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BACTERIOPHAGE.

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STUDIES OF THE "ACTIVE SITE" REGION
OF DIHYDROFOLATE REDUCTASE
SPECIFIED BY T4 BACTERIOPHAGE

by

John Sayers Erickson

A Dissertation Submitted to the Faculty of the
COMMITTEE ON BIOCHEMISTRY
In Partial Fulfillment of the Requirements
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THE UNIVERSITY OF ARIZONA

1972
I hereby recommend that this dissertation prepared under my direction by John Sayers Erickson entitled Studies of the "Active Site" Region of Dihydrofolate Reductase Specified by T4 Bacteriophage be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

Christopher K. Mathews  9/18/71
Dissertation Director  Date

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:

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*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.
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SIGNED: John D. Erickson
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ABSTRACT

Bacteriophage T4-specific dihydrofolate reductase (T4DR) and the host cell *Escherichia coli* dihydrofolate reductase (EDR) have been purified 6,000-fold and 27,000-fold, respectively. T4DR was initially purified by a many-step conventional method and subsequently purified by a simplified method involving affinity chromatography. EDR was also purified by the same method, which employs N\(^{10}\)-formylaminopterin linked to aminoethyl Bio-Gel P-150. Recoveries of 80% are obtained and the purified enzymes appear homogeneous by the criteria of acrylamide electrophoresis. The amino acid compositions of the two enzymes were analyzed and compared. Kinetic studies show that T4DR has a \(K_m\) for dihydrofolate (DHF) of 2.3 \(\mu M\) and for NADPH of 18 \(\mu M\). T4DR was shown to be protected against trypsin inactivation by NADPH to a much larger extent than by DHF. The effects of KSCN, NaCl, NaClO\(_4\), NaCl, KCl and KF on the enzymatic activity showed significant differences between the two enzymes. While all salts tested caused inhibition, the activity of T4DR appeared to be affected only by the anion. In the order of decreasing inhibition, the effect on T4DR was SCN\(^-\), ClO\(_4\)^-, Cl\(^-\), F\(^-\). The inhibition of EDR by KCl was significantly greater than for NaCl.
indicating that cation effects are also important in this system. Both enzymes have fluorescence emission maxima at about 340 nm. The excitation spectrum of EDR shows two maxima at 285 nm and 292 nm, while T4DR shows a single maximum at 285 nm with a shoulder at 292 nm. Both enzymes form complexes with NADPH which show the phenomenon of activation by energy transfer. This property is more pronounced with EDR than with T4DR. The effects of salts on protein fluorescence which has been quenched by substrate, cofactor, or inhibitors indicate that KSCN and NaClO$_4$ have large effects on the binding of both DHF and NADPH. NaCl and KCl have an identical, smaller effect on binding while KF is without effect. Little or no reversal of quenching is observed with the 4-amino compounds. The effects of salts in the EDR system are similar to those in the T4DR system but the changes are smaller. The T4DR-methotrexate complex is stable to 1 M guanidine-HCl but not 2 M guanidine-HCl, based on changes in protein fluorescence intensity. The same situation is true for the free enzyme. However, in 2 M guanidine-HCl, denaturation of the free enzyme is faster than denaturation of the complex, indicating that the latter is somewhat more stable. The T4DR-methotrexate complex has been shown not to involve stable covalent bond formation by treatment of the complex with sodium dodecyl sulphate and subsequent
resolution of enzyme and inhibitor by Sephadex G-25 chromatography. Difference spectroscopy of complexes of T4DR with methotrexate, aminopterin, and N^10-formylaminopterin show a marked resemblance to the difference spectra of the free inhibitors at acid versus neutral pH. This has been taken as evidence in support of the basicity hypothesis /Collin, R., and Pullman, B., Biochem. Biophys. Acta, 82, (1964) 232/ which explains the enhanced binding of 4-amino folate inhibitors on the basis of the greater basicity of these compounds compared to 4-hydroxyl compounds. The difference spectra of T4DR complexes with folate, dihydrofolate, and dihydroaminopterin were also obtained. These spectra do not resemble the acid-neutral spectra. The significance of the observed spectra for these complexes is not yet clear.
INTRODUCTION

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NAD(P) oxidoreductase, EC 1.5.1.3) is the enzyme which catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by transferring reducing equivalents from the coenzyme nicotinamide-adenine dinucleotide phosphate (NADPH). Throughout this work the abbreviation DR will be used to designate dihydrofolate reductase, regardless of source. (See Table IX, page 167, for list of abbreviations.) This enzyme plays a crucial supportive role in maintaining adequate THF levels for the numerous reactions which employ this coenzyme. While most of these reactions release the THF cofactor in its fully reduced form, the formation of thymidylic acid is an exception (1,2). The conversion of deoxyuridine monophosphate to thymidylate by thymidylate synthetase results in the oxidation of the THF cofactor (methylene THF in this case) and the production of a stoichiometric amount of DHF (3,4,5). Therefore, in the absence of an exogenous supply of a thymine compound, the concerted operation of DR as well as thymidylate synthetase is a requirement for the formation of thymidylate, and subsequently of deoxyribonucleic acid.
An understanding of these enzymatic processes, however, was not a necessary prerequisite for the employment of folate antagonists in certain types of chemotherapy. As early as 1948, Farber et al. (6) reported the treatment of leukaemia with the 4-amino analogue of folic acid, aminopterin. Nearly ten years elapsed after this before the observation was made that the target of the antifolates was probably DR.

**Enzymes That Reduce Dihydrofolate**

The potent inhibitory effect of aminopterin and its 10-methyl derivative methotrexate (amethopterin) towards DR was discovered independently by Futterman (7), by Osborn et al. (8) and by Zakrzewski and Nichol (9). The findings made by these groups stimulated interest in DR and in the past decade the enzyme has been examined from a number of sources (10, chap. 5). An examination of the resulting literature reveals that kinetic properties of DR in the presence of a vast array of inhibitors have been extensively investigated (10,11), while studies of the pure enzyme are quite limited. This is partially due to the fact that many of the laboratories that have studied DR are primarily interested in developing antineoplastic or antimalarial agents. However, an even more important reason for the emphasis on kinetics is related to the very low levels of DR found in naturally occurring tissues and the difficulty
encountered in its purification. These latter points can be illustrated with two examples. The chicken liver enzyme is considered homogeneous after an 8000 fold purification (12) but the recovery at this stage is less than 10%. Jarabak and Bachur have purified the enzyme from human placenta nearly 30,000 fold (13), but the material was probably not homogeneous and the recovery had dropped to 8%, leaving about 30 µg of protein. It is clear from these figures that studies of DR from a given source would be facilitated if considerably higher recoveries could be attained. For the DRs from two sources, T4-bacteriophage and its host cell *Escherichia coli*, simple methods of purification with high recoveries have been devised and will be described in detail in this work. Another approach to obtaining larger amounts of the pure enzyme is to induce high levels of the enzyme. This has been accomplished in bacteria for strains of *Streptococcus faecalis* (14), *Diplococcus pneumoniae* (15) and *Lactobacillus casei* (16) and in higher organisms with a mouse lymphoma (17) and finally in tissue culture with Sarcoma 180 (mouse) cells (18). In each case the approach was to conduct growth experiments in the presence of methotrexate, thereby selecting those cells which induced high levels of DR. In the case of the Sarcoma 180 cells it has been estimated that as much as 3 or 4% of the soluble protein was DR (18). However, even with these enormously increased levels of DR, the recovery
of enzyme by conventional purification procedures has retarded the study of the pure enzyme as judged by the lack of information in the literature.

An examination of the literature discloses considerable variation among the DRs from one source to another. Although most DRs have a molecular weight of about 20,000-30,000, the enzyme from *Plasmodium berghei* (mouse malaria) is much larger, showing a molecular weight of 190,000 as determined by gel filtration on Sephadex G-200 (19). Similarly, an amethopterin resistant mutant of *Diplococcus pneumoniae* has been reported to have a DR of molecular weight greater than 300,000 (20).

In general, the DRs from higher organisms can reduce folate as well as DHF but at a much slower rate at physiological pH, the pH optimum for folate reduction being about 5.0 (10). This activity towards folate is generally absent in procaryotic cells except for certain species of bacteria which have a folate growth requirement (10). But for these exceptions folate behaves as an inhibitor towards the DRs from procaryotic cells. Another area where the DRs from higher organisms differ from those of the bacterial sources is with respect to a group of substances which tend to activate the enzymes from the higher organisms while deactivating those from bacterial sources. However, the degree of activation is quite variable between the species
of higher organisms. For example, 0.6 M KCl causes an activation of approximately 3, 4 and 5 fold for DRs from human placenta, rat and mouse liver, respectively (13). Even different strains of the same species have shown marked difference with respect to KCl activation. Thus, Perone (21) found that a pyrimethamine (2,4-Diamino-5-(p-chlorophenyl)-6-ethylpyrimidine) resistant strain of Plasmodium berghei showed little response to a KCl concentration of 0.15 M whereas the pyrimethamine sensitive strain was activated 3 fold at this concentration. Other salts activate these enzymes as well and as pointed out by Reyes and Huennekens (22) the pattern of the salt effect is first an activation of activity, reaching a maximum at a certain optimum salt concentration and then a decrease in activity as the salt concentration is further increased. The optimum salt concentration for a given enzyme is quite variable with respect to the salt employed, showing greater dependence on the anion than on the cation. Activation by p-hydroxymercuribenzoate also produces a variable response. Whereas the chicken liver enzyme is activated several fold by the mercurial (23), the human placental enzyme shows no response (13). Inorganic iodine stimulated the chicken liver enzyme even more than the mercurial; as much as 10 fold (24). The effect was still attributed to reaction with sulfhydryl groups, however, as 2-mercaptoethanol could completely
reverse the activation. Curiously, the sulfhydryl reagents iodoacetamide, iodoacetic acid and N-ethylmaleimide neither inhibited nor activated the enzymatic activity.

In marked contrast to these types of activation displayed by the DRs from higher organisms, no bacterial enzyme has yet been shown to be significantly activated by any agent. Indeed, the usual case is deactivation by salts, sulfhydryl reagents and also urea or guanidine hydrochloride (10). (The last two reagents activate certain DRs from higher organisms.) As will be discussed in some detail in a later section, the T4 DR is particularly susceptible to deactivation by certain salts.

Other aspects of DRs, such as what is now understood about the mechanism of the enzymatic reaction, can best be described after a discussion of the substrate, cofactor and inhibitors. Therefore, these subjects will now be considered.

**Folic Acid Chemistry**

The discovery of pteroylglutamic acid as a new vitamin in the early 1940's served to focus considerable attention on the biochemical role played by this growth factor. The trivial name "folic acid" for the parent compound is now in common usage. As an aid to the biochemical investigations of the naturally occurring forms of
the vitamins, parallel studies were conducted on the chemical derivatives of folic acid (I).

\[\text{H}_2\text{N} - \text{N} - \text{N} - \text{N} - \text{N} - \text{N} - \text{N} - \text{N} - \text{N} \]

\[\text{H} - \text{N} \quad 9 \quad \text{CH}_2 \quad 10 \quad \text{N} - \text{H} \quad \text{C} - \text{NH} - \text{COOH} \]

\(\text{I) } \text{R}=\text{H} \)

\(\text{II) } \text{R}=\text{CHO} \)

\(\text{III) } \text{R}=\text{CH}_3 \)

This introduction will be limited to compounds pertinent to the present work on dihydrofolate reductase. Therefore, the numerous tetrahydrofolate derivatives involved in one-carbon metabolism will not be discussed. An inspection of the structural formula of folic acid shows a variety of functional groups, giving rise to numerous derivatives. \(\text{N}^{10}\)-formylfolic acid (II) is readily prepared by treatment of folic acid with concentrated formic acid (25) resulting in a quantitative conversion to the product. The \(\text{N}^{10}\) position also reacts with nitrous acid to produce the \(\text{N}^{10}\)-nitroso derivative (26). The synthesis of the \(\text{N}^{10}\)-methyl (III) compound will be discussed in connection with the total synthesis of folic acid.

The \(\text{N}^{10}\) position is also a reactive position with respect to chemical cleavage. Depending on the conditions,
three different types of cleavage can occur, all at the 9-10 position. Thus, aerobic alkaline hydrolysis produces pteridine 6-carboxylic acid (27), the presence of sulfurous acid produces pteridine 6-aldehyde (28) while zinc in acid produces 2-amino-4-hydroxy-6-methyltetrahydropteridine (28). In each case (p-aminobenzoyl) glutamate is a second degradation product. Folic acid is also decomposed by ultraviolet light, giving rise to numerous unidentified products (29).

Several halogenated derivatives of folic acid have been prepared (30,31) by substitution of the benzene ring. As could be expected, mono and di substitution takes place ortho (3' and 5') to the activating amino group (30).

Recently, Krumdieck and Baugh have developed a solid-phase synthesis of the polyglutamates of folic acid (31). The method, a modification of the Merrifield solid-phase peptide synthesis, results in peptide bond formation between the amino group of one glutamate moiety to the γ-carboxyl of the next glutamate moiety. Folic acids bearing a total of seven glutamates have been synthesized in this way. As will be discussed later, the penta or hexa-glutamate folic acid derivative should be of particular interest in connection with T-even phage DRs, as both of these entities (pteroyl polyglutamate and DR) have been found in the phage tail plates (32,33) and the identity between the soluble and particle bound enzyme established (34).
A number of folic acid derivatives have been prepared by means analogous to the total synthesis of folic acid. These include the 4-amino inhibitors methotrexate and aminopterin (35) and also a series of N\textsuperscript{10}-alkyl compounds (36). The synthesis of folic acid has been accomplished in a number of ways (see ref. 37 for a review) but most of the syntheses employ a simultaneous reaction between three components such as A, B and C.

\[
\begin{align*}
A & \quad \text{CHO} \\
& \quad \text{CHBr} \\
& \quad \text{CH}_2\text{Br}
\end{align*}
\]

If compound A, 2,4,5-triamino-6-hydroxypyrimidine is replaced by 2,4,5,6-tetraaminopyrimidine, the product is aminopterin rather than folic acid. There are, of course, numerous side reactions in either case (37). N\textsuperscript{10}-alkyl folic acids are synthesized by employing the appropriate secondary p-aminobenzoylglutamic acid for compound C. Therefore, either methotrexate or N\textsuperscript{10}-methylfolic acid can be synthesized by the reaction between the
appropriate pyrimidine, 2,3-dibromopropanal and N-methyl(p-aminobenzoyl)glutamic acid.

The reduction of folic acid compounds by chemical means has been accomplished by a number of means depending on the desired product. The dihydro series of compounds are usually prepared by reduction with dithionite (6,38,39). In certain cases, such as the reduction of 7-methylfolate, dithionite reduction fails. 7-methylfolate has been reduced to the dihydro compound by zinc in alkaline solution (40). Since these compounds are susceptible to air oxidation they are generally prepared in the presence of ascorbate or 2-mercaptoethanol and stored in sealed, deoxygenated vials. THP is generally prepared by catalytic hydrogenation in the presence of platinum in acid solution (41), although it has also been prepared by borohydride reduction (42). THP is considerably more susceptible to air oxidation than dihydro compounds, being oxidized to DHP in 9 min in a solution at pH 7.5 (43). If THF is first exposed to 2-mercaptoethanol its stability is considerably enhanced (44).

When folate is reduced by dithionite, the resulting DHF has been shown to have the 7,8 dihydro configuration (IV) by means of nuclear magnetic resonance and by tritium labeling studies (42,49). THF is assumed to have the 5,6,7,8-tetrahydro structure, an assumption supported by
the greater ease of reduction of pyrazines than pyrimidines.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{N} \quad \text{N} \quad \text{H} \\
\text{N} & \quad \text{H} \quad \text{CH}_2\text{-p-aminobenzoylglutamate} \\
\text{OH}
\end{align*}
\]

(IV)

When either folate or DHF is reduced to THF, a new asymmetric center is introduced at carbon 6. Chemically prepared THF is a mixture of the d,l stereoisomers whereas enzymatic reduction produces L,L-THF (L-glutamate) exclusively (46).

**Absorption and Fluorescent Properties of Folic Acid Compounds**

Folic acid is composed of two basic chromophores; a pterin moiety and an N-substituted p-aminobenzoic acid. The pterin moiety is in turn composed of the resonance-interacting pyrimidine and pyrazine rings. When the pterin is in the oxidized state, such as folic acid, the absorption spectra generally display characteristic maxima. As is shown in Fig. 1, folic acid has peaks at 282 nm and at 350 nm at pH 7.0 in 0.1 M potassium phosphate buffer (47). As has been pointed out by Huennekens and Osborn, the peak in the 350 nm region is a property of the pterin moiety
Fig. 1. Ultraviolet absorption spectrum of some folic acid compounds.

All spectra are for solution in 0.1 M potassium phosphate buffer, except for dihydroaminopterin.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Compound</th>
<th>pH</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Folic acid</td>
<td>7.5</td>
<td>47</td>
</tr>
<tr>
<td>A</td>
<td>N10-Methylfolic acid</td>
<td>7.0</td>
<td>48</td>
</tr>
<tr>
<td>A</td>
<td>N10-Formylfolic acid</td>
<td>7.5</td>
<td>47</td>
</tr>
<tr>
<td>B</td>
<td>DHF</td>
<td>7.5</td>
<td>47</td>
</tr>
<tr>
<td>B</td>
<td>N10-Methyl DHF</td>
<td>7.0</td>
<td>48</td>
</tr>
<tr>
<td>B</td>
<td>N10-Formyl DHF</td>
<td>7.0</td>
<td>49</td>
</tr>
<tr>
<td>C</td>
<td>THF</td>
<td>7.5</td>
<td>47</td>
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<tr>
<td>C</td>
<td>N10-Methyl THF</td>
<td>7.0</td>
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</tr>
<tr>
<td>C</td>
<td>N10-Formyl THF</td>
<td>7.5</td>
<td>47</td>
</tr>
<tr>
<td>D</td>
<td>Aminopterin</td>
<td>7.0</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>Methotrexate</td>
<td>7.0</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>Dihydroaminopterin</td>
<td>13.0</td>
<td>51</td>
</tr>
</tbody>
</table>
Fig. 1. Ultraviolet absorption spectrum of some folic acid compounds.
alone, while the high energy peak has contributions from both the pterin and the p-aminobenzoic acid portions. When the pH is shifted from 7.0 to 13.0, the 282 nm peak of folic acid is split (not shown) producing two maxima (52), one at about 255 nm, presumably due to the pterin, and a second near 285 due to the N-substituted p-aminobenzoic acid (47). Partial splitting of the high energy peak can be accomplished at neutral pH by alkylation of the N^10 position (35) as shown in Fig. 1 for N^10-methylfolate (48). Presumably, this is due to a shift in the absorption maxima of the N-substituted p-aminobenzoic acid portion, since the N^10 position is "insulated" from the pterin by the C^9-methylene group. When the N^10 position is substituted by the electron-attracting formyl group, the shift is in the direction opposite to N^10 alkylation, resulting in a single high energy peak blue shifted about 20 nm as compared to folic acid.

When the pyrazine ring is reduced to the dihydro level, absorption in the 350-400 nm region is altered. As is shown in Fig. 1, the reduction of folate to DHF eliminates the 350 nm maxima, producing a gently sloping shoulder at 304 nm (47). Further reduction of the pyrazine ring as in THF results in elimination of this shoulder, abolishing absorption at wave lengths greater than 350 nm (47).
similar stepwise change in absorption is seen upon reduction of $N^{10}$-methylfolate (48) and $N^{10}$-formylfolate (49).

Substitution of the 4-hydroxyl group of folic acid by a 4-amino group, as in aminopterin (Fig. 1, panel D), causes a blue shift in the high energy maxima of the pterin moiety (35). $N^{10}$-methyl substitution (methotrexate) results in a red shift for absorption by the p-aminobenzoic acid group (35), similar to folic acid, and reduction of the pyrazine ring results in likewise predictable absorbance changes (51).

The most thorough studies of the fluorescence of folate derivatives are those of Uyeda and Rabinowitz (53). These authors found that the fluorescence of folate compounds depended markedly on the pH. Thus folic acid is virtually non-fluorescent at pH 6.0 but does exhibit appreciable fluorescence at either higher or lower pH values, particularly the basic pH values. DHF also is more fluorescent at basic pH values, while THF is intensely fluorescent at acid pH values, reaching an optimum at pH 3.0. Reduction of the pyrazine ring causes decreases in both the excitation and emission maxima, being 363 nm and 450 nm for folate and 305-310 nm and 360 nm for THF. DHF holds an intermediate position with excitation and emission maxima of 317 and 425 nm respectively.
Spectral and Structural Properties of NADPH-Cofactor for Dihydrofolate Reductase

Reducing equivalents for the enzymatic reduction of DHF are provided by the pyridine nucleotides. Since the reaction with NADPH is several times faster than with NADH (10), NADPH is regarded as the natural cofactor. The absorption spectra of NADPH and NADP⁺ are shown in Fig. 2. The difference in the absorption of the reduced and oxidized forms at 340 nm, about 6,200 M⁻¹ cm⁻¹, has been used in the assay of a number of enzymes. There is also a difference in the absorption of DHF and THF at 340 nm. Since these absorption changes complement each other, the reaction

$$\text{DHF} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + \text{THF}$$ (1)

is accompanied by a total molar absorbancy change of 12,000 M⁻¹ cm⁻¹ (49). NADPH also possesses fluorescent properties which have made possible a study of its complex formation with various DHs. These will be discussed in detail in a later section.

Oxidation and reduction of the nicotinamide ring was shown to take place at the 4 position by Pullman, et al. (54). When NADP⁺ is converted to NADPH by dithionite reduction (54) the entering hydride can occupy one of two different positions with respect to the plane of the nicotinamide ring. Thus, when reduction takes place in
Fig. 2. Structure and ultraviolet absorption spectrum for NADP$^+$ and NADPH.
tritiated water, a mixture of the two isomers shown in the illustration is produced; the two sides being designated

\[ \text{A} \quad \text{B} \]

A and B (55). Almost all enzymatic reactions are specific for either the A or B side (56), the absolute configuration of which has been determined by Cornforth, et al. (57).

**Mechanism of the Enzymatic Reduction of DHF**

The reduction of DHF by NADPH according to Equation 1 is energetically favorable, having an equilibrium constant estimated to be in the range of \(0.84 \times 10^{11}\) to \(5.6 \times 10^{11}\) (49).

Attempts to demonstrate an ordered sequence of binding for various DRs have produced conflicting results. Fluorescence quenching studies of the L1210 DR have indicated that the folate analog triamterene (2,4,7-triamino-6-phenylpteridine) binds to the enzyme-NADPH complex 60 times greater than to free enzyme alone, but NADPH also binds to the enzyme-triamterene complex better than to free enzyme alone (58). This has been taken as evidence for a random addition of substrate and cofactor. However,
Blakley has pointed out that this ternary complex may have no kinetic significance (10). Kinetic evidence obtained by this author with the enzyme from \textit{S. faecalis} A indicates that NADPH binds to the enzyme before DHF. This conclusion was drawn from data which showed that inhibition by certain 2,4-diaminopyrimidines and by folate is competitive with respect to DHF and uncompetitive with respect to NADPH. Furthermore, plots of reciprocal velocity against reciprocal DHF concentration at various fixed concentrations of NADPH intersect instead of being parallel (10). Other evidence that the reaction has an ordered sequence with NADPH has been drawn from the finding that many DRs bind NADPH more tightly than DHF. In fact, for some DRs the binding of NADPH is sufficiently stable to permit the separation of the enzyme-NADPH complex from the free enzyme. Thus, the chicken liver enzyme has been reported to occur in multiple forms (59). The basis for this polymorphism was later found to be due to a portion of the enzyme occurring as a complex with NADPH; a similar situation obtains with the DR from \textit{L. casei} (16) and the L1210 lymphoma (17).

It is now known that the hydride ion transferred from the C4 position of NADPH to DHF occurs from the A side (Fig. 2) for DRs from chicken liver (60), mouse leukemia (60), \textit{S. faecalis} (61), and Sarcoma-180 cells (62). It will be shown that the T4 DR also has this specificity.
Some evidence has been presented by Zakrzewski (62) suggesting a possible arrangement of the nicotinamide ring with respect to the pterin moiety during the enzymatic (by Sarcoma 180 DR) transfer of the hydride ion. This author had prepared and separated the two stereoisomers of 7-methyl-DHF (V) and found that one isomer was a better substrate than the other by a factor of 60. Since both isomers were nearly equally effective inhibitors of the reduction of DHF, Zakrzewski concluded that both isomers were bound to DR with about the same strength. Therefore, the difference in the observed rates of reduction between the two isomers as substrates must be related to some other factor. As a possible explanation, Zakrzewski suggested "that the 7-methyl group (of one isomer) may interfere with the proper positioning of NADPH with respect to 7-methyl DHF." It was also suggested "that NADPH rests with its nicotinamide ring parallel and on top of the pyrazine ring" in the enzymatically productive complex, with the 7-methyl group of the less active isomer interfering with the transfer of the hydride ion from the A side of C4 of NADPH.
During the reduction of 7-methyl DHF (V) by dithionite or borohydride, Zakrzewski found that the product 7-methyl THF had lost considerable label, much of which was located at C7. On the other hand, enzymatic reduction resulted in a product with a specific activity equal to that of the substrate. Zakrzewski interpreted these facts to mean that chemical reduction was accompanied by considerable intramolecular rearrangement, while enzymatic reduction was not so accompanied and probably proceeded by a direct addition of hydride ion from NADPH to carbon 6 (62). The absence of a rearrangement during enzymatic reduction has also been demonstrated by NMR (63).

The Sarcoma 180 DR, being of mammalian origin, belongs to the class of DRs which can reduce either folate or DHF to THF. For these enzymes, the question arises: Is the NADPH binding site for the reduction of folate identical to that for the reduction of DHF, particularly with respect to positioning of the nicotinamide ring. Since intramolecular rearrangements appear to be excluded, it is generally assumed that hydride transfer directly to either carbon 6 or 7 can take place (10), but the relatedness of NADPH binding sites remains an open question.

So far, an understanding of the part played by the enzyme structure during the reduction of folate or DHF is quite limited. Baker has suggested that a histidine residue
could participate in proton transfer to $N^8$ and $N^5$ during the two-step reduction of folate to THF (64). According to this postulate, the imidazole ring would be placed "face-to-face" with the pyrazine ring of folate. Thus, one tautomer of the imidazole could transfer a proton to $N^8$ and the second tautomer could transfer a proton to $N^5$ as shown in Fig. 3.

Another feature of the binding site for folic acid which needs to be accounted for is the greatly enhanced binding that 4-amino-4-deoxyfolate compounds display towards all DRs which have been examined. This universal feature suggests that some indispensable enzyme structure is responsible for the observed enhancement of binding, an enhancement which has been estimated to be 10,000-fold (55) to 100,000-fold (65,66) in the case of aminopterin. Aminopterin has been estimated to be about 1,000 times more basic than folic acid (66). Although theoretical calculations showed $N^1$ was the most basic position in aminopterin (67), suggesting a linkage as shown in Fig. 4, A, Zakrzewski could find no relationship between basicity of a group of heterocycles and their affinity for DR (66). Baker has suggested that enhancement in binding could be partially accounted for by interaction between delocalized charge on aminopterin (and other 2,4-diaminoheterocycles) and partially ionized acidic groups on the enzyme (68,69). In addition to this he has suggested that the 4-amino
Fig. 3. Postulated involvement of a histidine residue in transferring protons to $N^6$ and $N^5$ during the reduction of folate and DHF, respectively.
Fig. 4. Some possible structures explaining the enhanced binding of 4-amino compounds to DH.
substitution alters the character of $N^5$ and $N^8$ to an extent that "quasi-ionic bonding" between these positions and an acidic function on the enzyme are possible with aminopterin but not with folic acid (64). Thus, the pyrazine portion also could make a contribution to the enhanced binding.

Still another suggestion to account for increased binding by the 2,4-diamino compounds postulates the existence of hydrogen bond formation (49,65,66). Since at physiological pH folic acid exists as the 4-keto form (Fig. 4, B), it could not participate in the type of hydrogen bond formation shown in Fig. 4, C (10,66). As has been pointed out by Mathews and Huennekens (49), a single hydrogen bond, particularly in a hydrophobic environment, could account for the enhancement of binding observed for aminopterin, at least, in the case of the chicken liver enzyme.

Bacteriophage Specific Dihydrofolate Reductase

The large rise in DR activity found in cells of *E. coli* infected with T4 phage was first reported by Mathews and Cohen (70). This 10 to 20-fold increase of reductase level was found to be induced by the T-even phage T2, T4 and T6 and by T5 (70). Subsequently, Mathews and Sutherland (71) showed that DR activity in uninfected cells precipitated in the 50-80% ammonium sulfate fraction, whereas the activity in infected cells could be separated into two
fractions, one precipitated by 50% of saturation with ammonium sulfate, and a much smaller fraction precipitating in the same region as that from the uninfected cells. Thus, the difference between the phage-induced enzyme and the host enzyme was established. However, it was not clear at this point whether the activity produced upon infection was a viral gene product or not. To answer this question, the DRs from T2, T4, T5, T6 and uninfected cells of E. coli were compared on the basis of heat stability, sedimentation behavior, and treatment with urea and inhibitors (72). These studies showed that the phage enzymes were more similar to each other with respect to inhibition by aminopterin or trimethoprim (2,4-diamino-5-(3',4',5'-trimethoxybenzyl) pyrimidine) than they were to the E. coli DR. On the other hand, treatment with urea or heat demonstrated marked differences between the phage enzymes. Thus, if the phage-induced enzymes were coded for by the E. coli genome, it would be necessary to postulate the induction of a different E. coli gene by each phage. The discovery of mutants in the phage DR gene discredit such a postulate.

Another point that was unclear concerned the need for an increase in an activity which already existed in the host cell. Since the product of the new enzyme was the same as the pre-existing enzyme (1, L-THF) the need for a special form of THF could be ruled out (73). Techniques
for the recognition of phage mutants with defects in pyrimidine metabolism by plaque assay (74) greatly aided the selection of mutants which are unable to induce phage DR (75,76). Studies with these mutants indicated that induction of the enzyme was useful but not necessary for phage production, with the mutant strain producing approximately two-thirds as many progeny phage per infected cell as the wild-type phage (75).

Recently the findings of Kozloff, et al., have disclosed another quite unexpected role for the phage DR (33). As already mentioned, this group has presented evidence that the phage base plate contains an unusual dihydropteroylpolyglutamate (32). It was therefore of interest to determine what phage protein bound this folate derivative. It was first found that phage ghosts (phage which have been osmotically shocked to remove their DNA) possessed a DR activity (77) which was subsequently localized in the base plate (33). The properties of this activity have many differences from the soluble enzyme. Thus, for phage ghosts to show DR activity they must be treated with 6 M urea or with 1.4 to 3.2 M formamide for various times and temperatures. The assay cell also contained significant amounts of urea or formamide. In the absence of treatment with urea or formamide the presence of an extremely limited activity was inferred by inactivation or reactivation of the phage
particle by NADPH and NADP as measured by changes in phage titer determined by the plaque assay. The conclusion was made that (i) the dihydro form of the folate compound bound to the phage particle was not released when converted to the level of either folate or tetrahydrofolate; (ii) the phage particle was viable only when the folate compound was in the dihydro form.

Although the existence of viable mutants in the phage DR gene which eliminates the increase of this activity in the soluble fraction would appear to contest the assumption of an identity between the soluble and particle-bound activities, it was a study of these mutants which first helped to establish this identity (33). When one such mutant designated T4Dwhll was grown on E. coli B, the progeny phage showed a heat inactivation curve that was strikingly different from the heat inactivation curve observed for T4Dwhll progeny grown on E. coli CR63, a bacterial strain which suppresses the amber mutation of the phage, permitting the synthesis of a functional, soluble DR. Furthermore, the progeny grown on E. coli CR63 were inactivated by heat in a fashion similar to the heat inactivation curve for the wild-type phage T4D. From these studies Kozloff, et al. concluded that "in a most unusual case of an amber mutation, in the wh gene where no suitable phage protein is produced, another DR (most likely the host enzyme) is
incorporated into the structure, producing a phage with altered phenotypic properties."

The identity of the gene coding for soluble and structural DRs has been confirmed by Mathews (34). Since the T6 soluble DR is much less heat stable than the T4 enzyme (72) it was expected that the heat inactivation curve for the T6 and T4 phage particles would have a predictable difference. While a marked difference was observed, it was in the reverse order (34). Thus, at 60°, T4 was inactivated about 100 times more rapidly than T6. On the other hand, when the gene for the T6 DR was inserted into a T4 genome with a mutation in its DR (wh) gene, by means of genetic recombination, followed by several backcrossings, a phage was obtained that showed the heat inactivation curves (for both phage particle and for soluble DR) displayed by T6 phage. While the identity for soluble and particle bound DR appears to be confirmed, the function of the particle bound DR is less clear and should provide a basis for some interesting future experiments.

Still another aspect of phage DR which deserves mention concerns the mechanism of its control. T4 DR belongs to a class of enzymes known as "early" because they are synthesized within a few minutes after infection of the host cell and approximately ten minutes after infection their synthesis ceases (78). However, T4 DR appears to
belong to a subgroup of these early enzymes. This conclusion can be drawn from a number of observations. There are a number of mutations which result in a large overproduction of several of the early enzymes but not of T4 DR (79). With these mutants T4 DR synthesis stops at the same time as in a wild-type infection, clearly indicating a difference in the control mechanism for T4 DR and the other early enzymes. The phage DR is also synthesized faster than the other early enzymes, reaching 50% of maximum by two minutes post infection (78,79). Recently, Lembach and Buchanan (80) have presented evidence that the synthesis of the messenger RNA coding for T4 DR is largely unaffected by the presence of chloramphenicol, added just before infection, while the synthesis of several other early enzyme messengers is severely limited. Thus, evidence is mounting that the T4 DR messenger is synthesized by the E. coli RNA polymerase found in uninfected cells. If other experiments could be designed to unequivocally establish this fact, it would be worthwhile to look for an amber mutant which overproduced T4 DR. Aside from providing a richer supply of T4 DR (worth the effort in itself), such a mutant might arise from a mutation in a control element of the transcriptive process and therefore be of fundamental interest from the standpoint of phage development (81, 82). Such a mutant would probably be of the "conditional
lethal" variety, complicating its selection. Although trial and error methods would probably have to be employed, an assay which could detect differential DR activity on a bacterial lawn "spotted" with different test mutants would be of great assistance. It might be possible to develop such an assay with the aid of the tetrazolium dye MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) which reacts with THF nonenzymatically, but not with DHF (59, 83). The rate at which the purple color accompanying this reaction develops should be a reflection of DR concentration. If a mixture of NADPH, DHF and MTT were sprayed onto a plate containing a bacterial lawn of the non-permissive host infected by a mutant phage which over-produces phage DR, a faster than normal color development might be expected to occur.

In the present work, studies of the soluble T4 DR have been extended in a number of directions. The enzyme has been purified to homogeneity, both by conventional means and subsequently by the powerful techniques of affinity chromatography. The availability of homogeneous enzyme has permitted a study of the interaction of the enzyme with substrate, cofactor and inhibitors by means of fluorescence and absorption techniques. In addition, a number of other properties of the enzyme have been determined, including its molecular weight, amino acid composition, and some kinetic properties. Since affinity
chromatography is a function specific purification process, it should, in principle, be possible to purify other DRs by this method. Indeed, the purification of the host cell enzyme by this technique will be described and the similarities and differences as compared to the phage enzyme discussed.

Interest in the phage-host cell DR system derives, in part, from the relative abundance and ease of preparation of the phage-induced DR, making this enzyme a convenient model for the study of this activity. In addition, the finding that T4DR is a structural element in the base plate of the T4 phage and that in certain cases the E. coli enzyme can substitute for the T4 enzyme makes a comparison of these enzymes of interest in its own right. The experiments described in this thesis are therefore designed to gain insight into the relatedness of the two enzymes and to discover what properties they have in common. It is also hoped that such a study would help to define some of the properties which are necessary features of this activity.
EXPERIMENTAL PROCEDURES

Materials

Sephadex G-100, G-25 and DEAE A-50 were obtained from Pharmacia. DEAE-cellulose, TEAE-cellulose, and Bio-Gel P-150 were from BioRad. Chemicals for electrophoresis on polyacrylamide gels were obtained from Canalco. Methotrexate (4-amino-10-aminopteroylglutamate) was the gift of Dr. J. M. Smith, Jr., of Lederle. Aminopterin (4-aminopteroylglutamate) was purchased from Nutritional Biochemical Co. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was purchased from Ott Chemical Co., Muskegon, Michigan. NADPH, streptomycin sulfate, sodium dodecyl sulfate, 2-hydroxy-5-nitrobenzyl bromide, p-chloromercuribenzoic acid, ω-chymotrypsinogen A, trypsin (bovine pancreas), bovine serum albumin, ovalbumin, and folic acid were obtained from Sigma. DHF was prepared by the method of Futterman as modified by Friedkin et al. (94) and, for routine assay work, stored in sealed ampoules as a dry powder under nitrogen. DHF used in binding studies was prepared on the day of its use. The aminoethyl derivative of Bio-Gel P-150 was prepared (Fig. 5) as described by Inman and Dintzis (85), by the direct reaction between Bio-Gel P-150 and ethylenediamine. The heating period was 3.5
hours. After the final wash the material was stored in distilled water at 4°. Dihydroaminopterin was prepared by dithionite reduction of aminopterin (51, 84). N\textsuperscript{10}-methylfolate was prepared by hydrolysis of methotrexate (36) and N\textsuperscript{10}-methyl DHF was prepared by dithionite reduction of N\textsuperscript{10}-methylfolate (48). Highly purified guanidine hydrochloride was obtained from Mann. Other chemicals used were obtained from local sources and were of reagent grade.

**Synthesis of N\textsuperscript{10}-formylaminopterin**

A method analogous to the synthesis of N\textsuperscript{10}-formyl- folic acid (25) was used. Two hundred mg of aminopterin was dissolved in a reagent composed of 20 ml of 98% formic acid and 5 ml of acetic anhydride and heated at 100° for one hour. Volatile reactants were removed in vacuo and the product was dissolved in 10 ml of 50% aqueous dimethylformamide. One to two ml of 3% NaHCO\textsubscript{3} may be required to complete the solution process. This solution was used directly in the coupling reaction.

**Purity of Folate Compounds Employed**

The purity of chemicals employed was checked by means of published absorption spectra or by paper chromatography and by thin layer chromatography on Silica gel G or cellulose powder. The spectra of methotrexate obtained from Lederle and aminopterin obtained from Nutritional
Fig. 5. Steps in the synthesis of the solid support used for affinity chromatography and its linkage to N10-formylaminopterin.
Biochemicals were identical to published spectra (36,50). Since chromatography on paper or thin layers (development was with 0.1 M tris-glycine buffer, pH 9.0) showed a single "quenching" spot (49), these substances were used without further purification. The purity of dihydroaminopterin, N°-methyl DHF and N°-formyl DHF was determined by published spectra after purification on DEAE-cellulose (refs. 86,48,49 respectively). The purity of N°-formylaminopterin was checked by paper chromatography in 0.1 M tris-glycine, pH 9.0.

**Coupling of N°-formylaminopterin and Methotrexate to Aminoethyl Bio-Gel P-150**

The conditions used in the coupling reaction are essentially those described by Cuatrecasas for coupling 3-0-succinylestradiol to aminoethyl Sepharose (87). Excess water was drained from the aminoethyl Bio-Gel P-150 by means of a filter funnel. About 50 ml of this slurry was transferred to a 250 ml TPX beaker (Nalge Co.). 30 ml of dimethylformamide was added, followed by the addition of about 50 mg of N°-formylaminopterin (2.5 to 3 ml). The pH was lowered to 4.7 with concentrated HCl while the material was stirred gently. Five hundred mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 5 ml of water was added over a 5-minute period. The reaction was allowed to proceed for about 18-24 hours at
room temperature with gentle stirring. The material was then transferred to a column, 6 x 15 cm, and washed with 100 ml of 50% aqueous dimethylformamide, followed by about 5 liters of 0.5 M NaCl dissolved in 1% NaHCO₃ over a period of 3 days. It is important that all glass coming in contact with the material be previously treated with Siliclad (85). Methotrexate was also readily linked, in large amounts, to aminoethyl Bio-Gel F-150 in exactly the same way by substituting methotrexate for N⁴⁰-formylaminopterin.

**Phage Production**

T₄amN82 (defective in gene 44) was prepared in two ten-liter batches at a time in a New Brunswick Fermentor. On E. coli B this DNA-negative phage synthesizes early but not late protein; consequently it is non-lysing. This property makes it useful in the continuous-flow production of cells as described below. The following medium was used, per liter: 7 g of Na₂HPO₄, 3 g of KH₂PO₄, 1.5 g of NH₄Cl, 10 mg of CaCl₂, 100 mg of MgSO₄, 5 mg of Fe(NH₄)₂(SO₄)₂·6H₂O, and 10 g of glucose. One liter of a saturated culture of E. coli CR63 was added to each fermentor jar. Cells were grown with forced aeration at 37° to a cell density of 6 x 10⁸ cells per ml, at which time the cells were infected at a multiplicity of infection of 0.1. The pH was maintained near 6.8 by addition of concentrated ammonium hydroxide until phage production was
complete, about 4 hours post infection. At this point aeration was discontinued and 1 ml of chloroform per liter of lysate was added and stirring continued for 10 minutes. The lysate was stored in the cold room and used without purification. Final phage titers obtained were of the order of $1 \times 10^{11}$ plaque-forming units per ml.

Wild-type T4D was grown in a similar fashion with the following exceptions: The host cell was *E. coli* B and the multiplicity of infection was 0.01. Yields on the order of $5 \times 10^{11}$ plaque-forming units per ml were obtained.

**Infected Cell Production**

The operation of the New Brunswick Fermentor of this type for the continuous-flow production of infected cells has been described (38). This method has been employed in the present case with the following modifications (Fig. 6): One hundred and ten liters of complete medium was autoclaved in two 60 liter stainless steel tanks. The medium was the same as used for phage production with the following exceptions: Glucose was replaced by glycerol, 4 g/liter of yeast extract was present and the pH of the media was raised to 7.8 with NaOH. Both fermentors were used for host cell production. A third fermentor was constructed for the infection vessel (Fig. 6). The medium was fed directly into the growth vessels by gravity at a flow rate of about 150 ml/min per jar and
Fig. 6. Schematic diagram of apparatus used in the large scale production of infected cells.
bacteria were pumped from the jars to the infection vessel at the same rate. Four phage particles per bacterium were pumped into the infection vessel. The level in the infection vessel was maintained by a glass tubing "stand pipe" of adjustable height which also served as the exit for infected cells. The volume of media in the infection vessel (about 9-10 liters) was determined so as to give the infected cells a mean residence time of about 12 to 15 mins (89). The stirring impeller of the infection vessel had a fixed speed of 450 rpm. No provision was made to sterilize this apparatus, however; the short period of mean residence time virtually eliminated any buildup of contaminants. The infected cells issuing from the infection vessel were collected in plastic buckets, cooled somewhat with a small amount of ice and fed into a refrigerated Sharples continuous-flow centrifuge operating at 50,000 rpm at a flow rate of 400 ml per minute.

**Preliminary Enzyme Purification Steps**

One thousand grams of frozen cells were stirred gently with sufficient 0.05 M tris-HCl, pH 7.4, initially at room temperature, to give a final volume of 5 liters. When the cell paste was almost completely melted, the suspension was transferred to the cold room, where it was blended to a smooth consistency with a Virtis homogenizer. All subsequent steps were performed at 0-4° except as
noted. The suspension was pumped at 40 ml per minute through a continuous-flow, ice-water-jacketed, sonication chamber of 100 ml capacity. Sonic oscillation was provided by the full power setting of a Branson sonifier. A second passage at the same rate completed the cell disruption. Cell debris was removed by centrifugation at 20,000 x g for 20 min. To the supernatant was added 0.3 volume of an 8% streptomycin sulfate solution. The milky suspension was clarified by centrifugation at 10,000 x g for 15 min.

Conventional Purification Scheme

To each liter of the streptomycin supernatant was added 313 g of solid ammonium sulfate. The resulting precipitate was stirred for 20 min. and collected by centrifugation. The supernatant was discarded, and the precipitate was dissolved in a minimal amount of 0.05 M tris-HCl buffer, pH 7.0 (buffer A) and dialyzed overnight against 20 liters of the same buffer.

DEAE-Cellulose Fractionation

DEAE-cellulose, 150 g, was suspended in buffer A. The fines were removed by decantation and the slurry was poured into a column 9.5 x 20 cm. The protein sample, about 35 g, was applied to the column at a rate of 5 ml per min. The column was then washed with buffer A until most nonadsorbing protein had been washed off the column.
and the absorbance at 280 nm ($A_{280}$) was about 0.5. The column was then washed with 0.2 M NaCl in buffer A at a rate of 15 ml per min. Fifty-ml fractions were collected in beakers until the $A_{280}$ was once more about 0.5. Fractions with a specific activity greater than the sample applied were pooled (pool 1).

Second Ammonium Sulfate Fractionation

To each liter of pool 1 was added 270 g of solid ammonium sulfate. The resulting precipitate was stirred for 20 min. and collected by centrifugation in four 250-ml centrifuge bottles. The supernatant was discarded. To each centrifuge bottle was added 200 ml of ammonium sulfate solution prepared by adding 245 g of solid ammonium sulfate and one ml of mercaptoethanol to one liter of buffer A. The precipitate in each bottle was suspended in the solution by means of a spatula. The settled precipitate was resuspended by stirring every 10 minutes for two hours and then recentrifuged. The supernatant was discarded and the extraction repeated with a similar solution prepared by adding 220 g of ammonium sulfate and 1 ml of mercaptoethanol to one liter of buffer A.

Sephadex G-100 Filtration

The precipitate from the final ammonium sulfate extraction was redissolved in 0.04 M potassium phosphate
buffer, pH 7.0 (buffer B). The sample was applied to a Sephadex G-100 column, 9.5 x 110 cm. An initial flow rate of 70 ml per hour was used. After 2000 ml of buffer B had passed through the column the flow rate was increased to 120 ml per hour. At this time 20-ml fractions were collected until a total of 5000 ml of buffer B had been applied to the column.

DEAE-Sephadex Chromatography

DEAE-sephadex A-50 (250 mg) was suspended in 200 ml of buffer B, in a one liter beaker, allowed to sediment and drained of excess liquid, after which pool 2 was poured into the beaker. The contents were stirred gently overnight by which time the enzyme was quantitatively adsorbed to the slurry. The slurry was transferred to a column, 1/2 x 6 cm and washed with 20 ml of buffer B. The enzyme was then eluted by a linear gradient of 0.04M - 0.40 M potassium phosphate, pH 7.0, formed by 150 ml of buffer B in the mixing chamber and 150 ml of 0.40 M potassium phosphate buffer pH 7.0 in the reservoir. Six-ml fractions were collected at a flow rate of 80 ml per hour.

TEAE-Cellulose Chromatography

TEAE-cellulose was suspended in buffer A, the fines were removed by decantation, and the remaining material was poured into a bed of final dimensions
1.2 x 30 cm. Pool 3 was concentrated to about 5 ml by means of an Amicon membrane concentrator, dialyzed against one liter of buffer for four hours, then against another liter of buffer A overnight. The sample was then applied to the column, followed by a linear gradient of 0-0.40 M NaCl formed by 250 ml of buffer A in the mixing chamber and 250 ml of 0.40 M NaCl dissolved in buffer A in the reservoir. Fractions of eight ml were collected.

Preparative Electrophoresis

Pool 4 was concentrated to about 10 ml by means of a membrane concentrator, dialyzed overnight against 2 liters of buffer A, and further concentrated to about one ml by means of placing the dialysis bag in solid sucrose. Preparative electrophoresis was conducted with apparatus manufactured by Canalco. A 4-cm separating gel and a 1-cm stacking gel were cast in the 2.2-cm diameter electrophoresis columns. The composition of the gels and buffer were those used for standard analytical purposes described by Davis (90) except that all gels were polymerized by riboflavin and light, and the separating gel contained 0.005% 2-mercaptoethanol. Electrophoresis conducted in gels polymerized by ammonium persulfate led to destruction of nearly all activity. Ice water was circulated through the cooling jacket, and no provisions were made for elution buffer. Two or three drops of tracking dye were
added to the sample before it was layered onto the top of the stacking gel. Electrophoresis was conducted at 5 ma for 3 hours, then at 9 ma for 9 hours, by which time the tracking dye had migrated 2.8 cm into the separating gel. At this point electrophoresis was discontinued, the gel column was removed from the apparatus and filled with buffer. A stopper, fitted with a tubing connected to a 20 ml syringe mounted in a syringe pump, was inserted into the opening of the electrophoresis column. The column was then inverted, the gel was loosened with a thin wire and the gel was extruded from the column. Slices of one to two mm were cut off with a clean razor blade as the gel emerged and were placed in separate 50 ml centrifuge tubes containing 10 ml of buffer A. After a 20-hour incubation the tubes were assayed for activity. The gels in the tubes showing activity were crushed and the incubation continued for another 24 hours at which time the tubes were centrifuged, and the supernatant from each tube was stored as a separate fraction at -20°.

Purification of T4DH
by Affinity Chromatography

70 g of DEAE-cellulose was equilibrated with 0.15 M NaCl in 0.04 M Tris-HCl, pH 7.0. The slurry was sedimented by centrifugation and the DEAE-cellulose pellet was stirred into the streptomycin sulfate supernatant which
had previously been brought to 0.15 M in NaCl. After 30 min the slurry was again removed by centrifugation. To each liter of the supernatant was added 270 g of ammonium sulfate. The precipitate was collected and redissolved in about 300 ml of 0.04 M potassium phosphate buffer, pH 7.0 (buffer B) and dialyzed overnight against 10 liters of buffer B. The sample was transferred to a TPX beaker, about 40 ml of \(^{10}\)-formylaminopterin aminoethyl Bio-Gel P-150 slurry was added and the mixture stirred gently overnight. The beaker and its contents were then brought to room temperature and the solid content of the beaker allowed to settle. After settling, the solution, followed by the slurry, was poured into a column (stopcock open) 2.8 cm in diameter. The column was then washed with buffer B until the absorbance at 280 nm (A280) was about 0.5 (about 300 ml). The flow rate of this and subsequent washes was 2.5 ml per min. The column was then washed with 0.4 M NaCl in buffer B until the A280 was less than 0.05 (about 100 ml). Then 20 ml of 0.4 M NaCl in 0.04 M potassium phosphate, pH 8.0, was applied to the column. This was followed by 0.20 M NaCl in 0.04 M potassium phosphate, pH 8.0, until the A280 was essentially zero (about 60 ml). The enzyme was eluted from the column by applying 0.2 mM dihydrofolate in 0.2 M NaCl, 0.04 M potassium phosphate, pH 8.0, 1% 2-mercaptoethanol. The
appearance of the enzyme in the effluent was signaled by a steep rise in the A280. The enzyme can be conveniently collected in a single 125 ml flask by collecting about 40 ml of effluent immediately upon the rise in A280 and then assaying the effluent and collecting it until the activity has dropped to a low level. The sample was concentrated to a few ml by means of dialysis against solid sucrose and then passed through a Sephadex G-25 column, 2.2 x 50 cm, equilibrated with buffer B to remove dihydrofolate. After use, the affinity material was washed overnight with five liters of 1.0 M NaCl in buffer B and brought to pH 9.0 with NaOH. The material was then washed with buffer B containing 0.02% sodium azide and transferred to a cold room for storage at 0-4°C. The material was prepared before reuse by washing with buffer B. The material has been reused several times and stored, as described, for periods up to eight months without noticeable changes in its ability to bind and purify the enzyme.

Purification of E. coli DR and T4DR from the Same Extract

The host cell DR can be obtained from the same crude extract used to prepare T4DR (Fig. 7). The two enzymes can be separated by ammonium sulfate precipitation (71). Since the E. coli DR (EDR) is partially inactivated by DEAE-cellulose (91), the DEAE-cellulose
STREPTOMYCIN SUPERNATANT

0-43% (NH₄)₂SO₄ FRACTION

DEAE-CELLULOSE EXTRACTION

SEDIMENTATION OF INSOLUBLE MATTER

55%-85% (NH₄)₂SO₄ FRACTION

ADSORPTION TO AFFINITY MATERIAL

ELUTION OF INACTIVE PROTEIN

ELUTION OF DR

REMOVAL OF DHF ON SEPHADEX G-25

PURIFIED ENZYME

Fig. 7. Flow sheet for the purification of both E. coli DR and T4DR from the same extract.
extraction of the streptomycin sulfate supernatant is omitted and a DEAE-cellulose extraction of the redissolved 0-43\% ammonium sulfate precipitate substituted. The redissolved 0-43\% ammonium sulfate fraction was brought to a volume of 500 ml by the addition of buffer B, then divided equally in 4 250 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 B. (This corresponds to about 55 g wet weight of DEAE-cellulose slurry, pelleted at 5,000 xg for 5 mins.) The slurry was stirred for 30 mins and the bottles were centrifuged at 5,000 xg for 5 mins and the supernatant collected. The slurry was extracted once with 100 ml per bottle of 0.15 M NaCl in buffer B, centrifuged and all supernatants combined. The combined supernatants were precipitated by the addition of 313 g/1 of solid ammonium sulfate. The precipitate was redissolved in 300 ml of buffer B and centrifuged at 20,000 xg for an hour to remove insoluble matter. The pH was adjusted to 6.5 with 1.0 M KH₂PO₄ and the T₄DR was purified by affinity chromatography as previously described.

To recover the EDR 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 collected and redissolved
in 300 ml of buffer B. The pH of this undialyzed solution was adjusted to 6.3 with 1 M KH$_2$PO$_4$ and the EDR was purified by the affinity chromatography steps previously described for the purification of T$_4$DR.

**Enzyme Assay**

The assay for dihydrofolate reductase has been described (75). Briefly, the assay is performed by observing the rate of change of absorbance in a cuvette containing 10$^{-4}$ M DHF, 10$^{-4}$ M NADPH, in .05 M potassium phosphate buffer, pH 7.0. 2-mercaptoethanol at a concentration of 0.01 M is present to retard oxidation of the reduced folate compounds. The reaction is carried out at 37°C in a final volume of 1.0 ml. A unit is defined as that amount of protein which will reduce one µmole of dihydrofolate per minute under the conditions of the assay. A molar absorbancy change of 12,000 M$^{-1}$ cm$^{-1}$ at 340 nm was used for calculation of units (49).

**Determination of Protein**

The concentration of impure DR fractions was determined by the biuret reaction or by absorption at 280 and 260 nm. The concentration of electrophoretically pure DR was determined by its absorption at 215 and 225 nm (92) or by the microbiuret reaction of Goa (93). In the latter method the color change is read at 330 nm at a pH of 13.
Fig. 8. Micro-biuret determination of protein standard curve.
(0.1 M NaOH). Fig. 8 shows the standard curve obtained with 3x crystalline bovine serum albumin. This determination was carried out only with enzyme dissolved in phosphate buffer. Tris-HCl buffer gave a high blank value.

**Amino Acid Analysis Methods**

The determination of the amino acid composition of T4DR and EDR was accomplished by methods based on those of Moore and Stein (94). The concentration of enzyme solutions was adjusted to approximately 600 μg/ml and dialyzed for 48 hours against three two-liter changes of distilled water. 0.5 ml aliquots of the solution were pipetted into pyrex ignition tubes, a small crystal of phenol (to preserve tyrosine) added (95), and 0.5 ml of concentrated HCl added. The tubes were deaerated and sealed under vacuum (less than 50 μ) and heated at 110° (boiling toluene bath) for 24 and 48 hours. Duplicate samples were analyzed on a Beckman model 120C automatic amino acid analyzer.

Cystine was determined as cysteic acid by the method of Moore (96).

Tyrosine and tryptophan were determined by a spectrophotometric method (97) which involves reading the absorbance of a known concentration of enzyme at 288 and 280 nm in a solution of 6 M guanidine hydrochloride. The number of each residue is then calculated from the equations:
\[ \varepsilon_{288} = (N_{\text{trp}})(4815) + (M_{\text{tyr}})(385), \quad (2) \]
\[ \varepsilon_{280} = (N_{\text{trp}})(5690) + (M_{\text{tyr}})(1280), \quad (3) \]

where \( N \) and \( M \) are the number of moles of tryptophan and tyrosine respectively.

The value for tyrosine obtained by the spectral methods was used as a base for calculations of the number of residues of the other amino acids. The number of cysteic acid and methionine sulfone residues was determined using aspartic acid as a standard for comparison (96).

**Purity of Enzymes**

Analytical gel electrophoresis was conducted with native enzyme or with sodium dodecyl sulfate (SDS) treated enzyme. For native enzyme the methods of Davis (90) were followed with some modifications. Specifically, the separating gel was polymerized with riboflavin (using the same concentration as for stacking gels) and also contained 0.005\% 2-mercaptoethanol. These modifications were found to be necessary in order to preserve enzyme activity (98, 99). The sample contained 20\% sucrose and was layered onto the surface of the stacking gel. Electrophoresis was at 3 ma per gel and was performed in the cold. Electrophoresis in SDS-containing gels was performed at pH 7.0 using buffers, stains and gel systems essentially identical to those described by Weber and Osborn (100). When using this method for molecular weight determinations,
the following marker proteins were used: bovine serum albumin (68,000), ovalbumin (43,000), carboxypeptidase A (34,600) and \( \alpha \)-chymotrypsinogen A (24,600). The molecular weights given are those listed by Weber and Osborn (100).

**Confirmation of the Structure of N\(^{\text{10}}\)-Formylaminopterin**

Evidence for the proposed structure of N\(^{\text{10}}\)-formylaminopterin (NFA, Fig. 39) is based on the following evidence: 1) The method of synthesis is analogous to that used for the quantitative conversion of folic acid to N\(^{\text{10}}\)-formylfolic acid (25). Since the N\(^{\text{10}}\) position of aminopterin is "insulated" from the pterin moiety by a methylene group as it is in folic acid, its reactivity should be about the same in the two compounds. 2) The 280 nm absorption peak of aminopterin is shifted to 260 nm (see Figs. 1 and 40) by formylation just as the 280 nm peak of folic acid is shifted to 260 nm by formylation. 3) Exposure of NFA to 0.1 N NaOH for a few min produces a compound with the same spectrum (pH 7.0) as aminopterin. Brief exposure of N\(^{\text{10}}\)-formylfolate to 0.1 N NaOH is known to produce folic acid (25). 4) Elemental analysis of NFA (Spang Microanalytical Laboratory, Ann Arbor, Michigan) agrees with the expected empirical formula calculated for \( \text{C}_{26}\text{H}_{20}\text{N}_{8}\text{O}_{6}\cdot\text{H}_{2}\text{O} \): H, 4.45%; C, 50.25%; N, 22.70%. Found: H, 4.45%; C, 49.89%; N, 22.40%.
NADPH Stereospecificity
of the T4DR Reaction

The methods used for the determination of the stereospecificity of hydride transfer from NADPH to DHF were those of Pastore and Friedkin (60). Briefly, NADP\(^{(4-\text{3}H)^+}\) was prepared by isotope exchange between unlabeled NADP\(^+\) and tritiated water (200 mCi/ml) (60, 101). The tritiated water was removed by lyophilization and the product NADP\(^{(4-\text{3}H)^+}\) purified on DEAE-cellulose (60). This labeled NADP\(^+\) was then reduced enzymatically with either glucose-6-phosphate dehydrogenase (102) to produce NADPH labeled on the A side (NADP\(^{3\text{H}}\cdot\text{H}-\text{A}\)) or with isocitrate dehydrogenase (103) producing NADPH labeled on the B side (NADP\(^{3\text{H}}\cdot\text{H}-\text{B}\)). The tritium labeled NADPH was purified on DEAE-cellulose (102) and then used for the reduction of DHF to THF by T4DR under conditions where all the NADPH was consumed (excess DHF). THF and NADP\(^+\) were isolated by DEAE-cellulose chromatography and the extent of tritium transfer from NADPH to THF determined.

Measurement of Radioactivity

All radioactive measurements utilized the commercial scintillation fluid Aquasol (New England Nuclear). This fluid will dissolve up to 20% water with good counting efficiencies. 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 spectrophotometer.

**Instrumentation and Spectral Measurements**

DR assays were conducted in a Gilford Automatic Spectrophotometer Model 2000 equipped with a Gilford-modified Honeywell recorder, which has a full scale calibration range adjustable over the 0-0.1 to 0-3.0 absorbance range. With this instrument it is possible to record absorbance changes on four different assay reactions in a "semi-simultaneous" fashion.

Routine absorption spectra were scanned with a Beckman Model DB double beam spectrophotometer.

Absorption difference spectra were obtained with a Cary 15 spectrophotometer. Difference spectra involving small molecule-protein interactions were obtained with the 0-0.1 absorbance unit slidewire setting and a dynode voltage setting of 2. Difference spectra involving free substrate or inhibitors at different pH values were obtained in the 0-1.0 absorbance unit range and a dynode voltage setting of 3. A set of four matched semi-micro rectangular quartz cuvettes was employed. The bottom of each cuvette was marked with a pencil so that each cuvette occupied the same position in the cell compartments throughout these
measurements. To check cuvette path length differences, a base line was established with only phosphate buffer in the cuvette. Then identical aliquots of a methotrexate solution sufficient to produce an absorption at 300 nm of 0.5 were added to one cuvette each in the sample and reference compartments and the baseline rechecked. This process was repeated for the second pair of cuvettes. In this way it was determined that path length differences were negligible for both slidewire settings.

All initial sample and blank volumes employed 0.80 ml of enzyme solution (in buffer B) or 0.80 ml of buffer B. The same 1.0 ml pipet was used throughout these measurements for transfer of buffers and solutions to insure as much as possible that identical volumes were placed in each cuvette. Enzyme solutions were centrifuged prior to use if turbidity was suspected. All transfers of solutions and all spectral measurements were performed at ambient temperatures (23 ± 1°C). To obtain difference spectra a buffer blank cuvette (placed first in the light path) and an enzyme solution cuvette were placed in each compartment and a base line established with the multipots. Identical aliquots (0.01 ml to 0.04 ml) of a small molecule (NADPH, DHF or a DR inhibitor) were delivered to the enzyme cuvette in the sample compartments and to the buffer blank cuvette in the reference compartment. A 0.1 ml Hamilton
syringe was used for this purpose. The syringe was rinsed with buffer B and an identical aliquot of buffer B was delivered to the enzyme cuvette in the reference compartment. In those cases where a dihydro compound required the presence of mercaptoethanol in the buffer, an identical aliquot of the mercaptoethanol buffer (see below) was added to the enzyme cuvette in the reference compartment and to the buffer cuvette in the sample compartment. Mixing was accomplished by cuvette inversion. Spectra were then recorded at a rate never exceeding 1.0 nm per second. All difference spectra involving enzyme interactions were performed at pH 7.0.

Difference spectra obtained on the 0-0.1 absorbance slidewire were subject to certain instrumental errors. It proved extremely difficult to obtain a perfectly straight base line over the entire spectral range studied, 240-420 nm. Fig. 39 demonstrates this problem. In order to make spectra more directly comparable, spectra were corrected for these fluctuations and presented relative to a straight base line. Since the base line fluctuations themselves were reproducible, this procedure seems justified. Another problem was due to small shifts in absorbance observed in repetitive scans. These shifts were either positive or negative and ranged up to 0.005 absorbance units. Because of this problem, difference spectra may contain errors
inversely proportional to their magnitude, estimated to be about 25% for an absorbance change of 0.01.

Below 240 nm spectra became increasingly unreliable because of high absorbances of the peptide bonds and decreased intensity of the light source, necessitating large slit widths.

All difference spectra involving pH changes (involving folate compounds only) employed only two cuvettes, one in each compartment. This simplification could be made since the various pH buffers were found to have only negligible absorbance differences. The reference pH was 7.0 (buffer B) in all cases. All folate compounds were dissolved in buffer B (plus 0.1 M mercaptoethanol in the case of DHF and dihydroaminopterin). The concentration of these solutions was such that 0.09 ml of this solution in 0.80 ml of buffer gave a final concentration of approximately $3 \times 10^{-5}$ M. To obtain pH difference spectra, a 0.1 M phosphate buffer of pH 5.4 and mixtures of this buffer and 0.1 M HCl were used to cover the pH range 1.0 to 5.6. Since the addition of the folate compound stock solution altered the initial buffer pH somewhat, the pHs reported are those determined after the spectra were obtained.

Fluorescence measurements were made in a Perkin-Elmer model MPF-2A Fluorescence Spectrophotometer. The light source on this instrument is a 150-watt xenon lamp.
Both excitation and emission monochromators consist of diffraction gratings (600 lines/mm). Both excitation and emission slits are continuously variable from 0.13 to 5.16 mm giving a bandpass range from 1.0 to 40 nm. The instrument is equipped with the Model QPD-33 recorder.

Emission spectra obtained on this instrument are not corrected for phototube response. Excitation spectra are also uncorrected for variation of light source intensity as a function of wavelength. While no attempt has been made to correct the spectra reported here, it was considered necessary to "standardize" the instrument with some fluorescent compounds whose spectra have been frequently reported in the literature in order that the reliability of the spectra reported here could be ascertained. The results of this standardization are shown in Table I. As can be seen, the maxima obtained for tryptophan, tyrosine, and a number of proteins are in excellent agreement with those reported in the literature. It is therefore concluded that the excitation and emission maxima here determined are close to their true values. On the other hand, the overall spectra may be less reliable. For example, the xenon arc lamp intensity varies widely over the 200-800 nm range of the MPF-2A. According to Brand and Wiltholt (104, citing a Hanovia Co. data file), the intensity in the 250-260 nm region is 0.05% of the electrical input whereas in the
350-360 nm region it is more than four times as much (0.21%). However, over the region where the high resolution tryptophan (in the enzyme) excitation spectra was examined (280-300 nm) the variation is only 10 to 12%, suggesting that errors due to this cause in protein excitation spectra are small.

Since many of the experiments reported here depend upon a linear relationship between fluorescence intensity and concentration of the fluorescing compound, a standard curve for this relationship was obtained with bovine serum albumin and tryptophan. This curve is shown in Fig. 9. The settings given are in the region most frequently employed for measurements of fluorescence-quenching experiments.

All fluorescence measurements were performed at room temperature (23 ± 1°C) in 1.0-cm square quartz cells using a sample volume of either 3.0 or 2.5 ml. Buffer B was commonly employed. Exceptions are noted in the Results section. 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
Table I

Standardization of the Perkin-Elmer MPF-2A by comparison to literature values for several fluorescent compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Emission Maxima (nm)</th>
<th>Excitation Maxima (nm)</th>
<th>Lit. Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPF-2A</td>
<td>Literature</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>350</td>
<td>303</td>
<td>105</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>303</td>
<td>303</td>
<td>105, 106</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>340</td>
<td>341</td>
<td>107</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>331</td>
<td>331</td>
<td>107, 108</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>341</td>
<td>342</td>
<td>107</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation Maxima (nm)</th>
<th>Lit. Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPF-2A</td>
<td>Literature</td>
</tr>
<tr>
<td>Tryptophan (270-300 nm region)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High energy peak</td>
<td>282</td>
<td>282b</td>
</tr>
<tr>
<td>Low energy peak</td>
<td>289</td>
<td>289b</td>
</tr>
</tbody>
</table>

a all values are for aqueous solutions.
d determined from graphical data.
Fig. 9. Relationship between relative fluorescence and concentration. — Aliquots of tryptophan or bovine serum albumin (BSA) were added to 2.5 ml of buffer B by means of 0.1 ml syringe. Each division of the abscissa represents .01 ml of titrant. Points are corrected for dilution. The final concentration for tryptophan is about 7 μM and about .08 mg/ml for bovine serum albumin. Excitation and emission wavelengths are 284 and 345 nm respectively and both slits were set at 10 nm. The sensitivity setting was 2.
mixed by means of a plastic dip stick. After the initial reading, titrant was transferred from a 0.1 ml Hamilton syringe (mounted on a ring stand by means of a clamp) to the cell by means of the plastic dip stick. Titrant concentration depended upon the affinity of the enzyme for the titrant and will be noted later. 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 solutions in water.

**Calculations**

The extent of quenching of T4DR and EDR at infinite substrate or coenzyme concentrations involves a plot of reciprocal fluorescence versus reciprocal concentration: \(1/(1-F_1)\) versus \(1/S\). Here \(F_1\) 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.

The calculation of the equilibrium association constant \(K_a\) is based on the assumption of 1 to 1 stoichiometry between enzyme and substrate, cofactor or inhibitor:

\[
DR + S \Rightarrow DR-S \tag{4}
\]

where DR is free enzyme and S is one of the small ligands.
It can be shown that the association constant \( K_a \) for equation (4) is given by the expression

\[
K_a = \left( \frac{\alpha}{[D]} \right) / (1-\alpha)
\]  

(5)

where \( \alpha \) is the ratio of bound S to total S.

Systematic errors in the determination of \( K_a \) due to dilution upon addition of S were never greater than 4%. Errors due to nonspecific quenching were difficult to determine but the maximum concentrations of S were usually less than 5 \( \mu \)M and in some cases considerably less. Test additions of DHF or NADPH to ovalbumin indicated that this type of error was about 10% at the maximum concentrations used.

The calibration of the micro syringe was checked spectrophotometrically with methotrexate.
RESULTS

Infected Cell Production

The continuous flow production of infected cells by the techniques described here was capable of producing 1.3-1.5 kg of infected cell paste from about 130 liters of medium in a period of about ten hours. However, several days of preparatory work are required prior to the actual run. Cell densities in the bacterial growth jars and in the infection vessel reached steady state values on the order of $4 \times 10^9$ cells/ml. At these high cell densities it was found that medium issuing from the infection vessel had a pH of 6.3 although the medium entering the bacterial growth vessels was at a pH of 7.8. Thus it is likely that the pH is a growth limiting factor under the conditions employed. Nevertheless, the amount of T4DR recovered from cells infected under these conditions was comparable to the amount of enzyme produced by cells infected at a lower cell density and at a pH closer to 7.0; the specific activity of crude extracts prepared from either source being about 0.025 units/mg protein. This result may be related to the very rapid rate at which T4DR production occurs subsequent to phage infection (79).
Results of the Purification of T4 and E. coli DR

In the conventional purification of T4DR it was found that the enzyme was nearly quantitatively precipitated at an ammonium sulfate concentration of 43% of saturation. As is shown in Table IIA, the 30 to 40% fraction contained the most activity, indicating a somewhat greater insolubility in ammonium sulfate solutions for the T4DR compared to the T6DR (71).

T4DR begins to slowly elute from DEAE-cellulose at about 0.12 M NaCl. While a number of elution schemes were attempted, no "sharp" elution band was ever achieved. Although the DEAE-cellulose step gave only a two-fold rise in specific activity (Table III), this procedure was useful in removing residual amounts of nucleic acids which remained after the streptomycin sulfate treatment and ammonium sulfate precipitation steps. The ratios of absorbance at 260 to 280 nm, before and after DEAE-cellulose fractionation, were about 1.6 and 0.7 respectively. Another feature of this step was a significant rise in the total amount of activity recovered, suggesting the removal of inhibitory substances. Experience gained in developing the conditions for this step proved valuable later when it was discovered that substances were present in the ammonium sulfate fraction which interfered with the binding of T4DR during affinity chromatography, which will be discussed below.
### Table II

#### A

Distribution of DR activity in ammonium sulfate fractions of the crude extract from T4 infected cells of *E. coli* B

<table>
<thead>
<tr>
<th>Fraction (% of saturation)</th>
<th>% of Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 30%</td>
<td>6.3</td>
</tr>
<tr>
<td>30 - 40%</td>
<td>80.5</td>
</tr>
<tr>
<td>40 - 50%</td>
<td>5.0</td>
</tr>
<tr>
<td>50 - 60%</td>
<td>0.2</td>
</tr>
<tr>
<td>60 - 70%</td>
<td>3.0</td>
</tr>
<tr>
<td>70 - 80%</td>
<td>4.9</td>
</tr>
</tbody>
</table>

#### B

0-43% Ammonium sulfate precipitate extraction data

<table>
<thead>
<tr>
<th>Ammonium Sulfate Concentration (% of saturation)</th>
<th>% Protein in Solution</th>
<th>% Activity in Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>37</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>20</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Table III
Conventional Purification of T4 Dihydrofolate Reductase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein (mg)</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Overall Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35,000</td>
<td>825</td>
<td>.024</td>
<td>1</td>
</tr>
<tr>
<td>Streptomycin sulfate super.</td>
<td>35,000</td>
<td>825</td>
<td>.024</td>
<td>1</td>
</tr>
<tr>
<td>0-55% AS</td>
<td>18,000</td>
<td>880</td>
<td>.048</td>
<td>2</td>
</tr>
<tr>
<td>DEAE-cellulose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9,100</td>
<td>937</td>
<td>.106</td>
<td>4.4</td>
</tr>
<tr>
<td>0-42% AS + AS extractions&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2,420</td>
<td>410</td>
<td>.166</td>
<td>7</td>
</tr>
<tr>
<td>G-100</td>
<td>100</td>
<td>680</td>
<td>6.8</td>
<td>275</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>34</td>
<td>540</td>
<td>15.8</td>
<td>632</td>
</tr>
<tr>
<td>TEAE-cellulose</td>
<td>15</td>
<td>440</td>
<td>30</td>
<td>1,140</td>
</tr>
<tr>
<td>Prep. disc-gel electrophoresis&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.7</td>
<td>167</td>
<td>94</td>
<td>3,600</td>
</tr>
</tbody>
</table>

a. Derived from 500 grams of cells

b. The DEAE-cellulose step consistently gave a slight increase in activity

c. This step consistently resulted in a decrease in activity, much of which could be restored by dialysis against buffer B or by the next step

d. Data are for fractions homogeneous on analytical gels
The results of the second ammonium sulfate fractionation step are shown in Table IIIB, and further demonstrate the relatively low degree of solubility of T4DR in ammonium sulfate solutions. While some of the enzyme remained in solution at 40% of saturation, once it was precipitated, very little T4DR could be redissolved by ammonium sulfate solutions at a concentration of 33% saturation. Because of this, nearly two-thirds of the inactive protein remaining prior to this step could be extracted from the precipitate. This extraction also proved to be vital to the success of the conventional purification procedure in that it removed inactive proteins which no subsequent step could entirely eliminate. However, this treatment also appeared to reduce the enzymatic activity by about one-half. This could be due to a lower turnover number for all enzyme molecules or to an actual inactivation of a fraction of the enzyme molecules. Whichever of these alternatives is correct was not determined, but the inactivation appeared to be largely reversible, as judged by the increased amount of activity recovered in the subsequent step.

The elution profile for the Sephadex G-100 step is shown in Fig. 10. A very large peak of inactive protein coincided with the void volume (2400 ml) indicating that much of the protein remaining from previous steps was
high molecular weight material. Aside from being the most effective purification step (Table III), the volume at which DR eluted from the G-100 column provided enzyme molecular weight information substantiating the molecular weight estimated by sucrose gradient centrifugation (71,72). Thus the peak of enzyme activity occurring at an elution volume of 4200 ml correlates with a molecular weight in the 30,000-dalton range (110).

The DEAE-Sephadex column profile shown in Fig. 11 discloses the interesting fact that the affinity of T4DR for the DEAE ion-exchange group can be altered by changing the matrix on which the DEAE group is supported. While the enzyme is eluted from DEAE-cellulose at a chloride concentration of 0.18 M, the peak of enzyme activity emerging from the DEAE-Sephadex column is at 0.26 M phosphate concentration. As will be discussed later, the DEAE-Sephadex step was used to help ascertain whether or not the T4DR purified by affinity chromatography contained an impurity contributing to the fluorescent excitation spectra of the enzyme.

Attempts to use phosphocellulose or carboxymethylcellulose in the purification of T4DR were unsuccessful. Even at buffer concentrations as low as 5 mM T4DR passed through columns of these cationic-exchangers with the bulk of the protein yielding virtually no purification. However, further purification subsequent to DEAE-Sephadex was
Fig. 10. Sephadex G-100 column profile.
attained by use of still another anion exchanger, TEAE-cellulose. The initial peak of protein eluting from this column (Fig. 12) proved inactive while the second peak showed a fair correlation between activity and protein concentration. Since this was the first step in the purification to show this relationship, the purity of the preparation was investigated at this point by means of disc gel-electrophoresis (Fig. 13). By the substitution of riboflavin and light for the polymerizing agent, enzymatic activity could be preserved, making it possible to slice up one gel and thereby identify the position of the enzyme band in another, stained gel. The distribution and density of the bands indicated the preparation to be about 50% pure at this stage. In addition to indicating that the enzyme migrates as a single band, this experiment suggested that preparative acrylamide electrophoresis could serve as the final step in the enzyme's purification. Usually four slices of the preparative electrophoresis gel showed enzyme activity. Of these, the top (as oriented in the electrophoresis column) two slices showed a single band when analysed by acrylamide gel electrophoresis. The two lower slices had decreasing specific activity and showed impurities on analytical gels. Attempts to bypass the TEAE-cellulose step resulted in impurities in all slices as judged by a reduced specific activity of the preparation obtained.
Fig. 12. TEAE-Cellulose column profile.
Fig. 13. Analysis of the TEAE-cellulose T4DR activity pool by acrylamide gel electrophoresis. Gels were run in duplicate as described in the experimental section. One gel was stained with aniline blue black while a second was sliced into the indicated segments. Each slice was crushed, incubated with one ml of buffer B and, after an hour, assayed for activity.
Results of Affinity Chromatography

The purpose of retaining the ammonium sulfate step in the affinity chromatography purification scheme is mainly to reduce the volume being worked with. The DEAE-cellulose step is more critical in that it removes substances which interfere with the binding of T4DR. In the instance where this was first noticed only about 10% of the activity present was bound to the affinity material. After the DEAE-cellulose step binding is essentially complete. The substances which interfere with binding have an $A_{260}/A_{280}$ ratio close to 2.0, travel with T4DR on Sephadex G25 and are non-dialyzable. They are partially removed from T4DR on Sephadex G-100 (50 x 2.5 cm column). These results would indicate that the material is of a nucleic acid nature but the exact composition of the material is not known. This material is apparently precipitated by an ammonium sulfate concentration of 55% of saturation, however, since the problem has not been encountered in the purification of the E. coli enzyme from the 55-85% ammonium sulfate fraction. This is fortunate, since extraction by DEAE-cellulose could have resulted in loss of EDR (91).

The purification of T4DR and EDR by affinity chromatography is summarized in Table IV. Approximately half (3,000-fold of a total of 6,000-fold, in the case of T4DR) of the overall purification factor was achieved by affinity chromatography alone.
Table IV

Purification of dihydrofolate reductase by affinity chromatography

**T4DR**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract<strong>b</strong>*</td>
<td>1,900</td>
<td>835</td>
<td>39,000</td>
<td>.024</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2,200</td>
<td>835</td>
<td>39,000</td>
<td>.024</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>2,100</td>
<td>815</td>
<td>31,000</td>
<td>.026</td>
</tr>
<tr>
<td>0-43% (NH₄)₂SO₄<strong>c</strong>*</td>
<td>280</td>
<td>750</td>
<td>15,000</td>
<td>.050</td>
</tr>
<tr>
<td>Affinity Chromatography</td>
<td>50</td>
<td>660</td>
<td>4.5</td>
<td>145</td>
</tr>
</tbody>
</table>

**EDR**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract<strong>b,c</strong>*</td>
<td>1,900</td>
<td>37</td>
<td>37,000</td>
<td>.001</td>
</tr>
<tr>
<td>Streptomycin<strong>c</strong>*</td>
<td>2,200</td>
<td>37</td>
<td>37,000</td>
<td>.001</td>
</tr>
<tr>
<td>55-85% (NH₄)₂SO₄<strong>d</strong>*</td>
<td>310</td>
<td>34</td>
<td>13,600</td>
<td>.0025</td>
</tr>
<tr>
<td>Affinity Chromatography</td>
<td>18</td>
<td>32</td>
<td>1.2</td>
<td>27</td>
</tr>
</tbody>
</table>

**a** EDR not recovered by this procedure.

**b** based on 500 g of cells.

**c** data is for uninfected cells.

**d** data is for either infected or uninfected cells.
T4DR samples prepared by affinity chromatography appeared homogeneous on the modified analytical gels of Davis and also on sodium dodecyl sulfate-containing gels. 20-μg samples showed a single band. Since these methods can easily detect a protein band containing 1 μg of protein (111) it is probable that any single protein contaminant, if any are present, is much less than 5% of the total protein. The recovery of activity in the final product is about four times higher for affinity chromatography than for the conventional purification scheme, being 80% as opposed to 20% (see Tables III and IV). About 4.5 mg of T4DR was obtained by affinity chromatography from 500 g of infected cells. The specific activity of 145 μmoles of DHF reduced per min per mg of protein is about 1.6 times that of the highest specific activity obtained by conventional methods. This apparent discrepancy can probably be explained by partial inactivation of the enzyme occurring during electrophoresis. This statement is supported by the almost total inactivation of enzyme electrophoresed on gels polymerized by ammonium persulfate and by the observation that the total recovery of activity from riboflavin-polymerized gels was never greater than 60% of the activity applied.

The overall purifications are 6,000-fold for the T4 enzyme and 27,000-fold for the EDR. Table IV also shows
that the recovery of EDR is as good as that for T4DR. About
1.2 mg of EDR can be obtained from 500 g of infected cell
paste by this method; this is true whether or not the cells
are infected with T4. The E. coli enzyme preparation has
been found to be homogeneous when electrophoresed on SDS-
containing gels (100). As will be shown later, more than
80% of the protein fluorescence can be quenched with
methotrexate, which would further indicate a high degree of
homogeneity.

**Molecular Weight and Subunit Determination of T4DR and EDR**

SDS-gel electrophoresis was also used to estimate
the molecular weights of the enzyme preparations. Fig. 14
shows the curve obtained when the log of the molecular
weight of several marker proteins is plotted against their
mobility. (Bovine serum albumin moved in an anomalous
fashion and its plot was ignored.) When the mobilities of
both DRs are placed on this curve a molecular weight of
29,000 ± 1,500 daltons is obtained for the T4 enzyme and
22,500 ± 1,100 daltons is obtained for the E. coli enzyme.
Since both of these values agree well with molecular weight
data obtained for the native enzymes (71, 72) it would
appear that each enzyme is composed of a single polypeptide
chain.
Fig. 14. Estimation of the molecular weights of T4DR and EDR by SDS acrylamide electrophoresis.—Marker proteins are: BSA, bovine serum albumin; OA, ovalbumin; CAR, carboxypeptidase; CY, chymotrypsinogen.
Substrate Binding Sites

The specific activity of the T4DR of 145 units per mg is one of the highest reported for DR from any source. Thus, it seemed possible that the enzyme possessed more than one catalytic site per molecule. This would imply the existence of more than one binding site for dihydrofolate per molecule. In order to determine the number of binding sites per molecule, the enzymatic activity was titrated with the stoichiometric inhibitor (21) methotrexate. As can be ascertained from Fig. 15, the data indicate the presence of but one binding site per molecule. This experiment also indicates the very potent inhibition which methotrexate (MTX) exerts on T4DR. The 5-μl samples used to assay remaining activity represent a 200-fold dilution (assay volume is 1.0 ml) of the enzyme-inhibitor complex. Thus the concentration of this complex in the assay cuvette is in the 10^{-9} M region. Furthermore, DHF, which could be expected to compete for the same binding site as MTX, is present at a 10,000-fold greater concentration than MTX. In spite of this, a reasonably straight line plot is obtained, indicating that the dissociation constant for the T4DR-MTX complex is much less than 10^{-9} M. As will be shown later, the loss in enzymatic activity also correlates well with MTX quenching of protein fluorescence.
Fig. 15. Titration of the activity of dihydrofolate reductase with methotrexate (MTX).—45μg of homogeneous T4DR in 3 ml of 0.04 M potassium phosphate buffer, pH 7.0 (corresponding to a molar concentration of 5.17 x 10^-7 M) was titrated with a solution of MTX, 5 x 10^-5 M. At the indicated points 5 μl of enzyme was removed and assayed for activity. The data are not corrected for the small volume changes due to additions and withdrawals. This error is less than 3%. An extrapolation of the linear portion of the curve intersects the abscissa at an MTX concentration of 5.2 x 10^-7 M, indicating the presence of only one binding site per molecule for this inhibitor.
The absorption spectra of T4DR and EDR, shown in Fig. 16, while those of simple proteins, demonstrate marked differences. Of the two, EDR is more similar to other DRs for which spectra have been published (16, 17). The maximum for T4DR is at 277 nm while that for EDR is at 281 nm. Also, the shoulder at 289 nm is much more pronounced in the E. coli enzyme. These properties indicate that tyrosine makes a greater contribution to the spectrum of T4DR compared to EDR.

Some early preparations of T4DR purified by affinity chromatography showed a significant absorption extending to and beyond 400 nm and appeared "brownish" to visual inspection. Since the enzyme prepared by conventional methods did not show this, an artifact of affinity chromatography was suspected. The problem was largely eliminated by preparing the DHF used for elution on the day of its use and removing the DHF from the eluted enzyme (Sephadex G-25 step) as soon as possible. This suggests that a decomposition product of DHF was responsible for the long wavelength absorption, but why this material would move with the enzyme on Sephadex G-25 is obscure. This substance was readily removed from the early preparation by means of the DEAE-sephadex procedure described in the experimental section.
Fig. 16. Absorption spectra of T4DR (solid line) and EDR (broken line). Spectra are for the enzyme solutions in buffer B.
Unlike the DR from *L. casei* (16), 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 involves their elution from a column by means of their substrate, DHF, would not be expected to show evidence of bound cofactor, unless NADPH were bound at a non-enzymatic site. There is no evidence for this.

When the protein concentration was determined by the microbiuret reaction, a 0.1% solution of T4DR in buffer B showed an $A_{280}$ of 1.20. Protein concentrations estimated by absorption at 215 and 225 nm gave values 10 to 15% lower than obtained by the microbiuret reaction. However, the latter method agreed well with concentrations determined by MTX titrations and was therefore considered more reliable. By this method a 0.1% solution of EDR was estimated to have an $A_{280}$ of 1.91.

As a means of estimating tryptophan content by the spectrophotometric method of Edelhoch (97), a portion of the ultraviolet absorption curve of T4DR was determined in 6 M guanidine-HCl (GuHCl). This is shown in Fig. 17. A known volume of a solution of T4DR of known concentration was added to a 1.0 ml volumetric flask (which contained 0.55 g of ultra pure (Schwartz-Mann) GuHCl to give a final
Fig. 17. The spectrum of T4DR in 6 M GuHCl, 0.04 M sodium phosphate buffer, pH 6.5.
volume of 1.0 ml. The \( A_{280} \) for a 0.1% solution of T4DR under these conditions (pH 6.5) was 1.21 or nearly the same as for the native enzyme.

**Results of Amino Acid Analysis**

Using a value of 30,000 for the molecular weight (the average of 29,000 determined by SDS-gel electrophoresis and 31,000 determined by sucrose density gradient, 71,72), the number of residues of tryptophan and tyrosine per T4DR molecule calculated by equations 2 and 3 was 4.6 and 9.0 respectively. In this connection it is interesting that the spectrum in 6 M GuHCl of trypsinogen, which has a molecular weight of about 24,000 and contains 4 tryptophan and 10 tyrosine residues per molecule (97), is very similar to the spectrum of T4DR in 6 M GuHCl.

The results of amino acid analysis of acid hydrolysates of T4DR and EDR are summarized in Table V. Both enzymes have aspartate and glutamate as the most plentiful amino acids. Since values for ammonia were unreliable it is not known how many of these acid residues occurred as asparagine or glutamine. 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. The results of the EDR analysis are presented in terms of the nanomolar
Table V
The amino acid composition of T4DR and EDR

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Nanomolar Composition of Amino Acid Hydrolysate</th>
<th>Residues per T4DR Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDR</td>
<td>T4DR</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.4</td>
<td>10.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>6.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.7</td>
<td>8.2</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>32.1</td>
<td>17.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.9(^a)</td>
<td>11.3</td>
</tr>
<tr>
<td>Serine</td>
<td>15.4(^a)</td>
<td>9.9</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>25.8</td>
<td>20.8</td>
</tr>
<tr>
<td>Proline</td>
<td>14.2</td>
<td>8.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>20.1</td>
<td>16.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>22.2</td>
<td>14.0</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>ND(^e)</td>
<td>1.60(^c)</td>
</tr>
<tr>
<td>Valine</td>
<td>19.3(^b)</td>
<td>16.0(^b)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.6</td>
<td>3.78(^c)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>15.7(^b)</td>
<td>11.1(^b)</td>
</tr>
<tr>
<td>Leucine</td>
<td>19.6(^b)</td>
<td>15.1(^b)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.6</td>
<td>6.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>9.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>----</td>
<td>----</td>
</tr>
</tbody>
</table>

\(^{a}\)extrapolated to 0 hours of hydrolysis.

\(^{b}\)based on 48 hours of hydrolysis.

\(^{c}\)based on values obtained from enzyme oxidized with per-formic acid before hydrolysis.

\(^{d}\)calculations based on a value of 9 tyrosine residues per 30,000 daltons of T4DR.

\(^{e}\)24-hour hydrolysis period except as noted.

\(^{\#}\)not determined.
composition of the hydrolysate due to the fact that determinations for tryptophan, methionine and cysteine were either not made or were not reliable. The T4DR analysis is presented this way too, but is also reported in terms of residues per enzyme molecule of 30,000 daltons. By using the value of 9 tyrosines per T4DR molecule determined spectrophotometrically as a basis of calculations, the number of each of the various amino acid residues was determined. The sum of the molecular weights of the residues came to about 27,400. This value is in reasonably good agreement with the molecular weight of 29,000 ± 1,500 determined by SDS-gel electrophoresis.

The number of lysine + arginine residues, 24, would suggest 25 tryptic peptides, while the 5 methionine residues indicate that 6 cyanogen bromide peptides should be produced. These two sets of fragments would probably be very useful in studies of the amino acid sequence of T4DR.

Michaelis Constants for DHF and NADPH

The kinetic constants for the binding of T4DR's substrate and cofactor were determined by means of the conventional double reciprocal procedure of Lineweaver and Burk (111). The reduction of DHF by NADPH can be considered a bisubstrate reaction. Although the kinetics properties
should therefore be treated as those of a bisubstrate reaction (such as by the equations of Alberty, 112), the methods of Lineweaver and Burk have been used in the vast majority of other studies on DR. Therefore, the methods of Lineweaver and Burk have been used in the present case in order that values obtained may be compared with those in the literature.

Constants for the T4DR were calculated by use of the equation: \( \text{slope} = \frac{K_m}{V} \), where \( V \) is the maximum velocity of the reaction. The values so derived for \( K_m \) were 2.3 \( \mu \)M for DHF and 18 \( \mu \)M for NADPH, based on line C of Figs. 18 and 19 respectively. These data indicate that the DHF concentration for a half maximal reaction rate is nearly an order of magnitude lower for the substrate than for cofactor.

Burchall and Hitchings (91) have reported Michaelis constants for EDR of 26 \( \mu \)M and 10 \( \mu \)M for DHF and NADPH respectively. Thus the order of higher and lower \( K_m \) values for the two enzymes are interchanged. While half maximal velocity for the T4 enzyme is attained at a DHF concentration of less than one-tenth that for EDR, the situation is reversed when the comparison is made to the well-studied mouse liver DR (\( K_m = 2 \times 10^{-7} \) M, 113) or the murine L1210 lymphoma (\( K_m = 4 \times 10^{-7} \) M, 55). The mouse liver enzyme is reported to be subject to substrate inhibition, however (113). Bertino et al. have reported a \( K_m \) of 1.3 \( \mu \)M for...
Fig. 18. Double reciprocal plot of the enzymatic activity of T4DR in the presence of a fixed concentration of NADPH and variable concentrations of DHF and N$^{10}$-formylaminopterin (NFA).
Fig. 19. Double reciprocal plot of the enzymatic activity of T4DR in the presence of a fixed concentration of DHF and variable concentrations of NADPH and NFA.

A. \([\text{NFA}] = 8.4 \times 10^{-7} \text{ M}\)
B. \([\text{NFA}] = 5.6 \times 10^{-7} \text{ M}\)
C. \([\text{NFA}] = 0\)

\([\text{DHF}] = 1 \times 10^{-4} \text{ M}\) IN ALL CASES
DHF using the Ehrlich ascites carcinoma as enzyme source (114), indicating that certain mammalian DRs do have values quite similar to those for T4DR.

Figs. 18 and 19 also show the kinetics of the T4DR reaction in the presence of the inhibitor NFA. This inhibitor is of particular interest since it is the ligand which was used so successfully in the affinity chromatography process previously discussed. As is indicated by the convergence of lines A and B in each figure, the inhibitor is competitive with respect to DHF and noncompetitive with respect to NADPH. A competitive $K_i$ of $1.8 \times 10^{-8}$ M can be calculated from the slopes of the lines in Fig. 18.

**Stereospecificity of T4DR-Promoted Hydride Transfer**

Previous studies have shown that the L1210 murine lymphoma DR (60) and the chicken liver DR (60) transferred the A side hydride of NADPH to DHF. Since T4DR is quite distant phylogenetically from these other DRs it could not simply be assumed that the phage enzyme had the same specificity. The methods of Pastore and Friedkin (see experimental section) were employed to answer this question. Fig. 20 shows the chromatographic separation of the products of the DR reaction, NADP$^+$ and DHF. In panel A NADP$^3$H-A was used to reduce DHF. The peak centered at fraction 16 was identified as NADP$^+$ by its ultraviolet
spectra and by its conversion to NADPH by glucose-6-phosphate dehydrogenase. The peak centered at fraction 20 was identified as THF by its spectrum. As can be seen, considerably more than half of the tritium label has been transferred to the product THF. In panel B where the NADP$^3$H-B was used to reduce DHF, no significant transfer of label to THF has occurred. Pastore and Friedkin (60) explained the tritium label occurring with the NADP$^+$ peak in panel A as being the result of tritium exchange between NADP$^+$ and tritiated water at positions other than the 4 position of the nicotinamide ring. The same explanation presumably would apply to the present case. Thus the conclusion of this experiment is that T4DR promotes transfer of the A side hydride, analogous to other DRs which have been examined.

**Stability of T4DR to Trypsin**

The T-even phage along with T5 provide a set of closely related enzymes (70,72) which could prove useful in comparative studies of the DR reaction. In this regard, there is interest in eventually obtaining peptide maps of these four enzymes. As a preliminary experiment to this study the stability of T4DR to trypsin degradation was examined. Although many proteins in their native conformation are resistant to the action of trypsin, it is clear from the data presented in Fig. 21 that native T4DR is
Fig. 20. Elucidation of the stereospecificity of T4DR-promoted hydride transfer as determined by DEAE-cellulose chromatography of the products.
Fig. 21. Trypsin inactivation of native T4DR and its protection by DHF and NADPH. — 0.2 ml aliquots of T4DR (90 μg/ml) in 0.02 M tris-HCl buffer pH 7.0 were distributed in three tubes. To one tube was added 0.01 ml of 10^{-4} M DHF, to a second tube was added 0.01 ml of 1 x 10^{-4} M NADPH, and to the third was added 0.01 ml of water. Then .01 ml of trypsin (100 μg/ml, diphenyl carbamyl chloride treated) dissolved in 0.001 N HCl. Digestion was at room temperature. Assays for enzymatic activity were performed at the indicated times. Open circles, NADPH present; solid circles, DHF present; triangles, free T4DR. Points for each curve were obtained from two separate experiments.
susceptible to this proteolytic enzyme. Using a weight-
to-weight ratio of T4DR to trypsin of 9/1, inactivation
of the native enzyme was 50% complete in 5 min. On the
other hand, a small amount of activity (10%) still remained
after a 30 min incubation period.

Burchall has studied the inactivation of EDR by
pronase (115) and found that it was rapidly inactivated by
this proteolytic enzyme. He also found, however, that
both DHF and NADPH could almost completely protect EDR
from inactivation by pronase. It was therefore of interest
to examine the protection afforded T4DR by its substrate
and cofactor in the presence of trypsin. As shown in Fig.
21, while both substrate and cofactor gave protection, only
NADPH showed a high degree of protection from the action of
trypsin. At a time when the free T4DR digest showed about
10% activity remaining, about 30% activity remained in the
presence of DHF while at the same time the digest containing
NADPH showed 85% of its initial activity. Although no
control experiment was performed here, it was known from
many previous experiments that no change in activity occurs
merely by allowing T4DR to stand at room temperature for
30 min.

These experiments with trypsin have the advantage
over those with pronase in that the activity of the former
enzyme is known to be much more specific. Thus, it seems
likely that T4DR has one or more peptide bonds involving arginine or lysine which is (are) "exposed" with regard to trypsin in the free enzyme but which is (are) much less "exposed" in the T4DR-NADPH complex. Whether this decreased exposure is due to a simple steric blocking of the action of trypsin by NADPH, to a NADPH-induced conformational change in the protein or to some unsuspected factor is not clear at this time.

Substrate Requirements of T4DR

It has been shown by Mathews and Sutherland (71) that T4DR could not use folic acid as a substrate. The range of substrate requirements was further examined by preparing two N\textsuperscript{10} folic acid derivatives, N\textsuperscript{10}-formylfolic acid (25, 49) and N\textsuperscript{10}-methylfolic acid (36). When these compounds were substituted for DHF in the assay reaction no change in absorbance at 340 nm (A\textsubscript{340}) occurred over a period of 5 min. These two compounds were then reduced with dithionite to form N\textsuperscript{10}-formyl-DHF (49) and N\textsuperscript{10}-methyl-DHF (48). These compounds did cause significant changes in the A\textsubscript{340}, when used in the assay in place of DHF. In the presence of equal amounts of T4DR and at substrate concentrations of 10\textsuperscript{-4} M the changes in the A\textsubscript{340} shown in Table VI were obtained. Presumably the product was the tetrahydro derivative in each case, although this was not rigorously determined.
Table VI

Substrate Specificity of T4DR

Assays were conducted as normal assays except that DHF was replaced by the folate compound listed (10^{-4} M in each case) and the recorder was calibrated for 2.0 absorbance units full scale against a water blank so that the full extent of the reaction could be followed. The reaction was considered complete when the absorbance change was less than 0.01/5 min.

<table>
<thead>
<tr>
<th>Compound Tested</th>
<th>Initial Activity $A_{340}$/min</th>
<th>Initial $A_{340}$</th>
<th>Final $A_{340}$</th>
<th>Time for 50% Completion of $\Delta A_{340}$</th>
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<tbody>
<tr>
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<td>0.18</td>
<td>59 sec</td>
</tr>
<tr>
<td>$N^{10}$-formylfolate</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$N^{10}$-methylfolate</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$N^{10}$-formyl-DHF</td>
<td>0.06</td>
<td>1.22</td>
<td>0.24</td>
<td>12 min</td>
</tr>
<tr>
<td>$N^{10}$-methyl-DHF</td>
<td>0.64</td>
<td>1.50</td>
<td>0.36</td>
<td>58 sec</td>
</tr>
</tbody>
</table>
The data in Table VI indicate that $N^{10}$-formyl-DHF is a much poorer substrate than DHF, a finding similar to that of Mathews and Huennekens in connection with the chicken liver enzyme (49). By contrast the substitution of a methyl group at the $N^{10}$ position has only minor effects on the rate of reaction.

The Effects of Various Salts on the Activity of T4DR and EDR

On the basis of inhibitor binding studies Baker has postulated that hydrophobic binding may account for as much as 80% of the free energy change accompanying the binding of DHF to DR (11, p. 204). Such an idea might be tested by agents which could weaken hydrophobic bonding. Recently Hatefi and Hanstein (116) have described the effects of certain anions, notably SCN$^-$ and ClO$_4^-$ on enhancing the solubility of certain organic compounds in aqueous solution. The increase in solubility of compounds such as adenine and riboflavin in aqueous solutions of NaSCN or NaClO$_4$ was ascribed to the ability of these salts (specifically the anions) to disrupt water structure. These authors point out that the direct consequence of such an effect would be to create a much more favorable entropy of transfer of an apolar molecule from a lipophilic surrounding to water. These considerations should also apply to folic acid compounds, since they too are organic compounds of limited
solubility in water (19 mg/l at 0° for folic acid, 48).
In addition, Baker's postulated hydrophobic bonding would
imply that the DHF complexed to DR exists, at least in part,
in a lipophilic environment.

Therefore it might be expected that chaotropic
agents like SCN⁻ or ClO₄⁻ could have a large effect on the
binding of DHF to DR and that this could be reflected in
the rate of the enzymatic reaction. To test this concept
T₄DR assays were conducted in the presence of several salts.
The results of these tests summarized in Fig. 22 clearly
indicate that SCN⁻ or ClO₄⁻ affect the rate of the reaction.
At as low a salt concentration as 0.04 M the activity has
decreased by about 50% compared to the initial value. At
these low concentrations SCN⁻ appears to be slightly more
effective than ClO₄⁻. A similar concentration of either
KCl or NaCl caused only a 5% decrease in the activity and
KF was virtually without effect. Since the order of
expected effectiveness was SCN⁻ > ClO₄⁻ > Cl⁻ > F⁻, the results
with the T₄ enzyme are consistent with Baker's hypothesized
hydrophobic bonding region.

One of the problems with this type of experiment is
related to the nature of intramolecular bonding in the
enzyme itself. Since it is possible that hydrophobic bond-
ing plays an important role in maintaining an enzymatically
active structure, the decrease in activity in the presence
The effects of certain salts on the enzymatic activity of T4DR. — Vo, reaction velocity in the absence of salts, was due to 0.025-0.08 units of enzyme. The ratios of V/Vo over this enzyme concentration range were identical, within experimental error. Assays were conducted at room temperatures, 23° ± 1°C in the presence of the indicated salt: KF (⊙), NaCl ( ), KCl (△), NaClO₄ (□), KSCN ( △ ).
Fig. 23. The effects of certain salts on the enzymatic activity of EDR. $V_o$, reaction velocity in the absence of salts, was due to 0.030 units of enzyme. Assays were conducted at room temperatures, $23^\circ \pm 1^\circ C$ in the presence of the indicated salt: KF (--○--), NaCl (--•--), KCl (--△--), NaClO$_4$ (--□--), KSCN (--▲--).
of these salts could be a reflection of protein denaturation. The following experiment shows that if denaturation occurs, it is a completely reversible denaturation (at least on the basis of activity). T4DR was incubated in the presence of 0.2 M and 0.4 M KSCN for a period of two hours at room temperature (pH 7.0). At various times aliquots (0.02 ml) were removed and tested for activity in a normal assay (i.e., chaotrope agent not present in the initial assay mixture). After corrections were made for the KSCN present in the enzyme sample, the activity was the same as that of a control assay. The results of test aliquots taken at the beginning of the two hour period were the same as those at the end of the two hour period. Thus these experiments show that the KSCN-induced inactivation of T4DR is not a time dependent process. Furthermore, it is a rapidly reversible process even when the enzyme is exposed to concentrations several times greater than the region where the change in activity is highly concentration dependent (0-0.10 M). In later experiments T4DR was found to be stable to concentrations of KSCN of 1.25 M at 0°C (pH 8.0).

By contrast to the results with T4DR, similar experiments with EDR are difficult to explain on the basis of changes in hydrophobic bonding. As is shown in Fig. 23, the pattern of effect between the two enzymes is completely
different. While inactivation occurs with all salts tested, the least effective salt was NaClO₄ and at low concentration KF was the most effective inactivator. The observation that KCl was more inactivating than NaCl indicates that differences in cation effects need to be considered in the salt inactivation of EDR. With T₄DR, NaCl and KCl produced identical effects (Fig. 22).

This type of experiment will, of course, not only reflect changes in substrate binding but also subtle changes which may occur in the catalytic efficiency of the enzyme. Furthermore, the cofactor NADPH which contains the adenine moiety in its structure might make important hydrophobic interaction with the enzyme. In order to separate changes in bonding interaction from other types of rate reducing effects a simplified system would be useful. Since previous work with a mammalian DR (58) had shown that the binding of both substrate and cofactor produce large changes in protein fluorescence, a study of the fluorescent properties of T₄DR and EDR was undertaken.
Characterization of the Fluorescence Properties of T4DR and EDR

The emission spectra of native and GuHCl denatured T4DR and EDR are shown in Fig. 24. 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 GuHCl the positions of the maxima are shifted in the long wavelength direction by 8 to 10 nm, to the position of the maximum emission of free tryptophan. By contrast, when 0.1% SDS is present (not shown) the position of the maximum is shifted in the shortwave direction 8 to 10 nm, close to the emission maximum of chymotrypsinogen (331 nm). On the basis of these results it would be difficult to distinguish T4DR from EDR.

By contrast to the emission spectra, the excitation spectra of the two enzymes possess marked differences (Fig. 25). With an excitation slit width of 3 nm the excitation spectrum of EDR shows 2 maxima, one at 285 nm and a second at 292 nm. The spectrum is quite similar to that of free tryptophan except that the peaks of the EDR spectrum relative to tryptophan are shifted 2 or 3 nm in the long wavelength direction. The T4DR excitation spectrum reveals only a single maximum (285 nm) and two shoulders, one at 281 nm and a second at 292 nm. Denaturation of the two enzymes with 6 M GuHCl has little effect on the spectrum of EDR.
Fig. 2b. Fluorescence emission spectra of T4DR and EDR. Excitation of T4DR in buffer B (—) or 6 M guanidine-HCl (—) was at 270 nm. Excitation of EDR in buffer B (—) or 6 M guanidine-HCl (—) was at 285 nm. T4DR emission spectra excited at 285 nm were the same as that at 270 nm except for intensities. Emission slit width was 4 nm. Intensities were not comparable.
Fig. 25. Fluorescence excitation spectra of T4DR and EDR. — Emission spectra of native T4DR (---) or EDR (-----) in buffer B were monitored at 340 nm. Emission spectra of 6 M guanidine-HCl denatured T4DR (----), EDR (--++) or tryptophan in buffer B (++++) were monitored at 350 nm. Intensities are not comparable.
Denaturation of the T4 enzyme abolishes the shoulder at 281 nm and converts the shoulder at 292 nm to a second peak although it is still less pronounced than the peak at 285 nm. In addition to this, the fluorescence intensity of T4DR denatured in 6 M GuHCl is reduced to approximately 40% of that of the native enzyme.

Perkins and Bertino (58) have shown that the binding of NADPH by the mouse lymphoma DR is accompanied by changes in both protein and NADPH fluorescence. Since these properties could prove useful to the present study, it was of interest to characterize the fluorescent properties of the NADPH complex with T4DR and EDR. 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 in connection with several dehydrogenases (117) will be referred to as NADPH-(ET)-Flu to indicate NADPH energy transfer fluorescence). As is shown in Fig. 26 the degree of protein quenching by NADPH is greater for EDR than for T4DR, about 60% and 40% respectively. Similarly, the NADPH-(ET)-Flu is weaker for T4DR than for EDR. It will be shown later that NADPH also binds to T4DR more weakly, than to EDR.

The excitation spectrum of the T4DR-NADPH complex is shown in Fig. 27, panel C. The fluorescence intensity
Fig. 26. Emission spectra of the T4DR-NADPH complex and the EDR-NADPH complex. — EDR concentration is .68 μM; T4DR concentration is .55 μM. In both cases 286 nm light (10 nm band slit for EDR and 6 nm for T4DR) was used for excitation. The emission slit was 10 nm for EDR, 6 nm for T4DR. The sensitivity setting was 3. NADPH concentrations are 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. Relative intensities for different NADPH concentrations are comparable for the same enzyme but not between enzymes. Further explanations are to be found in the text.
Fig. 26. Emission spectra of the T4DR-NADPH complex and the EDR-NADPH complex.
Fig. 27. Properties of the DR-NADPH and DR-NADPH-MTX complexes.— A. Increase in fluorescence of a blank solution —○— , T4DR solution —•— , or an EDR solution —x— , upon the addition of NADPH at the indicated concentration. Both enzymes were at a concentration of .68 μM. Excitation was at 340 nm (8.7 nm slit) and emission was at 460 nm (20 nm slit). Filter "43" was used in the emission beam to cut off scattered light of wavelength less than 430 nm.

B. Quenching of the fluorescence of the DR-NADPH complex by MTX. Quenching of the EDR-NADPH complex —○— was followed using the conditions described above for panel A. The T4DR-NADPH complex —— was excited at 284 nm and emission was monitored at 460 nm. C. Excitation spectra for the T4DR-NADPH and the T4DR-NADPH-MTX complex. Emission intensity was monitored at 460 nm (20 nm slit). An excitation slit of 8.7 nm was used and filter "43" was placed in the emission beam. T4DR concentration was .68 μM, NADPH concentration was 1.2 μM and the MTX concentration was 1.0 μM. Further explanations are to be found in the text.
Fig. 27. Properties of the DR-NADPH and DR-NADPH-MTX complexes.
at 460 nm observed for a T4DR solution (0.68 µM) alone is indicated by curve 2, while the fluorescence of the same T4DR 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 which has been described for certain other enzymes that bind pyridine nucleotides (117). The EDR NADPH-(ET)-Flu (Fig. 28) provides a better example of this, showing over a 100% increase in fluorescence. Fig. 28 also provides evidence that the increased fluorescence at 460 nm is due to light absorbed by enzyme. This suggestion can be drawn from the double peaks of the excitation spectrum which occur at the same positions as they do for free EDR emission measured at 340 nm (Fig. 25). The corresponding spectrum of the T4DR-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 (NADPH-(I)-Flu). Here light absorption is necessarily by NADPH. Once again, T4DR and EDR are qualitatively similar but quantitatively
Fig. 28. Excitation spectra of the EDR-NADPH complex monitored at 460 nm. The broken line indicates the fluorescence of 0.68 μM EDR alone. Solid line 1 indicates intensity after the addition of NADPH (to give 2.0 μM). Solid line 2 indicates the intensity after the addition of MTX (to give 0.8 μM). The dotted line indicates the fluorescence of 2.0 μM NADPH under these conditions. Excitation slit width was 3 nm.
different as illustrated in Fig. 27, panel A. When equal aliquots of NADPH are added to cells containing either buffer B or T^DR (0.68 μM) there is an identical increase in NADPH-(I)-Flu. When this experiment is performed with EDR of the same concentration the initial increase in NADPH-(I)-Flu is 50% greater in the cell containing EDR than in the cell containing buffer. Thus NADPH fluorescence is enhanced in the complex with EDR but largely unaffected in the complex with T^DR. This indicates that the 340 nm excitation peak of curve 1 in Fig. 27, panel C, is the same as that of free NADPH. However, the fluorescence of free NADPH cannot be quenched by MTX to the extent indicated by curve 3. The quenching of the two types of DR-NADPH fluorescence by MTX at 460 nm is shown in Fig. 27, panel B.

In each case the curve indicates the quenching process is a linear function of MTX concentration.

In general, the binding of MTX and other 4-amino folate compounds by T^DR and EDR results in a large degree of quenching not only of the NADPH fluorescence in the ternary complexes but of protein fluorescence as well. As is shown in Fig. 29, the quenching process with MTX shows a linear relationship between inhibitor concentration, decrease in fluorescence and also, by comparison with Fig. 15, enzymatic activity as well. While aminopterin also
Fig. 29. Quenching of protein fluorescence by some folate compounds and reversal of quenching by KSCN. Quenching of T4DR by MTX (— — —), EDR by MTX (— — —), T4DR by NFA (— Δ — ) and of T4DR by DHF (— X — ) is shown in the left hand panel and the reversal of quenching of the T4DR fluorescence is shown in the right hand panel. Protein concentration in the case of quenching by NFA was 0.3 μM and for DHF was 0.55 μM. The arrow indicates plot of fluorescence quenched by NFA concentration of 1.6 μM.
quenches protein fluorescence in a stoichiometric fashion, NFA binds less tightly, displaying a measurable equilibrium. Since MTX forms such tight complexes with EDR and T4DR it was of interest to characterize their spectra. As is shown in Figs. 30 and 31 the binding of MTX by T4DR or EDR produces small but reproducible changes in the excitation spectra of both enzymes. The T4DR-MTX complex shows a more pronounced shoulder at 292 nm than does the free enzyme. In addition, this shoulder now shows a greater intensity relative to the 285 nm peak. While the excitation spectrum of the T4DR complex with NADPH (not shown) is quite similar to the free enzyme, the ternary complex T4DR-MTX-NADPH does appear to be slightly altered compared to the T4DR-MTX complex, in that the relative intensity of the 292 nm peak is enhanced another degree, relative to the 285 nm peak. In the case of EDR the most significant change in the excitation spectra also involves the binding of NADPH to the EDR-MTX complex. However, in this case it is the 285 nm peak which is enhanced relative to the 292 nm peak. The fact that these changes for the two enzymes are of opposite "sign" indicates the changes are not due to trivial factors such as light absorption by the sample.

The protein emission spectra of the T4DR-MTX and T4DR-MTX-NADPH complexes are shown in Fig. 32. In order to detect shifts in the emission maxima the emission slit width was set at 3 nm. While the maximum for free enzyme is at
Fig. 30. Excitation spectra of the binary and ternary complexes of NADPH and MTX with EDR. - Spectra are: free EDR (---), EDR-NADPH (-----), EDR-MTX (----) and EDR-MTX-NADPH (----). The excitation slit width was 3 nm and fluorescence was monitored at 340 nm. Intensities are not comparable.
Fig. 31. Excitation spectra of the binary and ternary complexes of NADPH and MTX with T4DR. Spectra are: free T4DR (——), T4DR-MTX (---) and T4DR-MTX-NADPH (-----). The excitation slit width was 3 nm and fluorescence was monitored at 340 nm. Intensities are not comparable.
Fig. 32. Emission spectra of the binary and ternary complexes of MTX and NADPH with T4DR. - Spectra are: free T4DR (---), T4DR-MTX (—.), and T4DR-MTX-NADPH (---). Excitation was at 285 nm. Intensities are not comparable.
340-342 nm, the maxima for the two complexes, especially the ternary complex, appear to be shifted 2 to 4 nm in the short wavelength direction, possibly indicating a decrease in tryptophan solvation (108, p. 73-75) during complex formation. It should be pointed out, however, that these shifts are only of the same magnitude as the emission slit width and the radii of curvature of the arcs are quite "gentle" making the exact position of the maximum difficult to locate. Thus a firm decision concerning changes in tryptophan solvation cannot yet be made.

Equilibrium Binding Constants

As a preliminary to calculating binding constants, the limit of protein fluorescence quenching by several small molecules was determined by means of a double reciprocal plot (118), of fluorescence intensity (Fl) versus molar concentration. Four such plots are shown in Fig. 33. The intercept at infinite small molecule concentration was assumed to correspond to the fluorescence of the DR-small molecule complex. The extent of protein fluorescence quenching obtained from these plots is shown in Table VII. The extent of quenching by MTX and aminopterin could be determined directly, due to the very tight binding that these inhibitors display towards either DR. The data in Table VII indicate that all folate compounds tested result in quenching in the 75-80% range. The addition of NADPH
Fig. 33. Double reciprocal plot of substrate or cofactor concentration versus fluorescence intensity (Fl). A, T4DR-NADPH; B, T4DR-DHF; C, EDR-DHF; D, EDR-NADPH.
Table VII

Quenching of protein fluorescence by various compounds

<table>
<thead>
<tr>
<th>Complex</th>
<th>Quenching&lt;sup&gt;a&lt;/sup&gt; (%)</th>
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<tbody>
<tr>
<td>T4DR-DHF</td>
<td>76</td>
</tr>
<tr>
<td>T4DR-NADPH</td>
<td>61</td>
</tr>
<tr>
<td>T4DR-MTX</td>
<td>79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T4DR-MTX-NADPH</td>
<td>85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T4DR-aminopterin</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
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<td>82</td>
</tr>
<tr>
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<tr>
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<td>EDR-NADP</td>
<td>54</td>
</tr>
<tr>
<td>EDR-MTX</td>
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</table>

<sup>a</sup>determined by extrapolation to infinite substrate concentration, except as noted.

<sup>b</sup>determined directly by inspection of quenching curve.
to the T4DR-MTX complex results in an additional 5-7% quenching. (Thus about 85% of the fluorescence of the T4DR and EDR preparations can be quenched, substantiating previous data indicating the high degree of homogeneity of these preparations.) NADPH produces significantly less protein fluorescence quenching of T4DR than the folate compounds do. By contrast the quenching of EDR fluorescence by NADPH is nearly as great as that by the folate compounds.

The extent of quenching obtained by a number of other compounds was also investigated but these are not included in Table VII because the extent of quench was negligible. Thus NADP⁺ and NADH at concentrations of 4 and 0.4 μM respectively failed to give a detectable quenching of T4DR fluorescence at a concentration of 0.55 μM. 0.4 μM NADH also failed to detectably quench EDR fluorescence at a concentration of 0.35 μM. While folic acid was able to quench a small amount of T4DR fluorescence, at a concentration of 4 μM, the measurements were suspected of being highly inaccurate due to non-specific quenching or due to light absorption by the sample. In this connection, the quenching of EDR by NADP⁺, while listed in Table VII, may be in error by as much as 25-30%.

Data from quenching curves of the type shown in Fig. 29 were used to make a plot of \( \frac{\alpha}{[\text{DR}]} \) versus
1-\alpha (Fig. 34). From the slopes of these plots the association constants, $K_a$, for complex formation were determined. These are summarized in Table VIII, along with the corresponding standard free energy change of complex formation ($\Delta F^0$). For comparative purposes Table VIII also contains other types of binding data.

The results of the equilibrium binding experiments indicate that the substrate, DHF, binds nearly as well to both EDR and T4DR. By contrast the binding of the cofactor NADPH is significantly different. It is interesting that the more efficient catalyst (T4DR) makes the weaker interaction with NADPH.

The binding data also indicate that NFA, the ligand used in affinity chromatography, forms a complex with the T4 enzyme only a few times more tightly than does DHF. It is also interesting that the equilibrium-determined dissociation constant for NFA ($1/K_a$) 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 DR have also revealed significant differences between equilibrium and kinetic data (58).

The $I_{50}$ shown for folic acid indicates the relatively weak interaction that this compound makes with T4DR.
Fig. 34. Equilibrium binding of substrate, cofactor or inhibitor to T4DR and EDR. - Left and lower scales: T4DR-DHF (---), EDR-DHF (—-), T4DR-NADPH (—-); right and upper scales: T4DR-NFA (—□—), EDR-NADPH (—Δ—).
Table VIII

T^4DR and EDR binding constants for substrate, cofactor and inhibitors

### T^4DR

<table>
<thead>
<tr>
<th>Compound</th>
<th>$Ka$ (M)</th>
<th>$\Delta F^0$ (Kcal/mole)$^a$</th>
<th>$K_m$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHF</td>
<td>$2.0 \times 10^6$</td>
<td>-8.5</td>
<td>$2.3 \times 10^{-6}$</td>
</tr>
<tr>
<td>NADPH</td>
<td>$1.3 \times 10^6$</td>
<td>-8.3</td>
<td>$18 \times 10^{-6}$</td>
</tr>
<tr>
<td>NFA</td>
<td>$6.7 \times 10^6$</td>
<td>-9.2</td>
<td></td>
</tr>
</tbody>
</table>

### EDR

<table>
<thead>
<tr>
<th>Compound</th>
<th>$Ka$ (M)</th>
<th>$\Delta F^0$ (Kcal/mole)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHF</td>
<td>$2.4 \times 10^6$</td>
<td>-8.7</td>
</tr>
<tr>
<td>NADPH</td>
<td>$1.1 \times 10^7$</td>
<td>-9.5</td>
</tr>
</tbody>
</table>

$^a$ calculated from $\Delta F^0 = -RT\ln Ka$.

$^b$ as determined from the slopes of lines A and B of Fig. 18 from the equation: slope = $(K_m/V) [1 + (i/K_i)]$, (119), where $i$ is inhibitor concentration.

$^c$ inhibitor concentration required to reduce enzymatic activity by 50%.
and this probably explains the ineffective quenching of T4DR fluorescence by this compound.

Effect of Certain Salts on the Quenching of Fluorescence

As was previously shown, certain salts have a marked effect on the rate of the DR reaction. Since the reaction rate can be influenced by many factors, it could not be shown that the salts exerted their effect through decreased affinity of the enzyme for its substrate. Thus as a more direct approach to answering this question, the pattern of DR fluorescence quenching by substrate and cofactor was studied. Prior to these experiments the effects of KSCN and NaClO₄ on the fluorescence of free T4DR and subsequently EDR were investigated. Over the salt concentration range studied, 0-0.15 M, the maximum addition of 4 M KSCN or NaClO₄ (0.1 ml) to the enzyme solution (2.5 ml) produced a 3 or 4% decrease in the relative fluorescence. This change is what would be expected by dilution. Therefore it was assumed that any increase in fluorescence observed following addition of salts to one of the DR-complexes could be attributed to decreased binding. Fig. 35 shows the effects of several salts on the T4DR-DHF and the T4DR-NADPH complexes. Initially, each T4DR solution showed an identical relative fluorescence. These solutions were then quenched to within 70-80% of completion. The volume
Fig. 35. Reversal of the quenching of protein fluorescence of T4DR by varying salts. The fluorescence of T4DR (0.55 μM) was quenched to within 70 to 80% of completion by the addition of DHF (3.3 μM), left panel, or by NADPH (3.3 μM), right panel. The fluorescent 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.
of the quenching agent was such that the final volume of enzyme plus quenching agent was 2.5 ml. Subsequently, the effect of additions of a given salt was studied. Fig. 35 shows that the addition of either KSCN or NaClO₄ to the T₄DR-DHF complex is accompanied by a large increase in the relative intensity. The slope of the curve is very large in the 0-0.04 M region, corresponding to the same region where the enzymatic activity is changing most rapidly (Fig. 22). NaCl and KCl give patterns of quench reversal which are indistinguishable from each other, 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 that of KSCN and KF. The effect of this last salt on the binding of DHF to T₄DR appears to be completely negligible, as indicated in Fig. 35, by a plot of essentially zero slope.

Prior to these experiments, a futile attempt had been made to use KSCN to elute T₄DR from its complex with MTX which in turn was bound through peptide linkage to the solid support Bio-Gel P-150. It was therefore of interest to examine the effect of KSCN on the fluorescence of the T₄DR-MTX complex. As is shown in the right-hand panel of Fig. 29, there is no detectable change in the intensity of this complex in the 0-0.14 M concentration range. A
similar treatment of the T4DR-NFA complex does show a small increase in intensity but is still proportionately much less than the change in intensity produced in the T4DR-DHF complex. Some possible reasons for this difference will be discussed later.

Thus the pattern of effects of the various salts on the fluorescence intensity of the T4DR-DHF complex was the one anticipated on the basis of Baker's proposed hydrophobic bonding component (11,p.204). However, when a similar series of experiments was performed with the T4DR-NADPH complex the results were somewhat unexpected. As is shown in the right-hand panel of Fig. 35, the pattern of quench reversal for the T4DR-NADPH complex is strikingly similar to the pattern with T4DR-DHF. The same large slopes are seen for KSCN and NaClO₄, 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.

It is interesting to compare the change in intensity of the two complexes. In terms of per cent of quench reversal, at 0.2 M KSCN, the effect on the T4DR-NADPH complex is actually larger than that of the T4DR-DHF complex, 83 versus 71%, respectively. Thus, these data suggest that the decreased enzymatic activity of T4DR in the presence of KSCN or NaClO₄ can be accounted for, to a large extent, by decreased binding of both substrate and cofactor.
The effect of salts on EDR quench reversal (Fig. 36) showed a better correlation to the T4DR pattern just described than it did to the corresponding enzymatic activity curves (Fig. 23). However, in general the quench reversal at low salt concentration was much less for EDR than for T4DR, as can be seen by comparisons 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 EDR system than in the T4DR system, they still gave identical quench reversal patterns in the EDR system. In the case of the EDR-DHF complex, KCl and NaCl (along with KF) produced no effect at all. These results are interesting when compared to the marked differences observed for the EDR-enzymatic 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 NaClO₄ on quench reversal would not predict the effect of these salts on the enzymatic activity.

As a further demonstration that the increase in protein fluorescence observed in these studies was due to decreased binding, the effect of salts on the EDR-NADPH energy transfer fluorescence peak was examined (Fig. 36). Since this peak arises from complex formation, agents which
Fig. 36. Reversal of the quenching of protein fluorescence of EDR and of the fluorescence of the EDR NADPH (ET) Flu by various salts. - Left panel: The fluorescence of EDR (0.44 μM) was quenched with DHF (4.0 μM). Right panel: The fluorescence of EDR (0.35 μM) was quenched with NADPH (1.6 μM). The fluorescent 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 (ET) Flu (---), [EDR] 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 EDR-NADPH complex.
decrease the binding of NADPH to T₄DR should cause a
decrease in the intensity of this peak. As is shown in
Fig. 36, the curves obtained with KF and KSCN are just those
expected from the corresponding protein fluorescence curves.

Studies of the MTX-T₄DR Complex

The potent inhibition of T₄DR by MTX indicated by
stoichiometric inhibition of enzymatic activity and the
linear quenching of T₄DR fluorescence by MTX shows that the
binding of this inhibitor is unusually tight. Since the
studies with KSCN were unable to disclose any change in the
binding of MTX to T₄DR, it was decided to investigate the
stability of this complex in higher concentrations of
denaturing agents. Since it was known that denaturation
of free T₄DR by 6 M GuHCl resulted in a 50% decrease in
relative intensity, a sensitive method was at hand for
studying the relative stability of the free enzyme and the
MTX-T₄DR complex in this denaturing agent. As is shown in
Fig. 37, at 0 GuHCl concentration free T₄DR is about 5
times more fluorescent than the T₄DR-MTX complex. There‐
fore it seemed feasible to observe the denaturation of
either the free enzyme or the complex by a decrease or an
increase, respectively, of the fluorescence intensity,
assuming that denaturation of the complex resulted in the
same relative intensity as denaturation of the free
enzyme. Fig. 37 shows that the intensity of both the
Fig. 37. Comparative fluorescent intensity of free versus MTX-bound T4DR at various concentrations of guanidine-HCl. Two ml of T4DR (1.0 μM) in buffer B was inactivated with a small excess of MTX in .01 ml of buffer B. To a second aliquot of T4DR was added .01 ml of buffer B. 0.10 ml of each solution was transferred to 2.4 ml of guanidine-HCl solutions of the indicated concentrations. 3 min after mixing, the fluorescence intensity was recorded. The experiment was at room temperature. Excitation was at 285 nm and emission was monitored at 345 nm. Free T4DR (—o—), T4DR-MTX complex (—x—).
free enzyme and the complex is little altered by 1.0 M GuHCl. The initial intensity observed for both forms of the enzyme was unchanged after three min. However, at 2 M GuHCl, the initial intensity of both was changing, in the direction expected for denaturation. By 3 min the intensity had reached the indicated points, suggesting that denaturation of the free enzyme was nearly complete at a time when the complex was still roughly 50% intact. (Although no attempt was made to study the kinetics of the denaturation process, these techniques would provide a method for doing this.) At a concentration of 3.0 M GuHCl both forms of the enzyme rapidly came to the same fluorescence intensities indicated in Fig. 37. At higher concentrations of GuHCl the final intensity of both free enzyme and complex had been reached before the first measurement could be made.

These results indicate that the T4DR-MTX complex is somewhat more stable to GuHCl denaturation than is the free enzyme. It is also clear that essentially no reversal of MTX binding to T4DR is afforded by 1 M GuHCl, as indicated by only slight changes in the fluorescence intensity. The data also indicate (but do not establish) that it is the complex that is denaturing, rather than the complex being first resolved prior to enzyme denaturation. Thus, if the complex was first resolved into MTX and free
enzyme to a large degree, one might expect to see an initial fluorescent intensity considerably larger than was observed.

Because the inhibition of T4DR by MTX is so potent, the possibility existed that complex formation also involved the formation of a covalent bond. While the experiment just described showed that complex formation did not prevent denaturation by GuHCl, the question concerning covalent bond formation was left unsettled. To answer this question directly, the complex was treated with SDS and subjected to Sephadex G-25 chromatography as shown in Fig. 38. (GuHCl could not be used in this experiment because this substance created a precipitate in the scintillation fluid.) As a control for this experiment it was observed that free T4DR emerges from the column at a volume indicated by arrow A and free MTX elutes at the position indicated by arrow B. When T4DR is inactivated by a small excess of tritium labeled MTX and applied to the column, the eluate has the profile of radioactivity indicated by the solid line. The fraction at arrow A was found to be devoid of enzymatic activity and to have the fluorescent properties of the T4DR-MTX complex just described. Thus the complex emerges in the same position as the free enzyme.

When the T4DR-MTX complex is treated with SDS prior to chromatography the profile of radioactivity indicated by the broken line is obtained. Since control
Fig. 38. Sephadex G-25 Chromatogram of T\textsubscript{4}DR-MTX complex. 6.5 ml of homogeneous T\textsubscript{4}DR (3.3 units/ml) was treated with $3',5'-\text{H-MTX}$ (250 mcI/mM, Amersham/Searle) until inactive plus a small excess. A 0.5 ml aliquot (70,000 counts/min) was then applied to a Sephadex G-25 column (1.2 x 22 cm) equilibrated with buffer B. 5 ml fractions were collected at a flow rate of 50 ml/hour. A second aliquot was treated with 0.67% SDS and incubated for 30 min at 37° before chromatography. Radioactivity was measured as described in the experimental section with a counting efficiency of 15%. Further explanations are found in the text.
experiments in which free MTX treated with SDS gave a similar profile, the data indicate the resolution of the T4DR-MTX complex. The presence of protein at arrow A was again established by fluorescence. Thus it was concluded that the T4DR complex does not involve stable covalent bond formation.

The reasons for the shift in elution profile of MTX in the presence of SDS, as compared to MTX alone, was not established. However, SDS is believed to make an interaction with the hydrophobic side chains of proteins (120). It is possible that a similar interaction with MTX is occurring and that this is responsible for the observed shift in elution volume.

Characterization of the Absorbance Changes Accompanying the Binding of Substrate and Inhibitors

Heretofore, the changes in absorption spectra which accompany the binding of folate compounds to DR have not been described. Since the amounts of T4DR available by affinity chromatography were sufficient to permit a limited investigation of this type, the study of the difference spectra associated with the binding of DHF and several analogues to T4DR was undertaken.

One of the first compounds to be studied by this technique was NFA. The difference spectra obtained in the presence of T4DR with two different concentrations of this
compound are shown in Fig. 39. This figure also illustrates the difficulty in obtaining a satisfactory base line on the 0-0.10 absorbance setting of the Cary 15. Here lines 1 and 2 are shown relative to the original base line. Spectra in subsequent figures have been corrected in order that the spectra may be shown relative to a straight base line, facilitating comparisons. Line 2 represents an original trace indicating that the "noise" level was small.

The difference spectra obtained from the T4DR-NFA complex versus the free components indicate decreased absorption in the long-wavelength (greater than 380 nm) absorption region of NFA, a region of enhanced absorption in the 285-375 nm region and a second region of decreased absorption, forming a peak close to 260 nm. During the characterization of free NFA, the spectra of this compound were examined at pH 7.0 and in 0.1 N HCl (Fig. 40). After obtaining the T4DR-NFA difference spectra it was noticed that the changes observed when NFA binds T4DR were similar to the changes observed between the neutral and acid spectra of NFA. In order to make a better comparison, the difference spectra of NFA at several acid pH values versus NFA at pH 7.0 were scanned. As is shown in Fig. 39, the difference spectra obtained at acid versus neutral pH values bear a striking similarity to the spectra of the T4DR-NFA system.
Fig. 39. Difference spectra of the NFA-DR complex versus free components and difference spectra of NFA in acid versus neutral solution. Solid lines: (1), T4DR (2.8 μM) in the presence of 2.9 μM NFA; (2), 5.7 μM NFA; (1B), baseline for (1) and (2). (0-0.1 absorbance unit scale.) Broken lines: (3), NFA (2.7 x 10^{-5} M), pH 5.3 versus pH 7.0; (4), pH 2.8 versus pH 7.0; (2B), baseline for (3) and (4). (0-1.0 absorbance unit scale.)
Fig. 40. Acid and neutral absorption spectra of \( \text{N}^{10} \)-formyl-aminopterin.
Fig. 41. Difference spectra of the MTX-T4DR complex and of MTX in acid versus neutral solutions. The solid curve shows the difference spectra of 3.7 μM MTX in the presence of 2.8 μM T4DR obtained from the 0-0.1 absorbance unit scale. The broken curve is for 3.0 x 10^{-5} M MTX at pH 4.8 versus pH 7.0, obtained on the 0-1.0 absorbance unit scale.
Fig. 42. Difference spectra of the aminopterin-T4DR complex and of aminopterin in acid versus neutral solutions. The solid curve shows the difference spectra of 3.6 μM aminopterin in the presence of 2.8 μM T4DR obtained from the 0-0.1 absorbance unit scale. The broken curve is for 2.9 x 10^{-5} M aminopterin at pH 2.8 versus pH 7.0, obtained on the 0-1.0 absorbance unit scale.
There are some differences, however. One of the chief differences between the two systems is that the long-wavelength transition from + to - occurs at about 375 nm for the T4DR system as opposed to about 357 nm for the acid-neutral system. Another difference between the two is a 3 or 4 nm shift in the large negative peak near 260 nm. Here, also, the enzyme system is shifted in the long-wavelength direction relative to the acid-neutral system.

To see if the spectral changes observed for NFA applied to other 4-amino folate compounds, the study was extended to the more potent inhibitors MTX and aminopterin. Figs. 41 and 42 indicate that the main features of the spectral changes observed in the NFA system are also present in the difference spectra of MTX and aminopterin. Here again there are the same general features between the T4DR-inhibitor system and the acid-neutral system as noted with NFA. However, in going from NFA to aminopterin to MTX there is a deepening "trough" at about 315-320 nm, becoming a minor negative peak in the case of MTX. While the acid-neutral system of the three compounds shows a progressive change in a shoulder at about 310 nm, there are no negative absorption changes in this region.

As is indicated in Fig. 39, the magnitude of the acid-neutral difference spectra of NFA is a sensitive function of the acid pH. These changes in magnitude can
be most accurately determined from the large, negative difference peak near 260 nm. As a matter of convenience changes in this peak will be referred to as the $\Delta A_{260}$. (None of the three inhibitors produces a peak centered exactly at 260 nm.) As is shown in Fig. 39, about one-third of the final $\Delta A_{260}$ is obtained in going from pH 7.0 to pH 5.5 (relative to the $\Delta A_{260}$ between pH 7.0 and pH 2.8). That the $\Delta A_{260}$ peak is largely complete by about pH 4.0 is indicated by the change observed in going from pH 4.1 to pH 2.8 (Fig. 39). Since this also proved to be the case with MTX and aminopterin it was of interest to make a comparison of the $\Delta A_{260}$ observed in the enzyme system with that observed in the acid-neutral system. The following molar absorptivity changes for the $\Delta A_{260}$ were calculated: NFA, 8,600; MTX, 10,000 and aminopterin, 13,000. These $\Delta A_{260}$ values correspond to pH values in the 4.8-5.5 pH range for NFA and MTX and about pH 2.8 for aminopterin.

As was mentioned in the Introduction, MTX has been reported to be about 1,000 times more basic than folic acid (67). To see if the spectral changes observed for the 4-amino inhibitors also applied to 4-hydroxyl compounds, the spectral changes involving the binding of folate to T4DR and in the acid-neutral system were investigated. As is shown in Fig. 43, the main feature of the
Fig. 43. Difference spectra of the T4DR-folate complex and of folate in acid versus neutral solutions. Curves 1 and 2 show 8.0 μM T4DR in the presence of 8.5 and 17 μM folate, respectively, as obtained on the 0-.10 absorbance unit system. The broken and dotted curves show the difference spectra for folic acid at pH 2.8 versus 7.0 and 1.5 versus 7.0, respectively.
Fig. 44. Difference spectra of the T4DR-DHF complex. - Lines 1 and 2 show the difference spectra obtained for 5.9 μM T4DR in the presence of 4.1 μM DHF and 5.6 μM T4DR in the presence of 10.5 μM DHF, respectively.
Fig. 45. Difference spectra of the T4DR-dihydroaminopterin complex. Lines 1 and 2 are for 5.9 µM T4DR in the presence of 2.9 µM dihydroaminopterin and lines 3 and 4 are for 5.7 µM T4DR in the presence of 5.7 µM dihydroaminopterin. See text for further explanations. The inset shows the difference spectra of dihydroaminopterin at pH 0.3 versus pH 7.0, (86).
difference spectra of the enzyme-folic acid system is two peaks at about 248 and 303 nm, both of which are positive, plus a small peak at 353 nm. Since the interaction of folic acid with T4DR is weak, the T4DR concentration was increased to 8 μM and the final folic acid concentration was about 17 μM. Even with these relatively high concentrations the absorbance changes are rather small. Since the concentration of the T4DR-folate complex is not known, it cannot be determined whether the small absorbance changes are due to lack of complex formation or to small molar absorptivity changes.

As has been mentioned, the spectra of the 4-amino compounds are changing rapidly in the pH region 7 to 4.8. By contrast, the spectrum of folate is virtually unchanged in this region. As is shown in Fig. 43, the corresponding acid pH region where the folate spectrum is most sensitive to changes in pH is between about 2.8 and 1.5.

When folate difference spectra at acid pH values were determined, it was clear that the relationship between the enzyme and acid-neutral system observed for the 4-amino compounds did not extend to this 4-hydroxyl compound. While the most prominent peak in the acid-neutral system of folic acid is a negative peak (as is the case with the 4-amino compounds) only a small portion of the T4DR-folate system is negative.
The difference spectrum obtained from the system composed of T4DR and its substrate DHF is shown in Fig. 44. As with folate, the acid-neutral spectra of DHF (not shown) bear no resemblance to the enzyme system. The T4DR-DHF difference spectra are all positive, showing a large peak near 243 nm and a smaller one at about 305 nm. There is also a shoulder (or a maximum; it is difficult to say which) at about 355 nm. Thus the three peaks observed in the T4DR-folic acid system are also observed in the T4DR-DHF system although the relative intensities are different and the positions of the maxima are shifted slightly.

Based on a $K_a$ of $2 \times 10^6$, curve 2 is estimated to reflect the difference spectrum of a T4DR-DHF complex of 5.1 $\mu$M concentration. Therefore the 243 nm peak corresponds to a molar absorptivity increase of about 11,000, while the 305 nm peak is about half that.

Since neither folate or DHF gave difference spectra related to the corresponding acid-neutral spectra, the phenomena appeared to be a property of the 4-amino group. Therefore it was of interest to examine the difference spectra of a 4-amino inhibitor at the oxidation level of the substrate. Fig. 45 shows the difference spectra obtained with dihydroaminopterin. A comparison of the spectra obtained with this compound to the spectra of aminopterin and of DHF indicates that the spectra of the 4-amino,
dihydro inhibitor have more in common with the 4-hydroxyl dihydro compound (they both have positive peaks between 300-310 nm) than with its parent compound, aminopterin. Furthermore, the acid-neutral spectra for dihydroaminopterin does not resemble the enzyme-inhibitor system. Like the other folate compounds studied the acid-neutral spectra involve decreased absorption (86).

During the study of the T4DR dihydroaminopterin complex, repetitive scans of the difference spectra, at a single concentration of the enzyme-inhibitor complex, indicated substantial time-dependent alterations were taking place. An initial scan, curve 1 in Fig. 45, was followed about 30 min later by scan 2. During the time lapse a significant alteration in the spectra appears to have occurred, particularly in the wavelength region below 280 nm. Following the addition of a second aliquot of dihydroaminopterin, scan 3 was obtained. Scan 4, performed 2 hours after scan 3 (all cuvettes were removed from the light path during intervals between scans) indicates the emergence of what appears to be a peak at about 250 nm, with a magnitude which increases with time. Of all the compounds examined in this series of experiments only dihydroaminopterin showed these time-dependent spectral alterations. It is possible that the changes observed are due to decomposition of the dihydroaminopterin.
However, if this is the case, the magnitude of the spectral change observed would indicate a large degree of decomposition in a relatively short period of time. Kisliuk and Levine have found that 17% of the dihydroaminopterin incubated at room temperature in the presence of 2-mercapto-ethanol at neutral pH still remained after 48 hours (86). It is clear that, whatever the nature of the observed change may be, it represents differential properties of enzyme-bound versus free dihydroaminopterin.

While all of the folate compounds tested showed some type of difference spectra with T4DR, similar experiments with NADPH gave no results. Apparently the binding of NADPH by T4DR does not produce absorbance changes detectable by the present methods. Similarly the addition of NADPH to the various binary complexes just described gave no results except in one case. When NADPH was added to the T4DR-dihydroaminopterin complex, the peak at 305 nm was enhanced by about 25%. However, the instability of the spectra of the T4DR-dihydroaminopterin complex makes an assignment of this result to NADPH uncertain.
DISCUSSION

By means of the methods reported here it was possible to purify T4DR and EDR about 6,000-fold and 27,000-fold, respectively, with recoveries of about 80% in each case. Such high recoveries were possible only by the use of affinity chromatography, a method which is becoming relatively common (121) since the publications of Cuatrecasas on this subject (87,122). At the time that the present procedures were being developed (123), no successful affinity chromatography methods for the purification of DR, employing a solid support, had been reported in the literature. An attempt to purify DR from chicken liver by columns of MTX linked to aminoethyl cellulose met with no success—a suggested reason being the low degree of MTX linked to the support (124). A much greater degree of linkage of this inhibitor to soluble aminoethyl starch was attained; however, the soluble nature of the support required the use of time-consuming Sepha-
dex G-100 columns, and the reported recovery was low (124). During the preparation of this thesis an improved method for purification of the chicken liver enzyme by affinity chromatography was reported by Kaufman and Pierce (125). These investigators, following the lead of Cuatrecasas (87),
interposed either a six- or a twelve-carbon aliphatic diamine between the solid support, Sepharose 4B, and MTX, resulting in an affinity material that quantitatively adsorbed the chicken liver DR.

It is interesting that the affinity material described in this thesis successfully utilized a gel matrix (Bio-Gel P-150) which presumably is much less "open" than the more commonly used Sepharose and that only a two-carbon "arm" (ethylenediamine) was employed. The need for a long hydrocarbon arm is assumed to be related to steric factors associated with the approach of the enzyme to the binding ligand (87). Thus a ligand attached directly to the support matrix may be restricted to nonbinding interaction with the enzyme in question. Since no attempt has been made to purify the chicken liver DR by means of MTX-amoeneethyl Bio-Gel P-150, it is not known whether the need for the long hydrocarbon arm in the Sepharose system is due to differences in the nature of the matrix or is due to differences in properties of the chicken liver DR and T4DR.

When MTX was employed as the binding ligand in this study the problem was not in the lack of adsorption of the enzyme, but rather the inability to free the enzyme once bound. Thus, it would appear that the linkage of MTX to the insoluble polyacrylamide through a carboxyl group on the glutamate moiety does not interfere with the
stoichiometric and irreversible characteristics of this inhibition. This failure of MTX, which led to a search for a more suitable ligand, suggested that a "good" but reversible inhibitor of the enzyme would have the correct properties. Folic acid, as previously mentioned, was found to be a rather weak inhibitor. It has been reported that the substitution of a formyl group at the N\textsuperscript{10} position of folate enhanced binding of this analogue to DR from Ehrlich ascites carcinoma cells by a factor of about 600-fold (126). It was found, however, that N\textsuperscript{10}-formyl-folate was a weaker inhibitor of T\textsubscript{4}DR, by roughly a factor of 10 (based on I\textsubscript{50} data), rather than a stronger one. The immediate suggestion of this observation was that the substitution of a formyl group at the N\textsuperscript{10} position of aminopterin might produce the "good" but reversible inhibitor sought. As has been described in the Results section, this proved to be the case.

In each case where the N\textsuperscript{10} position of a folate compound was substituted by a formyl group the resulting product was either a poorer inhibitor (NPA or N\textsuperscript{10}-formyl-folate) or a poorer substrate (N\textsuperscript{10}-formyl-DHF, see Table VI) of T\textsubscript{4}DR. These results, at least in the case of the inhibitors, appear to be due to a decreased affinity of the formylated compound for T\textsubscript{4}DR. It is therefore interesting that N\textsuperscript{10}-formylfolate is more potent than
folate as an inhibitor of the Ehrlich ascites carcinoma enzyme (126). This same enzyme is also unable to utilize $N^{10}$-formyl-DHF as a substrate, although this is not due to a lack of binding for this compound (126). Since the chicken liver enzyme (like T4DR) utilizes $N^{10}$-formyl-DHF as a poor substrate (49), the result with the Ehrlich ascites carcinoma enzyme does not represent a universal property of DR.

The fluorescent emission maxima of T4DR and EDR at 340-342 nm (Fig. 24) are about the same as that for the L1210 mouse lymphoma (58) DR. Teale has shown that tryptophan-containing proteins show a fairly wide range of maxima from about 328 nm for edestin to 342 nm for bovine serum albumin (107). It is now generally assumed (108, p. 73-75) that the reason for this variation is related to the dielectric constant of the microenvironment of the various tryptophan residues of a given protein. Stated another way, the position of the emission maximum is to some degree a reflection of the extent of tryptophan exposure to the solution (assumed to be aqueous). Thus, denaturation of a protein with urea or GuHCl generally results in a shift in a protein's emission maximum to 348-350 nm, the same as that of free tryptophan (107). On the basis of these criteria, T4DR and EDR would appear to have a degree of tryptophan exposure as large as any observed in the proteins studied by Teale.
The excitation spectra of T^4DR and EDR and their complexes with MTX and NADPH are more difficult to interpret. The differences observed in the free proteins are most likely due to a combination of complex phenomena. A few of the possible contributing factors could be differences in energy transfer from tyrosine to tryptophan, differences in the arrangement of the chromophores with respect to each other, and variations in perturbations of the tryptophan residues by other protein groups (108, chap. 3). The excitation spectra of the MTX and NADPH complexes are equally difficult to interpret. Furthermore, since complex formation produced different spectral changes for the two enzymes, it is apparent that the excitation spectra of these complexes are not universal characteristics of DR. They are, rather, only characteristics of a specific DR. For the present purposes, therefore, the excitation spectra of T^4DR and EDR are most useful in further serving to differentiate the phage enzyme from the corresponding host cell enzyme.

The phenomenon of energy transfer, in which light absorbed by protein enhances the fluorescence of bound pyridine nucleotide, was first reported by Velick (117) during an investigation of the binding of NADH by several dehydrogenases. Perkins and Bertino (58) reported a virtually identical finding for the interaction between the L1210
lymphoma DR and NADPH. This type of fluorescence is also present in the T4DR and EDR systems. However, it is clear from an inspection of Figs. 26 and 27 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 Fig. 27, panel C. The reason for the difference in magnitude of this property of the two DRs is not clear at the present time.

The effects of various salts on the enzymatic activity have been noted in the past by a number of investigators (see page 5). In the present work these studies were extended by fluorescence techniques, which made it possible to determine if salts affected the fluorescence quenching properties of either substrate or cofactor. These studies indicate that certain salts, in particular the anions of KSCN and NaClO₄, do affect the quenching properties of both substrate and cofactor, as indicated by large increases in protein fluorescence by the NADPH and DHF complexes of T4DR and to a lesser extent by EDR (Figs. 35 and 36). The most reasonable explanation for these changes would appear to be a decrease in binding between the DRs and their substrate and cofactor. The magnitude of the changes observed decreases in the order SCN⁻, ClO₄⁻, Cl⁻, F⁻. Hatefi and Hanstein have presented evidence that anions such as SCN⁻ and ClO₄⁻ have a
"chaotropic" effect on water structure and on this basis these authors indicate that such anions could be expected to have an effect on hydrophobic bonding (116). Thus, in the present case, agents which are presumed to decrease hydrophobic bonding have apparently decreased the affinity of both DRs for NADPH and DHF. On the basis of the observed phenomena it is not yet possible to determine how these salts have decreased this affinity. While the data do not contradict Baker's postulated hydrophobic bonding region (11, p.204), they do not really support this conclusion, either. Another possible interpretation of the similarity of the quench reversal curves (Fig. 35) of T4DR 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 NaClO4. In view of the fact that the salt effect is known to be reversible, 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 (Fig. 29), which binds to DR competitively with DHF. A comparison of the quench reversal curve observed for DHF with that of NFA, for example, indicates only minor alterations in binding properties for NFA at a time when the affinity of DHF for T4DR is changing rapidly. One
possible explanation of these observations is that the 4-amino compounds are bound to T4DR by some fundamentally different mode than is DHF. Thus a given salt-induced change in enzyme structure could conceivably alter the binding determinants for DHF to a greater extent than the binding determinants for the 4-amino compounds. As mentioned earlier, these ideas are speculative.

The concept that salts can induce conformational changes in DR is not new. Reyes and Huennekens (22) have also studied the effects of SCN⁻, Cl⁻ and also Br⁻ on the activity of the L1210 lymphoma DR. With this enzyme, however, there is an initial increase in activity (as much as 5-fold with NaCl), which reaches a maximum at a certain concentration, depending on the salt. Further increases in salt concentration decrease the activation. To support their suggestion that the salt effects could be explained by alterations in enzyme conformation, these investigators offered the following evidence: 1) There is a large alteration in the relation between enzyme activity and pH. Thus, the pH-activity curve which had shown two maxima is reduced to a single pH maximum. 2) The activated enzyme has a considerably increased $K_m$ for NADPH.

The concept that 4-amino compounds bind DR by different modes than does DHF (or folate) is not a new idea, either. As mentioned in the Introduction, the
greatly-enhanced binding of aminopterin as compared to folate has been accounted for in two different fashions (see pages 21-24). One of these postulates that hydrogen bonding by the 4-amino group of aminopterin, an interaction that folate presumably cannot enter (66), could account for an increase in binding of 10,000-fold or greater (49, 65, 66). It is interesting in this regard that the studies of T4DR and T4DR-MTX complex, treated with GuHCl (a compound which is believed to disrupt hydrogen bonds, 127, 128) indicated that the latter is at least as stable as the native structure of T4DR itself (Fig. 37). This could mean that the postulated hydrogen bonds of the complex are inaccessible to this agent, or it could also be a reflection that hydrogen bonds do not make a large contribution to the stability of the complex.

The second school of thought suggests that the greater basicity of aminopterin and other 4-amino compounds can account for the enhanced binding. Thus, Baker has assumed that the 2-amino group of folic acid is one of the binding sites on the substrate which makes an interaction with the enzyme contributing to the stability of the complex (129). (Folic acid is a substrate of the DR to which he refers.) Since the amino group is basic, Baker assumes that the enzyme group in question is acidic. He further suggests that when the 4-hydroxyl group is replaced
by a 4-amino group, the 2-amino group becomes a much stronger base, and therefore makes a much stronger interaction with the acidic function of the enzyme. Due to the insoluble nature of pteridines, their ionization constants are determined by plotting absorption changes as a function of pH. By this technique Zakrzewski has confirmed the greater basicity of the 4-amino compounds (66). Thus, the ionization constants of a 6-methylpteridine and a 6-formylpteridine were found to be fully 3 pK_a units higher for the 2,4-diamino compounds (pK_a in the 5.0 area) as compared to the 2-amino-4-hydroxyl compounds (pK_a in the 2.0 area). Similar large differences in pK_a for oxidized 4-amino folate compounds versus folic acid can be implied from the data in Figs. 39, 41, 42 and 43. While pK_a data indicate greater basicity for the 4-amino compounds compared to 4-hydroxyl compounds as a whole, it does not indicate the group which is being protonated in either case. To explore this question further, Perault and Pullman determined the theoretical basicity (molecular orbital calculations) of each nitrogen atom of the pteridine moiety for a number of folate compounds (67). These calculations did indeed support the concept of a more basic 2-amino group in aminopterin as compared to folate. However, these calculations show that the most basic nitrogen atom in aminopterin is N_1, indicating that it is this group which is protonated at acid pH.
The marked similarity of the difference spectra of Figs. 39, 41 and 42 between the enzyme-inhibitor complex and the acid-versus-neutral system suggests that the inhibitor is undergoing similar changes in each system. If this is the case, the evidence from difference spectroscopy would suggest that in the case of NFA, aminopterin and MTX, an interaction between N₁ of these compounds and an acidic function on the enzyme is taking place. This concept is illustrated in Fig. 4, panel A. By contrast, difference spectra for the corresponding folate systems (Fig. 43) do not indicate a similar relationship.

The identity of the most basic nitrogen in folic acid is a controversial point. Perault and Pullman's calculations indicate it is N₈ (67). From studies of the model compound 2-amino-4-hydroxy-6-methylpteridine, Whiteley and Huennekens have suggested that either the 2-amino nitrogen or N₁ is the most basic (130). Of the two positions, they favored N₁, since positive charge resulting from protonation of N₁ could be more effectively delocalized than protonation at the 2-amino group. In view of these uncertainties, speculation as to the reason for the lack of correspondence of the difference spectra observed for the oxidized, 4-amino systems and of those observed in the folate system...
(4-hydroxyl) is of limited value. However, if the most basic nitrogen atom in folic acid is $N_1$, the basicity hypothesis would explain the lack of correspondence as a reflection of the failure of the acidic function of the enzyme to make more than a minor interaction with this position in folic acid.

It is interesting that the above-mentioned lack of correspondence also applies to the 4-amino compound at the dihydro oxidation level, as indicated in Fig. 45. (This conclusion is made from the portion of the difference spectrum obtained at wavelengths greater than 300 nm which were relatively stable. However, it can be concluded from the following observations that this lack of correspondence should not be attributed to a weaker binding of dihydroaminopterin as compared to aminopterin. It has been shown by Mathews and Sutherland (71) that dihydroaminopterin is a slightly poorer inhibitor of T6DR than is aminopterin, in terms of inhibitor concentration for 50% inhibition of enzymatic activity (0.004 μM and 0.009 μM for aminopterin and dihydroaminopterin respectively). T4DR shows nearly identical inhibition to T6DR with respect to two different inhibitors (72), suggesting that dihydroaminopterin would make a strong interaction with T4DR also. In support of this view it was observed that the increase in the 305 nm peak of Fig. 45 was nearly
complete when the inhibitor concentration was the same as T4DR. While dihydroaminopterin may bind less tightly to T4DR than aminopterin, the data in Fig. 29 clearly indicate that this is also true for NFA. (Fig. 29 shows the MTX and NFA quenching curve. Aminopterin quenches in an identical fashion to MTX.) Nevertheless, NFA shows a difference spectrum similar to that for aminopterin. Thus, the magnitude of the binding energy does not appear to be the determinant regarding the correlation of difference spectra between the enzyme system and the acid-neutral system. It therefore seems likely that the lack of correlation is due to the change in oxidation state of the 4-amino inhibitor. How this change in oxidation state alters the interaction of the inhibitor with the enzyme is not yet known.

The difference spectra of DHF (Fig. 44) also fail to make the correlation between the enzyme and acid-neutral system. This is not unexpected, however, since the most basic nitrogen in DHF is thought to be N5 (67). Perhaps the most striking feature of the DHF difference spectra is that the two peaks (at 243 nm and near 310 nm) correspond with "pre-existing" features of the absorption spectra of DHF. The absorption spectra of DHF at pH 7.0 is thought to be composed of two noninteracting chromophores, p-amino-benzoyl-glutamate and the pteridine portion (130). The p-amino-benzoyl group shows a single maximum at about
285 nm, which is a minimum in the DHF difference spectra. Thus the observed difference spectra appear to be enhancements of the 243 nm peak and 305 nm shoulder of the DHF absorption spectra, both of which contributions probably derive from the pteridine moiety. The significance of this in relation to enzymatic function, however, is not clear at the present time. It is possible that, in part, the DHF difference spectra reflect enzyme-induced electronic rearrangements of DHF which would "prepare" the substrate for hydride transfer from NADPH to carbon 6 of DHF. However, it would be premature to say that this is certainly the case, since the mechanism of the reduction of DHF and the order of substrate binding to the enzymatically productive complex is not yet known.

In summary, the difference spectra of NFA, MTX and aminopterin support the basicity hypothesis of Baker (127) and its restatement by Collins and Pullman (131). The difference spectra of DHF and folate are, at least, not contradictory with this hypothesis.
### Table IX

**Abbreviations Used**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>( A_{280} )</td>
<td>Absorbance at 280 nm</td>
</tr>
<tr>
<td>( \Delta A_{260} )</td>
<td>Change in absorbance at 260 nm</td>
</tr>
<tr>
<td>Buffer A</td>
<td>0.04 M Tris-HCl, pH 7.0</td>
</tr>
<tr>
<td>Buffer B</td>
<td>0.04 M potassium phosphate, pH 7.0</td>
</tr>
<tr>
<td>DHF</td>
<td>Dihydrofolate</td>
</tr>
<tr>
<td>DR</td>
<td>Dihydrofolate reductase</td>
</tr>
<tr>
<td>EDR</td>
<td>Dihydrofolate reductase of <em>Escherichia coli</em></td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine-HCl</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>NFA</td>
<td>( \text{N}^{10} )-formylaminopterin</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>T4DR</td>
<td>Dihydrofolate reductase of phage T4</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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REFERENCES CITED


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22. Reyes, P., and Huennekens, F. M., Biochemistry, 6, 3519 (1967).


121. Friedberg, F., Chromatographic Reviews, 14, 121 (1971).


130. Whiteley, J. M., and Huennekens, F. M., Biochemistry, 6, 2620 (1967).