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Dihydrofolate Reductase. Purification and Characterization of the Enzyme from an Amethopterin-Resistant Mutant of *Escherichia coli*†

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ABSTRACT: A convenient large-scale procedure for the isolation of dihydrofolate reductase from *Escherichia coli* B (strain MB 1428) based upon batchwise affinity chromatography is described. The purified enzyme exhibits an activity per milligram of protein 1400-fold enhanced over the crude cell lysate. The purified enzyme has been found to be homogeneous by the following criteria: (1) polyacrylamide gel electrophoresis, (2) cellulose acetate strip electrophoresis, (3) sedimentation velocity, (4) sedimentation equilibrium, (5) end-group analysis, (6) cyanogen bromide cleavage, and (7) amino acid analysis. The enzyme exhibits a most probable amino acid composition of 152 residues with a calculated molecular weight of 16,810. The molecular weight calculated from equilibrium edimentation data is 17,300 with an assumed partial specific volume of 0.69 cm^3/g. The enzyme contains two half-cystine, four tryptophan, five histidine, and four methionine residues per molecule. Cyanogen bromide cleavage of the enzyme gives five peptides. The enzyme exhibits a turnover number of 600 ± 50 moles of FAH_2 reduced per min per mole of methotrexate binding sites. The pH-activity profile of the purified enzyme exhibits a broad maximum at about pH 6.5 in 0.5 M KCl. There is a biphasic dependence of activity upon ionic strength for NaCl and KCl. The K_M values of NADPH, NADH, and dihydrofolate are 6.45 ± 0.9, 320 ± 30, and 0.44 ± 0.05 μM, respectively. Folic acid is not reduced by the enzyme. NADPH is 5.1 times more rapidly oxidized than NADH.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) which catalyzes the NADPH-dependent reduction of dihydrofolate holds considerable pharmacological interest as the target enzyme for a number of chemotherapeutic agents (Hitchings and Burchall, 1965). The enzyme has been partially purified from a number of organisms (reviewed by Huennekens, 1968), and has been purified to essential homogeneity from mouse L 1210 lymphoma (Perkins *et al.*, 1967), *Lactobacillus casei* (Dunlap *et al.*, 1971), T4 bacteriophage (Erickson and Mathews, 1971), chicken liver (Kaufman and Gardiner, 1966), calf thymus (Greenberg *et al.*, 1966), and *Diplococcus pneumoniae* (Sirotnak and Salser, 1971). It has been partially purified from *E. coli* B (Burchall and Chan, 1969; Mathews and Sutherland, 1965). We wish to report here a convenient four-step procedure based on the use of an affinity resin for the large-scale isolation and purification of dihydrofolate reductase as applied to a mutant strain of *E. coli* B, and further to describe a number of the chemical and physical properties of the highly purified enzyme obtained. This strain is methotrexate-resistant and contains a relatively high level of enzyme.

Hitchings and Burchall (1965) have proposed a detailed structure for the active site of dihydrofolate reductase, as determined from analysis of the activity:structure relationships among the inhibitors of the enzyme. We are using the highly purified mutant enzyme described herein to investigate and extend this hypothesis by a number of chemical and physical techniques, including amino acid sequence determination, magnetic resonance spectroscopy, and various optical spectroscopic methods.

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Materials and Methods

Materials. $NADP^+$, NADPH, and NADH were purchased from Calbiochem, folic acid from Cyclo, amethopterin (methotrexate, MTX)[1] from Nutritional Biochemicals Co., Sephadex G-75 and Sepharose 4B from Pharmacia, 2-mercaptoethanol from Aldrich, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Pierce Chemical Co., Rockford, Ill. Methotrexate-*3′,5′-t*, sodium salt, *i.e.*, tritiated in the 3′ and 5′ positions on the phenyl ring moiety, was purchased from Amersham-Searle, Arlington Heights, Ill. All other chemicals utilized were reagent grade.

Dihydrofolate (FAH_2) was prepared from commercial folic acid by the dithionite method of Futterman (1957) as modified by Blakley (1960), and stored at −20° under 5 mM HCl and 50 mM 2-mercaptoethanol. Standard buffers utilized were: buffer A, 0.05 M Tris-HCl (pH 7.2) at 23°–0.05 M KCl–0.01 M 2-mercaptoethanol–0.001 M EDTA; buffer B, 1 M NaCl–0.10 M Tris-HCl (pH 8.0) at 23°–0.01 M 2-mercaptoethanol–0.001 M EDTA.

Preparation of the Affinity Resin. The affinity resin MTX-AE-Sepharose was prepared from AE-Sepharose, which was prepared according to the method of Cuatrecasas (1970). Sepharose 4B (100 g) was activated with cyanogen bromide (25 g), washed, and then reacted with ethylenediamine (30 g) for 17 hr at 4°. Special effort was made for immediate reaction of cyanogen bromide activated Sepharose with ethylenediamine. Unless a freshly opened bottle of cyanogen bromide was used, the white resin picked up a yellow tint during the reaction with ethylenediamine. Next, 500 mg of MTX which was freshly ground by mortar and pestle was dissolved in 100 ml of H_2O at pH 9 and titrated to pH 6 with 1 M HCl. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (2.5 g) was added to the MTX solution. The AE-Sepharose was then washed repeatedly with distilled water and reacted for 20 hr at 23° with the MTX solution. The MTX-AE-Sepharose was transferred to a 2 × 100 cm column and allowed to settle. A solution containing 1 M NaCl was pumped through the affinity resin for 2 weeks at 23° until all unbound MTX was eluted. When tritiated MTX was used to follow the extent of binding, about 50 μmoles of the 1100 μmoles of MTX was found to be bound as MTX-AE-Sepharose; the rest of the MTX was quantitatively accounted for in the column washings.

Enzymatic Assay. Dihydrofolate reductase activity was measured by a spectrophotometric method involving the decrease in absorbance that occurs at 340 nm when NADPH and dihydrofolate (FAH_2) are reacted to form $NADP^+$ and tetrahydrofolate (FAH_4). Assays were performed at room temperature on a Cary 15 spectrophotometer set for synchronous drive on either a 0–0.1 or 0–1.0 OD slide-wire. For assay of crude enzyme preparations having relatively low activity and high absorbance at 340 nm, neutral density filters of either 0.5, 1.0, or 1.5 OD were used in the reference beam to enable the utilization of the 0–0.1 slide-wire. Standard assay conditions included 1.00 ml of buffer A, 0.10 ml of NADPH in buffer A weighed out to be 0.743 mg/ml (1 mM) and 0.01 ml of about 0.01 M FAH_2 in buffer A. Thus the standard assay medium contains 90 μM NADPH and about 90 μm FAH_2. Solutions of both NADPH and FAH_2 were prepared daily and stored at 4°. In assays of crude fractions, FAH_2 was added after a 5-min preincubation of enzyme with NADPH and the activity noted was corrected for FAH_2-independent NADPH oxidase activity. The preincubation was omitted when assaying highly purified enzyme. However, in the MTX titration, preincubation of the inhibitor with the purified enzyme was necessary. One unit of the enzyme is defined as the amount of enzyme which reduces 1 μmole of substrate/min under standard assay conditions (pH 7.2, 23°) using a molar extinction coefficient of 11,650 at 340 nm for the conversion of FAH_2 and NADPH to FAH_4 and $NADP^+$ (Kaufman and Gardiner, 1966). At 37° and pH 7.3, Hillcoat *et al.* (1967) report a molar extinction coefficient of 12,300.

Bacterial Growth and Harvesting Conditions. The mutant strain of *E. coli* selected for these studies was MB 1428 from the Merck Culture Collection, Merck & Co., Inc., Rahway, N. J. This strain will grow vigorously in the presence of 0.001 M MTX. Cells were grown at 37° in 150 gal. (467 l.) fermenters containing 1% dextrose, 0.6% Difco yeast extract, and 0.125 M potassium phosphate (pH 7.0); the pH of the growth medium was maintained above 6.7 by automatic addition of 10 M NaOH. The seed for the fermentation was prepared by inoculating 500 ml of broth from a 3-ml agar slant and incubating with shaking for 16 hr at 37° in a 2-l. shake flask; three 500-ml growths were inoculated into the fermenter using a sterile inoculum transfer procedure.

To determine the optimal harvest time one liter aliquots of the growth medium were taken at equal time intervals, and then assayed for dihydrofolate reductase activity as described above. The absorbance of the aliquots at 660 nm was recorded. The cells were centrifuged (1300*g* for 10 min at 4°), suspended in about 50 ml of 0.1 M Tris-HCl (pH 7.4) and washed three times by centrifugation (10,000*g* for 10 min at 4°), each time being resuspended in about 20 ml of the same buffer. The washed cells were resuspended in 5 ml of the buffer in a $^5/_8 \times 2.5$ in. cellulose nitrate tube (Beckman). This slurry, maintained in an ice bath, was sonicated for a total of 8 min with a Bronwill Biosonik oscillator. The temperature of the slurry which was monitored by a digital thermometer probe (United Systems Corp., Dayton, Ohio) varied between 3 and 10°. The sonicate was centrifuged (10,000*g* for 10 min at 4°), and the supernatant saved and assayed for dihydrofolate reductase activity.

Cell Breakage. In the purification procedures described below, the cells of two 150-gal., 6-hr growths were combined. The cells were harvested in a Sharples continuous-flow centrifuge operated at 15,000 rpm at 4° at a flow rate of 2 ml/min. The cells were suspended in two volumes buffer A and broken at 4° in a French pressure cell operated at 8000 psi (550 atm). The cell debris was removed in the Sharples at 4° at 15,000 rpm with a flow rate of 2 ml/min. The supernatant, which still contained appreciable particulate matter, was stored at 4°. This particulate matter was removed by the addition of 1 mole of NaCl/l., and centrifugation at 13,000*g* at 4° for 20 min or filtration under vacuum through Hyflo Super Cel (Johns Manville) layered over coarse filter paper on a Büchner funnel.

MTX-AE-Sepharose Precipitation and Elution. Binding to and elution from the affinity resin, MTX-AE-Sepharose, was done batchwise. All solutions used were kept at 4°. An aliquot of the filtrate or centrifugal supernatant of the salt-treated crude cell extract (1 l.), which is at pH 7.2, was mixed with 100 g of the affinity resin, prepared as described above, and stirred vigorously but without foaming for 20 min at 4°. The stirred material was poured onto a porcelain Büchner funnel (Coors 5, Ace Scientific, Linden, N. J.), fitted with filter paper (Whatman No. 31, 18.5 cm diameter) previously wetted with 1 M

[1] Nonstandard abbreviations used are: MTX, methotrexate or amethopterin; MTX-AE-Sepharose, the affinity resin methotrexate-aminoethyl-Sepharose.

NaCl, and filtered under vacuum until a cake formed. The vacuum was turned off and broken, and the cake resuspended with gentle stirring in about 700 ml of 1 M NaCl. This washing procedure was repeated until the filtrate was clear.

The washed filtered cake was then added to 400 ml of buffer B and 40 ml of thawed FAH_2 suspension containing 3.2 mg of FAH_2. After stirring vigorously for 30 min at 4°, the material was filtered under vacuum on a No. 4 porcelain Büchner funnel fitted with filter paper. The cake was then gently resuspended in about 200 ml elution buffer, and refiltered under vacuum. These filtrates were combined and 1 ml of 2-mercaptoethanol was added. The filtrate was then stored at 4°. The MTX-AE-Sepharose cake was washed three times with 500 ml of 1 M NaCl, and then suspended in 200 ml of 1 M NaCl for storage at 4° for reuse.

Filtration on Sephadex G-75. The affinity resin eluates were combined and concentrated by ultrafiltration at 4° (UM-10 membrane, 50 psi) in a 200-ml AMICON ultrafiltration cell equipped with a 2.75-l. reservoir. No significant reductase activity appeared in the ultrafiltrate. Roughly one-thousand units of activity in 1–2 l. of eluates were concentrated to 80 ml, centrifuged at 27,000*g* for 20 min at 4°, and the resulting deep red-brown supernatant pumped onto a 5 × 84 cm column packed with Sephadex G-75, previously equilibrated with buffer A at 4°. A peristaltic pump was used to distribute column eluate from the column into an automatic fraction collector. The column was eluted with buffer A; 650-sec fractions were collected with a volume of 14.5 ml each. Fractions containing reductase activity greater than 1 unit/ml were combined, ultrafiltered as before to 20 ml, and pumped onto a 2.5 × 89 cm column packed with Sephadex G-75 pre-equilibrated with buffer A at 4°. The column was eluted with buffer A, with fractions of 24 min or 9.4 ml, collected, and assayed for reductase activity. Fractions containing greater than 1 unit of activity/ml were combined. 2-Mercaptoethanol (1 ml) and $NADP^+$ (100 μl of 12.5 mg/ml) in buffer A were added to the combined fractions to stabilize the enzyme. The purified enzyme was stored at 4° in a tightly capped bottle; no significant loss of activity over a period of several months is detected under these conditions.

Electrophoresis Procedures. POLYACRYLAMIDE GEL. Varying amounts of purified enzyme (between 10 and 100 μg) were applied at one end of a polyacrylamide gel suspended in 0.2 M Tris–borate buffer (pH 8.9) at room temperature. Either 7.5 or 15% polyacrylamide gels were used, and electrophoresis was carried out for 90 min at 23° at constant current (2.5 mA) on a 5 × 40 mm cylinder in the Polyanalyst disc electrophoresis apparatus, Büchler Instruments Inc., Fort Lee, N. J. The gels were stained with Amido-Schwarz 10B.

CELLULOSE ACETATE STRIP. Electrophoresis was also done on cellulose polyacetate strips, 6.75-in. Sephaphore III, in a Electrophoresis Chamber No. 51170, Gelman Instrument Co., Ann Arbor, Mich. About 10 μg of purified enzyme was spotted on the strip and electrophoresed for 20 min at 4° and at 300 V in 0.1 M potassium phosphate or potassium acetate buffer. Electrophoresis was done at pH 5.0, 5.5, and 6.0 in phosphate buffer, and at pH 4.5, 4.7, and 4.8 in acetate buffer. The protein was stained with Ponceau S.

Ultracentrifugation. Both sedimentation velocity and sedimentation equilibrium measurements were carried out in a Spinco Model E analytical ultracentrifuge. A 2-ml solution of purified enzyme in buffer A containing about 110 μg of protein/ml was dialyzed for 3 hr against 100 ml of buffer A at 4°. The dialysate was utilized in the reference channel of the optical system of the ultracentrifuge. For the sedimentation velocity run at 64,000 rpm and 16°, the sample cell was monitored at 280 nm. For the sedimentation equilibrium run, the same sample and reference were used. Centrifugation proceeded for 20 hr at 20,000 rpm and 16°. After the 20-hr run, the rotor speed was increased to 60,000 rpm for 6 hr and then decelerated to 20,000 rpm to obtain a base-line correction. The sample was monitored at 298 and 310 nm. Calculations were done as described by Schachman (1959).

Amino Acid Analysis. Quantitative amino acid analysis was performed on an automated Spinco amino acid analyzer (Beckman Amino Acid Analyzer Model 120). For each analysis about 10 ml of purified enzyme solution containing roughly 1 mg of protein was dialyzed for 24 hr against distilled water at 4°, and redialyzed *vs.* fresh distilled water for 24 hr at 4°. For acid hydrolysis, the enzyme was lyophilized, and dissolved in 1 ml of 6 N HCl containing 0.01 M phenol. This solution was then placed in a small vial, purged extensively with nitrogen, and the vial sealed. Hydrolysis proceeded 20 hr at 110°. The hydrolysate was analyzed directly. The loss of serine and threonine under these hydrolysis conditions due to degradation, which is 10% for serine and 5% for threonine, is corrected for in the results reported. Cysteine was determined as cysteic acid from performic acid oxidized protein according to the method of Moore (1963). Tryptophan was determined from protein hydrolyzed in 4 M $Ba(OH)_2$. Lyophilized protein was transferred to a 19 × 120 mm Vycor test tube (Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.) and enough $Ba(OH)_2$ added to make 1 ml of solution 4 M in $Ba(OH)_2$. One ml of water was added, the test tube purged extensively with nitrogen, and the tube sealed. Hydrolysis proceeded for 70 hr at 110° in a forced air, hot air oven. After hydrolysis the solution was transferred to a round bottom flask, and the $Ba(OH)_2$ precipitated as $BaCO_3$ by passing CO_2 through the solution. The supernatant was transferred to another round-bottomed flask, as were the $BaCO_3$ washings. The supernatant and washings were concentrated to about 1 ml on a rotary evaporator, and then analyzed for amino acid content. When leucine and tryptophan were subjected to the alkaline hydrolysis procedure, and analyzed, they both exhibited 85% recovery.

Amino-Terminal End-Group Analysis. The amino-terminal residue of the enzyme was identified by the method of Gray (1967) using 0.1 mg of protein in 50 μl of 0.1% sodium dodecyl sulfate in 0.2 M $NaHCO_3$ instead of urea to solubilize the protein. After dansylation, the protein was precipitated with acetone and washed three times with acetone. Both the protein and acetone washes were hydrolyzed and assayed for dansyl-amino acids as described by Gottlieb *et al.* (1970).

Cyanogen bromide cleavage was performed on 0.9 mg of enzyme according to the method of Givol and Porter (1965). The reaction mixture was lyophilized. Thin-layer chromatography (silica gel G coated plates (Analtech) in the system 1-butanol–pyridine–acetic acid–water, 30:20:6:24, v/v) and cellulose acetate electrophoresis (1 mM sodium barbital buffer (pH 8.5), 100 V, 30 min) were performed upon the lyophilized material.

Protein Analysis. Crude cell lysate was assayed for protein content by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Purified enzyme was assayed by absorbance at 280 nm using the extinction coefficients for amino acids given by Mahler and Cordes (1966) and the amino acid content given in Table II.

Dependence of Enzyme Activity upon Substrate Concentration. Assays to determine the K_M values for FAH_2 and NADH and NADPH were run under standard assay conditions, ex-

TABLE I: Purification of Dihydrofolate Reductase from *E. coli* MB 1428.

Material	Vol (ml)	Protein Concn (mg/ml)	Sp Act. (Units/mg)	Purifcn	Total Act. (Units)	Recov
Crude cell lysate	14,200	60	0.026	(1)	21,970	(100)
Affinity resin eluate	11,100				17,000	77.4
First G-75 chromatography	1,600				12,640	57.6
Second G-75 chromatography	1,620	0.15	36.0	1400	9,120	41.5

cept that the concentration of each substrate was varied in turn. NADH and NADPH were standardized spectrometrically using ϵ (340 nm) = 6220 M^{-1} cm^{-1}, and FAH_2 standardized using ϵ (340 nm) = 6500 M^{-1} cm^{-1}, at pH 7.2 and 23°.

Inhibition of Enzyme Activity by MTX. Standard assay conditions were used with 2-μl aliquots of a highly purified enzyme preparation containing about 10 units of activity/ml. The enzyme was mixed with buffer and NADPH and varying amounts of MTX dissolved in buffer A, allowed to stand for 5 min at 23°, and then assayed by adding FAH_2. The number of micromoles of FAH_2 reduced per minute in the sample cuvet was plotted against the number of moles of MTX in the cuvet. This plot was then used to determine a turnover number by the method of McCullough and Bertino (1971). MTX was standardized optically at pH 13 using the following extinction coefficients: ϵ (257 nm) = 23,150 M^{-1} cm^{-1} and ϵ (302 nm) = 22,100 M^{-1} cm^{-1} (Seeger *et al.*, 1949).

Results

Enzyme Isolation and Purification. CELL HARVEST CONDITIONS. The optimal cell harvest time for the mutant *E. coli* strain was determined in the experiment summarized in Figure 1. The cells end their logarithmic phase of growth about 6 hr after inoculation; this is also the time of maximum total dihydrofolate reductase activity in the fermenter. Accordingly, cells were harvested after 6 hr in all subsequent growths.

AFFINITY CHROMATOGRAPHY. About 1 l. of crude extract containing 2000 units of enzyme activity was treated with 100 ml of MTX-AE-Sepharose resin. This represented about 3.0 μmoles of enzyme which is 6% of the number of moles of MTX on the resin. Thus the affinity resin should be capable of binding all the dihydrofolate reductase in the crude extract. Under these conditions 10–20% of the enzyme did not bind. Less than 5% of the activity, however, was removed by the washes with 1 M NaCl. It was found that the use of 1 M NaCl gave a large discrimination between specific enzyme–inhibitor binding and nonspecific binding due to the ion-exchange properties of the resin. High salt also facilitated the elution of the enzyme from the resin when dihydrofolate was added to the elution buffer (buffer B). This buffer quantitatively eluted the enzyme. The value reported in Table I is the total yield of 14 binding and elution steps. Enzyme recovery in the affinity chromatography step, including material in the washes, approached 100%.

SEPHADEX G-75 FILTRATION. A typical elution profile for filtration on Sephadex G-75 for the concentrated and centrifuged affinity resin eluate is graphically presented in Figure 2. The peak centered at fraction 66 in the elution profile corresponded to dihydrofolate reductase. When the fractions containing high activity were combined (fractions 61–75 in this case), concentrated, and rechromatographed on Sephadex G-75, the elution profile obtained is indicated in outline in Figure 3. The peak in Figure 3 centered near fraction 31 corresponded to dihydrofolate reductase. The combined active fractions (28–34 here) correspond to the material adjudged homogeneous by the criteria discussed below.

A representative tabular summary of the yields in the overall procedure for the isolation and purification of dihydrofolate reductase from two 150-gal. growths of *E. coli* MB 1428 is presented in Table I. An overall yield of 41.5% corresponding to about 260 mg of pure protein is obtained.

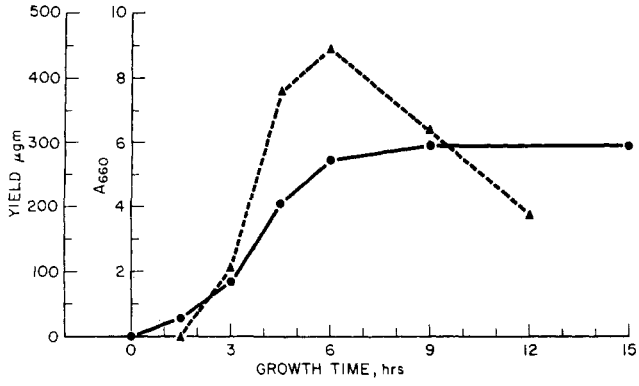

FIGURE 1: Time course of cell growth and dihydrofolate reductase content. Solid line, absorbance at 660 nm; dotted line, amount of enzyme in 1 l. of growth medium.

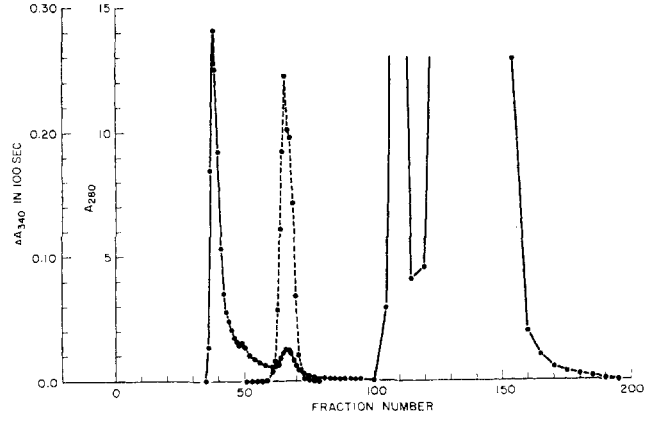

FIGURE 2: Elution profile of concentrated affinity-resin eluate on Sephadex G-75. Solid line, absorbance at 280 nm; dotted line, enzyme activity.

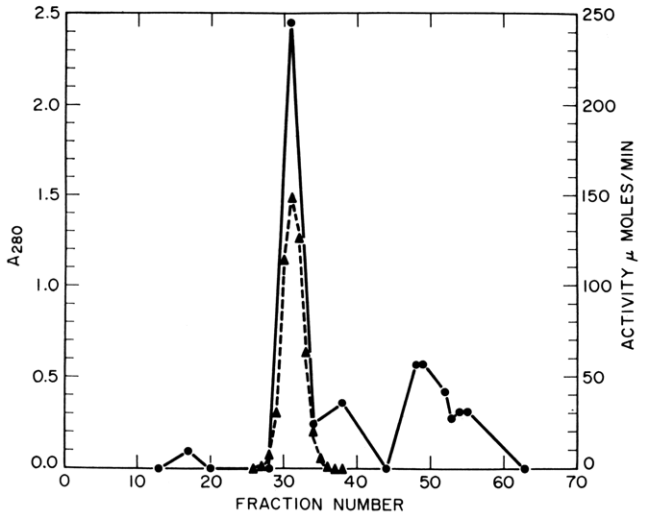

FIGURE 3: Second elution profile of concentrated column eluate (see Figure 2) on Sephadex G-75. Solid line, absorbance at 280 nm; dotted line, enzyme activity.

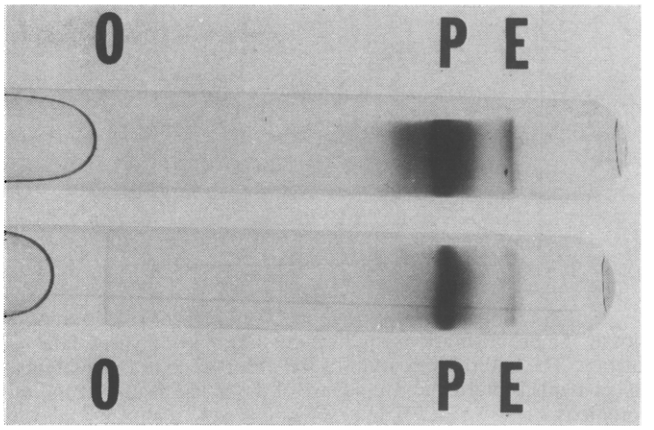

FIGURE 4: Electrophoresis of dihydrofolate reductase on polyacrylamide gel. Protein (50 and 100 μg) was used, respectively, in the lower and upper gels. Gels are suspended in a small test tube containing buffer. The origin and end of the gels are labeled "O" and "E," respectively. Both "O" and "E" were stained after the protein staining to make them more visible.

It seems reasonable that this procedure may be used with only minor alterations in a tenfold scale-up, and with other biological sources of dihydrofolate reductases.

Criteria of Purity. ELECTROPHORETIC MOBILITY. When the highly purified reductase was subjected to electrophoresis on polyacrylamide, a single protein band was observed. An example of the results obtained are the gels photographed for Figure 4. The gels were spotted with 100 and 50 μg of protein, respectively. Electrophoresis was from the origin, labeled "O," toward "E." Both "O" and "E" were stained *after* the protein stain. The only material visualized upon staining with Amido-Schwarz was at "P." The limit of detectability for proteins by the detection method used here was about 10 μg; thus no single proteinaceous impurity was present that amounted to 10% of the amount of reductase present.

Upon electrophoresis of the enzyme on cellulose polyacetate in potassium phosphate buffer at pH 5.0, two resolved but closely spaced, anodically migrating bands were found. Both bands exhibited MTX-inhibitable dihydrofolate reductase activity when redissolved in buffer A. If incubated with NADPH prior to spotting, the reductase exhibited only a single band. When electrophoresis was carried out on the highly purified protein at pH 4.8 in 0.1 M potassium acetate buffer, two bands were observed. One band, which corresponds to the band which disappears upon incubation of enzyme with NADPH prior to electrophoresis, migrates hardly at all. The second band moves a small amount in the cathodic direction. This microheterogeneity in electrophoresis due to the presence or absence of bound cofactor has been noted with dihydrofolate reductase as isolated from *L. casei* (Mell *et al.*, 1968a; Dunlap *et al.*, 1971), and L 1210 lymphoma cells (Harding *et al.*, 1970). The p*I* values, or isoionic pH, were determined by pH variation and were found for both forms of the *E. coli* enzyme to be near 4.8.

AMINO ACID ANALYSIS. Three different samples of the enzyme were subjected to acid hydrolysis; their hydrolysates exhibited reproducible ratios of amino acid contents. The calculated number of moles of amino acid produced per mole of protein hydrolyzed for the three samples is presented in Table II. The number of moles of protein present was calculated by assuming that the protein contained four tyrosine, five histidine, six phenylalanine, and six threonine residues per molecule protein. The reproducibility of the amino acid contents further confirms the purity of the protein preparation. When two samples of the enzyme were oxidized with performic acid, and subjected to acid hydrolysis, they exhibited 1.6 and 2.2 moles of cysteic acid per mole of protein, respectively, when standardized against all the other amino acids except Trp, Tyr, and Met. Two samples of the enzyme were subjected to alkaline hydrolysis, and exhibited 3.7 and 3.8 moles of tryptophan per 11 moles of leucine in the hydrolysate, respectively. The suggested amino acid content of *E. coli* MB 1428 dihydrofolate reductase is given in the fifth column of Table II. From this content and the p*I* of the protein it may be estimated that about 10 of the Glu and Asp residues are present as the respective amides. From this composition, a

TABLE II: Amino Acid Analysis of Dihydrofolate Reductase from *E. coli* B (Strain MB 1428).

Residue	Amino Acid Content of Acid-Hydrolyzed Protein			Suggested Content
Lys	8.07	7.38	7.39	7
His	4.61	4.82	4.91	5
Arg	7.48	9.15	8.30	8
Asp	17.22	18.94	18.29	18
Thr	6.10	6.24	6.19	6
Ser	7.69	9.29	9.18	9
Glu	16.32	16.11	16.13	16
Pro	9.32	9.93	9.46	9
Gly	9.74	10.85	10.54	10
Ala	11.92	13.27	12.66	13
$^1/_2$-Cys	0.49	Trace	0.96	2
Val	10.95	10.50	9.90	10
Met	3.81	3.40	3.47	4
Ile	8.66	9.93	9.42	9
Leu	10.19	11.42	11.39	11
Tyr	4.26	3.90	3.95	4
Phe	6.03	6.03	5.95	6
Trp				4
			Total	152

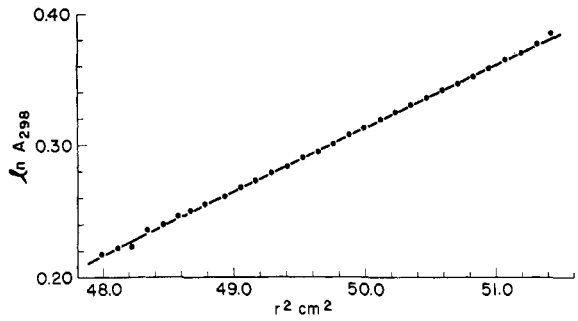

FIGURE 5: Sedimentation equilibrium data for dihydrofolate reductase. The natural logarithm of the absorbance of the analytical cell contents is plotted *vs.* the square of the radial distance from the rotor axis.

molecular weight of 16,810 may be calculated for the protein. The most interesting aspects of the amino acid composition are the presence of two half-cystines and the relatively high content of aromatic residues.

CYANOGEN BROMIDE CLEAVAGE. When subjected to cyanogen bromide cleavage, the enzyme exhibits five new components when chromatographed on silica gel and three new components when subjected to cellulose acetate electrophoresis. These results confirm the presence of four methionine residues per molecule protein, and further confirms the purity of the enzyme preparation.

ULTRACENTRIFUGATION. In the sedimentation velocity run only a single band corresponding to protein was observed, and the band sedimented with a velocity corresponding to 1.8–1.9 S at 64,000 rpm. In the sedimentation equilibrium run the data at 298 nm presented in Figure 5 were obtained. Similar data are obtained when the sample is monitored at 310 nm. If it is assumed that the partial specific volume of the protein is 0.69 cm^3/g and the density of suspending medium is 1.002 g/cm^3, the molecular weight of the protein is calculated to be 17,150 (data at 298 nm) and 17,500 (data at 310 nm) (Schachman, 1959). The presence of only one protein peak in the sedimentation velocity experiment, the linearity of the plot of ln A_{298} *vs.* r^2 (see Figure 5) in the sedimentation equilibrium experiment, and the agreement of the sedimentation equilibrium data at 298 and 310 nm all concur in assigning essential homogeneity to the enzyme preparation.

N-TERMINAL ANALYSIS. When the highly purified dihydrofolate reductase is dansylated and quantitatively analyzed for possible N-terminal amino acids, only a single amino acid residue, either leucine or isoleucine, is observed. No other significant end groups were found, although traces (less than 5% of the total N-terminal amino acids) of dansylaspartate, dansylglutamate, and dansylglycine were found. The acetone washes contained dansylglutamate, possibly derived from the bound FAH_2 often found associated with the purified enzyme, as found from ultraviolet absorption spectra.

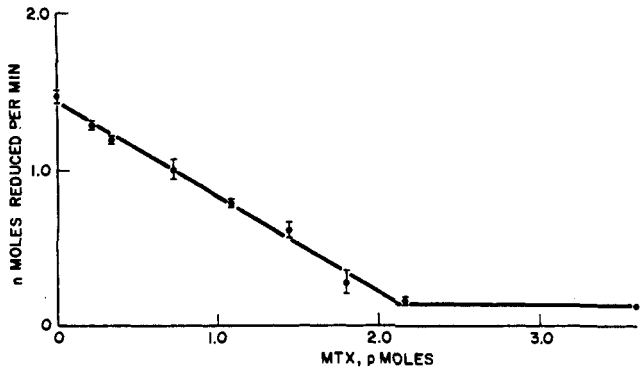

FIGURE 6: Inhibition of dihydrofolate reductase by methotrexate, MTX. Activity, given in nmoles of FAH_2 reduced per min in the sample cuvet, is plotted against number of pmoles of MTX in the cuvet. Enzyme (0.00145 unit) was used in each assay.

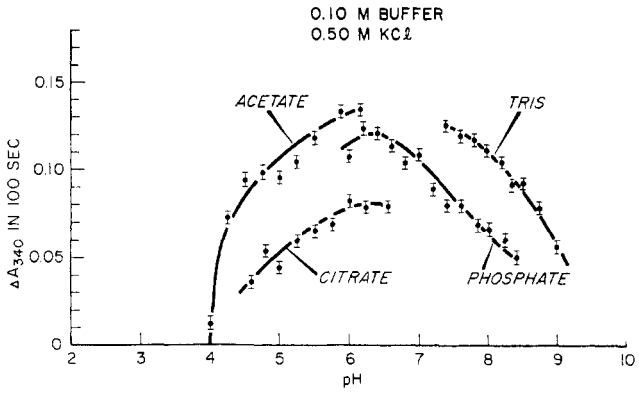

FIGURE 7: Dependence of dihydrofolate reductase activity upon pH at moderate ionic strength for four buffers. Assay medium contains 0.1 M buffer at indicated pH, 0.5 M KCl, 0.001 M NADPH, 0.0001 M FAH_2, and purified enzyme at 23°. Results are corrected for nonenzymatic rate of decline of absorbance, noted near pH 4 (Hillcoat *et al.*, 1967).

Properties of the Enzyme. INHIBITION OF ACTIVITY BY MTX. Despite the ability of *E. coli* MB 1428 to grow vigorously in a medium containing MTX, the purified dihydrofolate reductase from the bacterium was inhibited strongly by MTX at very low levels. This high affinity of MTX for the enzyme allowed its use as a stoichiometric inhibitor. The results of a titration of the enzyme with MTX are presented graphically in Figure 6. From the data presented in Figure 6, it may be calculated that there are 600 ± 50 moles of FAH_2 reduced per min per mole of MTX binding sites on the enzyme. This turnover number is about 5.7 times smaller where the assays are run without preincubation of the MTX with enzyme. If it is assumed that there is one high-affinity binding site for MTX on this reductase, the resulting calculated turnover number of 600 ± 50 moles of FAH_2 reduced per min per mole of protein may be used to convert enzyme activities into protein concentration.

pH–ACTIVITY PROFILE. The dependence of dihydrofolate reductase activity upon pH at moderate ionic strength is portrayed in the data summarized in Figure 7. The buffers utilized were potassium citrate, potassium acetate, Tris-HCl, and potassium phosphate. When the pH dependence of activity is investigated in buffer of low ionic strength, no simple pattern is noted. In 0.03 M Tris–acetate, activity increases about twofold in going from pH 4 to 7, and then stays roughly constant up to pH 9. Thus, the double pH optimum noted with dihydrofolate reductases from mouse liver and spleen (McCullough and Bertino, 1971), calf thymus (Greenberg *et al.*, 1966), and chicken liver (Kaufman and Gardiner, 1966) is not apparent. Moreover, this bacterial dihydrofolate reductase does not appear to reduce folic acid. The rate of enzyme-dependent decrease of absorbance at 340 nm is less than 0.001 as fast with folic acid as with dihydrofolic acid, at both pH 7.2 and 4.4.

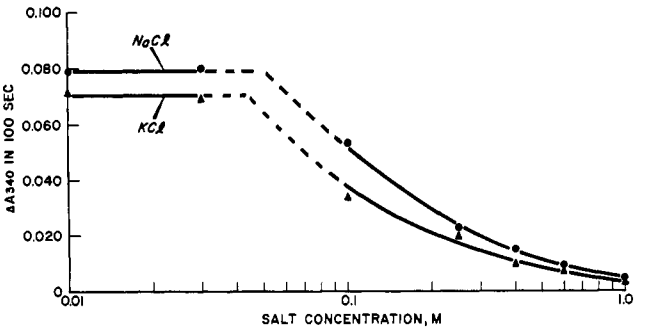

FIGURE 8: Dependence of dihydrofolate reductase activity upon salt concentration. Assay medium contains salt at indicated concentration, 0.03 M Tris–phosphate (pH 7.05), 0.001 M NADPH, and 0.0001 M FAH_2. Dotted lines indicate data interpolation not fully supported by presented data.

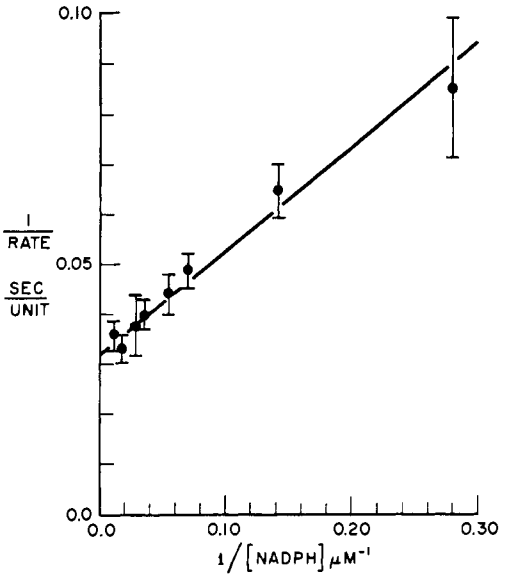

FIGURE 9: Double-reciprocal plot of dependence of dihydrofolate reductase activity upon NADPH concentration.

SALT INHIBITION. There is a simple dependence of enzyme activity upon ionic strength when sodium or potassium chloride is used in the assay medium, as shown in Figure 8. There is an interesting independence of activity up to a salt concentration of about 0.05 M and then a steady decrease with increasing salt concentration. This behavior is as yet unexplained. The inhibition of activity by high-salt concentrations is completely reversible; removal of salt by dialysis restores full activity.

DEPENDENCE OF ENZYME ACTIVITY UPON SUBSTRATE CONCENTRATION. The dependence of the rate of FAH_2 reduction upon the concentration of FAH_2, NADPH, and NADH obeys Michaelis–Menten kinetics at pH 7.2 and 23°. The linear time course of the reduction to substrate concentrations of the order of their K_M values implies that the reaction products, $NADP^+$ or NAD^+ and FAH_4, tetrahydrofolate, are relatively weak inhibitors of forward reaction catalyzed by the enzyme. A plot of the inverse of forward reaction rate *vs.* the reciprocal of the concentration of NADPH added is given in Figure 9. From analysis of slope and intercept, the K_M, or substrate concentration for half-maximal velocity, for NADPH was found to be 6.45 ± 0.9 μM (see Table III). Since the K_M for NADPH is relatively low, a significant fraction of the NADPH present at the lower concentrations assayed was utilized during the period of assay. Care was taken to use the initial rate of NADPH oxidation. This was no problem with NADH, but was a more serious difficulty in the K_M determination for FAH_2. In the K_M determination for FAH_2, the nonlinear portion of the recorder trace of absorbance at 340 nm *vs.* time near substrate exhaustion was utilized. Rates were calculated from the tangents to these curves, and absolute concentration using the extinction coefficient for the conversion of NADPH and FAH_2 to $NADP^+$ and FAH_4. The rate of FAH_2 reduction extrapolated to infinite pyridine nucleotide concentration with NADPH is 5.1 times that with NADH.

Discussion

Prior purification to essential homogeneity of dihydrofolate reductase from a number of sources have produced yields in the milligram range. The convenient large-scale procedure described herein produces essentially homogeneous protein with yields in the gram range, and opens the possibility of performing those detailed physicochemical procedures that require large quantities of material, such as amino acid sequence determination and nuclear magnetic resonance studies. The relatively good yields obtained from the mutant *E. coli* studied suggest that isotopically substituted versions of this enzyme can be made.

The dihydrofolate reductase isolated from strain MB 1428 of *E. coli* B appears to be somewhat different from the same enzyme in wild-type *E. coli* B as studied by Mathews and Sutherland (1965), Burchall and Hitchings (1965), and Burchall and Chan (1969). According to Burchall and Chan (1969), the wild-type enzyme has a molecular weight of 20,500 and K_M values of 10 and 8.4 μM for FAH_2 and NADPH, respectively. This compares to a molecular weight of 16,810 and K_M values of 0.44 and 6.45 μM for FAH_2 and NADPH, respectively, for the MB 1428 enzyme. Further, Burchall and Hitchings (1965) report that the pH of maximal activity is about 7.0 for the wild-type enzyme in comparison to 6.5 for MB 1428, while Mathews and Sutherland (1965) report no maximum in a plot of pH *vs.* activity. Since Mathews and Sutherland (1965) do not fully specify the conditions of their assays, it is difficult to assess the reason for the difference between their results and those of Burchall and Hitchings (1965). However, it is clear for the MB 1428 enzyme that the pH–activity profile depends strongly on the ionic strength of the assay medium. The differences between the various parameters reported for the wild-type enzyme and the MB 1428 enzyme may simply reflect slight differences in the exact experimental conditions used for the measurements, although the 20-fold difference in K_M for FAH_2 seems difficult to reconcile. Thus, the mutant enzyme is probably somewhat different from the wild-type enzyme, but this should be confirmed by analysis of a more highly purified wild-type enzyme.

TABLE III: Substrate K_M Values for *E. coli* MB 1428 Dihydrofolate Reductase at pH 7.2 and 23°.

Substrate	K_M (μM)	Rel V_{max}
FAH_2	0.44 ± 0.05	
NADPH	6.45 ± 0.9	1.00
NADH	320 ± 30	0.20

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A Kinetic Study of the Phospholipase A_2 (*Crotalus adamanteus*) Catalyzed Hydrolysis of 1,2-Dibutyryl-*sn*-glycero-3-phosphorylcholine†

Michael A. Wells

ABSTRACT: A detailed kinetic analysis of the *Crotalus adamanteus* phospholipase A_2 catalyzed hydrolysis of dibutyryllecithin has been carried out. The concentration of dibutyryllecithin chosen insured that the substrate was in the monomeric state. Initial velocity patterns as a function of Ca^{2+} and dibutyryllecithin concentration, as well as inhibition studies with Ba^{2+}, are consistent with an ordered addition of reactants to the enzyme. Ca^{2+} adds first and the K_{iCa} is 4×10^{-5} M. Dibutyryllecithin adds second with a $K_{DBL} = 0.032$ M. Product inhibition studies and dead-end inhibition studies with butryramide are consistent with an ordered release of products. The fatty acid is released first from the enzyme and the lysolecithin is released second. The K_i for lysobutyryllecithin is 0.075 M and for butyramide it is 0.2 M. Evidence is presented that butyric acid inhibits as butyrate at pH 8.0 and must be considered as a dead-end inhibitor. The pH–activity profile of the enzyme shows a maximum at pH 8.0–8.5. K_i for Ca^{2+} is independent of pH in the range of 7.0–9.0. V_m/K_{DBL} is nearly independent of pH in the same range. V_m is, however, pH dependent and evidence is presented that a group with a pK_{app} near 7.6 in the enzyme–substrate complex is involved in catalysis.

Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acid esterified to the number two position of 1,2-diacyl *sn*-phosphoglycerides, and has been used for several years to determine the positional distribution of fatty acids in phospholipids. Several reports have appeared in which various aspects of the enzymatic reaction have been investigated.

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