Both these predictions have been borne out experimentally. We find that the lifetime (τ_0) of the ^2E state of $Cr(phen)_3^{3+}$ [22] in N_2-purged dilute aqueous solution at 22 °C is 0.36 ms (compared to 0.063 ms for $Cr(bpy)_3^{3+}$). The anion effect on $Cr(phen)_3^{3+}$ and $Cr(bpy)_3^{3+}$ is seen by comparing these lifetimes with those in concentrated (11.7 M) $HClO_4$: $\tau_0 = 0.53$ and 0.67 ms for $Cr(bpy)_3^{3+}$ and $Cr(phen)_3^{3+}$, respectively. Thus, the maximum effect we have seen is almost a factor of 10 for $Cr(bpy)_3^{3+}$ but only a factor of 2 for $Cr(phen)_3^{3+}$.

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References and Notes

(1) Research supported by the National Science Foundation (Grant No. CHE 76-21050) and the North Atlantic Treaty Organization (Grant No. 658).
(2) A. W. Adamson, A. R. Gutierrez, R. E. Wright, and R. T. Walters, Twelfth Informal Conference on Photochemistry, National Bureau of Standards, Gaithersburg, Md., June 1976, Paper G1.
(3) The emission lifetime of the ^2E state of *t*-$Cr(en)_2(NCS)_2^+$ has been recently found to be ~10 μs (D. Sandrini, M. T. Gandolfi, L. Moggi, and V. Balzani, submitted for publication).
(4) The lowest excited state of $Cr(bpy)_3^{3+}$, ^2E, is in thermal equilibrium with the $^2T_1$ state; these two doublet states will be designated ^2E for simplicity.
(5) M. Maestri, F. Bolletta, L. Moggi, V. Balzani, M. S. Henry, and M. Z. Hoffman, *J. Chem. Soc., Chem. Commun.*, in press.
(6) F. Bolletta, M. Maestri, L. Moggi, and V. Balzani, *J. Chem. Soc., Chem. Commun.*, 901 (1975).
(7) R. Ballardini, G. Varani, F. Scandola, and V. Balzani, *J. Am. Chem. Soc.*, **98**, 7432 (1976).
(8) V. Balzani, personal communication of unpublished results.
(9) M. Maestri, F. Bolletta, L. Moggi, V. Balzani, M. S. Henry, and M. Z. Hoffman, submitted for publication.
(10) Φ_{rad} increases[11] in direct proportion to the increase in the lifetime of the ^2E state.[8,9]
(11) ClO_4^- has been shown to increase the fluorescence intensity from the S_1 state of 1-hydroxynaphthalene-2-sulfonate (R. M. C. Henson and P. A. H. Wyatt, *J. Chem., Soc., Faraday Trans. 2*, **71**, 669 (1975)).
(12) H. F. Wasgestian, R. Ballardini, G. Varani, L. Moggi, and V. Balzani, *J. Phys. Chem.*, **77**, 2614 (1973).
(13) F. M. Van Meter and H. M. Neumann, *J. Am. Chem. Soc.*, **98**, 1382 (1976).
(14) G. N. LaMar and G. R. Van Hecke, *Inorg. Chem.*, **12**, 1767 (1973).
(15) For $Fe(phen)_3^{2+} \cdots ClO_4^-$, $K_{ip} = 5.73$.[13] For $Cr(bpy)_3^{3+} \cdots ClO_4^-$, K_{ip} would be expected to be even larger for electrostatic reasons.
(16) G. J. Janz, B. G. Oliver, G. R. Lakshminarayanan, and G. E. Mayer, *J. Phys. Chem.*, **74**, 1285 (1970).
(17) R. Dannohl-Fickler, H. Kelm, and F. Wasgestian, *J. Lumin.*, **10**, 103 (1975).
(18) P. B. Merkel and D. R. Kearns, *J. Am. Chem. Soc.*, **94**, 7244 (1972).
(19) C. Conti and L. S. Forster, *J. Am. Chem. Soc.*, **99**, 613 (1977).
(20) J. R. Ferraro, "Low-Frequency Vibrations of Inorganic and Coordination Compounds", Plenum Press, New York, N.Y., 1971, pp 198–202.
(21) N. A. P. Kane-Maguire, J. Conway, and C. H. Langford, *J. Chem. Soc., Chem. Commun.*, 801 (1974).
(22) Φ_{rx} for $Cr(phen)_3^{3+}$ in neutral and basic solution[8] is ~0.01; thus τ_0^{-1} is a very good approximation to k_{nr}.

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^{19}F Nuclear Magnetic Resonance Investigation of the Ternary Complex Formed between Native Thymidylate Synthetase, 5-Fluoro-2′-deoxyuridylate, and 5,10-Methylenetetrahydrofolate

Sir:

The enzyme thymidylate synthetase catalyzes the reductive methylation of 2′-deoxyuridylate (dUMP) to form thymidylate (dTMP) while employing 5,10-methylenetetrahydrofolate (CH_2FH_4) as a coenzyme. The proposed involvement of de novo synthesis of thymidylate as a rate-determining factor in DNA synthesis and cell division has resulted in many attempts to elucidate the mechanism of action of thymidylate synthetase. Foremost among these efforts has been the identification and isolation of stable ternary complexes[1-7] between native thymidylate synthetase, its coenzyme CH_2FH_4, and the inhibitor 5-fluoro-2′-deoxyuridylate (FdUMP). We report here ^{19}F NMR data that provide direct physical evidence for the structure of this ternary complex formed with native enzyme isolated from amethopterin resistant *Lactobacillus casei*. The impact of these data with respect to previous studies involving model compounds[8] and fragments of the complex obtained by proteolytic degradation[9-10] is also discussed.

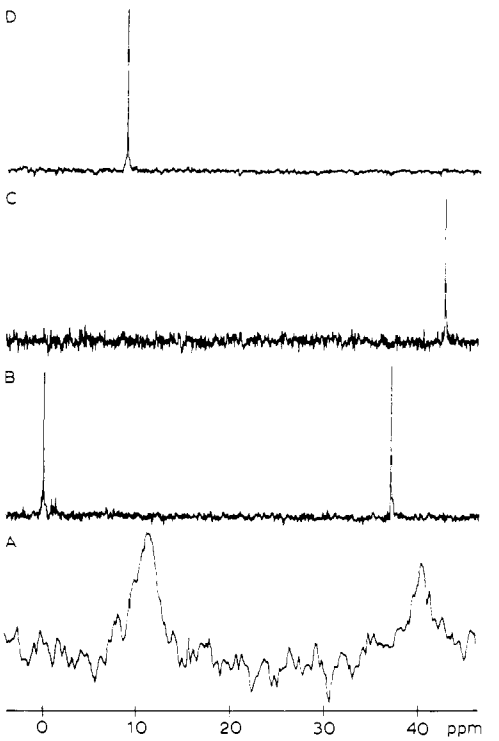
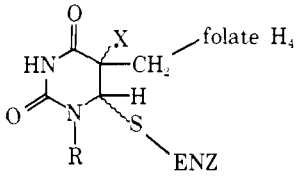


Figure 1. ^{19}F NMR spectra of the ternary complex (A), F_2dUMP (B), II (C), and III (D) (all spectra were recorded at 20 °C using 18-mm sample tubes in our homemade 18-mm probe:[22] spectra B–D were recorded under conditions of full proton decoupling): (A) ternary complex comprising 0.4 mM thymidylate synthetase, 0.8 mM F_2dUMP, and 4 mM CH_2FH_4 in 0.1 M Tris-SO_4, 50 mM $MgSO_4$, 20 mM β-mercaptoethanol, pH 7.3; (B) 0.010 M F_2dUMP in 0.1 M Tris-SO_4, pH 7.3; (C) 0.005 M II in 0.1 M Tris-SO_4, pH 7.3; (D) 0.005 M III in 0.1 M Tris-SO_4, pH 7.3.

Numerous studies have sought to elucidate the structural features in the ternary complex, with particular emphasis on the coordination of FdUMP and CH_2FH_4 to the enzyme. Danenberg et al.[9] and Pogolotti et al.[10] have reported procedures for the enzymatic degradation of the ternary complex to yield a small peptide fragment to which both FdUMP and CH_2FH_4 moieties remain bound, and Bellisario et al.[11] have presented evidence which suggests that the FdUMP is linked to a cysteinyl residue in a related peptide. These studies were based on radiolabeling techniques; hence there is no specific bonding information available to account for the association. Spectroscopic techniques,[12-15] used in attempts to determine the molecular nature of the binding of these moieties, provided inferences of the structural features. Danenberg and Heidelberger[16] have reported evidence based on chemical degradation which indicates that the point of covalent attachment for FdUMP to thymidylate synthetase occurs through nucleophilic attack of a cysteine sulfhydryl group on the pyrimidine ring. It is now generally accepted that the catalytic mechanism is initiated by attack of an active site nucleophile, most likely a cysteinyl sulfhydryl group, on carbon 6 of the pyrimidine ring to generate a carbanion which subsequently attacks CH_2FH_4

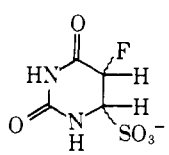


Ia, X = H
Ib, X = F
R = 5′-phospho-2′-deoxyribose

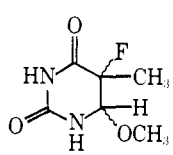
(or its iminium form) to yield a transient ternary complex (Ia). Methylation is then completed by the transfer of a proton from tetrahydrofolate to the methylene group and abstraction of the hydrogen from carbon 5 of the pyrimidine ring. The ultimate products are thus thymidylate, 7,8-dihydrofolate, and a free sulfhydryl form of the enzyme. The interaction of FdUMP and CH_2FH_4 with thymidylate synthetase, on the other hand, terminates with the formation of a stable covalent ternary complex whose proposed structure is Ib. Subsequent steps in the catalytic methylation are prevented, hence the inhibition by FdUMP, due to the fact that the fluorine cannot be readily abstracted from the ternary complex Ib.

Santi and coworkers[17,18] have reported the ^{19}F NMR spectrum of a peptide containing FdUMP isolated after proteolytic degradation of the ternary complex. This poorly resolved resonance was interpreted as a doublet of triplets arising from spin-spin coupling to the methylene protons of the coenzyme and a proton at the 6 position of the pyrimidine ring. Such a pattern would in fact be expected based on the proposed structure of the ternary complex. However, the resonance exhibited a chemical shift almost identical with that of FdUMP, which would seem to be inconsistent with the expected upfield shift upon conversion from an alkenyl fluorine to an alkyl fluorine. In attempting to elucidate the structure of this complex, it is pertinent to be very cautious of the inherent pitfall associated with chemical and enzymatic degradation procedures.

In order to avoid these difficulties, we have obtained the ^{19}F NMR spectrum of an intact ternary complex of native thymidylate synthetase. The inhibitor 5-fluoro-2′-fluoro-2′-deoxyuridylate (F_2dUMP) was used in the formation of the ternary complex. The additional fluorine at the 2′ position provides an internal standard for our ^{19}F NMR experiment. Reyes and Heidelberger[19] have shown previously that F_2dUMP is a good inhibitor of thymidylate synthetase. We have confirmed in our laboratory that this compound forms ternary complex quantitatively equivalent to that with FdUMP. Gel electrophoresis and spectral analysis indicate that the relative extent of formation of the 1:1:1 and 2:2:1 complexes (inhibitor:CH_2FH_4:thymidylate synthetase)[6,7,12] is the same for F_2dUMP as for FdUMP.



II



III

The ^{19}F NMR spectra of the ternary complex, the bisulfite adduct of fluorouracil (II),[20] F_2dUMP, and 5-fluoro-5-methyl-6-methoxy-5,6-dihydrouracil (III)[21] are shown in Figure 1; chemical shifts are reported relative to FdUMP. The assignment of the ^{19}F NMR spectrum of the ternary complex is made by comparison with the spectrum of the free ligand, F_2dUMP. The resonance due to the 2′-fluorine shifts upfield 2.4 ppm upon formation of the ternary complex. Such shifts have been observed for numerous CF_3-type ligands upon binding to proteins and indicate microenvironment changes, not bonding rearrangements. In contrast, the fluorine at the 5 position of the pyrimidine ring should reflect the change from an alkenyl to an alkyl system as exists in the proposed structure (Ib). Indeed the resonance due to this fluorine shifts upfield 11.9 ppm upon formation of the complex, clearly indicating a change in the bonding environment relative to free FdUMP. Comparison of spectra A and C reveals that II is not a good model of the ternary complex, probably due to its ionic nature and the lack of an additional substituent at the five position. To verify this hypothesis we show the ^{19}F NMR spectrum of III which is neutral and has both a fluorine and a methyl substituent at the five position. The spectrum exhibits a chemical shift of 9.5 ppm upfield of FdUMP which is analogous to the shift observed for the ternary complex. Although this shift may be due in part to the change from an ionic to a neutral species,[23] the effect of the methyl substituent, which creates a tertiary center rather than a secondary center at the 5 position, contributes significantly to the chemical shift difference between II and III. This result suggests that the native ternary complex also possesses a substituent at the five position.

We conclude that our data support the proposed structure (I) for the ternary complex of *native* thymidylate synthetase. The observed line widths for the ternary complex do not represent the natural line widths of the resonances, but, rather, they exhibit broadening due to protein association resulting from the high concentration of thymidylate synthetase employed. Under these conditions spin-spin coupling cannot be resolved. Further experiments are currently in progress both to improve the quality of the spectra and more closely delineate the structural features involved in the coordination of FdUMP and CH_2FH_4 to native thymidylate synthetase.

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References and Notes

(1) D. V. Santi and C. S. McHenry, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1855 (1972).
(2) J. Galivan, G. F. Maley, and F. Maley, *Biochemistry*, **13**, 2282 (1974).
(3) G. R. Capco, J. R. Krupp, and C. K. Mathews, *Arch. Biochem. Biophys.*, **158**, 726 (1973).
(4) D. V. Santi, C. S. McHenry, and H. Sommer, *Biochemistry*, **13**, 471 (1974).
(5) R. J. Langenbach, P. V. Danenberg, and C. Heidelberger, *Biochem Biophys. Res. Commun.*, **48**, 1565 (1972).
(6) J. L. Aull, J. A. Lyon, and R. B. Dunlap, *Microchem. J.*, **19**, 210 (1974).
(7) J. L. Aull, J. A. Lyon, and R. B. Dunlap, *Arch. Biochem. Biophys.*, **165**, 805 (1974).
(8) R. B. Dunlap, N. G. L. Harding, and F. M. Huennekens, *Ann. N.Y. Acad. Sci.*, **186**, 153 (1971).
(9) P. V. Danenberg, R. J. Langenbach, and C. Heidelberger, *Biochemistry*, **13**, 926 (1974).
(10) A. L. Pogolotti, K. M. Ivanetich, H. Sommer, and D. V. Santi, *Biochem. Biophys. Res. Commun.*, **70**, 972 (1976).
(11) R. L. Bellisario, G. F. Maley, J. H. Galivan, and F. Maley, *Proc. Natl. Acad. Sci.*, **73**, 1848 (1976).
(12) H. Donato, J. L. Aull, J. A. Lyon, J. W. Reinsch, and R. B. Dunlap, *J. Biol. Chem.*, **251**, 1303 (1976).
(13) R. K. Sharma and R. L. Kisliuk, *Biochem. Biophys. Res. Commun.*, **64**, 648 (1975).
(14) J. H. Galivan, G. F. Maley, and F. Maley, *Biochemistry*, **14**, 3338 (1974).
(15) D. V. Santi, V. A. Rena, and S. M. Lam, *Biochem. Biophys. Acta*, **438**, 324 (1976).
(16) P. V. Danenberg and C. Heidelberger, *Biochemistry*, **15**, 1331 (1976).
(17) D. V. Santi, A. L. Pogolotti, T. L. James, Y. Wataya, K. M. Ivanetich, and S. M. Lam in "Biochemistry Involving Carbon-Fluorine Bonds", R. Filler, Ed., American Chemical Society, Washington, D.C., 1976, p 57.
(18) T. L. James, A. L. Pogolotti, K. M. Ivanetich, Y. Wataya, S. M. Lam, and D. V. Santi, *Biochem. Biophys. Res. Commun.*, **72**, 404 (1976).
(19) P. Reyes and C. Heidelberger, *Biochem. Biophys. Acta*, **103**, 177 (1965).
(20) E. G. Sander and C. I. Deyrup, *Arch. Biochem. Biophys.*, **150**, 600 (1972).
(21) This compound was prepared by the addition of trifluoromethyl hypofluorite to thymine according to the method of M. J. Robins, M. MacCoss, S. R. Naik, amd G. Ramani, *J. Am. Chem. Soc.*, **98**, 7381 (1976), and was charac-

terized spectroscopically.
(22) R. A. Byrd and P. D. Ellis, *J. Magn. Res.*, **26**, 169 (1977).
(23) The fluorine resonance of the neutral compound 5-fluoro-6-methoxy-5,6-dihydrouracil in Me_2SO-d_6[21] occurs 5 ppm to lowfield of that for II. The influence of solvent is minimal as we have observed that the resonance for III shifts only 2 ppm on going from phosphate buffer to Me_2SO-d_6.
(24) Recipient of a Faculty Research Award (FRA-144) from the American Cancer Society.

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Catalysis of *p*-Nitrotrifluoroacetanilide Hydrolysis by an Imidazole Derivative of Polyethylenimine "Ghosts"

Sir:

We have recently prepared a cross-linked derivative of polyethylenimine (PEI) which could be useful in such areas as solid-phase organic synthesis, solid-phase sequencing of biopolymers, catalyst immobilization, and affinity chromatography. PEI ghosts are made in a three-step process (Figure 1). First, PEI is adsorbed to porous alumina beads. The PEI layer is then cross-linked. In the final step the inorganic core is removed by treatment with acid or base resulting in the formation of hollow polymer "ghosts". These structures have great chemical and mechanical stability. Other advantages include compatibility with a wide range of solvents, high capacity (1 mequiv of primary amine/g), and ease of preparation.

Soluble polymeric catalysts with enzyme-like characteristics have been reported by a number of workers.[1-5] We believe that the use of insoluble polymers in enzyme modeling studies is attractive for at least three reasons; the synthetic procedures are simplified, catalytic groups such as thiols or metal ions can not deactivate by forming disulfides or binuclear compounds, and the application of the catalyst in a heterogeneous system would be possible.

We report here the preparation of an effective catalyst of amide hydrolysis at pH 8.2. PEI ghosts containing lauroyl and histidyl groups catalyze the hydrolysis of *p*-nitrotrifluoroacetanilide more than 200 times faster than the rate brought about by imidazole alone.

PEI-600 (Dow, 400 mL of an 8.3% solution in methanol) was mixed with 100 mL of porous alumina beads.[6] After removal of trapped air, the mixture was gently agitated for 30 min. The beads were washed with five portions of methanol (200 mL each) and then dried in vacuo at room temperature. The beads (25 g) were reacted with 250 mL of 0.4% aqueous glutaraldehyde. Trapped air was removed and the reaction was continued for 30 min. The glutaraldehyde solution was then replaced with 100 mL of an 8.3% solution of PEI in methanol. After 15 min of gentle agitation, the PEI solution was replaced with 100 mL of methanol. A total of 2 g of $NaBH_4$ was added in small increments over a period of 30 min. The beads were washed and dried as described in the previous step. PEI ghosts were produced by treatment of PEI-alumina (10 g) with 100 mL of 1 N HCl. After ~15 min, the change in density is complete. The acid was decanted and replaced with a second 100 mL-portion of 1 N HCl. After 30 min, the PEI ghosts were washed with water and methanol and then dried in vacuo at room temperature.

Histidyl residues were introduced by reaction of PEI Ghosts with Boc-His(^{Im}DNP)-ONp. After treatment with 0.2% ethanolic KOH, the PEI ghosts (4-mL settled volume) were mixed with 100 mg of nitrophenyl ester and 2 equiv of triethylamine in 5 mL of dry dioxane. This mixture was rotated for

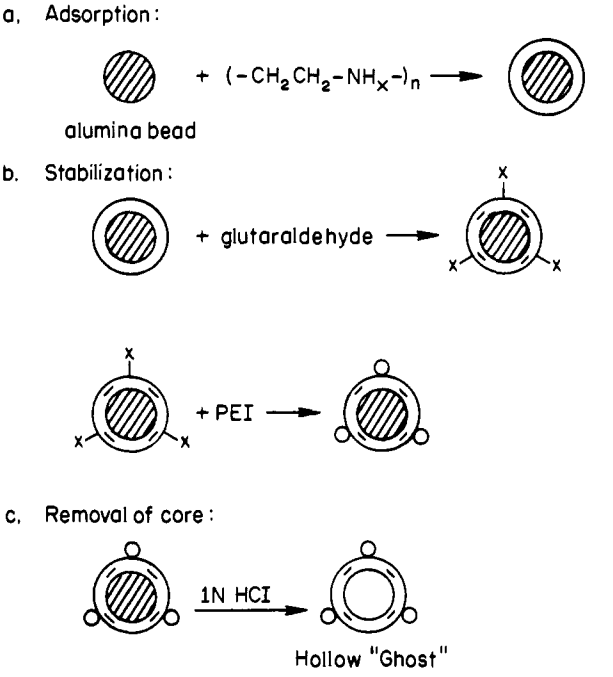

Figure 1. Preparation of PEI ghosts

12 h at 60 °C. The Boc group was removed with 30% TFA in $CHCl_3$ (5 mL, 12 h, at 25 °C). The DNP group was removed by thiolysis (0.1 M mercaptoethanol, pH 9, 12 h, 25 °C). After hydrolysis (6 N HCl, 100 °C, 24 h), amino acid analysis was done. The result was 0.4 mequiv of His/g. Lauroyl groups were then introduced by treatment with nitrophenyl laurate (500 mg in 25 mL of dioxane, containing 0.2 mL of triethylamine) for 72 h at 70 °C. Estimates based on loss of ninhydrin reactivity suggest incorporation of 0.2 mequiv of lauroyl groups/g. A sample which contained no histidyl residues was lauroylated in the same way and used as a control.

The hydrolysis of *p*-nitrotrifluoroacetanilide was followed at 410 nm (0.01 M *N*-ethylmorpholine-HCl buffer, pH 8.2; 40 mL of substrate, 10^{-4} M; 25 °C; 7 mg of catalyst). At timed intervals samples were removed for analysis by means of a syringe fitted with a tube covered by nylon net. The corrected second-order rate constants for hydrolysis catalyzed by imidazole and lauroylhisdidyl-PEI ghosts were estimated to be 0.0036 M^{-1} s^{-1} and 0.816 M^{-1} s^{-1}, respectively.[7] The rate constants were calculated on the basis of total imidazole content in both cases. PEI ghosts substituted only with lauroyl groups do not produce a rate enhancement over background. Therefore, the sizable rate enhancement of 230 times for the lauroylhistidyl preparation is probably the result of the lauroyl groups binding substrate in close proximity to the imidazole ring of histidine. Constant activity after repeated use with *p*-nitrotrifluoroacetanilide and after acylation with large excesses of nitrophenyl esters would suggest that the catalyst is regenerated.

Current investigations with the lauroylhistidyl catalyst are directed at the dependence of reaction rate on pH, substrate concentration, and temperature.

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References and Notes

(1) I. M. Klotz, G. P. Royer, and I. S. Scarpa, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 263-264 (1971).
(2) H. Moravetz, C. G. Overberger, T. C. Salamone, and S. Yaroslavsky, *J. Am. Chem. Soc.*, **90**, 651-656 (1968).
(3) R. L. Letsinger, and T. J. Savereide, *J. Am. Chem. Soc.*, **84**, 3122-3127