (0.1 N HCl) 268 nm (ϵ 8900); λ_{max} (0.1 N NaOH) 270 nm (ϵ 7730); pmr spectrum (D_2O) δ 6.62 (m, 4 H, aromatic), 7.60 (d, 1 H, J_{HF} = 8 Hz, 6 proton). *Anal.* Calcd for $C_{15}H_{16}N_3O_8PFBa_{0.5}\cdot 2H_2O$: N, 8.05; P, 5.94. Found: N, 7.93; P, 5.96.

5-Fluoro-2′-deoxyuridine 5′-(p-Aminophenyl phosphate) (III) Derivatives of Sepharose. (a) *Direct Linkage.* An aqueous solution (25 ml) of cyanogen bromide containing approximately 100 mg/ml was added to a stirred suspension of Sepharose (sedimented wet volume, 25 ml). The mixture was adjusted to pH 11 with 4 N NaOH and maintained at this pH for 10 min. The product was filtered rapidly, washed repeatedly with cold 0.1 M $NaHCO_3$ (pH 8.5), and then treated with a solution of barium 5-fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (0.032 g) in 15 ml of the same buffer. After the mixture had been stirred for 24 hr at 4°, the product was packed into a column and washed exhaustively with 0.5 M phosphate (pH 7) and water. The eluate was collected and the unbound was nucleotide determined by its absorption at 268 nm. Material bound was 0.030 g, or 58 μmol, corresponding to a capacity of 2.3 μmol/ml.

5-Fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII) was coupled to Sepharose by an identical procedure (capacity, 1.5 μmol/ml).

(b) *Extended Linkage.* Hexamethylenediamine (0.30 g) was reacted with Sepharose (25 ml) according to the procedure of Whiteley *et al.* (1972). This material was then treated with excess succinic anhydride (3.00 g) in the manner described by Cuatrecasas (1970). The succinylaminohexyl-Sepharose was suspended in water (30 ml) and barium 5-fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (0.040 g) and 1-ethyl-3(3′-dimethylaminopropyl)carbodiimide hydrochloride (0.580 g) were added. The pH was adjusted to 5.5 with 1 N HCl and the suspension was stirred for 48 hr. The solid was packed into a column and washed thoroughly with 1 M phosphate buffer (pH 7) and water. Coupled material was estimated as in (a) and corresponded to 1.4 μmol/ml.

(c) *Blocked Carboxyl Groups.* The matrix from (b) above (15 ml) was suspended in a 1:1 (v/v) mixture of dimethylformamide and 0.1 M cacodylate buffer (pH 4.5) (50 ml) containing glycinamide (1.10 g) (Chen *et al.*, 1972). To this suspension was added 1-ethyl-3(3′-dimethylaminopropyl)carbodiimide hydrochloride (2.00 g) dissolved in the same mixture (10 ml), and the reactants were stirred at room temperature for 12 hr. The product was washed repeatedly with 0.1 M acetic acid, water, and 1 M potassium phosphate buffer (pH 7) prior to use.

Results and Discussion

Although homogeneous thymidylate synthetases have been isolated from bacterial and mammalian sources by conventional procedures, the operations are lengthy and the overall yields are low (Dunlap *et al.*, 1971; Leary and Kisliuk, 1971; Lorenson *et al.*, 1967; Fridland *et al.*, 1971). With antifolate-resistant bacterial cell lines the elevated levels of the enzyme ensure recovery of the desired protein despite suboptimal conditions for isolation. However, the enzyme is present at much lower levels in mammalian tissues, making it mandatory to utilize efficient isolation procedures. It appeared, therefore, that affinity techniques similar to those developed for the purification of dihydrofolate reductase (Whiteley *et*

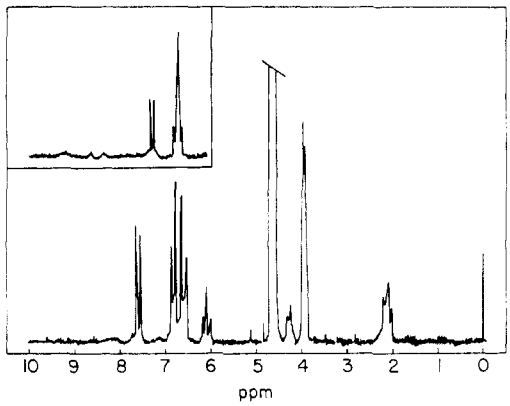

FIGURE 3: The 100-MHz proton magnetic resonance spectrum of III. Inset, a portion of the spectrum of VII.

al., 1972) might be advantageous for the isolation of the synthetase.[1]

Reaction 1 is strongly inhibited by 5-fluoro-2′-deoxyuridine 5′-phosphate (Cohen *et al.*, 1958; Bosch *et al.*, 1958). Recent reports from several laboratories have suggested that there is a covalent interaction between the pyrimidine portion of this substrate analog and thymidylate synthetase, provided the cosubstrate, 5,10-methylenetetrahydrofolate, is also present (Santi and McHenry, 1972; Langenbach *et al.*, 1972). It appeared likely, therefore, that column materials containing bound inhibitor might retain a diminished but still significant affinity for the enzyme (Whiteley, 1971). During our investigations into structural factors affecting the interaction of nucleotide analogs with the synthetase, it was determined that these compounds could be coupled to Sepharose, thereby providing the desired affinity column material. Because of the importance of the 5′-phosphate group in the interaction of the nucleotide with the enzyme, it appeared initially that a derivative linked from the 3′ position of the sugar (leaving a free 5′-phosphate group) would be preferable for retention of the enzyme.

Figure 1 illustrates the routes used for the preparation of a 5′-(*p*-aminophenyl phosphate) substituted derivative III of 5-fluorodeoxyuridine I and a similar 3′ derivative VII of 5-fluoro-2′-deoxyuridine 5′-phosphate; similar derivatives were described previously for the attachment of thymidine to Sepharose (Cuatrecasas *et al.*, 1969). 5-Fluoro-2′-deoxyuridine I was converted in high yield to the 3′,5′-diphosphate IV by a carbodiimide-promoted reaction with 2-(cyanoethyl phosphate), followed by hydrolysis. Diesterification was difficult to achieve until a 1:1 mixture of pyridine and dimethylformamide was used and the temperature was raised to 37°. In preliminary experiments, the level of substitution under various reaction conditions was monitored by pmr spectral measurements of the cyanoethyl phosphates which were isolated as their calcium salts.

The diphosphate was then converted to the di-*p*-nitrophenyl ester V by reaction with *p*-nitrophenol under similar forcing conditions to those used in the previous reaction. The diester V was also prepared by the direct interaction of *p*-nitrophenyl phosphate and 5-fluoro-2′-deoxyuridine I. The 5′-nitrophenyl group was removed with snake venom phosphodiesterase and the resulting 3′-*p*-nitrophenyl ester VI was reduced with hydrogen and palladium on charcoal to the desired 3′-(*p*-aminophenyl phosphate) 5′-phosphate derivative VII.

The 5′-monoester was obtained most readily by reacting a 1 M equivalent of *p*-nitrophenyl phosphate and 5-fluoro-2′-deoxyuridine I at ambient temperature. The nitrophenyl ester was reduced to the aminophenyl phosphate derivative III by hydrogenation over palladium on charcoal. Both III and VII were generally isolated as their barium salts by fractional crystallization from ethanol–water mixtures. The free acids tended to decompose rapidly; however, by precipitation with ether and rapid drying under reduced pressure, it was possible to obtain VII in this form for analytical purposes.

Figure 3 shows the pmr spectra of compounds III and VII. The following assignments for III were made by comparison with analogous nucleotide spectra. The two-proton multiplet at 2.2 ppm corresponds to the 2′ substituents, the 3-proton doublet at 4 ppm to the three hydrogens of the 4′ and the 5′ positions, and the 1-proton triplet at 6.1 ppm to the 1′ hydrogen. The 4-proton quartet centered at 6.6 ppm is the aromatic ester substituent and the doublet at 7.6 ppm is the hydrogen substituent at C^6 of the pyrimidine. It is of importance to note that the ratio of proton content corresponding to the pyrimidine C^6 hydrogen substituent and the phenyl ester group is 1:4 and that the single proton is a doublet showing that the fluorine atom is still present in the final product. It is noteworthy that the 3′ and 5′ derivatives appear as a triplet and quartet, respectively, in the two spectra.

Reaction of *p*-nitrophenol with the nucleotide diphosphate IV led to a variety of products which could be resolved by chromatography on DEAE-cellulose; the desired 3′,5′-diester (V) was obtained in a 19% yield. In contrast, the direct interaction of *p*-nitrophenyl phosphate and the nucleoside analog led to a 75% yield of the same compound (V). The two elution profiles recorded when the products from each of the preparative routes were chromatographed on DEAE-cellulose are illustrated in Figure 2.

Table I summarizes the kinetic constants obtained for inhibition of thymidylate synthetase by III and VII. The K_m value for the substrate, 2′-deoxyuridylate, was found to be 1.7×10^{-5} M in phosphate buffer (pH 7.2). This is somewhat higher than the value obtained by Dunlap *et al.* (1971), who

TABLE I: Inhibition Constants Derived for the Interaction of III, VII, and 5-Fluoro-2′-deoxyuridine 5′-Phosphate with Thymidylate Synthetase from Amethopterin-Resistant *L. casei*.[a]

Compound	pH	K_i
1. 5-Fluoro-2′-deoxyuridine 5′-phosphate	7.2	8.3×10^{-9}
2. 5-Fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (III)	6.5	3.5×10^{-6}
	7.2	8.0×10^{-6}
	8.5	8.5×10^{-6}
3. 5-Fluoro-2′-deoxyuridine 3′-(*p*-aminophenyl phosphate) 5′-phosphate (VII)	7.2	5.8×10^{-4}

[a] Enzyme and inhibitor were preincubated for 10 min at 30° in the presence of 5,10-methylene-5,6,7,8-tetrahydrofolate prior to the addition of substrate.

[1] While this work was in progress, Danenberg *et al.* (1972) reported that 2′-deoxyuridine 5′-phosphate attached to Sepharose *via* a 6-*p*-aminobenzamidohexyl group could be used for this purpose. However, these columns had a low capacity for the enzyme and gave a product with low specific activity. In addition, their synthetic procedure had the disadvantage that the product of spacer group and small molecular weight unit (2′-deoxyuridylate) required a mixed solvent system to ensure complete miscibility prior to coupling with the matrix.

used Tris-acetate buffer (pH 6.8). Inhibition by the 5′-nucleotide ester (III) varied little over a wide range of pH (6.5–8.5). Surprisingly, the 3′-*p*-aminophenyl ester derivative (VII) (which contained the free 5′-phosphate) showed a markedly decreased ability to inhibit the synthetase. For comparison, 5 fluoro-2′-deoxyuridine 5′-phosphate showed a K_i value of 8.3 $\times 10^{-9}$ M under these same experimental conditions. In addition, preliminary experiments showed that, when VII was coupled to Sepharose, no absorption of synthetase activity from cell-free extracts could be detected. It was decided, therefore, that superior affinity column materials would result from coupling the inert matrix to the 5′ position of the inhibitor and that the 3′ position should be unsubstituted.

Three types of column materials were prepared. (A) 5-Fluoro-2′-deoxyuridine 5′-(*p*-aminophenyl phosphate) (II) coupled directly to cyanogen bromide activated Sepharose. (B) Sepharose, activated with cyanogen bromide, coupled to diaminohexane and the product reacted with succinic anhydride. The resulting carboxyalkyl-Sepharose was then coupled with the nucleotide 5′-*p*-aminophenyl ester (III) in a carbodiimide-promoted reaction to give a column material in which the inhibitor was extended from the polysaccharide matrix. (C) This Sepharose derivative was prepared as in (B), but all free carboxyl groups which did not react with the inhibitor were blocked by treatment with excess glycinamide.

Each of the column materials was examined for its ability to adsorb the synthetase from cell-free extracts of amethopterin-resistant *L. casei*. The columns were preequilibrated at 4° with 0.01 M phosphate (pH 7.0) containing 0.2 M mercaptoethanol. After dialysis against the same buffer, the cell extract was applied to the columns. Column A would not retain the enzyme even when a mixture of column material and enzyme was preequilibrated in a batchwise procedure with 5,10-methylenetetrahydrofolate for 15 min at 37° prior to elution with 0.1 M phosphate. The inclusion of the cosubstrate in the eluting buffer did not promote adhesion either. It was concluded, therefore, that the large size of the thymidylate synthetase molecule (mol wt ~70,000) precluded interaction of the active site with the inhibitor since it did not protrude appreciably from the matrix. Column material B, which was developed to overcome this obstacle, was superior in several respects. Under conditions similar to those described above, considerable retention of enzyme occurred. Sequential washing of the column with pH 7 phosphate buffers of increasing concentration eluted extraneous protein and, with 0.2 M buffer, thymidylate synthetase was recovered quantitatively. However, the specific activity (1.3 units/mg) indicated that the enzyme was not yet pure and this was confirmed by gel electrophoresis. In an attempt to improve the procedure, two approaches were tried. The first involved recycling the preparation through the column; little additional purification was observed. In the second method, high levels of 2′-deoxyuridine 5′-phosphate were included in the eluent buffer in the hope that the substrate would facilitate a preferential elution of the enzyme; however, even at 10 mM substrate, the selective elution of the enzyme was not observed.

It seemed likely that the inability of material B to achieve complete purification of the enzyme in a single step was due to the ion-exchange capacity of the succinate carboxyl groups. Material C avoided this problem since the free carboxyl groups were blocked by reaction with excess glycinamide (Chen *et al.*, 1972). The validity of this approach is illustrated by the following representative experiment. A cell-free extract (37 ml), containing 0.36 unit/ml of synthetase activity (specific activity 0.017), was applied to a column with dimensions of

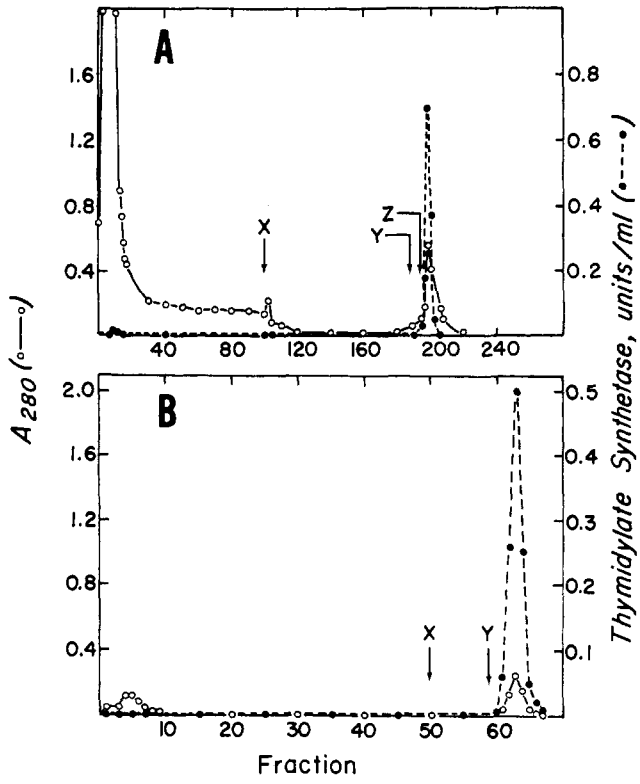

FIGURE 4: (A) Chromatography on affinity column C of a cell-free extract of amethopterin-resistant *L. casei*. Extract (37 ml; 0.36 unit ml) was applied and elution was begun with 0.01 M phosphate buffer (pH 7.0) containing 0.02 M mercaptoethanol. Stepwise increments in buffer concentration are indicated at points *x* (0.05 M), *y* (0.1 M), and *z* (0.2 M); 21-ml fractions were collected up to point *x* and thereafter the volume was reduced to 6 ml. (B) Rechromatography of peak fractions from (A) on the same column. Elution was initiated with 0.01 M phosphate buffer (pH 7.0) containing 0.02 M mercaptoethanol. Stepwise increments were applied at *x* (0.1 M) and *y* (0.3 M).

1.25 × 12 cm, representing 15 ml of packed material C. A separate experiment indicated that this column material had a capacity to absorb 2.32 units of synthetase/ml of packed volume. Stepwise elution with a series of phosphate buffers ranging from 0.01 to 0.2 M gave the profile depicted in Figure 4A. A repetition of the procedure (Figure 4B) led to pure material (specific activity 3.3). The sharpest band of enzyme and the highest specific activity was obtained when elution of the second column was carried out with 0.3 M phosphate buffer. These results are observed when the same column is used in both stages, however, for practical reasons, it has proven more convenient to use two identical columns in sequence. The purified enzyme showed a single band on electrophoresis (Figure 5) and, when stained for catalytic activity (Dunlap *et al.*, 1971), the fluorescent zone coincided with the protein stain. The overall recovery of enzymatic activity was greater than 50% (approximately 75% on each passage through the column), with a purification, in this preparation, of approximately 200-fold from the cell-free extract. Purified enzyme of the same high specific activity was obtained in each experiment; however, the specific activity of the crude cell extracts varied from 0.003 to 0.050, hence this overall purification is a variable figure.

If all enzyme-containing fractions are included in the eluted extract, the recovery of activity increases to 85% in each step; however, this gain is offset by a decrease in specific activity. Therefore, only the peak fractions are included in preparations requiring enzyme of the highest purity. Approximately 1 →

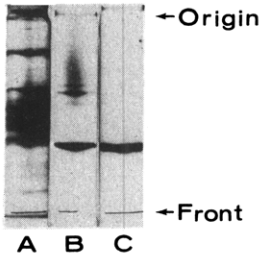

FIGURE 5: Polyacrylamide gel electrophoresis of: (A) cell-free extract; (B) preparation from the first column; and (C) preparation from the second column. The gels are stained for protein (see Experimental Section).

2% of the applied activity usually elutes with the first major peak (Figure 4A). The high specific activity of the final product, the repeated use of the same column materials over a 6-month period, and the lack of any 268-nm absorbing material being present in the effluxing buffers used to preequilibrate the column materials indicate that matrix C is stable and that hydrolysis of inhibitor is minimal.

The affinity column (material C) described above is now being used for the routine isolation of the amethopterin-resistant *L. casei* enzyme. In addition, these columns are being tested for their ability to isolate thymidylate synthetases from both the wild-type *L. casei* and from an amethopterin-resistant strain of L1210 mouse leukemia cells.

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