# Purification and Properties of Dihydrofolate Reductase from Ehrlich Ascites Carcinoma Cells*

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ABSTRACT: The enzyme dihydrofolate reductase has been purified over 500-fold from Ehrlich ascites carcinoma cells. A molecular weight of 20,200 and a turnover number of 270 have been calculated. The $K_m$ values for reduced nicotinamide–adenine dinucleotide phosphate (NADPH) and reduced nicotinamide–adenine dinucleotide are $5.6 \times 10^{-6}$ and $2.6 \times 10^{-4}$ M, respectively; oxidized nicotinamide–adenine dinucleotide phosphate has been found to be a competitive inhibitor ($K_i = 4.9 \times 10^{-6}$) of this enzyme reaction with respect to NADPH. The purified enzyme catalyzes the reduction of folate at a slower rate than the reduction of dihydrofolate, and only at an acid *p*H and with NADPH as the coenzyme. The ascites cell enzyme exhibited a broad specificity and reduced several pteroyl and dihydropteroyl compounds; $N^{10}$-methyldihydrofolate, dichlorodihydrofolate, and dibromodihydrofolate were reduced at faster rates than was dihydrofolate. Studies of several inhibitors indicated that the 2,4-diamino structure resulted in enhanced binding of the inhibitor to the enzyme, and that other portions of the molecule, including the glutamic acid portion, the *p*-aminobenzoic acid ring, and in particular the $N^{10}$-nitrogen are also important for binding. A $K_i$ value of $6.1 \times 10^{-9}$ for $N^{10}$-formylfolate was calculated; this unusually low dissociation constant may explain the failure of this compound and its dihydro derivative to act as a substrate for dihydrofolate reductase.

The enzyme dihydrofolate ($FH_2$)[1] reductase has been studied in bacterial (Blakley and McDougall, 1961; Burchall and Hitchings, 1964), avian (Mathews and Huennekens, 1963; Zakrzewski and Nichol, 1960), and mammalian tissues (Bertino *et al.*, 1960; Nath and Greenberg, 1962; Morales and Greenberg, 1964). The key role of this enzyme in thymidylate biosynthesis (Wahba and Friedkin, 1961) and its inhibition by the folate antagonists, methotrexate (amethopterin, $N^{10}$-methyl-4-aminopteroylglutamate, and aminopterin (4-aminopteroylglutamate) have been the subject of several studies (Werkheiser, 1961; Bertino *et al.*, 1964; Schrecker and Huennekens, 1964).

This report summarizes our studies concerned with the purification and properties of this enzyme obtained from a transplantable mouse tumor, the Ehrlich ascites carcinoma. A rapid, simple procedure is presented for the purification of this enzyme activity in high yield and of estimated 5–15% purity. Studies of the interaction of this enzyme with certain cations, thiourea, guanidine-HCl, and organic mercurials are presented in the accompanying paper (Perkins and Bertino, 1965).

## Materials and Methods

Folic acid was purchased from Nutritional Biochemicals Corp.; NADH, $NADP^+$, and NADPH were purchased from the Sigma Chemical Co.; 2-mercaptoethanol and DEAE-cellulose from the Eastman Chemical Company; Sephadex from the Pharmacia Co., and hydroxylapatite from Bio-Rad, Inc. Pteroyltriglutamate, 9-methylfolate, $N^{10}$-methylfolate, 3′,5′-dichlorofolate, 3′,5′-dibromofolate, $N^2$,$N^2$-dimethylfolate, 2-deamino-2-hydroxylfolate, pteroate, 2-amino-4-hydroxy-6-methylpteridine, and methotrexate were obtained from the Lederle Laboratories through the courtesy of Dr. J. M. Ruegsegger. Biopterin and 2,4,7-triamino-6-phenylpteridine (triamterene) were gifts of Dr. A. M. Maass of the Smith, Kline and French Laboratories. Pyrimethamine (Daraprim, 2,4-diamino-5-chlorophenyl-6-ethylpyrimidine) was a gift of Dr. George Hitchings of the Burroughs Wellcome and Co. laboratories. $N^{10}$-Formylfolate was synthesized from folic acid by the method of Gordon *et al.* (1948).

The folate compounds were purified by chromatography on DEAE-cellulose (Johns *et al.*, 1961); elution was accomplished by using solutions of ammonium carbonate of increasing molarity (0.004–0.4 M). The column effluent was monitored with a LKB Uvicord attached to a 1-ma recording instrument. The fractions containing the desired compound were identified by their ultraviolet spectra, flash evaporated to dryness

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[1] Abbreviations used in this work: $FH_2$, dihydrofolate; $FH_4$, tetrahydrofolate; NADH, reduced nicotinamide–adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphate, respectively.

at 37°, and brought into solution with a minimal amount of distilled water. The compound was then precipitated by bringing the *p*H to 3 with 1 N HCl. The precipitate was washed twice with 0.001 M HCl, and brought into solution by adjusting the *p*H to 7.5 with dilute KOH.

$FH_2$ was synthesized by the method of Futterman (1957) as modified by Blakley (1960). This compound was stored as a suspension for up to 2 weeks in a 10% solution of potassium ascorbate, *p*H 6.0, at 4°. For routine assays, an aliquot of the suspension was washed three times with $10^{-3}$ M HCl immediately before use, and brcught into solution with 0.05 M Tris buffer and made 0.1 M with respect to 2-mercaptoethanol. The final $FH_2$ concentration was adjusted to 1.0 μmole/ml using the molar extinction coefficient of 28,000 at 282 mμ (Blakley, 1960). When $FH_2$ was used for kinetic studies or compared as a substrate with other dihydro compounds, it was prepared daily by Friedkin's modification (Friedkin *et al.*, 1962) of the dithionite reduction. No differences in reaction rates were observed in $FH_2$ synthesized by the two different methods. Dihydropteroyltriglutamate, $N^{10}$-methyl-$FH_2$, 3′,5′-dichloro-$FH_2$, 3′,5′-dibromo-$FH_2$, $N^2$,$N^2$-dimethyl-$FH_2$, dihydropteroate, and $N^{10}$-formyl-$FH_2$ were synthesized by dithionite reduction (Friedkin *et al.*, 1962) from the compounds purified by DEAE-cellulose chromatography. The nonreduced as well as the dihydro compounds were checked for purity by thin-layer chromatography on cellulose. Approximately 1 hour was required for resolution when 0.05 M potassium phosphate buffer, *p*H 6.0, was used as the solvent. When the dihydro compounds were run, 0.01 M 2-mercaptoethanol was added to protect these compounds from oxidation. All compounds migrated as single spots except the dihydrodihalogenated compounds; in the latter, trace amounts of fluorescent impurities were noted.

The concentration of the pteridine compounds in solution was determined using published molar extinction coefficients; the extinction coefficients of the dihydropteroyl compounds were assumed to be similar to $FH_2$ (ε at 282 mμ = 28,000). Characteristic spectral changes similar to those occurring when folate is reduced to $FH_2$ occurred when these compounds were reduced by dithionite.

Assay of $FH_2$ reductase activity was performed by a spectrophotometric method utilizing the decrease in absorbance that occurs at 340 mμ when NADPH and $FH_2$ are converted to $NADP^+$ and $FH_4$, respectively. Absorbance readings were made at 15-second intervals with a Gilford attachment to a Beckman DU spectrophotometer. Each assay included a reference cuvet from which substrate was omitted. A similar spectrophotometric assay was used when nonreduced folate compounds were tested as substrates (Mathews and Heunnekens, 1963; Bertino *et al.*, 1964). Protein was determined by the biuret method in relatively crude extracts, and by absorbance readings at 280 mμ in more purified extracts using crystalline serum albumin as a standard. Specific activity, expressed as micromoles of substrate reduced per hour per milligram protein, was calculated from the combined decrease in absorbancy for NADPH (ε = 6200) and $FH_2$ (ε = 5800) at 340 mμ (Mathews and Huennekens, 1963). A unit of enzyme activity is defined as that amount reducing 1 μmole of substrate per hour under conditions of the standard assay (*p*H 7.5, 23°). Modifications of the standard assay procedure are indicated in the tables and figures.

*Purification of Dihydrofolate Reductase.* All operations were carried out at 4°. Unless otherwise indicated, centrifugations were performed for 20 minutes at 15,000 rpm in a Servall refrigerated centrifuge, Model RC-2.

*Preparation of Crude Extract.* One hundred mice bearing the Ehrlich ascites tumor were sacrificed 6 days after inoculation and the cells were harvested by the method of Sartorelli *et al.* (1964). The ascitic fluid was discarded after centrifugation (10 minutes, 2000 rpm) and the cell pack was washed with 0.15 M NaCl. The contaminating erythrocytes were lysed by adding 6 volumes of distilled water and mixing vigorously for 30 seconds. Two volumes of 0.6 M NaCl were then added to restore isotonicity. The cells were recovered after centrifugation and washed twice with 0.15 M NaCl, suspended in 4 volumes of 0.15 M NaCl, and lysed by homogenization for 2 minutes at 45,000 rpm in a Virtis "45." The resultant homogenate was then centrifuged and the supernatant solution was recovered.

*Fractionation by Precipitation at pH 5.1.* The *p*H of the supernatant fraction was adjusted carefully to 5.1 with 1 N HCl, the precipitated protein was removed by centrifugation, and the *p*H of the supernatant fluid was readjusted to 7.0 with 1 N KOH.

*Fractionation with Ammonium Sulfate.* Solid ammonium sulfate was slowly added to the above solution, and the *p*H of the solution was adjusted to 6.0; the protein precipitating after addition of 35 g of ammonium sulfate per 100 ml was discarded after centrifugation. More ammonium sulfate was then added (26 g/100 ml), the *p*H was adjusted to 6.0, and after mechanical stirring for 30 minutes the precipitate was collected by centrifugation and dissolved in 4.5 ml of 0.05 M Tris buffer, *p*H 7.5, containing 0.1 M KCl.

*Chromatography on Sephadex G-75.* A 2.5 × 35-cm column of Sephadex G-75 was prepared and washed with 0.5 M Tris, *p*H 7.5, in 0.1 M KCl. The 55–85% ammonium sulfate fraction described (4.5 ml) was applied to the column, and elution was accomplished with 0.05 M Tris buffer–0.1 M KCl, *p*H 7.5. An automatic fraction collector was employed to collect 4-ml fractions. The peak of enzyme activity appeared in the effluent between tubes 24 and 29.

*Chromatography on Hydroxylapatite.* A 2.2- × 10-cm column of hydroxylapatite was prepared and washed with 1 liter of 0.05 M potassium phosphate buffer, *p*H 7.0, which was also 0.1 M with respect to KCl. Positive pressure was maintained with the aid of compressed nitrogen (10 psi). Tubes 24–29 from the previous step were combined and the solution was allowed to adsorb onto the column. Elution was then carried out with batchwise portions of 0.05 M and 0.1 M phosphate buffers containing 0.1 M KCl, *p*H 7.0. The flow

TABLE I: Purification of $FH_2$ Reductase Activity from Ehrlich Ascites Cells.

| Fraction | Volume (ml) | Total Protein (mg) | Total Activity (μmoles/hr) | Specific Activity (μmoles/hr/mg) | Recovery (%) |
|---|---|---|---|---|---|
| (1) Crude homogenate | 98 | 1540 | 182 | 0.12 | |
| (2) *p*H 5.1 supernatant | 90 | 504 | 197 | 0.39 | 100 |
| (3) 55–85% saturated ammonium sulfate | 4.5 | 173 | 138 | 0.80 | 70 |
| (4) Sephadex G-75 tubes 25–28 | 16 | 12 | 84 | 7.0 | 47 |
| (5) Hydroxylapatite tubes 15–16 | 10 | 1.1 | 66 | 60.0 | 25 |

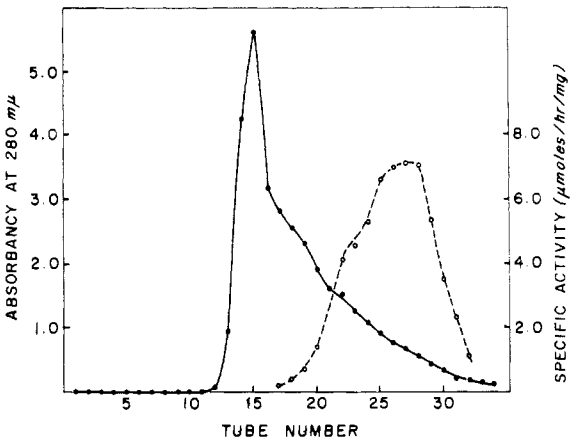


FIGURE 1: Chromatography of $FH_2$ reductase on Sephadex G-75. The solid line represents protein concentration as determined from 280-mμ absorption. The dashed line represents specific activity of the enzyme.

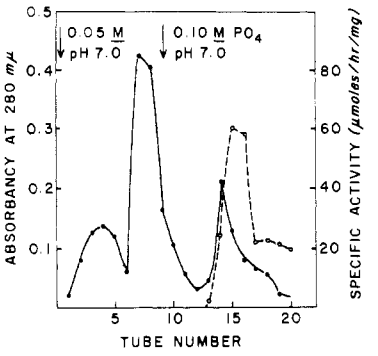


FIGURE 2: Chromatography of $FH_2$ reductase on hydroxylapatite. The solid line represents protein concentration as determined by absorption at 280 mμ, the dashed line represents specific activity of the enzyme. Stepwise changes in potassium phosphate buffer containing 0.10 M KCl are indicated by the arrows.

rate was maintained at 1 ml/minute with the aid of compressed nitrogen. An automatic fraction collector was employed and 5-ml fractions were collected. The greater part of the enzyme activity appeared in tubes 15 and 16.

## Results

*Purification of the Enzyme.* A summary of a typical purification is presented in Table I. The entire purification procedure can be carried out in one day, and the steps result in enzyme of high specific activity in good yield. Figure 1 shows a typical elution profile obtained when the enzyme is chromatographed on Sephadex G-75. The enzyme was eluted immediately after the hemoglobin peak. Chromatography on DEAE-cellulose (Sartorelli *et al.*, 1964) was abandoned in favor of gel filtration on Sephadex because of the marked loss in total activity (*ca.* 60%) that resulted when DEAE-cellulose was employed, despite a comparable increase in specific activity. The enzyme was eluted from the hydroxylapatite column with 0.10 M phosphate buffer after inert protein was removed with 0.05 M phosphate buffer (Figure 2).

The pooled active fractions from the hydroxylapatite column represent an increase in specific activity of about 500-fold over that of the supernatant fraction of the crude homogenate, with approximately 25% recovery of initial enzyme activity. Further chromatography of the highly purified enzyme obtained from hydroxylapatite on Sephadex G-75 or G-100 led to a marked loss in activity.

The purified enzyme in solution could be stored frozen (−5°) for several weeks or at 5° for several days without significant loss of activity. Rapid freezing or repeated freezing and thawing led to a considerable loss of activity.

When incubated at 37° at *p*H 6.0–8.0 (phosphate buffer, 0.05 M), the purified enzyme lost 50% of its activity in 30 minutes; the addition of either NADPH or $FH_2$ at $10^{-5}$ M prevented this inactivation.

*Molecular Weight and Turnover Number.* An estimate of the molecular weight of this enzyme was obtained by gel filtration on Sephadex G-100, by the method of Whitaker (1963). A molecular weight of 20,200 was

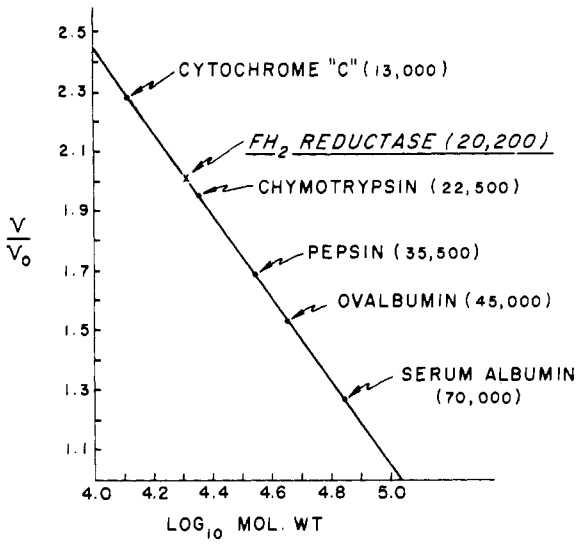


FIGURE 3: Determination of the molecular weight of $FH_2$ reductase by gel filtration on Sephadex. A column (1.6 × 50 cm) was packed with Sephadex G-100 suspended in 0.05 M potassium phosphate, *p*H 6.0, that was 0.2 M with respect to KCl. The procedure for packing was that used by Whitaker (1963). The flow rate was 0.6 ml/min and the void volume as determined with γ-globulin was 43 ± 1 ml. The molecular weights of the five standard proteins are those used by Whitaker (1963). The standard proteins were applied to the column in a volume of 2.5 ml (12.5 mg). Partially purified $FH_2$ reductase ("Sephadex" enzyme) was added to the column in a similar volume containing 0.8 unit of enzyme activity. NADPH, 0.0001 M, was included in the elution mixture to stabilize the enzyme. No change in void volume was caused by its inclusion. The point of exit of the standard proteins was determined by monitoring the effluent with an LKB Uvicord attached to a milliampere recorder. The point of exit of the reductase enzyme was determined by assay of measured volume fractions in the normal manner.

obtained by determining the ratio of the elution volume of this protein to the void volume ($V_0$) of the column after calibrating the column with other pure proteins of known molecular weight (Figure 3). This value is similar to the molecular weight of the chicken liver enzyme (15,000–20,000) obtained by Kaufman (1964) from studies of the behavior of the enzyme in a sucrose gradient.

*p*H *Optima.* The highly purified enzyme exhibited double *p*H optima with $FH_2$ as the substrate, as reported in an earlier communication, with a less pure enzyme (Bertino *et al.*, 1964). The present study has confirmed the finding that folate is reduced much less rapidly than $FH_2$, and to a significant extent only at an acid *p*H. Activity with NADH was observed only with $FH_2$ as a substrate in the acid range and was less than 10% of that observed with NADPH.

*Substrate Specificity.* The ability of the purified enzyme preparation to catalyze the reduction of several pteridine, pteroyl, and pteroylglutamate derivatives and their dihydro analogs was tested. The rates of reduction obtained with the nonreduced compounds as compared to folate are shown in Table II. Replace-

TABLE II: Analogs of Pteroyl-L-glutamate and Simple Pteridines as Substrates for $FH_2$ Reductase.[a]

| Substrate Tested | Δ Absorbance (340 mμ) |
|---|---|
| Folate | 0.103 |
| Pteroyltriglutamate | 0.080 |
| 9-Methylfolate | 0.048 |
| $N^{10}$-Methylfolate | 0.072 |
| $N^{10}$-Formylfolate | 0.005 |
| 3′,5′-Dichlorofolate | 0.071 |
| 3′,5′-Dibromofolate | 0.086 |
| Pteroate | 0.047 |
| $N^2$,$N^2$-Dimethylfolate | 0.005 |
| 2-Deamino-2-hydroxyfolate | 0.015 |
| Biopterin | 0.005 |
| 2-Amino-4-hydroxy-6-methylpteridine | 0.005 |

[a] Each reaction mixture contained: sodium citrate buffer, 100 μmoles, *p*H 5.0; KCl, 150 μmoles; NADPH, 0.06 μmole; and 0.7 ml of enzyme in a total volume of 1.0 ml. The substrate, 0.08 μmole, in a solution containing 10 μmoles of 2-mercaptoethanol, was added last to initiate the reaction. The final *p*H recorded was 5.25. Appropriate blanks (minus substrate and minus NADPH) were run for each substrate tested. The decrease in absorbance at 340 mμ was followed for 4 minutes; if no appreciable reaction was observed the reaction was followed for an additional 10-minute period.

ment of the 2-amino group by a hydroxyl group as in 2-deamino-2-hydroxyfolate, or substitution of the hydrogens of this amino group with methyl groups as in $N^2$,$N^2$-dimethylfolate, resulted in almost complete loss of activity even when these compounds were tested at the dihydro level (Table III), nor were these compounds capable of inhibiting enzyme activity (Johns *et al.*, 1964). Biopterin and 2-amino-4-hydroxy-6-methylpteridine did not have measurable substrate activity. Formylation of the nitrogen at position 10 of the folate molecule resulted in a compound with no measurable substrate activity even after reduction to the dihydro level (Table III). Of interest, however, was the ability of this compound to act as a powerful competitive inhibitor (Table IV). Pteroyltriglutamate, $N^{10}$-methylfolate, 3′-5′-dichlorofolate, and 3′,5′-dibromofolate had almost as much substrate activity as folate; 9-methylfolate and pteroate were reduced at approximately half the rate of folate.

TABLE III: Activity of $FH_2$ Reductase with Dihydropteridine Compounds.[a]

| Substrate Tested | Δ Absorbance (340 mμ) (−) KCl | (+) KCl |
|---|---|---|
| $FH_2$ | 0.125 | 0.200 |
| $N^{10}$-Methyl-$FH_2$ | 0.380 | 0.460 |
| $N^{10}$-Formyl-$FH_2$ | 0.000 | 0.000 |
| 3′,5′-Dichloro-$FH_2$ | 0.280 | 0.260 |
| 3′,5′-Dibromo-$FH_2$ | 0.240 | 0.225 |
| Dihydropteroate | 0.040 | |
| $N^2$,$N^2$-Dimethyl-$FH_2$ | 0.000 | 0.000 |
| $N^5$-Methyl-$FH_2$ | 0.000 | 0.000 |

[a] Each reaction mixture contained: Tris buffer, *p*H 7.5, 100 μmoles; KCl, 150 μmoles as indicated; enzyme, 0.1 ml; and 0.06 μmole of NADPH in a total volume of 1.0 ml. The substrate, 0.07 μmole, together with 2–4 μmoles of 2-mercaptoethanol, was added last to initiate the reaction. The decrease in absorbance at 340 mμ was recorded for 4 minutes.

TABLE IV: Inhibition of $FH_2$ Reductase by Folate Analogs.[a]

| Inhibitor | $K_i$ |
|---|---|
| Thymine | $>1 \times 10^{-4}$ |
| Pyrimethamine | $1.7 \times 10^{-8}$ |
| Adenine | $>1 \times 10^{-5}$ |
| Guanine | $>1 \times 10^{-5}$ |
| 2,6-Diaminopurine | $2.1 \times 10^{-6}$ |
| Xanthopterin | $>1 \times 10^{-4}$ |
| 6-Methyl-2-amino-4-hydroxypteridine | $>1 \times 10^{-4}$ |
| 2,4,7-Triamino-6-phenylpteridine[b] | $1.3 \times 10^{-8}$ |
| Pteroate | $8 \times 10^{-6}$ |
| Folate | $1.1 \times 10^{-7}$ |
| Dichlorofolate | $5 \times 10^{-8}$ |
| $N^{10}$-Formylfolate | $6.1 \times 10^{-9}$ |
| Methotrexate | $<6 \times 10^{-10}$[c] |

[a] Dissociation constants ($K_i$) for the enzyme-inhibitor complex (EI) were calculated from the slope obtained from a plot of $V/V_i$ versus increasing inhibitor concentration (Dawes, 1962; Zakrzewski, 1963). $V$ = maximal velocity without inhibitor; $V_i$ = velocity in the presence of inhibitor. Two different substrate concentrations were used for each compound tested; in every case the inhibition noted was competitive in nature. The reaction mixture contained Tris, *p*H 7.5, 100 μmoles; KCl, 150 μmoles; NADPH, 0.06 μmole; $FH_2$, either 0.02 or 0.06 μmole; hydroxylapatite enzyme, 0.1 ml; and inhibitor in a total volume of 1.0 ml. The substrate was added last to start the reaction after a 2-minute preincubation of the other components of the system. The $K_m$ value for $FH_2$ used in the calculation of $K_i$ was $1.3 \times 10^{-6}$ M (Bertino *et al.*, 1964). [b] Done at *p*H 6.5 with phosphate buffer instead of Tris. [c] Bertino *et al.*, 1964.

Several dihydropteroyl compounds were prepared as described under "Materials and Methods" and tested for activity as substrates; the rates obtained are compared with the rate of reduction of $FH_2$ (Table III). Of interest were the more rapid rates observed with $N^{10}$-methyl-$FH_2$, and the dihalogenated compounds as compared with $FH_2$. Inasmuch as further increases in substrate concentration did not increase the rates observed with these compounds, this effect could be attributed to a more rapid breakdown of the enzyme-substrate (ES) complex. The rates observed were dependent not only on the *p*H used, but also the buffer and salt (KCl) concentration. The most striking finding was the lack of stimulation afforded by 0.15 M KCl with the halogenated compounds as substrates; in fact, a decrease in activity was observed. These findings are presented in more detail in the accompanying paper. In agreement with the findings of Donaldson and Keresztesy (1962), 5-methyl-$FH_2$ was not reduced by $FH_2$ reductase.

*Coenzyme Specificity and Binding.* The $K_m$ values for NADPH and NADH for the purified enzyme were estimated from standard Lineweaver-Burk plots. The study of NADH was done at *p*H 5.5 with $FH_2$ as a substrate; under these conditions NADH could also act as a cofactor for $FH_2$ reduction. A much higher $K_m$ value was obtained for NADH ($2.6 \times 10^{-4}$ M) as compared to NADPH ($5.6 \times 10^{-6}$ M), explaining in part the slower rate noted with NADH as a coenzyme. However, extrapolated $V_{max}$ values for NADH were still less than the normal reaction velocity measured with NADPH.

*Product Inhibition.* $NADP^+$, but not $FH_4$, was found to inhibit significantly the reduction of $FH_2$. The inhibition of this enzyme by $NADP^+$ has not previously been noted; the demonstration that this reaction is reversible, although the equilibrium lies far toward the formation of $FH_4$ (Mathews and Huennekens, 1963), is evidence that some binding of products to the enzyme takes place. When the data obtained were plotted by the method of Dixon (1953), the inhibition with respect to NADPH was found to be competitive in nature, and a $K_i$ value of $4.9 \times 10^{-6}$ M was obtained.

*Enzyme Inhibition.* The importance of the 2,4-diamino structure for binding can be seen from the data presented in Table IV; if this structure is kept intact, significant binding will occur for pteridines such as 2,4,7-triamino-6-phenylpteridine, purines such as 2,6-diaminopurine, and even pyrimidines such as pyrimethamine. The finding that folate was a potent inhibitor of this enzyme activity, even at a *p*H at which it is an extremely poor substrate, has been noted previously (Zakrzewski and Nichol, 1960; Bertino *et al.*, 1964).

Pteroate, a relatively poor substrate for the enzyme as compared to folate, was found to have a higher dissociation constant than folate, while dichlorofolate had a slightly lower dissociation constant as compared to folate. $N^{10}$-Formylfolate, as mentioned previously, was not active as a substrate; however the $K_i$ value obtained is remarkably low for a compound that does not have a 2,4-diamino structure, and the $K_i$ value obtained is only about 10-fold greater than that obtained for methotrexate.

*Gel Filtration of the Enzyme-Inhibitor (E-I) Complex on Sephadex G-25.* The effect of *p*H and salt concentration on tightness of binding of methotrexate to the ascites cell enzyme was studied further by Sephadex chromatography of the E-I complex (Table V). Dissociation of the E-I complex was noted when chromatography was performed at *p*H 7.5 in the presence of KCl; without KCl less dissociation occurred. At *p*H 5.5, even in the presence of KCl, little dissociation was noted; when isotonic sucrose was utilized to elute the E-I complex, little if any free inhibitor was separated from the enzyme.

TABLE V: Separation of Methotrexate from Dihydrofolate Reductase on Sephadex G-25.[a]

| Buffer | 0.10 M KCl | Activity Regenerated (%) | Methotrexate Bound to Enzyme (%) |
|---|---|---|---|
| 0.05 M Tris-HCl, *p*H 7.5 | + | 60 | 34 |
| 0.05 M Tris-HCl, *p*H 7.5 | − | 26 | 62 |
| 0.05 M Sodium citrate, *p*H 5.5 | + | 29 | 70 |
| 0.05 M Sodium citrate, *p*H 5.5 | − | 26 | 66 |
| 0.25 M Sucrose | − | 0 | 100 |

[a] Methotrexate, $3.6 \times 10^{-4}$ μmoles, was added to 2-ml aliquots of enzyme (33 units/ml), in order to obtain 95% inhibition of enzyme activity (measured at *p*H 5.9 with citrate buffer (Bertino *et al.*, 1964), conditions otherwise as described in Table III with $FH_2$ as substrate). After incubation at room temperature for 5 minutes, 1.5 ml of the enzyme-methotrexate mixture was applied to a 2.2 × 5-cm Sephadex G-25 column, coarse grade, and elution was carried out with the indicated buffer. The entire protein peak was collected (6 ml) and assayed for dihydrofolate reductase activity as before. A 2-ml fraction of this material was then boiled for 3 minutes and assayed for methotrexate (Sartorelli *et al.*, 1964). The low molecular weight peak was also collected and assayed for methotrexate activity. Control runs of enzyme less methotrexate were performed under identical conditions and resulted in 93–100% recovery of enzyme activity.

## Discussion

The specific activity of the enzyme preparation obtained by the purification steps outlined in Table I has varied from 50 to 150 μmoles/hour per mg protein. The purity of the best enzyme preparations obtained from hydroxylapatite is estimated to be approximately 15%, if the following assumptions are made. Based on a molecular weight of 20,000, 1 mg of pure enzyme would contain $5 \times 10^{-8}$ mole. If each enzyme molecule contains one active site and one molecule of methotrexate can bind to each active site (Werkheiser, 1961; Bertino *et al.*, 1964), it would take $5 \times 10^{-8}$ mole of methotrexate to titrate completely 1 mg of enzyme. Since $5 \times 10^{-8}$ mole of methotrexate inactivates 1000 units of enzyme activity (Bertino *et al.*, 1964), then the specific activity of a completely pure enzyme would be 1000 μmoles/hour per mg. Thus an enzyme preparation with a specific activity of 150 μmoles/hour per mg would be 15% pure.

A turnover number of 270 (molecules of substrate reduced per minute per enzyme molecule at *p*H 7.5) can also be calculated. This value is in good agreement with values of 290 and 250 calculated for the enzyme from chicken liver and from a resistant subline (FR-8) of the L1210 tumor, respectively (Schrecker and Huennekens, 1964). Assuming that the turnover number of the enzyme obtained from *Streptococcus faecalis* R by Blakley and McDougall (1961) is similar to the value calculated for the ascites cell enzyme, then both preparations are of comparable purity, since the specific activity of the purified bacterial enzyme was 279 μmoles/hour per mg. Other highly purified enzymes have been obtained from chicken liver (63 μmoles/hour per mg) by Mathews and Huennekens (1963) and by Morales and Greenberg (1964), who obtained an enzyme of comparable specific activity.

The present studies of the substrate specificity of the ascites cell enzyme indicate also that this enzyme, like its counterpart in other tissues, has a broad specificity. Activity with folate as a substrate was observed at a reduced rate as compared with $FH_2$ and only at acid *p*H and with NADPH, but not with NADH. These findings are similar to those reported for avian (Mathews and Huennekens, 1963) and mammalian liver enzymes (Bertino *et al.*, 1963; Morales and Greenberg, 1964). In contrast, bacterial extracts capable of reducing $FH_2$ do not appear able to reduce folate (Blakley and McDougall, 1961; Sirotnak *et al.*, 1964; Burchall *et al.*, 1964). In agreement with studies of $FH_2$ reductase from chicken liver (Zakrzewski, 1960), activity with several nonreduced pteridines as substrates was found, including 9-methylfolate, $N^{10}$-methylfolate, and pteroate. The finding that $N^{10}$-formylfolate is a poor substrate for $FH_2$ reductase is in agreement with the results of Zakrzewski (1960) and Mathews and Huennekens (1963). $N^{10}$-Methylfolate, a weak folate antagonist

*in vivo* (Jukes *et al.*, 1950), is an excellent substrate for $FH_2$; indeed, Zakrzewski (1960) observed a greater $V_{max}$ for this compound than for folate.

The rate of reduction of $N^{10}$-methyl-$FH_2$ and the dichloro and dibromo derivatives of $FH_2$ was much more rapid than the rate of reduction of $FH_2$ when assayed as described in Table IV. Friedkin *et al.* (1962) previously observed that diiodo-$FH_2$ was more rapidly reduced by an enzyme from L1210 leukemia cells than was $FH_2$. Increasing the KCl concentration increases the activity observed with $FH_2$ as substrate; in contrast, high concentrations of KCl have little effect on the rate of reduction of $N^{10}$-methyl-$FH_2$ and actually inhibit the reduction of the halogenated dihydro compounds.

The relatively low affinity of NADH for the ascites cell $FH_2$ reductase ($K_m$ 2.6 $\times$ $10^{-4}$ M) is similar to the finding of Morales and Greenberg (1964), who obtained a $K_m$ value for this coenzyme of 0.8 $\times$ $10^{-4}$ M. Extrapolated $V_{max}$ values for this coenzyme did not give rates comparable to those obtained with NADPH, and in this respect these results differ from those of Morales and Greenberg (1964), who noted that extrapolated $V_{max}$ values for NADH gave rates similar to those obtained with NADPH for the sheep liver enzyme. The $K_m$ value of 5.6 $\times$ $10^{-6}$ M for NADPH obtained for the ascites cell enzyme is of the same order of magnitude as that reported by Morales and Greenberg (1964) for the sheep liver (1.7 $\times$ $10^{-6}$ M) and the calf thymus enzyme (4 $\times$ $10^{-6}$ M); these values may be compared to those obtained with the *Streptococcus faecalis* enzyme, which demonstrated less affinity for NADPH (4.6 $\times$ $10^{-5}$ M). The results of the experiments illustrated in Figure 4 demonstrating that $NADP^+$ is a potent competitive inhibitor of the $FH_2$ reductase from Ehrlich ascites cells emphasize the need for initial velocity measurements when Michaelis constants or dissociation constants are determined for this enzyme.

The replacement of the 4-hydroxyl group of folate or $N^{10}$-methylfolate by an amino group results in compounds (aminopterin and methotrexate) with at least 1000 times more affinity for the enzyme. The increase in affinity afforded by this substitution has allowed for the design of potent pteridine and even pyrimidine antagonists of $FH_2$ reductase (Baker *et al.*, 1964), which resemble aminopterin only in that the 2,4-diamino structure of the pyrimidine ring is kept intact. The additional substitution of an aromatic group, as in 2,4-diamino-5-chlorophenyl-6-ethylpyrimidine (pyrimethamine), further increases the binding to the enzyme. The importance of the 2,4-diamino structure for binding (2,6-diamino nomenclature in pyrimidines and purines) has been emphasized in previous studies (Zakrzewski, 1963; Baker *et al.*, 1964; Collin and Pullman, 1964), and is illustrated by the series of purines tested as analogs in Table IV. Thus, 2,6-diaminopurine had a $K_i$ of 2.1 $\times$ $10^{-6}$ M, while the $K_i$ values for adenine and guanine were greater than 1 $\times$ $10^{-5}$ M.

The importance of the 2-amino group for binding is apparent from the lack of affinity of 2-deaminofolate and $N^2$,$N^2$-dimethylfolate for the enzyme (Tables II and IV). The contribution of the glutamate portion of

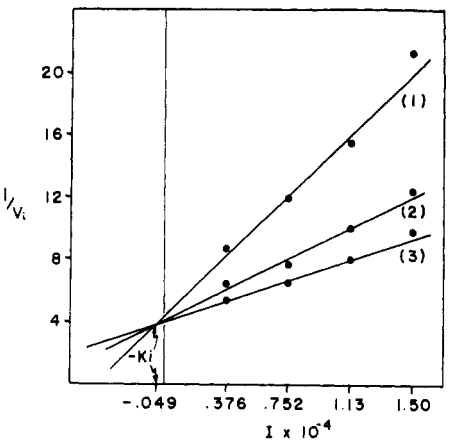


FIGURE 4: Kinetic analysis of the inhibition of $FH_2$ reductase by $NADP^+$. The assay mixture was the same as that described in Table IV except that the NADPH concentration was varied; curve (1) 3 $\times$ $10^{-5}$ M, curve (2) 6 $\times$ $10^{-5}$ M, and curve (3) 9.1 $\times$ $10^{-5}$ M. $NADP^+$ at the four concentrations indicated along the abscissa was the inhibitor used. Velocity was measured as the decrease in optical density occurring in 4 minutes at 340 m$\mu$. The reciprocal of the velocity in the presence of inhibitor ($V_i$) has been plotted against the inhibitor concentration ($I$) (Dixon, 1953).

the molecule to binding, although not a major one, nevertheless is significant as evidenced by the decrease in affinity observed with pteroate as an inhibitor. Removal of the *p*-aminobenzoic ring resulted in compounds of low affinity for the enzyme as compared to folate (e.g., 2-amino-4-hydroxy-6-methylpteridine). An apparent exception is the compound 2-amino-4-hydroxy-6-formylpteridine (Zakrzewski, 1960). The studies of Baker *et al.* (1964) show also that the 10-nitrogen is an important binding site. The substitution of a formyl group for a hydrogen at this position in the folate molecule results in a compound with a greater affinity for the enzyme than folate (Table IV). Thus, the inability of $N^{10}$-formylfolate and $N^{10}$-formyl-$FH_2$ to act as substrates is not a consequence of poor binding of these compounds to the enzyme.

The gel filtration studies with Sephadex indicate that both *p*H and salt concentration can effect binding of methotrexate to $FH_2$ reductase, and further support the previous data obtained by dialysis experiments (Bertino *et al.*, 1963; Schrecker and Huennekens, 1964). The lack of dissociation of the EI complex after passage through Sephadex also agreed with the studies of Werkheiser (1961), who noted that dialysis of aminopterin bound to rat liver $FH_2$ reductase against an isotonic sucrose solution did not result in the appearance of free inhibitor in the dialysate. These studies further point out the difficulty in comparing results of inhibition studies if the *p*H and salt concentrations are not similar. Studies of the effects of cations as well as other substances such as guanidine-HCl, thiourea, and organic mercurials on $FH_2$ reductase activity presented in the

accompanying paper indicate that these substances may cause conformational changes in the enzyme protein that in turn may affect the binding of substrates as well as inhibitors (Perkins and Bertino, 1964).

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