Dihydrofolate Reductase from *Lactobacillus leichmannii*. I. Purification and Characterization*

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ABSTRACT: Dihydrofolate reductase has been partially purified from extracts of *Lactobacillus leichmannii*. This enzyme is similar to the dihydrofolate reductase obtained from animal tissues in that it reduces both folate and dihydrofolate and utilizes either NADPH or NADH as the hydrogen donor.

In contrast to the animal reductases, this bacterial enzyme was not activated by urea, organic mercurials, or polyamines. The pH optimum of the purified bacterial enzyme is similar to the optimum for urea or mercuric ion "activated" enzyme from animal sources.

Tetrahydrofolic acid is involved in a variety of reactions of one-carbon metabolism (Friedkin, 1963). The last step in the biosynthesis of tetrahydrofolate is the reduction of dihydrofolate by the enzyme dihydrofolate reductase. A single enzyme, characterized from chicken liver (Zakrzewski and Nichol, 1960; Mathews and Huennekens, 1963), sheep liver (Morales and Greenberg, 1964), and Ehrlich ascites tumor cells (Bertino *et al.*, 1964), carries out the reduction of both folate and dihydrofolate to tetrahydrofolate. In contrast, dihydrofolate reductase, after purification from *Diplococcus pneumoniae* (Sirotnak *et al.*, 1964), and *Escherichia coli*, *Staphylococcus aureus*, and *Proteus vulgaris* (Burchall and Hitchings, 1964) reduced only dihydrofolate. Although folic acid was not a substrate, it inhibited the reduction of dihydrofolate by an enzyme preparation from *Streptococcus faecalis* (Blakley and McDougall, 1961).

To investigate a possible difference in folic acid metabolism between bacterial and animal systems, the properties of dihydrofolate reductase from *Lactobacillus leichmannii* were examined. This microorganism requires, for cell division, the addition of folic acid or the products of tetrahydrofolate-catalyzed reactions to the culture medium (Shive *et al.*, 1948; Roberts and Nichol, 1962). Growth is inhibited by folic acid antagonists.

In order to avoid interference by oxidases present in the crude homogenates (Roberts, 1961) enzyme activity was measured, in the early stages of purification, at an alkaline pH with dihydrofolate as substrate by the method of Bertino *et al.* (1960). The enzymatic reduction of folic acid could be measured during the later stages of enzyme purification.

Materials and Methods

Chemicals. Folic acid, nucleotides, and amino acids were obtained from Calbiochem and Mann Research Laboratories. Folic acid and methotrexate (4-amino-4-deoxy-N^{10}-methylpteroylglutamate) were purified on diethylaminoethyl- (DEAE-) cellulose columns (Oliverio, 1961). Peptone and yeast extract were purchased from Nutritional Biochemicals Corp. Dihydrofolic acid (DHFA) was prepared by the method of Friedkin *et al.* (1962). Solutions of nicotinamide–adenine dinucleotide phosphate, reduced (NADPH),[1] and nicotinamide–adenine dinucleotide, reduced (NADH), were prepared in pH 10 bicarbonate buffer and stored at 4° (Lowry *et al.*, 1961).

Growth of Cultures. L. leichmannii, ATCC 7830, was obtained from the American Type Culture Collection. Cultures were grown in medium containing, per liter, 10 g of dextrose, 7.5 g of peptone, 7.5 g of yeast extract, 2 g of potassium phosphate, and 1 ml of Tween 80. The medium was adjusted to pH 6.8 before autoclaving.

Purification of Formyltetrahydrofolate Synthetase. Formyltetrahydrofolate synthetase, E.C. 6.3.4.3., which is required in the coupled enzyme assay for dihydrofolate reductase, was prepared from frozen chicken liver. The liver was homogenized in four volumes of 0.1 M phosphate buffer at pH 7.0. After clarification by centrifugation at 15,000 × *g* for 20 min and by filtration through cheese cloth, the supernatant fluid was diluted with 0.3 volume of 2% protamine sulfate. The precipitate was removed by centrifugation, and the supernatant fluid was adjusted to pH 7.0 with 0.1 M NaOH and diluted to contain 10 mg of protein/ml. Ammonium sulfate was added to 35% of saturation. The resulting precipitate was washed once with a 35% solution of ammonium sulfate and dissolved in two volumes of 0.1

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[1] Abbreviations used in this work: DHFA, dihydrofolic acid; NADH and NADPH, reduced nicotinamide–adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate, respectively.

M Tris buffer at pH 7.0. The enzyme was stable at −20° for several months. No single portion was thawed and refrozen more than four times. The preparation has maximum formyltetrahydrofolate synthetase activity at pH 8.5, similar to the value reported for the enzyme from *Clostridium cylindrosporum* (Himes and Rabinowitz, 1962). Enzyme activity falls to 50% of maximum at pH 7.0 and 9.2.

Assays for Dihydrofolate Reductase

Method 1. Tetrahydrofolate, the product of the dihydrofolate reductase catalyzed reaction, was converted to N^{10}-formyltetrahydrofolate by formyltetrahydrofolate synthetase (Bertino *et al.*, 1960). The N^{10}-formyltetrahydrofolate was converted to N^5,N^{10}-methenyltetrahydrofolate by the addition of acid, and the absorbancy of this compound was measured at 355 mμ. When this method was applied to whole cells, toluene was included in the reaction medium to alter the permeability of the cells and to permit the substrate to penetrate the cell.

Method 2. The reduction of dihydrofolate and simultaneous oxidation of NADPH were measured spectrophotometrically by observing the decrease in optical density at 340 mμ, as described in the legend to Table II (Mathews *et al.*, 1963). Addition of excess mercaptoethanol failed to increase the rate of reduction of folate or dihydrofolate by the purified enzyme.

Method 3. The oxidation of reduced pyridine nucleotide during reduction of folate or dihydrofolate may be followed by measurement of the fluorescent product formed by alkaline degradation of the oxidized pyridine nucleotide (Lowry *et al.*, 1957).

Method 4. The reduction of folic acid could be measured by hydrolyzing the tetrahydrofolate to *p*-aminobenzoylglutamate (Werkheiser *et al.*, 1962) for measurement by the Bratten–Marshall assay. This assay cannot be used with dihydrofolate as substrate since dihydrofolate also forms *p*-aminobenzoylglutamate under the conditions of the assay (Mathews and Huennekens, 1963).

Results

Dihydrofolate Reductase in Whole Cells. Cells from aliquots of *L. leichmannii* cultures were collected by centrifugation and resuspended in the coupled enzyme assay mixture described in the legend to Table I. Reduction of dihydrofolate could be measured with 0.25 mg of wet cells. No reaction was detected if toluene, NADPH, *L. leichmannii*, or dihydrofolate were omitted from the assay medium. The endogenous level of formyltetrahydrofolate synthetase was rate limiting in the reaction unless the assay medium was supplemented with additional enzyme. Under the conditions of the assay, the rate of absorbancy change was proportional to the dihydrofolate reductase concentration. Methotrexate inhibited the reaction, and the complete system plus methotrexate was generally used as a blank for the enzyme reaction. Dihydrofolate contributed significantly to the blank. Cells from exponentially growing cultures reduced dihydrofolate at the rate of 1.0 mμmole/mg of wet cells per minute.

TABLE I: Dihydrofolate Reductase in *Lactobacillus leichmannii*.[a]

System	OD at 355 mμ
1. Complete	0.575
2. Omit toluene	0.300
3. Omit NADPH	0.330
4. Omit *L. leichmannii*	0.325
5. Omit dihydrofolate	0.085
6. Omit formyltetrahydrofolate synthetase	0.400
7. Add methotrexate	0.330

[a] Freshly collected or frozen cells (0.25 mg wet weight) were resuspended in 0.25 ml of medium containing 1 μl of toluene, 4 μl of a preparation of formyltetrahydrofolate synthetase from chicken liver, 5 μmoles of ATP, 50 μmoles of NH_4Cl, 20 μmoles of $MgCl_2$, 50 μmoles of sodium formate, 0.3 μmole of dihydrofolate, 0.4 μmole of NADPH, and 100 μmoles of Tris at pH 8.3. Tube 7 contained, in addition, 0.1 μg of methotrexate. After incubation at 37° for 30 min, 0.2 ml of 20% trichloroacetic acid was added to each tube; the denatured protein was removed by centrifugation, and the optical density of the product was measured, after 20 min, at 355 mμ.

Methotrexate, a folic acid antagonist, inhibited growth of cultures in B_{12} assay medium (Difco).[2] The bacterial dihydrofolate reductase of a culture was inhibited at a drug level of 10^{-9} g/ml (about 1000 molecules of drug/cell). At this drug level, the enzyme level was depressed by about 95% as measured by Method 1. Methotrexate was also an inhibitor of the enzyme in cell-free systems, as will be shown later.

Purification of Enzyme. For enzyme purification, large batches of cells were grown at 37° in 8-l. flasks. The cells were collected by centrifugation at 4°, washed with 0.9% NaCl, and frozen. The frozen cell paste was thawed and poured into 20 volumes of acetone at −15°, and 25 g of powder was collected by vacuum filtration on Whatman No. 54 paper and dried. Extracts of acetone powders, which contained all of the dihydrofolate reductase of whole cells, were used for purification. The acetone powder was extracted by stirring for 20 min with 10 volumes of 0.05 M phosphate buffer at pH 7.0. This, as well as all subsequent steps, was carried out at 4°. The extract was clarified by centrifugation at

[2] B_{12} assay medium contains vitamins, amino acids, and other nutrients necessary to support growth of *L. leichmannii*. Vitamin B_{12} (2 μg/l.) was added for growth of the cultures. The inhibition by methotrexate of cultures growing on this medium may be reversed to a large extent by supplementing the medium with thymine, 20 μg/ml.

TABLE II: Purification of Dihydrofolate Reductase.[a]

Procedure	Enzyme Recovery (%)	Total Protein (mg)	Enyzme Activity Dihydrofolate[b]	Enyzme Activity Folate[c]	Dihydrofolate[d]/ Folate
1. Intact cells	100		40	—	—
2. Acetone powder extract	100	4000	150	—	—
3. Streptomycin dialysis	95	1200	475	—	—
4. Ammonium sulfate 55-75%	50	300	1000	8	125
5. Sephadex G-100	20	35	3500	30	118
6. Sephadex G-100 (recycled)	14.5	11	7880	64	122
7a. DEAE Sephadex A-25	9	1.5	36100	295	123
7b. TEAE-cellulose	1.0	0.1	62500		

[a] Protein was estimated by measurement of optical density at 280 mμ. [b] Dihydrofolate: units/min/mg protein. One unit is defined as the quantity of enzyme necessary to cause a drop in absorbancy at 340 mμ, at 37°, of 0.001 per minute. Each cuvet contained 50 μmoles of phosphate buffer at pH 7, 0.05 μmole of dihydrofolate, and enzyme in a total volume of 300 μl. Reaction velocity was measured by observing the drop in optical density at 340 mμ in a Zeiss spectrophotometer PMQ II with the cell chamber at 37°. A blank value was obtained by incubating a similar cuvet with dihydrofolate omitted. [c] Folate: units/min/mg protein. One unit is defined as the quantity of enzyme necessary to cause an increase in absorbancy at 560 mμ of 0.001 per minute. Each cuvet contained 48 mμmoles of NADPH, 36 mμmoles of folate, 25 mμmoles of 3,3-dimethylglutarate, at pH 6.1, 2.5 μmoles of $MgCl_2$, 0.5 μmole of mercaptoethanol, and 2.5 μmoles of sodium citrate in a total volume of 250 μl. After incubation for 30 min at 37°, the mixture was treated with 250 μl of 20% trichloroacetic acid, the precipitated protein removed by centrifugation, and the supernatant fluid treated with successive addition, at 3-min intervals, of 20 μl each of 0.63% KNO_2, 2.5% ammonium sulfamate, and 0.5% *N*-(1-naphthyl)ethylenediamine dihydrochloride. After a final centrifugation, optical density was determined at 560 mμ. A control tube contained, in addition to the above materials, 1 μl of a 10 μg/ml solution of methotrexate. [d] Ratio of enzyme activity with these two substrates.

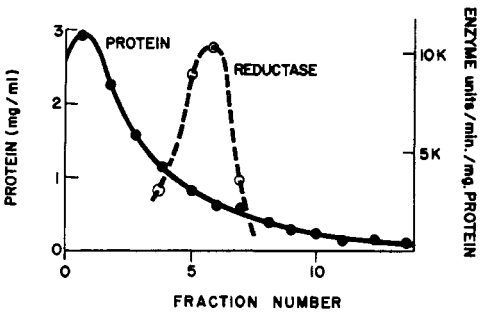

FIGURE 1: Elution profile of enzyme from Sephadex G-100. Dihydrofolate reductase was estimated by Method 2. Protein was eluted with 0.01 M phosphate buffer, pH 7.0.

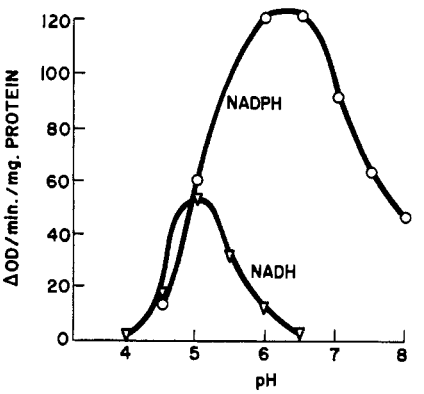

FIGURE 2: pH curve of the reduction of dihydrofolic acid as a function of the reduced pyridine nucleotide. Dihydrofolate reductase was estimated by Method 2.

20,000 × *g* for 20 min, and the supernatant fluid was treated with one-quarter volume of a 5% solution of streptomycin sulfate. The resulting mixture (300 ml) was dialyzed overnight against 10 l. of 0.01 M phosphate buffer at pH 7.0. The overnight dialysis resulted in denaturation and precipitation of a significant amount of protein. After a precipitate, which formed with dialysis, was removed by centrifugation at 20,000 × *g* for 20 min, ammonium sulfate was added to 55% saturation. The resulting precipitate was discarded, and ammonium sulfate was added to 75% saturation. This precipitate was collected and dissolved in 10 ml of 0.01 M phosphate buffer at pH 7.0. This preparation was fractionated on a 3 × 45 cm column of Sephadex G-100; the major portion of the protein was eluted with 0.01 M phosphate buffer at pH 7.0 (Figure 1). The eluate was collected in 10-ml fractions, and the tubes containing enzyme activity as judged by the assay of Method 2 were pooled and lyophilized. The enzyme was redissolved in 5 ml of water and recycled through a 2 × 15

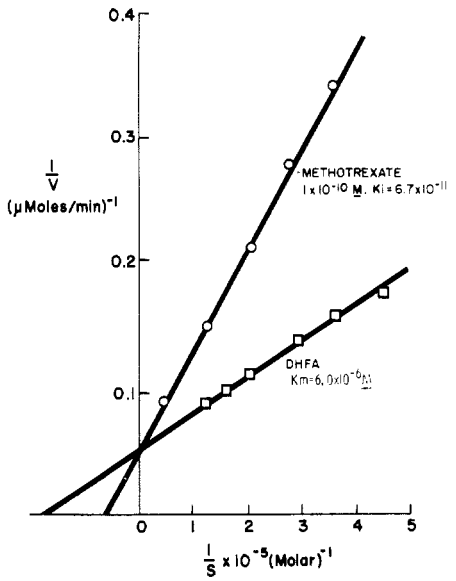

FIGURE 3: Lineweaver–Burk plots of purified dihydrofolate reductase with dihydrofolate (DHFA) as substrate, showing effect of methotrexate inhibition. The assay was by Method 4.

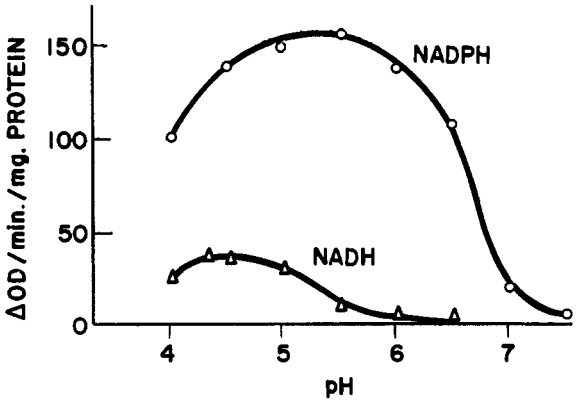

FIGURE 4: pH curve of the reduction of folic acid as a function of the reduced pyridine nucleotide. The assay was by Method 4.

cm column of Sephadex G-100. Protein was eluted with 0.001 M phosphate buffer at pH 7.0 and collected in 3-ml fractions.

The tubes containing enzymatic activity from the second Sephadex column were concentrated by lyophilization. The enzyme was redissolved in 1 ml of water and fractionated on a 1 × 15 cm column of DEAE-Sephadex A-25, previously equilibrated with 0.001 M phosphate buffer at pH 7.5. The dihydrofolate reductase was eluted with 5 ml of 0.001 M phosphate buffer at pH 7.5. Greater purification could be achieved on columns of triethylaminoethyl- (TEAE-) cellulose (Table II), but with considerably greater losses.

Coenzyme and Substrate Specificity. For Figures 2–6, enzyme purified through step 6 (Table II) was used. Dihydrofolate was optimally reduced at pH 6.3 with NADPH as the hydrogen donor. With NADH, optimal activity is at pH 5.2 (Figure 2). Both methotrexate and folic acid were competitive inhibitors of the reduction of dihydrofolate. The Lineweaver–Burk plot of enzyme activity at various concentrations of dihydrofolate with NADPH as hydrogen donor is shown in Figure 3, together with the data obtained with methotrexate present. The Michaelis constant for dihydrofolate was 6×10^{-6} M. Other reported K_m values are 4×10^{-6} M from *S. faecalis* (Blakley and McDougall, 1961), 5×10^{-7} M from chicken liver (Mathews and Huennekens, 1961), 1.2×10^{-6} M from sheep liver (Morales and Greenberg, 1964), 2×10^{-7} M from rat liver (Wang and Werkheiser, 1964), 2.5×10^{-5} M from three microorganisms (Burchall and Hitchings, 1964), and 1.5×10^{-6} M for Ehrlich ascites tumor cells (Bertino *et al.*, 1964).

With dihydrofolate as substrate, dihydrofolate reductase was inhibited by methotrexate ($K_i = 6.7 \times 10^{-11}$ M) and by folate ($K_i = 1.4 \times 10^{-5}$ M). With dihydrofolate as substrate, the Michaelis constant for NADPH was 3.8×10^{-5} M and for NADH it was 3×10^{-4} M. The following constants are offered for comparison: K_m for NADPH = 4.6×10^{-5} M (*S. faecalis*), $K_m = 1.7 \times 10^{-6}$ M (sheep liver); K_m for NADH = 0.8×10^{-4} M (sheep liver).

Enzymatic reduction of folate in crude extract was masked by enzymes which led to oxidative degradation of folate (Roberts, 1961). After partial purification of dihydrofolate reductase, the reduction of folate could be measured by Method 4. Reduction of folate by the purified reductase occurred optimally at pH 5.5, with NADPH as hydrogen donor (Figure 4). With NADH, optimal activity occurred at pH 4.5. The Michaelis constants for folate were calculated from Lineweaver–Burk plots. Values obtained were $K_m = 1.8 \times 10^{-5}$ M with NADPH as hydrogen donor (Figure 5); for comparison: $K_m = 7.9 \times 10^{-6}$ M for chicken liver (Zakrzewski and Nichol, 1960), $K_m = 7.4 \times 10^{-6}$ M for Ehrlich ascites tumor cells (Bertino *et al.*, 1964), $K_m = 1 \times 10^{-5}$ M for Sarcoma 180 (Hakala *et al.*, 1961). With folate as substrate, Michaelis constants for the pyridine nucleotides were obtained: K_m for NADPH = 8×10^{-5} M, K_m for NADH = 4×10^{-4} M. Reaction rates were measured at the pH optimum in each case. Stoichiometric inhibition of reductase activity by methotrexate was observed with folate as substrate as described with enzyme from rat liver (Werkheiser *et al.*, 1962).

Enzyme Activation. Some mammalian and avian dihydrofolate reductase preparations were activated, at pH 7.0, by 4 M urea (Kaufman, 1963), organic mercurials (Kaufman, 1964; Perkins and Bertino, 1964), and polyamines (Misra and Adamson, 1963). When effective, these agents raised the pH optimum of the enzyme from 5 to 7 and increased the V_{max} of the enzyme at the pH optimum. None of these compounds had any measurable effect on the reductase from *L. leichmannii*. Urea-treated enzyme from chicken liver (Kaufman,

TABLE III: Comparison of Kinetic Constants of Dihydrofolate Reductase.

Enzyme Source	Turnover Numbers[a]		Michaelis Constant (K_m)[b]	
	Folate	Dihydrofolate	Folate	Dihydrofolate
L. leichmannii	9.5	1180	180×10^{-7}	83×10^{-7}
Rat liver[c]	7.6	137	30×10^{-7}	2×10^{-7}

[a] Moles/min/mole methotrexate equivalent of enzyme. [b] Moles/liter. [c] K_m for dihydrofolate is from data of Wang and Werkheiser (1964); other data are from unpublished observations by D. Roberts.

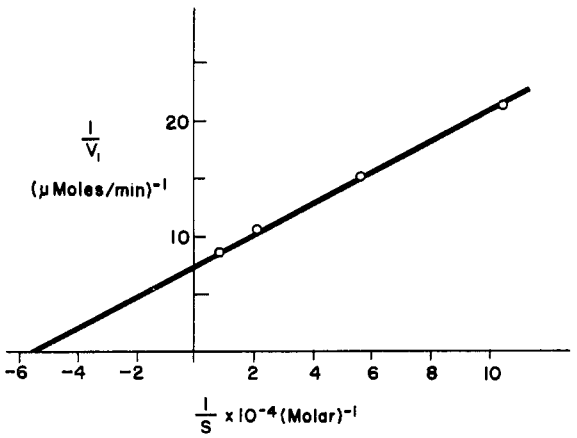

FIGURE 5: Lineweaver–Burk plot of purified dihydrofolate reductase with folic acid as substrate. The assay was by Method 2.

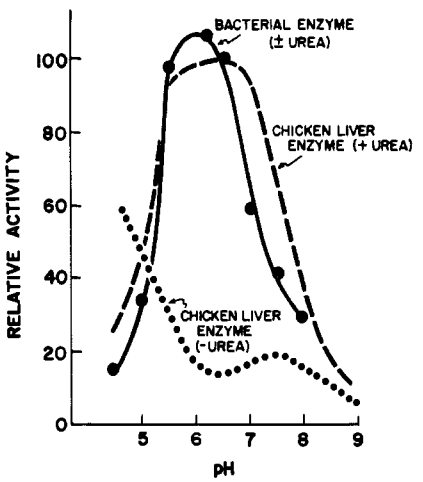

FIGURE 6: Comparison of pH optima and relative activity of bacterial and avian enzyme. The avian enzyme data is from observations by Kaufman (1963) and is reproduced with permission. The assay was by Method 2.

1963) exhibited a pH optimum remarkably similar to the untreated enzyme from *L. leichmannii* (Figure 6). Addition of a variety of deoxynucleotides[3] at 10^{-3} M failed to activate or inhibit the purified bacterial enzyme as measured by Methods 2 and 3. Addition of amino acids,[4] organic mercurials, fluorodinitrobenzene, iodoacetate, and spermine, at a variety of concentrations, was without effect. Addition of excess mercaptoethanol to a final level of as high as 0.1 M failed to activate the purified enzyme, nor did this potentiate urea or mercurial activation.

Estimation of Turnover Numbers. Although crystalline enzyme was not obtained, the turnover number per mole of methotrexate could be estimated by titration of the enzyme activity with methotrexate (Hakala *et al.*, 1961; Werkheiser *et al.*, 1962). Turnover numbers were calculated on the assumption that one molecule of enzyme binds one molecule of the drug. The turnover numbers are compared with results from a preparation of dihydrofolate reductase from rat liver in Table III.

[3] The nucleotides tested were deoxythymidine mono-, di-, and triphosphate, and the respective nucleotides of deoxyuridine and deoxycytidine.

[4] Glycine, serine, arginine, glutamine, glutamic acid, histidine, leucine, and phenylalanine were tested.

Kaufman observed a molecular weight of 15,000 from sedimentation studies of the chicken liver enzyme (Kaufman, 1964). The elution profile observed with Sephadex G-100 would suggest that the enzyme from *L. leichmannii* is of similar size.

Discussion

A preparation of dihydrofolate reductase from *L. leichmannii*, after partial purification, reduced both folate and dihydrofolate. A constant ratio of activity with these two substrates was found during the last five stages of purification, during which a 100-fold increase in specific activity was obtained. The Michaelis constant for folic acid as substrate (1.8×10^{-5} M) was almost identical with the constant (1.4×10^{-5} M) for folate as an inhibitor of dihydrofolate reduction. These data suggest that a single protein is responsible for reduction of both substrates. The reduction of folate proceeded at about 1% of the rate of dihydrofolate reduction.

Dihydrofolate reductase from *L. leichmannii* has many properties which were not observed with enzyme

from other bacterial sources but were found with enzyme from avian and mammalian sources. Both folate and dihydrofolate are substrates for reduction, and enzymatic activity occurs with either NADH or NADPH. Both the pH optimum and rate of the enzyme reaction are lowered when NADH is employed as the hydrogen donor. The enzyme isolated from *L. leichmannii* was not activated by urea, polyamines, or mercurials. Enzyme from *L. leichmannii* behaves like the "activated" mammalian and avian enzyme, with respect to pH optimum (Figure 6) and high turnover number for dihydrofolate (Table III). Like the bacterial enzyme, the avian dihydrofolate reductase was neither activated nor inhibited by a variety of deoxynucleotides or amino acids (Kaufman, 1964).

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