Purification and Properties of T2 Bacteriophage-Induced Thymidylate Synthetase†

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ABSTRACT: T2 bacteriophage-induced thymidylate synthetase was purified 4,000-fold to homogeneity, as revealed by sodium dodecyl sulfate acrylamide gel electrophoresis and sedimentation equilibrium measurements. With the latter method, a molecular weight of 64,400 ± 700 was obtained for the native enzyme, while sedimentation in 6.0 M guanidine hydrochloride and electrophoresis in sodium dodecyl sulfate acrylamide gels yielded a single homogeneous species with a molecular weight of 31,500 ± 600. The highest specific activity obtained for the native enzyme was 15 μmol of dTMP formed min^{-1} mg of $protein^{-1}$ at 30°. This value is considerably higher than those reported for homogeneous synthetases from *Lactobacillus casei*, Ehrlich ascites cells, and calf thymus. Antisera to the pure phage enzyme cross-reacted with the synthetases induced by bacteriophage T4 and T6, but not with the synthetases from *Escherichia coli*, *L. casei*, or regenerating rat liver. In the presence of 5,10-methylenetetrahydrofolate (5,10-CH_2H_4folate), the enzyme bound increasing amounts of 5-fluoro-2′-deoxyuridylate (FdUMP), which was associated with a proportionate decrease in enzyme activity. When the maximum binding ratio of 2 mol of FdUMP/mol of synthetase was attained, the enzyme was inhibited completely. The complex between FdUMP and synthetase was stable to 6 M guanidine hydrochloride, but approximately half the FdUMP was removed by 6 M urea. Although the FdUMP–enzyme complex was not formed in the absence of 5,10-CH_2H_4folate, intermediate levels of complex formation could be effected by other folate derivatives, *i.e.*, folate, dihydrofolate, tetrahydrofolate, and methotrexate. Extensive dialysis against buffer devoid of thiols resulted in the inactivation of thymidylate synthetase and a loss in its capacity to bind FdUMP. Both properties could be partially restored by 2-mercaptoethanol and suggest that enzyme cysteinyl groups may be involved in FdUMP binding and in the catalytic mechanism.

The terminal step in the synthesis of thymidylic acid is catalyzed by thymidylate synthetase according to the following reaction: 5,10-CH_2H_4folate[1] + dUMP → 7,8-H_2folate + TMP. The reaction is unique in that the methylene group is reduced by a hydrogen from the pteridine ring during its transfer to the C-5 position of deoxyuridylate (Friedkin, 1959). As subsequently shown, this hydrogen is derived from the C-6 position of the pteridine ring (Lorenson *et al.*, 1967). In spite of extensive studies on thymidylate synthetases from numerous sources, homogeneous preparations have only recently become available. The first of these was reported by Dunlap *et al.* (1971a) and Leary and Kisliuk (1971) who purified and crystallized the enzyme from methotrexate- and dichloromethotrexate-resistant *Lactobacillus casei*, respectively. More recently, the synthetase has been purified to homogeneity from Ehrlich ascites cells by Fridland *et al.* (1971) and from calf thymus by Horinishi and Greenberg (1972).

Barner and Cohen (1954) suggested that T2 bacteriophage might induce thymidylate synthetase in *E. coli* by demonstrating that thymine requiring strains of *E. coli* could provide thymine for phage DNA synthesis following phage infection. Flaks and Cohen (1957) and Barner and Cohen (1959) subsequently confirmed this hypothesis. Although thymidylate synthetase is present in uninfected *E. coli* (Friedkin and Kornberg, 1957), Greenberg *et al.* (1962) found the enzyme activity could be resolved into two fractions in infected cells, suggesting that the bacteriophage introduces its own structural gene for thymidylate synthetase into the host. These findings were substantiated by the enzymatic studies of Matthews and Cohen (1963). Genetic and immunological evidence (Shapiro *et al.*, 1965; Matthews, 1965; Krauss *et al.*, 1973) supported the conclusion that T2 and T4 bacteriophage contain structural genes for thymidylate synthetase. Uncontestable support for the above thesis has come from this laboratory (Trimble *et al.*, 1972) with the finding that T2 and T4 bacteriophage DNAs are templates for the *in vitro* synthesis of thymidylate synthetase. Krauss *et al.* (1973) have recently reported on the immunological properties of a crude preparation of T4 bacteriophage enzyme, while Capco *et al.* (1973) have presented similar studies on a partially purified preparation from the same source. The present study using T2 bacteriophage[2] infected *E. coli* describes for the first time the purification to homogeneity of a virus-induced thymidylate synthetase[3] and a detailed account of some of its properties.

Materials and Methods

Folate and dUMP were purchased from Sigma Chemical Co. 5-Fluoro[2-^{14}C]deoxyuridine was obtained from Schwarz/Mann. H_4folate was synthesized and stored according to Lorenson *et al.* (1967), except that 2,3-dimercaptopropanol was omitted. H_2folate was synthesized as described by Friedkin *et al.* (1962). FdUMP was prepared by the cyanoethyl

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[1] Abbreviations used are: dUMP, 2′-deoxyuridylate; FdUMP, 5-fluoro-2′-deoxyuridylate; TMP, thymidylic acid; 5,10-CH_2H_4folate, (±)-5,10-methylenetetrahydrofolate; H_4folate, (±)-tetrahydrofolate; H_2folate, dihydrofolate.

[2] The T2 bacteriophage used in this study is the (r^+) or lysis-inhibiting variety.

[3] For brevity, this enzyme will be referred to as T2 thymidylate synthetase.

phosphate method and purified according to the procedure of Tener (1961). The 2-^{14}C derivative was isolated by chromatography on Whatman 3 MM paper with 2-propanol–NH_3–H_2O (7:1:2) as developer. DEAE-cellulose and phosphocellulose were purchased from Schleicher and Schuell Co. The DEAE-cellulose was treated first with 0.5 N HCl and then with 0.5 N NaOH, each for 30 min. The phosphocellulose was treated similarly but in the reverse order. Both were washed with water, equilibrated with the buffers indicated in the purification, and stored as a thick slurry with chloroform as a preservative. Sephadex G-100 was purchased from Pharmacia and hydroxylapatite from Bio-Rad Laboratories. The hydroxylapatite was mixed in a 3:1 ratio (w:w) with Celite (Johns-Manville Co.) prior to use. Nitrocellulose filters, Type B6, were obtained from Schleicher and Schuell Co.

Thymidylate synthetase from dichloromethotrexate-resistant *L. casei* was provided as the dialyzed ammonium sulfate precipitate of fraction 2 (Leary and Kisliuk, 1971) by the New England Enzyme Center. Further purification and crystallization of the enzyme were achieved by a modification of the method of Leary and Kisliuk (1971).

Enzyme Assay. The enzyme was assayed spectrophotometrically essentially as described by Wahba and Friedkin (1961). A stock solution of 5,10-CH_2H_4folate maintained under a slow stream of argon contained H_4folate, 4 mM; formaldehyde, 20 mM; sodium bicarbonate, 30 mM; and 2,3-dimercaptopropanol, 10 mM. The following components were added to a 1-cm cuvet: 0.1 ml of the 5,10-CH_2H_4folate solution; 0.1 ml of 0.1 M 2-mercaptoethanol; 0.1 ml of 0.5 M potassium phosphate (pH 7.0); and enzyme to 0.98 ml. After the solution was equilibrated at 30° in a Gilford 2400-S spectrophotometer, the reaction was initiated with 0.02 ml of 50 mM dUMP. The change in absorbance was recorded at 340 mμ ($\epsilon = 6.4 \times 10^3$ M^{-1} cm^{-1}, Wahba and Friedkin, 1961) and corrected for the reaction that occurred in the absence of dUMP. Enzyme units were calculated on the basis of μmoles of dTMP formed/min under the above reaction conditions. Specific activity is expressed as units/mg of protein. Protein was determined by the method of Lowry *et al.* (1951).

Amino Acid Analyses. Dry, salt-free samples of enzyme were hydrolyzed in 1.0 ml of constant boiling HCl for 24, 48, and 96 hr at 110°. After removal of HCl by evaporation under reduced pressure, amino acid analyses were performed by the method of Spackman *et al.* (1958). Cysteine was determined as cysteic acid (Spencer and Wold, 1969), and the tryptophan content was analyzed by the technique of Liu and Chang (1971). A Model 5-AH amino acid analyzer (JEOL Inc.) was used for these analyses.

Sodium Dodecyl Sulfate Acrylamide Gel Electrophoresis. The procedure of Weber and Osborn (1969) was employed with the following alterations. The protein solution was heated at 90° for 10 min prior to the application of samples to the gels. Following electrophoresis, the gels were stained for 1 hr in a 2.5% (w/v) solution of Coomassie Blue in 5% acetic acid–50% methanol (v/v). The gels were destained in a solution of 7.5% acetic acid (v/v) and 5% methanol (v/v) for 1 hr in a Canalco destainer.

Ultracentrifugal Analysis. Sedimentation equilibrium analysis was performed by the meniscus depletion technique of Yphantis (1964). A Spinco Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanner was used. The partial specific volume, calculated from the amino acid analyses (Cohn and Edsall, 1943), was 0.74. Standard deviations are the average values of the standard deviations of the slopes from a least squares analysis of ln A *vs.* r^2, where A is absorbance and r is the distance from the axis of rotation. Sedimentation velocity measurements were obtained with a Spinco Model E ultracentrifuge using schlieren optics. Centrifugation was conducted at 59,740 rpm at 4°.

Preparation of Antibody. A thymidylate synthetase solution (1 mg/0.5 ml) was mixed with an equal volume of Freund's adjuvant and 0.25 ml was injected in each rear footpad and 0.5 ml in the cervical region of a rabbit. Intramuscular booster injections (0.5 mg each) were repeated three times on alternate weeks. The globulin fractions were purified by the technique of Sternberger and Petermann (1951).

Nitrocellulose Filtration. Isolation of [2-^{14}C]FdUMP enzyme complexes was carried out by the technique of Santi and McHenry (1972) with minor modifications. The filter was moistened with 0.05 M potassium phosphate (pH 7.0) and 0.01 M $MgCl_2$ and treated with 1 ml of the same solution containing 10 μg of bovine serum albumin. After application of the radioactive sample, unbound [2-^{14}C]FdUMP was removed from the filter with two 1-ml and one 4-ml successive washes of the above buffer. The filter was removed, dried, placed in 0.78% (w/v) 2-phenyl-5-(4-biphenyl)-1,3,5-oxadiazole (Packard Instrument Co., Inc.) in toluene, and counted in a Beckman LS-250 scintillation counter. The efficiency of counting under these conditions was 90%.

Circular Dichroism Spectra. The circular dichroism spectra were obtained with a Cary Model 61 spectropolarimeter. The samples were analyzed in 0.5-mm cylindrical quartz cells under constant nitrogen purging. The spectra were scanned at 3 nm/min with a pen period of 3 sec. Fluctuations in the recorder pen at the peak height were no greater than 1%. The data are reported as mean residue ellipticity in (deg-cm^2)/dmol which is given by

$$\theta = \theta^\circ M/10lc$$

where M is the mean residue molecular weight, determined as 115 from the amino acid analysis and the molecular weight, θ° is the observed ellipticity in degrees, l is the cell path length in cm, and c is the protein concentration in g/cm^3.

Purification of T2 Thymidylate Synthetase. All steps in the purification were carried out at 4°. The enzyme was purified from the supernatant of a streptomycin autolysate used in the purification of T2 deoxycytidylate deaminase (step 2, Maley *et al., 1972*).

Step 1. Ammonium Sulfate Precipitation. The supernatant fraction from the streptomycin autolysate was brought to 80% saturation by the addition of solid ammonium sulfate. The suspension was stirred for 10 min and then centrifuged for 15 min at 10,000g. The pellet was suspended in a minimum volume of 0.01 M potassium phosphate (pH 7.5) and 0.02 M 2-mercaptoethanol and stored at −55° for further purification.

Step 2. DEAE-Cellulose Chromatography. The DEAE-cellulose fractionation of Greenberg *et al.* (1962) was modified to effect a large-scale separation of the bacteriophage and *E. coli* thymidylate synthetases. Approximately 700 ml of the suspension from step 1 was stirred with 200 ml of 2.0 M K_2HPO_4 for 1 hr at room temperature. The resulting suspension was dialyzed against a solution of 0.02 M K_2HPO_4 and 0.05 M 2-mercaptoethanol in an Amicon DC2 hollow fiber concentrator with an HID × 50 cartridge. Two of the above dialyzed enzyme preparations were pooled and mixed with 1 l. of DEAE-cellulose which had been packed on a Büchner filter. After stirring for 30 min the mixture was filtered on a Büchner funnel, and the filtrate was discarded. The DEAE-cellulose was then washed with 1 l. of 0.05 M 2-mercaptoethanol. The

TABLE I: Purification of T2 Thymidylate Synthetase.

Step	Vol (ml)	Total Protein (mg)	Specific Activity (units/mg)	Total Units
1. Ammonium sulfate precipitation[a]	4800	1.02×10^5	0.0030	310
2. DEAE-cellulose	790	0.48×10^5	0.0037	180
3. Phosphocellulose I	190	1050	0.11	110
4. Phosphocellulose II	15	64	1.4	80
5. Sephadex G-100	18	20	3.6	72
6. Hydroxylapatite	13	2.9	16	46

[a] The supernatant fraction for this step was obtained from the streptomycin autolysate (step 2) as described in the purification of T2 deoxycytidylate deaminase (Maley *et al.*, 1972). It represents approximately 50% of the original T2 synthetase units in the cell extract, as half of the activity is lost during the streptomycin precipitation. The ammonium sulfate precipitate was assayed after the sample had been dialyzed in preparation for step 2.

T2 thymidylate synthetase was eluted from the DEAE-cellulose with 500-ml fractions of a solution containing 0.1 M NaCl–0.02 M potassium phosphate (pH 7.0)–0.05 M 2-mercaptoethanol. Fractions 2–4 were pooled and dialyzed overnight against 20 l. of 0.02 M potassium phosphate (pH 7.0) containing 0.05 M 2-mercaptoethanol and 20% ethylene glycol.

Step 3. Phosphocellulose Chromatography I. The dialyzed sample from step 2 was stirred for 30 min with 600 ml of packed phosphocellulose which had been equilibrated previously with 0.02 M potassium phosphate (pH 7.0). The suspension was filtered on a Büchner funnel and washed first with 1 l. of 0.1 M potassium phosphate (pH 7.0) and then with 800 ml of 0.14 M potassium phosphate (pH 7.0), each containing 0.05 M 2-mercaptoethanol. The phosphocellulose was then washed with several 250-ml volumes of a solution containing 0.26 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol. The enzyme was eluted in fractions 2–6. These fractions were pooled and dialyzed as in step 2.

Step 4. Phosphocellulose Chromatography II. The dialyzed solution from step 3 was passed through a 2.5 × 40-cm column of phosphocellulose which had been equilibrated with 0.02 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol. The column was washed with 500 ml of the same buffer and then with 500 ml of a solution containing 0.1 M potassium phosphate (pH 7.0), 20% ethylene glycol, and 0.05 M 2-mercaptoethanol. It was then eluted with a linear gradient from 0.1 to 0.25 M potassium phosphate (pH 7.0) (1000 ml in each reservoir). Each solution also contained 0.05 M 2-mercaptoethanol and 20% ethylene glycol. Ten-ml fractions were collected at a rate of 40–50 ml/hr. The enzyme emerged between fractions 110 and 150. The fractions containing more than 0.1 unit/ml were pooled, placed in a dialysis bag, and then concentrated by covering the bag with solid sucrose. The protein in the concentrated sample was precipitated by dialysis against 500 ml of saturated ammonium sulfate containing 0.1 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol. The precipitate was collected by centrifugation for 15 min at 20,000*g* and dissolved in a minimum volume of 0.2 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol.

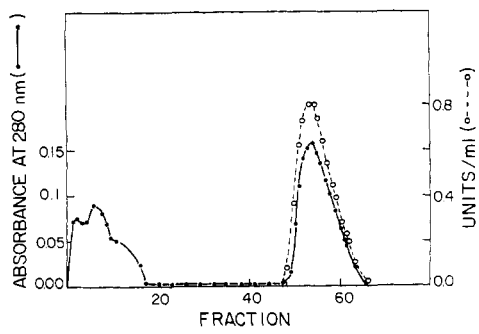

FIGURE 1: Chromatography of T2 thymidylate synthetase on hydroxylapatite (step 6); see Methods for details.

Step 5. Sephadex G-100 Gel Filtration. A Sephadex G-100 column (2.5 × 100 cm) was equilibrated with a solution containing 0.2 M potassium phosphate (pH 7.0), 0.05 M 2-mercaptoethanol, and 20% ethylene glycol. When more than 100 mg of protein was obtained from step 4, the protein solution was divided in half and two successive G-100 columns were run. Following application of the enzyme, the column was eluted with the same buffer at approximately 5 ml/hr. Fractions of 3 ml were collected. The synthetase was present in fractions 48–58 and was concentrated in dialysis tubing with solid sucrose as described in step 4. The concentrated sample (approximately 5 ml) was dialyzed for 3 hr against a solution of 0.01 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol in preparation for step 6.

Step 6. Hydroxylapatite Chromatography. The sample from step 5 was applied to a hydroxylapatite column (1 × 15 cm) which had been equilibrated previously with 0.01 M potassium phosphate (pH 7.0) and 0.05 M 2-mercaptoethanol. The column was washed with 110 ml of this buffer (Figure 1, fractions 1–10) at 30 ml/hr, and then eluted with a linear gradient consisting of 0.05 M potassium phosphate (pH 7.0) in the mixing chamber and 0.2 M potassium phosphate (pH 7.0) in the reservoir (300 ml of each). The concentration of 2-mercaptoethanol was 0.05 M throughout. The gradient was started after fraction 10 and thymidylate synthetase was eluted from the column between fractions 50 and 64 (Figure 1). The purified enzyme sample was concentrated to about 5 ml in a dialysis bag covered with sucrose. At this stage the enzyme could be stored in a concentrated solution for 2 weeks at 0° without a loss in activity.

Results

Enzyme Purification. The large apparent loss in total units and specific activity at step 2 (Table I) is in part due to the selective removal of the *E. coli* thymidylate synthetase which comprises about 20% of the units in step 1. The latter is bound more tightly to DEAE-cellulose than the T2 enzyme and could be recovered by washing the column (step 2) with a solution of 0.5 M NaCl and 0.05 M 2-mercaptoethanol. The

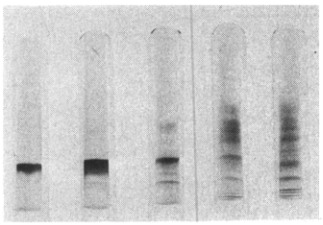

FIGURE 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of T2 thymidylate synthetase fractions. Protein concentrations are from left to right: phosphocellulose I, 20 μg; phosphocellulose II, 10 μg; Sephadex G-100, 6.3 μg; hydroxylapatite, 25 μg; and 0.01 M potassium phosphate (pH 8.0) precipitate, 10 μg.

purity of the T2 enzyme was followed throughout the purification procedure by sodium dodecyl sulfate acrylamide gel electrophoresis (Figure 2). Although the specific activity of the enzyme eluted from hydroxylapatite was constant throughout the peak, concentrated samples of enzyme from step 6 sometimes contained contaminants amounting to less than 5% of the total protein. At this stage, the enzyme could be separated from the contaminants by dialysis for 16 hr against a solution of 0.01 M potassium phosphate (pH 8.0) and 0.02 M 2-mercaptoethanol. The cloudy suspension which formed was centrifuged and the precipitate was dissolved in the same buffer containing 1.0 M NaCl. The contaminants observed in step 6 were removed by this technique but with a loss of more than 50% in enzyme activity. This enzyme preparation was used for amino acid analysis and for antibody production.

Molecular Weight. Enzyme from step 6 was subjected to sedimentation velocity centrifugation and a single component with an $s_{20,w}$ of 4.3 was observed. Sedimentation equilibrium analysis of the native enzyme yielded a molecular weight of 64,400 ± 700. When the same experiment was carried out in the presence of 6 M guanidine hydrochloride (Figure 3), a molecular weight of 31,500 ± 600 was obtained. The latter was substantiated by sodium dodecyl sulfate acrylamide gel electrophoresis with proteins of known molecular weight (Figure 4), suggesting that the native enzyme is composed of two subunits of equal molecular weight.

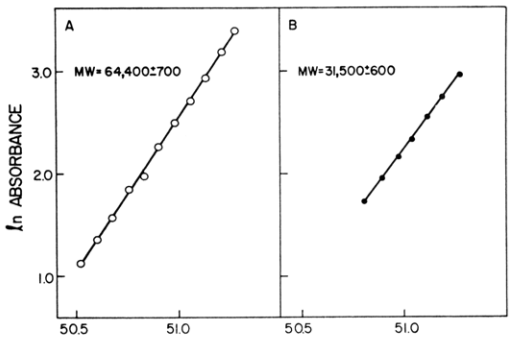

FIGURE 3: Sedimentation equilibrium centrifugation: (A) centrifugation of enzyme from step 6 (215 μg/ml) at 25,980 rpm and 4° in a solution containing 0.2 M potassium phosphate (pH 7.0) and 0.005 M 2-mercaptoethanol; (B) step 6 enzyme (190 μg/ml) was dialyzed for 16 hr against a solution containing 6 M guanidine hydrochloride, 0.05 M potassium phosphate (pH 7.0), and 5 mM 2-mercaptoethanol, and then centrifuged at 50,740 rpm; see Methods for further details.

Amino Acid Analysis. Table II presents the amino acid composition of the homogeneous enzyme. The cysteine content of 10 residues/mol is of particular interest since this value is 2.5 times higher than that reported for the *L. casei* synthetase (Dunlap *et al.*, 1971b) and may be related to the phage enzyme's greater susceptibility to inactivation when incubated in the absence of thiols. If the subunits are identical, as appears to be the case with the synthetase from *L. casei* (Loeble and Dunlap, 1972), there should be 5 cysteines/subunit.

Absorption Spectrum. The absorption spectrum of the enzyme (Figure 5) shows peaks at 282 and 276 nm with a distinct shoulder at 290 nm. The latter is indicative of the presence of tryptophan. An absorption minimum at 248–250 nm, a 280:260 ratio of 1.85, and a molar extinction coefficient at

TABLE II: Amino Acid Composition of T2 Thymidylate Synthetase.

Amino Acid	Residues/64,400 g of Protein[a]	Nearest Integer
Lys	44.82	45
His	14.17	14
Arg	26.34	26
Asp	61.76	62
Thr[b]	22.35	22
Ser[b]	22.73	23
Glu	55.45	55
Pro	27.24	27
Gly	39.93	40
Ala	28.01	28
Cys[c]	9.85	10
Val	33.94	34
Met	8.69	9
Ile	35.03	35
Leu	62.08	62
Tyr	29.37	29
Phe	22.86	23
Trp[d]	13.46	13

[a] Average of two determinations after hydrolysis in 6.0 N HCl at 110° for 24, 48, and 96 hr. [b] Values calculated by extrapolation to zero time. [c] Determined as cysteic acid by the method of Spencer and Wold (1969). [d] Determined by the method of Liu and Chang (1971).

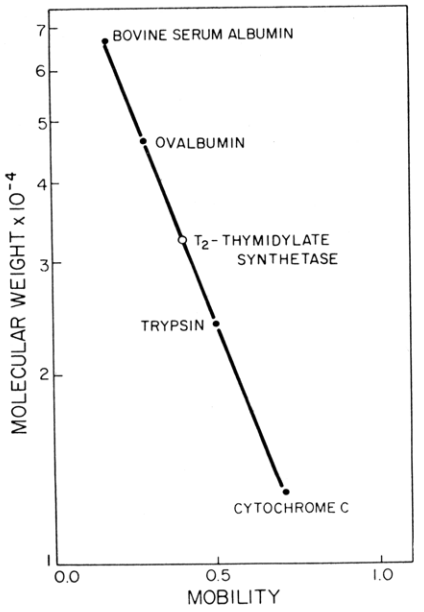

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of standard proteins and T2 thymidylate synthetase. The details of electrophoresis are described in Methods.

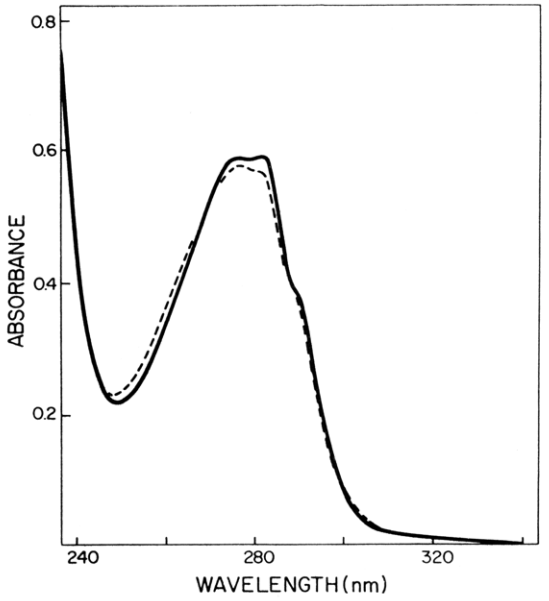

FIGURE 5: Absorption spectrum of thymidylate synthetase. The solid line represents the spectrum of T2 thymidylate synthetase (0.26 mg/ml), and the dashed line enzyme from a methotrexate-resistant *L. casei* (0.35 mg/ml). Both enzymes were dissolved in 0.1 M potassium phosphate (pH 7.0) and 0.02 M 2-mercaptoethanol. The spectra were taken with a Cary 14 spectrophotometer.

280 nm of 1.4×10^5 were obtained. The latter is based on the protein method of Lowry *et al.* (1951). The absorption spectrum of the *L. casei* synthetase is also included in Figure 5 to demonstrate the spectral similarity of the two enzymes.

Immunological Studies. Antibody prepared against purified T2 thymidylate synthetase formed a single precipitin line with the T2 enzyme but did not cross-react with the synthetase from *E. coli* or *L. casei* (Figure 6). As further proof of the purity of the T2 thymidylate synthetase, a single line was observed even with fractions prior to step 6 of Table I. The synthetases from T4 and T6 were completely inhibited by the antibody, while little or no effect on the synthetases from *E. coli*, *L. casei*, or regenerating rat liver was detected.

Catalytic Properties. The average specific activity of five preparations purified to homogeneity was 15 μmol min^{-1} mg of protein^{-1}. Protein concentrations determined by the Lowry procedure (Lowry *et al.*, 1951) were substantiated by calculations based on amino acid analysis. From these measurements, a turnover number of 960/mole of enzyme was obtained. A double reciprocal plot analysis revealed a K_m for dUMP of 6×10^{-6} M at pH 7.0, and for 5,10-CH_2H_4folate of 2×10^{-5} M. The enzyme had a broad pH maximum, between 6.8 and 8.7, and was unaffected by the presence or absence of Mg^{2+} when assayed in 0.05 M potassium phosphate (pH 7.0).

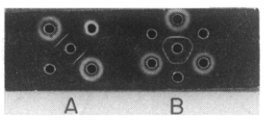

FIGURE 6: Reaction of antibody to T2 thymidylate synthetase with thymidylate synthetases from various sources. The center wells in (A) and (B) contain antibody to T2 thymidylate synthetase (see Methods). The upper left and lower right wells in (A) contain purified T2 thymidylate synthetase (step 6). The lower left well has enzyme from methotrexate-resistant *L. casei*, and the upper right well contains enzyme from *E. coli*. In (B) the uppermost well contains purified T2 enzyme (step 6). Continuing clockwise, the wells contain enzymes from G-100, step 6, phosphocellulose II, step 6, and phosphocellulose I.

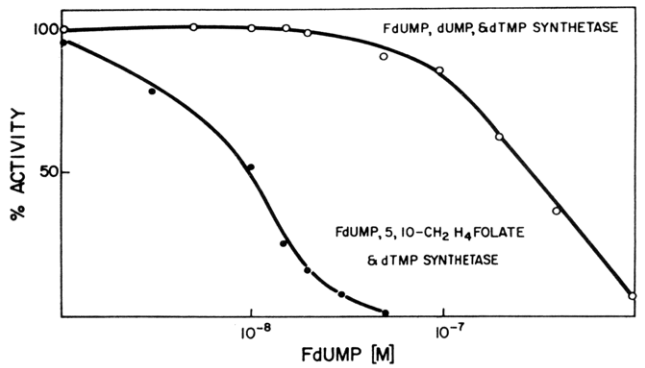

FIGURE 7: The effect of 5,10-CH_2H_4folate on the inhibition of T2 thymidylate synthetase by FdUMP. All reactions were at 30° and contained potassium phosphate (pH 7.0), 50 mM; 2-mercaptoethanol, 10 mM; 5,10-CH_2H_4folate, 0.4 mM; dUMP, 1 mM; enzyme (step 4), 2.5 μg; and FdUMP as indicated. The curve designated by (○) was obtained by preincubating FdUMP, dUMP, and enzyme for 15 min, then adding 5,10-CH_2H_4folate to a final volume of 1.0 ml to initiate the reaction. The curve designated by (●) was obtained by preincubating under similar conditions FdUMP, enzyme, and 5,10-CH_2H_4folate for 15 min, and then adding 1 μmol of dUMP to initiate the reaction.

FdUMP Inhibition. T2 thymidylate synthetase, like the enzyme from other sources, is markedly inhibited by FdUMP. In the experiment shown in Figure 7, FdUMP inhibited the synthetase by 50% at approximately 1×10^{-8} M, provided FdUMP, 5,10-CH_2H_4folate, and enzyme were incubated prior to the addition of dUMP. If FdUMP, dUMP, and enzyme were preincubated and the reaction was initiated with 5,10-CH_2H_4folate, the level of FdUMP required for 50% inhibition was almost two orders of magnitude higher. The finding that 5,10-CH_2H_4folate greatly enhances the inhibition of the T2 synthetase by FdUMP agrees with earlier studies on the synthetases from Ehrlich ascites cells (Reyes and Heidelberger, 1965), chick embryo (Lorenson *et al.*, 1967), and T4 bacteriophage (Capco *et al.*, 1973). Recent experiments by Santi and McHenry (1972) and Langenbach *et al.* (1972) suggest that FdUMP is covalently bound to thymidylate synthetase only in the presence of 5,10-CH_2H_4folate. Similar experiments were conducted with the T2 synthetase in which the binding of [2-^{14}C]FdUMP to this enzyme was measured (Figure 8) by the

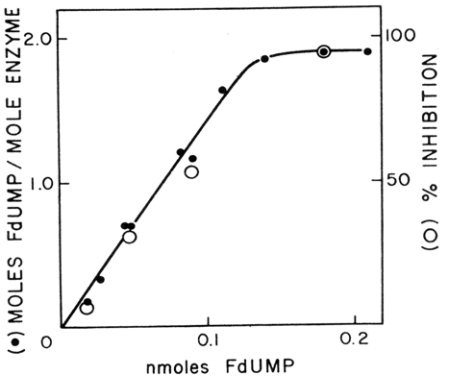

FIGURE 8: Titration of T2 thymidylate synthetase with FdUMP. Each reaction mixture contained in a volume of 0.05 ml: 5,10-CH_2H_4folate, 0.4 mM; 2-mercaptoethanol, 20 mM; potassium phosphate (pH 7.0), 100 mM; enzyme, 4.5 μg; and the indicated amount of [2-^{14}C]FdUMP (2.75×10^4 dpm/nmol). The sample was incubated at 30° for 30 min, after which 5 μl was assayed for enzymatic activity (○). The remaining solution was filtered through a nitrocellulose membrane and the enzyme bound radioactivity (●) measured (see Methods). The blank reactions did not contain 5,10-CH_2H_4folate.

TABLE III: Effect of Folate Derivatives on the Binding of FdUMP to T2 Thymidylate Synthetase.[a]

Compound	FdUMP Bound (moles/mole of enzyme)		
	Untreated	6.0 M Guanidine Hydrochloride	6.0 M Urea
None	0.0		
5,10-CH_2H_4folate	2.0	2.0	0.9
H_4folate	1.7	1.7	0.45
H_2folate	1.4	1.35	0.17
Folate	0.8	0.7	0.1
Methotrexate	0.5	0.5	0.1

[a] Binding of [2-^{14}C]FdUMP to enzyme was measured by nitrocellulose filtration (see Methods). The binding solutions were incubated at 30° for 30 min and contained in 0.05 ml: enzyme, 2.4 μg; [2-^{14}C]FdUMP (2.75×10^4 dpm/nmol), 2.5 μM; potassium phosphate (pH 7.0), 100 mM; 2-mercaptoethanol, 20 mM; and the folate derivative, 0.2 mM. Sufficient 8 M guanidine hydrochloride and 8 M urea were then added to separate solutions to yield final respective concentrations of 6 M. An equivalent volume of water was added to the untreated control solution. These solutions were then incubated for an additional 60 min at 30° before filtering through nitrocellulose membranes.

nitrocellulose filtration procedure (Santi and McHenry, 1972). As indicated, the synthetase activity can be completely titrated in the presence of 5,10-CH_2H_4folate, at which point the ratio of FdUMP to enzyme is 2:1. Santi and McHenry (1972) obtained nearly identical results with the *L. casei* synthetase. Further addition of FdUMP did not increase the amount of radioactivity retained by the enzyme. Of interest is the fact that the extent of inhibition of enzyme activity is directly proportional to the binding of [2-^{14}C]FdUMP. In the absence of 5,10-CH_2H_4folate, FdUMP did not bind to the enzyme. That the binding is extremely tight and possibly covalent is suggested from the finding that treatment of the enzyme–[2-^{14}C]-FdUMP complex with 6 M guanidine hydrochloride prior to filtration released little if any radioactivity (Table III). However, approximately one-half of the radioactivity could be released when the complex was treated with 6 M urea prior to filtration. The possibility that denaturing agents affect the efficiency of enzyme protein binding to the filter was ruled out by the observation that the retention of [^{35}S]methionine-labeled T2 synthetase by nitrocellulose filters (85%) is unaffected by the presence of 6 M urea or 6 M guanidine.

5,10-CH_2H_4folate was not the only folate derivative that would support FdUMP binding. Several analogs were tested and found to be effective but to a lesser degree than the substrate (Table III). Of these compounds, H_4folate was the most effective followed by H_2folate, folate, and then methotrexate. All of the complexes were stable to 6.0 M guanidine hydrochloride but, like 5,10-CH_2H_4folate, they were partially disrupted by 6.0 M urea.

Effect of Thiols. Since T2 thymidylate synthetase was dependent on the presence of thiols for maximal activity, 2-mercaptoethanol was included in all steps of the purification. On dialysis with 0.2 M potassium phosphate buffer (pH 7.0), for 16 hr in the absence of thiols, the enzyme lost 65% of its activity and after an additional 16 hr only 7% of the original activity remained. The latter sample could be reactivated to 30% on incubation with 0.3 M 2-mercaptoethanol for 10 min at 30° prior to assay. The presence of thiols was required also for FdUMP binding. As indicated earlier, thiol-depleted enzyme was much less susceptible to FdUMP inhibition than enzyme that had been reactivated by 2-mercaptoethanol (Galivan *et al.*, 1973). A related effect was reported by Kalman (1972), who observed that air-oxidized *L. casei* thymidylate synthetase was inhibited to a lesser extent by the dUMP analog, showdomycin, than dithiothreitol-treated synthetase. We extended these observations by directly measuring [2-^{14}C]-FdUMP binding to enzyme that had been inactivated by removal of thiols (Table IV). As indicated, the oxidized enzyme bound only negligible amounts of FdUMP. However, on addition of 2-mercaptoethanol, there was an increase of approximately 15-fold in enzyme activity and FdUMP binding was obtained.

TABLE IV: Thiol Requirement for Activity and FdUMP Binding of T2 Thymidylate Synthetase.[a]

Preincubation Conditions	Enzyme Activity (units/ml)	FdUMP Bound (nmoles/ml)
Plus 2-mercaptoethanol	0.39	1.15
Minus 2-mercaptoethanol	0.03	0.08

[a] Two ml (0.32 mg of thymidylate synthetase, step 6) was dialyzed against three 1-l. volumes of 0.2 M potassium phosphate (pH 7.0) for a total of 36 hr. A 0.5-ml aliquot was incubated for 20 min at 4° in the presence or absence of 0.3 M 2-mercaptoethanol and enzyme activity was measured spectrophotometrically. FdUMP binding to the treated and untreated enzyme was measured by nitrocellulose filtration as described in Methods. The incubation mixtures contained in a volume of 0.13 ml: enzyme, 16 μg; potassium phosphate (pH 7.0), 0.155 M; [2-^{14}C]FdUMP (2.75×10^4 dpm/nmol), 5.4 μM; and 5,10-CH_2H_4folate, 0.31 mM. The results are expressed for 1 ml of original enzyme solution (0.16 mg).

Circular Dichroism Studies. Freshly purified thymidylate synthetase showed two negative bands, one at 209–210 nm and the other at 225–227 nm (Figure 9). On dialysis for 16 hr

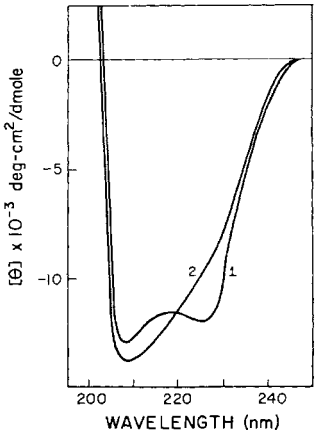

FIGURE 9: CD spectra of T2 thymidylate synthetase: curve 1, active enzyme in 0.2 M potassium phosphate (pH 7.0) and 0.005 M 2-mercaptoethanol; curve 2, enzyme inactivated by dialysis against distilled water for 48 hr.

against 0.2 M potassium phosphate (pH 7.0) in the absence of thiol, a decrease in the band at 225–227 nm occurred. When the enzyme was dialyzed against distilled water (Figure 9), a more dramatic decrease in ellipticity at 227 nm was observed, which was associated with a complete loss in enzyme activity.

Discussion

The homogeneous T2 thymidylate synthetase described in this paper possesses the highest specific activity reported for this enzyme to date. Five preparations of purified enzyme yielded an average specific activity of 15 μmol min^{-1} mg^{-1} at 30°, a value 30-fold higher than that of the pure enzyme from Ehrlich ascites cells (Fridland *et al.*, 1971), 45-fold higher than that from calf thymus (Horinishi and Greenberg, 1972), and fivefold higher than that from *L. casei* (Dunlap *et al.*, 1971a; Leary and Kisliuk, 1971). Capco *et al.* (1973) estimated the specific activity of T4 thymidylate synthetase from 50% pure preparations to be 3.8 μmol min^{-1} mg^{-1} at 37°. The molecular weight of the T2 enzyme (64,400) is in the same range as the thymidylate synthetases from a variety of animal sources (Lorenson *et al.*, 1967; Fridland *et al.*, 1971; Horinishi and Greenberg, 1972), from bacteria (Dunlap *et al.*, 1971a; Krauss *et al.*, 1973), and from T4 bacteriophage (Capco *et al.*, 1973). The T2 thymidylate synthetase is composed of two subunits of equal molecular weight which appears to be a common feature of this enzyme regardless of its biological origin (Dunlap *et al.*, 1971a; Roodman and Greenberg, 1971; Langenbach *et al.*, 1972; Capco *et al.*, 1973). Loeble and Dunlap (1972) have presented evidence indicating that the subunits are identical in the case of thymidylate synthetase from *L. casei*.

Although their molecular weights are similar, T2 thymidylate synthetase appears unrelated immunologically to the synthetases from *L. casei*, *E. coli*, and regenerating rat liver. Krauss *et al.* (1973) similarly found that antibody to T4 thymidylate synthetase did not cross-react with the same enzyme from *E. coli*. However, the T-even bacteriophage thymidylate synthetases are immunologically related as antibody to the T2 enzyme inhibited the enzymes induced by T4 and T6. This finding agrees with the observation of Capco and Matthews (1973) who reported that T4 antisera cross-reacted with the thymidylate synthetases induced by T2 and T6.

The requirement of thymidylate synthetase activity for thiols has been documented in several laboratories (Friedkin, 1973). The T2 enzyme is no exception, since dialysis against a thiol-free buffer causes a loss in activity which can be partially restored by the addition of thiols. By contrast, the enzyme from *L. casei* can be completely reactivated by thiols after exhaustive dialysis against a thiol-free buffer (Dunlap *et al.*, 1971a). The comparatively greater susceptibility of the T2 synthetase to irreversible inactivation may be a consequence of the greater number of cysteines associated with the T2 enzyme than with the *L. casei* synthetase (10 *vs.* 4).

The FdUMP T2 thymidylate synthetase binding ratio of 2:1 agrees with the data of Santi and McHenry (1972) on the binding of FdUMP to the *L. casei* synthetase. Although this complex is stable to 6.0 M guanidine hydrochloride, half of the bound FdUMP can be removed by 6 M urea. It has been suggested on the basis of inhibition studies (Langenbach *et al.*, 1972) that the FdUMP binding sites on the *L. casei* enzyme are not identical. Some support for this suggestion appears to be provided by the urea studies. Since Capco *et al.* (1973) have shown that FdUMP binding varies with the source of thymidylate synthetase, interspecies comparisons of FdUMP binding may be of limited value. This does not appear, however, to be the case for the *L. casei* and T2 thymidylate synthetases, for almost identical results on FdUMP binding were obtained when the *L. casei* synthetase was substituted for the T2 enzyme in the experiments described in Tables III and IV and Figure 8.

Our previous studies (Galivan *et al.*, 1973), combined with the FdUMP binding studies reported here (Table IV), demonstrate that T2 thymidylate synthetase inactivated by dialysis against thiol-free buffer is incapable of forming a stable complex with FdUMP. On the basis of this study, we cannot directly implicate cysteine in the binding of FdUMP, but these observations do not rule out this possibility. Several groups (Kalman, 1972; Langenbach *et al.*, 1972; Santi and McHenry, 1972) have postulated the presence of a covalent linkage between a nucleophile on the enzyme, possibly cysteine, and the C-6 position of FdUMP. Langenbach *et al.* (1972) have extended this proposal to suggest that FdUMP is also covalently linked by a methylene bridge through its C-5 position to 5,10-CH_2H_4folate. Our studies reveal that enzyme–FdUMP complex formation does not require a methylene bridge since folate analogs incapable of forming a methylene bridge can support FdUMP binding (Table III).[4] Circular dichroic spectra of thymidylate synthetase in the presence of FdUMP and the same folate analogs have yielded similar results (J. Galivan, G. F. Maley, and F. Maley, manuscript in preparation). It, therefore, remains to be determined whether a methylene bridge of the type suggested by Langenbach *et al.* (1972) is required for FdUMP binding in the presence of 5,10-CH_2H_4-folate.

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[4] After submission of this paper, similar findings on the binding of FdUMP in the presence of various folate analogs were reported by Santi *et al.* (1974).

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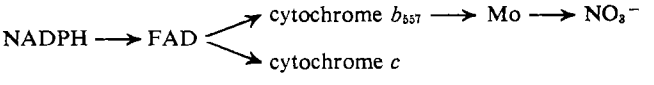
Purification and Properties of the Nitrate Reductase Isolated from *Neurospora crassa* Mutant *nit*-3. Kinetics, Molecular Weight Determination, and Cytochrome Involvement†

Alan D. Antoine

ABSTRACT: The nitrate reductase from *Neurospora crassa* mutant *nit*-3 exhibited reduced FAD–nitrate reductase and reduced methylviologen–nitrate reductase activities and the activities were nitrate inducible. The nitrate reductase was purified and characterized. The reduced FAD–nitrate reductase and reduced methylviologen–nitrate reductase activities of the mutant *nit*-3 enzyme remained associated following a 180-fold purification with the most highly purified fraction being homogeneous on disc gel electrophoresis. Both associated activities were relatively stable to treatment at 50° and inhibited by *p*-hydroxymercuribenzoate, had a pH optima between 7.0 and 8.0 depending on the type of buffer used and exhibited substrate affinities slightly different from the parent wild-type enzyme. The enzymatic activities reside in a protein complex having a sedimentation coefficient value of 6.8 ± 0.1 S, a Stokes radius of 56 ± 1 Å, and an estimated molecular weight of 160,000 ± 2000. The cytochrome associated with the homogeneous mutant enzyme exhibited a cytochrome b_{557} absorption spectrum. The properties of this mutant enzyme were compared to those of the partially purified enzyme from the parent wild-type *N. crassa* STA4.

Previous studies (Garrett and Nason, 1969) on the nitrate inducible nitrate reductase (NADPH:nitrate oxidoreductase, EC 1.6.6.2) of wild-type *Neurospora crassa* strain 5297a, including the identification and sequence of action of its components, have postulated the following electron-transport scheme for this enzyme complex



It has been further shown by Garrett and Nason (1969) that the *Neurospora crassa* nitrate reductase exists as a single protein complex with a molecular weight of approximately 228,000 and exhibits four nitrate inducible associated en-

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