Rosenberg, A., Theorell, H., and Yonetani, T. (1965), *Arch. Biochem. Biophys. 110*, 413.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Sober, H. A., Ed. (1968), Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co.
Theorell, H., and McKinley-McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1811.
Theorell, H., Taniguchi, S., Åkeson, Å., and Skursky, L. (1966), *Biochem. Biophys. Res. Commun. 24*, 603.
Theorell, H., and Winer, A. D. (1959), *Arch. Biochem. Biophys. 83*, 291.
Urnes P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 402.
Weber, G. (1960), *Biochem. J. 25*, 335.
Weber, G., and Daniel, E. (1966), *Biochemistry 5*, 1900.
Weiner, H. (1969), *Biochemistry 8*, 529.
Weiner, H., Iweibo, I., and Coleman, P. (1972), *in* Structure and Function of Oxidation-Reduction Enzymes, Åkeson, Å., and Ehrenberg, A., Ed., Oxford, Pergamon Press (in press).
Young, M. J., and Wang, J. H. (1971), *J. Biol. Chem. 246*, 2815.

Dihydrofolate Reductase from Amethopterin-Resistant *Lactobacillus casei*†

L. E. Gundersen,‡ R. B. Dunlap,§ N. G. L. Harding,¶ J. H. Freisheim,|| F. Otting, and F. M. Huennekens*

ABSTRACT: Two forms (I and II) of dihydrofolate reductase from sonically disrupted cells of an amethopterin-resistant strain of *Lactobacillus casei* have been isolated using a procedure that involves fractionation with ammonium sulfate and chromatography on hydroxylapatite and CM-Sephadex. As shown previously (Dunlap *et al.*, *Biochem. Biophys. Res. Commun. 42*, 772 (1971)), these forms differ by the presence of an equimolar amount of noncovalently bound TPNH in II. Homogeneity of I was established by polyacrylamide electrophoresis and ultracentrifugation. Form II was also homogeneous with respect to extraneous proteins but some preparations contained traces of I arising from the loss of TPNH. Form I had a molecular weight of 14,900 as judged by gel filtration, electrophoresis on sodium dodecyl sulfate–polyacrylamide and ultracentrifugation. Amino acid analyses of I revealed the presence of three tryptophan residues per molecule and an absence of cysteine residues. The enzyme was not activated by mercurials and only slightly activated by urea. At pH 6.5 and 30°, the turnover number was 180 moles of dihydrofolate reduced per min per mole of enzyme. Polyacrylamide electrophoresis was used to demonstrate the following interrelationships between the two forms of the enzyme: (a) the TPNH-dependent conversion of I → II; (b) the dihydrofolate-dependent conversion of II → I; (c) the TPN- and tetrahydrofolate-dependent conversion of I → II; and (d) the AP-TPN- and tetrahydrofolate-dependent conversion of II → I.

Using the procedures comparable to those employed by Kisliuk and coworkers (Crusberg *et al.*, 1970), an amethopterin-resistant strain of *Lactobacillus casei* has been selected (Dunlap *et al.*, 1971b) in which the levels of thymidylate synthetase and dihydrofolate reductase (EC 1.5.1.3) are elevated several 100-fold over the corresponding levels in the wild-type organism. In a preliminary communication (Dunlap *et al.*, 1971a), some of the properties of the two principal forms (I and II) of the dihydrofolate reductase from amethopterin-resistant *L. casei* have been described. These forms, which can be separated chromatographically or electrophoretically, differ only by the presence of an equimolar amount of noncovalently bound TPNH in II. The present paper further characterizes this enzyme,[1] particularly with respect to the interconversion of forms I and II. Subsequent communications from the laboratory will be concerned with the nature of the substrate binding sites of this enzyme and with the mechanism by which it catalyzes both the TPNH-dependent reduction of dihydrofolate and a novel transhydrogenation reaction (Huennekens *et al.*, 1970) between TPNH and AP-TPN.[2]

Experimental Section

Materials. Commerical materials included: TPN, TPNH, and AP-TPN (Sigma); Sephadex G-50 and CM-Sephadex (Pharmacia); cellulose CC31 (Whatman); Celite (Johns Manville); and Bio-Gel P-150 and hydroxylapatite (Bio-Rad Lab-

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received November 3, 1971.* This work was supported by grants from the American Cancer Society (P-302) and the National Cancer Institute, National Institutes of Health (CA 6522).

‡ Present address: Department of Scientific Information and Regulatory Affairs, Mead Johnson, Evansville, Ind. 47721.
§ U. S. Public Health Service Research Fellow. Present address: Department of Chemistry, University of South Carolina, Columbia, S. C. 29208.
¶ Present address: Postgraduate Medical School, Hills Road, Cambridge, England.
|| Present address: Department of Biological Chemistry, University of Cincinnati, Cincinnati, Ohio 45219.

[1] A similar dihydrofolate reductase has been isolated recently from another amethopterin-resistant strain of *L. casei* (Newbold and Harding, 1971).

[2] Abbreviations used are: AP-TPN, 3-acetylpyridine-TPN; SDS, sodium dodecyl sulfate.

oratories). The hydroxylapatite (100 g) was admixed with 40 g of Celite and the combined solids were washed with several changes of buffer (5 mM potassium phosphate–1 mM EDTA, pH 6.5) in order to remove finely divided material.

Amethopterin was obtained through the courtesy of Dr. Florence White, Drug Research and Development, Chemotherapy, National Cancer Institute, National Institutes of Health. Dihydrofolate (Blakley, 1960) and tetrahydrofolate (Hatefi *et al.*, 1960) were prepared by the indicated methods.

Methods. Dihydrofolate reductase activity was assayed spectrophotometrically by the following procedure. A 1-cm cuvet contained 0.17 μmole of TPNH, 55 μmoles of potassium phosphate (pH 6.5), and enzyme in a total volume of 1.2 ml. The absorbance change at 340 nm was followed for 3 min using a Gilford multisample absorbance recorder thermostatted at 30°; 0.11 μmole of dihydrofolate (in 50 μl) was then added and the absorbance change recorded over a 3-min period. The latter absorbance change was corrected for the dihydrofolate-independent blank rate and the resulting value was used in conjunction with a differential extinction coefficient of $12.3 \times 10^3\ M^{-1}\ cm^{-1}$ (Hillcoat *et al.*, 1967) to calculate the enzymatic rate. One unit of activity is defined as the amount required to reduce 1 μmole of dihydrofolate/min under these conditions. Specific activity is expressed as units per milligram of protein. During purification of the enzyme, protein was determined by the biuret method (Gornall *et al.*, 1949) using bovine serum albumin as the standard. Thymidylate synthetase activity was assayed by the spectrophotometric method described previously (Dunlap *et al.*, 1971b).

Electrophoresis of dihydrofolate reductase preparations on polyacrylamide and staining for protein and enzymatic activity were performed by the methods of Dunlap *et al.* (1971a). Molecular weight of the enzyme was determined by electrophoresis of form I on SDS–polyacrylamide according to the general method of Weber and Osborn (1969); the detailed procedure has been given previously (Dunlap *et al.*, 1971b). Relative mobility refers to the R_F value. Molecular weight determination, *via* filtration through a standardized Sephadex column, was carried out by the Whitaker (1963) method. In the latter procedure, a 2.5 × 115 cm column of Sephadex G-50 was equilibrated with 10^{-2} M phosphate buffer (pH 7.0) and small samples of the standard proteins and dihydrofolate reductase (form I) were applied to the column and washed through with the above buffer. For each protein, the relative mobility (ratio of its elution volume, v, to that of the void volume, v_0) was determined. In these methods for molecular weight determination the following proteins (molecular weights in parentheses) were used as standards: insulin (5700), cytochrome *c* (11,700), RNase (13,700), myoglobin (17,200), chymotrypsin (22,500), trypsin (23,000), glyceraldehyde-3-P dehydrogenase (37,000), and ovalbumin (43,000).

Ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge equipped with schlieren optics. $s_{20,w}$ values were determined by a sedimentation velocity technique (Schachman, 1959). Solutions of the enzyme at various concentrations were sedimented at 52,000 rpm in 0.1 M potassium phosphate (pH 7.0). A straight-line plot of the data ($s_{20,w}$ *vs.* protein concentration) was obtained by the least-squares method. We are indebted to Dr. V. deSaussure for carrying out these determinations.

Amino acid analyses followed the general procedures of Moore and Stein (1963). Weighed samples of enzyme were hydrolyzed in 6 N HCl at 110° for 24, 48, and 72 hr and the hydrolysates were analyzed using a Beckman Amino Acid Analyzer, Model 120C, with a Beckman Integrator, Model

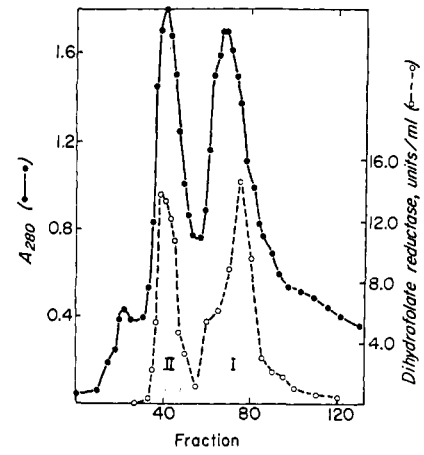

FIGURE 1: Separation of forms I and II of dihydrofolate reductase by chromatography on hydroxylapatite. Details are given in the Experimental Section. Protein (●) was measured by absorbance at 280 nm (A_{280}). Dihydrofolate reductase (○) was assayed as described in the Experimental Section and the results are expressed as units/ml.

125. Values for serine and threonine were extrapolated to zero time. Performic acid oxidized dihydrofolate reductase was hydrolyzed for 24 hr as above, and an aliquot was analyzed for cysteine and methionine. We are indebted to Dr. I. Crawford for his advice and help in carrying out these analyses. Tryptophan was measured by the methods of Barman and Koshland (1967), Goodwin and Morton (1946), and Edelhoch (1967).

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

Amethopterin-resistant *L. casei* was grown by the procedure of Dunlap *et al.* (1971b). Sonication of the cells and fractionation of the extract with ammonium sulfate were carried out according to steps 1 and 2 for purification of thymidylate synthetase (Dunlap *et al.*, 1971b), except that the procedure was scaled up to accommodate 320 g of frozen cells. The 35–65% ammonium sulfate precipitate was dissolved in 400 ml of buffer (50 mM Tris, pH 7.2, containing 50 mM KCl, 10 mM mercaptoethanol, and 1 mM EDTA) and the solution was dialyzed against the same buffer. The dialyzed material was then passed through a large column (14 × 170 cm) of Bio-Gel P-150 at a flow rate of 30 ml/hr. After discarding the void volume (3 l.), 18-ml fractions were collected. Thymidylate synthetase, which appeared between fractions 100 and 220, was purified further by the previous procedure (Dunlap *et al.*, 1971b). Dihydrofolate reductase was eluted between fractions 330 and 480. In various preparations, these combined fractions had a volume of about 2.5 l. and contained 1400–2100 units of activity and 2.0–2.5 g of protein.

The preparation of dihydrofolate reductase from the gel filtration step was treated with increments of solid ammonium sulfate until 90% saturation was reached. The precipitate was collected by centrifugation, dissolved in a minimum volume of 50 mM potassium phosphate–1 mM EDTA (pH 6.5), and dialyzed against the same buffer. The solution was then dialyzed against two separate portions of 5 mM potassium phosphate–1 mM EDTA (pH 6.5), and, after centrifugation, the small precipitate was discarded. The supernatant was then

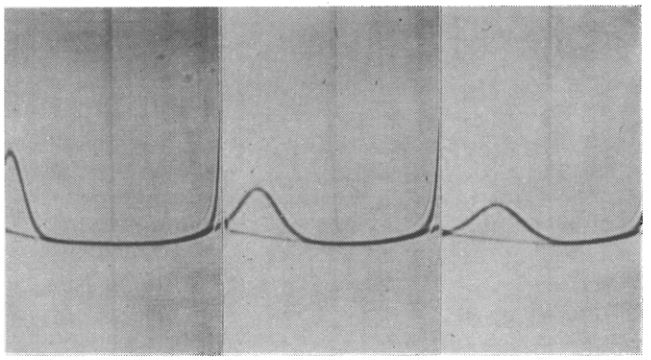

FIGURE 2: Sedimentation of dihydrofolate reductase. Enzyme (form I, specific activity 11.5 units/mg, 2.7 mg/ml) in 50 mM Tris buffer (pH 7.2) was centrifuged at 52,000 rpm in a Beckman analytical ultracentrifuge, Model E. Photographs (left to right) were taken at 48, 96, and 192 min.

applied to a 3 × 15 cm column of hydroxylapatite (see Materials). After washing the column with 400 ml of the 5 mM phosphate buffer, gradient elution was performed (750 ml of 20 mM potassium phosphate–1 mM EDTA (pH 6.5) in the mixing chamber and 750 ml of 150 mM potassium phosphate–1 mM EDTA (pH 6.5) in the reservoir). Fractions of 8 ml were collected. As shown in the elution profile (Figure 1), two distinct peaks of enzymatic activity were obtained. Fractions 34–51 contained form II of the enzyme (165 ml, 1160 units, 107 mg, specific activity 10.9 units/mg). Fractions 62–86 contained form I of the enzyme (320 ml, 1650 units, 139 mg, specific activity 11.9 units/mg). Enzyme having a specific activity of 11–12 units/mg of protein was found to be homogeneous by several criteria (see Results). When enzyme of lower specific activity was obtained by the above procedure, it was rechromatographed on hydroxylapatite or on CM-Sephadex; in the latter instance, elution was performed using a gradient of 1 l. of 20 mM potassium phosphate–1 mM EDTA (pH 6.0) in the mixing chamber and 1 l. of 80 mM potassium phosphate–1 mM EDTA (pH 6.0) in the reservoir.

Results and Discussion

Purification. The relative abundance and the stability of both forms I and II of dihydrofolate reductase from the amethopterin-resistant strain of *L. casei* facilitates purification of the enzyme from this source. As described in the Experimental Section, the cell sonicate was fractionated with ammonium sulfate and the 35–65% precipitate was passed through Bio-Gel P-150 in order to separate the low molecular weight reductase from thymidylate synthetase (mol wt 70,000); the latter enzyme can be further purified by the procedure described earlier (Dunlap *et al.*, 1971b). Fractions containing dihydrofolate reductase from the gel filtration step were pooled and chromatographed on hydroxylapatite. As shown in Figure 1, form II of the enzyme (the more negatively charged form at pH 6.5) was eluted first, followed by I. By excluding those fractions in which there was some overlap of the two forms, each was obtained in a highly purified state (specific activity 11–12 units/mg) from this step. Further purification, if necessary, was achieved by rechromatographing either form on hydroxylapatite or CM-Sephadex. Use of the latter exchanger for separation of the forms at an earlier stage in purification has been described elsewhere (*cf.* Figure 5 in Huennekens *et al.*, 1971).

In the present procedure, the overall purification was about 80-fold and the total recovery of activity was about 25%. Various batches of cells yielded approximately equal amounts of forms I and II[3] at the sonicate stage and this ratio was generally maintained during purification.

TABLE I: Amino Acid Composition[a] of Dihydrofolate Reductase from Amethopterin-Resistant *L. casei*.

Amino Acid	Residues/ 14,900 g	Residue Wt (μg/μmole)
Aspartic acid	14.2	1,598
Threonine	11.0	1,111
Serine	4.0	348
Glutamic acid	12.8	1,612
Proline	5.7	543
Glycine	7.9	439
Alanine	11.0	767
Valine	11.0	1,069
Methionine	1.6	210
Isoleucine	3.9	429
Leucine	10.9	1,209
Tyrosine	4.1	652
Phenylalanine	7.0	1,013
Lysine	7.9	986
Histidine	5.6	754
Arginine	6.7	1,030
Cysteine	0	0
Tryptophan[b]	(3)	555
Ammonia	9.3	155
	Total	14,480 (98%)

[a] Determination by Dr. Irving Crawford. [b] Average of three methods (see text).

Criteria of Purity. Form I of the enzyme having a specific activity[4] of 11–12 units/mg appeared homogeneous as judged by electrophoresis on polyacrylamide (*cf.* Figure 1 in Dunlap *et al.*, 1971a; and Figures 5 and 6 in this paper) and by its behavior in the ultracentrifuge (Figure 2). Freshly isolated or synthetically prepared II also showed a single band upon electrophoresis (*cf.* figures referred to above), but stored preparations of this form showed progressive breakdown to I. When II was examined in the ultracentrifuge, its sedimentation was very similar to that of I, thereby ruling out the possibility that II is a TPNH-induced aggregate of I.

Molecular Weight. The molecular weight of form I was determined by several methods. (A) Filtration through a calibrated column of Sephadex G-50: from the straight-line plot of the relative mobility values *vs.* log M_W for various standard proteins and for the reductase, an approximate molecular weight of 15,800 was obtained for the latter. (B) Electrophoresis on SDS–polyacrylamide: the straight-line plot of relative mobility values *vs.* log M_W for standard proteins and the reductase gave a minimum molecular weight of 14,800 for the latter. (C) Sedimentation in the ultracentrifuge: the

[3] Wild-type *L. casei* (amethopterin-sensitive) also contains both forms of the enzyme, but the activity is present at about a 200-fold lower level than in the amethopterin-resistant strain.

[4] In a previous communication (Dunlap *et al.*, 1971a), electrophoretically homogeneous enzyme was reported to have a lower specific activity, but this was due to the use of nonoptimal assay conditions.

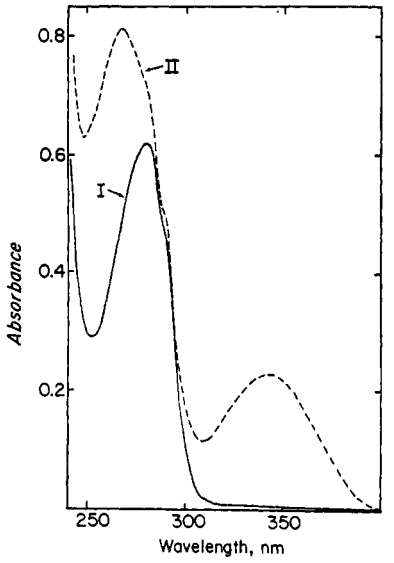

FIGURE 3: Absorbance spectra of forms I and II of the enzyme. Solvent, 0.05 M phosphate buffer (pH 7.0). Solid line, form I at 2.9×10^{-5} M. Dashed line, form II at 2.9×10^{-5} M (prepared as described in the text).

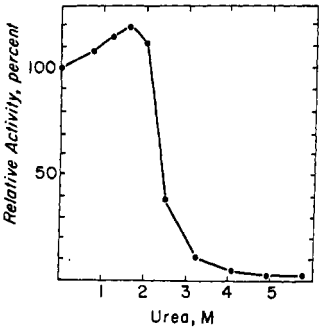

FIGURE 4: Effect of urea upon enzyme activity. Enzyme (form I, specific activity 11.5 units/mg, 0.73 μg) was assayed under standard conditions (see Experimental Section) with the indicated concentrations of urea present.

sedimentation velocity, determined at protein concentrations ranging from 2.2 to 11.1 mg per ml, was found to be essentially independent of protein concentration. At zero concentration, $s_{20,w} = 1.94$ S. For a globular protein, this value for the sedimentation coefficient would correspond to a molecular weight of 15,000. Since B and C are more accurate than A, 14,900 (an average of the values obtained by these methods) was used for the molecular weight of I in subsequent calculations.

Amino Acid Composition. Table I lists the amino acid composition of the enzyme. Based upon a molecular weight of 14,900, the recovery of amino acids was 98%. Of considerable interest is the fact that this dihydrofolate reductase contains no cysteine residues. This accounts for the inability of mercurials to activate the enzyme (see below). Because of their possible importance in substrate binding, tryptophan residues were determined by three independent methods. Values of 3.3, 3.0, and 2.9 residues per molecule were obtained, respectively, by the Barman and Koshland (1967), the Goodwin and Morton (1946), and the Edelhoch (1967) procedures.

Absorption Spectra. The absorption spectra of forms I and II, as isolated, were given in a previous communication (*cf.* Figure 2 in Dunlap *et al.*, 1971a). In view of the possibility that the sample of II used for that purpose may have lost some of its TPNH, it seemed worthwhile to redetermine the spectral characteristics of II by another method. Since I binds TPNH stoichiometrically (as discussed subsequently), admixing an equimolar amount of TPNH with pure I should result in the formation of pure II. Details of such an experiment are shown in Figure 3. The solid line is the spectrum of I at pH 7.0 and, using an extinction coefficient of 21.5×10^3 M^{-1} cm^{-1} at 278 nm which had been determined separately on a weighed sample of I, the concentration was calculated to be 2.9×10^{-5} M. The dashed line shows the spectrum of II produced by admixing TPNH with I so that each was present at 2.9×10^{-5} M. From this latter curve, extinction coefficients for II were calculated to be 27.6×10^3 and 7.2×10^3 M^{-1} cm^{-1} at 268 and 340 nm, respectively. The slightly enhanced absorbance of enzyme-bound TPNH at 340 nm ($\epsilon = 7.2 \times 10^3$ compared to 6.2×10^3 for free TPNH (Horecker and Kornberg, 1948)) is paralleled by an increase in the fluorescence of TPNH upon interaction with the protein (unpublished results from this laboratory).

Kinetic Parameters. Like several other dihydrofolate reductases of bacterial origin (reviewed in Huennekens, 1968; Blakley, 1969), the *L. casei* enzyme has a single pH optimum which, in this case, is centered at 6.5, K_m values for dihydrofolate and TPNH could not be determined conveniently with the present spectrophotometric assay since, in order to achieve velocities in the range of one-half V_{max}, the concentration of either substrate had to be less than 10^{-6} M. As a consequence, the absorbance changes at 340 nm were too small to be measured accurately. These observations indicate that K_m for TPNH with the *L. casei* is less than 10^{-6} M. A more direct measure of the affinity of the enzyme for TPNH (in the absence of the other substrate, dihydrofolate) can be achieved by fluorimetric techniques (unpublished results from this laboratory) which yield a value of $K_D = 10^{-7}$ M for the dissociation of the E·TPNH complex.

When assayed under optimal conditions of pH and substrate concentrations, various preparations of the *L. casei* enzyme had a specific activity of 11–12 μmoles of dihydrofolate reduced/min per mg of protein at 30°. Based upon a molecular weight of 14,900, the higher value corresponds to a turnover number of 180 moles of substrate transformed by each mole of enzyme per min. Under the same conditions, reduction of folate could not be detected. However, when the pH was lowered to 4.8, the rate of folate reduction was about 1% of that with dihydrofolate.

Response to Activators. Many dihydrofolate reductases have their catalytic activity increased in the presence of urea or mercurials (reviewed in Huennekens *et al.*, 1971; Huennekens, 1968; Blakley, 1969). The enzyme from amethopterin-resistant *L. casei*, however, is unresponsive to mercurials, as would be expected from the absence of cysteine residues in the protein. Urea (at 2 M) produced a small but definite activation (about 20%), as shown in Figure 4. Higher concentrations of urea inhibited the enzyme but this effect was reversible, *i.e.*, full catalytic activity was regained when an enzyme solution containing 8 M urea was subjected to dialysis or gel filtration.

TPNH-Dependent Conversion of Form I to Form II. In a previous communication (Dunlap *et al.*, 1971a), it was shown that form II differs from I only by the presence of an equimolar amount of noncovalently bound TPNH. This relationship can be verified by the TPNH-dependent conversion of I → II. In the experiment illustrated in Figure 5, I was incubated at neutral pH with 0, 0.25, 0.5, 1.0, and 5.0 mole equiv

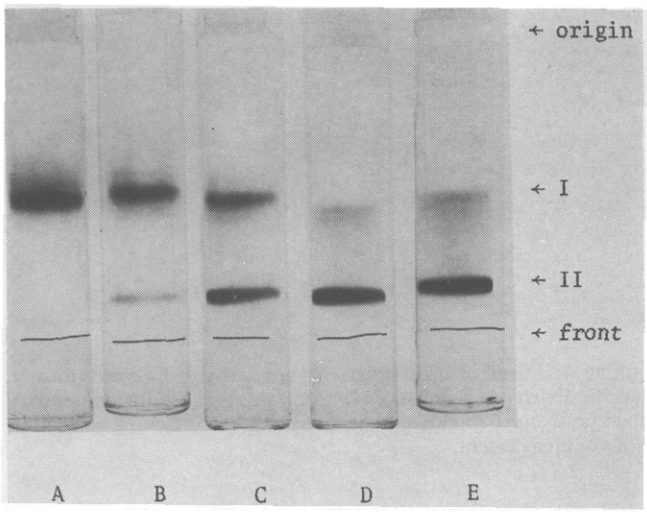

FIGURE 5: Electrophoretic demonstration of the TPNH-dependent conversion of forms I to II of the enzyme. Polyacrylamide gel electrophoresis and staining for protein was carried out as described in the Experimental Section. Form I (specific activity 11.5 units/mg, 1.5 nmoles) was admixed with 0, 0.38, 0.75, 1.5, and 2.5 nmoles of TPNH (A–E, respectively) prior to electrophoresis.

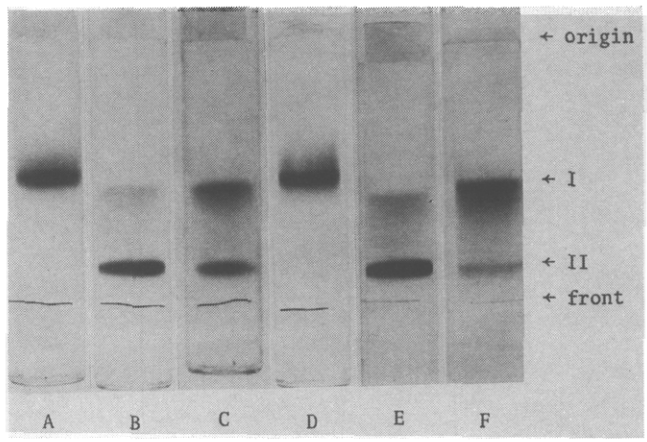

FIGURE 6: Electrophoretic demonstration of various methods for interconverting forms I and II of the enzyme. Experimental conditions as in Figure 5. The following materials (amounts in nmoles) were admixed prior to electrophoresis: A, I (1.5); B, II (1.5); C, I (1.5) + TPN (14) + tetrahydrofolate (42); D, II (1.5) + dihydrofolate (50); E, II (1.5) + AP-TPN (5); F, same as E plus tetrahydrofolate (3).

of TPNH and the resulting mixtures were examined electrophoretically. A progressive conversion of I → II is evident. Conversely, incubation of I with TPN, DPNH, DPN, dihydrofolate, or tetrahydrofolate does not alter the electrophoretic mobility of the protein.

Other Procedures for the Interconversion of Forms I and II. The conversion of form I to II can also be demonstrated in a novel manner by incubating the former with the products (TPN and tetrahydrofolate) of reaction 1. As shown in gels

$$\text{7,8-dihydrofolate} + \text{TPNH} + \text{H}^+ \rightleftarrows \text{tetrahydrofolate} + \text{TPN}^+ \quad (1)$$

A–C of Figure 6, TPNH produced by reversal of the reaction became firmly bound to the enzyme, thereby creating form II. This experiment proves that reaction 1 is inherently reversible and it provides an explanation for the difficulty (Osborn and Huennekens, 1958; Mathews and Huennekens, 1963; Blakley and McDougall, 1961; Nixon and Blakley, 1968; Mathews and Sutherland, 1965) in demonstrating reversibility when only *catalytic* amounts of the dihydrofolate reductase were present.

As stated previously (Dunlap *et al.*, 1971a), II can be converted to I by treatment of the former with adsorbents such as Dowex 1. It was also shown (*cf.* Figure 3 in Dunlap *et al.*, 1971a) that, when II was treated with dihydrofolate, there was a progressive loss of fluorescence as the bound TPNH was oxidized to TPN according to eq 1. Since the enzyme has a much lower affinity for TPN, this procedure would be expected to convert II → I. Electrophoretic evidence for this transformation is shown in gel D of Figure 6.

Several dihydrofolate reductases, including the *L. casei* enzyme, have been shown to catalyze the following transhydrogenation reaction (Huennekens *et al.*, 1970; J. H. Freisheim *et al.*, in preparation), provided that a catalytic amount of dihydrofolate or tetrahydrofolate is present. This reaction is

$$\text{TPNH} + \text{AP-TPN} \rightleftarrows \text{TPN} + \text{AP-TPNH} \quad (2)$$

usually followed spectrophotometrically by measuring the increase in absorbance at 380 nm due to the production of AP-TPNH. It can also be studied by polyacrylamide electrophoresis. When form II of the enzyme was incubated with AP-TPN (but in the absence of tetrahydrofolate), transhydrogenation could not occur and II remained unchanged (gel E in Figure 6). However, when the experiment was repeated with tetrahydrofolate present, an appreciable amount of I appeared (gel F). Although there is a conservation of *reduced* pyridine nucleotide in reaction 2, the enzyme has a lower affinity for AP-TPNH (unpublished results from this laboratory) than it does for TPNH and, thus, the experiment terminates with the enzyme present as a mixture of I and a "pseudo II," *i.e.*, enzyme·AP-TPNH.

Acknowledgments

The authors are indebted to D. Frazier, M. Schranner, A. Marvin, and K. Lintner for expert technical assistance.

References

Barman, T. E., and Koshland, D. E. (1967), *J. Biol. Chem. 242*, 5771.

Blakley, R. L. (1960), *Nature (London) 188*, 231.

Blakley, R. L. (1969), The Biochemistry of Folic Acid and Related Pteridines, Amsterdam, North-Holland, pp 139–187.

Blakley, R. L., and McDougall, B. M. (1961), *J. Biol. Chem. 236*, 1163.

Crusberg, T. C., Leary, R. P., and Kisliuk, R. L. (1970), *J. Biol. Chem. 245*, 5292.

Dunlap, R. B., Gundersen, L. E., and Huennekens, F. M. (1971a), *Biochem. Biophys. Res. Commun. 42*, 772.

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

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Gornall, A. G., Bardawill, C. S., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

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

Hillcoat, B. L., Nixon, P. F., and Blakley, R. L. (1967), *Anal. Biochem. 21*, 178.
Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.
Huennekens, F. M. (1968), *in* Biological Oxidations, Singer, T. P., Ed., New York, N. Y., Interscience, pp 439–513.
Huennekens, F. M., Dunlap, R. B., Freisheim, J. H., Gundersen, L. E., Harding, N. G. L., Levison, S. A., and Mell, G. P. (1971), *Ann. N. Y. Acad. Sci. 186*, 85.
Huennekens, F. M., Mell, G. P., Harding, N. G. L., Gundersen, L. E., and Freisheim, J. H. (1970), *in* Chemistry and Biology of Pteridines, Iwai, K., Akino, M., Goto, M., and Iwanami, Y., Ed., Tokyo, International Academic Printing Co. Ltd., pp 329–350.
Mathews, C. K., and Huennekens, F. M. (1963), *J. Biol. Chem. 238*, 3436.
Mathews, C. K., and Sutherland, K. E. (1965), *J. Biol. Chem. 240*, 2142.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Newbold, P. C. H., and Harding, N. G. L. (1971), *Biochem. J. 124*, 1.
Nixon, P. F., and Blakley, R. L. (1968), *J. Biol. Chem. 243*, 4722.
Osborn, M. J., and Huennekens, F. M. (1958), *J. Biol. Chem. 233*, 969.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, London, Academic Press.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Whitaker, J. R. (1963), *Anal. Chem. 35*, 1950.

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

Martin Poe,* Norma J. Greenfield, Jordan M. Hirshfield, Myra N. Williams, and Karst Hoogsteen

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.

† From the Department of Biophysics & Pharmacology, Merck Institute for Therapeutic Research, Merck & Co., Inc., Rahway, New Jersey 07065. *Received November 8, 1971.*