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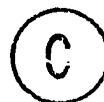
# CHARACTERIZATION OF DIHYDROFOLATE SYNTHETASE FROM *NEUROSPORA CRASSA* WILD TYPE AND ITS RESOLUTION FROM FOLYLPOLYGLUTAMATE SYNTHETASE ACTIVITY.

BY 

DARIN MCDONALD

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

PLANT BIOCHEMISTRY

DEPARTMENT OF BOTANY

EDMONTON, ALBERTA

FALL, 1993

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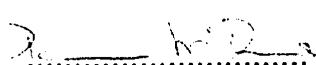

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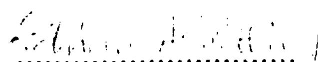

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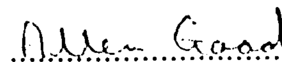

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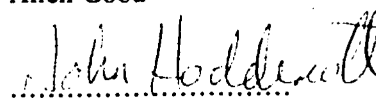

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## ABSTRACT

Dihydrofolate synthetase (DHFS), an enzyme involved in the *de novo* generation of folates, was partially purified over 1400-fold and characterized from mycelial extracts of *Neurospora crassa* wild type (FGSC 853). The organism was cultured in Vogel's liquid medium for 24 hours at 37°C. Mycelia were homogenized in 30 mM potassium phosphate buffer (pH 7.0) containing 50 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine. Following sonication, soluble protein was precipitated with ammonium sulfate. Enzyme activity was assayed by measuring the incorporation of $^3$H-glutamate into dihydrofolate in the presence of dihydropteroate ($H_2$Pte) and ATP. Activity was stabilized for approximately one month at 4°C by adding glycerol (20% v/v). The folate reaction product was characterized as a monoglutamate derivative by Zn-HCl cleavage to p-aminobenzoylglutamate followed by HPLC on Whatman Partisil 10 SAX. Purified *Lactobacillus casei* dihydrofolate reductase (BioPure) was used to confirm that dihydrofolate monoglutamate was the folate product of DHFS activity.

Two protocols were developed for enzyme purification. These included column chromatography on Heparin agarose, Matrex Green A, DEAE-Sephacel, and CM-Sephadex. Under the chromatographic conditions employed, DHFS and folylpolyglutamate synthetase (FPGS) activities were clearly separated by passage through columns of DE-52 cellulose. These proteins were also separated by chromatography on Matrex Green A, Reactive Green 5, and Heparin agarose. Gel filtration on Sephacryl S-200, in the presence of protease inhibitors, indicated apparent Mr values for DHFS and FPGS of 52 and 68 kD respectively. It is concluded that in *Neurospora*, these enzymes of folate biosynthesis are associated with distinct proteins. This is in contrast to their association as a bifunctional complex in *Escherichia coli*.

The major catalytic properties of *Neurospora* DHFS were examined and compared with those of the corresponding activity of *Pisum sativum, E. coli, Corynebacterium, and Serratia indica*. The purified *Neurospora* protein had maximal activity at pH 10.3, required $Mg^{2+}$, $K^+$, ATP (Km = 50 uM), $H_2$Pte (Km = 11 nM), and L-glutamate (Km = 290 uM). Inhibiton of product formation occurred in the presence of 10 to 500 uM dihydrofolate monoglutamate or dihydrofolate triglutamate. In contrast, pteroate, folic acid, [6R,S]-tetrahydrofolate monoglutamate, and [6S]-tetrahydrofolate triglutamate (up to 500 uM) did not affect the rate of dihydrofolate formation under the standard assay conditions.

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2. Pathway for the *de novo* Biosynthesis of Cellular Folate 11

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 10-HCO-$H_4Pte_n$ | 10-formyltetrahydrofolates |
| $^3H$ | tritium |
| $^3H$-Glu | tritiated glutamate |
| 5,10-$CH^+H_4PteGlu_n$ | 5,10-methenyltetrahydrofolates |
| 5,10-$CH_2H_4PteGlu_n$ | 5,10-methylenetetrahydrofolates |
| 5-$CH_3H_4PteGlu_n$ | 5-methyltetrahydrofolates |
| AICAR | 5-aminoimadazole-4-carboximide ribonucleotide |
| ATP | adenosine 5'triphosphate |
| BSA | bovine serum albumin |
| CM- | carboxymethyl- |
| DE-52 | diethylaminoethyl-celluose (Whatman) |
| DEAE- | diethylaminoethyl- |
| DHFR | dihydrofolate reductase |
| DHFS | dihydrofolate synthetase |
| DHPS | dihydropteroate synthetase |
| DMSO | dimethylsulfoxide |
| DPM's | disintegrations per minute |
| dUMP | deoxyuridine monophosphate |
| $FADH_2$ | flavin adenine dinucleotide (reduced form) |
| fmet-tRNA | formylmethionyl transfer-ribonucleic acid |
| FPGS | folylpolyglutamate synthtase |
| FPLC | fast performance liquid chromatography |
| GAR | Glycinamide ribonucleotide |
| GS | glycine synthase (decarboxylase) |
| $H_2Pte$ | dihydropteroate |
| $H_2PteGlu$ | dihydropteroylglutamic acid (dihydrofolate) |
| $H_2PteGlu_3$ | dihydrofolate triglutamate |
| $H_2PteGlu_n$ | dihydrofolates |
| $H_4PteGlu$ | tetrahydropteroylglutamate (tetrahydrofolate) |
| $H_4PteGlu_3$ | tetrahydrofolate triglutamate |
| $H_4PteGlu_n$ | tetrahydrofolates |
| HCl | hydrochloric acid |

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| KCl | potassium chloride |
| met-tRNA | methionyl transfer-ribonucleic acid |
| NaCl | sodium chloride |
| NADH | nicotinamide adenine (reduced form) |
| $NADPH_2$ | nicotinamide adenine phosphate (reduced form) |
| $NaNO_2$ | sodium nitrite |
| nm | nanometers |
| $pABAGlu_n$ | para-aminobenzoate glutamates |
| PMSF | phenylmethylsulfonyl fluoride |
| Pte | pteroate |
| PteGlu | pteroylglutamate (folate) |
| $PteGlu_n$ | folates |
| SAX | strong anion exchange |
| SHMT | serine hydroxymethyltransferase |

# INTRODUCTION

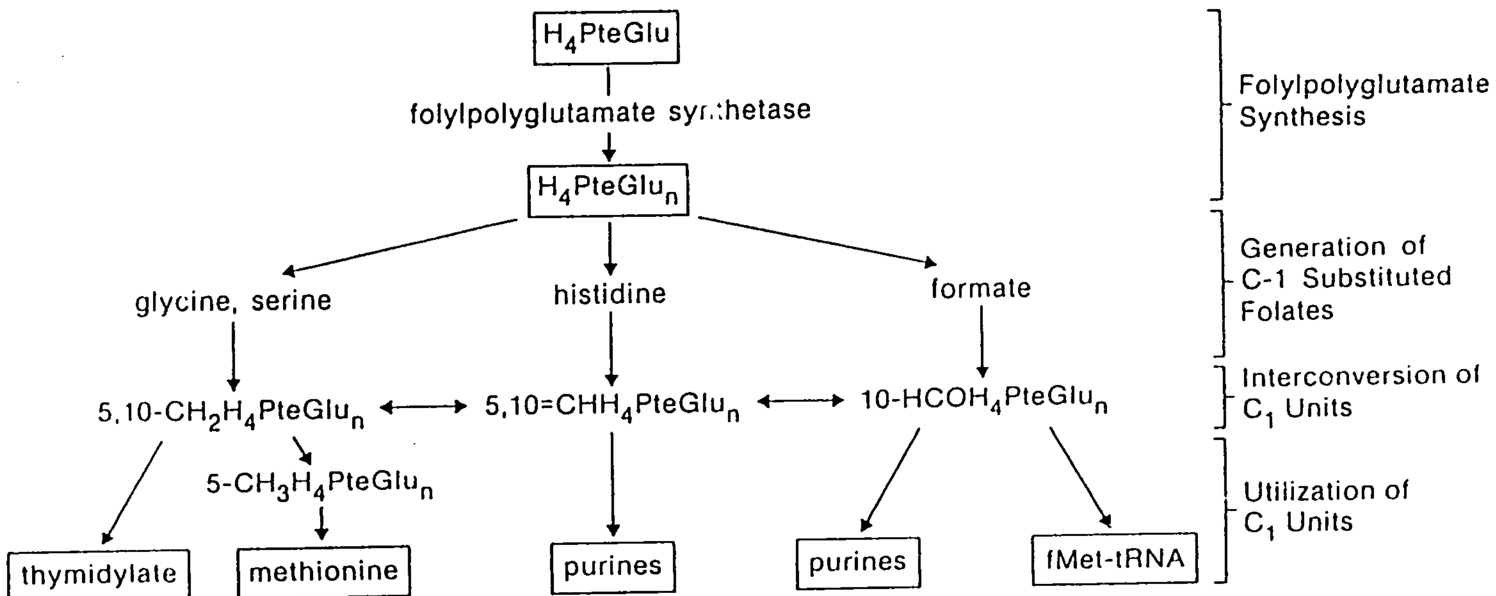
Dihydrofolate synthetase is an enzyme involved in the *de novo* generation of folates (Shiota, 1984). Folates are vitamins because of their requirement in the diet of mammals and are involved in a complex series of reactions referred to as one-carbon metabolism (Mackenzie, 1984). These pathways operate to produce a number of very important cellular components such as purines and pyrimidines for nucleic acid synthesis, and formylmethionyl tRNA for initiation of prokaryotic and organellar protein synthesis (Blakley, 1969). Folates are also instrumental in the synthesis of other constituents shown in Scheme 1. The pathways for the generation and utilization of $C_1$ units and folates as well as the importance of both in cellular metabolism will be discussed in the following sections of this Introduction. This will lead into a discussion of dihydrofolate synthetase as it relates to the research described in this thesis.

The Introduction to this thesis is therefore divided into three parts. First, the importance of one-carbon metabolism will be considered together with details of the principal folate-dependent enzymes that mediate these pathways. Second, the nature of folic acid and the pathway involved in the *de novo* synthesis of the physiologically active forms of this vitamin will be described. Third, a section, focused on the present study, investigates the possible relationship of two enzymes involved in the generation of tetrahydrofolate. These enzymes are dihydrofolate synthetase (DHFS) and folylpolyglutamate synthetase (FPGS). In certain organisms including *E. coli*, these proteins occur as a bifunctional complex encoded by the fol C gene (Bognar et al, 1985). However, the occurrence of this complex in eukaroytes that synthesize folate *de novo* has not been reported in the literature.

## The Biochemistry of One-Carbon Metabolism

This area of metabolism is very important in the generation of intermediates required for the synthesis of basic cellular constituents. The basis of these reactions is the addition of one carbon units to other compounds to yield the molecule of interest. Scheme 1 shows how these $C_1$ units are generated, the interconversion of the folate derivatives, and the incorporation of these $C_1$ units into new compounds.

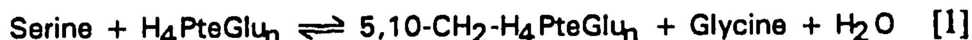
Scheme 1. Pathway Outlining the Reactions of One-Carbon Metabolism.




**Note:** Physiological folates are gamma-glutamyl conjugates (n = 4 to 7) of tetrahydropteroylmono-glutamate ($H_4$PteGlu). The principal one-carbon substituted folates serve as metabolic precursors of methylene ($CH_2$-), methenyl (CH=), formyl (HCO-) and methyl ($CH_3$-) groups. (from Cossins, 1987)

*The Generation of $C_1$ Units*

$C_1$ units can be generated by a number of routes shown in Scheme 1. The donation of $C_1$ units from histidine, and by purine degradation are thought to be insignificant. This is in contrast to the three major pathways of $C_1$ generation that center on serine, glycine and formate. In the first, serine is converted to glycine by the donation of a methylene group to folate to produce 5,10-methylenetetrahydrofolate, equation [1]. The enzyme serine hydroxymethyl-

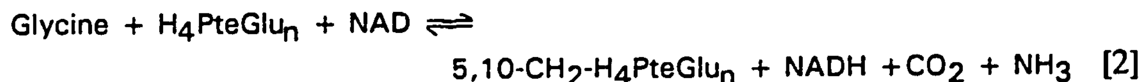


$$\text{Serine} + H_4PteGlu_n \rightleftharpoons 5{,}10\text{-}CH_2\text{-}H_4PteGlu_n + \text{Glycine} + H_2O \quad [1]$$

tranferase (SHMT) is responsible for this reaction and, because it is completely reversible (Schirch, 1984), either serine or glycine can be produced according to the demand.

This enzyme has been isolated in crystalline form from several animal tissues and was found to be composed of four identical subunits (Schirch, 1984). More recently this enzyme has been purified from *Neurospora crassa* (Kruschwitz et al, 1993). Some higher plants have also been studied and were found to contain SHMT isoenzymes (Cossins, 1980). These plants include cauliflower (Mazelis and Liu, 1967), mung bean (Rao and Rao, 1982) and soy bean (Mitchell et al, 1986) in which the enzyme appears to be similar to that of animals. In animals the enzyme is found both in the mitochondria and the cytosol. Both these enzymes exhibit similar substrate specificity (Schirch and Peterson, 1980; Ogawa and Fujioka, 1981; Schirch, 1982), whereas plants, appear only to have a mitochondrial form of the enzyme which may be present as numerous isoenzymes (Woo, 1979).

The second $C_1$ donor, glycine, can be involved in an oxidative cleavage reaction (Kikuchi, 1973), catalyzed by glycine synthase (GS), shown below in equation [2]. This was first described in bacteria by Sagers and Gunsalus



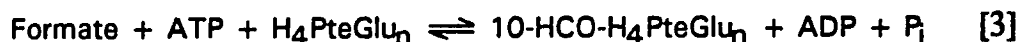
$$\text{Glycine} + H_4PteGlu_n + NAD \rightleftharpoons 5{,}10\text{-}CH_2\text{-}H_4PteGlu_n + NADH + CO_2 + NH_3 \quad [2]$$

(1961). Later work in eukaryotes, showed this to be an exclusively mitochondrial enzyme (Schirch, 1984). Glycine synthase of bacteria and animal mitochondria has been isolated and shown to be composed of P, H, L, and T

subunits (Schirch, 1984) each of which is responsible for a particular part of the reaction. P and H are involved in glycine decarboxylation; L is involved in the formation of NADH; and T in the formation of 5,10-methylenetetrahydrofolate (Schirch, 1984). This complex in pea leaf mitochondria is similar in that it is also composed of four different proteins with similar functions (Kikuchi, 1973; Walker and Oliver, 1986; Bourguignon et al, 1988). The P-protein binds the alpha-amino group and causes the release of $CO_2$; the H protein shuttles the remaining methylamine group to the T protein which carries out the transfer of the methylene carbon atom to tetrahydropteroylpolyglutamate releasing the amino nitrogen; the L protein catalyzes the reoxidation of the H protein lipoamide by sequential reduction of FADH and $NAD^+$ (Oliver et al, 1990).

Both SHMT and GS play very important roles in photorespiratory carbon metabolism (Keys, 1980; Tolbert, 1980). A great deal of work has been done on the enzymes, their regulation and physiological importance (for reviews see Cossins, 1980; Keys, 1980; Schirch, 1982, 1984).

Formate is the third donor of $C_1$, and appears to arise from glycolate and glyoxylate in plants (Cossins, 1980). Glyoxylate is decarboxylated to give rise to formaldehyde, which is then oxidized to formate (Davies and Corbett, 1969). Formate enters one-carbon metabolism via 10-formyltetrahydrofolate (10-HCO-$H_4$PteGlu) and the formyltetrahydrofolate synthetase reaction, equation [3].



$$\text{Formate} + \text{ATP} + H_4\text{PteGlu}_n \rightleftharpoons \text{10-HCO-}H_4\text{PteGlu}_n + \text{ADP} + P_i \quad [3]$$

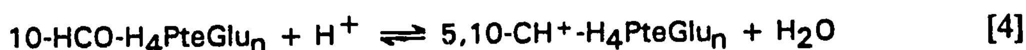
This enzyme is present in a wide variety of plants, and has been shown to resemble the corresponding animal and bacterial enzymes with respect to the activation by $Mg^{2+}$ and univalent cations, and the enhancement of formate binding to the active site by ammonium (Cossins, 1980).

*Interconversion of $C_1$ Substituted Folates*

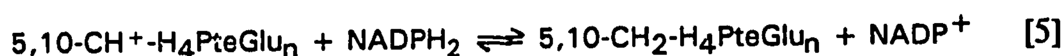
Since the interconversion of folate derivatives is an important key to what cellular components will be produced, a lot of work has been done on the enzymes from a variety of sources. These sources include certain eukaryotes such as mammals, yeast (MacKenzie, 1984; Appling and Rabinowitz, 1985) and spinach (Nour and Rabinowitz, 1988). Many other species have also been studied. For reviews of this topic the reader is referred to Mackenzie (1984) and

Appling (1991).

The reactions for interconversion of substituted folates are freely reversible and therefore allow for easy conversion from one form to another which is needed to form certain end products. The three enzymes catalyzing the reactions between 10-formyltetrahydrofolate and 5,10-methylenetetrahydrofolate are: formyltetrahydrofolate synthetase, equation [3], methylenetetrahydrofolate cyclohydrolase, equation [4], and methenyltetrahydrofolate dehydrogenase, equation [5].



$$10\text{-HCO-}H_4\text{PteGlu}_n + H^+ \rightleftharpoons 5,10\text{-CH}^+\text{-}H_4\text{PteGlu}_n + H_2O \qquad [4]$$

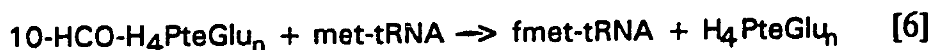


$$5,10\text{-CH}^+\text{-}H_4\text{PteGlu}_n + \text{NADPH}_2 \rightleftharpoons 5,10\text{-CH}_2\text{-}H_4\text{PteGlu}_n + \text{NADP}^+ \qquad [5]$$

Some organisms have all three activities on the same complex (trifunctional) called $C_1$ tetrahydrofolate synthase (Appling and Rabinowitz, 1985). Recent work with pea cotyledons has shown that the synthetase activity is separate from a cyclohydrolase-dehydrogenase bifunctional complex (Kirk et al, 1993). This work as well as previous studies (Ross et al, 1984; MacKenzie, 1984; and Rabinowitz, 1983) have shown that the preferred substrate for these enzymes are polyglutamyl folates. These proteins show an increased affinity for longer-chained polyglutamates and these substrates cause an increased binding affinity for formate and ATP (Strong and Schirch, 1989; Kirk et al, 1993).

*Utilization of $C_1$ Units*

As noted earlier, a number of very important cellular components are formed by the donation of $C_1$ units. One such product is formylmethionyl-tRNA, synthesized by the enzyme methionyl-tRNA transformylase, which transfers the formyl group from 10-formyl tetrahydrofolate to methionyl-tRNA (met-tRNA), equation [6].

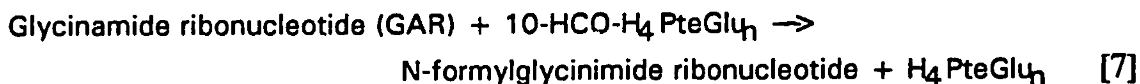


$$10\text{-HCO-}H_4\text{PteGlu}_n + \text{met-tRNA} \rightarrow \text{fmet-tRNA} + H_4\text{PteGlu}_n \qquad [6]$$

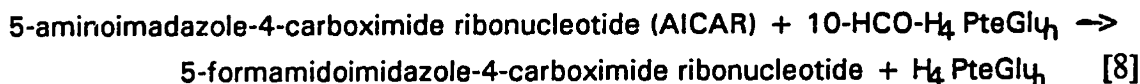
Formylmethionyl-tRNA (fmet-tRNA) is involved in the initiation of bacterial, mitochondrial and chloroplastic protein synthesis namely at the level of translation of mRNA into the amino acid sequence. Studies of yeast

mitochondria and *Euglena* chloroplasts have clearly implicated 10-formyltetrahydrofolate in organellar protein synthesis (Bianchetti et al, 1977; Lucchini and Bianchetti, 1980). Studies using 5-formyltetrahydrofolate, a potent competitive inhibitor of the transformylase, showed that protein synthesis was also inhibited indicating the specificity and requirements of the transformylase reaction (Lucchini and Bianchetti, 1980). Confirmation of the involvement of a $C_1$ folate transfer was obtained in another study using pea and peanut mitochondria to show an absolute requirement for 10-formyltetrahydrofolate (Coffin and Cossins, 1986).

10-formyltetrahydrofolate is also an important $C_1$ donor in the production of purines and histidine. A number of complex reactions are involved in the biological production of purines and two of these reactions involve the donation of $C_1$ units by 10-formyltetrahydrofolate (Rowe, 1984). The first gives rise to the carbon #8 and the second supplies the carbon #2 of the purine ring, equations [7] and [8] respectively.



Glycinamide ribonucleotide (GAR) + 10-HCO-$H_4$PteGlu$_n$ —>
N-formylglycinimide ribonucleotide + $H_4$PteGlu$_n$ [7]



5-aminoimadazole-4-carboximide ribonucleotide (AICAR) + 10-HCO-$H_4$PteGlu$_n$ —>
5-formamidoimidazole-4-carboximide ribonucleotide + $H_4$PteGlu$_n$ [8]

Both of the above reactions are catalyzed by specific transformylases (Rowe, 1984). These are glycinamide ribonucleotide (GAR) transformylase and aminoimidazole carboximide ribonucleotide (AICAR) transformylase (equations 7 & 8, respectively).

Histidine is formed by a series of reactions, the first of which combines ATP and phosphoribosyl pyrophosphate and closely resembles the methodology of the salvage pathway for purine biosynthesis (Shane and Stokstad, 1984). Since ATP is an endproduct of the pathway involving the two transformylases, (equations 7 and 8), histidine is thought to be produced by the donation of $C_1$ units from 10-formyltetrahydrofolate (for a review see Shane and Stokstad, 1984).

Thymidylate is another compound formed by the transfer of $C_1$ units and results from the donation of $C_1$ units from 5,10-methylenetetrahydrofolate to

deoxyuridine monophosphate, equation [9]. Thymidylate synthase catalyzes this reaction which is of importance in DNA synthesis. The

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + dUMP \rightleftharpoons \text{thymidine monophosphate} + H_2PteGlu_n \quad [9]$$

enzyme is present in low amounts, leading to the conclusion that it may be regulatory (Reddy, 1982). A wide range of organisms have now been studied and this protein has now been isolated from bacteria, such as *Lactobacillus casei* (Dunlap et al, 1971; Leary and Kisliuk, 1971) and *E. coli* (Haertle et al, 1979; Maley and Maley, 1981; Slavik and Slavikova, 1980), mammals (Staben and Rabinowitz, 1984) and plant cell suspension cultures (Nielson and Cella, 1988).

Methionine is also a product of $C_1$ metabolism. This amino acid is produced by a combination of two reactions. The first, catalyzed by methylenetetrahydrofolate reductase, producing 5-methyltetrahydrofolate from 5,10-methylenetetrahydrofolate, is physiologically irreversible, equation [10], and represents the only route for *de novo* formation of the methyl group of methionine. C1 units from this step are essentially committed to methionine synthesis, as 5-methyltetrahydrofolate has no other known metabolic fate.

The second reaction yields methionine and tetrahydrofolate as shown in equation [11] and is catalyzed by the enzyme methionine synthase.

$$5,10\text{-}CH_2\text{-}H_4PteGlu_n + FADH_2 \rightarrow 5\text{-}CH_3\text{-}H_4PteGlu_n + FAD \quad [10]$$

$$5\text{-}CH_3\text{-}H_4PteGlu_n + \text{L-Homocysteine} \rightleftharpoons \text{Methionine} + H_4PteGlu_n \quad [11]$$

The methionine synthase of mammals and some bacteria is a cobalamin requiring enzyme (Matthews, 1984). Other organisms including plants produce methionine via a cobalamin-independent methionine synthase (Cossins, 1987). The biosynthetic pathway leading to methionine in higher plants has been investigated. However, the final transmethylation reaction and the pathway for generation of one-carbon units has not been studied in detail. As a result, these areas remain to be studied.

## The Nature of Folates

Folate is the general term used to describe a number of different folate

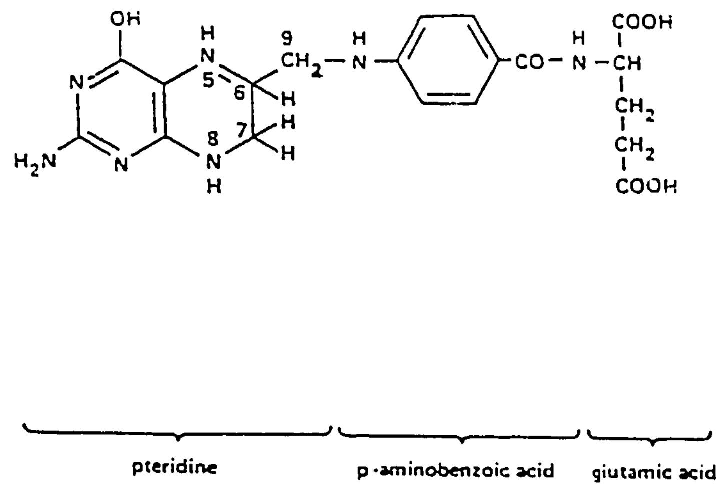
derivatives from 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate polyglutamate including a number of $C_1$ substituted forms. The term folic acid was first used by Mitchell et al (1941) to describe a *Streptococcus faecalis R* nutritional factor, isolated from spinach. Another name for the same compound, pteroylglutamic acid, was used by Angier et al (1946), to describe the structure they proposed for folic acid (Figure 1).

Folic acid was subsequently isolated from a number of different sources and found to have importance in growth and nutrition. One study used folic acid extracted from yeast and liver to treat tropical macrocytic anemia (Wills, 1931) as well as prevent macrocytic anemia in monkeys (Wills and Stewart, 1935; Day et al, 1938). Other studies, using folic acid isolated from yeast, alfalfa and wheat bran found that it stimulated growth of chicks (Hogan and Parrot, 1940; Pfiffner et al, 1943). Essential nutrients for the growth of lactic acid bacteria, later identified as folates, were also present in liver, yeast, and spinach extracts (Stokstad, 1943; Mitchell, 1941, 1944).

It is now clear that folates are ubiquitous to all tissues and organisms. They have been studied in mammalian organs such as liver, intestine, kidney, and spleen; in physiological fluids such as human milk and blood serum; in microrganisms, and plants (Blakley, 1969; Cossins, 1984). Another more exotic source is the gas gland of *Physalia*, more commonly referred to as Portuguese man o' war. This organ produces carbon monoxide when supplied with L-serine *in vitro* and is rich in folate derivatives (Blakley, 1969).

The types and quantity of folate derivatives in these tissues have been studied in a number ways. In the standard microbiological assay of folate three microorganisms which can only grow in the presence of certain forms of folate and are unable to utilize other derivatives are generally employed. *Lactobacillus casei* (ATCC 7469) grows on all forms of folate when oxidation is prevented by ascorbate. This organism is used to measure total folate in biological extracts. *Streptococcus faecalis* (ATCC 8043) does not respond to methylated folates and so can be used to determine the amount of this compound when compared to the growth of *L. casei*. *Pediococcus cerevisiae* (ATCC 8081) responds only to highly reduced but not methylated derivatives (Blakley, 1969). By using standard growth curves constructed for all three organisms the amount and types

**Figure 1.** Structure of Folic Acid.



of folates can be determined. Information on the levels of polyglutamyl folates is generally obtained after enzyme hydrolysis (Cossins, 1984).

Other methods for determining folate amounts and types include: paper chromatography, competitive radioassay techniques, ion exchange chromatography, high performance liquid chromatography (HPLC) and gel filtration (Cossins, 1984).

Although folate is found in, and required by all organisms, not all are able to synthesize this vitamin. In mammals, folate is obtained from the diet and from the internal flora of microorganisms in the gut (Stryer, 1988). Mammals ingest folates that are largely polyglutamates and impermeable to the cellular membrane. The longer chain folates are first hydrolyzed by gamma-glutamyl hydrolase enzyme and then transported into the cell where the glutamate chain is extended by folylpolyglutamate synthetase. The resulting polyglutamates can then be used in the reactions of $C_1$ metabolism.

Bacteria and plants are capable of synthesizing their own folate and do so by the pathway outlined in Scheme 2. The synthesis involves the conversion of guanosine triphosphate to 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine. This compound is then converted to a pyrophosphate intermediate by a reaction involving ATP and $Mg^{2+}$. The pyrophosphate intermediate is then reacted with p-aminobenazoate to yield dihydropteroate. Both of the previous steps are catalyzed by dihydropteroate synthetase (DHPS) as described by Shiota et al (1964).

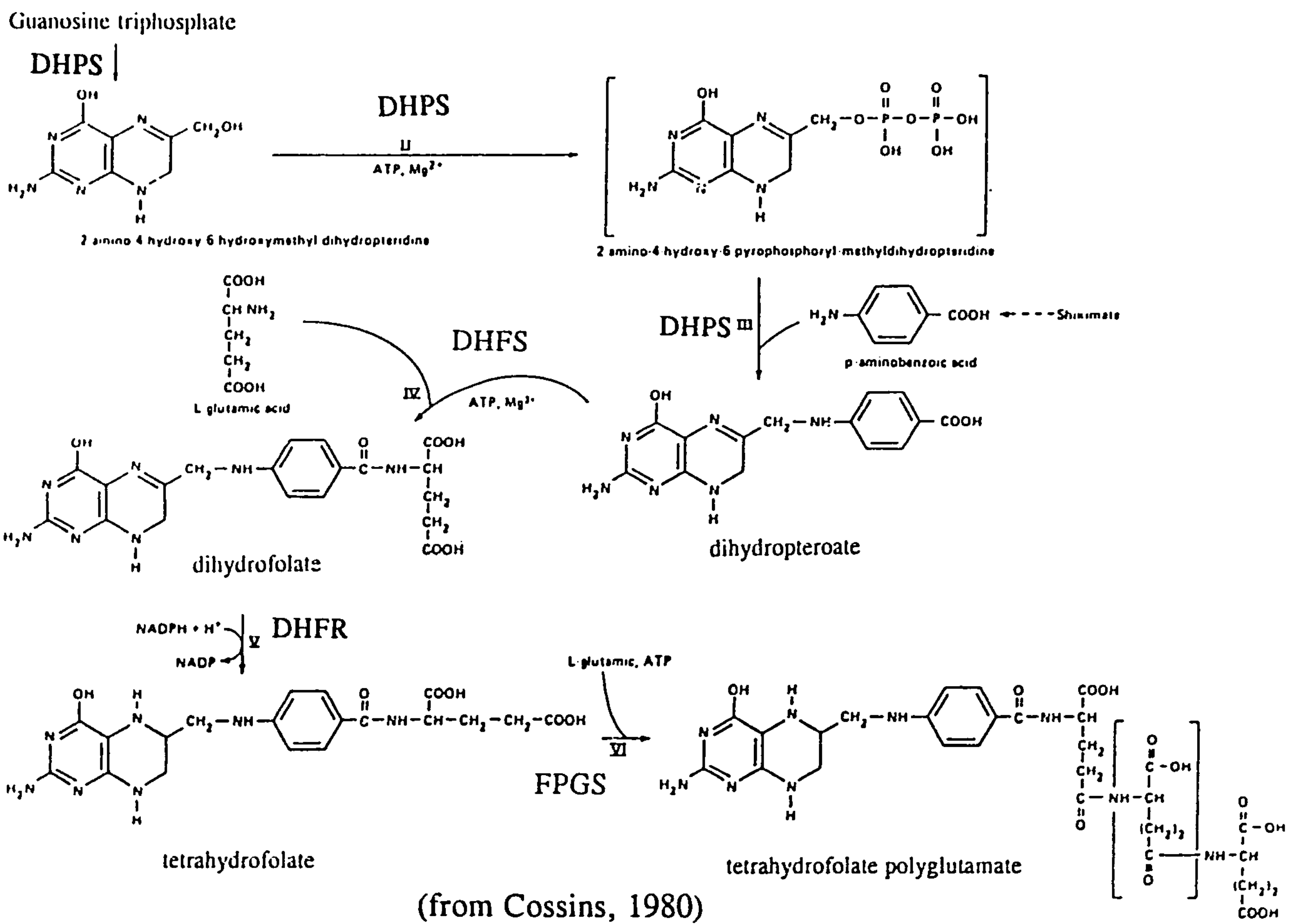
DHFS catalyzes the generation of dihydrofolate, equation [12]. This enzyme is discussed in more detail (see below).

$$H_2Pte + Mg^{2+} + ATP + glu \rightleftharpoons H_2PteGlu + ADP + Pi \quad [12]$$

Dihydrofolate produced by DHFS is converted to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR). This enzyme has been studied in great detail including work on mammals, bacteria (Blakley, 1969; Brown, 1970) and plants (Suzuki and Iwai, 1970; Reddy and Rao, 1976; Ratnam et al, 1987; Hynes, 1991). Inhibition of this reaction by antifolates such as methotrexate and aminopterin has been a subject of considerable study (Blakley, 1984).

Folylpolyglutamate synthetase mediates the polyglutamylation of tetrahydrofolate and methylenetetrahydrofolate to the chain length predominant

**Scheme 2.** Pathway for the *de novo* Biosynthesis of Cellular Folate.




(from Cossins, 1980)

for each organism. FPGS has also been studied from a wide range of sources and has been purified to homogeneity from hog liver (Cichowicz and Shane, 1987), *E. coli* (Ferone and Warskow, 1983; Bognar et al, 1985), and *Corynebacterium* (Shane, 1980).

Evidence for the pathway outlined in Scheme 2 has been provided by studying the rates and folate products formed when cell extracts were supplied with different substrates (Iwai, Okinaki, and Suzuki, 1968; Griffin and Brown, 1964). The enzymatic synthesis of dihydrofolate was also studied in bacteria, plants and fungi (Ikeda, Iwai, and Fujino, 1977). Work was also done using inhibitors to different enzymes of the pathway (Webb and Ferone, 1976; Iwai and Kobashi, 1975).

The distribution of DHFS has been studied for a variety of species including plants (Ikeda and Iwai, 1970), bacteria such as *Corynebacterium* (Shane, 1980), *Serratia indica* (Ikeda and Iwai, 1976), and *E. coli* (Brown et al, 1961; Bognar et al, 1985) and many other prokaryotes and eukaryotes (Griffin and Brown, 1964; Webb and Ferone, 1976; Ferone and Warskow, 1981). Ikeda and Iwai (1975) showed that although DHPS and DHFR were present in mammalian tissues, DHFS was lacking. This inability to generate dihydrofolate accounted for the dietary requirement for folates mentioned earlier.

**The Present Study:**

A great deal of work has been done on the association of DHFS and FPGS in prokaryotes. It is known that both activities are encoded by a single gene (fol C) in *E. coli* (Bognar et al, 1985) and the two activities are clearly associated in *Corynebacterium* sp. (Shane, 1980). The present study was initiated to determine if the two activities in *Neurospora* were on the same protein or whether they were catalyzed by two separate proteins. This study also involved work on the possible association of DHFS and FPGS in pea. Pea DHFS has previously been purified from mitochondria but its possible association with FPGS was not examined (Iwai and Ikeda, 1975). *Neurospora* was chosen to study FPGS and DHFS enzyme relationships for a number of reasons. The most important being that mutants are available that have genetic lesions affecting the FPGS activity (Cossins and Chan, 1984). By studying the DHFS activity in these mutants a relationship between the two activities could be proposed. In this regard the *met 6* and *mac* mutants both contain mutant FPGS

activities but the levels of DHFS activity have not been studied. *Neurospora* DHFS has not been studied other than to confirm the presence of the activity in cell free extracts (Griffin and Brown, 1964) so work was initiated.

Preliminary studies by Cossins and Shane (unpublished) using FPLC analyses showed that DHFS and FPGS activities could be separated, leading to the conclusion that the enzymes were separate proteins. It was also shown by these workers that DHFS was mainly cytosolic, which is in contrast to the pea enzyme which was purified from mitochondria (Ikeda and Iwai, 1975). Cossins and Shane also showed that the *Neurospora* DHFS was extremely labile but could be stabilized by adding glycerol, PMSF and benzamidine. Later work showed that the addition of BSA and DMSO to the assay system also help stabilize the activity.

In the present study anion exchange chromatography was used to isolate both the pea and *Neurospora* DHFS enzymes from the corresponding FPGS activity. Affinity chromatography, and gel filtration were also used to separate the two activities in *Neurospora*. Further work was also done on *Neurospora* to partially purify (1500 fold) and characterize this DHFS protein. The characterization involved: molecular sizing by gel filtration; kinetic studies to determine Michaelis-Menten constants for the substrates of the reaction; and possible inhibition of the reaction by endproducts, as reported for DHFS from other sources (Iwai and Ikeda, 1975; Ikeda and Iwai, 1976; Webb and Ferone, 1976).

# MATERIALS AND METHODS

## Chemicals

Reagent-grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA), Fisher Scientific, Baxter-Canlab, and BDH Chemicals (all located in Edmonton, Alberta). Whatman DE-52 cellulose was purchased from Rose Scientific, Edmonton. Matrex Green A was purchased from Amicon, Oakville, Ontario. CM-Sephadex was purchased from BioRad Laboratories, Mississauga, Ontario. Heparin-agarose, Reactive Green 5, DEAE-Sephacel, pteroate and dihydrofolate reductase were purchased from Sigma. Dihydrofolate reductase, used for the generation of tetrahydrofolate from dihydrofolate, was purchased from BioPure, Boston, MA. Sephacryl S-200 and molecular weight markers were purchased from Pharmacia, Baie Durfe, Quebec. The [U-$^3$H]L-glutamate ($^3$H-glu) was purchased from Amersham Canada Ltd., Oakville, Ontario. Dr. B. Schircks Laboratories, Jona, Switzerland supplied the pteroyltriglutamate and p-aminobenzoylpolyglutamate markers for high performance liquid chromatography (HPLC). The HPLC anion exchange column, Whatman Partisil 10 SAX, was purchased from Terochem. Bray's solution (Bray, 1960) was prepared from scintillation-grade chemicals from Sigma and Fisher Scientific.

## Fungal and Plant Material

*Neurospora crassa* Lindegren A wild type (FGSC 853) was cultured in defined liquid medium using freshly harvested conidiospores as inoculum (Cossins and Chan, 1984). Seeds of pea (*Pisum sativum* L. cv. Homesteader) were obtained from Apache Seeds Ltd., Edmonton. The seeds were imbibed in tap water overnight and those which were damaged or did not imbibe were removed before continuing the extraction.

## Preparation of Dihydropteroate Substrate

Dihydropteorate ($H_2$Pte) was prepared by dithionite reduction of pteroate, following a method used for the reduction of folate to dihydrofolate (Futterman, 1957). Pteroate (10mg) was dissolved in 2 mL of 0.1 N NaOH and added to 5 mL of 10% (w/v) sodium ascorbate (pH 6.0). Next, 222 mg of sodium dithionite were added slowly with stirring which was continued for an additional

30 minutes. After this, the pH of the solution was slowly lowered to pH 3.0 by dropwise addition of 1 N HCl while stirring in an ice bath. The solution was stirred for an additional 5 minutes after which time the precipitate ($H_2Pte$) was removed by centrifugation. After resuspension in 2 mL 0.1 N NaOH and 5 mL 10% ascorbate, the process was repeated. The product was finally recovered by centrifugation and washed by repeated (10 times) resuspension in 10 mL of 1 N HCl containing 100 mM 2-mercaptoethanol and centrifuging. After this washing, the product was resuspended in 10 mL of the same solution and stored as 1 mL aliquots at -20$^{o}$C.

The amount of $H_2Pte$ formed was measured using a molar extinction coefficient of 28,400 at a wavelength of 282 nm. This is the same value as reported for dihydrofolate (Blakley, 1969). The yield of dihydropteroate was approximately 5 micromoles or approximately 15% of the theoretical value.

**Preparation of Substrates for Inhibitor Studies**

Dihydrofolate was prepared as described above for dihydropteroate. Dihydrofolate triglutamate ($H_2PteGlu_3$) was prepared from pteroyltriglutamate ($PteGlu_3$) by dithionite reduction followed by purification using BioGel P2 column chromatography according to the methods of (Stover and Schirch, 1992). Tetrahydrofolate triglutamate ($H_4PteGlu_3$) was prepared from dihydrofolate triglutamate using Biopure DHFR (Stover and Schirch, 1992). To prepare tetrahydrofolate ($H_4PteGlu$), folic acid was catalytically hydrogenated in the presence of platinum oxide (Reid and Friedkin, 1973).

**Measurement of DHFS Activity**

The enzyme was assayed by measuring the incorporation of tritiated glutamate into dihydrofolate ($H_2PteGlu$). The method for assay was essentially that used by Shane (1980) for FPGS, with minor modifications. These modifications included: the use of a different substrate, $^3H$ instead of $^{14}C$ labelled glutamate, no dithiothrietol, a total volume of 1.0 mL, and a pH of 10.3. The major steps of this assay are summarized in Table 1. In order to account for background levels of radioactivity, controls were set up as for the standard assays but lacking a key component (ATP, $H_2Pte$ or enzyme).

Table 1. Major steps in the assay of *Neurospora crassa* DHFS activity.

| Step | Reaction and Isolation Procedures |
|---|---|
| 1. | Incubation of reaction system* at 37$^{o}$ for 2 hours |
| 2. | Termination by addition of 3ml cold 30 mM 2-mercaptoethanol |
| 3. | Reaction mixture applied to columns (3 x 1 cm) of Whatman DE-52 cellulose (with a thin layer of sand on top to prevent gel disturbance), to absorb folate product and washed with 6 x 5 mL aliquots of wash buffer (80 mM NaCl, 10 mM Tris-HCl buffer pH 7.5) to remove unincorporated $^{3}$H-Glu (McGuire et al, 1980). |
| 4. | Elution of labelled dihydrofolate with 6 x 1 ml of 0.1 N HCl collected as one 6mL fraction. |
| 5. | 1 mL aliquot of the HCl wash was assessed for radioactivity by liquid scintillation counting. |

*The standard reaction system (1.0 mL) contained: 5 nanomoles dihydropteroate; 0.1 mg bovine serum albumin (BSA); 30 ul reagent dimethylsulfoxide (DMSO); 30 umoles KCl; 10 umoles $MgCl_2$; 0.3 mmoles Tris buffer (pH 10.3 at 22$^{o}$C); 4.5 umoles ATP; 1.5 umoles L-glutamate containing 2.5 uCi of $^{3}$H; and 200 ul of enzyme.

## Preparation of Cell Free Extracts

After 24 hours growth at 30°C in a water bath, *Neurospora* mycelia were harvested using vacuum filtration through Whatman #1 filter paper. Only cultures yielding clear filtrates were processed further. The mycelia pads were weighed and homogenized (Sunbeam Osterizer 8) in buffer A (30 mM potassium phosphate, 50 mM 2-mercaptoethanol, 20% glycerol, 1 mM PMSF and 1 mM benzamidine, pH 7.0) using a 1:1.5 ratio (grams of tissue:mL of buffer A). The homogenate was passed through a continuous flow sonicator (Branson Sonic, Sonic Cell Disruptor 350) and then centrifuged at 4500 x g for 10 minutes to give Step 1 protein (supernatant).

In experiments with peas, the seeds were imbibed as described earlier and

then the testas and embryos were removed. The cotyledons were thoroughly washed with distilled water, weighed and then homogenized (Sunbeam Osterizer 8) in buffer A. Homogenate preparation and all further steps were performed at 4°C. The homogenate was filtered through four layers of cheesecloth prior to centrifugation at 4500 x g for 10 minutes.

**Purification of DHFS from *Neurospora***

Initial studies showed that DHFS protein was present in low amounts and was rapidly and irreversibly inactivated during ion-exchange and molecular exclusion chromatography. In order to minimize these losses a number of chromatographic media and conditions were examined. This resulted in the development of the two purification protocols shown below.

*Protocol #1:*

The homogenate (Step 1) was treated with streptomycin sulfate (final concentration of 1%) and the solution was stirred at 10 minute intervals for 1 hour in total. The solution was then centrifuged at 6500 x g for 10 minutes to remove precipitated protein and nucleic acids. The supernatant (Step 2) was then fractionated using ammonium sulfate as described by Chan et al (1991). The first precipitate (0-45% of saturation) was removed after 1 hour of stirring by centrifugation at 10,000 x g for 10 minutes and discarded. Further additions of ammonium sulfate were then made to increase the supernatant to 70% saturation. The solution was stirred for 1 hour and then the precipitate was collected by centrifugation at 10,000 x g for 10 minutes. The pellet was resuspended in Buffer A and desalted by passage through 1.6 x 12 cm BioGel P6-DG columns pre-equilibrated with Buffer A to give Step 3 protein. Step 3 protein was immediately applied to a 5 x 5 cm column of DE-52 cellulose. DHFS was not retained under these conditions and was collected in the column wash (Step 4 protein) as 10 mL fractions. Further purification of DHFS protein was achieved by applying Step 4 protein to a 2.5 x 10 cm column of heparin agarose (Type I, Sigma) pre-equilibrated with Buffer A (pH 6.0). DHFS activity was eluted by a linear gradient (0 to 0.5 M KCl in Buffer A at pH 6.0) and collected in 10 mL fractions. After concentrating using an Amicon 200 mL stirred cell under pressure from an argon cylinder, and desalting, according to methods stated earlier, the resulting Step 5 protein was applied to a 5 x 5 cm

column of CM Sephadex pre-equilibrated with Buffer A (pH 5.0). DHFS protein was eluted by a linear gradient (0 to 0.8 M KCl in Buffer A at pH 5.0) and fractions were collected as before. The active fractions were then combined, concentrated by ultrafiltration, and desalted (as before) in 30 mM Tris buffer, 100 mM mercaptoethanol, 20% glycerol, 1 mM PMSF and 1 mM benzamidine (pH 9.0) (Buffer B) to yield Step 6 protein. This protein was applied to a 5 x 5 cm DEAE Sephacel column pre-equilibrated in the same buffer. A linear gradient of KCl (0 to 0.75 M in this buffer) was applied to elute DHFS which was collected in 10 mL fractions. Protein was concentrated by ultrafiltration and desalted as before to yield Step 7 protein which was applied to a 2.0 x 10 cm column of heparin-agarose in Buffer A (pH 7.0) and subsequently eluted with an increasing linear gradient of KCl and ATP (0 mM KCl and 0 mM ATP to 300 mM KCl and 50 mM ATP) in Buffer A to yield Step 8 protein. Three milliliter fractions were collected.

*Protocol #2*:

Steps 1 - 4 of Protocol #1 were followed without modification and the resulting Step 4 protein was applied to a 5 x 5 cm column of DEAE-Sephacel pre-equilibrated in Buffer B. A linear gradient of KCl (0 to 0.5 M in this buffer) was applied to elute DHFS. Protein was concentrated by ultrafiltration, desalted (Step 5 protein) and applied to a 2.5 x 10 cm column of heparin agarose (Sigma) pre-equilibrated with Buffer A (pH 6.0). DHFS was eluted by a gradient (0 to 0.5 M KCl in Buffer A at pH 6.0). After concentrating and desalting, Step 6 protein was applied to a 2.5 x 10 cm column of Matrex Green A in Buffer A (pH 8.0) and subsequently eluted with a linear gradient (0 to 1 M NaCl in Buffer A) to give Step 7 protein.

**Separation of DHFS and FPGS Activities**

*A. Neurospora crassa*

To examine the possible copurification or resolution of distinct DHFS and FPGS proteins a number of different approaches were taken. One approach used DE-52 cellulose and Step 3 protein which contained both activities. The column was developed with an increasing salt gradient from 0 to 100 mM KCl.

Other column media used in such studies included: Matrex Green A, heparin-agarose, and Reactive Green 5. All were contained in 1.5 x 10 cm

columns. Step 3 protein was applied in each case, the column was washed with 100 mL of Buffer A and the proteins were eluted with increasing linear salt gradients (0 to 0.8 M KCl) in the same buffer.

*B. Pisum sativum*

To study pea DHFS and FPGS activities and their possible association, the cell free extract (homogenate) was treated with streptomycin sulfate and fractionated using ammonium sulfate as described for the *Neurospora* protein with one exception; the precipitate collected was a result of 40-65% saturation. This pellet was resuspended in Buffer A, desalted and applied to a 5 x 5 cm DE-52 cellulose column which was developed with a linear gradient of KCl from 0 to 0.8 M.

**Identification of Dihydrofolate by HPLC**

The folate reaction product from DHFS assays was cleaved to p-aminobenzoyl polyglutamates ($pABAGlu_n$) and analyzed by HPLC as described below. The method followed was that of Shane (1986).

*Cleavage Procedure:*

The HCl wash from the assay columns was adjusted to a pH 0.5 by the addition of 5 N HCl and left overnight at 4 $^oC$. The solutions were then treated with a zinc dust suspension (0.05 vol; 1 g Zn in 4mL 0.5% gelatin with stirring). This resulted in quantitative cleavage of $H_4PteGlu_n$ and $H_2PteGlu_n$ to $pABAGlu_n$. The mixtures were shaken intermittently for 10 minutes to convert any remaining $PteGlu_n$ to $pABAGlu_n$. The zinc was removed by centrifugation and supernatant was lyophilized to reduce volume (Shane, 1986). The $pABAGlu_n$ was then purified by conversion to azo dyes of naphthylethylenediamine (Brody et al, 1979).

*Formation and Purification of Azo Dye Derivatives:*

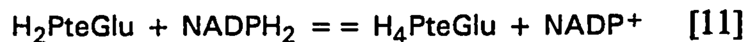
The product was first resuspended, after lyophilization, in 2 mL of 1 N HCl. Next, 0.3 mL of 0.5% $NaNO_2$ was added to generate the diazonium salts. After 2-3 minutes, 0.3 mL of 2.5% ammonium sulfamate was added with mixing to destroy excess $HNO_2$. After 2-3 minutes, 0.5 mL of 0.1% naphthylethylenediamine was added and the solution was left for 30 minutes to ensure complete conversion to the azo dye derivatives. This solution was then

applied to a 4 x 0.7 cm Econo-column of Bio-Gel P2 (200-400 mesh) pre-equilibrated in 0.1 N HCl. The column was washed with 6 x 1 mL of 0.1 N HCl to remove any pterin cleavage products. The azo dye derivatives were eluted with a wash of 1 mL potassium phosphate (pH 7) followed by five 1 mL aliquots of water. The final 1 mL of HCl, in addition to the buffer and water washes were collected and $pABAGlu_n$ were regenerated by adding 0.02 vol 5 N HCl and 0.05 vol of a Zn dust suspension (1 g in 50 mL 0.5% gelatin). This solution was shaken intermittently for 30 minutes to remove all the pink color. The zinc was then removed by centrifugation and the solution lyophilized. The product was resuspended in 2 mL of water and passed through a 0.45um filter to remove particulate material that might block the HPLC column. The solution was then applied to a Whatman Partisil 10 SAX anion exchange column pre-equilibrated with 25 mM ammonium acetate buffer (pH 6.5) and the $pABAGlu_n$ were eluted (flow rate of 1mL/min) with a gradient of ammonium acetate buffer pH 6.5 up to 500 mM. Fractions (1 minute) are collected from the time of sample application up to 60 minutes. The fractions were then assessed for radioactivity by liquid scintillation counting, as described below, to determine the position of the labelled product. The retention times of authentic $pABAGlu_n$ standards were determined in separate HPLC analyses.

**DHFR Analysis of the Assay Product**

Dihydrofolate reductase was also used to characterize the DHFS product. This assay (Stover and Schirch, 1992) was carried out in a cuvette containing 750 uL of 20 mM $K^+$Bes buffer pH 7.5, 100 uL freshly prepared DHFR (0.35 mg/mL, 3.7 units/mg) from Sigma, 50 uL $H_2PteGlu_n$ sample, and 100 uL of 1 mM $NADPH_2$ (freshly prepared solution). The reaction was started by the addition of the $NADPH_2$ from which time the change in absorbance at 340 nm was recorded for a total period of 5 minutes.

This enzyme causes a change in absorbance at 340 nm due to the conversion of $NADPH_2$ to NADP+ only if all components in the reaction outlined below in equation [11] are present. Separate analyses showed that this



$$H_2PteGlu + NADPH_2 == H_4PteGlu + NADP^+ \quad [11]$$

change was dependent on the presence of all substrates including dihydrofolate.

On this basis, the product of DHFS activity should cause a decrease in absorbance under these assay conditions.

### Measurement of Radioactivity

Radioactive samples containing $^3H$ were measured by liquid scintillation counting. A Beckman LS 6000TA Liquid scintillation system was routinely used to measure the number of DPM's (disintegrations per minute) in 1 mL aliquots of the 6 mL of acid wash from the DE-52 cellulose assay columns. The instrument was also used to measure $^3H$ recovered in fractions during HPLC.

### Protein Determination

The amount of protein was measured colormetrically at 595 nm as described by Bradford (1976). Bovine serum albumin was used to construct standard curves for macro- and microassays in this colormetric procedure.

# RESULTS

## DHFS Assay

In earlier studies DHFS activity was measured in terms of folic acid equivalents formed per unit time using *L. casei* and microbiological assay (Hebert, 1961; Griffin and Brown, 1964). In the present study, DHFS was assayed by a method similar to that of Shane (1980). The assay involved the incorporation of $^{3}$H-glutamate into dihydropteroate to form radio-labelled dihydrofolate (equation 12), which was quantified using liquid scintillation counting. The separation of unincorporated $^{3}$H-glutamate from radio-labelled dihydrofolate was achieved by passage of the reaction mixture through a 1 x 3 cm DE-52 cellulose column as outlined in the Materials and Methods. Figure 2 shows a typical elution profile from the DE-52 cellulose assay column.

As *Neurospora* DHFS had not previously been studied it was necessary to determine the optimal conditions and time for this enzyme assay. Table 2 summarizes the absolute requirements for the reaction as well as the effect of varying the concentrations of reaction components on product formation. The absolute requirements included enzyme, ATP, dihydropteroate, KCl, and $MgCl_2$. The optimum concentrations for BSA (0.1 mg/assay), DMSO (30 uL/assay), KCl (30 mM) and $MgCl_2$ (10 mM) were determined using Step 7 protein from protocol #1 under standard assay conditions (see Materials and Methods). The reaction rate of DHFS was also examined for linearity over a 2 hour assay period. The results are shown in Figure 3.

## Identification of DHFS Assay Product

To determine that the product collected from the assay columns was actually dihydrofolate monoglutamate, it was subjected to cleavage, an azo-dye treatment and purification (see Materials and Methods). HPLC analysis was then used to determine the glutamate chain length. The retention times for six $pABAGlu_n$ standards and the DHFS assay product are listed in Table 3. It was clear from these experiments that the assay product was a monoglutamate derivative as expected. A DHFR assay was also conducted (see Materials and Methods). It also confirmed that dihydrofolate was the product as a decrease in absorbance at 340 nm was dependent upon the addition of the DHFS assay product to the reaction mixture (data not shown).

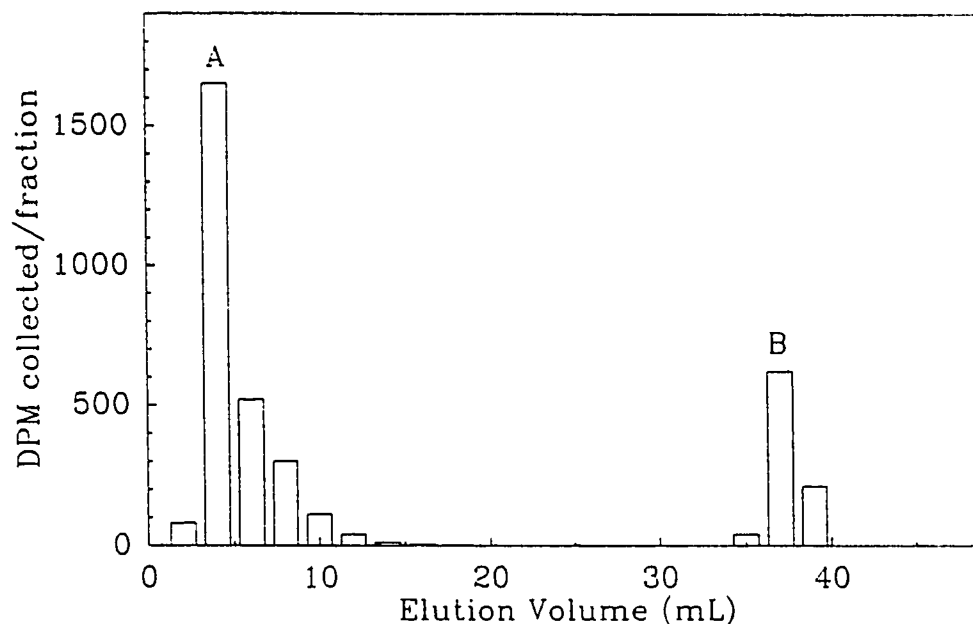



**Figure 2**

**CHROMATOGRAPHIC SEPARATION OF $^3$H-GLUTAMATE FROM RADIO-LABELLED DIHYDROFOLATE.**

Standard reaction mixtures (see Materials and Methods) were applied to a 1 x 3 cm DE-52 cellulose column pre-equilibrated with 30 mM Tris-HCl buffer pH 7.5 containing 10 mM NaCl. A, elution of unincorporated $^3$H-glutamate (dpm x $10^{-4}$). B, elution of $^3$H dihydrofolate (dpm x 10). Fraction 35 was the point at which the acid was added to the column.

**Table 2.** ***Optimal conditions for the assay of*** *Neurospora* ***DHFS activity.***

| Deletions or Additions to Standard Reaction System | nmoles Glutamate incorporated into $H_2$PteGlu |
|---|---|
| Complete* | 4.9 |
| -enzyme | 0 |
| -ATP | 0 |
| -$H_2$Pte | 0 |
| -KCl | 0 |
| +20 mM KCl | 3.4 |
| +30 mM KCl | 4.9 |
| +40 mM KCl | 3.6 |
| -$MgCl_2$ | 0 |
| +5 mM $MgCl_2$ | 3.6 |
| +7.5 mM $MgCl_2$ | 4.0 |
| +10 mM $MgCl_2$ | 4.9 |
| +12 mM $MgCl_2$ | 4.8 |
| +20 mM $MgCl_2$ | 4.5 |
| -BSA | 4.1 |
| +50 ug/assay | 4.6 |
| +100 ug/assay | 4.9 |
| +150 ug/assay | 4.4 |
| -DMSO | 4.3 |
| +10 uL reagent DMSO | 4.4 |
| +30 uL reagent DMSO | 4.9 |
| +50 uL reagent DMSO | 4.3 |

*Standard reaction conditions were employed (see Materials and Methods) with the exception that certain components were omitted or their concentrations altered as indicated. Step 7 protein from Protocol #1 was used as a source of DHFS activity. This protein lacked FPGS activity.

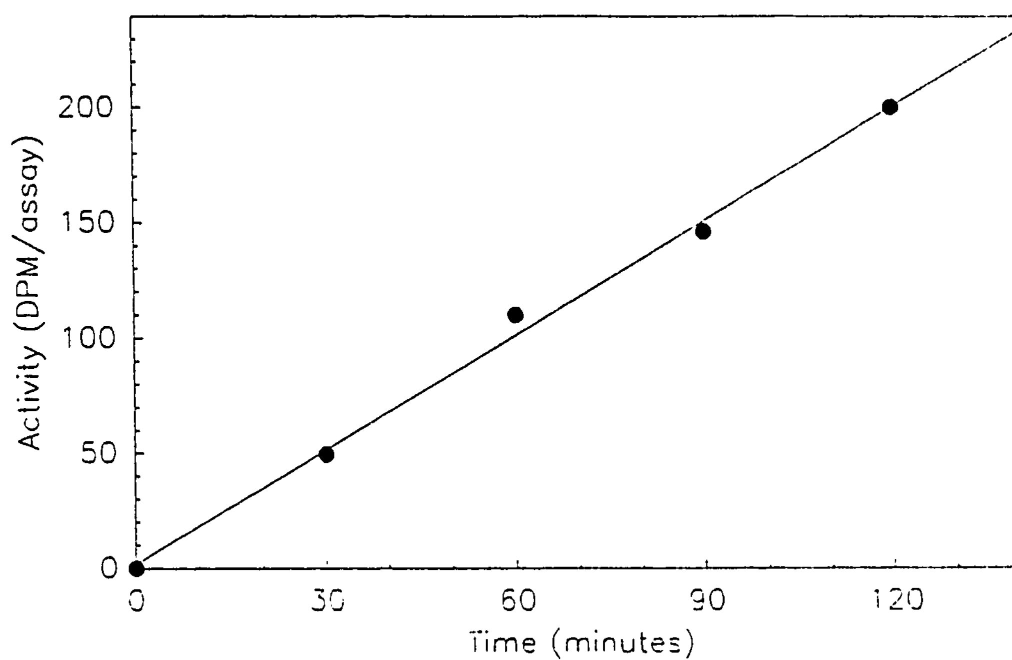



**Figure 3**

**DIHYDROFOLATE FORMATION AS A FUNCTION OF TIME.**

Step 4 protein was assayed for time periods indicated using standard assay conditions (see Materials and Methods). The above values were the average of three determinations.

**Table 3.** ***Retention times of pABAGlu$_n$ standards during HPLC analysis.***

| Sample or Standard added to HPLC column | Retention Time* (minutes) |
|---|---|
| Standard: | |
| pABA-Glu$_1$ | 10 |
| pABA-Glu$_2$ | 25 |
| pABA-Glu$_3$ | 32 |
| pABA-Glu$_4$ | 38 |
| pABA-Glu$_5$ | 42 |
| pABA-Glu$_6$ | 47 |
| DHFS Assay Product** | 10 |

*Elution times were determined as the amount of time from sample injection to collection of the peak fraction. These times are the averages of three sample runs.

**The DHFS assay product was converted to a pABAGlu$_n$ derivative by acid cleavage prior to HPLC. This time was the result from three separate runs.

**Separation of DHFS and FPGS Activities**

*A. Neurospora crassa*

A number of methods were used to separate DHFS and FPGS activities but the one that was used most often and was included in both purification protocols was DE-52 cellulose column chromatography. This anion exchanger retained the FPGS activity but DHFS washed through in the void volume as shown in Figure 4.

To ensure that the separation was due to the isolation of separate proteins and not simply the resolution of a bifunctional complex, Step 1 and 3 proteins, containing protease inhibitors, were applied on separate occasions to Sephacryl S-200. The results were the same and are presented in Figure 5. FPGS activity eluted with a Ve corresponding to a molecular weight of approximately 68 kD whereas DHFS activity eluted later with a Ve corresponding to a molecular weight of about 52 kD.

The two activities were also separated by a number of other chromatographic media including Matrex Green A, Heparin agarose, and Reactive Green 5 (Figures 6, 7 and 8 respectively). FPGS protein was retained by these three media and subsequently eluted when the gradient reached 0.6 M, 0.20 M and 0.6 M KCl in Buffer A respectively. DHFS protein in Buffer A was retained by Matrex Green A and Heparin agarose columns but was eluted when the gradient reached 0.2 M and 0.10 M KCl in Buffer A respectively. DHFS protein in Buffer A was not retained by the Reactive Green 5 column.

*B. Pisum sativum*

Preliminary assays were carried out with 5 day seedlings (cotyledons removed). Under the standard assay conditions employed, DHFS activity was not detected in these extracts. However, partially purified extracts of 1 day cotyledons (see Materials and Methods) which were incubated under standard assays conditions for 24 hours synthesized low levels (about 2000 DPM of glutamate incorporated) of dihydrofolate. Using such extracts, a separation of pea DHFS and FPGS activities was achieved on DE-52 cellulose as shown in Figure 9.

**Partial Purification of DHFS Activity**

In attempts to purify DHFS activity, two separate protocols were developed as summarized in Tables 4 and 5 respectively. The first four

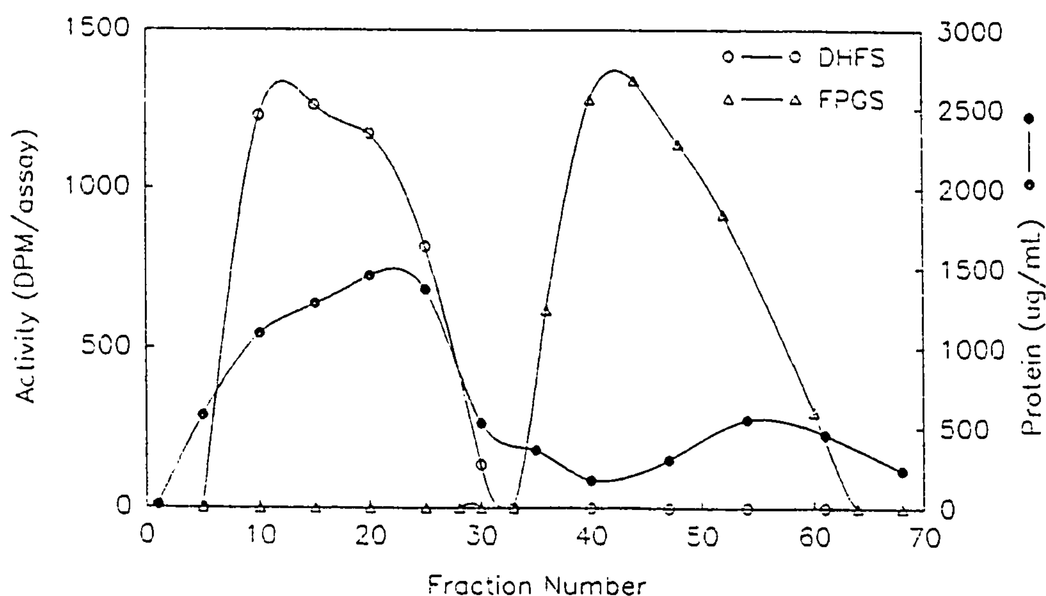



**Figure 4**

**DE-52 CELLULOSE CHROMATOGRAPHY TO SEPARATE *NEUROSPORA* DHFS AND FPGS ACTIVITIES.**

Step 3 protein (up to 300 mL) was applied to a DE-52 cellulose column (5 x 5 cm) pre-equilibrated with Buffer A. The column was washed with 200 mL of Buffer A and developed with a linear gradient (400 mL) of KCl (0 to 0.1 M) in Buffer A. The gradient was applied starting at tube 30. Fractions were assayed for protein as well as FPGS and DHFS activities.

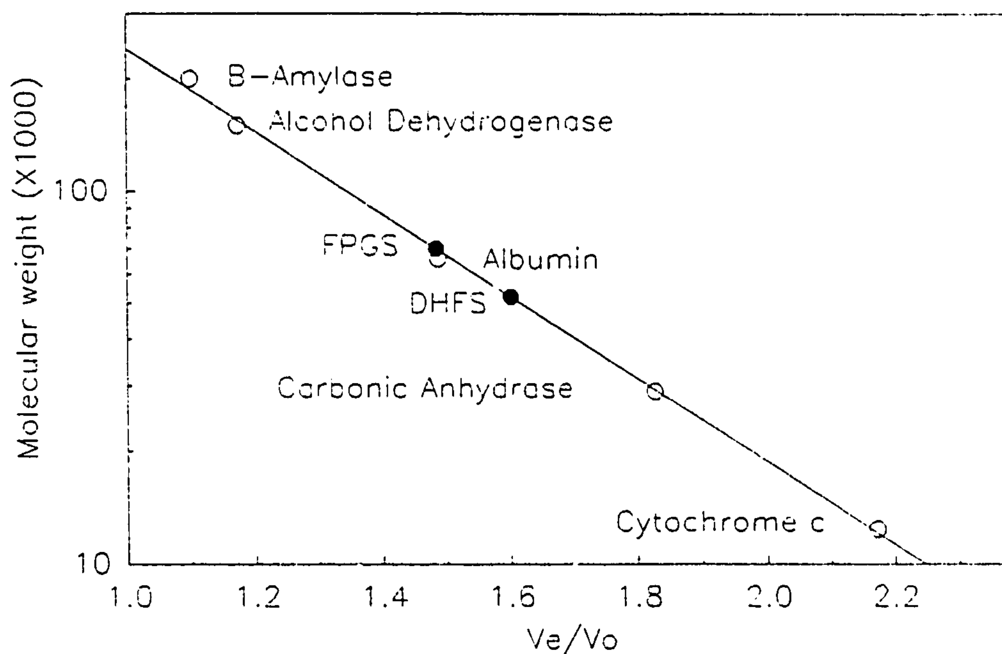



**Figure 5**

**DETERMINATION OF THE MOLECULAR WEIGHT OF NEUROSPORA DIHYDROFOLATE SYNTHETASE AND FOLYPOLYGLUTAMTE SYNTHETASE.**

Molecular Weight Standards were applied to a 2.6 x 80 cm column of Sephacryl S-200. The volume of buffer required to elute each protein (Ve) was determined by reading the absorbance at 280 nm for each fraction, or, in the case of DHFS and FPGS, by performing standard assays. The void volume (Vo) was determined by measuring the elution volume of Blue Dextran.

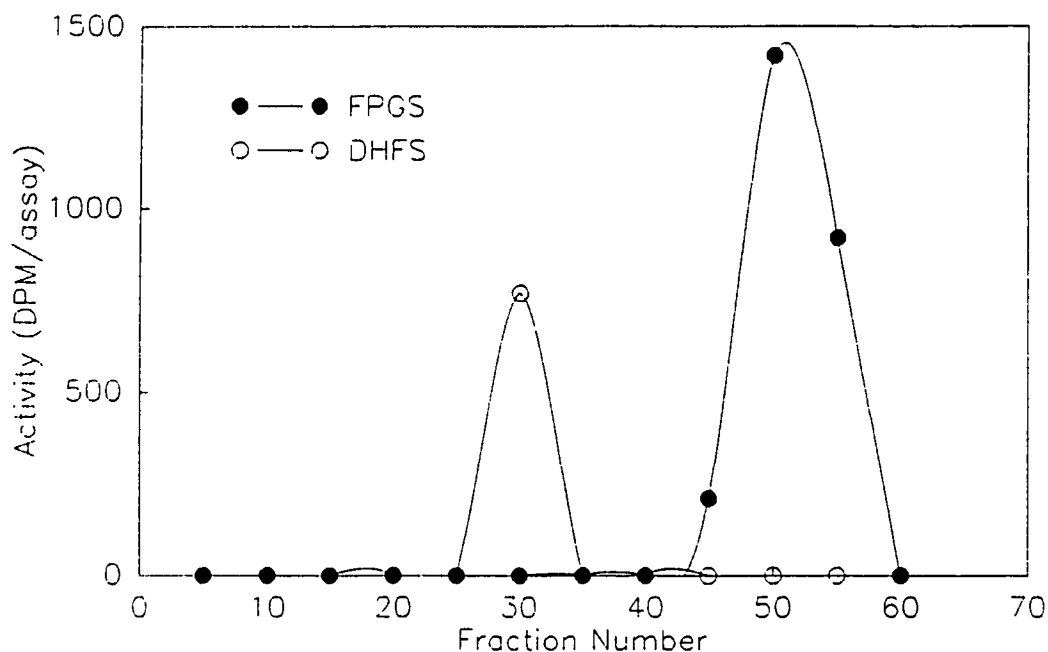



**Figure 6**

**MATREX GREEN A CHROMATOGRAPHY TO SEPARATE DHFS AND FPGS ACTIVITIES.**

Step 3 protein was applied to a Matrex Green A column (2.5 x 10 cm) pre-equilibrated with Buffer A (pH 7.0). The column was washed with 100 mL of Buffer A and developed with a linear gradient (200 mL) of a KCl (0 to 0.8 M) containing Buffer A. The gradient was applied starting at fraction 20. Fractions were assayed for DHFS and FPGS activity.

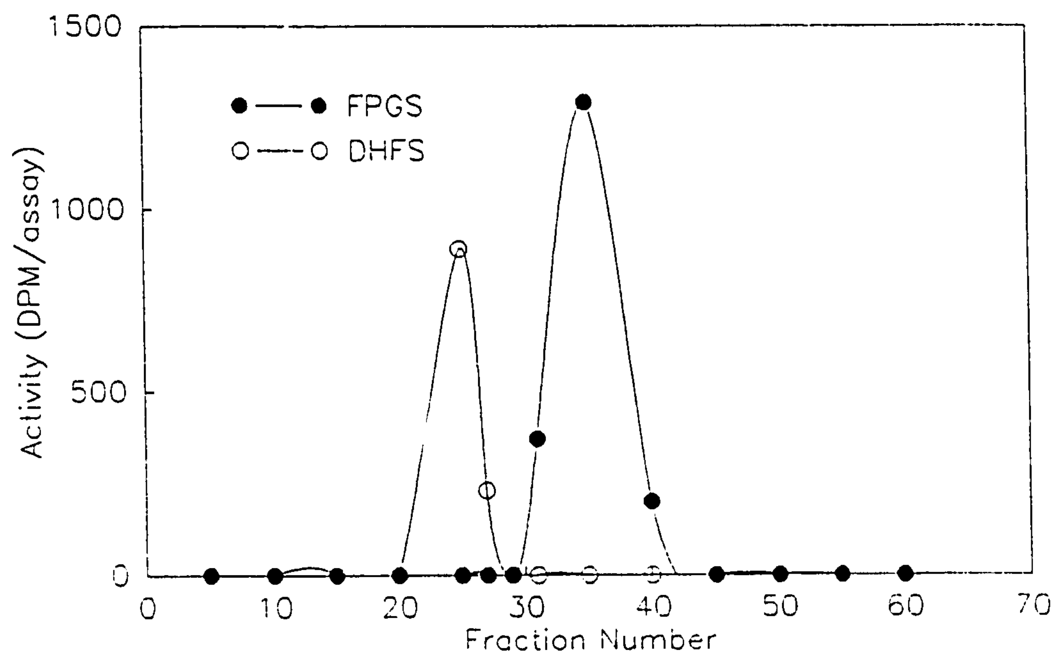



**Figure 7**

**HEPARIN AGAROSE CHROMATOGRAPHY TO SEPARATE DHFS AND FPGS ACTIVITIES.**

Step 3 protein was applied to a Heparin agarose column (2.5 x 10 cm) pre-equilibrated with Buffer A (pH 7.0). The column was washed with 100 mL of Buffer A and developed with a linear gradient (200 mL) of a KCl (0 to 0.8 M) containing Buffer A. The gradient was applied starting at fraction 15. Fractions were assayed for DHFS and FPGS activity.

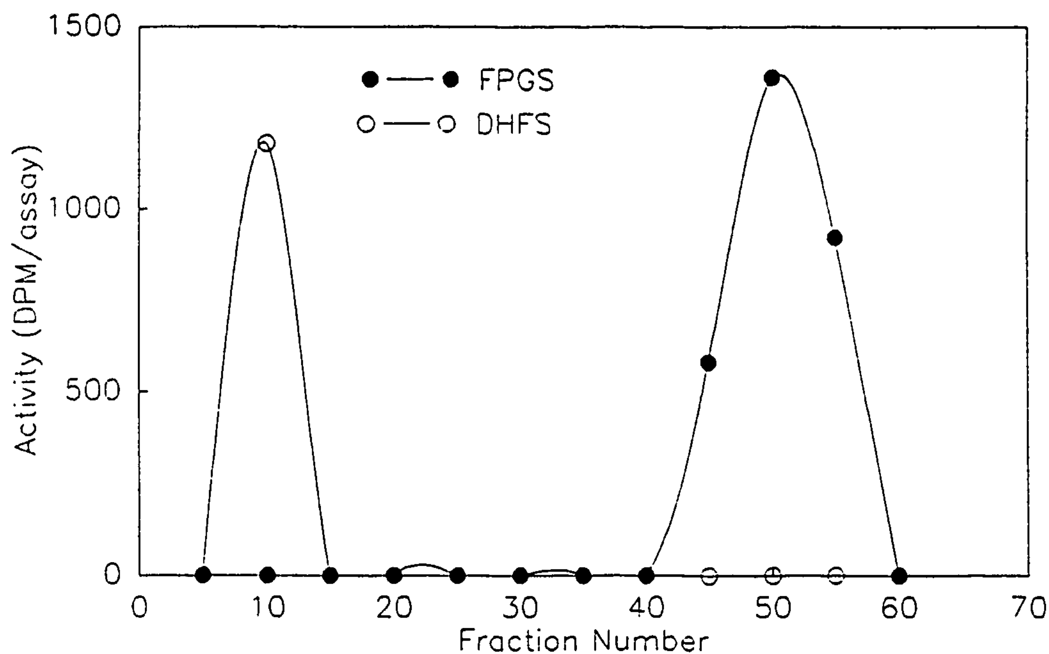



**Figure 8**

**REACTIVE GREEN 5 CHROMATOGRAPHY TO SEPARATE DHFS AND FPGS ACTIVITIES.**

Step 3 protein was applied to a Reactive Green 5 column (2.5 x 10 cm) pre-equilibrated with Buffer A (pH 7.0). The column was washed with 100 mL of Buffer A and developed with a linear gradient (200 mL) of a KCl (0 to 0.8 M) containing Buffer A. The gradient was applied starting at fraction 25. Fractions were assayed for DHFS and FPGS activity.

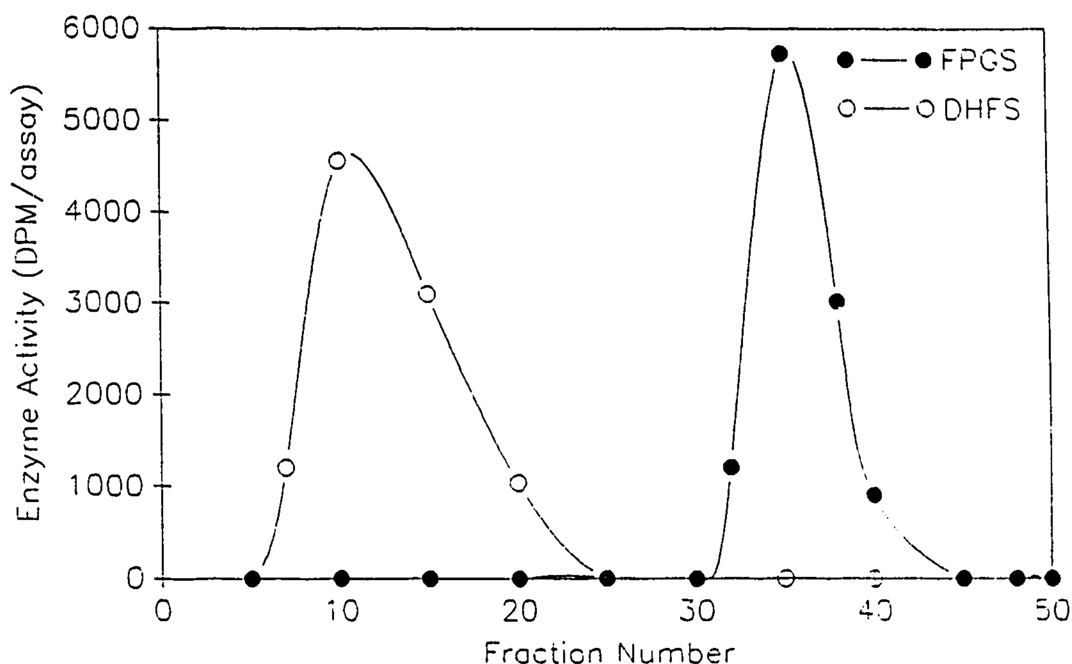



**Figure 9**

**SEPARATION OF PEA DHFS AND FPGS ACTIVITIES USING DE-52 CELLULOSE CHROMATOGRAPHY.**

The ammonium sulfate pellet resulting from 40-65% saturation was desalted and applied to a DE-52 cellulose column (5 x 5 cm) pre-equilibrated with Buffer A. The column was washed with 200 mL of Buffer A and developed with a linear gradient (400 mL) of KCl (0 to 0.8 M) in Buffer A. The gradient was applied starting at fraction 25. Fractions were assayed for FPGS and DHFS activities.

Table 4.

***Protocol #1*: Partial Purification of Neurospora DHFS.**

| Fractionation step | Volume (ml) | DHFS (units/ml) | Protein (ug/ml) | Spec. Act. (units/mg) | Purification (x-fold) | Yield (%) |
|---|---|---|---|---|---|---|
| 1. Crude extract | 800 | 5.49 | 1800 | 3.1 | 1.0 | 100 |
| 2. Streptomycin $SO_4$ | 825 | 4.81 | 1400 | 3.4 | 1.1 | 90 |
| 3. 45-70% $(NH_4)_2SO_4$ | 300 | 6.35 | 2800 | 2 | 0.7 | 59 |
| 4. DE-52 Cellulose | 375 | 7.02 | 500 | 14 | 4.7 | 82 |
| 5. Heparin Agarose | 105 | 14.23 | 44 | 323 | 126 | 34 |
| 6. CM Sephadex | 220 | 3.65 | 10.5 | 630 | 210 | 18 |
| 7. DEAE Sephacel | 50 | 9.26 | 10.4 | 890 | 296 | 10.5 |
| 8. Heparin Agarose (ATP) | 19 | 5.94 | 1.3 | 4569 | 1473 | 2.6 |

DHFS activity is expressed in nanomoles glutamate incorporated/hr.

**Table 5.**

***<u>Protocol #2</u>: Partial Purification of Neurospora DHFS.***

| Fractionation step | Volume (ml) | DHFS (units/ml) | Protein (ug/ml) | Spec. Act. (units/mg) | Purification (x-fold) | Yield (%) |
|---|---|---|---|---|---|---|
| 1. Crude extract | 800 | 6.43 | 3060 | 2.1 | 1.0 | 100 |
| 2. Streptomycin $SO_4$ | 820 | 5.83 | 2600 | 2.2 | 1.1 | 93 |
| 3. 45-70% $(NH_4)_2SO_4$ | 410 | 7.80 | 4300 | 1.8 | 0.9 | 62 |
| 4. DE-52 Cellulose | 450 | 10.14 | 840 | 12.1 | 5.8 | 89 |
| 5. DEAE-Sephacel | 100 | 19.5 | 530 | 36.8 | 18 | 38 |
| 6. Heparin Agarose | 60 | 21.43 | 120 | 179 | 85 | 25 |
| 7. Matrex Green A | 15 | 40.66 | 18 | 2259 | 1075 | 12 |

DHFS activity is expressed in nanomoles glutamate incorporated/hr.

purification steps were common to both protocols. The first step involved sonication of the cell suspension followed by centrifugation to remove cellular debris (see Materials & Methods). The supernatant was adjusted to 1% streptomycin sulfate to remove nucleic acids, and other polyanionic proteins, and to narrow the ammonium sulfate range used to recover the enzyme. Without this step the amount of contaminating protein was found to be higher and the range of ammonium sulfate concentrations required to precipitate the enzyme varied between extractions.

The third step involved the precipitation of DHFS by slow addition of ammonium sulfate (see Materials and Methods). The precipitate was resuspended and desalted before being applied to DE-52 (Figure 4). The combined DHFS-containing fractions showed an increase from 59 to 82 in % yield as well as an increase from 2 to 14 in specific activity which may be a result of the removal of a specific DHFS inhibitor or ATPases and other proteins that may compete with DHFS for substrates.

The combined DE-52 fractions were then applied to a column of Heparin Agarose (Fig. 10). DHFS was eluted with a increasing linear KCl gradient which resulted in an increase in the specific enzyme activity to 323 units/mg of protein. The active fractions were pooled and concentrated, using an Amicon 200 mL stirred cell fitted with a membrane having a 10 kD exclusion limit. After desalting, the protein was applied to a CM-Sephadex column. This column retained DHFS protein which was eluted with an increasing linear salt gradient (Fig. 11). The removal of more protein resulted in a further increase in specific activity to 630 units/mg of protein. The active fractions were concentrated and desalted as described earlier, before being applied to a DEAE-Sephacel column (Fig. 12). DHFS was eluted with a salt gradient and resulted in an increase in specific enzyme activity to 890 units/mg of protein. A second Heparin Agarose column was used as the final step, to which the concentrated and desalted protein off the previous column was applied, in this case the protein was eluted with an increasing ATP/salt gradient (Fig. 13). This column resulted in the greatest increase in specific activity and purification but the final yield of DHFS activity was only 2.6% of that present initially.

In Protocol 2, fractions containing DHFS activity were pooled and applied to DEAE-Sephacel instead of Heparin agarose. In this step and each successive step, the enzyme was eluted using a salt gradient then concentrated, desalted and

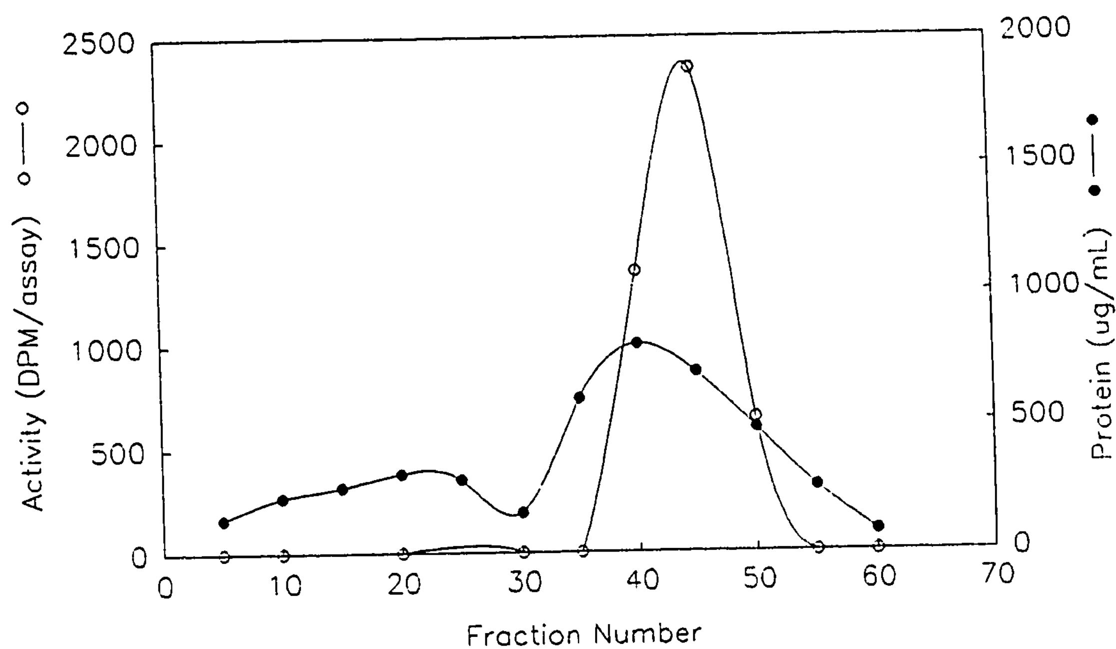



**Figure 10**

**HEPARIN AGAROSE CHROMATOGRAPHY OF DHFS PROTEIN.**

Step 4 protein was applied to a Heparin agarose column (2.5 x 10 cm) pre-equilibrated with Buffer A (pH 6.0). The column was washed with 100 mL of the above buffer and developed with a linear gradient (200 mL) of KCl (0 to 0.5 M) in Buffer A, pH 6.0. The gradient was applied starting at fraction 20. Fractions were assayed for protein and DHFS activity.

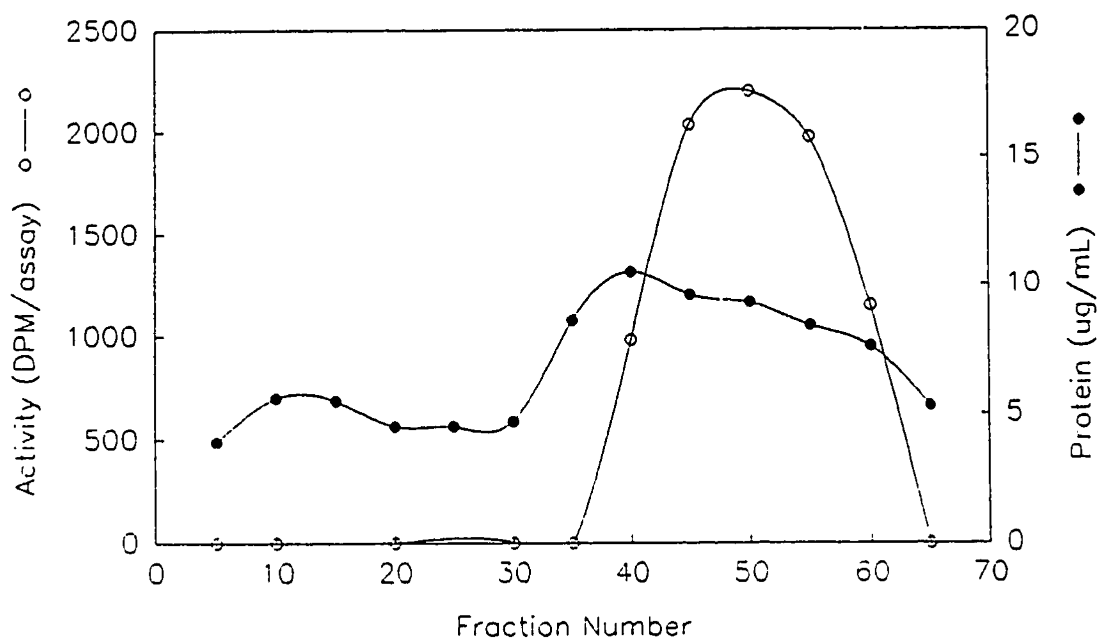



**Figure 11**

**CHROMATOGRAPHY OF DHFS PROTEIN ON A CATION EXCHANGE COLUMN (CM-SEPHADEX).**

Step 5 protein was applied to a CM-Sephadex column (5 x 5 cm) pre-equilibrated with Buffer A (pH 5.0). The column was washed with 200 mL of the above buffer and developed with a linear gradient (400 mL) of KCl (0 to 0.8 M) in Buffer A, pH 5.0. The gradient was applied starting at fraction 20. Fractions were assayed for protein and DHFS activity.

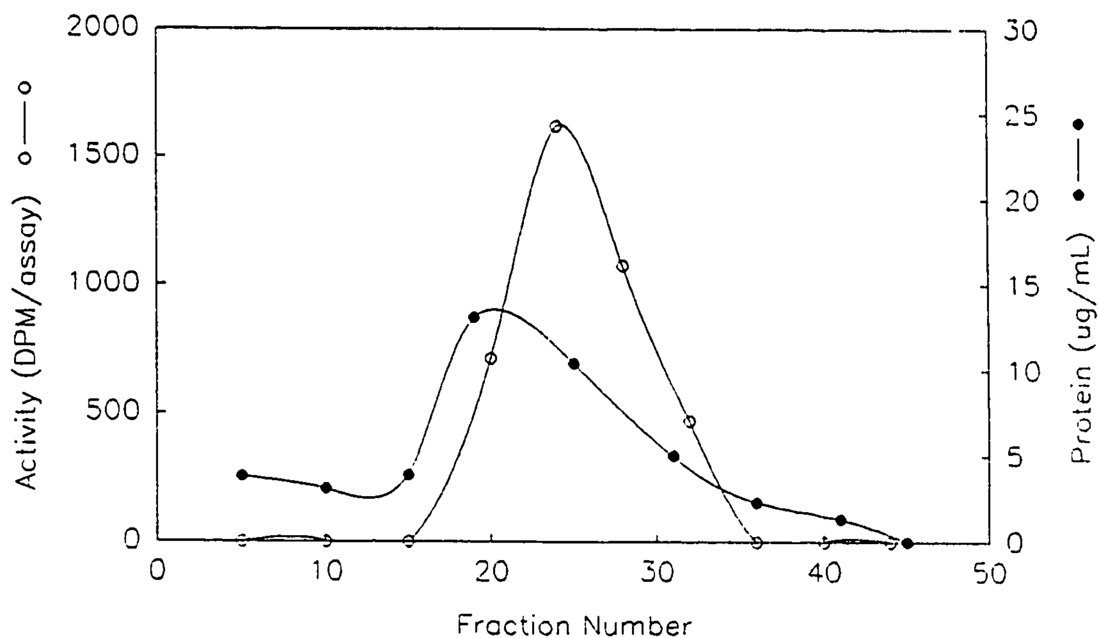



**Figure 12**

**ANION EXCHANGE CHROMATOGRAPHY OF DHFS PROTEIN ON DEAE-SEPHACEL.**

Step 6 protein was applied to a DEAE-Sephacel column (5 x 5 cm) pre-equilibrated with Buffer B (pH 9.0). The column was washed with 200 mL of Buffer B and developed with a linear gradient (400 mL) of KCl (0 to 0.75 M) in Buffer B. The gradient was applied starting at fraction 10. Fractions were assayed for protein and DHFS activity.

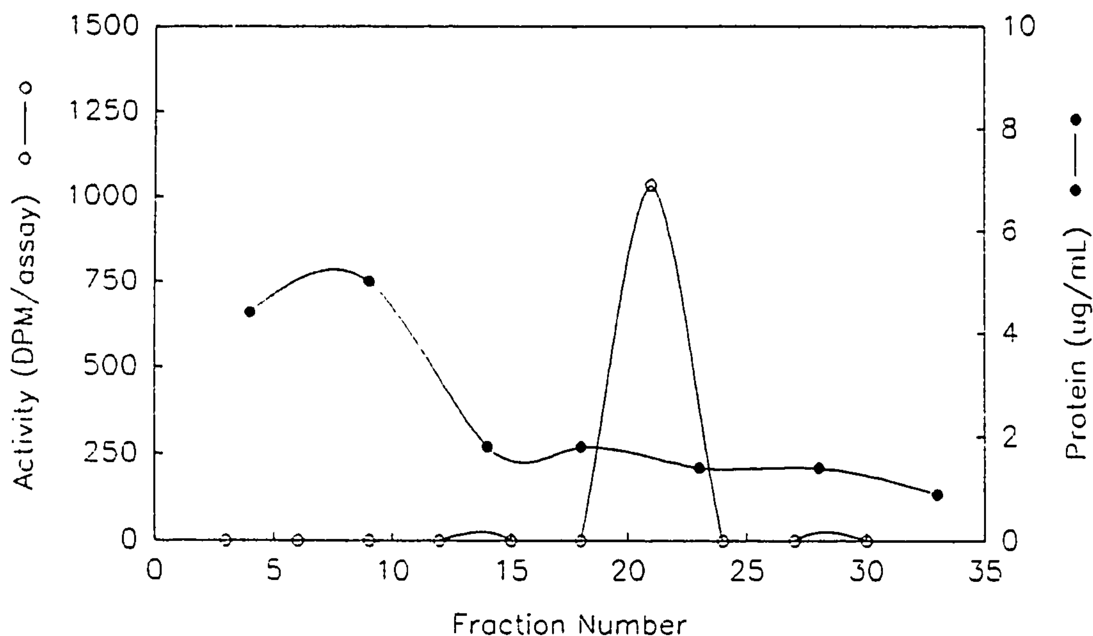



**Figure 13**

**CHROMATOGRAPHY OF DHFS PROTEIN ON HEPARIN AGAROSE USING ATP AND SALT FOR ELUTION OF THE ENZYME.**

Step 7 protein was applied to a Heparin agarose column (2.5 x 10 cm) pre-equilibrated with Buffer A (pH 6.0). The column was washed with 100 mL of Buffer A and developed with a linear gradient (200 mL) of a KCl (0 to 0.5 M) and ATP (0 to 50 mM) containing Buffer A, pH 6.0. The gradient was applied starting at fraction 10. Fractions were assayed for protein and DHFS activity.

applied to the next column. The column following DEAE-Sephacel was Heparin agarose, which was followed by Matrex Green A. The specific enzyme activity increased from 36.8 to 179 to 2259 following DEAE-Sephacel, Heparin agarose, and Matrex Green respectively as summarized in Table 5. The x-fold purification after each step as well as % yield are also summarized in Table 5 with the final step again being the most effective but only 12% of the initial enzyme acitvity was recovered.

The protein at different steps in the purification exhibited different stability. After desalting, the enzyme activity was virtually stable at $4^oC$ for at least 3 months. However, the enzyme following DE-52 chromatography had to be immediately applied to the next column as it lost approximately 50% of the activity overnight and was completely inactivated after 48 hours. After further purification steps the enzyme again became more stable. For example the enzyme after STEP 7 (protocol #1) was almost as stable as the desalted ammonium sulfate precipitate. In this case Step 7 protein retained DHFS activity for at least one month when stored at $4^oC$.

## Properties of *Neurospora* DHFS

DHFS protein (Protocol 1, Step 7) was characterized with respect to Michaelis-Menten constants (Km), molecular size, pH optimum, and possible inhibition by a number of different biological substrates. In order to determine the Km values for each substrate it was first necessary to determine whether reaction rates were a linear function of substrate concentration. The results of these experiments as well as the corresponding Lineweaver-Burk plots for ATP, dihydropteroate, and glutamate are shown in Figures 14, 15, and 16 respectively. The Km values for ATP (50 uM), dihydropteroate (0.11 uM) and L-glutamate (290 uM) were determined from the corresponding Linweaver-Burk plots and are summarized in Table 6 with published data for other species. The Km values were the average of three separate determinations.

The molecular weight of DHFS, based on three separate determinations, was estimated to be approximately 52 kD by gel filtration on Sephacryl S-200 (Andrews, 1964). The calibration curve, based on 2 separate determinations, was constructed using blue dextran to determine void volume (Vo) and a series of protein molecular weight standards (Figure 5). Steps 8 and 7 proteins (Protocols 1 and 2 respectively) were subjected to SDS-PAGE and silver

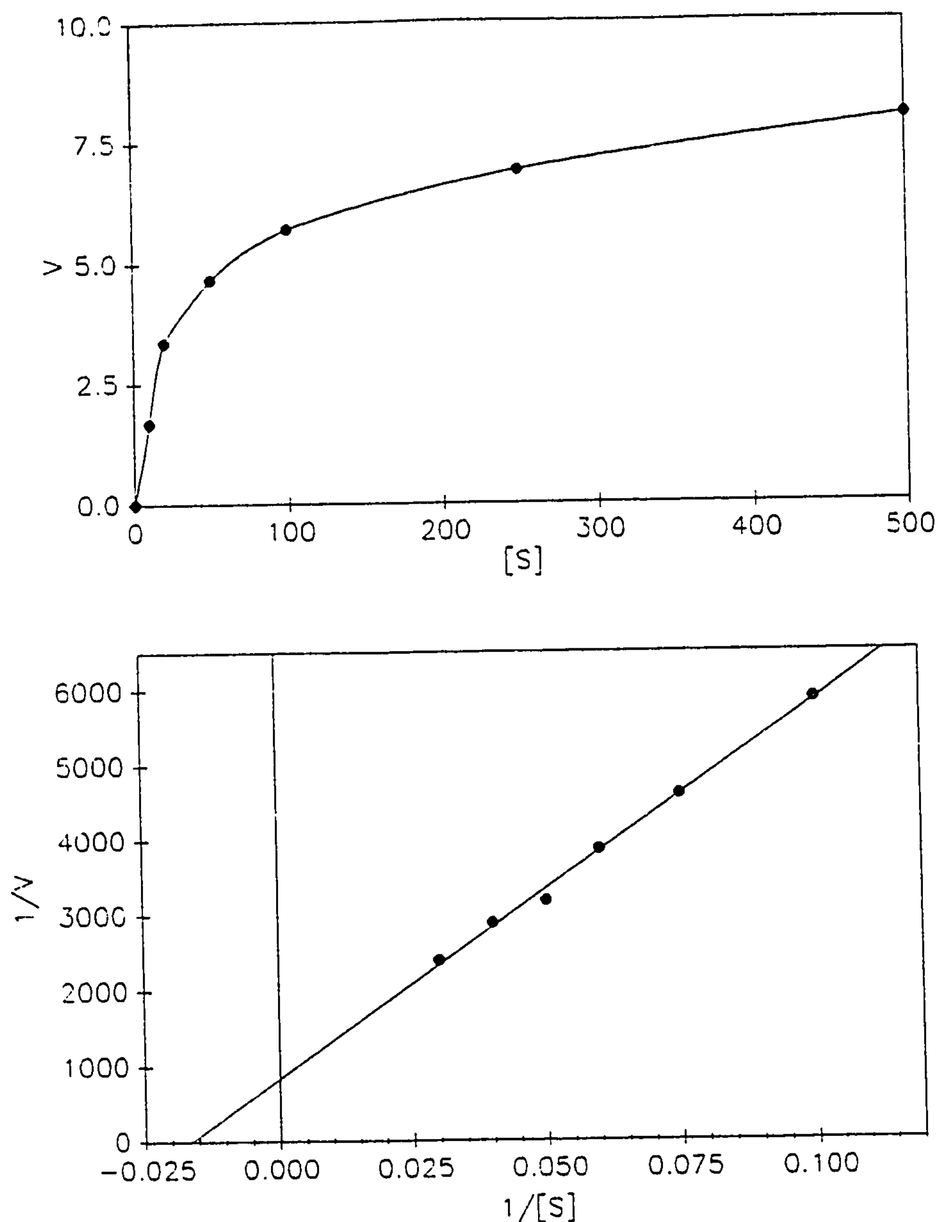



**Figure 14**

**EFFECT OF ATP CONCENTRATION ON THE RATE OF THE DHFS REACTION.**

Step 7 protein from protocol 1 was used in a standard assay except with varying concentrations of ATP (umol) represented in A. B, represents the Lineweaver-Burk plot of 1/V versus 1/[S] from which a Km of 50 uM was calculated.

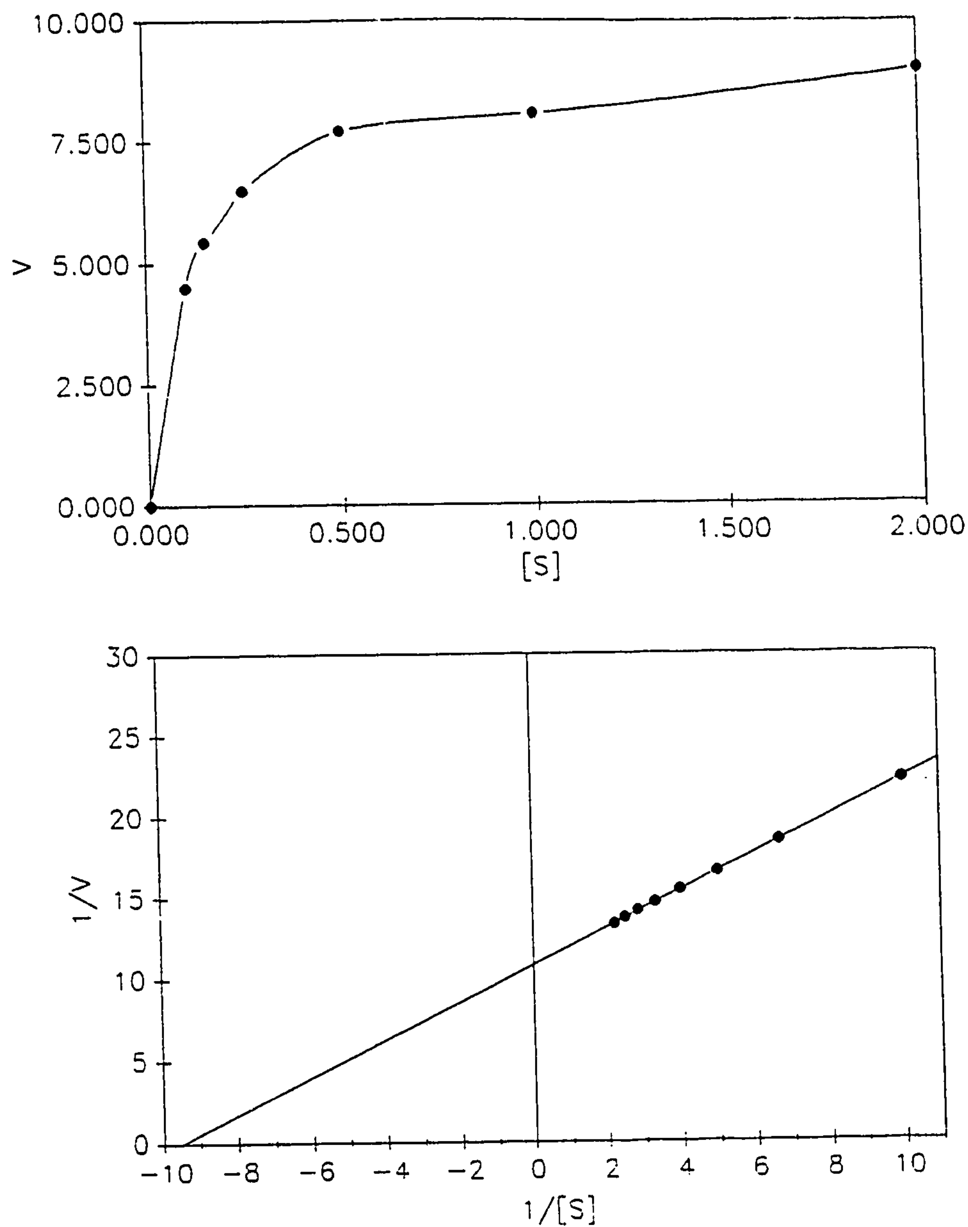



**Figure 15**

**EFFECT OF DIHYDROPTERОATE CONCENTRATION ON THE RATE OF THE DHFS REACTION.**

Step 7 protein from protocol 1 was used in a standard assay except with varying concentrations of dihydropteroate, represented in A. B, represents the Lineweaver-Burk plot of 1/V versus 1/[S] from which a Km of 0.11 uM was calculated.

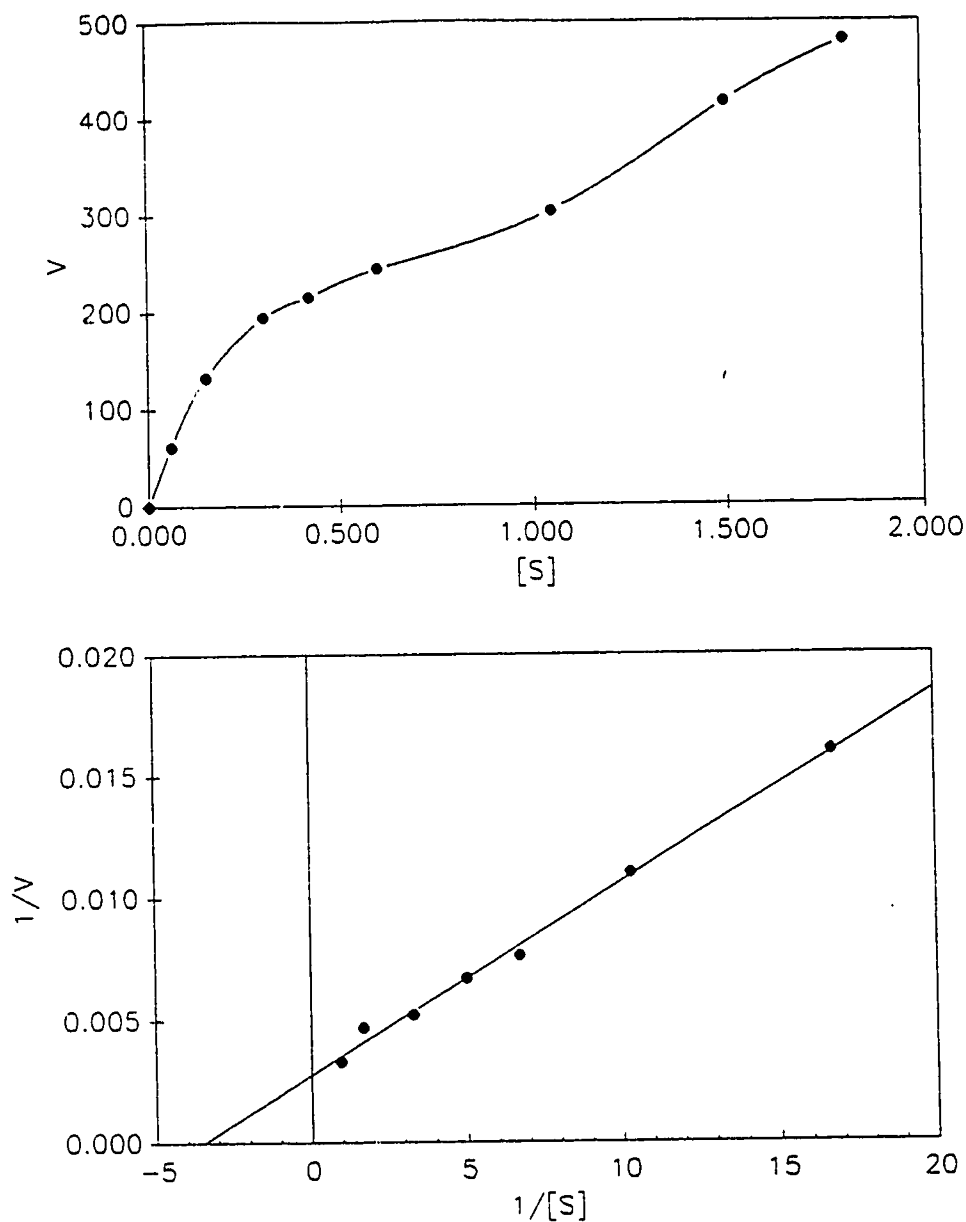



**Figure 16**

**EFFECT OF GLUTAMATE CONCENTRATION ON THE RATE OF THE DHFS REACTION.**

Step 7 protein from protocol 1 was used in a standard assay except with varying concentrations of glutamate, represented in A. B, represents the Lineweaver-Burk plot of 1/V versus 1/[S] from which a Km of 290 uM was calculated.

**Table 6.** ***Properties of Dihydrofolate Synthetase from Different Sources.***

| | Source | | | | |
|---|---|---|---|---|---|
| **Property** | *Escherichia coli* | Pea seedling | *Corynebacterium sp.* | *Serratia indica* | *Neurospora crassa* |
| Molecular weight (kD) | 54 | 56 | 51 | 47 | 52 |
| Km (uM) | | | | | |
| 7,8-$H_2$Pte | 1.9 | 1.0 | <0.4 | -- | 0.11 |
| L-Glutamate | 3.5 | 1500 | 1380 | -- | 290 |
| ATP | -- | 100 | -- | -- | 50 |
| $Mg^{2+}$ | -- | 1100 | -- | -- | -- |
| Mg.ATP | 8.1 | -- | 2.9 | -- | -- |
| $Mn^{2+}$ | -- | 63 | -- | -- | -- |
| pH optimum | 8.9 | 8.8 | 9.5 | 9.0 | 9.8 |
| References | Bognar et al (1985) | Iwai and Ikeda (1975) | Shane (1980) | Ikeda and Iwai (1976) | |

NOTE: All of the above DHFS enzyme preparations require $Mg^{2+}$ and a monovalent cation for optimal activity.

staining. Four bands of protein were detected on the gels ranging in molecular weight from about 20 to 60 kD (data not shown). The major band at a molecular weight of approximately 50 kD. The intensity of this band appeared to increase across the peak of DHFS activity from the Matrex Green A and Heparin agarose columns (data not shown).

The pH optimum for DHFS activity was determined to be approximately 10.3 at 22°C and is represented in Figure 17. Higher or lower pH values caused a decrease in activity. The Tris buffers were adjusted to pH values up to 10.3 by the addition of HCl; NaOH was added to obtain pH values between 10.3 and 11.0. In addition, carbonate and Tris\glycine buffers were used in place of the above assay buffer but no activity could be detected under standard assay conditions.

A variety of biological compounds were tested to study possible inhibition by products of the reaction as well as by compounds such as $H_4$PteGlu which would normally be present *in vivo*. The results of these studies are summarized in Table 7. Dihydrofolate and dihydrofolate-triglutamate both inhibited enzyme activity at micromolar concentrations. AMP and ADP inhibited enzyme activity at concentrations equal to 50 mM but at 1 to 10 mM concentrations DHFS activity appeared to increase. Tetrahydrofolate ($H_4$PteGlu), the corresponding triglutamate derivative ($H_4$PteGlu$_3$), folic acid (PteGlu), and pteroic acid (Pte) had no effect on enzyme activity under the conditions employed.

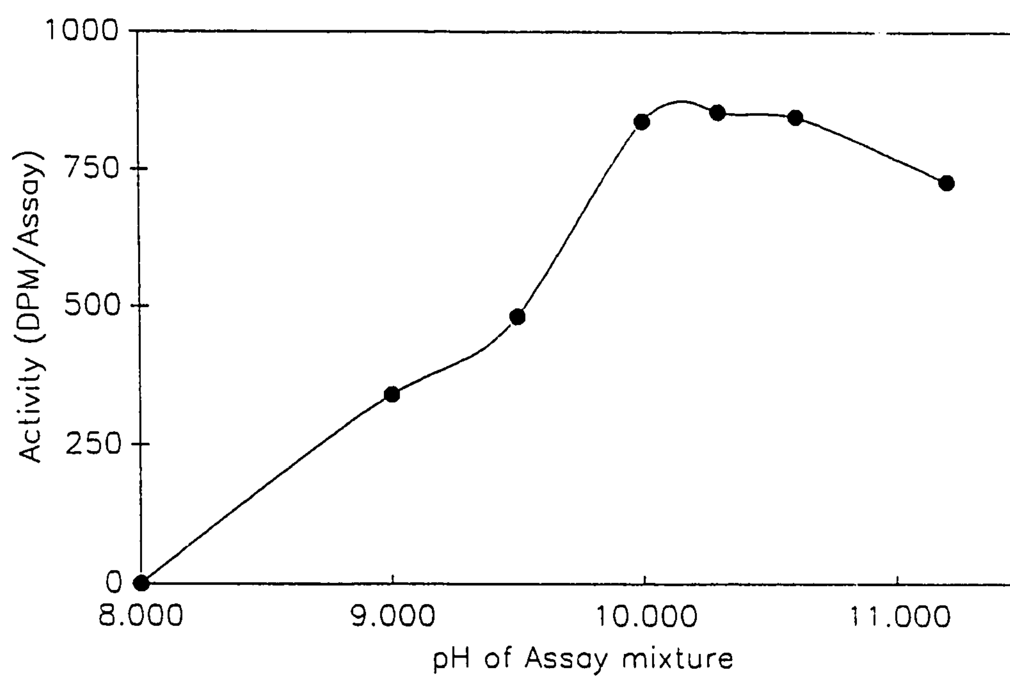



**Figure 17**

**DETERMINATION OF THE PH OPTIMUM FOR DHFS ACTIVITY.**

Step 7 protein (protocol 1) was used under standard assay conditions except that the Tris buffer was adjusted to pH 10.3 and lower with HCl and to pH values higher than 10.3 with NaOH as indicated in the Results. The above values were the average of two separate determinatons.

**Table 7.** ***Biological compounds used to study inhibition of DHFS activity.***

| Possible Inhibitor | Inhibitor Concentration (uM) | Relative Activity (%)* |
|---|---|---|
| 1. Pte | <500 | 100 |
| 2. PteGlu | <500 | 100 |
| 3. $H_2$PteGlu | 10 | 64 |
| | 100 | 42 |
| | 500 | 34 |
| 4. $H_2$PteGlu$_3$ | 10 | 98 |
| | 100 | 68 |
| | 500 | 66 |
| 5. $H_4$PteGlu | <500 | 100 |
| 6. $H_4$PteGlu$_3$ | <500 | 100 |
| 7. ADP** | 0.1 | 100 |
| | 1 | 115 |
| | 10 | 111 |
| | 50 | 73 |
| 8. AMP** | 0.1 | 100 |
| | 1 | 126 |
| | 10 | 122 |
| | 50 | 90 |

*Relative activity was measured in relation to a standard assay system (see Materials and Methods)

**[ADP] and [AMP] were in mM not uM

# DISCUSSION

This part of the thesis deals with different five different aspects of the research and their relationships to earlier studies. The first section discusses the importance and distribution of DHFS in eukaryotic and prokaryotic species. It deals with previous work that described the folate requirements of different organisms and how this relates to the expression of DHFS activity. The second section describes purification protocols for DHFS from different sources emphasizing the various methodologies and media used. Problems encountered in devising a suitable protocol for the purification of *Neurospora crassa* DHFS are also discussed and compared to these earlier methods. The third part of this Discussion deals with the reported association of FPGS and DHFS activities in some species and the lack of such association in others. Some possible reasons for the differences are suggested. Section four compares the properties of DHFS from different sources in terms of pH optima, Michaelis-Menten constants and the requirements for dihydrofolate synthesis *in vitro*. The final section outlines suggestions for further research with commentary on how such studies might contribute to an better understanding of folate biosynthesis.

## Importance and Distribution of DHFS

The first studies of DHFS focussed on the identity of pathway intermediates and steps for the biosynthesis of folate (Brown et al, 1961; Shiota et al, 1964). These initial studies tended to use crude extracts and concentrated on the involvement of certain precursors in the formation of dihydrofolate and tetrahydrofolate. Griffin and Brown (1964) were among the first to study DHFS activity from a number of sources. These workers partially purified this enzyme activity from *E. coli* using ammonium sulfate fractionation, calcium phosphate gel treatment, and DEAE-cellulose chromatography. The distribution of DHFS activity was also examined using cell-free extracts of *Corynebacterium* species, *Saccharomyces cerevisiae*, *Bacillus megaterium*, *Neurospora crassa* as well as several other microorganisms (Griffin and Brown, 1964). These studies confirmed that DHFS was present in all of these organisms. Later work showed that DHFS was also present in numerous species of plants including pea, lettuce, cabbage, swiss chard, and spinach (Ikeda and Iwai, 1970).

It is now clear that the organisms examined in these studies are capable of

synthesizing folate *de novo* and do not require additional folate supplements for normal growth. In contrast a number of other organisms require folate supplements. These include mammals and birds and some bacteria such as *Lactobacillus casei* and *Pediococcus cerevisiae*. These folate-requiring species all lack DHFS activity, a major contributing factor leading to their nutritional requirement for exogenous folates (Iwai, Ikeda and Fujino, 1977).

**Purification of DHFS from Different Sources**

Although DHFS plays a key role in the biosynthesis of folate and is widely distributed in plants, fungi, and prokaryotes relatively few detailed studies of this enzyme have been reported. Thus the sources studied to date include only a few organisms such as *E. coli*, (Bognar et al, 1985), *Corynebacterium* species (Shane,1980), *Serratia indica* (Ikeda and Iwai, 1976), pea (Iwai and Ikeda, 1975), and *Neurospora crassa* (this study). The techniques used to isolate the enzyme from these sources have included molecular cloning of the *E. coli* gene and partial purification from a mitochondrial source as in the case of pea seedlings. Although all of these studies involved the use of ion exchange chromatography and affinity chromatography on different media, the approach used for the isolation of *Neurospora* DHFS was distinct from the earlier work.

*E. coli* DHFS was purified by Bognar et al (1985) using molecular cloning techniques to isolate the fol C gene which encodes for a bifunctional protein containing FPGS activity in addition to DHFS. The purification protocol for the DHFS/FPGS protein involved ammonium sulfate fractionation, DEAE-Sephadex, Sephadex G-100, and a Mono S HPLC column. The resulting protein was monomeric, with an Mr=47 kD and was purified 93-fold. Purification of DHFS from *Corynebacterium* (Shane, 1980) involved ammonium sulfate fractionation, DEAE-cellulose, Sephadex G-150, butyl-agarose, and AMP-agarose column chromatography. The resulting 51 kD protein had both FPGS and DHFS activities and was purified approximately 7000-fold. DHFS was also purified (130-fold) from *Serratia indica* (Ikeda and Iwai, 1976) by ammonium sulfate fractionation, DEAE-Sephadex column chromatography, Sephadex G-200 gel filtration, and DEAE-cellulose chromatography. The enzyme was apparently homogeneous as judged by ultracentrifugal analysis and ultraviolet absorption spectrophotometry. This protein had a molecular size of approximately 47 kD.

The possible association of DHFS and FPGS activities was not examined. Pea DHFS was isolated from a mitochondrial fraction by Ikeda and Iwai (1975) and then subjected to ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-200 filtration, and hydroxylapatite column chromatography. This resulted in about 2000-fold purification of a 56 kD protein (Iwai and Ikeda, 1975). It was not determined if this plant DHFS protein also catalyzed the FPGS reaction. In common with the DHFS proteins of pea and *Serratia* DHFS the *Neurospora* enzyme was also very labile and lost activity rapidly during purification. Consequently, it was first necessary to find a means of stabilizing the activity. This was partially accomplished by the addition of 20% glycerol and protease inhibitors (PMSF and benzamidine) to the extraction and chromatographic buffers. The addition of BSA and DMSO to the assay system also enhanced product formation. The search for the best method that would minimize losses of activity and maximize protein purification led to the development of the two protocols summarized in Tables 4 and 5. The first column used in both protocols, after ammonium sulfate fractionation, was DE-52 cellulose. This column was important in the present purification because it clearly separated FPGS and DHFS activities. It also appeared to enhance the yield of DHFS activity. This may have been due to the removal of other proteins that compete with DHFS for substrates. Conceivably, this step may also remove inhibitors of DHFS activity. A proteinaceous inhibitor of FPGS activity has been reported for several strains of *Neurospora* (Cossins and Chan, 1988) but its action against DHFS has not been ascertained. Further work on the possible presence of a specific DHFS inhibitor appears warranted as such a compound could play a role in controlling this important reaction of folate biosynthesis.

Protocols 1 and 2 resulted in 1473 and 1075-fold purifications respectively with the final steps (Heparin agarose and Matrex Green A) being the most effective even though both steps resulted in significant decreases in the yield of enzyme activity (Tables 5 and 6 respectively). This may be the result from very tight binding of DHFS protein to the column that the subsequent elution caused damage to the protein resulting in losses of activity. After chromatography of DHFS on Matrex Green A the total activity recovered in the combined active fractions appeared to be less than the activity after concentrating this solution. This could be due to inactivation of the enzyme or perhaps reflects

an instability of the protein during assay in dilute solution. The inactivation may be a result of a conformational change in the enzyme during binding or elution from the column in such a way that the active site is altered. Upon concentration, the enzyme may be able to reconfigure itself and therefore an apparent increase in recovered actvity was observed. An attempt to include Matrex Green A as the final step in Protocol #1 resulted in complete loss of enzyme activity. Som[illegible] explanations are given above but another may be that chromatograph[illegible] Green A simply reduced the activity to an undetectable level.

The final preparati[illegible] from both protocols when subjected to SDS-PAGE and silver staining gave approximately four bands of protein. The intensity of a 50 kD protein appeared to correspond to the DHFS activity of the peak fractions isolated by both protocols. This 50 kD size is in agreement with the value obtained from the gel filtration studies (Fig. 5) which suggests that the DHFS protein may be monomeric. This would be in agreement with the DHFS proteins isolated from other sources (Bognar et al, 1985; Shane, 1980; Iwai and Ikeda, 1975).

**Association of DHFS and FPGS Activities**

The reactions catalyzed by DHFS and FPGS are very similar in nature. Both activities result in the additon of glutamate to a reduced pteroyl compound in an ATP-dependent process. These similarities and the close association of these reactions in the biosynthesis of folate imply that some homologies may exist in their amino acid sequences. The association of these activities in certain organisms supports this possibility. As mentioned earlier, DHFS and FPGS activities of *E. coli* are associated with a single protein encoded by the fol C gene (Bognar et al, 1985). It is believed that *Corynebacterium* also has both activities associated with one protein (Shane, 1980). In order to be sure that the separation of these two activities in *Neurospora* was not due to proteolytic cleavage, protease inhibitors (PMSF and benzamidine) were included in all buffers. It is clear from the Results (Figures 4-8) that separation of the two activities in *Neurospora* was achieved on a number of different chromatographic media ranging from gel filtration to affinity and anion exchange. Studies using gel filtration determined that the DHFS and FPGS had different elution volumes and therefore different molecular sizes. Separation of the *Neurospora* activities

on Heparin agarose, Matrex Green and Reactive Green 5 confirms these chromatographic differences between the two enzymes and strengthens the proposal that they are separate proteins. Anion exchange chromatography used to separate the pea and *Neurospora* DHFS from their corresponding FPGS activities (Figures 4 and 9 respectively) showed similarities between these proteins in that pea and *Neurospora* DHFS activities came through in the column wash whereas both FPGS activities were only eluted by applying a gradient of increasing salt concentration.

The conclusion that they are separate proteins in *Neurospora* is also supported by work on the *met 6* mutant, which lacks normal FPGS activity (Cossins and Chan, 1984). Analyses showed that it contained comparable DHFS activity to the wild type on a per gram of mycelium basis. Thus although FPGS activity was reduced by this mutation, the level of DHFS activity was unaffected.

Recent work on *Saccharomyces cervisiae* has shown that FPGS and DHFS are encoded by separate genes (Brenner et al, 1993). These workers used molecular cloning techniques to isolate the DHFS-encoding fragment of yeast DNA. The clone that encodes DHFS activity was sequenced and mapped. As a result, the DHFS gene was shown to be separate and distinct from the gene encoding FPGS activity. This recent study provides additional support for the above conclusions that DHFS and FPGS in *Neurospora* and pea are separate proteins encoded by separate genes.

The distinct nature of DHFS and FPGS may relate to the phylogenetic status of bacteria, fungi, and plants. This change from one protein to separate proteins may allow control of activity at a transcriptional level. Mammals and birds must have taken a different evolutionary branch because they lack the DHFS gene.

**The Properties of DHFS from Different Sources**

The properties of DHFS from *Neurospora*, *Serratia indica*, *E. coli*, pea and *Corynebacterium* sp. are summarized in Table 6. These proteins tend to be similar in terms of their size, pH optimum and reaction requirements. These enzymes all have an alkaline pH optimum ranging from 8.8 for *Pisum* to 9.9 for *Serratia*. DHFS from these sources have molecular weights of approximately 50 kD; ranging from 47 for *Serratia* to 56 for the pea seedling. Each of the DHFS

proteins requires dihydropteroate, glutamate, ATP, magnesium as well as a monovalent cation for optimal activity. *Neurospora* DHFS has an absolute requirement for potassium and magnesium (Table 2) a feature also noted for the pea enzyme.

The apparent Km values for glutamate are the lowest for *E. coli* at 3.5 uM and highest for pea seedling at 1500 uM. The latter value is very close to 1380 uM reported for *Corynebacterium* sp. The corresponding value for *Neurospora* is 290 uM. The Km values for dihydropteroate do not vary as much and range from 0.11 uM for *Neurospora* to 1.9 uM for *E. coli*. Pea and *Neurospora* have Km values calculated for ATP that are quite similar while the Km (Mg·ATP) for other species are also in reasonably close agreement.

Studies of possible inhibitors for *Neurospora* DHFS revealed product inhibition by dihydrofolate and ADP (Table 6). *Serratia indica*, pea, and *E. coli* DHFS proteins all demonstrate product inhibition by ADP. ADP is also inhibitory at concentrations above 2.5 mM for *Serratia* and pea DHFS, whereas for the *E. coli* enzyme 50% inhibition occurs at 116 uM. These studies therefore demonstrate further similarities between the DHFS from these different biological sources.

**Suggestions for Further Research**

A possible direction for *Neurospora* DHFS research could be the cloning of the gene and its mapping to study its possible linkage to the FPGS gene. It may also be of interest to study the similarities between the DHFS from different sources in terms of immunological properties. Such a study, based on the cross-reactivity to specific antibodies may provide information on possible sequence homology. If the protein sequence was determined the amount of conservation within and outside the active site between species could also be studied.

Other studies might focus on compartmentalization of both DHFS and FPGS in eukaryotes that express both activities. Such intracellular localization may have a role in the control of enzyme activity by regulating substrate availability. Other levels of control that need to be studied are transcriptional and translational as well as enzyme turnover. These studies could lead to an understanding of whether this enzyme is a key regulatory step in the pathway of folate biosynthesis *de novo*.

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