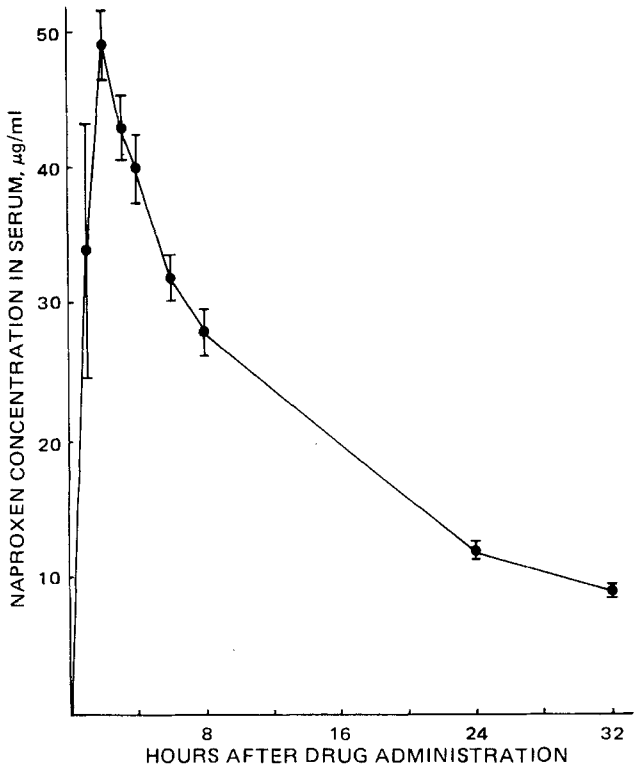

Figure 3—*Mean (±SEM) serum levels of naproxen in humans (n = 6) following single-dose oral administration of 250 mg of drug as a compressed tablet.*

of the linearity of the standard curve, serum concentrations up to 250 μg/ml can be measured using 0.1 ml of sample.

Several drugs at normal serum concentrations were examined for interference with this fluorometric assay of naproxen. Basic drugs were removed in the extraction procedure and caused no interference. The following acidic drugs also did not interfere: chlorpropamide, furosemide, ibuprofen, indomethacin, mefenamic acid, phenobarbital, phenylbutazone, phenytoin, sulfamethoxazole, and warfarin. Interference was observed with salicylic acid; 200 μg of salicylic acid/ml of serum gave a fluorescence value equivalent to about 10 μg of naproxen/ml. Therefore, this fluorometric assay method is not suitable for the determination of serum levels of naproxen in patients simultaneously receiving salicylates.

Interference by metabolites of naproxen was not studied, because the concentrations of metabolites in serum after naproxen administration are negligible (3).

The precision of the method was determined at three different concentrations. Naproxen solution was added to serum to give final concentrations of 5, 50, and 200 μg/ml. Ten 0.1-ml samples of each serum solution were analyzed by the proposed method at the same time. The relative standard deviations obtained were 2.6, 1.4, and 1.1%, respectively.

To test the accuracy of the method, 32 serum samples containing unknown concentrations of naproxen were assayed by both the present method and the GLC method (2). The results (Fig. 2) showed excellent correlation between the two methods.

To demonstrate the utility of this fluorometric method, serum concentrations of naproxen were measured in six normal human subjects after oral administration of a single dose of 250 mg of naproxen (one 250-mg tablet). The resulting serum levels (Fig. 3) agreed well with the serum levels of naproxen obtained by other workers using the GLC method (2).

The described fluorometric method is simple, rapid, and sensitive and can serve as a useful tool in the study of biopharmaceutics and pharmacokinetics of naproxen.

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Fluorescent Inhibitors of Thymidylate Synthetase

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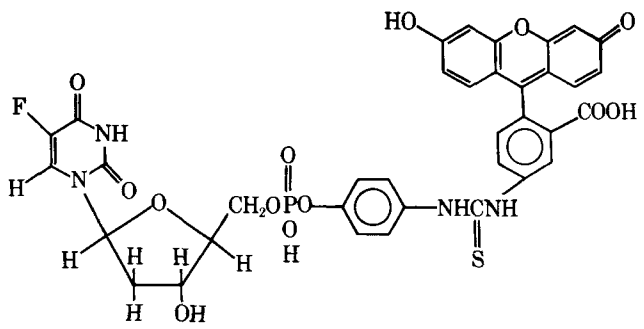
Abstract □ Two fluorescent derivatives of 2′-deoxy-5-fluorouridine 5′-*p*-aminophenyl phosphate were prepared by treatment of this compound with fluorescein isothiocyanate in dimethyl sulfoxide or 5-(dimethylamino)naphthalenesulfonyl chloride in pyridine. The products of the reactions were isolated by diethylaminoethylcellulose chromatography and were shown to be homogeneous by polyacrylamide electrophoresis and TLC. Confirmation of the structure was provided by elemental analysis, absorption and fluorescence spectra, PMR measurements, and liberation of nucleotide upon hydrolysis with snake venom phosphodiesterase. The fluorescent derivatives are good competitive inhibitors ($K_i \simeq 10^{-6}$ *M*) of thymidylate synthetase from a methotrexate-resistant strain of *Lactobacillus casei*.

Keyphrases □ Thymidylate synthetase—inhibition by fluorescent derivatives of 2′-deoxy-5-fluorouridylate ester evaluated □ 2′-Deoxy-5-fluorouridylate ester—fluorescent derivatives synthesized, inhibition of thymidylate synthetase evaluated □ Enzyme inhibitors—fluorescent derivatives of 2′-deoxy-5-fluorouridylate ester synthesized, effect on thymidylate synthetase

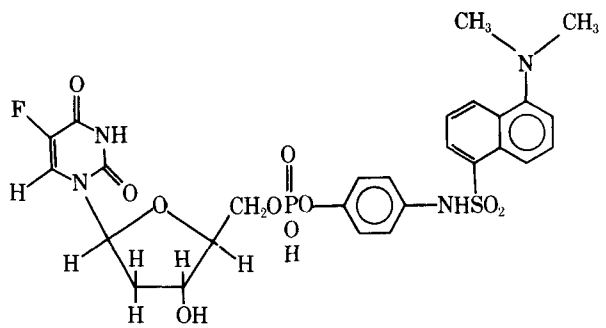
The *de novo* enzymatic synthesis of thymidylate (2′-deoxy-5-methyluridylate) is achieved by transfer of the methylene substituent of 5,10-methylenetetrahydrofolate to 2′-deoxyuridylate with the associated conversion of the hypothetical methylene intermediate (1) to methyl using the inherent reducing power of the folate cofactor (Scheme I). A metabolic cycle is completed when the ensuing dihydrofolate is reduced to the tetrahydro level in the presence of dihydrofolate reductase, and an additional one-carbon unit is reintroduced enzymatically (2). Because

5,10-methylenetetrahydrofolate + 2′-deoxyuridylate $\xrightarrow{\text{thymidylate synthetase}}$ dihydrofolate + thymidylate

Scheme I



I



II

thymidylate is a required precursor of DNA, these two enzymes are essential for cellular replication; their interrelationship and mechanism of action have been of considerable interest during the past decade (3, 4).

Earlier experiments showed that fluorescein isothiocyanate coupled *via* a diaminopentyl group to methotrexate gives a compound that exhibits significant fluorescence changes in the presence of dihydrofolate reductase but retains its inhibitory properties and coelectrophoreses with this enzyme on polyacrylamide gels (5). As a result of this observation and earlier reports by Stryer (6) who demonstrated how variations in fluorescence intensity could be used as indicators of the nature of the heme-binding site in myoglobin, it appeared possible that similar fluorescent derivatives of the potent inhibitor of thymidylate synthetase, 2′-deoxy-5-fluorouridylate[1], might be useful as probes for the active site of this enzyme.

The syntheses and properties of the fluorescein and 5-(dimethylamino)naphthalenesulfonyl derivatives of 2′-deoxy-5-fluorouridylate are described in this paper. The two compounds (I and II) were obtained by condensing equimolar quantities of 2′-deoxy-5-fluorouridine 5′-*p*-aminophenyl phosphate (III), a compound previously synthesized in this laboratory (7), with fluorescein isothiocyanate[2] in dimethyl sulfoxide or 5-(dimethylamino)-naphthalenesulfonyl chloride[2] in pyridine. In each case, the desired fluorescent products were purified by chromatography on diethylaminoethylcellulose[3] and isolated by lyophilization. Further purification was achieved by fractional crystallization of the barium salts from ethanol–water. When I and II were irradiated at 492 and 360 nm, respectively, they showed strong fluorescence maxima at 523 and 543 nm, respectively, and the intensity varied markedly with the polarity of the solvent. Both compounds were good competitive inhibitors of thymidylate synthetase from a methotrexate-resistant strain of *Lactobacillus casei* (4).

EXPERIMENTAL[4]

Materials and Methods—Quantum yields were determined by comparison with quinine sulfate as a standard. Two systems were used for TLC: A, cellulose layers developed with 1-butanol–5 *N* acetic acid (2:1); and B, diethylaminoethylcellulose layers developed with 0.5 *M* ammonium acetate.

Thymidylate synthetase was isolated from a methotrexate-resistant strain of *L. casei* according to the procedure of Whiteley *et al.* (7) and was assayed spectrophotometrically (8). 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. Disc electrophoresis on 7.5% (w/v) polyacrylamide gels was carried out in 0.6 × 6-cm tubes. Enzymatic hydrolysis of the fluorescent derivatives was achieved by incubating an aliquot of the sample with snake venom phosphodiesterase[5] in 0.5 *M* tromethamine hydrochloride[2] buffer, pH 9, at ambient temperatures for 1 hr. The nucleotide product was identified by comparison with an authentic standard on TLC using System A.

Preparation of Fluorescent Derivatives of III—*Fluorescein Derivative (I)*—Fluorescein isothiocyanate (61.8 mg, 0.159 mmole) and the barium salt of III (89.7 mg, 0.159 mmole) were dissolved in dimethyl sulfoxide (1 ml). After standing for 4 hr at room temperature, the solution was applied to a 2 × 40-cm column of diethylaminoethylcellulose, which was then washed with 0.3 *M* ammonium acetate (2 liters). The product (I) was recovered by eluting with a 0.6 *M* solution of the same buffer, followed by lyophilization (126 mg, 99%). The product was either isolated as the free acid at this stage by trituration with 1-propanol and filtration or converted to the barium salt by dissolving in water and adjustment of the resulting solution to pH 8 with saturated barium hydroxide.

After lyophilization, the residue was taken up in the minimum quantity of water, precipitated with absolute ethanol, filtered, and dried; TLC: System A, R_f 0.69; System B, R_f 0.22; λ_{max} (0.1 *M* HCl) 251 and 438 nm (ϵ 41,200 and 40,000); λ_{max} (0.1 *M* potassium phosphate, pH 7) 236, 262, and 492 nm (ϵ 48,200, 35,300, and 53,900); λ_{max} (0.1 *M* NaOH) 237, 260, and 488 nm (ϵ 56,700, 34,600, and 67,700); fluorescence maximum (0.1 *M* potassium phosphate, pH 7): 492 nm (excitation) and 523 nm (emission) (quantum yield = 0.51); PMR (D_2O): δ 2.1 (2H, m) (2′-protons), 4.0 (3H, m) (4′- and 5′-protons), 4.3 (1H, m) (3′-proton), 6.0 (1H, m) (1′-proton), and 6.2–7.8 (broad multiplet containing the remaining 14 nonexchangeable proteins attached to unsaturated carbon).

Anal.—Calc. for $C_{36}H_{28}FN_4O_{13}PS \cdot 5.5\ H_2O$: C, 47.74; H, 4.34. Found: C, 47.48; H, 3.93.

5-(Dimethylamino)naphthalenesulfonyl Derivative (II)—A mixture of 5-(dimethylamino)naphthalenesulfonyl chloride (59.4 mg, 0.22 mmole) and the barium salt of III (113 mg, 0.2 mmole) dissolved in pyridine (5 ml) was allowed to react at ambient temperatures for 20 hr. The solution was then applied directly to a 2 × 40-cm column of diethylaminoethylcellulose. Application of water (300 ml) followed by 0.2 *M* ammonium bicarbonate (2 liters) eluted the gold fluorescent product (II). The principal fractions were combined and lyophilized (111 mg, 86%). The free acid was purified by dissolving the residue in a minimum quantity of methanol, followed by precipitation with ether. To obtain the barium salt, the material was taken up in water (15 ml), adjusted to pH 8 with barium hydroxide, filtered, and lyophilized.

The residue was dissolved in a minimum quantity of water, and the nucleotide was precipitated by the addition of acetone; TLC: System A, R_f 0.60; λ_{max} (0.1 *M* HCl) 275 and 320 nm (ϵ 13,300 and 2850); λ_{max} (0.1 *M* potassium phosphate, pH 7) 248 and 320 nm (ϵ 17,800 and 4040); λ_{max} (0.1 *M* NaOH) 244 and 318 nm (ϵ 22,400 and 5240); fluorescence maximum (0.1 *M* potassium phosphate, pH 7): 360 nm (excitation) and 543 nm (emission) (quantum yield = 0.02); PMR (D_2O): δ 2.0 (8H, m) (*N*-dimethyl and 2′-protons), 3.9 (3H, broad s) (4′- and 5′-protons), 4.2 (1H, m) (3′-proton), 6.0 (1H, m) (1′-proton), and 6.3–8.2 (broad multiplet containing the remaining 11 nonexchangeable protons attached to unsaturated carbon).

Anal.—Calc. for $C_{27}H_{28}FN_4O_{10}PS \cdot 2.5H_2O$: C, 46.62; H, 4.78. Found: C, 46.63; H, 5.03.

[1] Floxuridine is the USAN name for 2′-deoxy-5-fluorouridine.
[2] Sigma Chemical Co.
[3] Whatman Biochemicals, Ltd.

[4] Absorption, fluorescence, and PMR spectra were recorded with Cary model 14, Turner model 210, and Jeol model JNM-PS-100 (courtesy of Dr. J. Rivier, Salk Institute for Biological Studies) spectrometers, respectively.
[5] Worthington Biochemical Corp.

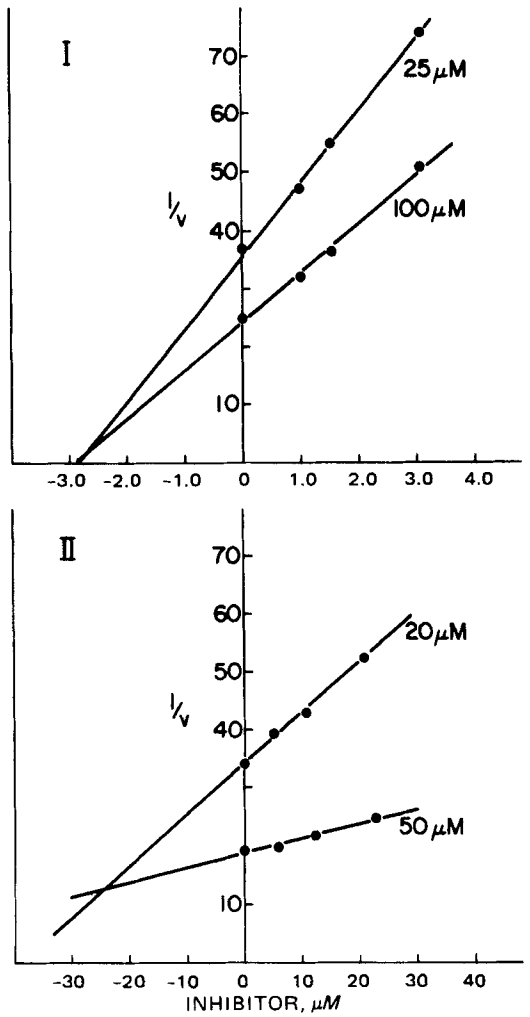


Figure 1—*Inhibition of thymidylate synthetase by I and II. The activity of the synthetase from a methotrexate-resistant strain of* L. casei *(specific activity = 3; 1.2 μg of protein) was measured as described under* Experimental. *Enzyme, 5,10-methylenetetrahydrofolate, and inhibitor were incubated together for 10 min prior to the addition of the indicated concentrations of 2′-deoxyuridylate to initiate the reaction.*

RESULTS AND DISCUSSION

The two fluorescent derivatives (I and II) were shown to be homogeneous by polyacrylamide electrophoresis and TLC. Structure confirmation was afforded by hydrolysis with snake venom phosphodiesterase to liberate free 2′-deoxy-5-fluorouridylate, elemental analysis, and PMR and absorption spectra. The absorption spectrum of each product contained the chromophore of the fluorescent moiety superimposed on that of the nucleotide ester; however, the absorptivity of the fluorescein derivative at 492 nm (pH 7) was only 54,000. This figure is 26% lower than the value recorded for free fluorescein under the same conditions (9), which suggests that there may be some interaction between the pyrimidine and fluorescein components.

This inference is supported (Table I) by the lower quantum yield (0.51) recorded for this compound when compared with the quantum yield (0.64) of free fluorescein at pH 7 (10). In the presence of ethanol, the quantum yield was depressed a further 40%, suggesting that the interaction of the two chromophores is increased in the less polar solvent. Derivative II showed a much smaller efficiency of fluorescence (quantum yield = 0.019, *cf.*, Table I); but in the presence of increasing amounts of ethanol, solutions of this compound showed a progressive increase in quantum yield. These disparate observations suggest that the two derivatives may complement each other when used as probes for the active site of the enzyme.

Table I—Quantum Yields for I and II

Derivative	Solvent	Quantum Yield
I	0.1 *M* PO_4, pH 7.0	0.51
	0.1 *M* PO_4–25% ethanol	0.31
	0.1 *M* PO_4–50% ethanol	0.32
II	0.1 *M* PO_4, pH 7.0	0.019
	0.1 *M* PO_4–25% ethanol	0.047
	0.1 *M* PO_4–50% ethanol	0.10

The inhibition constants (K_i) derived at pH 7 for the interaction of purified thymidylate synthetase with I, II, and III were 2.8, 24, and 8 μM, respectively. Clearly, introduction of the fluorescent groups made little change in the inhibitory properties. In fact, the large fluorescein moiety slightly enhanced the inhibitory capacity of the nucleotide ester, although a small decrease was observed for II. That the inhibition (for both I and II) was competitive with the normal substrate of the reaction is shown in Fig. 1, where the reciprocal velocity is plotted *versus* the inhibitor concentration at different concentrations of the substrate (2′-deoxyuridylate).

The strongly inhibitory properties demonstrated by I and II, coupled with their observed variation in fluorescence intensity with solvent polarity, suggest that these compounds may be useful aids for probing the active site of thymidylate synthetase. The compounds may provide information concerning the interaction of the nucleotide substrate or its fluorinated analog with the synthetase and may also have an additional function as visual protein markers during enzyme purification.

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