Hull, W. E., and Sykes, B. D. (1975a), *J. Chem. Phys. 63,* 867-880.
Hull, W. E., and Sykes, B. D. (1975b), *J. Mol. Biol. 98,* 121-153.
Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1969), *Methods Enzymol. 14,* 43-50.
Prescott, D. J., and Vagelos, P. R. (1972), *Adv. Enzymol. 36,* 269-311.
Ray, T. K., and Cronan, J. E., Jr. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 4374-4378.
Schulz, H. (1975), *J. Biol. Chem. 250,* 2299-2304.
Schulz, H. (1977), *FEBS Lett. 78,* 303-306.
Schulz, H., Weeks, G., Toomey, R. E., Shapiro, M., and Wakil, S. J. (1969), *J. Biol. Chem. 244,* 6577-6583.
Spassov, S. L., Griffith, D. L., Glazer, E. S., Nagarajan, K., and Roberts, J. D. (1967), *J. Am. Chem. Soc. 89,* 88-94.
Spencer, A. K., Greenspan, A. D., and Cronan, J. E., Jr. (1978), *J. Biol. Chem. 253,* 5922-5926.
Takagi, T., and Tanford, C. (1968), *J. Biol. Chem. 243,* 6432-6435.
Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968), *J. Biol. Chem. 243,* 6420-6431.
Wagner, G., and Wüthrich, K. (1975), *J. Magn. Resonance 20,* 435-445.

Effects of Polyoxyanions on Sulfhydryl Group Modification of Thymidylate Synthetase†

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ABSTRACT: The reactivity of the catalytic cysteines of thymidylate synthetase in the presence and absence of polyoxyanions was studied by inactivation of the enzyme with methyl methanethiolsulfonate (MMTS) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). When measured in various buffers, the rate of the MMTS-dependent inactivation decreased in the series Tris-Cl > Tris-acetate > piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) > Tris-AsO_4 > Tris-SO_4 > phosphate > Tris-PO_4 and the analogous series for DTNB-dependent inactivation is Tris-Cl > Tris-SO_4 > phosphate > Tris-phosphate. This buffer dependence of the rates is interpreted in terms of a polyoxyanion interaction with the enzyme. MMTS inactivation of thymidylate synthetase was characterized by incorporation of 1.5-1.6 mol of [^{14}C]CH_3S- per mol of enzyme dimer using [^{14}C]MMTS, with concomitant loss of both enzymatic activity and ability to form covalent ternary complexes. Protection by pyrimidine derivatives and folates in the presence and absence of polyoxyanions was also investigated. The pH dependence of MMTS inactivation of Pipes buffer is in qualitative agreement with our earlier results in phosphate (Munroe, W. A., et al. (1978) *Biochem. Biophys. Res. Commun. 80,* 355-360). Observation of a finite reaction at low pH is consistent with activation of the catalytic cysteines by an as yet unidentified general base.

Thymidylate synthetase isolated from amethopterin resistant *Lactobacillus casei* catalyzes the reductive methylation of dUMP to form dTMP employing the coenzyme CH_2H_4folate.[1] This reaction is believed to proceed through a transient covalent ternary complex involving nucleophilic addition of a catalytic cysteine to carbon 6 of the uracil ring and attachment of the methylene portion of the coenzyme to carbon 5 of the uracil ring (see Danenberg, 1977, and references therein). This information has been inferred from studies of the stable covalent ternary complex composed of the enzyme, coenzyme, and the substrate analogue FdUMP. Recent ^{19}F NMR studies have delineated the relative stereochemistry of the ternary complex in the active site of the enzyme (Byrd et al., 1977, 1978; Byrd, 1977). The role of the catalytic cysteines has been further elaborated by correlation of extent of thiol modification with loss of enzyme activity and ability to form ternary complexes (Plese & Dunlap, 1977). Our recent studies have focused on the activation of the catalytic cysteine residues through interaction with an as yet unidentified general base residue in the active site as demonstrated by the pH dependence of the rate of inactivation of thymidylate synthetase by MMTS and DTNB (Munroe et al., 1978).

In order to further our understanding of the activation of the cysteine residues, we have extended our studies of MMTS inactivation of thymidylate synthetase. We have investigated the protection from MMTS inactivation of the enzyme by substrates and products and their analogues under a variety of conditions and have determined the stoichiometry of inactivation by this reagent. During these studies we observed that the presence of polyoxyanions had a pronounced effect upon the rate of inactivation and the degree of protection afforded by the substrate dUMP. In view of the observed effect we have extended our studies of the pH dependence of MMTS inactivation of thymidylate synthetase to include Pipes buffer which appears to be a noninteracting buffer.

Materials and Methods

Thymidylate synthetase was purified in the presence of exogenous thiols from amethopterin resistant *Lactobacillus*

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[1] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); dUMP, deoxyuridylate; dTMP, deoxythymidylate, FdUMP, 5-fluorodeoxyuridylate; dCMP, deoxycytidylate; dU, deoxyuridine; (±)-CH_2H_4folate, (±)-5,10-methylene-5,6,7,8-tetrahydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; H_2folate, 7,8-dihydrofolate; MMTS, methyl methanethiolsulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol.

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casei by the procedure of Lyon et al. (1975). The enzyme used in all experiments except those done in 50 mM phosphate buffer was further purified by DEAE-Sephadex chromatography as described by Byrd et al. (1978). This enzyme has been shown to be free of bound phosphate by ^{31}P NMR.[2] The enzyme was shown to be greater than 95% homogeneous by polyacrylamide gel electrophoresis. The enzyme was activated prior to use by dialysis against 25 mM 2-mercaptoethanol in 50 mM buffers at 0–5 °C for 24 h. Activated enzyme was dethiolated on Sephadex G-10 columns immediately prior to use, as previously described (Plese & Dunlap, 1977). Assays for thymidylate synthetase activity were performed spectrophotometrically by monitoring the increase in absorbance at 340 nm due to the production of H_2folate (ϵ = 6400 M^{-1} cm^{-1}). Enzyme activity was measured in either 0.1 M potassium phosphate, pH 6.8, or 0.1 M Tris-Cl, pH 7.4, and contained 10^{-4} M dUMP and 2×10^{-4} M (±)-CH_2H_4 folate. All assays, except those pertaining to the reactivation studies, were performed in the absence of exogenous thiols.

MMTS was prepared by the hydrogen peroxide oxidation of dimethyl disulfide in 77% yield by the procedure of Smith et al. (1975). [^{14}C]MMTS was prepared by the alkylation of sodium methanethiolsulfonate (Kenyon & Bruice, 1977) by [^{14}C]methyl bromide (New England Nuclear, 1.7 mCi/mmol) following the procedure of Smith et al. (1975), with purification by silica gel column chromatography as described by Currier & Mautner (1977). [^{14}C]MMTS was obtained in 49% yield with a specific activity of 0.54 mCi/mmol and exhibited a 90-MHz ^{1}H NMR spectrum identical with that of unlabeled material (δ 3.29 (s) and 2.69 (s)).

Inactivation of thymidylate synthetase by MMTS was examined under a variety of experimental conditions, in each case utilizing activated, dethiolated enzyme in the appropriate buffer system. In all studies a fresh aqueous stock solution of MMTS was prepared daily. The concentration dependence of the rate of inactivation was studied in 50 mM potassium phosphate, pH 6.8, at 0 °C, employing molar ratios of MMTS to enzyme of 2 to 25.

Reactivation of thymidylate synthetase which had been inactivated by a tenfold excess of MMTS was studied using 2-mercaptoethanol and DTE. In each case the inactivated enzyme, without removal of excess MMTS, was incubated in phosphate buffer, pH 6.8 at 25 °C and assayed at various times in the presence of either 25 or 50 mM 2-mercaptoethanol or 36 or 72 mM DTE.

Quantitation of the labeling of thymidylate synthetase by MMTS was accomplished by incubating the enzyme with varying molar ratios of [^{14}C]MMTS (0.25- to 20-fold excess) at pH 8.0 in 50 mM potassium phosphate for 30 min. Each sample was then assayed for activity and passed down a Sephadex G-10 column to remove excess reagent. Protein concentration of the eluent was determined spectrophotometrically at 280 nm ($A_{1\%}$ = 15.5), and the radiolabel was quantitated by liquid scintillation counting in Bray's solution using a Nuclear Chicago Isocap 300. Polyacrylamide gel electrophoresis was also performed on the labeled enzyme to show that ternary complex forming ability was lost as the radiolabel was incorporated (Aull et al., 1974a,b). In order to ensure that the phosphate buffered system was not an unusual case, in view of the strong buffer effects to be described, maximal [^{14}C]-CH_3S- incorporation was also determined in 50 mM Pipes, 1 mM EDTA at pH 7.0 using a threefold molar excess of reagent to enzyme.

[2] R. Byrd, P. D. Ellis, & R. B. Dunlap, unpublished results.

Protection of thymidylate synthetase from inactivation by MMTS was investigated using dUMP, dTMP, dCMP, dU, CH_2H_4folate, H_4folate, and H_2folate. Folate protection studies were carried out in 50 mM phosphate, pH 6.8, while the nucleotide and nucleoside protection studies were performed in 50 mM Tris-Cl, 7.4. Additional studies using dUMP were done in a variety of buffers including: phosphate, malate, Tris-acetate, Tris-AsO_4, Tris-PO_4, and Pipes. During the course of these studies it was observed that the rate of inactivation was very sensitive to the presence of polyoxyanions. To further investigate this effect, the rate of inactivation was studied in 50 mM Tris-Cl at pH 7.4 with increasing amounts of phosphate added.

In order to determine if polyoxyanions were affecting the enzyme or the reagent, MMTS, the rate of DTNB inactivation of thymidylate synthetase was investigated at pH 7.4 in phosphate, Tris-Cl, Tris-SO_4, and Tris-PO_4 buffer. dUMP protection from inactivation was also studied in Tris-PO_4 buffer. The extent of modification by DTNB was determined using ϵ = 13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1959).

The pH dependence of MMTS inactivation of thymidylate synthetase was investigated in 50 mM Pipes buffer. Inactivations were performed in the pH range 5.6 to 8.3 using a 1.75- to 6-fold excess of MMTS to catalytic cysteine.

All chemicals were reagent grade and were used without further purification unless specified. Ultraviolet-visible spectra and enzyme assays were recorded on either a Beckman Acta III or Acta CV instrument. ^{1}H NMR spectra were recorded on a Perkin-Elmer R-32 at 90 MHz.

Results

Examination of the concentration dependence of the MMTS inactivation of thymidylate synthetase permitted evaluation of the most suitable conditions for rapidly inactivating the enzyme. Low molar excesses of this reagent were found to produce rapid loss of enzymatic activity as shown in Figure 1. At 0 °C in phosphate buffer, a tenfold excess of MMTS over enzyme (approximately sixfold MMTS per catalytic cysteine residue) led to complete loss of enzymatic activity within 10 min. Enzyme inactivated under these conditions was shown to regain at least 85% of its original activity when exposed to thiols. DTE, 36–72 mM, produced a 98% recovery of activity within 12 min, while 25–50 mM 2-mercaptoethanol generated only 86% of the original activity over 20 min. The ability of DTE to affect reactivation at lower concentrations results from the presence of 2 thiol equiv per mol compared with 1 from 2-mercaptoethanol. With both reagents, up to 50% of original activity was regained within 5 min, but maximal recovery required as long as 20 min.

The quantitation of incorporation of [^{14}C]CH_3S- into inactivated, dethiolated thymidylate synthetase yielded a value of 1.6 mol per mol of enzyme (see Figure 2). It should be noted that in the experiments where very high excesses of labeled reagent were employed, the ratio approached 2.0 mol of [^{14}C]CH_3S- per mol of enzyme, but this was found to be due to incomplete separation of the radiolabeled enzyme from the large excess of unreacted reagent. Polyacrylamide gels of the labeled enzyme clearly demonstrated that the MMTS-dependent inactivation was paralleled by a loss in ability to form ternary complexes. Maximal incorporation performed in Pipes buffer yielded a value of 1.5 mol of [^{14}C]CH_3S- per mol of enzyme.

Protection from inactivation was not exhibited by H_4folate or H_2folate. Initially, (±)-CH_2H_4folate appeared to show protection, but, when MMTS and (±)-CH_2H_4folate were

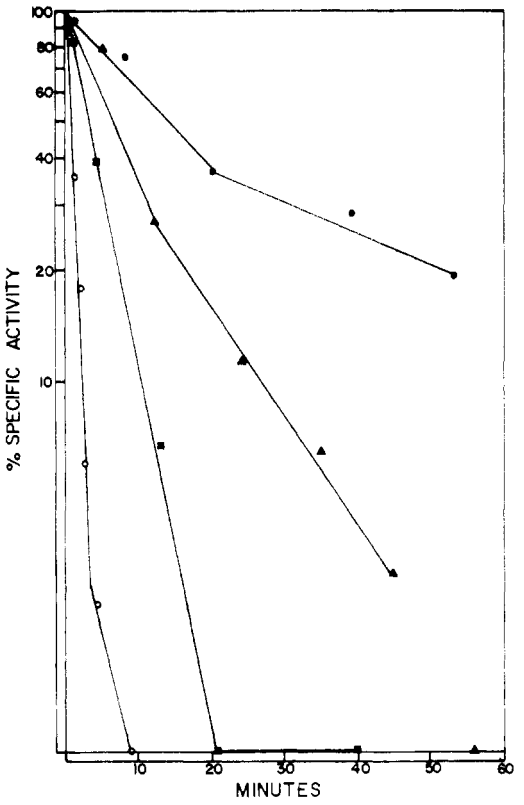

FIGURE 1: Concentration dependence of the loss of thymidylate synthetase (10^{-6} M) activity in the presence of a 1.3- (●), 3.4- (▲), 6.3- (■), and 16.8- fold (○) excess of MMTS per mol of catalytic cysteine, assayed at pH 6.8 in 0.1 M potassium phosphate.

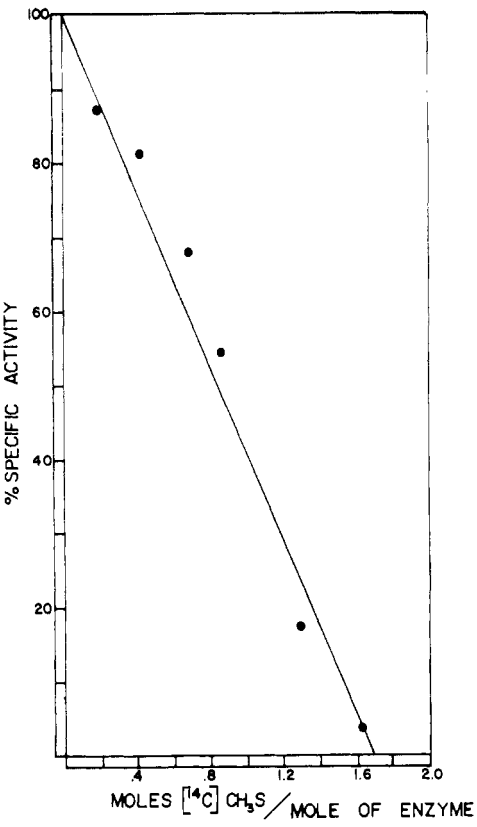

FIGURE 2: Correlation of incorporation of $[^{14}C]CH_3$-S- into thymidylate synthetase with loss of enzymatic activity. Enzyme (4.8×10^{-5} M) was incubated with 0.25-2.0-fold molar excesses of [^{14}C]MMTS at pH 8.0 in 50 mM potassium phosphate at 30 °C for 30 min, assayed, and chromatographed on Sephadex G-10 columns. The final protein concentration was determined by A_{280}, and the amount of label present was quantitated by liquid scintillation counting.

preincubated and then used to inactivate the enzyme, the potency of the MMTS appeared to have been greatly diminished. Therefore, the observed apparent protection by (±)-CH_2H_4folate is due to an actual reduction in the concentration of viable MMTS rather than a competition for binding in the active site. The product of the reaction between MMTS and (±)-CH_2H_4folate was not further characterized. Deoxyuridine did not protect against inactivation by MMTS, a fact which is consistent with its inability to serve as a substrate for the enzyme. Deoxyuridylate exhibited good protection against inactivation yielding a second-order rate constant of 730 M^{-1} s^{-1} when the nucleotide was present in a 10.7-fold excess over the enzyme, which can be contrasted with a k_2 of 19 000 M^{-1} s^{-1} for the rate of inactivation of the unprotected enzyme. dTMP was slightly less effective as a protecting agent and yielded a k_2 = 1400 M^{-1} s^{-1} when present in an 8.4-fold excess over the enzyme. Surprisingly, a 7.9-fold excess of dCMP slowed the rate of inactivation to 9030 M^{-1} s^{-1}. Previous studies by Danenberg et al. (1974) and Leary et al. (1975) have shown that dUMP can protect the enzyme from sulfhydryl group modification by iodoacetamide.

Inactivations performed in buffers other than Tris-Cl as controls for the dUMP protection studies were found to proceed much more rapidly at substantially lower concentrations of MMTS than in phosphate buffer. The second-order rate constants for the rapid phase of these inactivations are presented in Table I. The presence of polyoxyanions appeared to slow the inactivations. The rate of inactivation at pH 7.4 was fastest in Tris-Cl and decreased in the order Tris-acetate > Pipes > Tris-AsO_4 > Tris-SO_4 > PO_4 > Tris-PO_4. The rate of inactivation at pH 6.0 was fastest in Pipes and approximately the same in phosphate and malate. Inactivations in the various buffers were all observed to be biphasic. The degree

TABLE I: Buffer Effects on Rate of MMTS-Dependent Inactivation of Thymidylate Synthetase.

buffer and pH	[MMTS]/[enzyme dimer]	k_2 (M^{-1} s^{-1})
Tris-Cl, pH 7.4	3	19 000
Tris-acetate, pH 7.4	3	13 500
Pipes, pH 7.4	3	10 500
Tris-AsO_4, pH 7.4	3	3 270
Tris-SO_4, pH 7.4	3	2 630
PO_4, pH 7.4	10	1 600
Tris-PO_4, pH 7.4	10	1 200
Pipes, pH 6.0	10	362
PO_4, pH 6.0	10	117
malate, pH 6.0	10	95

of protection afforded by the substrate dUMP also varied as a function of buffer. Second-order rate constants for the rapid phase of those inactivations in these various buffers in the presence and absence of dUMP are presented in Tables I and II. In general, the faster the rate of inactivation, the better the protection observed in the presence of dUMP. Pipes buffer is an exception to the above trend since the protection exhibited by dUMP is better in this buffer than that observed in Tris-Cl and Tris-acetate, even though the rates of inactivation are faster in the latter buffers (Table II).

The effect of added phosphate upon the rate of inactivation at two different MMTS to enzyme ratios is shown in Figure 3. It should be noted that, when the ln k_2 is plotted against phosphate concentration as in Figure 3, the lines drawn do not

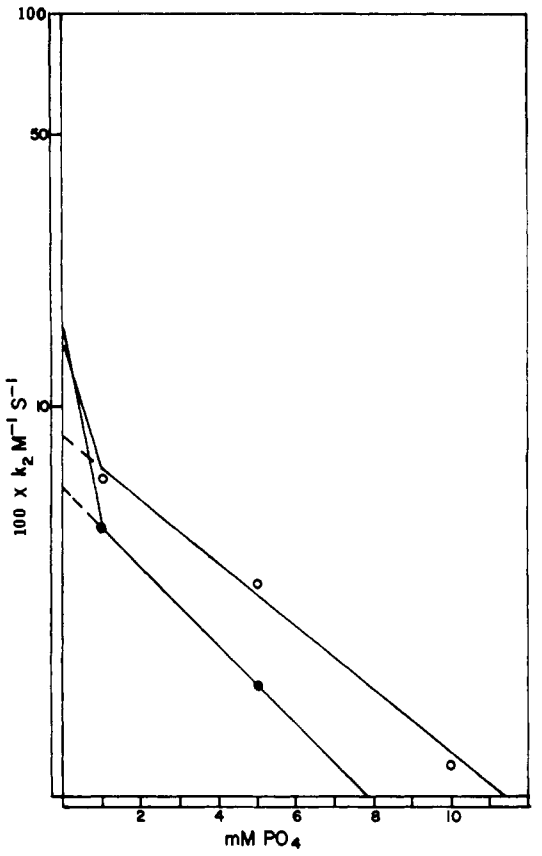

FIGURE 3: Effect of added phosphate on the rate of MMTS-dependent inactivation of thymidylate synthetase in 50 mM Tris-Cl, pH 7.4. Enzyme (4×10^{-6} M) was inactivated with either a 3- (●) or 5-fold (○) molar excess of MMTS in the presence of added sodium phosphate.

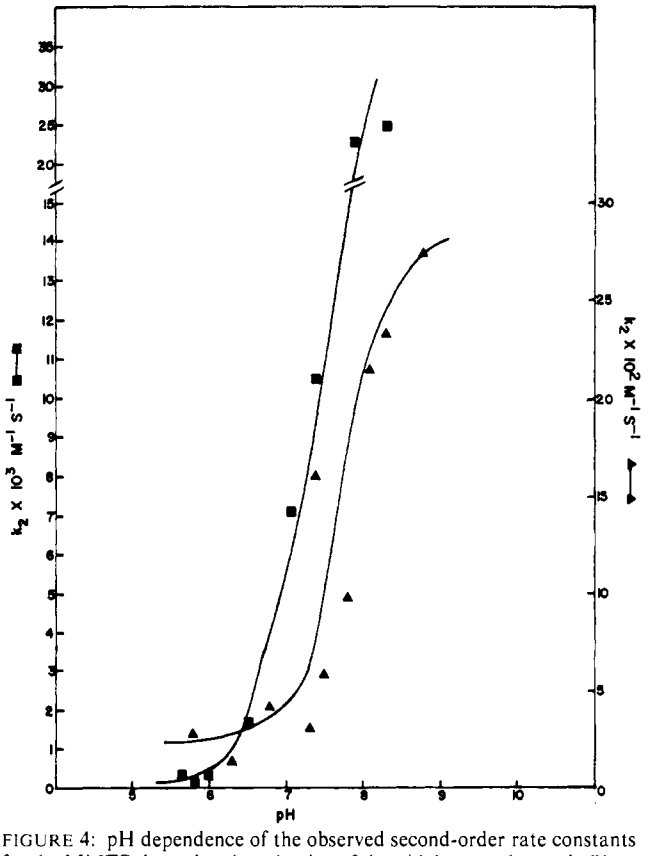

FIGURE 4: pH dependence of the observed second-order rate constants for the MMTS-dependent inactivation of thymidylate synthetase in Pipes (■) and potassium phosphate (▲) buffers. Enzyme (10^{-6} M) in 50 mM buffer at 0 °C was inactivated with a 3-fold molar excess of MMTS in Pipes and a 10-fold molar excess in potassium phosphate. The phosphate data are taken from Munroe et al. (1978).

TABLE II: Buffer Effect on dUMP Protection against MMTS-Dependent Inactivation of Thymidylate Synthetase.

buffer and pH	MMTS/ enzyme dimer	dUMP/ enzyme dimer	k_2 (M^{-1} s^{-1})
Tris-Cl, pH 7.4	3	10.7	730
Tris-Cl, pH 7.4	3	2.0	2670
Tris-acetate, pH 7.4	3	2.0	1253
Pipes, pH 7.4	3	2.0	1366
Pipes, pH 7.4	3	10.5	0
Tris-AsO_4, pH 7.4	3	2.0	894
Tris-SO_4, pH 7.4	3	2.0	335
PO_4, pH 7.4	10	2.0	1470
Tris-PO_4, pH 7.4	10	2.92	530
Pipes, pH 6.0	10	2.2	1.0
PO_4, pH 6.0	10	2.0	110
malate, pH 6.0	10	2.0	10.3

TABLE III: Buffer Effects on Rate of DTNB-Dependent Inactivation of Thymidylate Synthetase.

buffer and pH	DTNB/ enzyme dimer	k_2 (M^{-1} s^{-1})
Tris-Cl, pH 7.4	50	157
Tris-SO_4, pH 7.4	68	105
PO_4, pH 7.4	100	50
Tris-PO_4, pH 7.4	100	40
Tris-PO_4 + 2 × dUMP	100	17
Tris-PO_4 + 10 × dUMP	100	10

extrapolate back to the experimental values determined for zero concentration of phosphate.

To ensure that the polyoxyanions were not interacting with MMTS to affect the rates of inactivation, DTNB inactivation was studied in several buffers (see Table III). A threefold rate enhancement of DTNB dependent inactivation was observed in Tris-Cl over PO_4 at pH 7.4. The rate of inactivation was observed to decrease from Tris-Cl > Tris-SO_4 > PO_4 > Tris-PO_4 exactly as observed for the MMTS inactivation rates. Modification of 1.6–1.7 sulfhydryls per dimer was determined with this reagent. The substrate dUMP also afforded moderate protection in Tris-PO_4 buffer.

It was not possible to determine a complete pH dependence for MMTS inactivation in Pipes buffer due primarily to the extremely rapid rate of inactivation at higher pH (>25 000 M^{-1} s^{-1} at pH 8.0). However, we were able to demonstrate that the behavior is in qualitative agreement with the previously reported pH dependence in phosphate buffer (Munroe et al., 1978). Figure 4 shows a comparison of the Pipes results and the phosphate results (note the different scales).

Discussion

Methyl methanethiosulfonate has been demonstrated to be a useful reagent for inactivating thymidylate synthetase by blocking the catalytic sulfhydryls with a CH_3S- group. This reagent has previously been used to great advantage by Lewis et al. (1976) in studying ionization of active site residues in papain. Modification of thymidylate synthetase required low molar excesses of MMTS, is rapid, and readily reversible by addition of thiols. The inactivation occurs through the modification of the catalytic cysteine residues of the enzyme, which

serve as the nucleophile which initiates the enzymatic reaction by attacking C-6 of the uracil ring. This conclusion is supported by observation of the incorporation of 1.5 to 1.6 mol of [^{14}C]CH_3S- into thymidylate synthetase when the enzyme is inactivated using [^{14}C]MMTS. Modification of 1.5–1.7 sulfhydryl groups with MMTS and DTNB is consistent with the results of the previous report of Plese & Dunlap (1977), where *n*-ethylmaleimide, *p*-chloromercuribenzoate, iodoacetamide, and DTNB were used to determine the number of catalytic sulfhydryl groups per enzyme dimer for enzyme prepared in the presence of exogenous thiol. These values correlate well with the extent of ternary complex formation as described by Aull et al. (1974a), Donato et al. (1976), and Plese & Dunlap (1977). The mixed population of enzyme capable for forming 1:1:1 (30%) and 2:2:1 (70%) ternary complexes leads to a predicted average sulfhydryl group content of 1.7 per enzyme dimer. As described above, labeling of these cysteines results in the parallel loss of enzymatic activity and ability to form ternary complexes (Plese & Dunlap, 1977).

Evaluation of protection afforded by several pyrimidines was performed in Tris-Cl at pH 7.4, conditions free from effects of polyoxyanions. The greater protection observed for dUMP over dTMP is consistent with the K_m values reported by Daron & Aull (1978). Slight protection exhibited by dCMP may indicate very weak affinity of this pyrimidine for the active site of thymidylate synthetase. The importance of the 5′-phosphate in nucleotide binding is indicated by the absence of protection by dU.

Addition of phosphate to inactivations performed in Tris-Cl at pH 7.4 dramatically decreased the rate of inactivation (Figure 3). This effect has also been observed at phosphate concentrations as low as 0.1 mM if the MMTS concentration is also low (1.75 mol of MMTS/mol of catalytic cysteine). Unfortunately, these data do not lend themselves to classical treatment to determine if phosphate is a competitive or noncompetitive inhibitor or to permit the evaluation of its inhibition constant, K_i. If these anions were simply competing for the 5′-phosphate portion of the dUMP binding site, one would expect that a plot such as Figure 3 would yield a line which extrapolated through the k_2 experimentally determined at zero concentration of phosphate. The observed extrapolation to a value much lower than that actually observed is inconsistent with a simple competitive interaction of polyoxyanions with the enzyme. It is not clear how this effect relates to the apparent competitive inhibition of the enzymatic reaction by phosphate alluded to by Daron & Aull (1978). The break in the plot in Figure 3 may suggest that phosphate interacts at more than one site or exhibits two affinities for the enzyme. This effect, observed at low concentration of phosphate (<1 mM), is probably distinct from the protection effects exhibited by high concentrations of phosphate (50 mM) against proteolytic inactivation as reported by Galivan et al. (1977); however, the linear decrease in the rate of inactivation at intermediate phosphate concentrations (1–10 mM) may be reflecting a similar interaction with the enzyme.

In view of the dramatic polyoxyanion effects described above, the pH dependence of the inactivation of thymidylate synthetase by MMTS was extended to include Pipes buffer. Our results show that Pipes appears to be relatively noninteracting and is an effective buffer over the pH range examined. The entire pH profile could not be determined since extremely rapid rates of reaction were observed at high pH. The resulting data (Figure 4) are in qualitative agreement with our earlier results obtained in phosphate buffer, which indicated a pK_a of approximately 8.0. Although we were not able to complete the profile and hence cannot determine a pK_a in Pipes buffer, examination of the data suggests that the pK_a may be slightly lower than 8.0 but not less than 7.5. More important, however, is the fact that the rate of inactivation levels off at low pH, which is consistent with activation of the catalytic cysteine by a general base as proposed earlier (Munroe et al., 1978). Therefore, the activation of the catalytic cysteines appears to be a general phenomenon and not an effect due to the phosphate buffer used in the previous study.

Polyoxyanions present in the inactivation mixtures greatly decreased the rates of inactivation of thymidylate synthetase by MMTS and DTNB. Such an effect was quite unexpected since the enzyme is most stable and displays high specific activities over a broad pH range in phosphate buffers[3] (Dunlap et al., 1971). Observation of pronounced polyoxyanion effects for both sulfhydryl reagents suggests that these anions are exerting their influence on the enzyme, rather than on the reagents. It should also be noted that this effect is on sulfhydryl group modification and is not dependent upon nucleotide binding. The smaller relative decreases in rate of DTNB-dependent inactivation than those observed for MMTS probably reflect the larger size, charged nature and decreased reactivity of the disulfide compared with the thiolsulfonate (Kenyon & Bruice, 1977).

The degree of protection afforded by dUMP in the presence of different polyoxyanions decreased in the same order as the decreasing rates of sulfhydryl group modification. The decreased protection in the presence of polyoxyanions is consistent with the previous studies of Galivan et al. (1976); however, the observed parallel trend suggests that these polyoxyanions may be doing more than competing for the nucleotide binding site. Pipes buffer does not fit entirely into this trend (compare Tables I and II). This may be due to the sulfonate portion of the Pipes molecule interacting with the enzyme in a manner different from a sulfate molecule.

A priori the polyoxyanion effect described above might be explained as a simple competitive interaction of these anions with the 5′-phosphate portion of the dUMP binding site, which could be sufficiently close to the catalytic cysteine to affect its reactivity. Several observations suggest that this is not the case. Circular dichroism studies by Leary et al. (1975) have been interpreted to suggest that dUMP is bound to the enzyme in the syn conformation, such that the 2-keto group is projected toward the 5′-phosphate. This conformation places carbon-6, the site of the nucleophilic attack by the sulfhydryl, at the greatest distance from the phosphate. Binding of free phosphate or the other polyoxyanions at such a distant site is unlikely to account for the observed effects. The specific activity of the enzyme is as high in 0.1 M potassium phosphate as measured in 0.1 M Tris-Cl, also arguing against simple competition. Preliminary experiments describing arginine modification and inactivation of thymidylate synthetase have been reported by Cipollo & Dunlap (1978). Arginine is postulated to provide the cationic counterion for binding of the 5′-phosphate of dUMP. The arginines modified in these studies are protected from modification by dUMP, but not by folates, suggesting that their role is in the nucleotide binding domain. If the 5′-phosphate portion of the nucleotide binding site can bind inorganic phosphate, then the presence of phosphate should be expected to decrease the rate of arginine modification. However, modification by 2,3-butanedione in borate buffer is not affected by added phosphate and phenylglyoxal modification of arginine in phosphate buffer occurs rapidly.[4] This evidence, taken together with the data of Figure 3,

[3] J. L. Aull, M. P. Murray, & R. B. Dunlap, unpublished results.
[4] K. L. Cipollo & R. B. Dunlap, unpublished results.

suggests that the effect of these anions is both not at the 5′-phosphate portion of the dUMP binding site and also not a simple competitive effect.

The most interesting aspect of the results presented here is the decreased rate of sulfhydryl group modification in the presence of polyoxyanions. This is particularly interesting in light of the many previous studies[3] (Dunlap et al., 1971; Galivan et al., 1977; Daron & Aull, 1978) indicating that the enzyme is highly active and very stable in phosphate buffer, even though the reactivity of the catalytic cysteines is diminished. The fact that the anions show an analogous effect on the protection afforded by dUMP suggests that these two properties may be monitoring the same effect on the enzyme. This may suggest that the polyoxyanions bind in the dUMP binding portion of the active site in close proximity to the catalytic cysteines. This idea is not unreasonable if one considers the general base residues which are proposed to be in the active site. We have proposed a general base residue which activates the catalytic cysteine (Munroe et al., 1978) and general base residues have been proposed to abstract the proton from C-5 and to serve as a binding locus for the 4 keto group of dUMP (Reyes & Heidelberger, 1965; Santi & Sakai, 1971, 1972; Danenberg & Danenberg, 1978). These residues in the pyrimidine portion of the nucleotide binding site may be involved in binding of polyoxyanions to the enzyme. It is not possible to conclude from the available data whether the presence of phosphate in this portion of the active site results in competitive inhibition or exerts a more complex effect on the enzyme; however, the results do indicate that the anions bind in close proximity to the catalytic cysteines and have dramatic effects on the reactivity of the thiol nucleophiles.

References

Aull, J. L., Lyon, J. A., & Dunlap, R. B. (1974a) *Arch. Biochem. Biophys. 165,* 805–808.

Aull, J. L., Lyon, J. A., & Dunlap, R. B. (1974b) *Microchem. J. 19,* 210–218.

Byrd, R. A. (1977) Ph.D. Thesis, University of South Carolina.

Byrd, R. A., Dawson, W. H., Ellis, P. D., & Dunlap, R. B. (1977) *J. Am. Chem. Soc. 99,* 6139–6141.

Byrd, R. A., Dawson, W. H., Ellis, P. D., & Dunlap, R. B. (1978) *J. Am. Chem. Soc.* (in press).

Cipollo, K. L., & Dunlap, R. B. (1978) *Biochem. Biophys. Res. Commun. 81,* 1139–1144.

Currier, S. F., & Mautner, H. G. (1977) *Biochemistry 16,* 1944–1948.

Danenberg, P. V. (1977) *Biochim. Biophys. Acta 473,* 73–92.

Danenberg, P. V. & Danenberg, K. D. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* Abstract No. 156.

Danenberg, P. V., Langenbach, R. J., & Heidelberger, C. (1974) *Biochemistry 13,* 926–933.

Daron, H. H., & Aull, J. L. (1978) *J. Biol. Chem. 253,* 940–945.

Donato, H., Jr., Aull, J. L., Lyon, J. A., Reinsch, J. W., & Dunlap, R. B. (1976) *J. Biol. Chem. 251,* 1303–1310.

Dunlap, R. B., Harding, N. G. L., & Huennekens, F. M. (1971) *Biochemistry 10,* 88–97.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82,* 70–77.

Galivan, J., Maley, F., & Baugh, C. M. (1977) *Arch. Biochem. Biophys. 184,* 346–354.

Kenyon, G. L., & Bruice, T. W. (1977) *Methods Enzymol. 47,* 407–430.

Leary, R. P., Beaudette, N., & Kisliuk, R. L. (1975) *J. Biol. Chem. 250,* 4864–4868.

Lewis, S. D., Johnson, F. A., & Shafer, J. A. (1976) *Biochemistry 15,* 5009–5017.

Lyon, J. A., Pollard, A. L., Loeble, R. B., & Dunlap, R. B. (1975) *Cancer Biochem. Biophys. 1,* 121–128.

Munroe, W. A., Lewis, C. A., Jr., & Dunlap, R. B. (1978) *Biochem. Biophys. Res Commun. 80,* 355–360.

Plese, P. C., & Dunlap, R. B. (1977) *J. Biol. Chem. 252,* 6139–6144.

Reyes, P., & Heidelberger, C. (1965) *Mol. Pharmacol. 1,* 14–30.

Santi, D. V., & Sakai, T. T. (1971) *Biochemistry 10,* 3598–3607.

Santi, D. V., & Sakai, T. T. (1972) *Biochem. Biophys. Res. Commun. 46,* 1320–1325.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14,* 766–771.