Dihydrofolate Reductases of *Escherichia coli* and Bacteriophage T4. A Spectrofluorometric Study†

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ABSTRACT: The dihydrofolate reductase of *Escherichia coli* B has been purified 27,000-fold by affinity chromatography and its properties compared to those of phage T4 dihydrofolate reductase, whose purification has been previously reported. The amino acid composition of each enzyme has been determined. Disc gel electrophoresis in sodium dodecyl sulfate shows each enzyme to be a single polypeptide. The T4 enzyme transfers hydride to dihydrofolate from the "A side" of NADPH. While the enzyme activity of each dihydrofolate reductase is inhibited by chaotropic salts, including SCN^-, ClO_4^-, Cl^-, and F^-, the magnitudes of inhibition vary considerably. Equilibrium binding constants (K_d) for *E. coli* and T4 dihydrofolate reductases, based on quenching of protein fluorescence, are 4.2×10^{-7} and 5.0×10^{-7} M^{-1}, respectively, for dihydrofolate, and 0.1×10^{-7} and 7.7×10^{-7} M^{-1}, respectively, for NADPH. Quenching of protein fluorescence by dihydrofolate or NADPH could be reversed by SCN^-, ClO_4^-, or Cl^-, but the effects were different for each enzyme. F^- was without effect. Little or no quench reversal was observed when protein fluorescence was quenched by 4-amino-4-deoxy-N^{10}-formylpteroylglutamate (N^{10}-formylaminopterin) or 4-amino-4-deoxy-N^{10}-methylpterolyglutamate (methotrexate). Quench reversal has been interpreted as a decreased affinity of the enzyme for substrate and cofactor.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase, EC 1.5.1.3.) catalyzes the reduction of dihydrofolate to tetrahydrofolate by transferring reducing equivalents from the coenzyme NADPH. Due to the crucial role played by this enzyme in providing adequate levels of reduced folate cofactors, it has long been recognized as a susceptible site for chemotherapy of neoplastic disorders, malaria, and certain other diseases (Blakley, 1969). However, because dihydrofolate reductase is not abundant in most target tissues or organisms (*e.g.*, human (Jarabak and Bachur, 1971), plasmodia (Ferone, 1970)), it has so far been virtually impossible to explore at the molecular level the interaction of the enzyme with chemotherapeutic agents.

The bacterial and phage-coded dihydrofolate reductases present attractive systems for study of this enzyme at the molecular level, because of their relative abundance and ease of preparation. The large rise in dihydrofolate reductase activity found after infection of *Escherichia coli* with T2, T4, T5, or T6 phage was first reported by Mathews and Cohen (1963). Subsequently, Mathews and Sutherland (1965) demonstrated that the increase in activity was due to an enzyme not present in uninfected cells. Recently we have reported a simple method for purification of the T4 enzyme by affinity chromatography (Erickson and Mathews, 1971). As reported here, this method, which recovers up to 80% of the enzyme in an electrophoretically homogeneous form, has now been extended to the purification of the host cell dihydrofolate reductase. In addition to the ease of purification and relative abundance of the phage dihydrofolate reductase, the ready availability of mutants in the structural gene for the T4 reductase (the *wh* gene; Hall *et al.*, 1967; Mathews, 1967a) makes this a particularly attractive system for studying the relation between protein structure and enzyme function; in principle, our purification scheme can be applied to mutationally altered, catalytically inactive proteins. Analysis of such proteins should lead to identification of amino acid residues essential for catalytic activity.

Recently the findings of Kozloff *et al.* (1970) have disclosed another, quite unexpected, role for the phage dihydrofolate reductase. These authors have demonstrated a dihydrofolate reductase activity associated with the base plate of T-even phages. We have presented data (Mathews, 1971) in support of the idea that this structural enzyme is the same as the soluble dihydrofolate reductase which we have purified from extracts of phage-infected cells. The specific role of the particle-bound enzyme is not yet clear, and the problems involved in obtaining large amounts of purified base plates are formidable. Of particular interest is the suggestion (Kozloff *et al.*, 1970) that under certain conditions, namely, infection by an *amber wh* mutant under nonsuppressing conditions, where the soluble T4 activity does not appear, the host cell enzyme can substitute for the phage enzyme in the base plate. The possibility (however remote) of such a substitution implies a degree of similarity between the two enzymes. Partly to explore this idea, we have determined and compared, for the T4 and *E. coli* enzymes, the amino acid composition, absorption spectra, fluorescence emission spectra, substrate and inhibitor binding data, and the effects of certain salts on enzymatic activity and binding constants. In addition, the availability of homogeneous enzyme preparations has made possible a variety of immunological studies on the role of the structural reductase (C. Male, J. Erickson, C. Mathews, and L. Kozloff, to be published).

Experimental Procedures

Materials. DEAE-cellulose and Bio-Gel P-150 were obtained from Bio-Rad. Chemicals for electrophoresis on polyacrylamide gels were obtained from Canalco. Methotrexate was the gift of Dr. J. M. Smith, Jr., of Lederle. Aminopterin

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(4-aminopteroylglutamate) was purchased from Nutritional Biochemical Co. NADPH, streptomycin sulfate, sodium dodecyl sulfate, α-chymotrypsinogen A, bovine serum albumin, ovalbumin, carboxypeptidase A, and folic acid were obtained from Sigma. Dihydrofolate was prepared by the method of Futterman as modified by Friedkin *et al.* (1962) and, for routine assay work, stored in sealed ampoules as a dry powder under nitrogen. Dihydrofolate used in binding studies was prepared on the day of its use. [4-^{3}H]NADP$^+$ was prepared by isotope exchange between unlabeled NADP$^+$ and tritiated water (200 mCi/ml, Amersham-Searle), and purified on DEAE-cellulose (Pastore and Friedkin, 1962). The synthesis of N^{10}-formylaminopterin, the affinity material used for purification of T4 and *E. coli* dihydrofolate reductase, has been described (Erickson and Mathews, 1971). Other chemicals used were obtained from local sources and were of reagent grade.

Methods. The methods used for production of large amounts of infected cells, determination of protein, and enzyme assay were as previously described (Erickson and Mathews, 1971). Methods for determining the stereospecificity of hydride transfer from NADPH to dihydrofolate were those of Pastore and Friedkin (1962). All measurements of radioactivity utilized the commercial scintillation fluid Aquasol (New England Nuclear). For monitoring column effluents, 0.5-ml samples were mixed with 5 ml of Aquasol in scintillation vials and swirled until complete solution occurred. Radioactivity was measured in a Beckman liquid scintillation spectrometer.

Purification of E. coli Dihydrofolate Reductase and T4 from the Same Extract. The host cell dihydrofolate reductase can be obtained from the same crude extract used to prepare dihydrofolate reductase since the two enzymes can be separated by ammonium sulfate precipitation (Mathews and Sutherland, 1965). The DEAE-cellulose extraction of the streptomycin sulfate supernatant of the previously described method is omitted, and a DEAE-cellulose extraction of the redissolved 0–43% ammonium sulfate precipitate (containing T4 dihydrofolate reductase) is substituted. The redissolved 0–43% ammonium sulfate fraction was brought to a volume of 500 ml by the addition of buffer A (0.04 M potassium phosphate, pH 7.0), then divided equally in four 250-ml centrifuge bottles. To each bottle was added 6 g (dry weight) of DEAE-cellulose which had been washed with 0.15 M NaCl in buffer A. The slurry was stirred for 30 min; the bottles were centrifuged at 5000*g* for 5 min, and the supernatant was collected. The slurry was extracted once with 100 ml/bottle of 0.15 M NaCl in buffer and centrifuged. Protein in the combined supernatants was precipitated by the addition of 313 g/l. of solid ammonium sulfate. The precipitate was redissolved in 300 ml of buffer A and centrifuged at 20,000*g* for 1 hr. The pH of the supernatant was adjusted to 6.5 with 1.0 M KH_2PO_4, and the T4 dihydrofolate reductase was purified by affinity chromatography as previously described (Erickson and Mathews, 1971).

To recover *E. coli* dihydrofolate reductase, the ammonium sulfate content of the crude extract was brought to 55% of saturation and the resulting precipitate discarded. The supernatant was brought to 85% of saturation with solid ammonium sulfate and the resulting precipitate was collected and redissolved in 300 ml of buffer A. The pH of this undialyzed solution was adjusted to 6.3 with 1 M KH_2PO_4, and the *E. coli* dihydrofolate reductase was purified by the affinity chromatography steps, previously described (Erickson and Mathews, 1971).

Amino Acid Analytical Methods. Determination of the amino acid composition of T4 and *E. coli* dihydrofolate reductases was accomplished by methods based on those of Moore and Stein (1960). A small crystal of phenol was added to each hydrolysis tube to preserve tyrosine (Sanger and Thompson, 1963). Duplicate samples were analyzed on a Beckman Model 120C automatic amino acid analyzer. Cystine was determined as cysteic acid by the method of Moore (1963). Tyrosine and tryptophan were determined by a spectrophotometric method (Edelhoch, 1967).

Fluorescence Measurements. Fluorescence measurements were made in a Perkin-Elmer Model MPF-2A fluorescence spectrophotometer. The light source on this instrument is a 150-W xenon lamp. Emission spectra obtained on this instrument are not corrected for phototube response. However, the instrument was "standardized" with some fluorescent compounds whose spectra have been frequently reported in the literature. The emission maxima obtained for tryptophan, tyrosine, chymotrypsinogen, and bovine serum albumin are in excellent agreement (within 1 nm) with the values obtained by others (Donovan, 1969; Teale and Weber, 1957; Teale, 1960; Konev, 1967). All spectral measurements were made at instrumental settings which gave a linear relationship between fluorescence intensity and concentration of the fluorescing compound.

All fluorescence measurements were performed at room temperature (23 ± 1°) in 1.0-cm square quartz cells, using a sample volume of either 3.0 or 2.5 ml. Except as noted in Results, buffer A was employed. Enzyme concentrations were never greater than 0.80 μM, minimizing errors due to light absorption by the sample.

To obtain protein fluorescence measurements, the light scattering from a cell containing only buffer was determined (in most cases this was negligible). An appropriate aliquot of enzyme solution was added and the sample was mixed with a plastic dip stick. After the initial reading, titrant was transferred from a 0.1-ml Hamilton syringe to the cell by means of the plastic dip stick. In experiments which examined the effect of various salts on the quenched protein fluorescence, these salts were also delivered from the Hamilton syringe in the form of 4.0 M aqueous solutions.

The extent of quenching of T4 and *E. coli* dihydrofolate reductases at infinite substrate or coenzyme concentrations was determined from a plot of reciprocal fluorescence *vs.* reciprocal concentration: $1/(1 - Fl)$ *vs.* $1/[S]$. Here Fl is defined as F/F_0, where F_0 is the initial protein fluorescence intensity and F is the fluorescence intensity at a given substrate or cofactor concentration.

Calculation of the equilibrium dissociation constant K_d is based on the assumption of 1:1 stoichiometry between enzyme and ligand (substrate, cofactor, or inhibitor)

$$DR + S \rightleftharpoons DR - S$$

where DR is free enzyme and S is a ligand. One can show that the association constant K_d for the above equation is given by the expression

$$K_d = (1 - \alpha)/(\alpha/[DR])$$

where α is the ratio of bound S to total S (Scatchard *et al.*, 1957).

Systematic errors in the determination of K_d due to dilution upon addition of S were never greater than 4%. Errors due to nonspecific quenching were difficult to determine, but

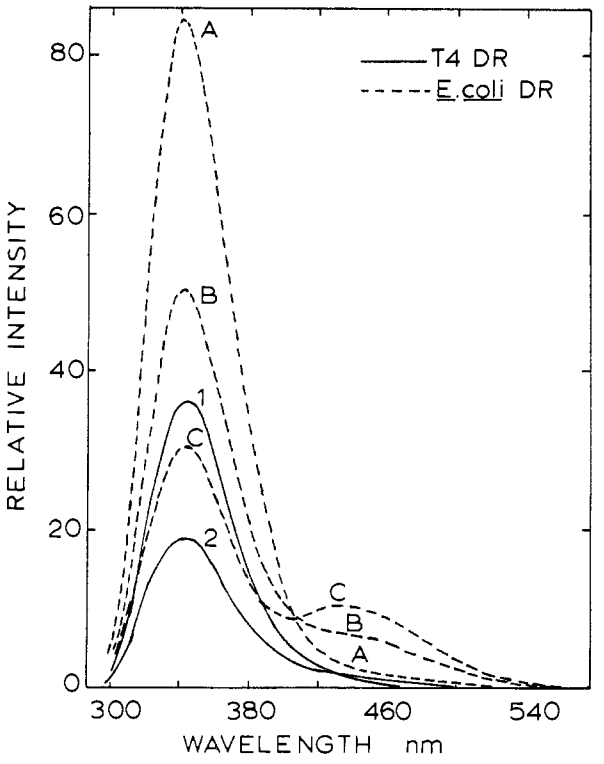

FIGURE 1: Absorption spectra of T4 dihydrofolate reductase (0.65 mg/ml) and *E. coli* dihydrofolate reductase (0.24 mg/ml), both in buffer A. "DR" on the figure signifies dihydrofolate reductase.

the maximum concentrations of S were usually less than 5 μM and in some cases considerably less than that. Test additions of dihydrofolate or NADPH to ovalbumin indicated that this type of error was about 10% at the maximum concentrations used.

Calibration of the microsyringe was checked spectrophotometrically with methotrexate.

Results

The purification of *E. coli* dihydrofolate reductase by affinity chromatography is summarized in Table I. The overall purifications are 6000-fold for the T4 enzyme (Erickson and Mathews, 1971) and 27,000-fold for *E. coli* dihydrofolate reductase. The *E. coli* enzyme preparation is homogeneous by the criterion of disc gel electrophoresis on sodium dodecyl sulfate containing gels (Weber and Osborn, 1969). As will be shown later, more than 80% of the protein fluorescence of both reductase preparations can be quenched with methotrexate, which would further indicate a high degree of homogeneity (since contaminating proteins would probably not bind methotrexate and their fluorescence would presumably not be quenched). Other evidence for the purity of the T4 enzyme has been presented previously (Erickson and Mathews, 1971).

Molecular Weight and Subunit Determination. The molecular weight of the enzymes as determined by sodium dodecyl sulfate gel electrophoresis were found to be 29,000 ± 1500 daltons for the T4 enzyme and 22,500 ± 1100 daltons for the *E. coli* enzyme. Since both of these values agree well with molecular weight data obtained for the native enzymes (Mathews and Sutherland, 1965; Mathews, 1967b), it would appear that each enzyme is composed of a single polypeptide chain.

TABLE I: Purification of *E. coli* Dihydrofolate Reductase by Affinity Chromatography.

Purification Step	Vol (ml)	Total Act. (Units)	Total Protein (mg)	Sp Act. (Units/mg)
Crude extract[a]	1900	37	37,000	0.001[b]
Streptomycin	2200	37	37,000	0.001
55–85% $(NH_4)_2SO_4$	310	34	13,600	0.0025[c]
Affinity chromatography	18	32	1.2	27[c]

[a] Based on 500 g (wet weight) of cells. [b] Data are for uninfected cells. [c] Data are for either infected or uninfected cells.

Ultraviolet Absorption Spectra. The absorption spectra of T4 and *E. coli* dihydrofolate reductases, shown in Figure 1, while characteristic of simple proteins, demonstrate marked differences. The maximum for T4 dihydrofolate reductase is at 277 nm, while that for *E. coli* dihydrofolate reductase is at 281 nm. Also, the shoulder at 289 nm is much more pronounced in the spectrum of the *E. coli* enzyme. These properties indicate that tyrosine makes a greater contribution to the spectrum of T4 dihydrofolate reductase than to that of the bacterial enzyme.

Unlike the dihydrofolate reductase from *Lactobacillus casei* (Dunlap *et al.*, 1971) the spectrum of the T4 enzyme purified by conventional methods did not show any evidence of bound NADPH. The enzymes purified by affinity chromatography, which involved their elution from a column by means of their substrate, dihydrofolate, would not be expected to show evidence of bound cofactor, unless NADPH were bound at a noncatalytic site. There is no evidence for this. Moreover, in the fluorescence studies (see below), there was no significant activation of fluorescence around 340 nm, as would have been expected if either enzyme contained bound NADPH.

When the protein concentration was determined by the microbiuret reaction (Goa, 1953), a 0.1% solution of T4 dihydrofolate reductase in buffer A showed an A_{280}[1] of 1.20. By this method a 0.1% solution of *E. coli* dihydrofolate reductase was estimated to have an A_{280} of 1.91.

Results of Amino Acid Analysis. Using a value of 30,000 for the molecular weight (the average of 29,000 determined by sodium dodecyl sulfate electrophoresis and 31,000 determined by sucrose gradient analysis, Mathews and Sutherland, 1965; Mathews, 1967b), we calculated the number of residues of tryptophan and tyrosine per molecule of T4 dihydrofolate reductase to be 4.6 and 9.0, respectively. The results of amino acid analyses of the T4 and *E. coli* reductases are summarized in Table II. Both enzymes have aspartate and glutamate as the most plentiful amino acid. Asparagine and glutamine contents were not determined. The hydrophobic residues are also well represented in both proteins. Cysteine, determined as cysteic acid, is the least abundant amino acid in the T4 enzyme, with two residues per molecule. By using the value of nine tyrosines per T4 dihydrofolate reductase molecule as a basis of calculations, the number of each of the various amino

[1] The following abbreviations are used: I_{50}, inhibitor concentration required to reduce enzymatic activity by 50%; A_{280}, absorbance at 280 nm.

TABLE II: Amino Acid Composition of T4 and *E. coli* Dihydrofolate Reductases.

Amino Acid	nM Composition of Amino Acid Hydrolysate[e]		Residues/T4 Enzyme Molecule[d]
	E. coli Enzyme	T4 Enzyme	
Lysine	12.4	10.0	13
Histidine	6.0	3.8	5
Arginine	12.7	8.2	11
Aspartic acid	32.1	17.8	23
Threonine	11.9[a]	11.3	16
Serine	15.4[a]	9.9	13
Glutamic acid	25.8	20.8	27
Proline	14.2	8.9	12
Glycine	20.1	16.0	21
Alanine	22.2	14.0	18
Half-cystine	ND[g]	1.60[c]	2
Valine	19.3[b]	16.0[b]	21
Methionine	0.6	3.78[c]	5
Isoleucine	15.7[b]	11.1[b]	15
Leucine	19.6[b]	15.1[b]	20
Tyrosine	6.6	6.9	9
Phenylalanine	9.8	6.8	9
Tryptophan[f]			4–5

[a] Extrapolated to zero hours of hydrolysis. [b] Based on 48 hr of hydrolysis. [c] Based on values obtained from enzyme oxidized with performic acid before hydrolysis. [d] Calculations based on a value of 9 tyrosine residues per 30,000 daltons of T4 dihydrofolate reductase. [e] 24-hr hydrolysis period except as noted. [f] Not determined for the *E. coli* enzyme because of insufficient material. [g] Not determined.

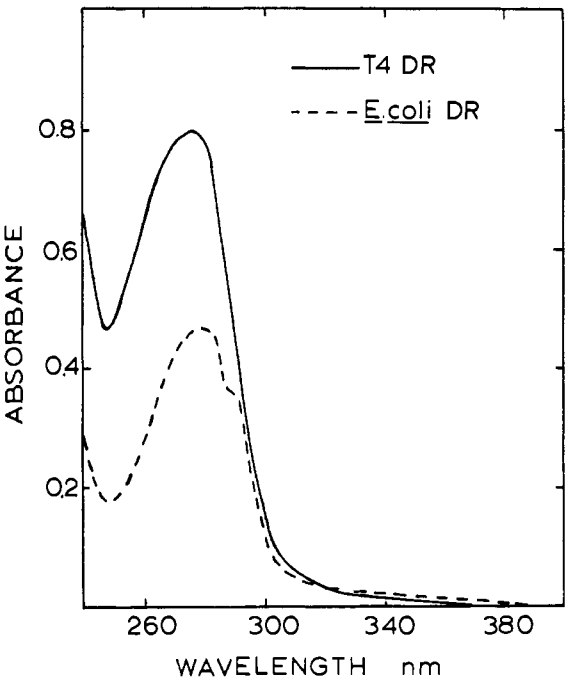

FIGURE 2: Fluorescence emission spectra for T4 and *E. coli* dihydrofolate reductases and their NADPH complexes. T4 enzyme was at 0.55 μM and *E. coli* enzyme was at 0.68 μM. Excitation was at 286 nm (10-nm band slit for *E. coli* enzyme and 6 nm for T4 enzyme). The emission slit was 10 nm for *E. coli* enzyme and 6 nm for T4 enzyme and the sensitivity setting was 3. NADPH is 0, 1.6 μM, and 4.0 μM for curves A, B, and C, respectively, and 0 and 3.3 μM for curves 1 and 2, respectively. "DR" connotes dihydrofolate reductase.

acid residues was determined. The sum of the molecular weights of the residues came to about 27,400.

Kinetic Constants of T4 Dihydrofolate Reductase. Michaelis constants for T4 dihydrofolate reductase were determined from double-reciprocal plots by the methods of Lineweaver and Burk (1934). At an NADPH concentration of 1×10^{-4} M, the K_m for dihydrofolate was found to be 2.3×10^{-6} M. In the presence of a constant dihydrofolate concentration of 1×10^{-4} M, the K_m for NADPH was found to be 1.8×10^{-5} M. The kinetics of the T4 dihydrofolate reductase reaction in the presence of the inhibitor N^{10}-formylaminopterin was also examined. This inhibitor is of particular interest since it is the ligand which was used successfully in the affinity chromatography process used to purify the enzymes described here. From the kinetics of the reaction it was found that inhibition by N^{10}-formylaminopterin is competitive with respect to dihydrofolate and noncompetitive with respect to NADPH. A competitive K_i of 1.8×10^{-8} M was determined.

Stereospecificity of T4 Dihydrofolate Reductase Promoted Hydride Transfer. Previous studies have shown that dihydrofolate reductases of L1210 murine lymphoma and chicken liver transferred the A side hydride of NADPH to dihydrofolate (Pastore and Friedkin, 1962). A similar result was observed for this enzyme from *Streptococcus faecium* (Blakley *et al.*, 1963). Since T4 dihydrofolate reductase is quite distant phylogenetically from these other enzymes, one could not assume that the phage enzyme had the same specificity. By the methods of Pastore and Friedkin (1962) we determined that in the reduction of dihydrofolate by the T4 enzyme only the NADPH prepared with glucose-6-phosphate dehydrogenase transferred significant label to produce tetrahydrofolate. Thus, the conclusion of this experiment is that T4 dihydrofolate reductase promotes transfer of the A side hydride, analogous to other DR's which have been examined.

Flourescence Properties. Perkins and Bertino (1966) have shown that in the binding of substrates or inhibitors by the mouse lymphoma dihydrofolate reductase is accompanied by quenching of protein fluorescence, a phenomenon which made it possible to determine equilibrium binding constants. Since knowledge of these properties could prove useful to the present study, it was of interest to characterize the fluorescent properties of T4 and *E. coli* dihydrofolate reductase and of their complexes with NADPH.

The emission spectra of native T4 and *E. coli* dihydrofolate reductases are shown in Figure 2 (curves 1 and A), respectively. Both native enzymes show emission maxima close to 340 nm when excited in the region 270–290 nm. When the enzymes are denatured in 6 M guanidine-HCl (not shown) the positions of the maxima are shifted in the long-wavelength direction by 8–10 nm, to the position of the maximum emission of free tryptophan.

NADPH quenches protein fluorescence of both enzymes, but at the same time enhances fluorescence near the NADPH emission maximum of 440 nm, excited by light in the 270–290-nm region. This type of fluorescence, first reported by Velick (1958), is referred to as NADPH energy-transfer fluorescence. As shown in Table III the degree of protein fluorescence quenching by NADPH is greater for *E. coli* dihydrofolate reductase than for T4 enzyme. Similarly, the

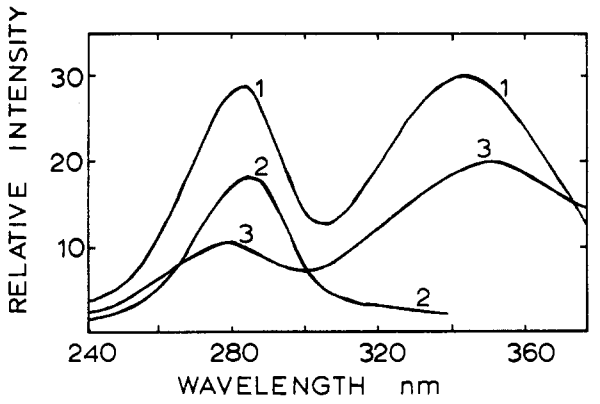

FIGURE 3: Fluorescence excitation spectra for the T4 dihydrofolate reductase–NADPH complex (curve 1), free T4 enzyme (curve 2), and the enzyme–NADPH–methotrexate complex (curve 3). Emission intensity was monitored at 460 nm (20-nm slit). An excitation slit of 8.7 nm was used, and a "43" filter was placed in the emission beam to reduce the intensity of scattered light below 430 nm. T4 enzyme was at 0.68 μM, NADPH was at 1.2 μM, and methotrexate was at 1.0 μM.

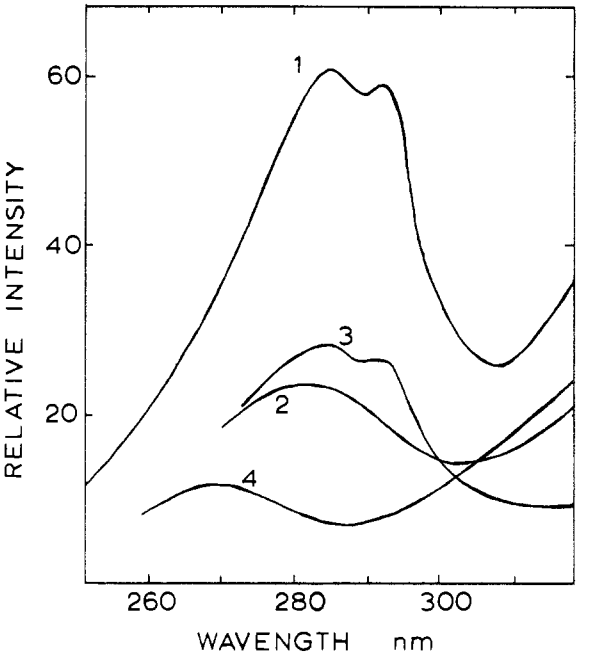

FIGURE 4: Fluorescence excitation spectra of the *E. coli* dihydrofolate reductase–NADPH complex (curve 1), the *E. coli* enzyme–NADPH–methotrexate complex (curve 2), free *E. coli* enzyme (curve 3), and free NADPH. Emission intensity was monitored at 460 nm (20-nm slit). An excitation slit of 3 nm was used. *E. coli* enzyme was at 0.68 μM, NADPH was at 2.0 μM, and methotrexate was at 0.8 μM.

NADPH energy-transfer fluorescence is weaker for T4 dihydrofolate reductase than for the bacterial enzyme.

The excitation spectrum of the T4 dihydrofolate reductase–NADPH complex is shown in Figure 3. The fluorescence intensity at 460 nm observed for a T4 dihydrofolate reductase solution (0.68 μM) alone is indicated by curve 2, while the fluorescence of the same T4 enzyme solution in the presence of 1.2 μM NADPH is shown by curve 1. NADPH fluorescence alone, excited at 280 nm (an NADPH excitation minimum) cannot account for the entire 50% increase in the 280-nm peak. The difference appears to be due to the phenomenon of activation by energy transfer (Velick, 1958). The *E. coli* NADPH energy-transfer fluorescence (Figure 4) provides a better example of this, showing over a 100% increase in fluorescence. Figure 4 also provides evidence that the increased fluorescence at 460 nm is due to light absorbed by enzyme. This conclusion can be drawn from the double peaks of the excitation spectrum, which occur at the same positions as they do for the free enzyme. The corresponding spectrum of the T4 dihydrofolate reductase–NADPH complex shows only a single maximum, as does the free enzyme; however, this is a weak complex where two-thirds of the intensity can be accounted for by free protein fluorescence.

Still another type of fluorescence displayed by these binary complexes is that of NADPH fluorescence excited at 340 nm and monitored at 460 nm. Here light absorption is necessarily by NADPH. Once again, T4 dihydrofolate reductase and the *E. coli* enzyme are qualitatively similar but quantitatively different as illustrated in Figure 5. When equal aliquots of NADPH are added to cells containing either buffer A or T4 dihydrofolate reductase (0.68 μM), there is an identical increase in fluorescence intensity at 460 nm. When this experiment is performed with *E. coli* enzyme of the same concentration, the initial increase in fluorescence intensity is 50% greater in the cell containing enzyme than in the cell containing buffer. Thus, NADPH fluorescence is enhanced in the complex with *E. coli* dihydrofolate reductase but largely unaffected in the complex with T4 dihydrofolate reductase. This indicates that the 340-nm excitation peak of curve 1 in Figure 3 is the same as that of free NADPH. However, the fluorescence of free NADPH cannot be quenched by methotrexate at this concentration, by more than a few per cent. Quenching at 460 nm of the two types of dihydrofolate reductase–NADPH fluorescence by methotrexate also occurs. In each case the curve (not shown) indicates that the extent of quenching is a linear function of methotrexate concentration.

In general, the binding of methotrexate and other 4-aminofolate compounds by T4 and *E. coli* dihydrofolate reductases results in a large degree of quenching not only of the NADPH fluorescence in the ternary complexes, but of protein fluorescence as well. The quenching process with either methotrexate or aminopterin (data not shown) gives a linear relationship between inhibitor concentration and decrease in

TABLE III: Quenching of Protein Fluorescence by Various Compounds.

Dihydrofolate Reductase	Ligand	Quenching[a] (%)
T4	Dihydrofolate	76
T4	NADPH	61
T4	Methotrexate	79[b]
T4	Methotrexate + NADPH	85[b]
T4	Aminopterin	80[b]
T4	N^{10}-Formylaminopterin	82
E. coli	Dihydrofolate	78
E. coli	NADPH	73
E. coli	NADP	54
E. coli	Methotrexate	83[b]

[a] Determined by extrapolation to infinite substrate concentration, except as noted. [b] Determined directly by inspection of quenching curve.

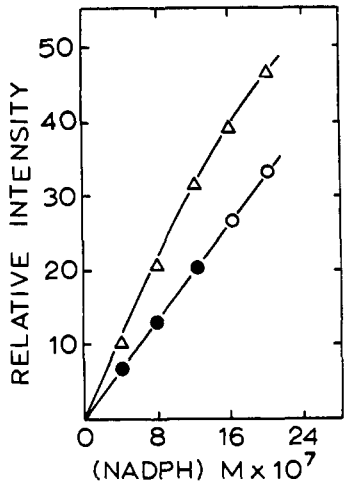

FIGURE 5: Enhancement of NADPH fluorescence by binding to *E. coli* dihydrofolate reductase but not to T4 dihydrofolate reductase. Both enzymes were at 0.68 μM in buffer A. Excitation was at 340 nm (8.7-nm slit), and emission was at 460 nm (20-nm slit), with filter "43" in the emission beam. Increments of NADPH were added to a blank of buffer A (○), T4 enzyme (●), or *E. coli* enzyme (△).

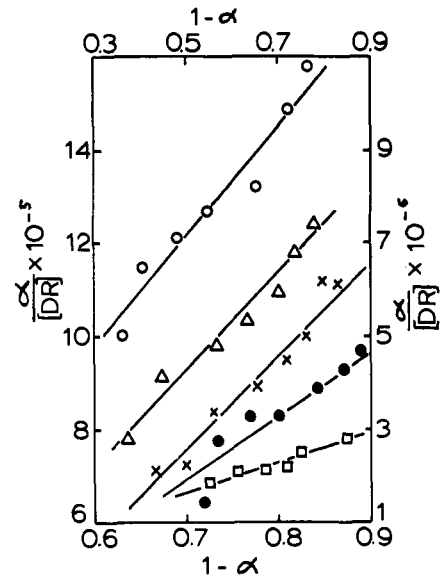

FIGURE 6: Equilibrium binding of substrate, cofactor, or inhibitor to T4 dihydrofolate reductase and *E. coli* dihydrofolate reductase. Left and lower scales: T4 dihydrofolate reductase–dihydrofolate (×), *E. coli* dihydrofolate reductase–dihydrofolate (○), T4 dihydrofolate reductase–NADPH (●); right and upper scales: T4 dihydrofolate reductase–N^{10}-formylaminopterin (□), *E. coli* dihydrofolate reductase–NADPH (△). "DR" connotes dihydrofolate reductase.

fluorescence. N^{10}-Formylaminopterin binds less tightly, displaying a measurable equilibrium.

Equilibrium Binding Constants. Preliminary to calculating binding constants, the limit of protein fluorescence quenching by several small molecules was determined by means of a double-reciprocal plot, of fluorescence intensity (*Fl*) *vs.* molar concentration. The intercept at infinite small molecule concentration was assumed to correspond to the fluorescence of the dihydrofolate reductase–small molecule complex. The extent of protein fluorescence quenching obtained from these plots is shown in Table III. The extent of quenching by methotrexate and aminopterin could be determined directly, due to the very tight binding that inhibitors display toward either enzyme. The data in Table III indicate that all folate compounds listed result in quenching in the 75–80% range. The addition of NADPH to the T4 dihydrofolate reductase–methotrexate complex results in an additional 5–7% quenching. Thus, about 85% of the fluorescence of the T4 and *E. coli* dihydrofolate reductase preparations can be quenched, substantiating the high degree of homogeneity of these preparations, since presumably other proteins would not bind methotrexate and their fluorescence would not be quenched. NADPH produces significantly less protein fluorescence quenching of the T4 enzyme than do the folate compounds. By contrast the quenching of *E. coli* dihydrofolate reductase fluorescence by NADPH is nearly as great as that by the folate compounds.

$NADP^+$ and NADH at concentrations of 4 and 0.4 μM, respectively, failed to give detectable quenching of T4 dihydrofolate reductase fluorescence at a concentration of 0.55 μM. NADH (0.4 μM) also failed to detectably quench *E. coli* dihydrofolate reductase fluorescence at a concentration of 0.35 μM. While folic acid could quench a small amount of T4 dihydrofolate reductase fluorescence, at a concentration of 4 μM, the measurements were suspected of being inaccurate due to nonspecific quenching or to light absorption by the sample. In this connection, the quenching of *E. coli* dihydrofolate reductase by $NADP^+$, while listed in Table III, may be in error by as much as 25–30%.

Data from quenching curves were used to make a plot of α/[DR] *vs.* $1 - \alpha$. From the slopes of these plots (shown in Figure 6) the dissociation constants K_d, for complex formation were determined (Table IV). For comparative purposes Table IV also contains other types of binding data.

Results of the equilibrium binding experiments indicate that the substrate, dihydrofolate, binds about equally well to both *E. coli* and T4 dihydrofolate reductases. By contrast the binding of NADPH is significantly different. It is interesting

TABLE IV: Dissociation Constants for Substrate, Cofactor, and Inhibitors.

T4 Dihydrofolate Reductase			
Compound	K_d (M^{-1})	E^0 (kcal/mol)[a]	K_m (M)
Dihydrofolate	5.0×10^{-7}	8.5	2.3×10^{-6}
NADPH	7.7×10^{-7}	8.3	18×10^{-6}
N^{10}-Formylaminopterin	1.5×10^{-7}	9.2	
	K_i[b]		I_{50} (M)[c]
N^{10}-Formylaminopterin	1.8×10^{-8}		8×10^{-7}

E. coli Dihydrofolate Reductase		
	K_d	F^0 (kcal/mol)[a]
Dihydrofolate	4.2×10^{-7}	8.7
NADPH	9.1×10^{-8}	9.5

[a] Calculated from $\Delta F^0 = -RT \ln K_d$. [b] As determined from the slopes of double reciprocal plots from the equation: slope $= (K_m/V)[1 + (i/K_i)]$, where i is inhibitor concentration. [c] Inhibitor concentration required to reduce enzymatic activity by 50%.

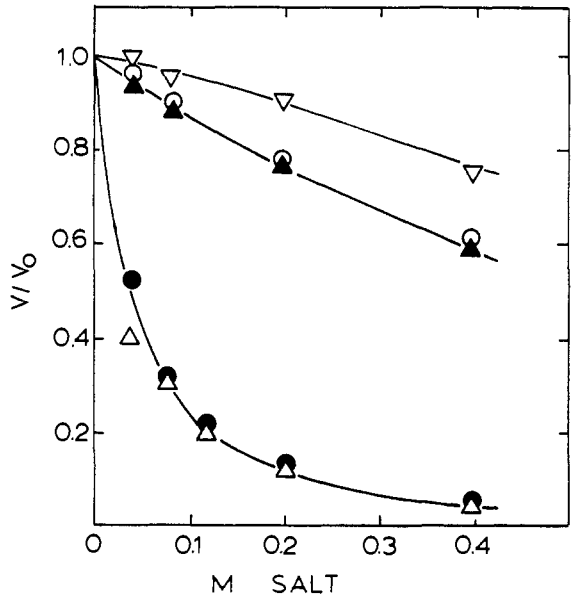

FIGURE 7: Effects of salts on the enzymatic activity of T4 dihydrofolate reductase. V_0, reaction velocity in the absence of salts, was determined with 0.025–0.08 unit of enzyme activity. Within this range of enzyme concentrations, V/V_0 ratios for a given salt were identical at the same salt concentration, within experimental error. Assays were carried out at 23° in the presence of KF (▽), NaCl (○), KCl (▲), $NaClO_4$ (●), and KSCN (△).

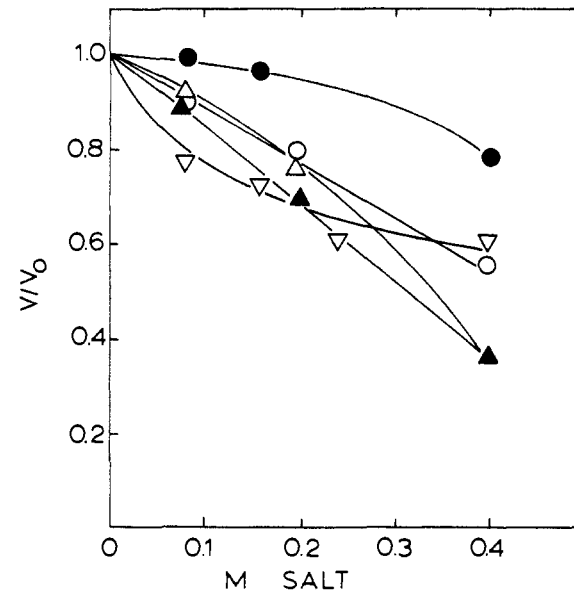

FIGURE 8: Effects of salts on the enzymatic activity of *E. coli* dihydrofolate reductase. Enzyme (0.030 unit) was used for each assay, all of which were carried out at 23°, in the presence of KF (▽), NaCl (○), KCl (▲), $NaClO_4$ (●), and KSCN (△)

that the more efficient catalyst (T4 dihydrofolate reductase) has the weaker interaction with NADPH.

The binding data also indicate that N^{10}-formylaminopterin, the ligand used in affinity chromatography, complexes with the T4 enzyme only a few times more tightly than does dihydrofolate. It is also interesting that the equilibrium-determined dissociation constant for N^{10}-formylaminopterin is significantly larger than the kinetically determined K_i. By contrast the kinetic data for substrate and cofactor would indicate a weaker interaction with the enzyme than is indicated by the equilibrium techniques. Other studies on dihydrofolate reductase have also revealed significant differences between equilibrium and kinetic data (Perkins and Bertino, 1966).

Effects of Chaotropic Ions on the Activity of T4 and E. coli Dihydrofolate Reductase. As a means of comparing the T4 and *E. coli* enzymes with each other and with dihydrofolate reductases from other sources, we studied the effects on enzymatic activity of KF, KCl, NaCl, $NaClO_4$, and KSCN. All five salts gave inactivation of both microbial reductases. Results of these studies for the T4 enzyme are summarized in Figure 7. At ClO_4^- or SCN^- concentration of 0.04 M the activity was reduced by about 50%. At these low concentrations SCN^- appears to be slightly more effective than ClO_4^-. A similar concentration of either KCl or NaCl caused only a 5% decrease in the activity, and KF was virtually without effect.

As shown in Figure 8, the *E. coli* enzyme is quite different in its responses to this series of salts. While inactivation occurs with all salts tested, the least effective salt in this case was $NaClO_4$, and at low concentration KF was the most effective inactivator. The observation that KCl inactivated more rapidly than NaCl indicates that differences in cation effects need to be considered in the salt inactivation of *E. coli* dihydrofolate reductase. With T4 dihydrofolate reductase NaCl and KCl produced identical effects (Figure 7).

Effects of Salts on the Quenching of Fluorescence. In order to separate changes in bonding interaction from other types of rate-reducing effects, we studied the effects of the above salts on dihydrofolate fluorescence quenching by substrate and cofactor. In preliminary experiments it was found that KSCN and $NaClO_4$, up to concentrations of 0.15 M, were without effect on the fluorescence of the free enzymes. Therefore, it was assumed that any increase in fluorescence observed following addition of salts to one of the dihydrofolate reductase complexes could be attributed to decreased binding. Figure 9 shows the effects of several salts on the T4 enzyme–dihydrofolate and the enzyme–NADPH complexes. Initially, each enzyme solution showed the same relative fluorescence. These solutions were then quenched to within 70–80% of completion. The volume of quenching agent was such that the final volume of enzyme plus quenching agent was 2.5 ml. Subsequently, we observed the effect of additions of a given salt. Figure 9 shows that the addition of either KSCN or $NaClO_4$ to the T4 enzyme complex is accompanied by a large increase in fluorescence intensity. The slope of the curve is very large in the 0–0.4 M region, corresponding to the same region where the enzymatic activity is most sensitive to changes in salt concentration (Figure 7). NaCl and KCl give indistinguishable patterns of quench reversal, indicating that it is the anion which is responsible for the observed changes. As with the enzymatic activity, the NaCl and KCl quench reversal pattern indicates an intermediate effect between those of KSCN and KF.

When KSCN was added to the T4 dihydrofolate reductase–methotrexate complex *no* change in fluorescence intensity was observed (0–0.14 M KSCN concentration range). Similar treatment of the T4 dihydrofolate reductase–N^{10}-formylaminopterin complex does show a small increase in intensity, but it is still proportionately much less than the change in intensity produced in the T4 dihydrofolate reductase–dihydrofolate complex. Some possible reasons for this difference will be discussed later.

Similar experiments performed with the T4 dihydrofolate reductase–NADPH complex showed a pattern of quench re-

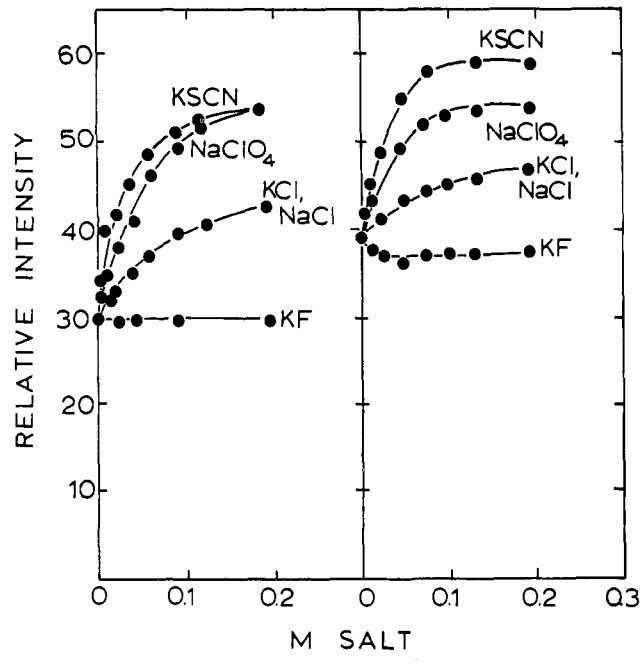

FIGURE 9: Reversal of the quenching of protein fluorescence of T4 dihydrofolate reductase by salts. The fluorescence of T4 enzyme (0.55 μM) was quenched to within 70–80% of completion by the addition of dihydrofolate (3.3 μM), left panel, or by NADPH (3.3 μM), right panel. The fluorescence intensity at the indicated salt concentrations was then observed. All data are based on an initial unquenched protein fluorescence with a relative intensity of 62. Excitation was at 285 nm and emission was monitored at 340 nm.

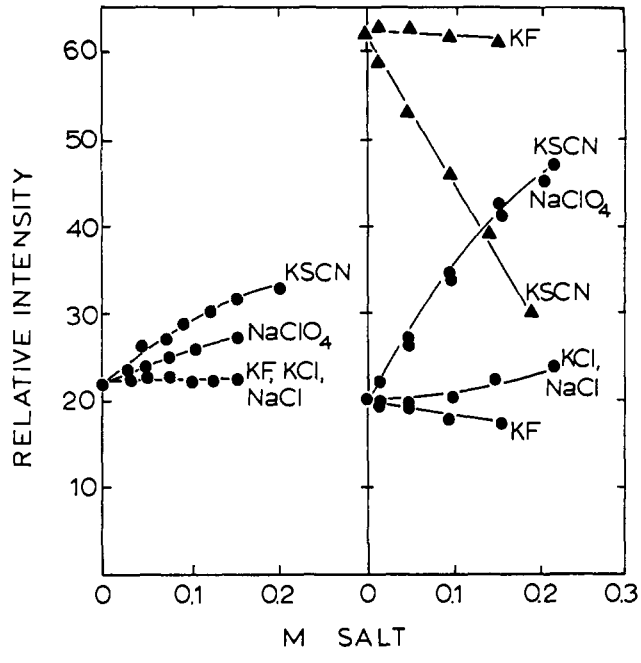

FIGURE 10: Reversal of the quenching of protein fluorescence of *E. coli* dihydrofolate reductase and of its energy-transfer fluorescence. Left panel: The fluorescence of *E. coli* enzyme (0.44 μM) was quenched with dihydrofolate (4.0 μM). Right panel: The fluorescence of *E. coli* enzyme (0.35 μM) was quenched with NADPH (1.6 μM). The fluorescence intensity at the indicated salt concentrations was then observed. For the reversal of quenching (●) excitation was at 285 nm and emission monitored at 340 nm. These data are based on an initial unquenched intensity of 62. For the reversal of the NADPH energy-transfer fluorescence (▲), *E. coli* enzyme was 0.35 μM and NADPH was 2.0 μM. Excitation was at 284 nm and emission at 460 nm. The initial intensity of 62 is that due only to the enzyme–NADPH complex.

versal strikingly similar to that seen with the T4 enzyme–dihydrofolate complex (Figure 9, right-hand panel). The same large slopes are seen for KSCN and $NaClO_4$, in the 0–0.04 M concentration range, and the effects of KCl and NaCl are identical. In this case, however, KF appears to give a small initial decrease in fluorescence followed by no change.

In general the quench reversal at low salt concentration was much less for *E. coli* dihydrofolate reductase (Figure 10) than for T4 dihydrofolate reductase as seen by comparison of the slopes of the KSCN curves below 0.05 M. The qualitative order of effectiveness of the salts was nearly the same for both enzymes. Although KCl and NaCl were less effective in the *E. coli* dihydrofolate reductase system than with the T4 enzyme, they still gave identical quench reversal patterns for *E. coli* dihydrofolate reductase. In the case of the *E. coli* enzyme–dihydrofolate complex, KCl and NaCl (along with KF) produced no effect at all. These results are in contrast to the marked differences observed for the *E. coli* reductase–*activity* curve in the presence of KCl and NaCl, indicating that these salts affect the enzymatic rate by some other factor than altered substrate or cofactor binding. Similarly, the effect of KSCN and $NaClO_4$ on quench reversal would not predict the effect of these salts on the enzymatic activity.

To further demonstrate that the increase in protein fluorescence observed in these studies was due to decreased binding, we examined the effect of salts on the *E. coli* dihydrofolate reductase–NADPH energy-transfer fluorescence peak (Figure 10). Since this peak arises from complex formation, agents which decrease the binding of NADPH to *E. coli* dihydrofolate reductase should cause a decrease in the intensity of this peak. As shown in Figure 10, the curves obtained with KF and KSCN are just expected from the corresponding protein fluorescence curves.

Discussion

By the methods reported here it was possible to purify the T4 and *E. coli* dihydrofolate reductases about 6000-fold and 27,000-fold, respectively, with recoveries of about 80% in each case. Such high recoveries were possible only with the use of affinity chromatography. Our procedures demonstrate that it is possible to recover, in a homogeneous form, the minute quantities of dihydrofolate reductase that are found in uninduced systems. Recently, Poe *et al.* (1972) described a method for the large-scale purification of dihydrofolate reductase from a methotrexate-resistant strain of *E. coli* B. *A priori* one might expect this enzyme to be the same as our *E. coli* dihydrofolate reductase—just more abundant. Since Poe *et al.* reported a molecular weight of about 17,000, considerably less than the 22,500 obtained in our experiments on the *E. coli* reductase, it seems possible that their methotrexate-resistant strain makes a different dihydrofolate reductase.

The phenomenon of energy transfer, in which light absorbed by protein enhances the fluorescence of bound pyridine nucleotide, was first reported by Velick (1958). Perkins and Bertino (1966) reported a virtually identical finding for the interaction between the L1210 lymphoma dihydrofolate reductase and NADPH. This type of fluorescence is also present within the T4 and *E. coli* dihydrofolate reductase. However, it is clear from an inspection of Figures 2 and 3 that the extent of this transfer is significantly less for the phage enzyme, being most perceptible in the increase in intensity of the 280-nm excitation peak of Figure 3. The reason for this is not yet clear.

Previous workers have noted the effects of various salts on the enzymatic activity of dihydrofolate reductase (Reyes and Huennekens, 1967). In the present work these studies were extended by fluorescence techniques, which allowed us to determine if salts affected the fluorescence quenching properties of either substrate of cofactor. Certain salts, in particular the anions of KSCN and $NaClO_4$, do affect the quenching properties of both substrate and cofactor, as indicated by large increases in protein fluorescence by the NADPH and dihydrofolate complexes of T4 dihydrofolate reductase and to a lesser extent by *E. coli* dihydrofolate reductase. These changes appear to reflect a decrease in binding between each enzyme and its substrate and cofactor. On the basis of the observed phenomena we cannot yet determine how these salts have decreased this affinity. The anions of KSCN and $NaClO_4$ are thought to affect hydrophobic bonding (Hatefi and Hanstein, 1969). In addition, Baker has presented evidence that hydrophobic bonding plays an important role in binding between dihydrofolate and the reductase (Baker, 1967). While the data do not contradict Baker's postulated hydrophobic binding region, they do not really support this conclusion, either. Another possible interpretation of the similarity of the quench reversal curves (Figure 9) of T4 dihydrofolate reductase for *both* substrate and cofactor would be that progressive subtle changes in protein structure (and therefore of substrate and cofactor binding sites) are occurring in response to an increased concentration of KSCN or $NaClO_4$. The salt effect is known to be reversible, thus the hypothetical changes in protein structure would also have to be reversible. One apparent difficulty with this hypothesis is the very limited reversal of quenching observed for the 4-amino compounds, which bind to dihydrofolate reductase competitively with dihydrofolate. A comparison of the quench reversal curve observed for dihydrofolate with that of N^{10}-formylaminopterin at a time when the affinity of dihydrofolate for T4 dihydrofolate reductase is changing rapidly. One possible explanation of these observations is that the 4-amino compounds are bound to T4 dihydrofolate reductase by some fundamentally different mode than is dihydrofolate. Thus, a given salt-induced change in enzyme structure could conceivably alter the binding determinants for dihydrofolate to a greater extent than the binding determinants for the 4-amino compounds. Evidence for this latter explanation is presented elsewhere (Erickson and Mathews, 1972).

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