(1968) who reports that protein synthesis in *Rana pipiens* is unaffected by variations in pool size. Our preliminary experiments suggest that exogenous leucine has little effect on the measured rate of synthesis in tobacco callus.

Acknowledgments

The authors thank Dr. C. D. Upper for his constructive suggestions, Dr. J. P. Helgeson for kindly providing us with tobacco callus, and Mr. J. N. Campbell and Mr. F. Vojtik for their excellent technical assistance.

References

Berg, W. E. (1968), *Exp. Cell Res. 49*, 379.
Bidwell, R. G. S., Barr, R. A., and Steward, F. C. (1964), *Nature (London) 203*, 367.
Cowie, D. B., and McClure, F. T. (1959), *Biochim. Biophys. Acta 31*, 236.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Eagle, H., Piez, A., and Fleishman, R. (1957), *J. Biol. Chem. 238*, 847.
Fry, B. J., and Gross, P. R. (1970), *Develop. Biol. 21*, 125.
Halvorson, H. O., and Cohen, G. N. (1958), *Ann. Inst. Pasteur Paris 95*, 73.
Helgeson, J. P., Krueger, S. M., and Upper, C. D. (1969), *Plant Physiol. 44*, 193.
Helgeson, J. P., and Upper, C. D. (1970), *Plant Physiol.* (in press).
Kipnis, D. M., Reiss, E., and Helmreich, E. (1961), *Biochim. Biophy. Acta 51*, 519.
Linsmaier, E. M., and Skoog, F. (1965), *Physiol. Plantarum 18*, 100.
Rutter, W. J. (1967), *in* Methods in Developmental Biology, Wilt, F. H., and Wessells, N. K., Ed., New York, N. Y., Crowell, pp 671–683.
Smith, D. (1968), *Develop. Biol. 18*, 232.
Stephenson, M. L., Thimann, K. V., and Zamecnik, P. C. (1956), *Arch. Biochem. Biophys. 65*, 194.
Steward, F. C., and Bidwell, R. G. S. (1966), *J. Exp. Bot. 17*, 726.
Virtanen, A. I., Laine, T., and Toivonen, T. (1940), *Z. Phys. Chem. 266*, 193.
Wright, B. E., and Anderson, M. L. (1960), *Biochim. Biophy. Acta 43*, 67.

Thymidylate Synthetase from Amethopterin-Resistant *Lactobacillus casei**

R. B. Dunlap,† N. G. L. Harding,‡ and F. M. Huennekens§

ABSTRACT: Thymidylate synthetase has been isolated from sonically disrupted cells of amethopterin-resistant *Lactobacillus casei* using a procedure that involves fractionation with ammonium sulfate, filtration through Sephadex G-100, and successive chromatography on CM-Sephadex, hydroxylapatite, and DEAE-Sephadex. The enzyme was crystallized by dialysis against ammonium sulfate. Homogeneity of the enzyme was established by disc electrophoresis on polyacrylamide gel and by ultracentrifugal analysis. The molecular weight of the protein (approximately 70,000) was obtained by polyacrylamide electrophoresis and gel filtration. Electrophoresis on sodium dodecyl sulfate–polyacrylamide revealed that the protein contains two subunits, each having a molecular weight of approximately 35,000. In Tris-acetate buffers, the enzyme displays a broad pH optimum between 6.5 and 6.8 when dUMP is the substrate (K_m = 5.1 × 10^{-6} M and 6.8 × 10^{-7} M, respectively, in the presence and absence of Mg^{2+}). The K_m value for *dl*,L-methylenetetrahydrofolate (in the presence of Mg^{2+}) is 3.2 × 10^{-5} M. The enzyme also utilizes UMP as a substrate, although at a reduced rate (*ca.* 40%) compared to dUMP. The UMP-dependent reaction also has a different pH optimum (5.9) and is not stimulated by Mg^{2+}. The catalytic activity of the enzyme, at all stages of purification, is increased severalfold by inclusion of thiols in the assay system. The enzyme is inhibited by *p*-mercuribenzoate, iodoacetate, *N*-ethylmaleimide, cyanide, H_2O_2, tetrathionate, 2-hydroxyethyl disulfide, and 5,5′-dithiobis(2-nitrobenzoic acid). All catalytic activity is lost when one of the four accessible sulfhydryl groups on the protein has reacted with *p*-mercuribenzoate.

Thymidylate synthetase catalyzes a novel reaction (eq 1) in which a methylene group undergoes a reduction while being transferred from tetrahydrofolate to C-5 of deoxyuridylate. Reduction of the one-carbon group is accommodated by oxidation of the tetrahydrofolate to dihydrofolate (Humphreys and Greenberg, 1958; Friedkin, 1959; McDougall and Blakley, 1960). During this oxidoreduction reaction, the hydrogen from C-6 of tetrahydrofolate is transferred without dilution to the methyl group of thymidylate (Friedkin, 1959;

5,10-methylenetetrahydrofolate + deoxyuridylate ⟶ dihydrofolate + thymidylate (1)

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Lorenson *et al.*, 1967). The continuous synthesis of thymidylate is accomplished *via* a "thymidylate synthesis cycle", consisting of reaction 1, followed by reaction 2, catalyzed by dihydrofolate reductase, and a third reaction in which 5,10-

$$\text{dihydrofolate} + \text{TPNH} + \text{H}^+ \longrightarrow \text{tetrahydrofolate} + \text{TPN}^+ \quad (2)$$

methylenetetrahydrofolate is regenerated from tetrahydrofolate.

Thymidylate synthetases have been purified to varying degrees from diverse sources such as *Escherichia coli* (Friedkin *et al.*, 1962), calf thymus (Jenny and Greenberg, 1963), *Streptococcus faecalis* R (Blakley, 1963), chronic myelocytic leukemic leukocytes (Silber *et al.*, 1963), chick embryo (Lorenson *et al.*, 1967), T-even bacteriophage (Greenberg *et al.*, 1962), and Ehrlich ascites cells (Revelle, 1968; Fridland and Heidelberger, 1970). In each instance, the relatively low level of thymidylate synthetase in the starting material made it difficult to obtain appreciable quantities of the purified enzyme. Recently, however, it has been found that, in dichloroamethopterin-resistant *Lactobacillus casei* (Crusberg and Kisliuk, 1969; Leary *et al.*, 1970) and in regenerating liver in amethopterin-treated rats (Labow *et al.*, 1969), the level of thymidylate synthetase is increased considerably. This result is somewhat surprising since dihydrofolate reductase, rather than thymidylate synthetase, is believed to be the target enzyme for this inhibitor, and it has been shown in several different systems that the reductase increases markedly in response to amethopterin (reviewed by Huennekens, 1968).

In the present investigation the baseline level of thymidylate synthetase in *L. casei* has been increased approximately 200-fold by making these cells resistant to amethopterin. A procedure has been developed for the isolation in good yield of pure thymidylate synthetase from this source and some general properties of the enzyme are described. The availability of the highly purified enzyme, in addition to its primary value in facilitating studies on the mechanism of reaction 1, also provides a means for following other enzymatic reactions (*e.g.*, methionine synthetase) in which tetrahydrofolate is a product (Galivan *et al.*, 1970).

Experimental Section

Materials. Commercially available materials included: *L. casei* var. *rhamnosus* (ATCC 7469) (American Type Culture Collection); lactobacilli broth AOAC and enzyme-hydrolyzed casein (Bacto-Casitone) (Difco Laboratories); ammonium sulfate, reagent grade (Merck); Sephadex G-100, CM-Sephadex C-50, and DEAE-Sephadex A-50 (Pharmacia); DEAE-cellulose and CC31 cellulose (Whatman); hydroxylapatite and Bio-Gel P-150 (Bio-Rad Laboratories); Celite (Johns Manville Co.); butyl-PBD (Packard); and Bio-Solv BBS2 (Beckman). All other chemicals were of reagent grade. Glass-distilled water was used routinely for preparation of solutions, except those used in the 150-l. fermentations, which were prepared with deionized water.

Tricine[1] was synthesized by the method of Good *et al.* (1966). dUMP-5-*t* (0.56 Ci/mmole) was obtained from Calatomic; the purity of each sample was checked by thin-layer chromatography on MNCEL 300 (Machterey Nagel and Co., Düren, Germany) using concentrated ammonium hydroxide–*t*-butyl alcohol–methyl ethyl ketone–water (40:20:12:8, v/v) as the solvent system. This material was diluted with unlabeled dUMP to yield a 10^{-3} M stock solution having a specific activity of 36 μCi/μmole; prior to use, this solution was diluted 10-fold with 10^{-3} M dUMP. The stock solution was stored frozen at −20° in order to minimize the nonenzymic loss of tritium into water. When necessary, dUMP-5-*t* was repurified by lyophilization or by adsorption on washed Norit A, followed by elution with ethanol–ammonia–water (60:5:35, v/v). 5-Fluorodeoxyuridine was a gift from Dr. W. E. Scott, Hoffman-LaRoche, Inc. 5-Fluorodeoxyuridine 5′-phosphate was kindly provided by Dr. J. Whiteley. Amethopterin, obtained from the Cancer Chemotherapy National Service Center, was further purified by chromatography (N. G. L. Harding, unpublished method) on DEAE-cellulose and CC31 cellulose and stored as a lyophilized preparation in the dark at 5°. Solutions of the ammonium salts of folic acid and amethopterin in 0.05 M Tris-chloride (pH 7.5) were sterilized by filtration through Millipore membranes (HAWG 025 00), and stored in sealed ampoules at −20°. *dl*,L-Tetrahydrofolic acid was prepared by catalytic hydrogenation of folic acid in deoxygenated acetic acid (Hatefi *et al.*, 1960) and the product was lyophilized. Small portions (about 10 mg) of the resultant white powder were transferred rapidly into 10-ml serum bottles (fitted with split rubber stoppers) and the latter were evacuated (*ca.* 50 μ) for 6 hr in a Virtis vacuum-stoppering chamber attached to a Virtis lyophilizer. The stoppers were secured with crimped aluminum seals. When stored under these conditions at −20°, tetrahydrofolate remains stable for at least 1 year.

A stock solution (2×10^{-3} M) of *dl*,L-5,10-methylenetetrahydrofolate was prepared by dissolving 6 mg of *dl*,L-tetrahydrofolate in 5 ml of a solution containing 0.05 M $NaHCO_3$, 0.07 M HCHO, and 0.25 M β-mercaptoethanol (or 0.2 M DTE). This solution was stored frozen in a sealed tube under argon for no longer than 1 week. When being used in assays, the solution was kept in an ice bath under a gentle stream of argon for no more than 24 hr.

Norit A (Matheson Coleman & Bell) was purified by the following method. The material (25 g) was stirred with several changes of 1 M HCl until the supernatant solution was colorless. The material was then washed successively with water, ethanol–ammonia–water (60:5:35, v/v), and water, and finally dried at 100°.

Methods

Spectrophotometric Assay of Thymidylate Synthetase (adapted from the procedure of Wahba and Friedkin (1961)). The following components were added to a 1-cm cuvet: 0.1 ml of 2×10^{-3} M *dl*,L-5,10-methylenetetrahydrofolate (see Materials); 0.2 ml of 0.5 M potassium phosphate buffer (pH 6.8); enzyme; and water to make the total volume 0.9 ml. The dUMP-independent change in absorbance at 340 mμ was followed for 3 min using a Gilford multisample absorbance recorder (set at 0–0.200 full scale) thermostatted at 30°; 0.1 ml of 10^{-3} M dUMP was then added and the absorbance change at 340 mμ due to reaction 1 was recorded over a 5–10-min period. The net absorbance change, after being corrected for the dUMP-independent blank rate, was used in conjunction with the differential extinction coefficient of 6.4×10^3 M^{-1} cm^{-1} at 340 mμ (Wahba and Friedkin, 1961) to calculate the enzymatic rate. One unit of enzyme activity is defined

[1] Abbreviations used are: DTE, dithioerythritol; *p*-MB, *p*-mercuribenzoate; SDS, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; ME, β-mercaptoethanol; $LipSH_2$, dihydrolipoate.

as the amount required to synthesize 1 μmole of thymidylate/min under these conditions. Specific activity is expressed as units per milligram of protein. Protein was determined by the biuret (Gornall *et al.*, 1949) or the Lowry *et al.* (1951) methods, using bovine serum albumin as the standard.

Tritium-Release Assay of Thymidylate Synthetase (adapted from the procedure of Lomax and Greenberg (1967)). The following components were added to a 10 × 100 mm test tube: 0.05 ml of 2×10^{-3} M *dl*,L-5,10-methylenetetrahydrofolate (see Materials), 2.5 μmoles of potassium fluoride, 50 μmoles of potassium phosphate (pH 6.8), enzyme, and water to make the total volume 0.5 ml. The reaction was initiated by adding 0.05 ml of 10^{-3} M dUMP-5-*t* (3.6 μCi/μmole). After an incubation period of 10 min at 30° under argon, the reaction was stopped by the addition of 0.1 ml of 1.0 M HCl. (Alternatively, the solution can be lyophilized at this point and the radioactivity determined on an 0.2-ml aliquot of the water fraction.) Washed Norit A (40 mg) suspended in 0.5 ml of water was added and, after filtration through a Millipore membrane to remove the Norit which had adsorbed the unreacted dUMP-5-*t*, an 0.2-ml aliquot of the fitrate was added to 10 ml of scintillant (5 g of butyl-PBD, 100 ml of Bio-Solv BBS2, and 900 ml of toluene) and assayed for tritium at 37–42% efficiency in a Beckman scintillation counter, Model LS233. A correction was made for a control in which the enzyme was omitted or inactivated by HCl prior to addition of the substrates.

Chromatographic Procedures. The following buffer systems, freshly prepared, were used in the various chromatographic steps (concentrations are millimolar: G-100 (pH, 7.2; Tris (as the free base), 50; KCl, 50; mercaptoethanol, 10; EDTA, 1), CM-1 (pH, 6.5; Tris, 50; KCl, 50; mercaptoethanol, 10; EDTA, 1), CM-2 (pH, 7.7; Tris, 50; KCl, 700; mercaptoethanol, 10; EDTA, 1), DEAE-1 (pH, 7.2; Tris 25; mercaptoethanol, 5; $MgCl_2$, 10), DEAE-2 (pH, 6.2; Tris, 25; mercaptoethanol, 5; $MgCl_2$ 200), HA-1 (pH, 7.5; KH_2PO_4, 10; mercaptoethanol, 10), and HA-2 (pH, 7.5; KH_2PO_4, 500; mercaptoethanol, 10).

Sephadex gels and ion exchangers (particle size, 40–120 μ) were swollen with water, washed at room temperature (with the initial buffer to be used in the chromatographic procedure), and then stored at 5°. Columns were packed at room temperature and then transferred to a cold room (*ca.* 5°) for chromatography. Elution gradients were determined by conductivity measurements on the fractions (at 5°) using a Radiometer conductivity meter CDM2d equipped with cell CDC114.

Electrophoretic Methods. Electrophoresis on cellulose acetate membranes was performed with the Beckman microzone apparatus (3–7°, 150 V, 20–30 min) using Tris-maleate and potassium phosphate buffers (Γ/2, 0.075), containing 1 mM EDTA, at the indicated pH values. Protein was visualized by treatment of the membrane with Beckman fixative dye, followed by destaining with 5% acetic acid.

Disc electrophoresis on polyacrylamide gels was performed according to the procedures of Ornstein (1964) and Davis (1964). Unless otherwise specified, the tubes (0.6 × 6 cm) contained 7.5% w/v acrylamide and were developed at 0–5° and at 11–14 mA/cm² until the bromophenol blue marker had migrated about 5 cm. Immediately after being removed from the tube, the position of the dye was marked with a nylon bristle. Protein was visualized in polyacrylamide gels by staining with Amido Black (Davis, 1964) and in SDS polyacrylamide gels by staining with coomassie blue (Weber and Osborn, 1969). R_F values are relative to the bromophenol blue marker. Thymidylate synthetase activity was visualized by placing the polyacrylamide gel in a 6-ml screwcapped tube with 5 ml of an assay mixture that contained 1.0 M Tris-acetate (pH 6.8), 0.025 M mercaptoethanol, 0.010 M HCHO, 2×10^{-4} M dUMP, and 5×10^{-4} M *dl*,L-tetrahydrofolate. After standing for 15 min at 37° in this solution, the gel was washed with deoxygenated, argon-saturated 0.1 M potassium phosphate buffer (pH 6.8). The gel was then examined under ultraviolet light (365 mμ) in a Chromatovue and the fluorescent zone due to the dihydrofolate produced (*cf.* reation 1) was recorded photographically using a Polaroid camera MP3 with high contrast, type 51 film. When the gel was preincubated for 10 min with 10^{-6} M 5-fluorodeoxyuridine 5′-phosphate (a potent inhibitor of the enzyme (Hartmann and Heidelberger, 1961)) before exposure to the assay solution, no fluorescence was observed.

Molecular weight determinations on the intact protein were carried out by the Sephadex method of Whitaker (1963), and the polyacrylamide gel techniques of Zwaan (1967) and Hedrick and Smith (1968). Molecular weight determinations of the subunits were carried out by the SDS polyacrylamide method of Weber and Osborn (1969).

Ultracentrifugal Analysis. $s_{20,w}$ values were determined by a sedimentation velocity technique (Schachman, 1959) using a Beckman Model E analytical ultracentrifuge equipped with schlieren optics (rotor speed, 52,000 rpm; temperature, 2°).

Preparation of Amethopterin-Resistant L. casei. L. casei was grown by the following modification of the previous procedure of Harding (1966). One-hundred grams of enzyme-hydrolyzed casein (Bacto-Casitone) was suspended in 1 l. of water containing 50 g of KH_2PO_4, and the pH was adjusted to 4.9 with acetic acid. This solution was autoclaved for 30 min at 15 psi and, while still hot, was stirred for 10 min with 1 g of washed Norit A. K_2HPO_4 (50 g) and sodium acetate (120 g) were added, and the hot solution was stirred for 15 min. Charcoal was then removed by filtration through Celite and the filtrate was stored at 5°.

The complete medium was prepared by mixing 50 ml of the above charcoal-treated casein hydrolysate with 910 ml of water containing (in grams): L-tryptophan, 0.1; guanine, 0.01; adenine sulfate, 0.01; xanthine, 0.01; uracil, 0.01; L-asparagine-HCl, 0.30; L-cysteine-HCl, 0.10; L-ascorbic acid, 1.00; $MgSO_4 \cdot 7\ H_2O$, 0.80; $FeNH_4(SO_4)_2$, 0.056; and $MnSO_4 \cdot H_2O$, 0.146. The pH was adjusted to 6.8 with 5 M NaOH and, if solid medium was required, agar (1 g/100 ml of medium) was added. The medium was then autoclaved for 15 min at 15 psi and stored at 5° for no longer than 10 days. Prior to inoculation, each 957 ml of medium was mixed with 40 ml of a 50% solution of glucose, 2.5 ml of a folic acid solution (2 μg/ml in 0.05 M Tris-chloride, pH 7.5, sterilized by Millipore filtration), and 1 ml of a mixture that contained (in milligrams): riboflavin, 0.50; thiamine-HCl, 0.20; biotin, 0.01; nicotinic acid, 0.40; *p*-aminobenzoic acid, 1.00; and pyridoxine-HCl, 2.00.

The amethopterin-resistant strain of *L. casei* was obtained by the following procedure: 5 ml of a log-phase culture of *L. casei* (obtained by transfer to the above medium of the freeze-dried organism from ATCC suspended in Lactobacilli broth) was used to inoculate 200 ml of the above medium supplemented with amethopterin (1.3×10^{-10} M) contained in flask 1 (500 ml, with a side arm for a Klett photometer; Bellco; Vineland, N. J.). After 4-hr log-phase growth at 37°, the concentration of amethopterin was increased to 5.4×10^{-10} M. No significant decrease in growth rate was apparent during the next 4 hr. The amethopterin concentration was then ele-

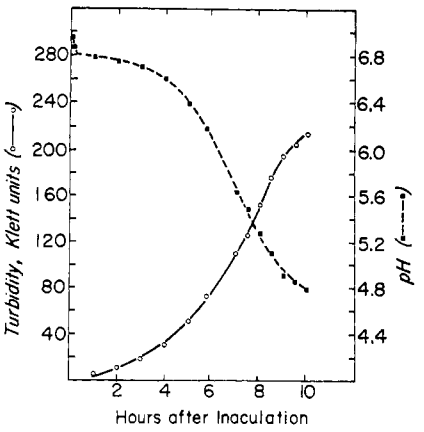

FIGURE 1: Growth characteristics of amethopterin-resistant *L. casei*. Cells were grown in 150 l. of medium, as described in the Experimental Section. Samples were withdrawn at the indicated times and the turbidity determined in a Klett photometer equipped with a red 66 Filter. pH values were taken at 5° with a glass electrode.

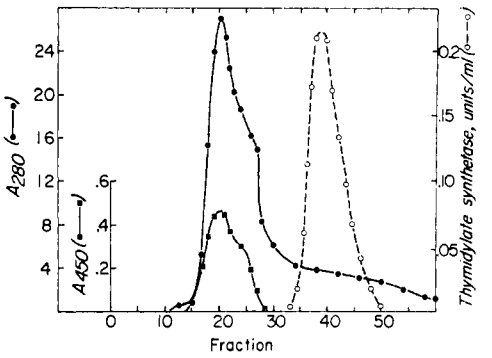

FIGURE 2: Filtration of thymidylate synthetase preparation through Sephadex G-100 (see Experimental Section for details). Protein was measured by absorbance at 280 mμ (A_{280}) and flavin by absorbance at 450 mμ (A_{450}). The dashed line indicates thymidylate synthetase activity measured by the spectrophotometric assay and expressed as units per milliliter in each fraction.

vated to 5.0×10^{-9} M. When growth had reached the stationary phase, flask 2 containing fresh medium with amethopterin at 4.5×10^{-9} M was inoculated with 5 ml of the culture from flask 1; the cells in flask 2 were allowed to grow to stationary phase without further addition of amethopterin. Flask 3 had an initial amethopterin concentration of 4.5×10^{-9} M and was inoculated with 5 ml of stationary-phase culture from flask 2; at the mid-log phase of growth the concentration of amethopterin in flask 3 was increased to 1.4×10^{-8} M and grown to stationary phase. This cycle was repeated six times until the cells were able to tolerate an amethopterin concentration of 1.0×10^{-5} M.

Large-scale cultures of the resistant organism were grown at 37° (without aeration and with mechanical stirring) in a stainless steel tank (Fermentation Design Inc., Pa.). The complete medium (150 l., without amethopterin) was inoculated with 1.5 l. of a log-phase culture of the amethopterin-resistant organism and growth was monitored by absorbance in the Klett Photometer and by pH changes (Figure 1). After approximately 10 hr (see Figure 1), the cells (650–750 g wet weight) in late-log phase were harvested by centrifugation (flow rate, 1.4–1.8 l./min) in a refrigerated Sharples centrifuge, Model AS16P (Pennsalt Chemicals Corp., Pa.), and stored in 40-g portions in polyethylene bags at −20°.

Purification of Thymidylate Synthetase. Unless otherwise stated, all operations were carried out at 0–5°. Centrifugations were performed in a Sorvall refrigerated centrifuge, Model RC-2, using the GSA or SS34 heads. In the chromatographic steps, fractions were collected automatically and were monitored for protein, enzyme activity, and conductivity.

STEP 1. CELL-FREE EXTRACT. Frozen cells (40 g) of amethopterin-resistant *L. casei* were suspended in 7 volumes of G-100 buffer and the pH was adjusted to 7.2 with 5 M NaOH. Suitable aliquots of this suspension were placed in a 400-ml rosette flask immersed in ice–water and the cells were lysed with a Branson Sonifier, Model S125 (Danbury, Conn.), at a setting of 8, for five 5-min periods. During sonication, the temperature was not allowed to rise above 10°. Centrifugation at 21,000*g* for 50 min yielded a pale yellow supernatant solution (290 ml).

STEP 2. FRACTIONATION WITH SOLID AMMONIUM SULFATE. Solid ammonium sulfate was added slowly and with stirring to the cell-free extract until 35% saturation had been reached (19.6 g/100 ml). The suspension was stirred for 20 min, centrifuged at 21,000*g* for 50 min, and the precipitate was discarded. Solid ammonium sulfate was then added to the supernatant (18.6 g/100 ml) to achieve 65% saturation. Centrifugation at 21,000*g* for 50 min yielded about 16 g of precipitate, which was then dissolved in a minimal volume (40–50 ml) of G-100 buffer and dialyzed overnight against 6 l. of the same buffer.

STEP 3. FILTRATION THROUGH SEPHADEX G-100. Sephadex G-100 was packed into a 4-cm column to a height of 115 cm and equilibrated with G-100 buffer. The dialyzed fraction (60 ml) from step 2 was filtered through the column (flow rate 10–20 ml/hr) using the same buffer and 8.5-ml fractions were collected (*cf.* Figure 2). Most of the protein and considerable amounts of flavin (indicated by the A_{450} values) appeared in the early fractions (tubes 15–30). Fractions containing thymidylate synthetase (tubes 35–45) were pooled and adjusted carefully to pH 6.5 with 1 M HCl. It should be noted that dihydrofolate reductase, which appears later in the profile (centered approximately at tube 100), can also be obtained from this step. On larger scale preparations (*i.e.*, starting with 320 g of cells), step 3 can be carried out most conveniently using a 14 × 170 cm column of Bio-Gel P-150.

STEP 4. CHROMATOGRAPHY ON CM-SEPHADEX. A 3.5-cm column was packed to a height of 52 cm with CM-Sephadex and equilibrated with CM-1 buffer. The pooled material (80–90 ml) from step 3 was applied to this column (Figure 3) and elution with the same buffer was carried out (9.5-ml fractions; flow rate, *ca*, 50 ml/hr) until the effluent was color-

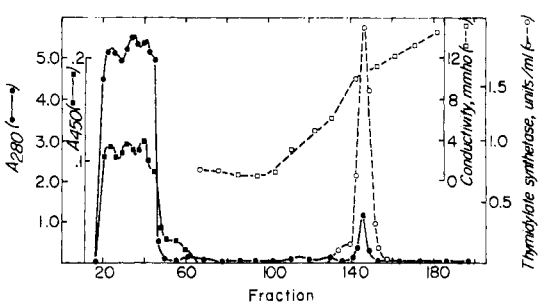

FIGURE 3: Chromatography of thymidylate synthetase preparation on CM-Sephadex (see Experimental Section and legend for Figure 2 for details).

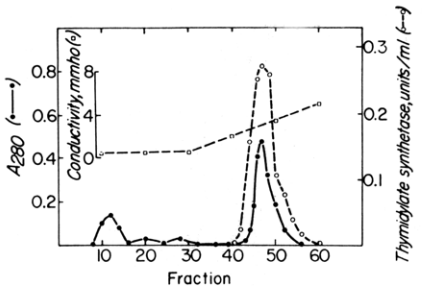

FIGURE 4: Chromatography of thymidylate synthetase preparation on hydroxylapatite (see Experimental Section and legend for Figure 2 for details).

less (*ca.* tube 60). A gradient of increasing pH and salt concentration was then applied using 1.0 l. of CM-1 buffer in the mixing flask and 1.0 l. of CM-2 buffer in the reservoir (flow rate 30 ml/hr; 7.5-ml fractions). Fractions containing thymidylate synthetase activity (tubes 130–150) were pooled. It should be noted that this step can also be carried out using a batchwise elution procedure.

STEP 5. CHROMATOGRAPHY ON HYDROXYLAPATITE. The pooled fractions from step 4 (90–100 ml) were dialyzed against HA-1 buffer and applied to a 2.5 × 10 cm column of hydroxylapatite. The column was washed with about 120 ml of the above buffer and then eluted (flow rate not greater than 10 ml/hr) with a linear gradient of increasing potassium phosphate concentration (0.5 l. of buffer HA-1 in the mixing vessel and 0.5 l. of buffer HA-2 in the reservoir). Fractions (6 ml) were collected. Thymidylate synthetase appeared in fractions 40–55 (Figure 4).

STEP 6. CHROMATOGRAPHY ON DEAE-SEPHADEX. A 1-cm column was packed to a height of 25 cm with DEAE-Sephadex and equilibrated with DE-1 buffer. The enzyme preparation from step 5 was dialyzed against several changes of DE-1 buffer and, if turbid, was centrifuged before application to the DEAE-Sephadex column. Elution was performed by means of a linear gradient (250 ml of DE-1 buffer in the mixing vessel and 250 ml of DE-2 buffer in the reservoir). The flow rate was ca. 15 ml/hr and 8-ml fractions were collected. As shown on the elution profile (Figure 5), the symmetrical peak of thymidylate synthetase eluted between tubes 65 and 78. The minor protein component immediately following thymidylate synthetase (tubes 80–90) had the same R_F value

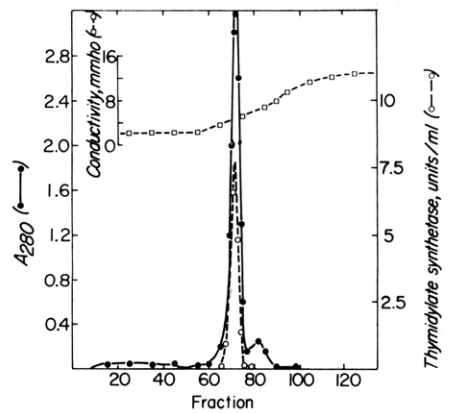

FIGURE 5: Chromatography of thymidylate synthetase on DEAE-Sephadex (see Experimental Section and legend for Figure 2 for details).

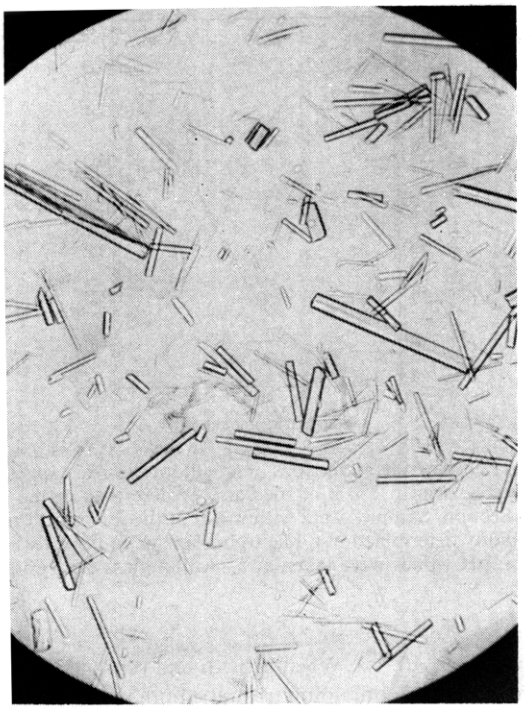

FIGURE 6: Crystals of thymidylate synthetase suspended in 50% ammonium sulfate (pH 6.0). Magnification (100×).

(0.62) as the enzyme when subjected to electrophoresis on polyacrylamide, but it showed no activity with either dUMP or UMP as substrate.

STEP 7. CRYSTALLIZATION. Visking dialysis tubing (previously boiled in 1% mercaptoethanol) was washed thoroughly with water and filled with 10 ml of thymidylate synthetase from step 6 (1 mg/ml in 0.3 M KCl containing 50 mM Tris-chloride (pH 7.0) and 10 mM mercaptoethanol). An ammonium sulfate solution (70 ml, 50% saturated and pH 6.0 at 0°—measured with a glass electrode and not compensated for salt concentration) was placed in a 2.5 × 25 cm glass cylinder and carefully overlaid with 20 ml of 50 mM Tris-chloride (pH 7.9). The dialysis sac was carefully suspended in the upper layer in such a manner that the interface was not disturbed. The cylinder was then capped and diffusion allowed to occur at 5°. Crystals of the enzyme, which appeared within 4 weeks on the walls of the sac, were allowed to grow for several additional weeks before being harvested by centrifugation at 200*g* for 10 min. The crystals were then washed by resuspension and centrifugation in 20 ml of ammonium sulfate (50% saturation, pH 6.0). Figure 6 shows the appearance of these crystals when suspended in cold 50% saturated ammonium sulfate solution (pH 6.0). They readily dissolved in this solution, however, when the temperature was raised to 37°.

Recrystallization of the enzyme was accomplished by a stepwise increase in salt concentration, accompanied by a reduction of the pH toward the isoelectric point. The enzyme (10 mg in 10 ml of 50 mM Tris-chloride, pH 7.5) was placed in washed dialysis tubing and dialyzed sequentially against solutions A–E (Table I). In each instance, dialysis was carried out against two successive 90-ml portions of the solution. Solutions were changed at 3-day intervals, except that dialysis against solution C was continued for 10 days during each of the two changes in order to facilitate the initial, slow growth of the crystals.

TABLE I: Ammonium Sulfate Solutions Used in the Recrystallization of Thymidylate Synthetase.

Solution[a]	$(NH_4)_2SO_4$ (g)	1.0 M Tris[b] (ml)	1 M HCl (ml)	pH[c]
A	18	10	8.2	8.0
B	36	5	4.5	7.5
C	54	2.5	2.4	7.0
D	72	1.25	1.24	6.5
E	90	0.03	0	6.1

[a] Water added to make a final volume of 250 ml. [b] Free base. [c] Uncorrected values obtained at 5° using a glass electrode.

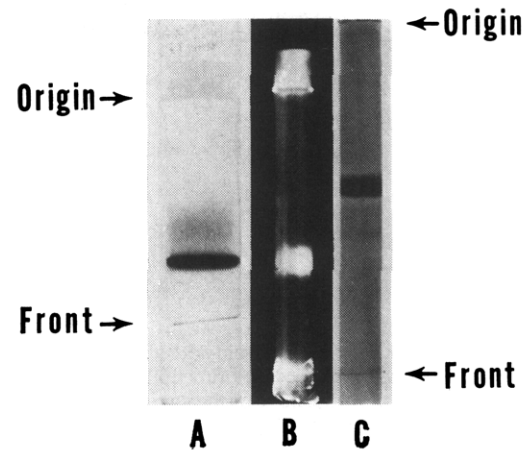

FIGURE 7: Electrophoresis of thymidylate synthetase on polyacrylamide gels. Panels A and B show 100 μg of enzyme (specific activity 2.5 units/mg) electrophoresed on polyacrylamide gel (see Experimental Section for details). (A) Stained for protein. (B) Stained for enzymatic activity (and photographed under ultraviolet light in a Chromatovue). Panel C, 100 μg of enzyme (specific activity 2.5) was dialyzed at 37° for 2 hr against a solution containing 1% w/v SDS and 1% v/v mercaptoethanol. Electrophoresis was performed on 10% w/v acrylamide, 0.2% w/v methylenebisacrylamide and 0.1% w/v SDS. Protein, in each case, migrated toward the positive electrode.

Results and Discussion

Assay Systems. During the course of this investigation two different methods were used to assay thymidylate synthetase activity. The spectrophotometric method, modified slightly from the procedure of Wahba and Friedkin (1961), is rapid and convenient. It was used routinely with enzyme concentrations greater than 6.7×10^{-4} unit/ml, except where other substances present (*e.g.*, certain inhibitors) would have interfered. The tracer method, adapted from the procedure of Lomax and Greenberg (1967), is extremely sensitive and was used with more dilute preparations of the enzyme. It is also useful in mechanistic studies involving labilization of the hydrogen at C-5 of deoxyuridylate.

Amethopterin-Induced Increase in Enzyme Level. The level of thymidylate synthetase in amethopterin-sensitive *L. casei*, harvested in late-log phase, is about 10^{-4} unit/mg of protein. Although this is considerably higher than the level of thymidylate synthetase in mammalian tissues, it is still not entirely satisfactory for purification purposes. However, Kisliuk and coworkers (Crusberg and Kisliuk, 1960; Leary *et al.*, 1970) have shown that the level of thymidylate synthetase in *L. casei* is elevated when these cells are made resistant to dichloroamethopterin. Essentially the same technique, except for the substitution of amethopterin for dichloroamethopterin, has been used in the present investigation to enhance the level of thymidylate synthetase in *L. casei* by several 100-fold (*i.e.*, $2\text{–}7 \times 10^{-2}$ unit/mg of protein in different preparations).

The drug-resistant organism is similar to the parent strain with respect to its requirement for folic acid and its morphological and colonial characteristics. The sensitive organism, however, tends to aggregate in the late phases of growth, whereas the resistant strain remains in a homogeneous suspension.

Purification Procedure. By growing amethopterin-resistant *L. casei* on a relatively large scale (150 l.) and by using a large column for gel filtration (step 3), it is possible to prepare about 20–30 mg of highly purified enzyme/week. Representative data for the purification procedure are summarized in Table II. Following step 6 (chromatography on DEAE-Sephades), the enzyme has a specific activity of 2.5 units/mg of protein and is recovered in about a 30% overall yield. At this stage, the preparation shows only a single protein band, which coincides with enzyme activity, when subjected to disc electrophoresis on polyacrylamide gel (see below). Although crystallization of the enzyme raises the specific activity slightly, the procedure is somewhat lengthy and was not used routinely. All of the subsequent experiments reported in this paper were performed with enzyme that had been processed through step 6.

The stability and activity of the enzyme are markedly dependent upon the presence of thiol-containing compounds in the medium (see below). For this reason, all buffers used during purification contained mercaptoethanol. The enzyme is stable for months when stored at 5° under argon in a solution that is 0.1 M in potassium phosphate (pH 6.8) and 0.025 M in mercaptoethanol. The crystalline enzyme can be kept as a suspension in 50% ammonium sulfate (pH 6.0) under argon at 5°.

Criteria of Purity. Following step 6 in the purification procedure, the enzyme shows only a single band after being electrophoresed on polyacrylamide gel (panel A in Figure 7). Coincidence of enzyme activity with the protein could be shown by excising the segment of an identical polyacrylamide column, extracting the gel with CM-1 buffer, and assaying the extract spectrophotometrically; or, preferably, by placing an identical polyacrylamide column in the enzyme-visualization

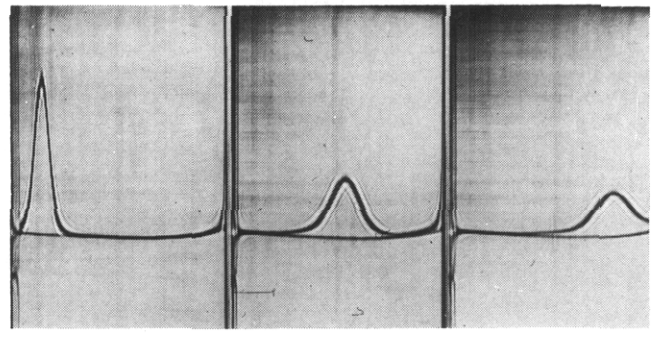

FIGURE 8: Sedimentation of thymidylate synthetase. Enzyme (specific activity 2.5 units/mg; 13 mg/ml) in 0.05 M Tris-chloride (pH 7.5) which also contained 0.05 M $MgCl_2$, was centrifuged at 59,000 rpm in a Beckman analytical ultracentrifuge, Model E. Photographs (left to right) were taken at 48, 200, and 306 min.

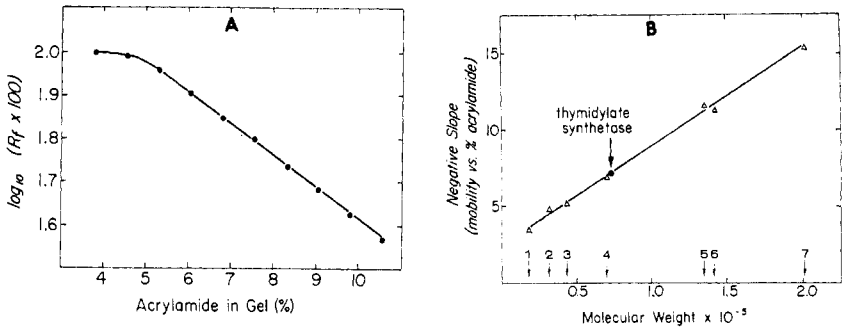

FIGURE 9: Molecular weight of thymidylate synthetase by electrophoresis on polyacrylamide gels of varying porosity. Panel A, log of R_F values of thymidylate synthetase as a function of per cent acrylamide in gel. Panel B, comparison of the mobility of thymidylate synthetase (shown as 100× the negative slope of the straight line portion of the plot in A) to mobilities of proteins of known molecular weight (determined individually in the same manner). Marker proteins and their molecular weights are: 1, myoglobin (17,500); 2, pepsin (34,000); 3, ovalbumin (43,000); 4, bovine serum albumin monomer (68,000); 5, bovine serum albumin dimer (136,000); 6, lactate dehydrogenase (140,000); 7, bovine serum albumin trimer (204,000).

TABLE II: Purification of Thymidylate Synthetase.

Step	Fraction	Vol (ml)	Protein (mg/ml)	Act. (Units/ml)	Sp Act. (Units/mg)	Total Units	Recov (%)
1	Cell-free extract	285	7.2	0.47	0.065	134	100
2	Ammonium sulfate (35–65%)	92	8.0	1.21	0.15	111	83
3	Sephadex G-100	175	1.1	0.49	0.45	86	64
4	CM-Sephadex	97	0.3	0.59	2.0	57	44
5	Hydroxylapatite	80	0.27	0.63	2.33	50	37
6	DEAE-Sephadex	70	0.24	0.61	2.5	43	32

medium (see Experimental Section) and photographing the gel under ultraviolet light (panel B in Figure 7).

Homogeneity of the enzyme was also demonstrated by two other techniques, namely, immunophoretic analysis using an antibody prepared from rabbit serum (R. Reid, B. Dunlap, and N. G. L. Harding, unpublished results), and ultracentrifugal analysis. In the latter method, the protein sedimented as a single, symmetrical peak (Figure 8) and gave an $s_{20,w}$ value of 5.1 S.

Molecular Weight. The molecular weight of the *L. casei* thymidylate synthetase has been determined by two different methods. Filtration of the enzyme through a Sephadex G-100 column, standardized with proteins of known molecular weight (Whitaker, 1963), gave a value of 70,000. Electrophoresis of the enzyme on polyacrylamide gels of increasing porosity (Zwaan, 1967) gave a molecular weight of 72,000 when compared to proteins of known molecular weight (Figure 9).

When the enzyme was dialyzed against mercaptoethanol and SDS, and then subjected to electrophoresis on polyacrylamide gel containing SDS (Weber and Osborn, 1969), a single, enzymatically inactive protein band was observed (panel C in Figure 7). The molecular weight of this material was estimated to be 34,000–36,000 by comparison to mobilities of similarly treated proteins of known molecular weight. The same result was obtained when mercaptoethanol was omitted from the dialysis step. From these data, the molecular weight of thymidylate synthetase is assumed to be approximately 70,000 and to consist of two 35,000 molecular weight subunits. It is not yet known whether these are identical or nonidentical subunits.

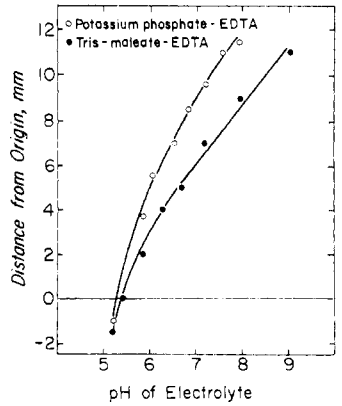

FIGURE 10: Electrophoretic mobility of thymidylate synthetase on cellulose acetate as a function of pH. Enzyme, 100 μg; other details given in Experimental Section.

Isoelectric Point. Figure 10 illustrates the electrophoretic mobility of the enzyme at various pH values. Although the mobility of the enzyme was greater in potassium phosphate–EDTA buffers than in Tris-maleate–EDTA, the isoelectric point in either system occurred at approximately the same pH, *i.e.*, 5.3–5.4.

Absorption Spectrum. The absorption spectrum of the enzyme in 0.1 M potassium phosphate (pH 7.0) shows a single absorption maximum at 275 mμ and a minimum at 248 mμ (Figure 11). The absorbance ratios at 275:248 and 280:260 mμ are 1.90 and 1.28, respectively. At higher protein concen-

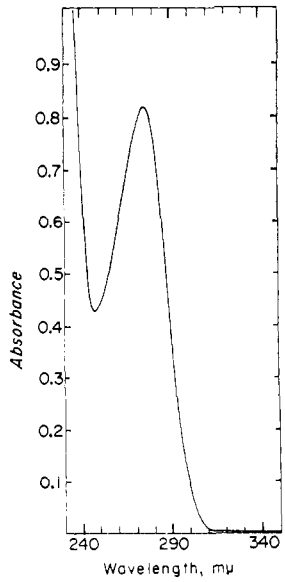

FIGURE 11: Absorption spectrum of thymidylate synthetase. Absorption spectrum of the enzyme (specific activity 2.5 units/mg; 0.66 mg/ml (biuret determination) in 0.1 M potassium phosphate, pH 7.0) determined using a Cary recording spectrophotometer, Model 14, against a buffer blank.

trations (5 mg/ml), an additional absorption band of low intensity is seen in the 330-mμ region (absorbance ratio 275:330 $\simeq$ 250). Based upon a molecular weight of 70,000, the molar extinction coefficient for the enzyme at 275 mμ is calculated to be 8.6×10^4 M^{-1} cm^{-1}. There was no apprciable change in the spectrum of the enzyme in the presence of formaldehyde (4×10^{-3} M) or dUMP (2×10^{-5} M).

Kinetic Constants. At the highest stage of purity, and when tested under optimal conditions, the enzyme catalyzes the formation of 2.5 μmoles of thymidylate/min per mg of protein at 30°. Based upon a molecular weight of 70,000, this corresponds to a turnover number of 175 (molecules of product synthesized per min by each molecule of enzyme).

The effect of pH upon the activity of thymidylate synthetase is complex and depends, to a considerable degree, on other factors such as the nature of the buffer, the presence or absence of Mg^{2+} and the substrate employed. In phosphate buffer (Figure 12, left panel), the pH optimum occurs in the range of 6.5–6.8 when dUMP is the substrate. Mg^{2+} has little effect in this system. The activity with UMP as substrate is barely detectable in phosphate buffer and, again, Mg^{2+} is without effect. Quite different results are obtained, however, in Tris-acetate buffer (Figure 12, right panel). The pH optimum for dUMP again falls slightly on the acid side of neutrality and there is an additional, smaller optimum at pH 5.2–5.4. In this buffer the requirement for Mg^{2+} is more pronounced. The enzyme is relatively specific for Mg^{2+}. Of a number of other cations tested, only Mn^{2+} showed a small stimulatory effect (*ca.* 10%, compared to Mg^{2+}) under these conditions. Somewhat unexpectedly, the enzyme shows an appreciable activity with UMP in this system, the single pH optimum occurs at about 5.9 and there is no appreciable effect of Mg^{2+}. The enzyme, however, shows no activity with 5-hydroxymethyl-dUMP (kindly provided by Dr. F. Maley), dUDP, dUTP, UDP, UTP, or with a uridine-rich tRNA (kindly provided by Dr. G. D. Novelli).

Michaelis constants for the substrates were determined in 0.05 M Tris-acetate buffer (pH 6.8). Under these conditions,

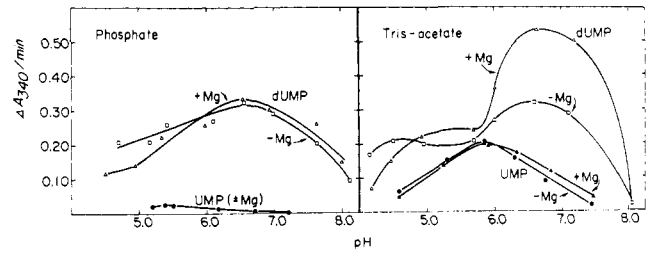

FIGURE 12: pH–activity relationships of thymidylate synthetase with dUMP and UMP as substrates. Left panel, phosphate buffers (0.05 M); right panel, Tris-acetate buffers (0.05 M with respect to Tris). Spectrophotometric assay with indicated substitutions or deletions. UMP, 1×10^{-3} M; Mg^{2+}, 1×10^{-2} M. Each cuvet contained 23 μg of enzyme.

the K_m for dUMP was 5.1×10^{-6} and 6.8×10^{-7} M, respectively, in the presence and absence of Mg^{2+} (10 mM). The K_m value for UMP (at its pH optimum of 5.9) was much higher (*ca.* 10^{-4} M) and was unaffected by Mg^{2+}.

The other substrate, 5,10-methylenetetrahydrofolate, is most conveniently prepared *in situ* by the nonenzymatic interaction of tetrahydrofolate with HCHO. Since the HCHO is usually added in excess, it was necessary to determine its effect upon the reaction. Using the spectrophotometric assay (*dl*,L-tetrahydrofolate at 2×10^{-4} M), optimal activity was observed when the HCHO concentration varied between 5×10^{-3} and 1×10^{-2} M. At higher concentrations of HCHO, a linear inhibition was observed, *e.g.*, at 3×10^{-2} M the activity had decreased to about 70% of that observed at the maximum. Based upon these results, the K_m for methylenetetrahydrofolate was measured by varying the concentration of tetrahydrofolate, while keeping the HCHO concentration constant at 7×10^{-3} M. Under these conditions, the K_m values for *dl*,L-methylenetetrahydrofolate were 3.2×10^{-5} M and 1.2×10^{-5} M, respectively, in the presence and absence of Mg^{2+} (10 mM).

Activation and Inhibition Related to Sulfhydryl Groups. Previous investigators (McDougall and Blakley, 1961; Wahba and Friedkin, 1962; Jenny and Greenberg, 1963; Reyes and Heidelberger, 1965; Lorenson *et al.*, 1967; Revelle, 1968) have observed that thymidylate synthetases from various sources are stabilized and their activities stimulated appreciably by thiols. The same general effects were also encountered with the *L. casei* enzyme. Accordingly, mercaptoethanol was present at all stages in the purification and it was also included in the assay systems. Superficially it would appear as if the thiol were protecting a sensitive sulfhydryl group(s) on the protein but the function of these agents is complex, as shown by the following experiments.

In order to test the effect of added thiols or inhibitors, it was necessary to use enzyme from which exogenous thiols had been removed. This could be accomplished by exhaustive dialysis against 0.1 M potassium phosphate buffer (pH 6.8). Using the criteria described below, the same results were obtained when the enzyme was dialyzed: (a) under argon; (b) under oxygen; or (c) in the presence of 5 mM arsenite. When the thiol-free enzyme, designated E_p, was tested in the *absence* of thiols in the spectrophotometric assay system (Figure 13), a linear response was seen for several minutes, but thereafter the rate of the reaction declined markedly. However, addition of mercaptoethanol (final concentration 0.025 M) caused the original linear rate to be reestablished within 2 or 3 min.

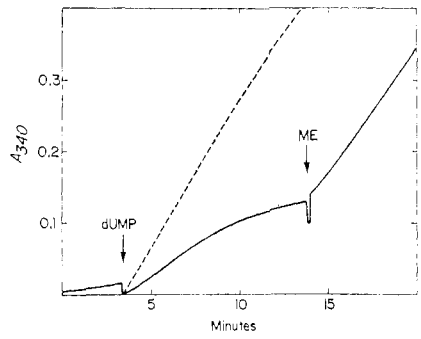

FIGURE 13: Effect of mercaptoethanol on thiol-free thymidylate synthetase (E_p). Spectrophotometric assay with mercaptoethanol omitted from methylenetetrahydrofolate solution. At time 0, the cuvet contained enzyme (32 μg) and normal assay ingredients with the exception of mercaptoethanol and dUMP. At the indicated times, dUMP (0.2 μmole) and 25 μl of 1 M mercaptoethanol were added (continuous line). The dashed line represents the rate obtained using the same amount of enzyme in the standard assay. The enzyme from step 6 was exhaustively dialyzed against argon-bubbled 0.1 M KPO_4, pH 6.8, prior to use.

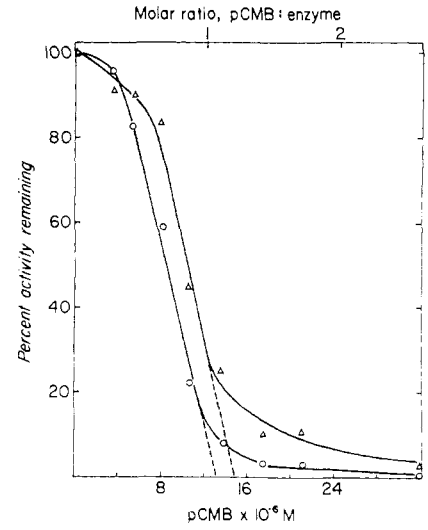

FIGURE 15: Effect of *p*-MB on activity of thymidylate synthetase. Spectrophotometric (○) and tritium release (△) assays were performed in the absence of added thiols following incubation for 10 min at room temperature with the indicated concentrations of *p*-MB. Enzyme concentration, 1.23×10^{-5} M in each case.

The effect of various thiols upon the activity of E_p is shown in Figure 14. Maximum stimulation was about 3-fold, leading to an activated state, designated E_a. It should be noted that specific activities given in Table II were obtained with this form of the enzyme. The shape of the activity–concentration curves varied, however, for the different thiols. Mercaptoethanol, glutathione, and DTE behaved similarly, while cysteine activated maximally at lower concentrations and dihydrolipoate failed to achieve the same degree of activation as the other thiols. It should be noted that maximum activation requires thiol concentrations that are several orders of magnitude larger than that of the enzyme.

The above results suggested that the *L. casei* thymidylate synthetase contains one or more readily oxidizable sulfhydryl groups that serve in a structural or functional role. This hypothesis was further investigated by testing the sensitivity of the enzyme to various inhibitors. The latter were preincubated with the E_p form of thymidylate synthetase for 15 min at 37° in 0.1 M potassium phosphate (pH 7.5) prior to the assay. Because of interference with the spectrophotometric assay, the following inhibitors were tested using the tritium release assay: iodoacetamide, iodoacetate, *N*-ethylmaleimide, DTNB, and cyanide. At 6 mM, iodoacetate, iodoacetamide, *N*-ethylmaleimide, and *p*-MB completely inhibited the enzyme. The enzyme was also inhibited completely by cyanide (6 mM), tetrathionate (5 mM), H_2O_2 (1 mM), and by certain disulfides, notably DTNB (6 mM) and 2-hydroxyethyl disulfide (4×10^{-3} mM). Oxidized glutathione, however, shows no inhibitory effect, even at 10 mM. Inhibition by the disulfide of mercaptoethanol offers an explanation for the loss of activity that is observed when enzyme preparations are purified or stored with aged buffers containing mercaptoethanol.

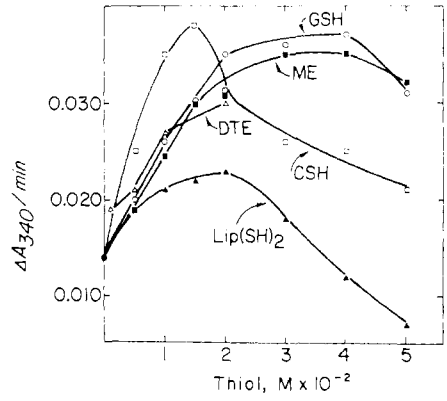

FIGURE 14: Effect of thiols on activity of thymidylate synthetase (E_p). For details, see Experimental Section and legend for Figure 13. Enzyme (30 μg) was incubated with the thiols for 1 min prior to addition of dUMP. ΔA_{340}/min taken from the initial linear portion of the reaction trace.

Titration of the E_p form of the enzyme with *p*-MB (Boyer, 1954) indicates that 3 to 4 sulfhydryl groups per molecule are exposed at pH 7 (in the absence of urea). In order to assess the importance of these groups to catalytic activity, the latter was measured as a function of added mercurial (Figure 15). Complete loss of activity occurs approximately at an equimolar ratio of mercurial to enzyme. Under these conditions (*i.e.*, when only one sulfhydryl group of the protein has reacted with *p*-MB), treatment with mercaptoethanol completely reactivates the enzyme. The fact that the tritium release assay gives results identical with the spectrophotometric assay suggests that a sulfhydryl group on the protein is involved in the mechanism by which hydrogen is labilized at C-5 of the pyrimidine.

The operational relationship between E_a and E_p, and inactive (but not denatured) form(s) of the enzyme, designated collectively as E_i, is shown in Figure 16. It should be noted

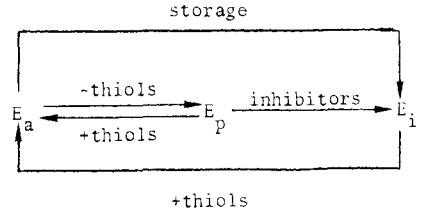

FIGURE 16: Interrelationship of different catalytic states of thymidylate synthetase.

that all three forms of the enzyme behave similarly upon polyacrylamide electrophoresis or gel filtration and that, at present, they can be differentiated only on the basis of activity.

Acknowledgments

The authors are indebted to Dr. I. Crawford, Miss V. Gage, and Mr. William Lee for their advice in the microbiological aspects of this problem and to Miss D. Frazier and Messrs. R. Barrett and J. Etchison for expert technical assistance. Drs. V. deSaussure and D. Jacobsen kindly carried out the ultracentrifugal analyses and SDS polyacrylamide gel electrophoreses, respectively.

References

Blakley, R. L. (1963), *J. Biol. Chem. 238*, 2113.
Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.
Crusberg, T. C., and Kisliuk, R. L. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 473.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Fridland, A., and Heidelberger, C. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 878.
Friedkin, M. (1959), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 18*, 230.
Friedkin, M., Crawford, E. J., Donovan, E., and Pastore, E. J. (1962), *J. Biol. Chem. 237*, 3811.
Galivan, J., Murphy, S., and Jacobsen, D. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 334.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. and Singh, R. M. M. (1966), *Biochemistry 5*, 467.
Gornall, A. G., Bardawill, C. S., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Greenberg, G. R., Somerville, R. L., and DeWolf, S. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 242.
Harding, N. G. L. (1966), Ph.D. Thesis, Oxford University.
Hartmann, K. U., and Heidelberger, C. (1961), *J. Biol. Chem. 236*, 3006.
Hatefi, Y., Talbert, P., Osborn, M. J., and Huennekens, F. M. (1960), *Biochem. Prepn. 7*, 89.
Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.
Huennekens, F. M. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Interscience, p 87.
Humphreys, G. K., and Greenberg, D. M. (1958), *Arch. Biochem. Biophys. 78*, 275.
Jenny, E., and Greenberg, D. M. (1963), *J. Biol. Chem. 238*, 3378.
Labow, R., Maley, G. F., and Maley, F. (1969), *Cancer. Res. 29*, 366.
Leary, R., Crusberg, T. C., and Kisliuk, R. L. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 339.
Lomax, M. I. S., and Greenberg, G. R. (1967), *J. Biol. Chem. 242*, 109.
Lorenson, M. G., Maley, G. F., and Maley, F. (1967), *J. Biol. Chem. 242*, 3332.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McDougall, B. M., and Blakley, R. L. (1960), *Biochim. Biophys. Acta 39*, 176.
McDougall, B. M., and Blakley, R. L. (1961), *J. Biol. Chem. 236*, 832.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Revelle, P. (1968), Ph.D. Thesis, Cornell University, Ithaca, N. Y.
Reyes, P., and Heidelberger, C. (1965), *Mol. Pharmacol. 1*, 14.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, London, Academic Press.
Silber, R., Gabrio, B. W., and Huennekens, F. M. (1963), *J. Clin. Invest. 42*, 1913.
Wahba, A. J., and Friedkin, M. (1961), *J. Biol. Chem. 236*, PC11.
Wahba, A. J., and Friedkin, M. (1962), *J. Biol. Chem. 237*, 3794.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.
Zwaan, J. (1967), *Anal. Biochem. 21*, 155.