Fluorescent Analogues of Methotrexate: Characterization and Interaction with Dihydrofolate Reductase†

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ABSTRACT: The dansylated derivatives of lysine and ornithine analogues of methotrexate exhibit fluorescence properties characteristic of the dansyl moiety with an excitation at 328 nm and an emission maximum at 580 nm in aqueous media. As in the case of dansyl amino acids, the fluorescence emission is dependent upon the polarity of the medium. In solvents of low dielectric constant there is an enhancement of the dansyl fluorescence intensity as well as a shift to shorter wavelengths. The dansylated analogues show a reduction in the quantum yields as compared to N^{ϵ}-dansyl-L-lysine and 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonic acid. The absorption spectra of the two dansyl analogues are similar to the spectra of the parent basic amino acid precursors but with reduced molar extinction values. The two fluorescent analogues of methotrexate were found to be potent inhibitors of purified dihydrofolate reductases from *Lactobacillus casei* and from chicken liver. The binding of these fluorescent analogues to either dihydrofolate reductase resulted in 10–15-nm blue shift of the ligand emission maxima and a 2–5-fold enhancement of the emission. These fluorescent properties of the bound ligands indicate a possible interaction of the dansyl moiety with a region on the enzyme molecule which is more hydrophobic relative to the surrounding solvent.

The classical folic acid antagonist methotrexate (MTX)[1] is one of the most widely used clinical antitumor agents and has been employed in the treatment of leukemias, lymphomas, psoriasis, and other clinical disorders (Bertino & Johns, 1972). A number of MTX derivatives have been synthesized and characterized with respect to in vitro inhibition of dihydrofolate reductase (DHFR), transport into normal and drug-resistant cells grown in culture, and in vivo antitumor activity (Chaykovsky et al., 1975; Rosowsky et al., 1977; Montgomery et al., 1979 and references cited therein). The binding of MTX to its primary intracellular target, DHFR, has been very well characterized. X-ray crystallographic studies of enzymes from *Lactobacillus casei* and *Escherichia coli* (Matthews et al., 1977, 1978) as well as that from chicken liver (Volz et al., 1982) reveal that certain invariant residues in the primary sequence of DHFR play an important role in the binding of MTX and other folate antagonists at the active site. A specific charge interaction between the α-carboxylate of the L-glutamate moiety of MTX and an invariant arginine residue has been implicated to be important in the binding of MTX to DHFR. The importance of free α-carboxylate on the MTX molecule for binding has been further substantiated from the studies with α- and γ-carboxyl-modified MTX derivatives (Piper & Montgomery, 1979; Piper et al., 1982; Rosowsky et al., 1981a,b). These studies indicate that the presence of various groups on the γ-carboxylate of MTX does not significantly alter the binding of these MTX derivatives to DHFR as evidenced from the inhibition studies. For example, the MTX-γ-benzylamide derivative, despite the bulkiness of the benzyl ring, was shown to be a good inhibitor of L1210 DHFR (Piper et al., 1982). However, the α-carboxyl-substituted derivatives were found to be considerably less inhibitory as compared to MTX. Under physiological conditions, MTX is converted to γ-polyglutamyl derivatives (Baugh et al., 1973). These derivatives seem to possess the same affinity for DHFR as MTX (Galivan, 1980; Fry et al., 1982), further indicating that substituents on the γ-carboxyl group of MTX do not significantly alter enzyme binding.

Recently we reported the synthesis and properties of lysine and ornithine analogues of MTX (Kempton et al., 1982; Kumar et al., 1983a). These analogues possess the essential free α-carboxyl group required for binding to the DHFR and, in addition, contain a terminal amino group instead of a carboxylate group. In spite of the charge difference, both analogues are potent inhibitors of DHFR. Furthermore, the presence of a free amino group provides an excellent attachment point for additional substituent groups. As a result, we have synthesized two fluorescent analogues of MTX using the lysine and ornithine analogues and dansyl chloride (DNS-Cl) (Kumar et al., 1983a,b). Gapski et al. (1975) have reported the synthesis of a fluorescein conjugate of MTX (MTX-F) in which fluorescein–aminopentane was linked to the α- and/or γ-carboxyl group(s) of MTX by using a carbodiimide coupling procedure. The MTX-F conjugate has been used in recent years as an intracellular marker for DHFR although its transport into cells is extremely slow (Kaufman et al., 1978; Henderson et al., 1980). An important consequence of carbodiimide coupling during the synthesis of MTX-F is the possible generation of the α-carboxyl conjugate and also racemization of the glutamate moiety. During the synthesis of α- and γ-glutamate peptides of MTX, Rosowsky & Yu (1978) observed that the α-substituted isomer was the major product in direct condensation reactions using peptide bond forming reagents such as *N*,*N*′-dicyclohexylcarbodiimide. For these reasons, a direct reaction of DNS-Cl with the ϵ- or

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[1] Abbreviations: MTX, 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid; FAH_2, 2-amino-4-hydroxy-7,8-dihydropteroylglutamic acid; APA-Orn, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-ornithine; APA-Lys, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine; APA-Orn-DNS, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{δ}-[5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonyl]-L-ornithine; APA-Lys-DNS, N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ϵ}-[5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonyl]-L-lysine; *Q*, quantum yield; DNS-Cl, 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonyl chloride; DNSA, 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonic acid; DHFR, dihydrofolate reductase.

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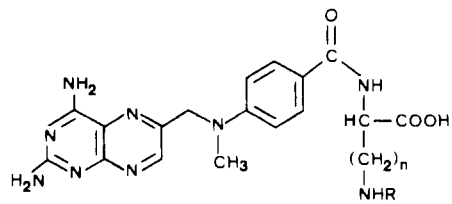

COMPOUND	n=	R=
APA-Orn	3	H
APA-Orn-DNS	3	DNS
APA-Lys	4	H
APA-Lys-DNS	4	DNS

FIGURE 1: Structures of methotrexate analogues.

δ-amino groups on lysine or ornithine analogues of MTX provides fluorescent analogues of MTX with free α-carboxyl groups. In this communication we report the characterization and interaction of these dansyl analogues with dihydrofolate reductase from bacterial and avian sources.

Materials and Methods

Methotrexate (MTX) was obtained from Lederle Laboratories (Pearl River, NY). NADPH from P-L Biochemicals. Dihydrofolic acid (FAH_2) was prepared by the dithionite reduction of folic acid according to the method of Blakely (1960) and stored in 1 mM HCl. Methanol, 1-propanol, and 1-butanol (HPLC grades) were from Burdick and Jackson Laboratories (Muskegon, MI). N^{ϵ}-Dansyl-L-lysine was purchased from Sigma Chemical Co. (St. Louis, MO).

The analogues of MTX (Figure 1) were prepared by methods published earlier (Kempton et al., 1982; Kumar et al., 1983a) and were purified by high-performance liquid chromatography (Kumar et al., 1983b). Dihydrofolate reductases from chicken liver (Kaufman & Kemerer, 1977) and *L. casei* (Dann et al., 1976), purified by affinity chromatography, were used in these studies. Protein concentration was determined by absorbance at 280 nm or by MTX titrations. The enzyme was assayed for activity as described previously (Kumar et al., 1983a). The enzyme (ca. 10^{-7} M) was preincubated for 2 min with inhibitor and the remaining enzyme activity determined by measuring the decrease in absorbance at 340 nm following the addition of NADPH (100 μM) and FAH_2 (98 μM). The residual activity in the presence of inhibitor is expressed as a percent of the activity observed in the absence of inhibitor. The enzyme assays as well as the absorption spectra of the inhibitors were performed by using a Cary 219 recording spectrophotometer (Varian Instruments). The sample compartment was maintained at 20 °C by using a circulating water bath.

Fluorescence measurements were carried out by using a Perkin-Elmer Model MPF-44A spectrofluorometer operated in the ratio mode. The emission and the excitation monochromators were set at 6.0 nm. A Perkin-Elmer microprocessor-controlled differential corrected spectra unit (DCSU) was employed to obtain corrected emission and excitation spectra. The emission and excitation modes were calibrated by following the manufacturer's instructions using Rhodamine 6B in ethylene glycol. The samples were scanned at a rate of 120 nm/min, and the absorbance of the samples was not allowed to exceed a value of 0.2 at the exciting wavelength. Fluorescence quantum yields (Q) were determined by comparing the fluorescence of the unknown samples with that of a sample of known quantum yield. The reference standards employed were quinine sulfate in 0.1 N sulfuric acid (Q = 0.55) (Parker & Rees, 1960) and 5-(N,N-dimethylamino)-1-naphthalenesulfonic acid in 0.1 M sodium bicarbonate (Q = 0.36) (Chen, 1966).

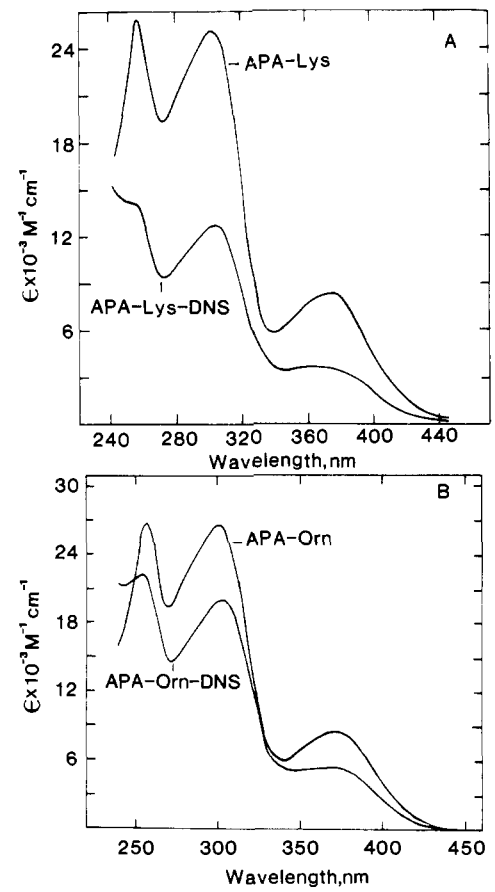

FIGURE 2: Absorption spectra of lysine and ornithine analogues of methotrexate. (A) Absorption spectra of APA-Lys and APA-Lys-DNS in 0.1 N KOH. (B) Absorption spectra of APA-Orn and APA-Orn-DNS in 0.1 N KOH.

Results

Characterization of Methotrexate Analogues. (1) Spectral Properties. The absorption spectra of the two fluorescent analogues APA-Lys-DNS and APA-Orn-DNS together with their precursors APA-Lys and APA-Orn in 0.1 N KOH are shown in Figure 2. The four compounds exhibit absorbance maxima in the wavelength regions 250, 300, and 370 nm similar to that of the parent compound methotrexate. An examination of the absorption spectrum of N^{ϵ}-dansyl-L-lysine in 0.1 N KOH (results not shown) indicated that it has a maximum at 314 nm near the absorption maximum of APA-Lys-DNS (303 nm). Determination of molar extinction (ϵ_M) values for the dansyl analogues gave values of 13 200 and 20 200 for APA-Lys-DNS and APA-Orn-DNS, respectively, at 304 nm. These ϵ_M values are lower than the values for corresponding precursors, viz, APA-Lys (24 800) and APA-Orn (25 600) at 302 nm. The decreased ϵ_M values for the dansyl analogues were observed at all three wavelength regions of maximum absorption (250, 300, and 370 nm) and in neutral (pH 7.0) as well as acidic (pH 1.8) media.

(2) Fluorescence Properties. The corrected fluorescence excitation and emission spectra of APA-Lys-DNS and APA-Orn-DNS in 0.1 M potassium phosphate, pH 7.0, indicate that both analogues exhibit identical emission spectra with a broad emission peak in the region 560–590 nm. The emission maximum was centered at ca. 580 nm for both compounds in aqueous media. The two dansyl analogues have similar

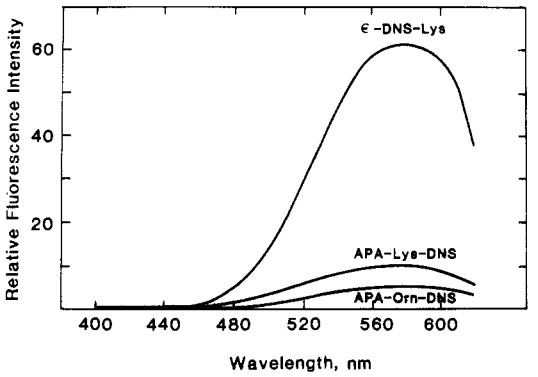

FIGURE 3: Relative fluorescence emission of N^{ϵ}-DNS-Lys, APA-Lys-DNS, and APA-Orn-DNS. Equimolar concentrations (27.7 μM) of N^{ϵ}-DNS-Lys, APA-Lys-DNS, and APA-Orn-DNS in 0.1 M potassium phosphate, pH 7.0, were excited at 328 nm (22 °C), and corrected emission spectra were recorded from 400 to 620 nm.

Table I: Fluorescence Quantum Yields and Fluorescence Maxima of N^{ϵ}-DNS-L-Lys, APA-Lys-DNS, and APA-Orn-DNS[a]

compound	fluorescence: excitation max (nm)	fluorescence: emission max (nm)	relative quantum yields: Q^b	relative quantum yields: Q^c
N^{ϵ}-DNS-L-Lys	328	580	0.055	0.056
APA-Lys-DNS	328	580	0.0067	0.0065
APA-Orn-DNS	328	580	0.0035	0.0035

[a] Fluorescence spectra were determined in 0.01 M Tris-HCl, pH 7.0, at 25 °C. [b] Based on quinine sulfate in 0.1 N sulfuric acid as the standard (Parker & Rees, 1960). [c] Based on DNSA in 0.1 M sodium bicarbonate as the standard (Chen, 1966; Seiler et al., 1973).

excitation spectra with a maximum at 328 nm. An examination of the fluorescence properties of N^{ϵ}-dansyl-L-lysine in 0.1 M potassium phosphate, pH 7.0, revealed that this compound also has an excitation maxima at 328 nm. Chen has reported a wavelength of 332 nm for the excitation maxima of dansyl amino acids in 0.01 M Tris-HCl, pH 7.0 (Chen, 1967). The excitation spectrum for N^{ϵ}-dansyl-L-lysine (results not shown) was identical with that exhibited by the two dansyl analogues of MTX in 0.1 M potassium phosphate, pH 7.0. The individual corrected fluorescence emission spectra of equimolar amounts of N^{ϵ}-dansyl-L-lysine, APA-Lys-DNS, and APA-Orn-DNS in 0.1 M potassium phosphate, pH 7.0, are shown in Figure 3. The compounds were excited at 328 nm and the corrected emission spectra were recorded from 400 to 620 nm. The results indicate that there is a loss of intrinsic fluorescence of the dansyl moiety when attached to APA-Lys or APA-Orn. Furthermore, the extent of the decrease in fluorescence is different for the two analogues (8-fold for APA-Lys-DNS and 15-fold for APA-Orn-DNS) as compared to N^{ϵ}-dansyl-L-lysine. This intrinsic quenching of the dansyl fluorescence emission is further reflected in the low values obtained for quantum yields for the two analogues. Table I summarizes the relative quantum yield values for N^{ϵ}-dansyl-L-lysine, APA-Lys-DNS, and APA-Orn-DNS as compared to standards. The corrected emission spectra for the three test compounds were recorded in 0.01 M Tris, pH 7.0, and the total fluorescence emission in the region 400–620 was used for the determination of quantum yields. The results again indicate that the relative quantum yield for APA-Lys-DNS (0.0065) was about 12% of that of N^{ϵ}-dansyl-L-lysine (0.055) and for APA-Orn-DNS (0.0035) the value was 6% of that of N^{ϵ}-dansyl-L-lysine. Chen (1967) has reported a value

Table II: Fluorescence Emission of APA-Lys-DNS and APA-Orn-DNS in Solvents of Decreasing Dielectric Constant[a]

solvent	APA-Lys-DNS: emission max (nm)	APA-Lys-DNS: fluorescence	APA-Orn-DNS: emission max (nm)	APA-Orn-DNS: fluorescence	dielectric constant (D)
0.01 M Tris, pH 7.0	580	4	580	2	
methanol	530	32	530	18	32.6
ethanol	524	41	523	28	24.3
1-propanol	520	50	521	33	20.1
1-butanol	518	56	520	36	17.1
acetone	520	11	510	5	20.7
ethyl acetate	502	30	505	19	6.0
dioxane	497	67	502	42	2.2

[a] Stock solutions of APA-Lys-DNS and APA-Orn-DNS in 20 μL of ethanol were diluted to 3.0 mL with the indicated solvent such that the final concentrations were 10.6 and 8.0 μM, respectively. Corrected fluorescence emission spectra were obtained by exciting the samples at 328 nm and at 25 °C.

of 0.053–0.091 for the quantum yields of dansyl amino acids in 0.01 M Tris-HCl, pH 7.0. A quantum yield value of 0.026 has been quoted for N^{ϵ}-dansyl-L-lysine in water (Seiler & Demisch, 1977), a value slightly lower than that determined in these studies.

The fluorescence emission of the dansyl moiety is dependent upon the polarity of the environment surrounding the dansyl group. Chen (1967) reported an enhancement as well as a blue shift in the emission maximum for dansyl tryptophan when the dansyl amino acid was excited in solvents of decreasing dielectric constant. The fluorescence emission of the two dansyl analogues of MTX was examined in solvents of decreasing dielectric constant. The results, shown in Table II, indicate an enhancement of the fluorescence as well as shift to shorter wavelengths in the emission maximum for both analogues. The maximum enhancement and blue shift were observed in dioxane. For APA-Lys-DNS the enhancement of the fluorescence was 17-fold and the shift in the emission maximum was 83 nm. APA-Orn-DNS fluorescence in dioxane was enhanced 21-fold with a 78-nm blue shift in the emission maximum.

Interaction of MTX Analogues with Dihydrofolate Reductase. (1) Inhibition of Dihydrofolate Reductase. Methotrexate is a very potent, stoichiometric inhibitor of dihydrofolate reductase (Werkheiser, 1961). The analogues APA-Orn-DNS and APA-Lys-DNS contain the essential structural binding features of MTX such as the 2,4-diaminopyrimidine grouping and the free α-carboxyl group of the L-Glu moiety which are necessary for potent enzyme inhibition. Our preliminary experiments have shown that the precursors of these two analogues are also potent inhibitors of dihydrofolate reductase (Kempton et al., 1982; Kumar et al., 1983a). For determination of the relative potencies of these two fluorescent analogues, compared to MTX, as inhibitors of dihydrofolate reductase, homogeneous dihydrofolate reductases from chicken liver and from *L. casei* were used. The results are shown in Figure 4. Both enzymes were inhibited by MTX in a linear, stoichiometric manner up to 10% of the original activity at pH 7.0. The relative efficiencies of the two derivatives to completely inhibit both the enzymes was obtained by extrapolating the linear portion of the inhibition curve. The results indicate that a 1.3-fold excess of APA-Orn-DNS and a 2-fold excess of APA-Lys-DNS were able to cause total inhibition of the bacterial dihydrofolate reductase. On the other hand, the complete inhibition of the avian dihydrofolate reductase required a 1.4-fold excess of APA-Orn-DNS and

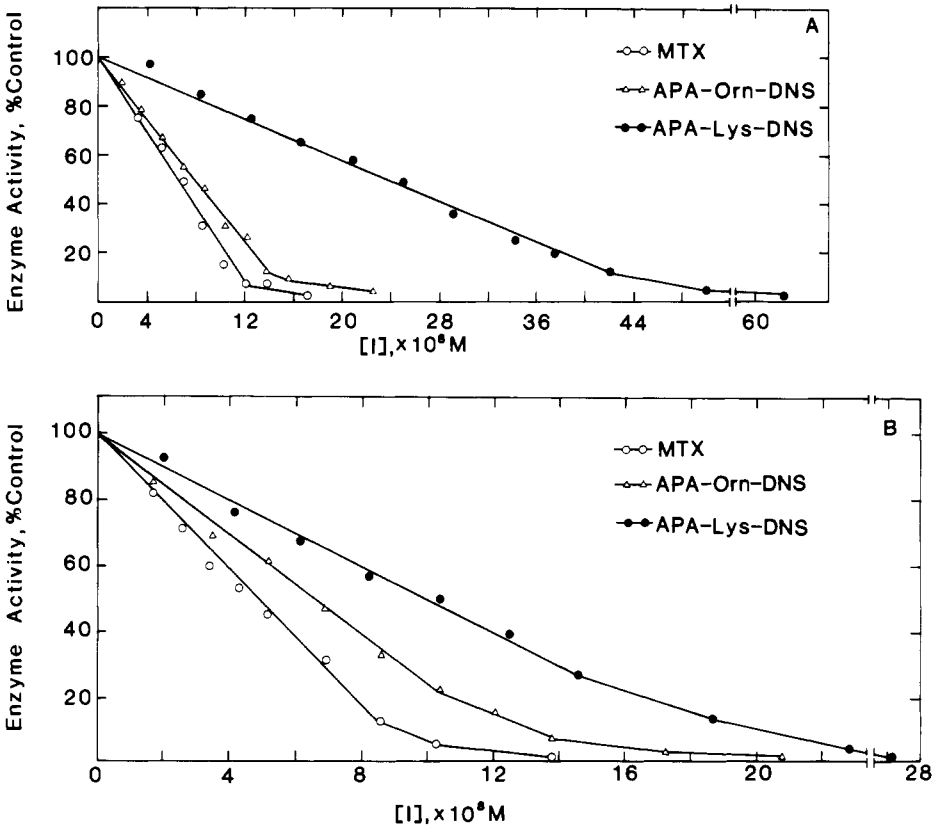

FIGURE 4: Inhibition of dihydrofolate reductase by MTX and MTX analogues. (A) Inhibition of chicken liver dihydrofolate reductase (1.1×10^{-7} M) and (B) *L. casei* dihydrofolate reductase (1.0×10^{-7} M) by MTX (○), APA-Orn-DNS (△), and APA-Lys-DNS (●). The enzymes were incubated with the indicated concentrations of the inhibitor for 2 min in 0.1 M Tris-HCl, pH 7.0, at 20 °C. The residual enzyme activity was determined following addition of NADPH (100 μM) and FAH_2 (98 μM). The enzyme activity was determined by measuring the rate of decrease of the absorbance at 340 nm with a Cary 219 recording spectrophotometer.

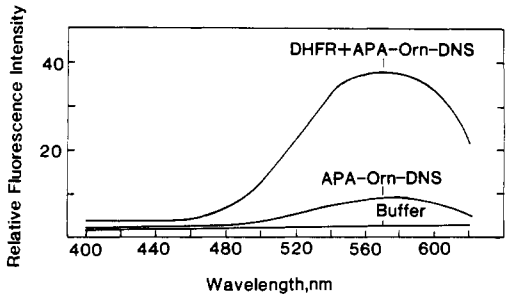

FIGURE 5: Enhancement of dansyl fluorescence emission on binding to dihydrofolate reductase. Corrected emission spectra of free APA-Orn-DNS (1.99×10^{-6} M) and its complex with *L. casei* dihydrofolate reductase (2.93×10^{-6} M) in 0.1 M potassium phosphate, pH 7.0, at 22 °C. The samples were excited at 328 nm.

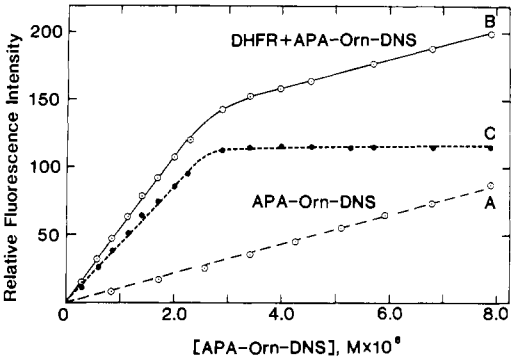

FIGURE 6: Fluorescence titration of *L. casei* dihydrofolate reductase with APA-Orn-DNS. Fluorescence emission of increasing concentrations of APA-Orn-DNS in the absence of (A) and the presence (B) of *L. casei* dihydrofolate reductase (2.75×10^{-6} M). (C) The resultant corrected enhancement on binding to dihydrofolate reductase. Buffer: 0.1 M potassium phosphate, pH 7.0 (22 °C). The samples were excited at 328 nm.

a 4-fold excess of APA-Lys-DNS. Thus, APA-Orn-DNS was more efficient as an inhibitor of both dihydrofolate reductases and was only slightly less potent as an inhibitor of the enzyme from both sources as compared to MTX.

(*2*) *Fluorescence Enhancement on Binding to Dihydrofolate Reductase.* The fluorescence emission spectra of APA-Orn-DNS in 0.1 M potassium phosphate buffer, pH 7.0, with and without added *L. casei* dihydrofolate reductase are shown in Figure 5. The addition of the enzyme to the fluorescent analogue results in a 5-fold enhancement of the fluorescence as well as a 10-nm blue shift in the emission maximum. A similar effect was observed with both fluorescent analogues on interaction with either bacterial or avian dihydrofolate reductase. The interactions of APA-Lys-DNS and APA-Orn-DNS with avian dihydrofolate reductase result in 3- and 2-fold enhancement of the fluorescence emission, respectively, and a 15-nm blue shift in the emission maximum (results not shown). On the other hand, addition of *L. casei* dihydrofolate reductase to APA-Orn-DNS or APA-Lys-DNS results in a 5- or 3-fold enhancement of fluorescence emission with a 10-nm blue shift in the emission maximum. The result of adding increasing concentrations of APA-Orn-DNS to *L. casei* dihydrofolate reductase in 0.1 M potassium phosphate buffer, pH 7.0, are indicated in Figure 6. This figure is a representation of the fluorescence emission of free APA-Orn-DNS and the enzyme bound APA-Orn-DNS when excited at 328 nm. Also indicated in Figure 6 is the resultant fluorescent enhancement observed at each concentration of APA-Orn-DNS, correcting for the fluorescence of free ligand. Similar

results were obtained during the titration of APA-Lys-DNS with *L. casei* dihydrofolate reductase and APA-Lys-DNS as well as APA-Orn-DNS with avian dihydrofolate reductase. The enhancements of fluorescence obtained from the titration experiments at equimolar concentrations of APA-Lys-DNS and APA-Orn-DNS with chicken liver and *L. casei* dihydrofolate reductases were 2, 2, 3, and 5, respectively.

Discussion

The fluorescent analogues of MTX, APA-Lys-DNS, and APA-Orn-DNS were synthesized by condensing APA-Lys and APA-Orn with DNS-Cl (Kumar et al., 1983a,b). The two analogues, purified by HPLC, exhibit typical pteridine spectral characteristics in 0.1 N KOH (cf. Figure 2). A striking feature of the absorption spectra is the low molar absorptivities exhibited by both dansyl derivatives as compared to their precursors at 252, 304, and 370 nm. APA-Lys-DNS showed the largest hypochromic effect of the two analogues with molar extinction (ϵ_M) values of almost half those for APA-Lys under identical conditions. In an attempt to explain the observed hypochromic effect, the following three possible explanations were considered. An intermolecular stacking involving two molecules of APA-Lys-DNS leading to lowered absorption of light was ruled out since very dilute solutions of the compound also gave ϵ_M values far lower than predicted. This was true at extremes of pH as well as in the presence of chaotropes (e.g., 8 M urea). An intramolecular stacking of the naphthyl ring of the DNS moiety and the pteridine ring was considered but may not be responsible for the lowered ϵ_M values, even though the flexible nature of the four carbon methylene groupings between the two bulky ring systems might allow such an intramolecular stacking without steric hindrance between the sulfonyl group and *p*-aminobenzoyl groups (from model building studies). Finally, a chemical modification of the pyrazine ring of the pteridine moiety during the synthesis of the compound as a probable cause for the hypochromic effect was discarded since the observed mass spectrum of the compound was identical with that predicted (Kumar et al., 1983a). An examination of the absorption spectra of APA-Orn-DNS reveals that the hypochromic effect with this analogue was not as pronounced as observed with APA-Lys-DNS. One interesting explanation could be that the shorter three carbon methylene bridge might prevent a proper intramolecular stacking of the two ring systems. The latter argument is supported from an examination of the space-filling model. In the absence of further evidence for intramolecular stacking, it is equally probable to assume that the lowered ϵ_M values for the two compounds are an intrinsic property of these two analogues.

APA-Lys-DNS and APA-Orn-DNS both exhibit fluorescence properties characteristic of the dansyl moiety. Similar to N^{ϵ}-DNS-L-Lys the two analogues absorb at 328 nm in aqueous media and show fluorescence emission in the 540–600-nm range. The corrected fluorescence emission spectra are broad with maxima centered at 580 nm. The fluorescence emission and its intensity are sensitive to the polarity of the environment surrounding the dansyl moiety (Table II), a property well established for aminonaphthalene (Li et al., 1975) and dansyl amino acids (Chen, 1967). As with dansyl amino acids, the maximum enhancement of the emission intensity and blue shift of the emission maximum for the two analogues are observed in dioxane. A difference in the emission intensities was noted between APA-Lys-DNS and APA-Orn-DNS; a comparison with N^{ϵ}-DNS-L-Lys (Figure 3) revealed that the APA-Lys-DNS emission intensity was approximately 8-fold lower than the N^{ϵ}-DNS-L-Lys emission under identical conditions. APA-Orn-DNS emission was approximately 15-fold lower than that of N^{ϵ}-DNS-L-Lys. As reported previously (Li et al., 1975) in the case of DNSA, even amide formation as in 5-(*N*,*N*-dimethylamino)-1-naphthalenesulfonamide or derivatization to DNS amino acids (Chen, 1967) leads to a reduction in quantum yields (Q) for dansyl derivatives. Determination of Q values for APA-Lys-DNS and APA-Orn-DNS from corrected emission spectra (Table I) again indicates a decrease in the quantum efficiency of the dansyl moiety of the two analogues as compared to either N^{ϵ}-DNS-L-Lys or DNSA.

The interaction of the two analogues of MTX with dihydrofolate reductase was studied both by investigating their effect on enzyme activity and by following the fluorescence emission of the dansyl moiety in the presence and absence of the enzyme. Like MTX, APA-Lys-DNS and APA-Orn-DNS are potent inhibitors of bacterial and avian dihydrofolate reductases with inhibition profiles similar to that of MTX (Figure 4). In the studies reported here the molar excess of the inhibitor required for complete inhibition was used as an index to evaluate these analogues. A more rigorous kinetic index for the evaluation of these new analogues as potent inhibitors of dihydrofolate reductase would be a comparison of the K_i values for these compounds. Determination of K_i for a stoichiometric inhibitor such as MTX ($K_i \simeq 10^{-12}$ M) is very difficult as evidenced by the various methods employed (Jackson et al., 1976; Domin et al., 1982). In the present investigation the main emphasis has been on the characterization of the fluorescence properties of these two analogues and changes in these properties on interaction with the enzyme. Our inhibition studies clearly demonstrate that replacement of the γ-carboxylate of the L-glutamate moiety of MTX by an amino group (as in APA-Lys and APA-Orn) and further derivatization with the introduction of a bulky dansyl group does not significantly alter the ability of these compounds to inhibit dihydrofolate reductase.

The fluorescence emission of the two analogues was enhanced on binding to dihydrofolate reductase with a blue shift of 10–15 nm in the emission maxima (Figure 5). This enhancement in the intensity as well as a shift in the emission maximum is similar to the effects observed with the free compounds in nonpolar solvents, indicating the contribution of hydrophobic interactions at the binding site. In recent years a good deal of information has accumulated regarding the active site of the dihydrofolate reductase and the mode of binding of MTX at the active site (Matthews et al., 1977, 1978). In addition, the γ-carboxylate of MTX can be modified with a number of substituents without adversely affecting the overall potency of the MTX molecule (Rosowsky et al., 1981b; Piper et al., 1982). Our results with APA-Lys and APA-Orn further substantiate the lesser importance of the γ-carboxyl group since a change from a negative charge (COO^-) to a positive charge (NH_3^+) in APA-Lys and APA-Orn does not alter the inhibitory potency of the MTX molecule significantly. From the available information on the binding of MTX to the reductase and structural similarities between the two analogues and MTX, one would predict that the dansyl group in APA-Lys-DNS and APA-Orn-DNS would be near the surface of the enzyme exposed to solvent molecules as is the case of the γ-carboxylate of MTX. However, since there is an enhancement of the dansyl fluorescence on binding to the enzyme, the dansyl group could interact with hydrophobic residues near the surface of the enzyme due to a folding of the liganded molecule as a result of the flexible nature of the $(CH_2)_n$–NH grouping. The binding of these two fluorescent

analogues at the active site of dihydrofolate reductase appears to be similar to that of MTX. Such a tight binding also makes any possible determination of binding constants extremely difficult. The linear nature of the dependence of fluorescence enhancement as a result of binding of the APA-Orn-DNS analogue to the *L. casei* enzyme (cf. Figure 6) on ligand concentration and the nature of the saturation phenomena observed made it very difficult to arrive at a meaningful K_D value. We estimate that the binary K_D value is less than 10^{-10} M. Similar results were obtained with APA-Lys-DNS and also with the avian enzyme. Although the fluorescence enhancements observed were different for each ligand with the two enzymes, the enhancement profiles were similar to that described in Figure 6. Both APA-Lys-DNS and APA-Orn-DNS have been shown to be excellent fluorescence probes of dihydrofolate reductase. In addition, APA-Lys-DNS is transported into murine L1210 cells as efficiently as MTX,[2] providing yet another analytical tool for examining and comparing dihydrofolate reductase levels within cells by using the fluorescence-activated cell sorter.

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Registry No. APA-Lys-DNS, 83416-29-9; APA-Orn-DNS, 83966-27-2; APA-Lys, 80407-56-3; APA-Orn, 80407-73-4; N^{ϵ}-dansyl-L-lysine, 1101-84-4; DHFR, 9002-03-3.

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