Bovine Liver Dihydrofolate Reductase: Purification and Properties of the Enzyme†

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ABSTRACT: A purification procedure is reported for obtaining bovine liver dihydrofolate reductase in high yield and amounts of 100–200 mg. A key step in the procedure is the use of an affinity gel prepared by coupling pteroyl-L-lysine to Sepharose. The purified reductase has a specific activity of about 100 units/mg and is homogeneous as judged by analytical ultracentrifugation, polyacrylamide gel electrophoresis, and titration with methotrexate. The products of the first step of Edman degradation indicated a minimum purity of 79%. The reductase has a molecular weight of about 21500 on the basis of amino acid composition and 22100 ± 300 from equilibrium sedimentation. It is not inhibited by antiserum to the *Streptococcus faecium* reductase (isoenzyme 2). Unlike the reductase of many other vertebrate tissues, the bovine enzyme is inhibited by mercurials rather than activated and it has a single pH optimum at both low and high ionic strength. However, the position of the pH optimum is shifted and the activity increased by increasing ionic strength. Automatic Edman degradation has been used to determine 34 of the amino-terminal 37 amino acid residues. Considerable homology exists between this region and the corresponding regions of the reductase from *S. faecium* and from *Escherichia coli*. This strengthens the idea that this region contributes to the structure of the binding site for dihydrofolate.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase EC 1.5.1.3) is a central enzyme in one-carbon metabolism. The enzyme has been isolated from a wide variety of sources and shown to consist of a single polypeptide chain of molecular weight 15000–30000, depending on the source (Blakley, 1969). This small molecular size makes it ideal for detailed structural and mechanistic studies. However, previous studies of the sequence (Bennett, 1974; Gleisner et al., 1974) have been limited to reductase from bacterial sources because of the difficulty in obtaining large quantities of the enzyme from mammalian tissue.

Although a purification procedure for bovine dihydrofolate reductase has been previously reported, only small amounts of enzyme with relatively low specific activity were obtained (Rowe and Russel, 1973). This study reports the development of a purification procedure for obtaining nearly homogeneous dihydrofolate reductase from bovine liver in quantities of 50–100 mg at a time. We also report some of the properties of the purified enzyme.

Experimental Section

Materials

Fresh beef liver was obtained from Wilson Co., Cedar Rapids, Ia. NADPH, folic acid, Sephadex G-75, Sepharose 4B-200, and *p*-chloromercuribenzoate were obtained from Sigma, sequenator reagents (sequenal grade) from Pierce Chemical Co., methotrexate was from Nutritional Biochemicals Corp., ϵ-*tert*-butoxycarbonyllysine *tert*-butyl ester hydrochloride was from Cyclo Chemical, and ethyl mercuribromide was from K and K Laboratories. Dihydrofolate was prepared according to the method of Blakley (1960). Iodoacetate (Pierce) was recrystallized from boiling petroleum ether–ethyl ether (Goren et al., 1968).

Methods

Enzyme Assay. Spectrophotometric assay of bovine liver dihydrofolate reductase was carried out at 37°C with a Cary Model 14 recording spectrophotometer. The standard reaction mixture contained 33 μM dihydrofolate, 50 μM NADPH, 5 mM mercaptoethanol, 0.50 M sodium acetate buffer (pH 6.0), and 0.6 M KCl in a total volume of 3.0 ml. A value of 12300 was used as the molar extinction charge at 349 nm (Hillcoat et al., 1967). One unit of activity was defined as that catalyzing the reaction of 1 μmol of each substrate/min under the experimental conditions.

Protein Determination. Protein concentrations were determined according to the biuret method of Gornall et al. (1949) with bovine serum albumin as the standard, or from amino acid analysis. Before applying the biuret method to most samples, the protein was precipitated with 5% trichloroacetic acid, and the precipitate was washed once with 7% trichloroacetic acid and finally dissolved in 1 N NaOH. If hemoglobin was present it was also necessary to correct the absorbance obtained, by subtracting the absorbance of a sample treated with reagent containing all ingredients except the copper sulfate.

Preparation of the Affinity Gel. Pteroic acid was prepared by the method of Levy and Goldman (1965) with some slight modifications. The organism (ATCC 25301) was grown for 4 days at 30–32°C in 30 l. of medium containing 30 g of folic acid. The suspended material was partly removed by centrifugation at 13700g for 30 min and the remainder by filtration on a Buchner funnel through Hyflo-supercel. The recovered mixture of cells and pteroic acid was extracted with a total of 3 l. of 2% sodium carbonate containing 0.05 N NaOH and 0.5% sodium ascorbate, and

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filtered as before. As soon as possible the extract was adjusted to pH 7 with 5 *N* HCl and kept overnight at 0°C to precipitate pteroic acid. The precipitate was collected by centrifugation and washed with a total of 3 l. of ice-cold water. On readjusting the pH of the supernatant wash liquid to pH 6.5–7 by addition of 7% acetic acid some additional material precipitated and was collected by centrifugation. After drying 16 hr at room temperature in vacuo over P_2O_5, 19.6 g of dry solid was obtained. Paper chromatography showed very little contamination by ultraviolet absorbing or fluorescing materials and purification as carried out by Houlihan et al. (1972) was unnecessary.

Pteroic acid was converted to the 2-acetyl-10-trifluoroacetyl derivative by the method of DeGraw et al. (1965) with the modification that the acetylation was carried out by refluxing with acetic anhydride for 30 min. The 2-acetyl-10-trifluoroacetylpteroic acid was condensed with ϵ-*tert*-butoxycarbonyllysine *tert*-butyl ester hydrochloride by the method of Plante et al. (1967; see also Pastore et al., 1974) with the modification that material eluted from DEAE-cellulose was evaporated and worked up with water rather than with ether. From 9.6 g of pteroic acid, 8.9 mmol (3.9 g) of pteroyllysine was obtained.

Preparation of activated Sepharose 4B-200 was carried out according to the procedure for obtaining high activity gel of Porath et al. (1973). To 770 g of washed, activated gel was added a solution of 2.38 mmol of pteroyllysine (vol. 450 ml) which had been adjusted to pH 10.4. After the mixture was stirred overnight at 4° the gel was filtered and washed successively with 2 l. of 0.1 *N* NaOH, 2 l. of water, 2 l. of 0.1 *N* NaOH, and water until neutral. The absorption spectra of the filtrates indicated a coupling efficiency of 86% based on the total pterin. However, the spectra indicated that some of the uncoupled material was unconjugated pterin, which does not combine with the activated gel.

Purification of Bovine Dihydrofolate Reductase. All steps were carried out at 4°C unless otherwise indicated.

(1) Preparation of the Liver Homogenate. A quantity of 16–20 kg of fresh beef liver was used in a single preparation. A 1-gal Waring Blendor was chilled before use and 1500-g portions of liver were homogenized for 1 min at a medium speed setting in 2 l. of 0.09 *M* sodium acetate buffer (pH 4.42) containing 1.8 m*M* EDTA and 0.02% sodium azide. The homogenate was centrifuged for 20 min at 9500*g* in a Sorval GS3 rotor. The supernatant solution was adjusted to pH 5.2, if necessary, by addition of 5 *N* acetic acid, and freed of lipid particles by pouring through glass wool. A sample was kept for activity and protein determination and a neutral solution of disodium folate was added to the remainder to bring the final folate concentration to 5 m*M*.

(2) Heat Treatment. The heating of the extract was carried out by pumping the extract through a stainless steel coil (0.07 × 5.5 m) immersed in a 53°C water bath and then through a second stainless steel coil (0.07 × 3 m) immersed in ice-water, at a flow rate of 150–200 ml/min. This resulted in the extract reaching 49–51°C at the outlet of the heating coil, and cooling to less than 10°C at the outlet of the cooling coil. The heat-treated extract was then centrifuged at 9500*g* for 20 min. The precipitate was discarded and the pH of the supernatant solution was readjusted to 5.2 if necessary.

(3) Ammonium Sulfate Precipitation. Sufficient 0.25 *M* EDTA (pH 7.0) was added to the supernatant to bring the final EDTA concentration to 10 m*M*. Solid ammonium sulfate (277 g/l.) was added to the supernatant over a 30-min period. The mixture was stirred for 30 min after all the sulfate had dissolved and then centrifuged at 9500*g* for 20 min. The precipitate was discarded and solid ammonium sulfate (210 g/l.) was added to the supernatant solution. The mixture was again stirred for 30 min after the crystals had dissolved, before centrifuging at 9500*g*. The supernatant solution was discarded and the precipitate suspended in a minimum volume (about 350 ml) of 0.05 *M* sodium acetate buffer (pH 6.0) containing 10 m*M* EDTA and 0.02% sodium azide. The suspension was dialyzed for a total of 16 hr against 20 l. of this same buffer, the buffer being replaced after 2 and 4 hr. A small amount of precipitate was removed from the dialyzed solution by centrifugation.

(4) Gel Filtration on Sephadex G-75. The dialyzed solution (2–3 l.) was divided into halves which were applied respectively to two G-75 Sephadex columns (16 × 128 cm, 1–1.5 l./column) which had been equilibrated with 0.05 *M* sodium acetate buffer (pH 6.0) containing 10 m*M* EDTA and 0.2% sodium azide. Elution was carried out with the same buffer and fractions of approximately 300 ml were collected. Fractions containing activity of 0.1 unit or more per ml were pooled.

(5) Affinity Chromatography. The pooled enzyme solution from the two Sephadex G-75 columns (total volume 9–10 l.) was applied to a column of pteroyllysine-Sepharose (6 × 44 cm) at a rate of 200–250 ml/hr. The column was then washed: first with 4 l. of 0.1 *M* Tris-HCl buffer (pH 8.5) containing 0.02% sodium azide, secondly, with 4 l. of 0.5 *M* Tris-phosphate buffer (pH 8.5) containing 0.02% sodium azide; and thirdly, with 0.1 *M* Tris-HCl buffer (pH 8.5) containing 0.02% sodium azide, until the absorbance at 280 nm had fallen to below 0.005 (about 14 l.). The dihydrofolate reductase was then eluted with a gradient of sodium folate in 0.1 *M* Tris-HCl buffer (pH 8.5) containing 0.02% sodium azide. The mixing chamber contained 4 l. of buffer and the reservoir 4 l. of 2 m*M* folate in the buffer.

(6) Concentration and Removal of Folate. The enzyme eluted from the affinity column was concentrated in an Amicon ultrafiltration cell fitted with a UM 10 membrane. The folate was removed either by passage of the enzyme solution through a Sephadex G-25 column at pH 9.5 in dilute ammonium hydroxide, or by passage at pH 6 to 8.5 through a Dowex 1-X2 chloride column (1 ml bed volume per 50 ml of enzyme solution).

Amino Acid Analysis. Protein samples were hydrolyzed in sealed evacuated tubes containing 1.0 ml of constant boiling HCl for 24, 48, 80, and 120 hr, at 110°C. Amino acid analyses were then performed on a JEOL JLC-6AH automatic amino acid analyzer. Tryptophan was determined by amino acid analysis after alkaline hydrolysis according to the method of Hugli and Moore (1972). Performic acid oxidation was performed according to the method of Hirs (1967a). Reduction and carboxymethylation was performed according to the method of Hirs (1967b). Free sulfhydryl groups were determined by the method of Ellman (1959) as follows. The protein (about 40 nmol) in 1.1 ml of 0.18 *M* Tris-phosphate buffer (pH 8.1) containing 1.1% sodium dodecyl sulfate was treated with 0.1 ml of 10 m*M* 5,5′-dithiobis(2-nitrobenzoic acid) in 0.05 *M* Tris-phosphate buffer (pH 8.1). The absorbance at 412 nm was measured in cells with 1-cm light path, 4-mm width, in a Cary 14 recording spectrophotometer when color development was a maximum (about 30 min after reagent addition).

Table I: Purification of Bovine Liver Dihydrofolate Reductase.[a]

Purification Step	Volume (ml)	Protein Concn (mg/ml)	Activity		Yield (%)
			Concn (units/ml)	Specific (units/mg)	
1. Homogenate supernatant	21250	25.2	1.17	0.046	100
2. Heat	19400	11.8	0.55	0.047	43
3. Ammonium sulfate	3120	41.7	3.07	0.074	39
4. G-75 Sephadex	9850	2.66	1.08	0.41	43
5. Affinity column	1900	0.0404[b]	4.12	102	31
6. Ultrafiltration	221	0.338[b]	34.8	103	31

[a] Weight of liver used in this preparation was 18.8 kg. [b] Determined from amino acid analysis on a hydrolysate, assuming the composition shown in Table II, and a molecular weight of 21500.

Sequence Determination. The Edman procedure was applied to the enzyme by the use of a JEOL JAS 47-K automatic sequence analyzer as previously described (Gleisner et al., 1975).

Preparation of Antiserum. Antiserum against the "mutant" dihydrofolate reductase (isoenzyme 2) from *Streptococcus faecium* var *Durans* strain A (Nixon and Blakley, 1968) was prepared by injection of 5 mg of enzyme in 1:1 saline–Freund's complete adjuvant into the hind feet of a rabbit. A second injection of 5 mg of enzyme in saline was given after 3 weeks. The rabbit was bled after 10 additional days. Serum was collected after the blood was allowed to clot for 2 hr at room temperature.

Molecular Weight Determination. Sedimentation equilibrium experiments were performed at both high speed (Yphantis, 1964) and low speed (Richards et al., 1968). The data were obtained at 20°C in a Beckman Model E Analytical ultracentrifuge equipped with a RTIC unit and interference optics. An epon-charcoal double sector centerpiece or a capilliary type, synthetic boundary, epon-aluminum double sector centerpiece was used. Each was fitted with sapphire windows and all runs were performed in an AN-H rotor. Fluorochemical FC-43 was omitted since precipitation of dihydrofolate reductase occurred in its presence. Interference patterns were photographed on Kodak Type 11-G spectroscopic plates and fringe displacements read from the plates with a Nikon microcomparator Model 6C.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in the triethanolamine *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer system of Orr et al. (1972). Staining for reductase activity was carried out by incubating the gel at 5°C for 30–60 min in a reaction mixture containing 0.3 m*M* NADPH, 0.2 m*M* dihydrofolate, 0.16 m*M* thiazolyl blue (Schwarz/Mann), 0.05 *M* sodium acetate buffer (pH 6) and 0.6 *M* KCl.

Results

Purification of the Enzyme. Table I summarizes the data for a typical purification of dihydrofolate reductase from 18.8 kg of bovine liver.

During homogenization the pH reaches 5.2 and as a result there is considerably more precipitation and better packing of the precipitate during centrifugation than when homogenization is carried out in neutral buffer. A clear, deep red supernatant solution is obtained. The folate, which is added to this solution to stabilize the enzyme during subsequent steps of the purification, causes marked (but reversible) inhibition of the enzyme, so that the apparent activity values for the heated extract (step 2) and even for the ammonium sulfate fractionated enzyme (step 3) are lower than the true values.

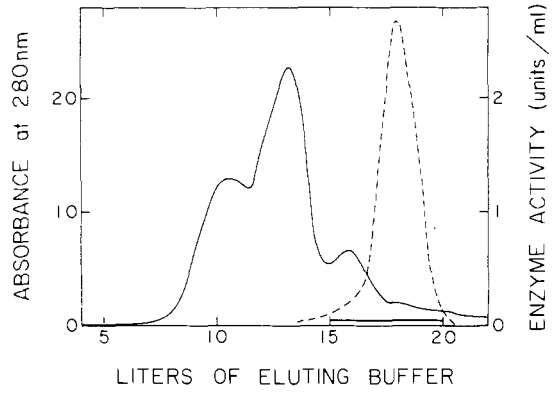

FIGURE 1: Purification of bovine liver dihydrofolate reductase by gel filtration on Sephadex G-75. The sample applied was one-half of that obtained at step 3 in the preparation described in Table I (volume 1560 ml). Fractions indicated by the bar were pooled. Other details are given in the Experimental Section.

The heat and ammonium sulfate steps each remove about 50% of the protein present at the preceding stage and the latter step serves to reduce the volume sufficiently (to about 2–3 l.) for application to the G-75 Sephadex columns. Gel filtration (Figure 1) gives about sixfold purification and is essential for proper functioning of the affinity column subsequently. Attempts to eliminate this step failed, the enzyme subsequently binding poorly to the affinity column because of the presence of large amounts of extraneous protein.

When the pooled G-75 Sephadex fractions were applied directly to the affinity column at pH 6.0 the enzyme bound strongly, and was not subsequently eluted by high concentrations of salt (up to 2 *M* sodium acetate (pH 6.0)) or by increasing the pH to 8.5 with 0.1 *M* Tris-HCl as buffer. These steps do, however, remove extraneous protein (Figure 2). Tris-phosphate buffer, 0.5 *M*, at pH 8.5, elutes a small amount of activity and a large amount of extraneous protein. The column was therefore washed with this buffer and then with 0.1 *M* Tris-HCl (pH 8.5) until protein elution (detected by absorbance at 280 nm) had ceased. Enzyme was then eluted by a folate gradient. It will be noted that the enzyme activity profile showed a small peak ahead of the main peak. Since this extra peak was not always observed, and even in the best preparations was poorly separated, the material in it has not been obtained in adequate amounts for characterization as yet. It is conceivable that it is due to a buffer complex of the type reported for reductase in mammalian cells in culture by Gauldie et al. (1973) or to

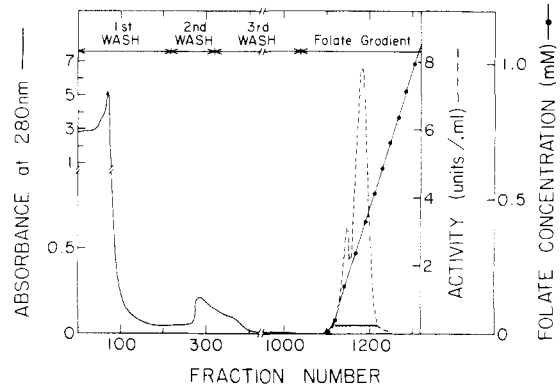

FIGURE 2: Purification of bovine liver dihydrofolate reductase by affinity chromatography. The combined fractions from the two G-75 Sephadex columns (described in step 4 of Table I) were applied. Collection in fractions (19 ml) was commenced after loading was complete. During loading, the absorbance at 280 nm was about 3. Folate concentration refers to the concentration in the effluent as measured by the absorbance at 346 nm. Other details are given in the text. Fractions indicated by the bar were pooled.

an $NADP^+$ complex of the kind observed by Gunderson et al. (1972) and by Harding et al. (1970).

Since the enzyme from the affinity chromatography was very dilute (0.1–0.15 mg/ml), this solution was concentrated about tenfold by ultrafiltration. No significant loss of activity occurred during this step. The solution thus obtained, containing folate and azide, could be stored for several months at 4°C without loss of activity. However, after removal of folate the preparation lost its activity in a few days. At earlier steps in the purification procedure the enzyme was also less stable, particularly after the gel filtration of Sephadex G-75 when no folate is present.

After use, the affinity column was stripped by washing at room temperature with 1 l. of 8 *M* urea, and then 1 l. of water. After returning to the cold room it was prepared for use by passage of 4 l. of 0.05 *M* sodium acetate buffer (pH 6.0) containing 10 m*M* EDTA and 0.02% sodium azide.

Amino Acid Composition. The composition shown in Table II after converting to closest integers and assuming about 20 of the acid residues are present as amides, corresponds to a molecular weight of about 21500. When compared to the *S. faecium* isoenzyme 2, the sequence of which we have previously reported (Gleisner et al., 1974), there are about 19 additional residues in the bovine enzyme, the greatest increases being in proline (14 compared with 6) and valine (17 compared with 9). The fact that there are only two residues each of His, Trp, and Cys makes this an interesting enzyme in which to study the role of these residues in the catalytic site.

Amino-Terminal Sequence. Approximately 0.5 μmol of enzyme was reduced, carboxymethylated, and subjected to 40 steps of automatic Edman degradation. A total of 34 of the first 37 residues were identified, although it is uncertain whether residue 11 is serine or cysteine.[1] The sequence obtained and its homology with sequences in reductase from other sources is discussed later.

Analytical Ultracentrifugation. The enzyme preparation was monodisperse in all measurements in the ultracentrifuge. Only one symmetrical peak was seen by schlieren optics as the boundary traversed the length of the cell, and linear plots of the logarithm of fringe displacement vs. r^2 were obtained in all runs both at low and at high speed. For calculations of the molecular weight the partial specific volume was calculated from the amino acid composition given in Table II. A value of 0.737 was obtained. Four estimates of the molecular weight ranged from 21257 ± 448 to 22937 ± 261 with a mean of 22078 ± 303. The protein concentration was 0.4 mg/ml in three determinations and 0.76 mg/ml in the fourth. The value obtained is in reasonable agreement with that calculated from the amino acid composition.

Table II: Amino Acid Composition of Bovine Liver Dihydrofolate Reductase.

Residue	Mole Ratio[a]	Residue	Mole Ratio[a]
Tryptophan	2.1	Glycine	11.9
Lysine	17.1	Alanine	7.7
Histidine	2.0	Half-cystine	1.6[b]
Arginine	8.5	Valine	17.0
Aspartic acid	20.2	Methionine	4.2
Threonine	6.0	Isoleucine	9.0
Serine	10.1	Leucine	14.4
Glutamic acid	24.0	Tyrosine	6.8
Proline	14.3	Phenylalanine	10

[a] Based on 10 phenylalanine. [b] Determined as cysteic acid.

Polyacrylamide Gel Electrophoresis. When enzyme samples of 20–200 μg were electrophoresed the gels all showed only a single band on staining with naphthol blue and specific staining for reductase activity on similar gels showed that the only detectable activity was associated with the same protein.

Oxidation State of Cystine Residues. Determination of the number of cysteine residues by the action of Ellman's reagent in the presence of 1% sodium dodecyl sulfate on a sample that had been freed of folate gave a value of 1.1 cysteine residues/molecule. It is possible that this rather low value (cf. 1.6 cysteic acid found) is due to partial oxidation. However, when the preparation was treated with 0.1 *M* mercaptoethanol at pH 8, first for 1 hr at 20° and then for 1 hr at 37° no increase in activity occurred, so that if the preparation had been partially oxidized this did not seem to be responsible for activity loss.

Treatment of another sample of enzyme with Ellman's reagent and sodium dodecyl sulfate without removal of folate from the preparation gave a value of 1.4 cysteine residues per molecule.

In the absence of denaturants the cysteine did not react with either Ellman's reagent or iodoacetate so that these residues must be relatively inaccessible to these reagents.

Effect of Antiserum to S. faecium Dihydrofolate Reductase. The effect of antibody to *S. faecium* reductase isoenzyme 2 on the activity of *S. faecium* reductase isoenzymes 1 and 2 and on bovine liver reductase is shown in Figure 3. Whereas the activity of isoenzyme 2 of *S. faecium* was markedly inhibited by quite small volumes of serum, there was only slight inhibition of isoenzyme 1 by much larger volumes of serum and no inhibition of bovine liver reductase at all, indicating significant differences in structure between the three enzymes.

Effect of pH and Ionic Strength on Activity. Figure 4 shows the effect of pH and ionic strength on the activity of the bovine liver enzyme. In buffers of low ionic strength (0.05 *M* sodium acetate) the enzyme exhibits a single pH

[1] Methods of identifying residues were as previously used (Gleisner et al., 1975): the yield at each step has been examined by the referees and is available from the authors on request.

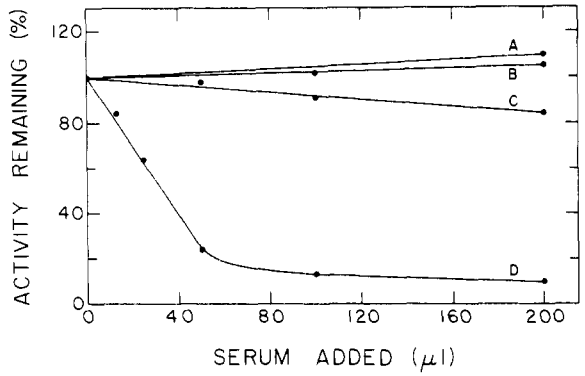

FIGURE 3: The effect of rabbit antiserum prepared against *S. faecium* dihydrofolate reductase isoenzyme 2 on the activity of reductase isoenzymes 1 and 2 from *S. faecium* and bovine liver reductase. Each enzyme sample (0.01 unit of activity in 0.01 ml) was incubated at room temperature for 20 min in 0.2 ml of various dilutions of antiserum or of normal serum. Dilutions were performed with 0.05 *M* potassium phosphate buffer (pH 7.0). From the incubated samples a 0.1-ml portion was used to assay for enzyme activity. (A) Bovine liver reductase treated with antiserum; (B) *S. faecium* isoenzyme 2 treated with normal rabbit serum; (C) *S. faecium* isoenzyme 1 treated with antiserum; (D) *S. faecium* isoenzyme 2 treated with antiserum.

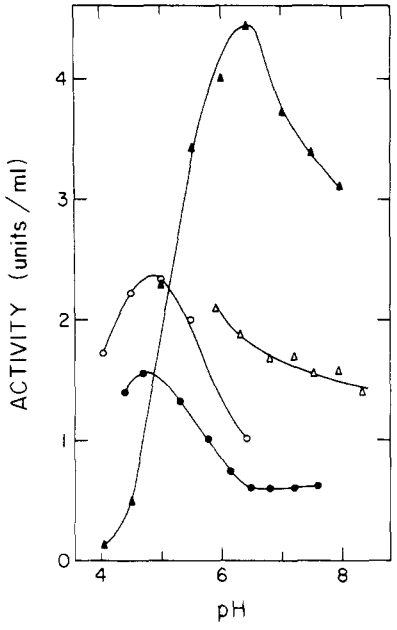

FIGURE 4: The effect of pH and ionic strength on the activity of bovine liver dihydrofolate reductase. Assay mixtures contained 0.05 *M* buffer, 50 μ*M* NADPH, 33 μ*M* dihydrofolate, and (where indicated) 0.6 *M* KCl in a total volume of 3.0 ml. (○) Sodium acetate buffer; (△) potassium phosphate buffer; (▲) KCl and sodium acetate buffer (pH 4–6) or potassium phosphate buffer (pH 6–8); (●) citrate buffer (citric acid adjusted to pH with disodium phosphate).

optimum between 4.8 and 5.0. This optimum is shifted to pH 6.2 in the presence of 0.6 *M* KCl, and in addition the activity is increased three- to four-fold under the latter conditions. These results are consistent with those reported by Rowe and Russel (1973). However, contrary to the observations of the latter authors, a maximum of activity was observed at only a single pH in solutions of low ionic strength. This distinguishes the bovine liver enzyme from the reductase of a number of other vertebrate tissues (Blakley, 1969).

Effects of Urea and Mercurials on the Enzyme Activity. *p*-Chloromercuribenzoate, mercuric chloride, and ethyl mercuribromide at a concentration of 0.1 m*M* were found to inhibit bovine liver dihydrofolate reductase, the extent of inhibition being 85, 100, and 71%, respectively. This inhibition could not be reversed by the addition of 10 m*M* mercaptoethanol, or even (in the case of mercuribenzoate) 0.3 *M* mercaptoethanol. Urea (4 *M*) initially activates the enzyme, but on continued incubation at 0°C the activity decreases, as described by Rowe and Russel (1973).

Titration with Methotrexate. Figure 5 shows the effect of increasing amounts of methotrexate on enzyme activity at pH 6.0 and pH 8.0. At pH 6.0 the inhibitor is bound essentially stoichiometrically, allowing the determination of the enzyme concentration, whereas at pH 8.0 the binding is significantly weaker.

On the assumption that each molecule of enzyme binds only 1 molecule of methotrexate, the abscissa intercept gives the number of paramoles of enzyme present in the reaction mixture, from which the molar concentration of the stock solution was calculated. This was in good agreement with the calculation based on protein concentration and molecular weight and hence confirmed both the essential purity of the enzyme and the 1:1 stoichiometry of the inhibitor–enzyme complex.

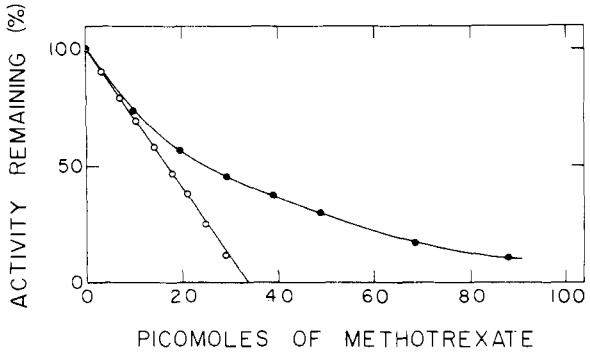

FIGURE 5: Titration of bovine liver dihydrofolate reductase with methotrexate. The enzyme was incubated at 37° for 5 min with 50 μ*M* NADPH and 0.6 *M* KCl in either 0.5 *M* sodium acetate buffer (pH 6.0) or 0.5 *M* Tris-HCl buffer (pH 8.0) and various amounts of methotrexate. The enzyme reaction was then initiated by addition of 33 μ*M* dihydrofolate and the activity determined. (○) Activity at pH 6; (●) activity at pH 8.0.

Discussion

Investigation of the interaction of mammalian dihydrofolate reductases with methotrexate and other antifolates is more relevant to the clinical use of these compounds than studies with bacterial reductases, but has been difficult to pursue because of the problem of obtaining adequate quantities of pure mammalian enzyme. The reductase content of most mammalian tissues that have been examined is quite low, so that extensive purification from large amounts of tissue is necessary. The purification procedure we have devised gives a good yield of enzyme in substantial amounts (50–100 mg) in a period of a few days, but requires equipment suitable for handling large volumes of the preparation. The essential feature of the procedure is affinity chromatography on pteroyl-L-lysine-Sepharose. This behaves satisfactorily only after large amounts of protein are removed by isoelectric precipitation, heat treatment, salting out, and gel filtration. The overall purification is about 2000-fold.

By a number of criteria the enzyme obtained was close to homogeneous. In the ultracentrifuge all preparations examined were found to be monodisperse. Titration with methotrexate at pH 6.0 gave a linear plot from which an enzyme

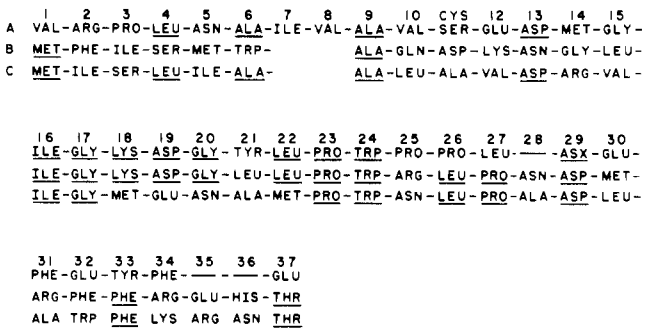

FIGURE 6: Comparison of the amino-terminal region of the amino acid sequence of dihydrofolate reductase from bovine liver (A), *S. faecium* (B), and *E. coli* (C; Bennett, 1974). Residues identical at the same position in two or three sequences are underlined. Blank spaces denote deletions. Dashes indicate unidentified residues.

molar concentration was calculated that agreed with the value from the protein concentration and molecular weight. The first step of Edman degradation gave 170 nmol of Val and small amounts of eight other amino acids which together totaled 87 nmol, so that the minimum purity must be 66%, although it could be significantly higher if the residues other than Val are due to adventitious contamination rather than additional end groups. In fact in a "zero step" cycle in which no phenylisothiocyanate was added but an otherwise complete cycle was carried out, 42 nmol of adventitious amino acids was found. After correction for this the minimum purity becomes 79%. The largest amount of any residue other than Val obtained in step 1 was 29 nmol (Phe), so that the maximum possible contamination by any single protein is 14%.

The specific activity of the final preparation, 103 units/mg, is higher than the values reported for reductase from other vertebrate tissues (Blakley, 1969) in part because of the activating effect of KCl on the bovine enzyme. An exception must be made in the case of methotrexate-resistant lines of neoplastic cells, such as the sarcoma 180 (Hakala and Ishihara, 1962) some of which have remarkably high levels of dihydrofolate reductase.

The bovine liver reductase differs from the enzyme from many vertebrate tissues (Blakley, 1969) in that it is not activated by mercurials and indeed is strongly, and apparently irreversibly, inhibited by these agents. Moreover, even the activation by urea, which it does have in common with the other vertebrate reductases, is transitory, the activation by 4 *M* urea lasting for only 30 min at 0°C. Evidently the transition into a more favorable, stable conformer that occurs in the case of other vertebrate enzymes does not happen with the bovine liver enzyme. It is noteworthy that Greenberg et al. (1966) found similar lack of stimulation of the calf thymus reductase by organic mercurials.

Homology of the Amino-Terminal Region with That of the Bacterial Reductases. Figure 6 compares the bovine liver reductase enzyme (A) with that of the reductases from *S. faecium* (B) and *E. coli* (C). It may be seen that treating Ile-7 and Val-8 of the bovine liver sequence as insertions maximizes the number of residues in the bovine sequence that are identical with a corresponding residue in at least one of the bacterial sequences. We have not undertaken calculations to determine which positions for the insertions (or deletions in the bacterial sequences) give the minimum number of base changes per codon when the bovine and bacterial sequences are interconverted.

Examination shows considerable homology between the bovine and bacterial sequences. The minimum number of base changes per codon for interconversion of the bovine liver sequence and *E. coli* sequences is 0.875, the same as required for interconversion of these regions of the *E. coli* and *S. faecium* sequences. The homology between the *S. faecium* and bovine liver sequences is somewhat less, requiring a minimum of 1.1875 base changes per codon for interconversion. This degree of homology is consistent with our argument (Gleisner et al., 1974) that the amino-terminal region is involved in the dihydrofolate binding site. If this argument is true then it is of interest to examine the residues common to all three sequences for any that might have characteristics fitting it for participation in the catalytic process. Only Asp-29 seems a possible candidate among the residues examined, although it is still uncertain whether this is an amide or acid residue in the bovine liver sequence.

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Purification and Properties of *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: Dihydrofolate reductase has been purified 40-fold to apparent homogeneity from a trimethoprim-resistant strain of *Escherichia coli* (RT 500) using a procedure that includes methotrexate affinity column chromatography. Determinations of the molecular weight of the enzyme based on its amino acid composition, sedimentation velocity, and sodium dodecyl sulfate gel electrophoresis gave values of 17680, 17470, and 18300, respectively. An aggregated form of the enzyme with a low specific activity can be separated from the monomer by gel filtration; treatment of the aggregate with mercaptoethanol or dithiothreitol results in an increase in enzymic activity and a regeneration of the monomer. Also, multiple molecular forms of the monomer have been detected by polyacrylamide gel electrophoresis. The unresolved enzyme exhibits two pH optima (pH 4.5 and pH 7.0) with dihydrofolate as a substrate. Highest activities are observed in buffers containing large organic cations. In 100 m*M* imidazolium chloride (pH 7), the specific activity is 47 μmol of dihydrofolate reduced per min per mg at 30°. Folic acid also serves as a substrate with a single pH optimum of pH 4.5. At this pH the K_m for folate is 16 μM, and the V_{max} is 1/1000 of the rate observed with dihydrofolate as the substrate. Monovalent cations (Na^+, K^+, Rb^+, and Cs^+) inhibit dihydrofolate reductase; at a given ionic strength the degree of inhibition is a function of the ionic radius of the cation. Divalent cations are more potent inhibitors; the I_{50} of $BaCl_2$ is 250 μM, as compared to 125 m*M* for KCl. Anions neither inhibit nor activate the enzyme.

Dihydrofolate reductase [5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase (EC 1.5.1.3)] is the target enzyme of several important drugs. We have previously shown that dihydrofolate reductases from bacterial and mammalian sources can be distinguished from each other on the basis of their relative ability to bind a series of small molecule analogues of dihydrofolate. These differences in binding explain how the drugs can function as potent and nontoxic antibacterials even though their target is common to both host and parasite (Burchall and Hitchings, 1965).

Dihydrofolate reductase has been purified to homogeneity from a variety of sources, including *Lactobacillus casei* (Pastore et al., 1974; Gundersen et al., 1972), *Streptococcus faecium* (Nixon and Blakley, 1968; D'Souza et al., 1972), T4 bacteriophage (Erickson and Mathews, 1973), and a methotrexate-resistant strain of *Escherichia coli* (Poe et al., 1972). Previously, the enzyme was partially purified in our laboratories from a strain of *Escherichia coli* resistant to 128 μg/ml of trimethoprim (Burchall, 1970). However, larger quantities of pure enzyme were needed to physically characterize the protein and its ligand complexes. In this paper, we describe the properties of dihydrofolate reductase purified to homogeneity from a strain of *E. coli* B (RT 500) resistant to 500 μg/ml of trimethoprim. The RT 500 *E. coli* produces at least 300-fold more enzyme than the wild type and tenfold more than the strain resistant to 128 μg/ml of trimethoprim. Sufficient dihydrofolate reductase was isolated for structural studies (Stone et al., 1975) and investigations of enzyme–ligand interactions (Pattishall et al., 1975).

Experimental Procedure

Materials. Folic acid was purchased from Calbiochem. Dihydrofolate was prepared by the method of Futterman (1957), as modified by Blakley (1960), and stored as a suspension in 5 m*M* HCl at −70°. NADPH was from P.L. Biochemicals; methotrexate was supplied by Nutritional Biochemicals; and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was purchased from Pierce Chemical Company. AH-Sepharose 4B, Sephadex G-50, and Sephadex G-100

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