Purification and Properties of Folate Reductase, the Cellular Receptor of Amethopterin

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(Received May 2, 1966)

SUMMARY

Folate reductase has been purified from an amethopterin-resistant subline of cultured Sarcoma-180 cells. The molecular weight of this enzyme was estimated to be 21,000. The specific activity of the purified reductase was 3.1×10^{-8} mole of the enzyme per milligram of protein, and on this basis the preparation was calculated to be 65% pure. During free boundary electrophoresis, however, the enzyme migrates as a single peak toward the anode at pH 8.7. Both folate and dihydrofolate are substrates for this reductase, dihydrofolate being reduced much faster than folate at 30°. The Michaelis constant (K_m) and the turnover number with dihydrofolate as substrate are independent of pH between pH 4.5 and 6.3. When folate is the substrate, both K_m and the turnover number decrease with increasing pH. The enthalpy of activation is +14.7 kcal for dihydrofolate and +5.7 kcal for folate. The properties of the reductase of Sarcoma-180 are compared with those of other preparations of this enzyme from different sources.

INTRODUCTION

The reduction of folic acid is essential for the utilization of this vitamin in many metabolic reactions involving the participation of the tetrahydrofolate cofactors (1). Folate reductase (also known as dihydrofolate reductase and tetrahydrofolate dehydrogenase, EC 1.5.1.3) is inhibited by several chemotherapeutic drugs, such as the antileukemic agent amethopterin (Methotrexate) and the antimalarial agent pyrimethamine (Daraprim) (2). Although the basis for the selective action of these drugs is incompletely understood, their activity reflects the indirect inhibition of nucleic acid and protein biosynthesis ensuing from their interference with folate reductase. Thus, considerable attention has been directed in recent years to the isolation of this enzyme from different sources and to the study of its biochemical properties and physical characteristics. Only recently have others succeeded in preparing highly purified solutions of folate reductase (3–5). In our laboratory, an amethopterin-resistant subline of Sarcoma-180 has served as a useful source since these cells contain 300–400 times more folate reductase than the parent cell line (6, 7). The purification of folate reductase and estimation of its molecular weight are described in this paper, and some of the properties of the enzyme are compared with those described by others concerning the same enzyme from different sources. Some of these findings were presented in a preliminary report (8).

METHODS

Source of folate reductase. The origin and maintenance of the subline of mouse Sarcoma-180 cells (AT/3000) has been described (7). These cells are highly resistant to amethopterin (3000-fold) and contain 300–400 times more folate reductase than

the parent strain from which it was developed. These cells provide excellent starting material for isolation of folate reductase since this enzyme comprises about 4–6% of the protein in cell extracts. The cells (AT/3000) were grown for 10–12 days in amethopterin-free medium lacking folate but supplemented with hypoxanthine (100 μM), thymidine (30 μM), and glycine (30 μM) (9).

Purification of the enzyme. Cells were scraped from the glass surface of the culture flasks by means of rubber policemen, collected, and centrifuged for 5 min at 500 rpm. The cell pellet was then washed three times with twice its volume of balanced salt solution and finally centrifuged at 2000 rpm for 10 min. These "packed cells" were stored at —75°. For preparation of the cell extract, the pellet was covered with two-thirds of its volume of ice-cold saline and allowed to thaw at 5°. This caused disruption of the cells, and the debris was then removed by centrifugation at 30,000 *g* for 30 min.

The first purification step was chromatography of the crude cell extract on a column of Sephadex G-75 at 5°. Before use, the column was washed with a neutral solution of folate, 100 μg/ml, and the same solution was used also for elution of the column. The inclusion of folate in the solvent prevented partial inactivation of the enzyme. A typical elution pattern is shown in Fig. 1. Most of the protein in the cell extract appeared in the void volume. The fact that folate reductase is retarded on this column indicates that its molecular weight is much below 100,000. A column of the size described in Fig. 1 has ample capacity for cell extracts containing 60–70 mg of protein. This procedure was scaled up so that cell extracts containing close to 800 mg of protein were purified on a column 10 cm in diameter and about 35 cm long. The latter column was prepared using 200 g of Sephadex G-75 "new bead form" (Pharmacia, Uppsala, Sweden) giving a flow rate of about 10 ml/min. Thus, this fractionation could be completed in about 4 hr. For further purification, the active fractions were lyophilized and the dry powder was dissolved in 0.05 M sodium citrate to give a concentration of about 2 mg of protein per milliliter. This solution was fractionated with ammonium sulfate; the protein fraction which precipitated between 70 and 100% saturation contained all the enzymic activity.

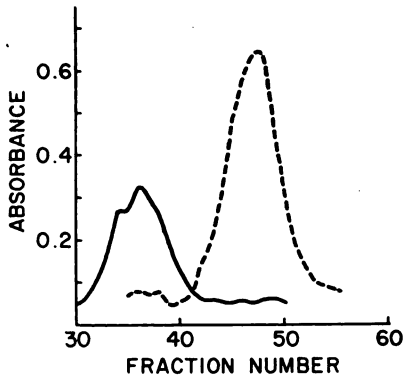

FIG. 1. *Purification of folate reductase on Sephadex G-75 column*

Column was 4.6 cm in diameter, 27 cm long; flow rate 0.35 ml/min; fractions 4.9 ml each. The solid line represents protein (absorbance at 750 mμ), the broken line the enzyme activity (absorbance at 560 mμ).

Determination of specific activity. Based on the stoichiometric binding of amethopterin by folate reductase (10), the concentration of this enzyme at different stages of purification was determined by "titration" with amethopterin as described elsewhere (6). The specific activity of the enzyme is expressed in terms of moles of amethopterin bound per milligram of protein. In these experiments, folate was used as the substrate and the rate of its reduction was determined from the amount of diazotizable amine released spontaneously from tetrahydrofolate (11). Protein was determined by the method of Lowry *et al.* (12).

Kinetic studies. Either folate or dihydrofolate was used as the substrate, and in each case TPNH was the hydrogen donor. The rate of reduction was measured by following the decrease in absorbancy at 340 mμ (11). The molar extinction coefficients (E_{340}) of 18.3×10^3 and 13.2×10^3 used with folate and dihydrofolate as substrates, respectively, were calculated from the fol-

lowing: E_{340} for the oxidation of reduced triphosphopyridine nucleotide = 6.22×10^3 (13); E_{340} for reduction of dihydrofolate = 7.2×10^3; E_{340} for reduction of folate = 5.9×10^3. The turnover numbers and Michaelis constants (K_m) were estimated by the method of Lineweaver and Burk (14).

Estimation of the molecular weight. The procedure described by Whitaker (15) was applied to the estimation of the molecular weight of folate reductase. The sources of the reference proteins were as follows: crystalline serum albumin, Nutritional Biochemicals Corporation; crystalline ribonuclease, Worthington Biochemicals Corporation; and rabbit γ-globulin, Pentex Incorporated. Ovalbumin was a gift from Dr. A. Nisonoff, Department of Microbiology, University of Illinois, Urbana, Illinois.

Electrophoresis. The electrophoresis of folate reductase was performed at 0° in a Perkin-Elmer instrument Model 38A at 200 volts and 10 ma. Sodium barbiturate of ionic strength 0.1 and pH 8.7 was used as the buffer.

RESULTS

Purification of folate reductase. The purification procedure is summarized in Table 1. The crude supernatant contained 1.7 ± 0.3 × 10^{-9} mole of folate reductase per milligram of protein. A single passage of the cell extract through the Sephadex column accomplished about a 10-fold purification. The peak portion of the reductase fractions had a specific activity 2.5 × 10^{-8} M and was estimated to be more than 50% pure. The total yield at this step was 70–80% and the purity was 36%. The eluate could be lyophilized without any loss of activity. At this stage of purification, the enzyme could be dialyzed against 0.05 M sodium citrate solution overnight at 4° to remove the folic acid without loss of activity.

Fractionation of this solution with ammonium sulfate increased the specific activity about 2-fold, but at the same time, considerable loss of activity occurred (Table 1). At this stage, the preparation had a specific activity of 3.1 × 10^{-8} mole per milligram of protein and was 65% pure. When the enzyme obtained by ammonium sulfate fractionation was redissolved in 0.05 M sodium citrate and stored frozen at −5° or −80°, or lyophilized, no loss of activity was noted for a period of 3 weeks. After 2 months, the frozen preparations lost about 25%, and the lyophilized preparations about 10%, of their activity. Repeated freezing and thawing or dialysis against 0.05 M sodium citrate, however, inactivated the enzyme rapidly at this stage of purification.

Estimation of molecular weight and purity. The elution pattern of several proteins of known molecular weight from the Sephadex G-100 column is shown in Fig. 2. γ-Globulin is not retarded on this column, and thus its elution volume represents the void volume. This peak is followed by that of serum albumin (mol. wt. 70,000), ovalbumin (mol. wt. 45,000), and then folate reductase followed by ribonuclease

TABLE 1
Purification of folate reductase from S-180 subline AT/3000

Sample	Specific activity (molar equivalents of amethopterin per mg of protein)	Yield (%)	Purity[a] (%)
Original cell extract	1.7 ± 0.3 × 10^{-9}	100	3.6
Folate reductase from Sephadex G-75 column	1.7 ± 0.4 × 10^{-8}	70–80	36
$(NH_4)_2SO_4$ precipitate, at 70–100% saturation	3.1 ± 0.7 × 10^{-8}	30–40	65

[a] Assuming one binding site for amethopterin per mole of enzyme and molecular weight of 21,000, the specific activity for pure folate reductase is 4.77 × 10^{-8} mole/mg protein.

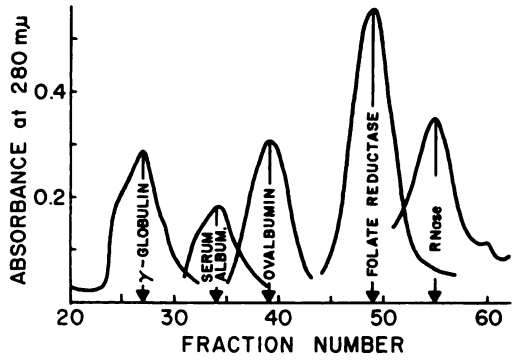

FIG. 2. *Elution pattern from Sephadex G-100 column of several proteins with known molecular weight compared with that of folate reductase*

Column was 1 cm in diameter, 92 cm long; flow rate: 0.25 ml/min; fractions 1 ml each.

(mol. wt. 13,600). A plot of V/V_0 (V = elution volume and V_0 = void volume) versus the logarithm of molecular weight is presented in Fig. 3. The molecular weight of folate reductase can be estimated from this plot to be 21,000. The limit of accuracy of this procedure is imposed by the volume of the fractions collected. Thus, the designation ± 10% indicated that if the peak were shifted by one fraction in either direction, the molecular weight would be estimated to be 19,000 or 23,000, respectively. This molecular weight agrees very well with that determined by Kaufman (23,000) for folate reductase of chicken liver (3) and by Bertino (20,200) for reductase from Ehrlich ascites cells (5). If the molecular weight and the specific activity (moles of amethopterin per milligram of protein) are known, the degree of purity of this folate reductase preparation can be estimated. If 1 mole of amethopterin is bound per mole of enzyme, then the specific activity of pure folate reductase would be $1/21{,}000 = 4.77 \times 10^{-5}$ mole of amethopterin per gram or 4.77×10^{-8} mole per milligram of protein. The specific activity of the enzyme purified by ammonium sulfate fractionation was determined to be 3.1×10^{-8} mole per milligram of protein. On this basis, the enzyme preparation is calculated to be 65% pure.

Electrophoretic purity. The electrophoretic pattern of the purified folate reductase is seen in Fig. 4. The enzyme moved toward the anode as a single peak with a mobility of 3.2×10^{-5} cm^2 sec^{-1} volt^{-1} indicating that it has a net negative charge. The photographic examination of the electrophoretic pattern indicates that the protein present is essentially homogeneous. Thus, there is an apparent discrepancy with the results presented above, which indicate that the prep-

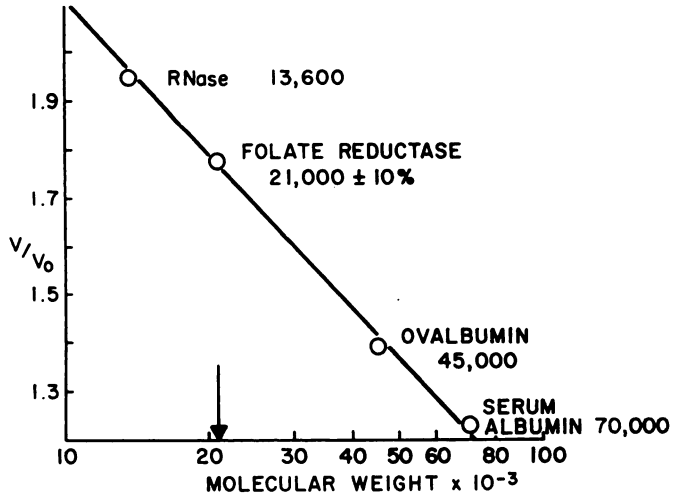

FIG. 3. *Plot V/V_0 versus log molecular weight*

V_0 = void volume, V = elution volume.

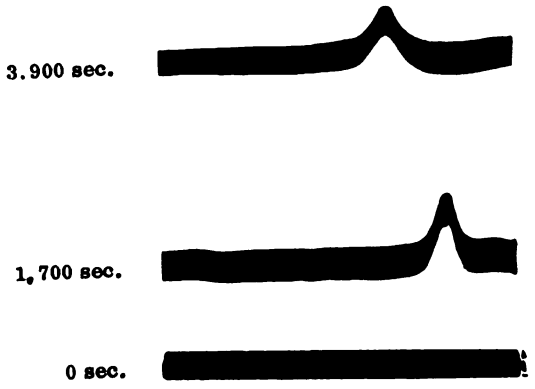

FIG. 4. *Electrophoretic pattern of folate reductase, descending boundary*

For conditions see Methods.

aration was 65% pure. One likely explanation is that the enzyme preparation is homogeneous but about one-third of its enzymic activity has been lost. The other, less likely, possibility is that the enzyme preparation is contaminated by various proteins of different mobilities, each in quantity too small to appear as a peak. The former explanation is favored because of the observed instability of the purified enzyme.

Effect of pH. Because of the instability of the highly purified enzyme preparation, all kinetic experiments were performed with the partially purified preparation (36% pure, Table 1) which was freed from folate by dialysis against 0.05 M sodium citrate. In Figs. 5 and 6 are presented double reciprocal plots at different hydrogen ion concentrations with dihydrofolate and folate as substrates. When dihydrofolate was the substrate the $1/v$ values obtained at different hydrogen ion concentrations were scattered along a single line. Thus, it appears that both K_m and turnover number of dihydrofolate are independent of pH between pH 4.5 and 6.3. The average values of K_m and turnover number were calculated to be 6.1×10^{-6} M and 226 min^{-1}, respectively. In contrast, when folate was the substrate the values of both $1/v$ and $1/K_m$ varied considerably with pH. The calculated K_m and turnover number values for folate are presented in Table 2. It is apparent that dihydrofolate is reduced at a much faster rate than folate under any of these conditions.

Effect of temperature. An attempt was made to determine the K_m and turnover number values for folate and dihydrofolate at different temperatures. At lower tem-

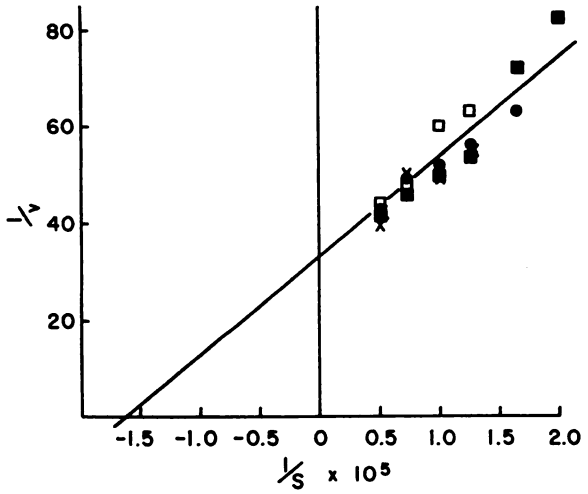

FIG. 5. *Double reciprocal plots at different pH values with dihydrofolate as substrate*

The reactions were carried out in Beckman cuvettes, 1 ml at 30° in 0.05 M phosphate-citrate buffer with 1×10^{-4} M TPNH and 1×10^{-6} M folate reductase. S = M dihydrofolate; v = decrease of absorbance at 340 mμ per minute. × = pH 4.5, ■ = pH 5.35, □ = pH 6, ● = pH 6.3.

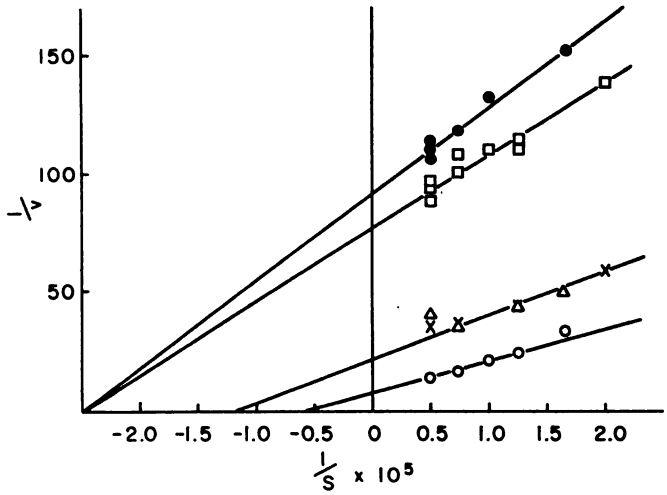

FIG. 6. *Double reciprocal plots at different pH values with folate as substrate*

Conditions as in Fig. 5 except that the concentration of TPNH was 2×10^{-4} M and that of folate reductase 1×10^{-7} M. S = M folate; v = decrease of absorbance at 340 mμ per minute. ○ = pH 4, × = pH 4.5, △ = pH 4.85, □ = pH 6, ● = pH 6.3.

TABLE 2

Michaelis constants (K_m) and turnover numbers for folate at different pH values[a]

pH	K_m (M × 10^6)	Turnover number (min^{-1})
4.0	17.9	68.2
4.5	8.7	24.8
4.9	8.7	24.8
6.0	4.0	7.2
6.3	4.0	5.2

[a] The experimental conditions are described in the legend to Fig. 6.

peratures (10°–20°), the slope of the plots of $1/v$ versus $1/S$ was very shallow within the concentrations of substrate tested (5 to 20×10^{-6} M) indicating that either the K_m values at these temperatures became indeed very small or that slight substrate or product inhibition occurred at higher substrate concentrations. Because of this uncertainty, the K_m values at 10–20° were not evaluated. However, the turnover number values could be estimated (Table 3).

The logarithms of the turnover numbers were plotted against the reciprocal of the absolute temperature for both substrates,

TABLE 3

Turnover number of folate and dihydrofolate at different temperatures

The reactions were carried out in Beckman cuvettes (1 ml) in 0.05 M phosphate-citrate buffer pH 5.35. TPNH and enzyme concentrations were: 2×10^{-4} M and 1×10^{-7} M, respectively, with folate as substrate or 1×10^{-4} M and 1×10^{-8} M, respectively, with dihydrofolate as substrate.

Temperature (C°)	Turnover number (min^{-1})	
	Folate	Dihydrofolate
10.7	6.45	50
20.3	10.93	99
30.3	10.93	269
40.0	16.10	623

folate and dihydrofolate (Fig. 7). The enthalpy of activation (ΔH_a) was calculated from the slopes of the lines according to the equation:

$$\Delta H_a = \text{slope} \times 2.303R$$

The entropy of activation (ΔS_a) was calculated according to the Eyring equation:

$$k = \frac{RT}{Nh} \times e^{\Delta S_a/R} \times e^{\Delta H_a/RT}$$

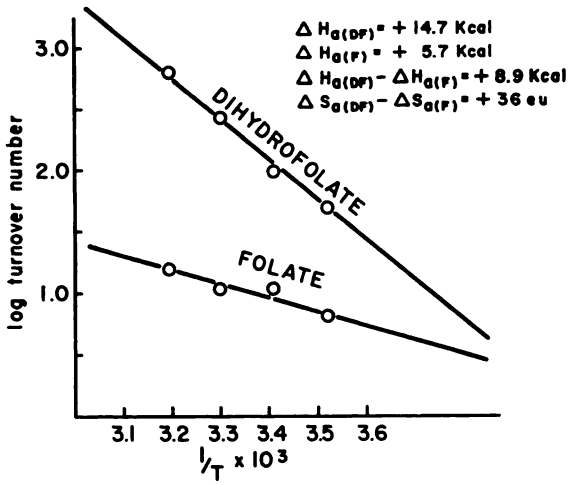

FIG. 7. *Plot of logarithm of turnover number versus reciprocal of absolute temperature (T)*

$\Delta H_a^{\ddagger}$ = enthalpy of activation, $\Delta S_a^{\ddagger}$ = entropy of activation; subscript (DF) = dihydrofolate as substrate, subscript (F) = folate as substrate; eu = entropy units.

where k is the rate constant (turnover number), R is the gas constant, h is Planck's constant, N is Avogadro's number, and T is the absolute temperature. Although at temperatures above 0°, dihydrofolate is reduced much faster than folate, nearly 9 kcal more is required to activate dihydrofolate than folate. More energy than needed is supplied by a large positive change in the entropy of activation.

DISCUSSION

Folate reductase is now recognized as the cellular receptor of the antileukemic drug, amethopterin, and other diamino antagonists of folic acid. The initial identification of the metabolic site of action of amethopterin as the impairment of the reduction of folic acid both *in vivo* and *in vitro* (16) was followed by the finding that the particular reaction inhibited by this drug is the transfer of hydrogen from TPNH to folate (11, 17) or dihydrofolate (18). At first it appeared that two enzymes may be inhibited by this drug and this impression was accentuated by the use of different names for the enzyme in published reports, related primarily to the choice of the substrate used in different laboratories. Evidence was soon found, however, indicating that a single enzyme mediates the reduction of both folate and dihydrofolate (19). Some insight into the mechanism of this reaction was brought about by experiments involving tritium and deuterium labeling. These experiments indicate that the enzymic reduction of 7,8-dihydrofolate involves an intramolecular rearrangement presumably to 5,8-dihydrofolate which is followed by the transfer of a hydride ion from TPNH to position 7 of dihydrofolate (20). Thus, it is suggested that when folate is the substrate the first step of the reduction is the transfer of a hydride ion to position 7 of folate to form 7,8-dihydrofolate. Subsequently rearrangement takes place and the second hydride ion from TPNH is transferred again to position 7 to form tetrahydrofolate (20). Thus, the enzyme would catalyze the same transfer reaction twice to accomplish the two-step reduction of folate to tetrahydrofolate. There is substantial evidence that all the amethopterin retained in tissues (10) and cell cultures (6) exposed to this drug can be accounted for completely by the amount bound to folate reductase. Thus, in addition to its intimate involvement with the mode of action of certain antileukemic and antimalarial drugs, folate reductase is a particularly good model for the detailed study of this enzyme molecule as a drug receptor in different types of cells.

Folate reductase from S-180 cells resembles closely reductases obtained from other mammalian, avian, or some bacterial cells (2–5, 21, 22). The different preparations of folate reductase which have been studied in detail have the following common properties: (a) the proteins are of relatively low molecular weight (about 20,000); (b) both folate and dihydrofolate are substrates, the latter being reduced more rapidly than the former; (c) TPNH rather than DPNH is used as the hydrogen donor; and (d) 4-amino-analogs of folate are strong inhibitors. From the point of view of cancer chemotherapy, it is of significance that no striking differences in the sensitivity of the reductase from different species, as well as from normal and malignant tissues of the same species, toward the 4-amino analogs of folate have been reported. A more detailed examination of folate reductases from various sources, reveals differences which may be of significance in achieving selective, therapeutic effects by other types of folate antagonists.

The inhibitory effect of various substituted 2,4-diaminopyrimidines on folate reductase preparations from different bacterial and mammalian cells was studied by Hitchings and Burchall (2). Their extensive studies led to the conclusion that the binding sites which interact with the 2,4-diaminopyrimidine structure are common to all the reductases. An additional binding region seemed to be species specific. Geographically it corresponds to the area in the vicinity of the *p*-aminobenzoyl group of folate. Thus, 2,4-diamino-5,6-disubstituted pyrimidines and triazines which differed from each other with respect to the substituents at positions 5 and 6 exhibited different inhibitory activity for reductases from different species. In certain cases, very large differences (up to 10,000-fold) in the inhibition constants were found when bacterial and mammalian enzymes were compared. Recently, Sirotnak *et al.* (23) developed some antifolic resistant strains of *Diplococcus pneumoniae*. The reductases in these strains differed to some extent in the kinetics of inhibition by 4-amino analogs of folate.

Another type of difference not necessarily related to the binding site of the enzyme becomes apparent by comparing the present results with investigations reported by others. For the enzyme from S-180 cells, the turnover number and K_m for dihydrofolate are independent of pH. This is in contrast to the results obtained with the reductase of chicken liver, for which the K_m value for dihydrofolate decreases with increasing pH (at pH 5.2 $K_m = 8.6 \times 10^{-6}$ and at pH 7.5 $K_m = 5 \times 10^{-7}$) (2). Although no other studies concerning dependence of K_m and turnover number on pH have come to our attention, the relationship between enzyme activity and pH has been extensively investigated, and in each case the activity varied with pH (1, 24–26).

Two groups of investigators have described a 4- to 5-fold stimulation of dihydrofolate reductase activity by organic mercurials in enzyme preparations of avian and mouse origin (3, 4, 27, 28). In contrast, folate reductase of S-180 cells lost both its activity and amethopterin binding capacity when treated with *p*-chloromercuribenzoate (29). Since all these studies were made under different conditions, a direct comparison is difficult, and it seems premature on this basis to suggest differences between the enzymes. That the SH group(s) involved in the inactivation by *p*-chloromercuribenzoate is indeed located at the active site is indicated by its protection with substrate, coenzyme, or inhibitor (29, 30).

Another interesting characteristic of the reductase from S-180 is apparent when the activation enthalpy for folate and dihydrofolate are compared (Fig. 7). Whether this difference in the energy required for activation is unique for the reductase from S-180 cells is not known at present, since no comparable studies with the reductases from other sources have been described. The low enthalpy of activation for folate (5.7 kcal) in the present system, however, is in sharp contrast to the enthalpy of activation observed for folate (18 kcal) with the reductase enzyme from chicken liver (S. F. Zakrzewski, unpublished data).

Obviously, more comparative data are needed in order to ascertain differences

between the reductases from normal and malignant tissues. On the basis of the evidence presently available, it appears that although most of the reductases from different sources are grossly similar, subtle differences are indicated which might be exploited as a basis for chemotherapy. It is suggested that in addition to the inhibitors which interact with substrate binding sites on the enzyme, other inhibitors which may affect the conformation of the enzyme should also be developed and investigated.

ACKNOWLEDGMENT

The authors are indebted to Dr. Peter Stelos for performing the free boundary electrophoresis and to Mrs. Jadwiga Drobniak and Mrs. Marcia Berggren for their capable technical assistance. This investigation was supported in part by research grants (CA-02906 and CA-04175) from the National Cancer Institute of the United States Public Health Service.

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