Lim, V. I. (1974a), *J. Mol. Biol. 88,* 857–872.
Lim, V. I. (1974b), *J. Mol. Biol. 88,* 873–894.
Magnusson, S., Peterson, T. E., Sottrup-Jensen, L., and Claeys, H. (1975), in Proteases and Biological Control, Reich, E., Rifkin, D. B., Shaw, E., Eds., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, pp 123–150.
Magnusson, S., Sottrup-Jensen, L., Claeys, H., Peterson, T. E., and Zajdel, M. (1976), in Abstracts, The Eighth Miami Winter Symposia, Ribbons, D. W., Brew, K., Ahmad, F., Schultz, J., Eds., Miami, Florida, University of Miami, pp 30–34.
Majerus, P. W. (1968), *Science 159,* 428–430.
Matthews, B. W. (1975), *Biochim. Biophys. Acta 405,* 442–451.
Moffat, K., Fullmer, C. H., and Wasserman, R. H. (1975), *J. Mol. Biol. 97,* 661–664.
Nagano, K. (1973), *J. Mol. Biol. 75,* 401–421.
Nagano, K. (1974), *J. Mol. Biol. 84,* 337–372.
Nelsestuen, G. L., Broderius, M., Zytkovicz, T. H., and Howard, J. B. (1975), *Biochem. Biophys. Res. Commun. 65,* 233–240.
Potter, J. D., and Gergely, J. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1465.
Rossmann, M. G., Moras, D., and Olsen, K. W. (1974), *Nature (London) 250,* 194–199.
Sauer, F., Pugh, E. L., Wakil, S. J., Delaney, R., and Hill, R. L. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1360–1366.
Schulz, G. E., Barry, C. D., Friedman, J., Chou, P. Y., Fasman, G. D., Finkelstein, A. V., Lim, V. I., Ptitsyn, O. B., Kabat, R. A., Wu, T. T., Levitt, M., Robson, B., and Nagano, K. (1974), *Nature (London) 250,* 140–142.
Schulz, H. (1972), *Biochem. Biophys. Res. Commun. 46,* 1446–1453.
Stenflo, J. (1974), *J. Biol. Chem. 249,* 5527–5535.
Stenflo, J., Fernlund, P., Egan, W., and Roepstarff, P. (1974), *Proc. Natl. Acad. Sci., U.S.A. 71,* 2730–2733.
Szent-Gyorgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 47–53.
Szent-Gyorgyi, A. G., Szentkiralyi, E. M., and Kendrick-Jones, J. (1973), *J. Mol. Biol. 74,* 174–203.
Tufty, R. M., and Kretsinger, R. H. (1975), *Science 187,* 167–169.
Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968a), *J. Biol. Chem. 243,* 6409–6419.
Vanaman, T. C., Wakil, S. J., and Hill, R. L. (1968b), *J. Biol. Chem. 243,* 6420–6431.
Van Eerd, J., and Takahashi, K. (1975), *Biochem. Biophys. Res. Commun. 64,* 122–127.
Weber, A., and Murray, J. M. (1973), *Physiol. Rev. 53,* 612–673.
Weber, M. M., Gaffin, S. L., and Oplatka, A. (1972), *J. Mechanochem. Cell Motil. 1,* 91–96.
Weeds, A. G. (1967), *Biochem. J. 105,* 25C–27C.
Weeds, A. G., and Frank, G. (1972), *Cold Spring Harb. Symp. Quant. Biol. 37,* 9–16.
Weeds, A. G., and McLachlan, A. D. (1974), *Nature (London) 252,* 646–649.

Interactions of Platinum Complexes with the Essential and Nonessential Sulfhydryl Groups of Thymidylate Synthetase†

John L. Aull,* Allison C. Rice,‡ and Laura A. Tebbetts

ABSTRACT: Thymidylate synthetase (methylenetetrahydrofolate:deoxyuridylate *C*-methyltransferase) from *Lactobacillus casei* was progressively inactivated when incubated at 25 °C, pH 6.8, in the presence of *trans*-$Pt(NH_3)_2Cl_2$. The inhibition appeared to be irreversible, and the rate of activity loss was dependent on the inhibitor concentration. The corresponding cis isomer was incapable of inhibiting the enzyme under the same conditions. The presence of 2-mercaptoethanol protected the enzyme from inhibition, but did not reactivate enzyme preparations which had been inhibited prior to the addition of the thiol. The interactions of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ with the enzyme's sulfhydryl (-SH) groups were inferred from the results of spectrophotometric titrations of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-hydroxymercuribenzoate. The results suggested that the cis isomer reacted with an average of 1.3 of the enzyme's 4 -SH groups and that these were not essential for catalysis. The trans isomer reacted with a total of approximately 2.5 -SH groups, 1.2 of which are essential for catalysis. Neither the trans isomer nor a combination of both isomers was able to react with 1.2 of the 4 -SH groups. Further evidence that the Pt complexes are interacting with enzyme's -SH groups was obtained by reversibly blocking the -SH groups of thymidylate synthetase, and demonstrating the resistance of these preparations to inhibition by the trans Pt complex. Possible explanations for the preferential inhibition of thymidylate synthetase by only one of the two geometric isomers of $Pt(NH_3)_2Cl_2$ are considered.

Thymidylate synthetase catalyzes the reductive methylation of 2′-deoxyuridylate by (+)-5,10-methylenetetrahydrofolate to form 2′-deoxythymidylate and 7,8-dihydrofolate (Huennekens, 1968; Blakely, 1969) as shown in eq 1.

$$\text{dUMP} + (+)\text{-5,10-CH}_2\text{H}_4\text{-folate} \rightarrow \text{dTMP} + \text{dihydrofolate}^{1} \quad (1)$$

† From the Department of Chemistry, Auburn University, Auburn, Alabama 36830. *Received June 23, 1976.* Supported by a grant from Research Corporation and an Auburn University Grant-in-aid.

‡ Present address: Department of Microbiology, Vanderbilt University, Nashville Tennessee.

[1] Abbreviations used are: 5,10-CH_2H_4-folate, 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridylate; pHMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); H_4-folate, 5,6,7,8-tetrahydrofolate; $NaDodSO_4$, sodium dodecyl sulfate.

This reaction is of special interest because the compound 5-fluorouracil ultimately exerts its antitumor properties by inhibiting thymidylate synthetase, and depleting the cells' supply of the thymidylate required for DNA synthesis (Friedkin, 1973).

Lactobacillus casei thymidylate synthetase has a molecular weight of 70 000 (Dunlap et al., 1971) and consists of two subunits having the same N terminus and molecular weight (Loeble and Dunlap, 1972). Recent results have strongly indicated that the apparently identical subunits of thymidylate synthetase do not interact symmetrically to form two equivalent active sites. Circular dichroic spectral measurements have indicated that 1 mol of dUMP was bound per mol of enzyme in the absence of folates (Leary et al., 1975). Galivan et al. (1976) have recently used equilibrium dialysis to show that single site binding was exhibited by dUMP, dTMP, (+)-5,10-CH_2H_4-folate, and several other folates under a variety of conditions. On the other hand, these studies indicated approximately two nonequivalent binding sites for FdUMP. Fluorescence and circular dichroic spectral measurements (Donato et al., 1976a) as well as gel electrophoresis studies (Aull et al., 1974) have shown that thymidylate synthetase has two nonequivalent binding sites for both FdUMP and (+)-5,10-CH_2H_4-folate when all three components are present in the reaction mixtures.

Investigations of the role of the sulfhydryl groups of thymidylate synthetase in accomplishing catalysis have also yielded results which are inconsistent with the two active site hypothesis. It has been reported that 1 mol of enzyme is completely inactivated by either 1 mol of pHMB (Dunlap et al., 1971), DTNB, or iodoacetamide (Leary et al., 1975).

Rosenberg et al. (1969) have shown that certain Pt–amine complexes exhibit antitumor activity, and it appears that the major effect of these compounds on tumor cells is to inhibit DNA synthesis by cross-linking the complementary strands of nucleic acids (Roberts and Pascoe, 1972). Other researchers have reported that the cis and trans isomers of $Pt(NH_3)_2Cl_2$ are inhibitors of enzymes (Melius et al., 1972), particularly those containing reactive sulfhydryl groups (Friedman and Teggins, 1974). It was these observations which prompted the present investigation into the interactions of Pt complexes with the sulfhydryl groups of thymidylate synthetase.

Experimental Procedures

Materials

Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei* (Dunlap et al., 1971) was purified in the presence of 10 mM 2-mercaptoethanol by a procedure involving ammonium sulfate fractionation of cell-free extracts of sonicated cells, followed by successive chromatography on carboxymethyl-Sephadex and hydroxylapatite (Lyon et al., 1976). Enzyme preparations which were homogeneous, as evidenced by analytical polyacrylamide gel electrophoresis, had specific activities ranging from 2.7 to 3.9 units/mg when assayed spectrophotometrically in the presence of 25 mM 2-mercaptoethanol (Dunlap et al., 1971). One unit of thymidylate synthetase is defined as that amount of enzyme catalyzing the formation of 1 μmol of thymidylate per min. The present study utilized pure thymidylate synthetase which had been activated by dialysis at 5 °C for 12 to 24 h against 0.1 M potassium phosphate buffer, pH 6.8, containing 25 mM 2-mercaptoethanol. Mercaptoethanol-free enzyme solutions were subsequently prepared for use in the experiments described herein.

cis-$Pt(NH_3)_2Cl_2$ was prepared and analyzed by the method of Ramberg (1913) as modified by Reishus and Martin (1961). *trans*-$Pt(NH_3)_2Cl_2$ was prepared and analyzed by the method of Drew (1932) as modified by Bauer (1953).

H_4-folate was prepared by the catalytic hydrogenation of folic acid in acetic acid (Hatefi et al., 1960) and was stored at −50 °C as a lyophilized powder under argon in sealed serum bottles. Stock solutions of (±)-5,10-CH_2H_4-folate were prepared by dissolving H_4-folate in a solution consisting of 0.05 M sodium bicarbonate, 0.07 M formaldehyde, and 0.25 M 2-mercaptoethanol (Dunlap et al., 1971).

Other chemicals were obtained as follows: folic acid, dUMP, urea, DTNB, pHMB, and $NaDodSO_4$ (Sigma Chemical Co.); Sephadex G-100 and G-25 and carboxymethyl (C50)-Sephadex (Pharmacia Fine Chemicals); hydroxylapatite (Bio-Rad Laboratories).

Methods

Enzyme Preparations and Assays. Mercaptoethanol-free solutions of thymidylate synthetase were prepared by diluting enzyme stock solutions which contained mercaptoethanol and 0.1 M phosphate, with cold distilled water to give a final phosphate concentration of 0.05 M. The diluted solution was applied to a small carboxymethyl-Sephadex column (approximately 1 ml of swollen gel per 5 mg of protein applied) equilibrated in 0.05 M potassium phosphate buffer, pH 6.8. The column was then washed with ten times the column volume of cold equilibration buffer, which had been previously deoxygenated by boiling and bubbling with argon. The protein was eluted from the column with cold, deoxygenated 0.1 M potassium phosphate buffer, pH 6.8, which contained 0.5 M KCl. The thiol-free enzyme solutions were stored under argon at 5 °C in screw-capped tubes. These solutions were diluted with the elution buffer to give the desired enzyme concentration.

Thymidylate synthetase was assayed spectrophotometrically (Wahba and Friedkin, 1962) at 25 °C, as previously described (Dunlap et al., 1971). Unless otherwise stated, the assays were performed in the presence of 25 mM 2-mercaptoethanol. Enzyme concentrations were established by using extinction coefficients at 278 nm of 105 000 M^{-1} or 1.55 ml mg^{-1} (Lyon et al., 1976), or by the method of Lowry (Lowry et al., 1951) using pure thymidylate synthetase as a standard. All spectral measurements were made on a Gilford Model 250 spectrophotometer equipped with an automatic cuvette changer, recorder, and wavelength scanner.

Measurement of Inhibition of Thymidylate Synthetase by Pt Complexes. Mercaptoethanol-free solutions of the enzyme in 0.1 M potassium phosphate buffer (pH 6.8)–0.5 M KCl were prepared as described. Stock solutions of the Pt complexes ranging in concentration from 1 to 8 mM were prepared by dissolving the crystalline solids in 0.08 M sodium pyrophosphate buffer, pH 8.8. Dissolution was accomplished by heating the suspensions to 60 °C with vigorous shaking for about 10 min. The resulting solutions were stored at 5 °C in the dark for no longer than 5 days prior to use. Typically, the inhibition reactions were begun by adding the appropriate volume (5–100 μl) of an inhibitor stock solution to an aliquot of enzyme (1–5 ml) solution to give the desired concentration of inhibitor and enzyme. In experiments where the inhibitor concentration was the variable, the concentrations of all other components of the reaction mixtures were kept constant by adding appropriate volumes of 0.01 M sodium pyrophosphate buffer, pH 8.8, to the samples. The inhibition reaction mixtures were incubated

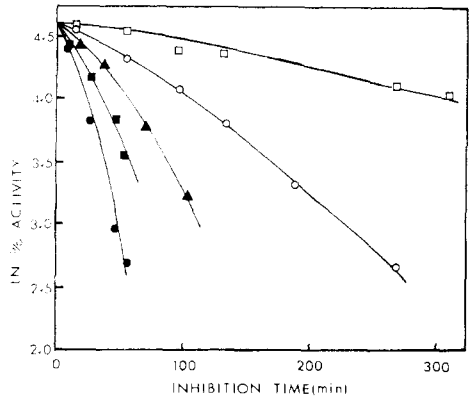

FIGURE 1: Inhibition of thymidylate synthetase by *trans*-$Pt(NH_3)_2Cl_2$. Enzyme concentration: 2.0×10^{-6} M. Inhibitor concentrations: 1.90×10^{-4} (□), 3.81×10^{-4} (○), 5.22×10^{-4} (▲), 6.96×10^{-4} (■), 1.04×10^{-3} M (●). Inhibition carried out at 25 °C in 0.1 M potassium phosphate, pH 6.8.

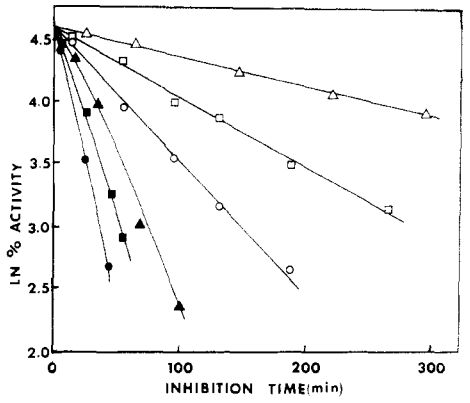

FIGURE 2: Inhibition of *cis*-$Pt(NH_3)_2Cl_2$-pretreated thymidylate synthetase by *trans*-$Pt(NH_3)_2Cl_2$. The enzyme was preincubated with the *cis* Pt complex prior to addition of the trans Pt complex. (See text for details.) Enzyme concentration: 2.0×10^{-6} M. *trans*-$Pt(NH_3)_2Cl_2$ concentrations: 9.52×10^{-5} (△), 1.90×10^{-4} (□), 3.81×10^{-4} (○), 5.22×10^{-4} (▲), 6.96×10^{-4} (■), 1.04×10^{-3} M (●). Inhibition carried out at 25 °C in 0.1 M potassium phosphate, pH 6.8.

at 25 °C and aliquots were withdrawn for assay and/or sulfhydryl group titration after various inhibition times.

Titration of the Sulfhydryl Groups of Thymidylate Synthetase. The DTNB titrations were carried out essentially by the method previously described (Ellman, 1959). Typically, 25 μl of a 0.01 M DTNB solution was added to a cuvette containing 0.5 ml of an enzyme solution in 0.1 M potassium phosphate buffer, pH 6.8. The color was developed for 10 min and read at 412 nm against an enzyme solution containing no DTNB. A reagent blank was subtracted from the apparent absorbance to give the net absorbance. A molar absorptivity value of 13 600 M^{-1} cm^{-1} (Ellman, 1959) was used for calculation of the sulfhydryl content of the enzyme solution in the absence of denaturing reagents. The sulfhydryl content of the enzyme solution was determined in the presence of 5 M urea by the addition of 240 mg of solid urea to each of the three cuvettes containing the sample, enzyme blank, and reagent blank, respectively. The net absorbance at 412 nm was determined, and the volume of each cuvette was measured for use in recalculating the enzyme concentration in the sample. A molar absorptivity of 13 600 M^{-1} cm^{-1} was determined for nitromercaptobenzoate in 0.1 M potassium phosphate (pH 6.8)–5 M urea, using a standard solution of 2-mercaptoethanol. In the absence of urea, the standard 2-mercaptoethanol solution gave a molar absorptivity value of 13 200 M^{-1} cm^{-1}, which is in close agreement with the published value of 13 600 M^{-1} cm^{-1}.

Titrations with pHMB were performed in 0.1 M phosphate buffer, pH 6.8, as described (Stadtman, 1957) using a molar absorptivity of 7300 M^{-1} cm^{-1} at 250 nm.

The separation of thymidylate synthetase from excess reagents in the various reaction mixtures was accomplished by gel filtration at 5 °C on columns of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 6.8.

Electrophoretic Techniques. Electrophoresis was performed at 5–8 °C on 7.5% polyacrylamide essentially as described (Ornstein, 1964; Davis, 1964). No stacking gels were used. Gels were stained for protein using Coomassie brilliant blue (Chrambach et al., 1967).

Results and Discussion

Inhibition of Thymidylate Synthetase by trans-$Pt(NH_3)_2Cl_2$. The progressive inhibition of thymidylate synthetase observed when the enzyme was incubated in the presence of various concentrations of *trans*-$Pt(NH_3)_2Cl_2$ is shown in Figure 1. The same inhibition rates were observed whether the enzyme assays were performed in the presence or absence of 25 mM 2-mercaptoethanol. However, the inclusion of 25 mM 2-mercaptoethanol in the inhibition reaction mixtures completely prevented the inhibition of the enzyme. Therefore, mercaptoethanol can apparently prevent the interaction between the enzyme and Pt complex, but cannot readily reverse the inhibition process once it has occurred. The inhibition experiments were carried out under pseudo-first-order conditions to facilitate kinetic analyses; however, the curved semilogarithmic plots indicate that the kinetics are complex. The fact that the rate of inhibition was not proportional to the inhibitor concentration further substantiates that the inhibition is not a simple, first-order process.

Treatment of thymidylate synthetase with up to a 530-fold molar excess of *cis*-$Pt(NH_3)_2Cl_2$ for periods as long as 24 h did not result in inhibition of enzyme activity. However, enzyme preparations which were first pretreated with the *cis*-$Pt(NH_3)_2Cl_2$ and subsequently inhibited with the *trans*-$Pt(NH_3)_2Cl_2$ showed inhibition patterns (Figure 2) which were markedly different from those shown in Figure 1. The data summarized in Figure 2 are taken from a typical experiment which involved preincubation of the enzyme for 5 h in the presence of a 500-fold molar excess of *cis*-$Pt(NH_3)_2Cl_2$, followed by the addition of various amounts of *trans*-$Pt(NH_3)_2Cl_2$ to aliquots of the pretreated enzyme preparation to initiate the inhibition process. A comparison between Figures 1 and 2 indicates that, although the cis Pt complex is not an inhibitor of thymidylate synthetase, it does interact with the enzyme in such a way as to render it more susceptible to inhibition by the trans Pt complex. At any given inhibitor concentration, enzyme preparations which had been pretreated with the cis Pt complex were inhibited at a faster rate than those which had not been pretreated.

Interaction of Pt Complexes with Thymidylate Synthetase Sulfhydryl Groups. Attempts to use amino acid analysis to identify the amino acid residues in thymidylate synthetase which were interacting with the Pt complexes failed, apparently because of the instability of the enzyme–Pt derivatives. The interaction of the Pt complexes with cysteine residues was thus inferred indirectly by determining the concentration of free sulfhydryl (–SH) groups in enzyme solutions both before and after treatment with Pt complexes. The concentration of

TABLE I: DTNB and pHMB Titrations of Thymidylate Synthetase before and after Treatment with Cis and Trans isomers of $Pt(NH_3)_2Cl_2$.[a]

Sample	Sp. Act. (units/mg)	[-SH] M/[Enz] M (DTNB) − Urea	[-SH] M/[Enz] M (DTNB) + 4 M Urea	[-SH] M/[Enz] M (pHMB) −Na-DodSO$_4$	[-SH] M/[Enz] M (pHMB) + 2% NaDodSO$_4$
Native enzyme	3.90	1.0	3.7	4.0	3.9
cis-$Pt(NH_3)_2Cl_2$ treated	3.95	1.0	2.4	3.2	2.7
trans-$Pt(NH_3)_2Cl_2$ treated	0	0	1.2	1.2	1.5

[a] The enzyme was incubated at 25 °C in the presence of 7.3×10^{-4} M Pt complex for 6 h and then isolated by gel filtration on Sephadex G-25 prior to titration. The final enzyme concentrations ranged from 7.3×10^{-6} to 8.7×10^{-6} M.

-SH groups was determined by spectrophotometric titrations with DTNB and pHMB both in the presence and absence of denaturing reagents, as described. The data shown in Table I represent the average of three separate experiments, all of which yielded values within 5% of those given in the table. Specific activities of the native and Pt-treated enzyme samples were measured before gel filtration and shown to be unchanged after gel filtration on Sephadex G-25, indicating that only the trans Pt complex inhibits, and that the inhibition process is not readily reversible. DTNB titrations performed in the presence of excess Pt complex (before gel filtration) gave essentially the same results as those shown in Table I, indicating that the Pt complexes do not interfere with the results of the titration by interacting with the DTNB. The DTNB titrations performed in the absence of urea indicate that native thymidylate synthetase contains one exposed -SH group per enzyme dimer, which is in agreement with previous reports (Leary et al., 1975). In the presence of 4 M urea, approximately 3 (2.7) additional -SH groups are capable of reacting with DTNB. The cis isomer of the two Pt complexes used in these studies interacts with the enzyme in such a way as to prevent the DTNB reaction with 1.3 of the enzyme's 3.7 -SH groups. The -SH groups blocked by the cis Pt complex are apparently not essential for enzyme activity, and do not correspond to the exposed -SH groups which react with DTNB in the absence of denaturing agents. The trans isomer prevents the DTNB reaction with approximately 2.5 -SH groups, one of which corresponds to the exposed -SH group that reacts with DTNB in the absence of denaturant. The inhibition of activity by the trans isomer suggests that at least one of the -SH groups blocked by this reagent is essential for catalysis. The results obtained using pHMB as the -SH group titrant were similar to those obtained with DTNB, except that about 4 -SH groups are exposed to pHMB both in the presence and absence of denaturing agent (Table I). The cis Pt complex blocked the pHMB titration of 0.8–1.2 -SH groups, and the trans Pt complex blocked 2.4–2.8 -SH groups.

When the enzyme was treated either sequentially or simultaneously, with both of the Pt complexes, the -SH group titrations indicated that 1.2–1.5 -SH groups were not blocked by either of the two compounds.

Correlation between the Loss of Titratable -SH Groups and the Inhibition of Thymidylate Synthetase. In these experiments, both the time-dependent loss of activity and the loss of titratable -SH groups was followed when solutions of thymidylate synthetase were incubated in the presence of the Pt

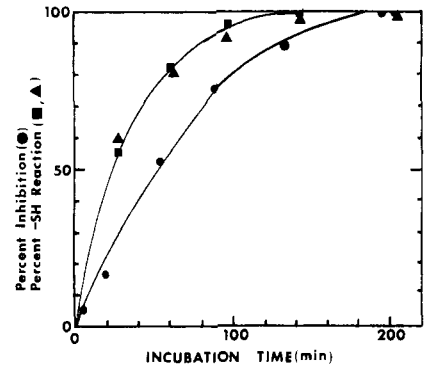

FIGURE 3: Correlation between the progressive inhibition of thymidylate synthetase by *trans*-$Pt(NH_3)_2Cl_2$ and the blocking of -SH groups as evidenced by DTNB titrations performed in the presence of 4 M urea. Enzyme concentration, 1.13×10^{-5} M; inhibitor concentration, 7.3×10^{-4} M. Percent inhibition after various incubation times (●). Sulfhydryl groups blocked by the inhibitor, expressed as percentages of the total number of -SH groups (2.5) blocked after prolonged incubation times (■, ▲) (two independent titrations).

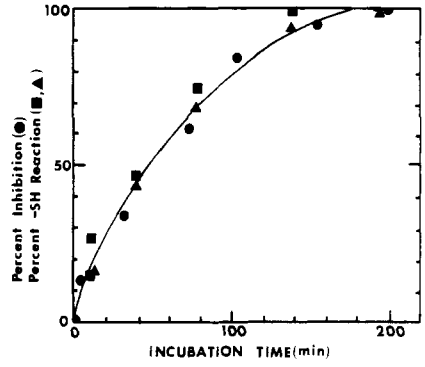

FIGURE 4: Correlation between the progressive inhibition of *cis*-$Pt(NH_3)_2Cl_2$-pretreated thymidylate synthetase by *trans*-$Pt(NH_3)_2Cl_2$ and the blocking of -SH groups as evidenced by DTNB titrations in the presence of 4 M urea. Enzyme samples were preincubated for 160 min with 7.3×10^{-4} M *cis*-$Pt(NH_3)_2Cl_2$ prior to addition of the trans Pt inhibitor. Enzyme concentration, 1.03×10^{-5} M; inhibitor concentration, 7.3×10^{-4} M. Percent inhibition after various incubation times (●). Sulfhydryl groups blocked by the inhibitor expressed as percentages of the total number of additional -SH groups (1.3) blocked after prolonged incubation times with the trans Pt complex (■, ▲) (two independent titrations).

complexes. Results obtained using *trans*-$Pt(NH_3)_2Cl_2$ are summarized in Figure 3. Under the conditions employed in the experiments (see figure legend), this inhibitor gave 100% inhibition in less than 200 min and blocked the titration of a total of 2.5 -SH groups per enzyme molecule (expressed as 100% in Figure 1) within 140 min. An additional 1.2 -SH groups were not blocked even after a 12-h inhibition time. There was little correlation between activity loss and loss of titratable -SH groups, indicating that at least some of the -SH groups blocked by the trans Pt complex are not essential for catalysis.

In similar experiments *cis*-$Pt(NH_3)_2Cl_2$ (7.3×10^{-4} M) was shown to rapidly block the titration of approximately 1.4 -SH groups per enzyme molecule within 15 min, without resulting in inhibition of activity. Approximately 2.3 -SH groups were not blocked by this compound. However, when enzyme samples which had been previously exposed to the cis Pt complex were subsequently treated with the trans Pt complex, both inhibition and further loss of titrable -SH groups resulted (Figure 4). Addition of the trans Pt complex to enzyme samples which had been pretreated with the cis isomer resulted in complete inhibition and the loss of an additional 1.3 titratable

TABLE II: Protection of Thymidylate Synthetase from *trans*-$Pt(NH_3)_2Cl_2$ Inhibition by Reversibly Blocking the Enzyme's -SH Groups with DTNB and pHMB.[a]

Sample	Sp Act. (units/mg) + Mercaptoethanol		Sp Act. (units/mg) − Mercaptoethanol	
	Before Adding Pt Complex	After Adding Pt Complex	Before Adding Pt Complex	After Adding Pt Complex
Untreated enzyme	3.40	0	1.20	0
pHMB-treated enzyme	2.75	2.12	0	0
DTNB-treated enzyme	2.14	2.23	0	0

[a] Enzyme samples were treated with an excess of DTNB or pHMB and the resulting modified enzyme was isolated by gel filtration on Sephadex G-25 and assayed in the presence and absence of 0.1 M 2-mercaptoethanol. Subsequently, the samples were incubated for 4 h in the presence of 1.1×10^{-3} M inhibitor and assayed again, with and without mercaptoethanol. Protein concentrations were determined by the Lowry method using pure thymidylate synthetase as a standard.

-SH groups within 200 min. Approximately 1.1 -SH groups were not blocked by either of the two Pt complexes.

In these experiments (Figure 4), the correlation between activity loss and loss of -SH groups is quite good and suggests that the 1.3 -SH groups affected by the trans isomer are at or near the active site of the enzyme.

Protection of Thymidylate Synthetase from Inhibition by Reversibly Blocking -SH Groups. Previous studies have shown that thymidylate synthetase is completely inactivated by both DTNB and pHMB, but regains full activity when assayed in the presence of 0.1 M 2-mercaptoethanol (Dunlap et al., 1971; Leary et al., 1975). These studies have also shown that the activity of the purified enzyme is increased by a factor of about 3 when mercaptoethanol is included in the assay mix.

If the inhibition of thymidylate synthetase by the trans Pt complex is a result of interactions between the inhibitor and the enzyme's -SH groups, then reversibly blocking these groups should afford protection to the enzyme from inhibition. The results shown in Table II indicate that the DTNB- and pHMB-treated enzyme samples are resistant to inhibition by the trans Pt complex. Although we were never able to fully reactivate the DTNB- or pHMB-treated enzyme preparations, they were nevertheless not as susceptible to inhibition by the Pt complex as the untreated enzyme. Neither of the treated enzyme preparations was active when assayed in the absence of mercaptoethanol, which indicated that the essential -SH groups were blocked by the sulfhydryl reagents. It is noteworthy that DTNB, which reacted with 1.0–1.3 of the enzyme's -SH groups, afforded protection from inhibition which was at least equal to that afforded by pHMB, which reacted with approximately 4 -SH groups.

Structural Changes of Thymidylate Synthetase Resulting from Treatment with Pt Complexes. Enzyme samples which had been incubated in the presence of 5.0×10^{-4} M *cis*- and/or *trans*-$Pt(NH_3)_2Cl_2$ for time periods ranging from 4 to 24 h were subjected to gel filtration on Sephadex G-100 and polyacrylamide gel electrophoresis. When compared with native enzyme samples, no dissociation into subunits or agglomeration was in evidence for any of the Pt-treated samples. However, samples which were treated with *trans*-$Pt(NH_3)_2Cl_2$ and subjected to electrophoresis showed a somewhat diffuse single band, rather than a single sharp band which is normally observed. This observation may be indicative of partial unfolding of the polypeptide chains; however, the position of this band with respect to the dye front was essentially the same as the native enzyme.

Conclusions

In this study, *trans*-$Pt(NH_3)_2Cl_2$ was shown to inhibit purified thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*, whereas the cis isomer of the same Pt complex was not an inhibitor when tested under the same conditions. However, a synergistic effect on the rate of inhibition by the trans Pt complex was observed when the enzyme samples were preincubated with the noninhibitory cis Pt complex prior to the addition of the trans inhibitor. Both isomers of the Pt complex interact with the enzyme to block the titration of -SH groups with DTNB and pHMB; however, the trans isomer blocks an average of 2.5 of the enzyme's 4 -SH groups, whereas the cis isomer blocks only 1.3. An average of 1.2 -SH groups were unaffected by either the trans isomer, or by a combination of both isomers. The results suggest that both isomers interact with the same nonessential -SH groups, but that only the trans isomer interacts with additional -SH groups which are essential for enzyme activity. So far as we know, the Pt complexes are unique in that the cis isomer interacts only with nonessential -SH groups of thymidylate synthetase and the trans isomer interacts with all but 1.2 of the enzyme's 4 -SH groups.

The mode of interaction between the Pt complexes and the -SH groups of thymidylate synthetase has not yet been determined, but a logical explanation would appear to be the displacement of one or both of the halide ligands by sulfur atoms of the enzyme's cysteine side chains. The apparently different specificities shown by the two Pt complexes in interacting with the enzyme's -SH groups is difficult to visualize. Square planar Pt complexes containing trans Cl ligands are kinetically more reactive in chloride displacement reactions than the corresponding cis isomers. It follows that the reaction of the enzyme's -SH groups with the trans isomer would be kinetically more favorable than with the cis cisomer. However, this reasoning alone does not explain the rapid disappearance of 1.3 titratable -SH groups when the enzyme was treated with the cis Pt complex. A second possibility is that the dipole moment of the cis isomer may result in its exclusion from certain apolar regions of the enzyme which are accessible to the trans isomer alone. However, this does account for the fact that at least one of the enzyme's -SH groups is blocked by both the cis and the trans Pt complexes.

Another possibility is that two -SH groups, in a single enzyme molecule, are capable of forming a bidentate complex between the enzyme and the trans Pt complex, but not with the cis isomer. This situation could exist if the two -SH groups were far enough apart to bridge the trans complex and displace both Cl ligands, and yet too far apart to displace both Cl ligands in the cis complex.

It should be pointed out that we have purposely not expressed the [-SH]M/[enzyme]M ratios as whole numbers in these studies. A growing body of evidence obtained in our laboratories, as well as those of other researchers (Donato et al., 1976a,b), suggests that, in the absence of denaturing agents, the -SH groups of thymidylate synthetase do not react with all sulfhydryl group reagents in a completely independent manner. This suggests that the reaction of some of the -SH groups may affect the ability of the other -SH groups to react when the enzyme is in its native conformation.

It should also be emphasized that the present studies do not

preclude the possible interaction of Pt complexes with amino acid side chains other than cysteine. We are currently in the process of determining the actual stoichiometry of binding between thymidylate synthetase and several Pt–amine complexes.

Acknowledgment

We thank Dr. John E. Teggins of Auburn University at Montgomery for synthesizing the Pt complexes used in these studies.

References

Aull, J. L., Lyon, J. A., and Dunlap, R. B. (1974), *Arch. Biochem. Biophys. 165,* 805.

Bauer, A. (1953), Handuch der Praparativen Anorganischen Chemie, E. Stuttgart, p 1181.

Blakely, R. L. (1969), in The Biochemistry of Folic Acid and Related Pteridines, New York, N.Y., Wiley, p 232.

Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, I. J. (1967), *Anal. Biochem. 20,* 150.

Davis, B. J. (1964), *Ann. N.Y.. Acad. Sci. 121,* 404.

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

Donato, H., Plese, P. C., Reinsch, J. W., Hopper, W. E., and Dunlap, R. B. (1976b), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 2031.

Drew, H. D. K. (1932), *J. Chem. Soc. 14,* 2328.

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

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

Friedkin, M. (1973), *Adv. Enzymol. Relat. Areas Mol. Biol. 38,* 235.

Friedman, M. E., and Teggins, J. E. (1974), *Biochim. Biophys. Acta 350,* 263.

Galivan, J. H., Maley, G. F., and Maley, F. (1976), *Biochemistry 15,* 356.

Hatefi, Y., Talbert, P. T., Osborn, M. J., and Huennekens, F. M. (1960), *Biochem. Prep. 7,* 89.

Huennekens, F. M. (1968), in Biological Oxidations, Singer, T. P., Ed., New York, N.Y., Interscience p 439.

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

Loeble, R. B. and Dunlap, R. B. (1972), *Biochem. Biophys. Res. Commun. 49,* 1671.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 195,* 265.

Lyon, J. A., Pollard, A. L., Loeble, R. B., and Dunlap, R. B. (1976), *Cancer Biochem. Biophys. 1,* 121.

Melius, P., Teggins, J. E., Friedman, M. E., and Guthrie, R. W. (1972), *Biochim. Biophys. Acta 268,* 194.

Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.

Ramberg, L. (1913), *J. Inorg. Chem. 83,* 33.

Reishus, J. W., and Martin, Jr., D. S. (1961), *J. Am. Chem. Soc. 83,* 2457.

Roberts, J. J., and Pascoe, J. M. (1972), *Nature (London) 235,* 282.

Rosenberg, B., Van Camp, L., Trosko, J. E., and Mansour, V. H. (1969), *Nature (London) 222,* 385.

Stadtman, E. R. (1957), *Methods Enzymol. 3,* 931.

Wahba, A. J., and Friedkin, M. (1962), *J. Biol. Chem. 237,* 3794.