A Trifunctional Folate-Dependent Enzyme

by

© Larry Ung Leong Tan

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Department of Biochemistry
McGill University
Montreal, Que.
Canada

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To my parents

and

my wife, Ying-Yu

ABSTRACT

Methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase activities have been co-purified more than 500-fold from pig liver. The preparation yields a single protein band on electrophoresis in dodecyl sulfate gels and has an amino terminal sequence of alanine-proline-alanine. It is concluded that the three activities are properties of a multifunctional protein, a single type of polypeptide capable of catalysing more than one enzyme reaction. The trifunctional protein is a dimer of identical subunits of 100 000 daltons. A dehydrogenase-cyclohydrolase fragment and a synthetase fragment with subunit molecular weights of 33 000 and 67 000 could be prepared by tryptic and chymotryptic digestion respectively. While the dehydrogenase and cyclohydrolase activities appear to be closely associated, possibly located within a single domain within the trifunctional protein, double immunodiffusion studies demonstrate the synthetase to be derived from a different region of the polypeptide. Based on N-terminal sequence analysis, the dehydrogenase-cyclohydrolase activities are deduced to be at the N-terminal region of the polypeptide.

RESUME

Les activités déshydrogénase méthylènetétrahydrofolate-cyclohydrolase méthènyltétrahydrofolate-synthétase formyltétrahydrofolate ont été co-purifiées plus de 500 fois à partir d'extraits de foie de porc. L'analyse de la préparation par électrophorèse sur gel de polyacrylamide en présence de dodécylsulfate de sodium montre une seule bande protéique dont la séquence de l'extrémité N-terminale est alanine-proline-alanine. Il est conclu que les trois activités sont la propriété d'une protéine multifonctionnelle étant donné qu'elles sont catalysées par une seule chaîne polypeptidique. La protéine multifonctionnelle est un dimère de sous-unités identiques de 100 000 daltons. La digestion par la trypsine permet de préparer un fragment de 33 000 daltons possédant les activités déshydrogénase-cyclohydrolase tandis que la digestion par la chymotrypsine libère un fragment d'un poids moléculaire de 67 000 ayant l'activité synthétase. Les activités déshydrogénase et cyclohydrolase semblent intimement liées et sont possiblement situées dans une même région de la protéine multifonctionnelle. Par contre, les études d'immunodiffusion double démontrent que l'activité synthétase est attribuable à une région différente du polypeptide. L'analyse de la séquence de l'extrémité N-terminale de la protéine permet de déduire que les activités déshydrogénase-cyclohydrolase sont localisées dans cette région du polypeptide.

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PUBLICATION OF THE WORK PRESENTED IN THIS THESIS

1. Methylenetetrahydrofolate Dehydrogenase-Methenyltetrahydrofolate Cyclohydrolase-Formyltetrahydrofolate synthetase. A Multifunctional Protein from Porcine Liver. L.U.L. Tan, E.J. Drury and R.E. MacKenzie, J. Biol. Chem. 252, 1117-1122 (1977).

2. Methylenetetrahydrofolate Dehydrogenase, Methenyltetrahydrofolate Cyclohydrolase and Formyltetrahydrofolate Synthetase from Porcine Liver. Isolation of a Dehydrogenase-Cyclohydrolase Fragment from the Multifunctional Enzyme. L.U.L. Tan and R.E. MacKenzie, Biochim. Biophys. Acta 485, 52-59 (1977).

3. Methylenetetrahydrofolate Dehydrogenase-Methenyltetrahydrofolate Cyclohydrolase-Formyltetrahydrofolate Synthetase from Porcine Liver. Location of the Activities in Two Domains of the Multifunctional Polypeptide. L.U.L. Tan and R.E. MacKenzie, Can. J. Biochem. ("C.H. Best Memorial" issue), 1979.

4. Methylenetetrahydrofolate Dehydrogenase-Methenyltetrahydrofolate Cyclohydrolase-Formyltetrahydrofolate Synthetase. R.E. MacKenzie and L.U.L. Tan, Methods in Enzymology 66E, 1979.

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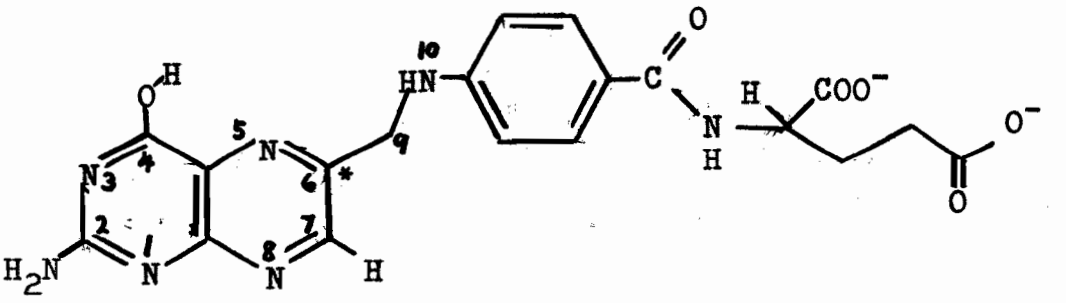
CHAPTER I

INTRODUCTION

(A) FOLATE-MEDIATED METABOLISM

Many of the tetrahydrofolate-dependent reactions in mammals are catalyzed by enzymes that have been found to be relatively unstable <u>in vitro</u>[1], and consequently have been difficult to study. This property has hampered the development of our understanding of the molecular events involved in the interconversion of "active" one-carbon units, including the regulation of this important area of metabolism.

The central character in one-carbon metabolism is folic acid which has the following structure:



2-amino-4-hydroxy-6-methylpterin | p-aminobenzoic acid | glutamic acid

FOLIC ACID

The active form of folic acid is tetrahydrofolic acid (THF) obtained by hydrogenation at positions 5,6,7, and 8 giving rise to an asymmetric carbon at position 6. The one-carbon units carried by THF are on nitrogen atoms 5 or 10, or form a bridge between the two nitrogens.[2]

Inside the cell, folate derivatives are present mainly in the polyglutamyl forms,[2-6] where the glutamates in tandem are linked by γ peptide bonds[7]. The most abundant polyglutamyl chain length in mammals is believed to be the pentaglutamate[3]. There is evidence which suggests polyglutamates are the natural substrates for the many folate-dependent enzymes.[8,9] These observations include lower Km's for polyglutamates for some enzymes and absolute requirements for polyglutamates for others. An example of the former is the Clostridial 10-formylTHF synthetase which has a 200-fold lower Km for the triglutamyl than the monoglutamyl derivative. The Escherichia coli methylcobalamin independent enzyme which methylates homocysteine to form methionine can use only the polyglutamyl forms of the folate substrate. In addition, the presence of polyglutamyl moieties may be important in the retention of folate derivatives within the cell[5,10].

Following ingestion of food containing folates

which are mainly polyglutamates, the lumen absorbs the vitamin and degrades the polyglutamates into monoglutamates[11-13] by the action of intracellular conjugase (γ-glutamyl carboxypeptidase) as well as that present in some foods. The product is then reduced if necessary, methylated, and released into the blood stream as 5-methylTHF.[10,14] Upon intracellular localization, the synthesis of polyglutamates from 5-methylTHF requires its obligatory demethylation,[15,16] accomplished by the vitamin B_{12} dependent enzyme, methylTHF: homocysteine methyltransferase.[17,18] Glutamates are then attached to the freed THF in a stepwise fashion[19] catalyzed by the polyglutamate synthetase (ligase) to give the polyglutamyl forms of the THF. The THF polyglutamates can now take part in active one-carbon transfer reactions as shown in figure 1 for vertebrate systems.

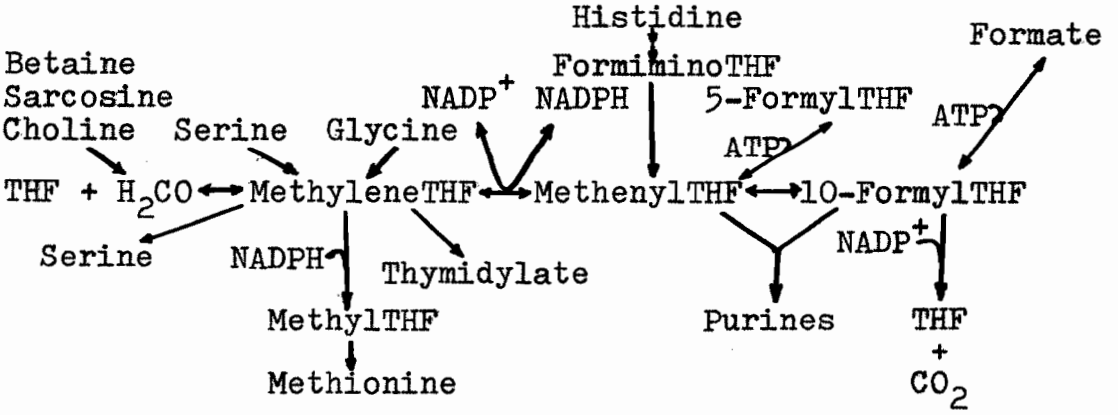


Figure 1

The main sources of one-carbon units are from the degradation of the amino acids serine and glycine, with histidine and tryptophan (via formate) being quantitatively less important. The various forms of one-carbon units can then be interconverted by means of methyleneTHF dehydrogenase and methenylTHF cyclohydrolase to the proper oxidation level for the various biosynthetic steps. These biosynthetic steps, which include purine, thymidylate, methionine and serine syntheses utilize one-carbon THF derivatives. One-carbon THF produced in excess over biosynthetic requirements may be oxidized through 10-formylTHF dehydrogenase as recently proposed by Krebs and Hems.[20]

The reaction which transfers the β carbon from serine to THF is catalyzed by serine transhydroxymethylase[21] (EC 2.1.2.1) according to equation 1.

$$\text{Serine} + (-),1\text{-THF} \longleftrightarrow (+),1\text{-methyleneTHF} + \text{glycine} + H_2O \quad (1)$$

This enzyme is widespread occurring in livers of mammals, birds, fish and amphibia[22] as well as many tissues of man and other vertebrates as well as invertebrates.[23,24] Serine transhydroxymethylase was partially purified from the livers of sheep,[25] beef[26] and to homogeneity from rabbit liver.[27] The rabbit liver enzyme is inhibited by 5-methylTHF and 5-formylTHF.[28]

The influx of one-carbon units from glycine occurs by decarboxylation as shown in equation 2.[29-31,2]

$$\text{Glycine} + \text{THF} + \text{NAD}^+ \longrightarrow \text{methyleneTHF} + \text{NADH} + \text{CO}_2 + \text{NH}_4^+ \quad (2)$$

Four proteins from Peptococcus glycinophilus[32,33] designated P_1, P_2, P_3 and P_4 are required for the catalysis of the reactions shown in equation 2. However, in rat liver,[29] the reactions are catalysed by a single multienzyme complex. The proteins P_1 and P_3 from P. glycinophilus are pyridoxal phosphate and FAD requiring enzymes respectively,[34,35] while P_2 is a thiol-containing protein.[33,36] The P_4 enzyme has not been well studied.[2] The scheme for the decarboxylation of glycine is shown in figure 2.[33]

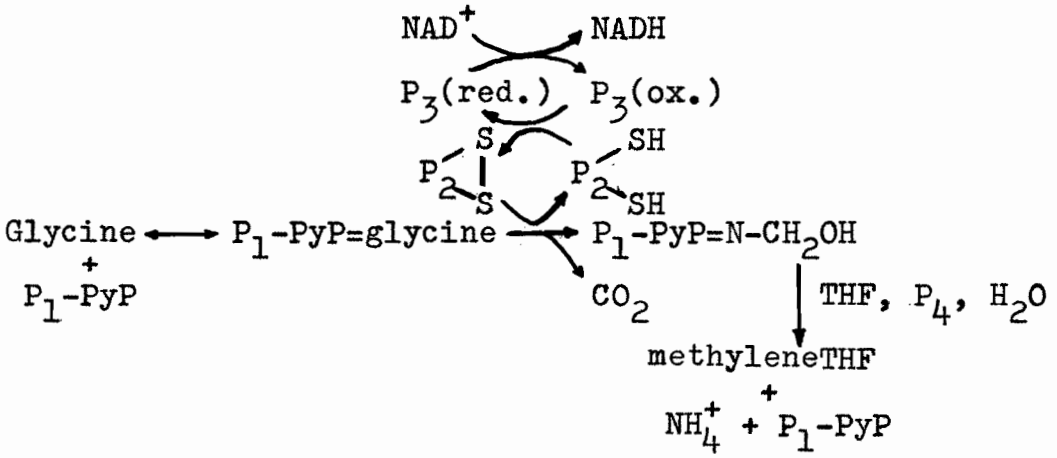


Figure 2

Degradation of histidine results in the formation of N-formiminoglutamate. The formimino group can then be

transferred from glutamate to THF by formiminoglutamate: THF 5-formiminotransferase (EC 2.1.2.5) according to equation 3. Following the transfer, the formiminoTHF is deaminated and cyclized by the formiminoTHF cyclodeaminase as shown by equation 4.[2]

$$\text{Formiminoglutamate} + \text{THF} \longrightarrow \text{5-formiminoTHF} + \text{glutamate} \qquad (3)$$

$$\text{5-FormiminoTHF} + H^{+} \longrightarrow \text{5,10-methenylTHF} + NH_3 \qquad (4)$$

The formiminotransferase and cyclodeaminase from liver acetone powder were found to copurify through 700-fold.[37] The distribution of these two activities are ubiquitous among mammalian livers and kidneys but are absent in human epidermis, skeletal muscles or small intestine.[23] The formiminotransferase from man and rat appears to be regulated by hormones; while glucagon and epinephrine increased, insulin decreased transferase activity.[38]

The degradation of tryptophan results in the production of formate which can be incorporated into the one-carbon pool by means of the 10-formylTHF synthetase (EC 6.3.4.3) catalyzed reaction according to equation 5.

$$\text{THF} + \text{HCOOH} + \text{ATP} \longleftrightarrow \text{10-formylTHF} + \text{ADP} + \text{Pi} \qquad (5)$$

Another source of formate is the bacterial breakdown of carbohydrates in the gut.[20] FormylTHF synthetase is a common enzyme found in almost all tissues including erythrocytes

of various species,[39] all organs of guinea pig examined[40] and in most tissues of 36 animals from 11 phyla.[24] The best characterized formylTHF synthetases are those from bacterial sources where it was found that monovalent cations NH_4^+, K^+ and Rb^+ stimulate activity.[41] Binding of the substrate ATP, requires the presence of divalent cations Mg^{2+} or Mn^{2+}. It appears the active substrate for the synthetase is the complex $MgATP^{2+}$ or $MnATP^{2+}$ rather than ATP.[41,42] The enzymes from Micrococcus aerogenes and Clostridium cylindrosporum are inhibited by both ADP and inorganic phosphate.[43,44] While the Clostridial enzyme is stable and has been purified and crystallized,[45] the beef liver enzyme is highly unstable, enabling only partial purification.[1]

5,10-MethyleneTHF: $NADP^+$ oxidoreductase (EC 1.5.1.5) commonly referred to as methyleneTHF dehydrogenase, first observed by Jaenicke,[46] and later confirmed by Uyeda and Rabinowitz[47] catalyses the reaction represented in equation 6.

$$\text{5,10-MethyleneTHF} + NADP^+ \longleftrightarrow \text{5,10-methenylTHF} + NADPH + H^+ \qquad (6)$$

MethyleneTHF dehydrogenase is commonly distributed among animals and in all tissues of vertebrates except muscle.[24,48] While the majority of species contain $NADP^+$-dependent enzyme, an NAD^+-linked dehydrogenase was found in the Ehrlich ascites

cell.[49] MethyleneTHF dehydrogenases from both Salmonella typhimurium and E. coli were found to be inhibited by ATP, GTP, ITP and GDP.[50-52] The labile enzymes from beef liver and calf thymus, unlike the bacterial dehydrogenases, could be inhibited by divalent cations.[1,2,53] The latter enzyme also was inactivated by sulfhydryl reagents.

The enzyme, 5,10-methenylTHF 5-hydrolase (decyclizing) (EC 3.5.4.9) commonly known as 5,10-methenylTHF cyclohydrolase catalyses the reaction shown in equation 7.

$$5,10\text{-MethenylTHF} + H_2O \longleftrightarrow 10\text{-formylTHF} + H^+ \qquad (7)$$

The reaction proceeds spontaneously[2] and under acidic conditions, the equilibrium shifts in favor of methenylTHF while at neutral and basic pH, an opposite shift occurs.[54] The cyclohydrolase from mammalian sources, similar to methyleneTHF dehydrogenase and 10-formylTHF synthetase, is widely distributed and labile.[24,37,48,55] This enzyme had been partially purified from livers of rabbit, beef and pig.[37,55,56] In the latter case, the cyclohydrolase copurified 100-fold with methyleneTHF dehydrogenase and 10-formylTHF synthetase. The beef liver enzyme appears to be a sulfhydryl enzyme that can be inhibited by both sulfhydryl reagents and polyvalent cations.[55]

5-FormylTHF cyclodehydrase[2] catalyses the reaction

in equation 8.

$$5\text{-FormylTHF} + ATP \xleftrightarrow{Mg^{2+}} 5,10\text{-methenylTHF} + ADP + Pi \quad (8)$$

The enzyme had been purified 400-fold from sheep liver and it appears to be a sulfhydryl-dependent enzyme.[57]

10-FormylTHF deacylase catalysing the reaction in equation 9 has been observed in liver and ovaries of several species[24] and was purified about 10-fold from beef liver.[58,59] The enzyme appears to require catalytic amounts of $NADP^+$ for activity.[2]

$$10\text{-FormylTHF} + H_2O \xleftrightarrow{NADP^+} HCOOH + THF \quad (9)$$

An enzyme, 10-formylTHF dehydrogenase catalyzing the reaction according to equation 10, has been purified 200-fold from pig liver.[60,61]

$$10\text{-FormylTHF} + NADP^+ + H_2O \longrightarrow THF + CO_2 + NADPH + H^+ \quad (10)$$

The enzyme also catalyses the reaction in equation 9, implying the 2 enzymes to be identical.[60] The 10-formylTHF dehydrogenase was found to be insensitive to 5-methylTHF and 5-formylTHF but is inhibited at low (1 μM) concentrations of THF.[60]

The synthesis of purines requires participation of one-carbon units in transformylation reactions to provide carbons 2 and 8 in the purine ring. The transformylation of carbon 8 is catalyzed by the enzyme, 5,10-methenylTHF:

2-amino-N-ribosylacetamide-5'-phosphate transformylase (glycinamide ribonucleotide transformylase) according to the reaction in equation 11.

5,10-MethenylTHF + glycinamide ribonucleotide ⟷ THF + N-formylglycinamide ribonucleotide (11)

The activity was first observed by Goldthwait et al[62] and Hartman et al[63] and shown to require a folate derivative[64] demonstrated to be 5,10-methenylTHF.[65] The glycinamide ribonucleotide transformylase from chicken liver was later purified 58-fold by Warren and Buchanan[66] and subsequently to homogeneity by Caperelli et al.[67]

The second transformylation providing carbon 2 of the purine ring requires the enzyme, 10-formylTHF: 5-amino-1-ribosyl-4-imidazolecarboxamide-5'-phosphate transformylase (AICAR transformylase). The reaction goes according to equation 12 and was deduced by Buchanan and Greenberg.[65,68-70]

10-FormylTHF + AICAR ⟷ THF + 5-formamido-imidazole-4-carboxamide ribonucleotide (FICAR) (12)

The AICAR transformylase from chicken liver was found to copurify with inosinicase,[71] an enzyme catalyzing ring closure (equation 13).

FICAR ⟷ IMP + H_2O (13)

The two enzyme activities from bacteria appear to be derived

from the same protein since genetic studies show a single mutation in Enterobacteriaceae destroys both activities.[72]

Thymidylate synthetase catalyses the transfer of the one-carbon group (equation 14) from methyleneTHF to deoxyuridine monophosphate (dUMP)[2,73] with a concomitant reduction by THF using the hydrogen at carbon 6.[74]

$$\text{dUMP} + \text{5,10-methyleneTHF} \longleftrightarrow \text{dTMP} + \underset{(\text{DHF})}{\text{dihydrofolate}} \qquad (14)$$

The concentration of thymidylate synthetase from submaxillary gland and thymus of the rat is high, but negligible amounts are observed in the liver, spleen and lung of adults.[75] However, livers from partially hepatectomized rats[76] and 18 day rat embryos[77] contain significantly higher concentrations of the enzyme than those from adults. Elevated thymidylate synthetase is also found in human leucocytes of most leukaemia patients.[78] Because of the low level of synthetase in normal adult tissues, it was postulated that regulation of this enzyme also regulates DNA synthesis.[77]

Thymidylate synthetase was purified 10-fold from E. coli[79] and leucocytes[78] and to a greater extent from calf thymus[80] and chick embryo.[74] The E. coli[81] and leucocyte[78] enzymes, in in vitro studies, showed strong inhibition by 5-fluorodeoxyuridine monophosphate but not 5-fluorouracil

and 5-fluorodeoxyuridine respectively. However, in vivo studies demonstrated the ability of 5-fluorouracil to inhibit thymine biosynthesis[81] probably by conversion into 5-fluorodUMP. Because of the thymidylate requirement in DNA synthesis, the halogenated pyrimidines are used in cancer chemotherapy in hope of arresting the growth of tumours. 5-Fluorodeoxyuridine was shown effective in the inhibition of Sarcoma 180 tumours in mice with no toxic effects, but was ineffective against other forms of tumours.[82] Fluorouracil, in combination with amethopterin (methotrexate), showed an enhanced effect against L1210 leukaemia in mice.[83] However, while these drugs prolong life, a cure for cancer has yet to be found.[84] Other inhibitors of thymidylate synthetase include a host of pyrimidine and folate analogues.[2]

The dihydrofolate (DHF) generated in the thymidylate synthetase reaction is reduced to THF by dihydrofolate reductase (EC 1.5.1.3) as shown in equation 15. This enzyme is also active in reducing folate to THF although at a slower rate.[2]

$$DHF + NADPH + H^+ \longrightarrow THF + NADP^+ \qquad (15)$$

The reductase is ubiquitous among mammalian tissues, with the concentration being highest in liver and kidney and lowest in brain, lung and skeletal muscle.[2] Like thymidylate synthetase, the reductase in leucocytes is elevated in

leukaemia patients[48] and embryonic tissues have higher enzyme levels than adults.[48,85] In contrast with thymidylate synthetase, the liver reductase does not appear to increase after partial hepatectomy.[76]

Dihydrofolate reductase had been highly purified from calf thymus[86] and guinea pig liver [87] and purified to homogeneity from chicken liver.[88] The reductase is strongly inhibited by aminopterin and amethopterin under slight acidic conditions to an extent that it is essentially irreversible.[2] However, at higher pH, there appears to be some reversibility. The enzyme from some amethopterin-resistant mutants of Diplococcus pneumoniae, shows complete reversibility.[2] Because of the requirement for dihydrofolate reductase in continuous thymidylate synthesis, a search was initiated for a compound which could inhibit the reductase from either bacterial or neoplastic sources but at the same time, would not interfere with host cell reductases. Such a compound would have possibilities as bacteriostatic agent or as a drug in chemotherapy. Although no one compound was totally effective in this respect, based on these studies, numerous inhibitors of dihydrofolate reductase were synthesized based on the skeletal structures of pyrimidine, triazine and 2,4-diamino-4-deoxypterin.[2]

MethyleneTHF reductase (EC 1.1.1.68) from vertebrate livers catalyses the reaction depicted in equation 16.

$$\text{5,10-MethyleneTHF} + \text{NADPH} + \text{H}^+ \longrightarrow \text{5-methylTHF} + \text{NADP}^+ \qquad (16)$$

The enzyme was purified 20-fold from pig liver [89] and 300-fold from rat liver.[90] The rat liver enzyme appears to contain bound FAD with a specific preference for NADPH as the reducing agent. The enzyme is inhibited by S-adenosylmethionine.[90,91] The equilibrium of the reaction in equation 16 is so far to the right that it is essentially irreversible.[3,92]

5-MethylTHF: homocysteine methyltransferase from vertebrate sources catalysing the reaction in equation 17 is a cobalamin dependent enzyme.[93,94]

$$\text{5-MethylTHF} + \text{homocysteine} \xrightarrow[\text{S-adenosylmethionine}]{\text{cobalamin}} \text{THF} + \text{methionine} \qquad (17)$$

In addition, catalytic amounts of S-adenosylmethionine and $FADH_2$ seem necessary to activate the enzyme.[95] In animals with vitamin B_{12} deficiency, the methyl transfer from 5-methylTHF to homocysteine, which is the only major pathway for the release of THF from 5-methylTHF,[17,96] is blocked. Because the reduction of methyleneTHF to 5-methylTHF is essentially irreversible, it was hypothesized that there is a gradual conversion of most of the one-carbon units into the now useless 5-methylTHF pool.[3,20] This explanation for

the effect of vitamin B_{12} deficiency constitutes the "methyl-folate trap hypothesis".[3] The condition becomes more detrimental since mammalian cells are normally presented only 5-methylTHF (monoglutamate) from the blood stream. The inability to demethylate results in little or no formation of polyglutamates since the substrate for polyglutamate synthetase reactions is free THF.[3] This depletion of useful folate derivatives in vitamin B_{12} deficiency may account for clinical symptoms similar to folate deficiency.[20]

Deficiency of folate or some folate dependent enzymes at an early stage of development frequently expresses itself as megaloblastic anemia and mental retardation, possibly due to a defect in purine and/or thymidylate biosynthesis in the brain.[97] However, a folate deficiency in adults leads only to sleeplessness, forgetfulness, irritability and eventually megaloblastic anemia.[98]

(B) MULTIFUNCTIONAL PROTEINS

The reactions in folate metabolism, as with other metabolic pathways, are catalyzed by multiple enzymes. It had been observed that functionally integrated enzymes in a particular pathway have occasionally been found to be covalently

linked to form multifunctional proteins;[99,100] defined here as a single polypeptide capable of catalysing more than one enzyme reaction. This definition is distinct from a single catalytic site with wide substrate specificity. Multifunctional proteins are analogous to multienzyme complexes except the different activities are covalently linked within a polypeptide. Some examples include the bifunctional formiminotransferase and cyclodeaminase from pig liver[101,102] and the trifunctional DNA polymerase I from E. coli.[103] Both of these multifunctional proteins demonstrate autonomy for the component activities. In the case of formiminotransferase-cyclodeaminase, the cyclodeaminase activity is selectively destroyed by chymotrypsin, while at high pH, it is the formiminotransferase which is inactivated.[37] As for the DNA polymerase I, partial proteolysis produces two fragments, one with polymerase and 3' to 5' exonuclease activity,[104,105] while the other has 5' to 3' exonuclease activity.[106,107]

The advantages of having functionally related enzymes in an aggregated and/or multifunctional protein are well summarized by Kirschner and Bisswanger[99] and by Welch.[100] They include; firstly, the localization and ultimately the compartmentalization of substrates and intermediates which in effect increases the working substrate concentrations for enzymes.[100] At the same time, the lack of uniform dispersion

of intermediates preserves the solvent property of the bulk cell sap and prevents interference by intermediates among different metabolic pathways. Localization also reduces the " transient time" for metabolites; that is the time required between their release and binding to the next enzyme within the metabolic chain.[100] The reduction of transient time could result in an overall increased rate of catalysis. Secondly, the aggregation of sequential enzymes may be for the orientation of active sites in such a way that the product of the first enzyme reaction, without having to be released into the medium, is channelled into the next enzyme in the metabolic chain. This ability to channel intermediates circumvents wasteful accumulation, and would be particularly useful with labile intermediates.[100] In addition, channelling offers quick response to effectors since no prior accumulation of intermediate is necessary. Another advantage to channelling may be the lowering of Km's for the sequential enzyme.[100] This occurs if the first enzyme presents to the sequential enzyme an intermediate which is in a favorable orientation for catalysis, thus eliminating the orientation factor in the collision-binding process of a freely diffusible system. Thirdly, by having certain enzymes in the metabolic chain together in one protein, a single effector may simultaneously regulate all the reactions catalysed by this protein.[99] In terms of enzyme synthesis, the multifunctional protein

would certainly be more advantageous than its analogue, the multienzyme complex. While multienzyme complex synthesis requires the coordinated synthesis of each of its components, none is required for a multifunctional protein.

An example of a multifunctional protein includes anthranilate synthetase[108-110] which is composed of two non-identical subunits. Whereas subunit I contains anthranilate synthetase activity using NH_3 as substrate, subunit II which contains glutamine amidotransferase (GAT) allows anthranilate synthetase to use glutamine as the ammonia donor. The subject of interest is the subunit II with GAT activity. Whereas the GAT from Pseudomonas and Bacillus subtilis is monofunctional,[111] this activity from other sources was found to be covalently linked to other activities of the tryptophan biosynthetic pathway.[108] For example, the enzyme from E. coli and S. typhimurium, is a single polypeptide containing both GAT and phosphoribosylanthranilate (PRA) transferases. However, the yeast GAT activity is associated with indole-3-phosphate synthetase while in the fungi, it is associated with both indole-3-phosphate synthetase and PRA isomerase. The GAT and PRA transferase from E. coli and S. typhimurium had been shown to be located in different domains of the polypeptide. Deletion of the first third of the gene in E. coli[109] retained PRA transferase activity with loss of GAT activity, while

tryptic digestion of the enzyme from S. typhimurium destroys the C-terminal two-thirds of the polypeptide, with a concomitant loss of PRA transferase, but retention of GAT activity.[110]

An example of compartmentalization by channelling of substrate into specific pathways is demonstrated by the carbamyl phosphate (CAP) utilization in arginine and pyrimidine biosynthetic pathways in Neurospora.[112] Neurospora contains 2 types of carbamyl phosphate synthetases, one of which is associated with ornithine transcarbamylase (OTCase) while the other is with aspartate transcarbamylase (ATCase). These transcarbamylases are branch points for arginine and pyrimidine synthesis respectively, utilizing the common substrate, CAP. From genetic studies, it appears the CAP_{pyr} synthetase and ATCase are parts of a bifunctional protein. On the other hand, indirect evidence suggests CAP_{arg} synthetase is associated with OTCase. To demonstrate compartmentalization, Davis[112] selected mutants with one or more defects in the genes coding for CAP_{pyr} synthetase, ATCase, CAP_{arg} synthetase, and OTCase. The observation was that a defect in either CAP_{pyr} or CAP_{arg} synthetase resulted in pyrimidine or arginine requirement respectively. Since both synthetases produce a common product, CAP, this would suggest that the product of each synthetase was channelled

specifically into its respective pathway and not accumulated as a common CAP pool. That the segregation was not due to membrane bound structures was shown by the use of double mutants with defects in both CAP_{pyr} synthetase and OTCase. This mutant was found to have no requirement for pyrimidine despite the defect in CAP_{pyr} synthetase. However, if this mutant was grown in arginine, the pyrimidine requirement reappears. An explanation for the observed events was that the normal channelling of CAP from CAP_{arg} synthetase to OTCase was uncoupled by the defect in the OTCase, resulting in the release of CAP which could now be utilized by ATCase for pyrimidine synthesis. When the mutant is grown in arginine, feedback mechanisms inhibit CAP_{arg} formation, resulting in insufficient CAP overflow for pyrimidine synthesis. A similar response was observed in the CAP_{arg} synthetase and ATCase double mutant, thus reinforcing this explanation.

Tryptophan synthetase from yeast and fungi is another interesting bifunctional protein[113] containing regions A and B catalysing the following equations.

Indole glycerol-3-P $\longleftrightarrow$ indole + glyceraldehyde-3-P (A)

Indole + serine $\longrightarrow$ tryptophan (B)

Normally, the indole generated by reaction A never gets released from the protein, but channels into reaction B to form tryptophan. However, a mutant in yeast was found which

had poor interactions between regions A and B.[114] As a result, the mutant exhibited drastic growth lag probably due to its inability to retain indole within the cell. The effect due to the loss of the ability to channel indole demonstrates the importance of this function.

Aspartokinase I: homoserine dehydrogenase, a bifunctional protein from E. coli catalysing non-sequential reactions shows coordinated feedback inhibition of both activities by the end product, threonine.[99,115] The two activities could be separated by isolating termination mutants[116] or by partial proteolysis of the bifunctional protein.[117]

Mechanisms resulting in multifunctional protein formation may include gene duplication, gene translocation, gene fusion and covalent peptide linkage of polypeptides.[99] In 1975, Zipkas and Riley[118] reported the interesting observation that functionally related genes of the E. coli genome are clustered at 90^{o} and 180^{o} from each other on the genetic map. The authors then concluded that the present day genome is probably the result of twice duplication of a primordial genome followed by fusion of the DNA in a circular array.

Gene fusion has been observed in a number of instances

with little or no effect on the individual gene product functions. The his D and his C genes from S. typhimurium normally give rise to separate proteins, histidinol dehydrogenase and aminotransferase respectively. However, mutants have been isolated whereby the his D and his C genes were fused resulting in both activities being on a single polypeptide.[119,120] Similarly, mutants of E. coli could be obtained whereby the lac repressor gene was fused to the β-galactosidase gene[121] giving rise to a single polypeptide with both functions.

In addition to derivation of multifunctional proteins by the process of gene fusion, it appears another mechanism, that of protein splicing may also be possible. Implication for the presence of protein splicing is the result of studies on β-galactosidase fragments and the processing of λ phage head proteins.

Termination and restart mutants at the z (β-galactosidase) gene of E. coli had been known to produce polypeptide fragments from different regions of the β-galactosidase enzyme.[122] Under certain circumstances and favorable pairings, in vitro studies demonstrated the fragments could complement each other non-covalently to form active proteins.[123,124] However, in vivo studies by Apte and Zipser,[122] using double

gal$^-$ diploids of E. coli obtained by sexduction, showed that in a number of cases, wild type β-galactosidase protein of a single type of polypeptide was obtained. That the fusion of fragments was not the result of gene recombination or suppression of a nonsense codon was proven. Although the process of recombination at the level of mRNA had not been ruled out, the authors did not think it probable. The only other probable explanation was that the protein fragments were specifically aggregated, spliced and covalently linked into a single polypeptide.

A second possible example of protein ligation was observed in the processing of proteins for head assembly in λ phage infection of E. coli. The head region of the λ genome is composed of 9 known genes, two of which are the C and E genes, physically separated by the nu3 and D genes. In the assembled λ head, 2 minor protein components, X_1 and X_2, were found to be present. From peptide mapping and molecular weight determination, the protein X_2 was concluded to be a proteolytic product of X_1.[125] More interestingly, the peptide map of X_1, when compared to those of C and E proteins, seemed to indicate X_1 to be derived partly from C and partly from E proteins. From molecular weights, X_1 could not be precursors of both C and E proteins. The part originating from the E protein was confirmed, since a single amino acid

mutation in the E protein caused an identical change in the peptide maps of both the mutated E protein and its corresponding X_1 protein. The requirement for C gene product in X_1 synthesis was confirmed when phage carrying amber mutation in C gene resulted in neither X_1 nor X_2 proteins. The probable explanation was not a read through at the DNA and mRNA level because such products should contain nu3 and D proteins. Discrete C proteins were observed in the lysate with no attached nu3 or D proteins. The authors then finally concluded the C and E proteins were probably ligated at the protein level and processed by proteolytic enzyme(s) to give the X_1 and X_2 minor proteins.

The evolution of multifunctional proteins appears to start from separate proteins to multienzyme complex to multifunctional protein.[99] This is illustrated by tryptophan synthetase which has 2 activities; an A activity where indole is liberated from indoleglycerol-3-phosphate and a B activity where indole is covalently attached to serine to give tryptophan. The organisms, B. subtilis and E. coli have the two activities on separate polypeptides aggregated into a protein of non-identical subunits. The synthetase from yeast and fungi, in contrast, is composed of a single type of polypeptide incorporating both activities.[113] The enzyme from B. subtilis could be dissociated into individual

polypeptides, retaining full B activity. The A polypeptide, however, requires interactions with the B polypeptide for maximal activity. In the case of the E. coli enzyme, both activities were reduced upon dissociation. A similar interdependence appears to exist between the two activities from yeast and fungi, although in this case, the interaction is at the level of intrapolypeptide and not at the intersubunit level. From these observations, Bonner et al[113] postulated that tryptophan synthetase probably originated from a protein with independently functioning subunits. Through evolution, the subunits developed partial dependence (B. subtilis), then total dependence (E. coli) and finally, the subunits were linked to form a multifunctional protein (yeast and fungi).

(C) STATEMENT OF THE PROBLEM

In 1973, MacKenzie[56] reported the partial purification of methyleneTHF dehydrogenase, methenylTHF cyclohydrolase and 10-formylTHF synthetase from pig liver. The point of interest was that the three activities copurified through 100-fold purification yet retained a relatively constant ratio of activities throughout the purification scheme. This is in contrast with data obtained from

Clostridia where the three activities were separable proteins.[45,47,126] Since at the time, these three folate dependent enzymes had not been purified to homogeneity from any vertebrate source, the pig liver system offered the opportunity to complete the purification of the three enzymes so that the nature of the association could be defined. Should the nature of association be multifunctional, this would place these three activities among the limited number of known cases of multifunctional proteins. In addition, understanding the interrelationship among these three activities catalyzing reactions midway between one-carbon regeneration and utilization, may give an insight into the regulation of one-carbon metabolism.

CHAPTER II

MATERIALS & METHODS

MATERIALS

Sepharose 4B, DEAE-Sephadex A-50 and A-25, 2',5'-ADP-Sepharose, Sephadex G-25, thyroglobulin and low molecular weight standards for dodecyl sulfate gel electrophoresis were obtained from Pharmacia; phosphocellulose P11 was from Whatman Biochemicals Ltd. Electrophoresis grade acrylamide, bisacrylamide, tetramethylethylenediamine, 2-mercaptoethanol, imidazole, trifluoroacetic acid, 3,5-dinitrosalicylic acid and hydrazine (+95%) were products of Eastman Chemical Co.; diethyladipate, formaldehyde, and sodium periodate were obtained from British Drug Houses. Ovalbumin, bovine serum albumin, yeast hexokinase, yeast alcohol dehydrogenase, rabbit muscle aldolase, γ-chymotrypsin (bovine pancreas), bovine liver catalase, horse spleen apoferritin, folic acid, ATP, glycylglycine, hydrazine sulfate, asparagine, soluble starch, dithiothreitol, NAD^+, $NADP^+$, Trizma base, phenylmethylsulfonylfluoride, riboflavin, dansyl chloride and dansyl amino acids were supplied by Sigma. Difco was the source for Noble agar and Freund adjuvant. Merthiolate and dimethyl suberimidate dihydrochloride were from Aldrich. ICN Pharmaceuticals and Otto C. Watzka and Co. Ltd. supplied

phenylisothiocyanate and Hyland immunodiffusion plates respectively. Polyamide sheets and dithiobis(succinimidyl propionate) were obtained from Pierce. Worthington Biochemicals was the source for trypsin, soybean trypsin inhibitor and horse liver alcohol dehydrogenase. Acetonitrile, magnesium chloride, potassium phosphate and polyethylene glycol 6000 were obtained from J.T. Baker Co., while all other common chemicals were supplied by Fisher Scientific Co.

METHODS

(A) ENZYME ASSAYS

MethyleneTHF dehydrogenase was assayed at 30°C in an incubation mixture containing 100 mM potassium phosphate, pH 7.3, 200 mM 2-mercaptoethanol, 0.2 mM (±)-tetrahydrofolate, 2.3 mM formaldehyde, and 0.2 mM $NADP^+$ in a volume of 1 ml. The reaction was terminated by addition of 1 ml of either 7% trichloroacetic acid or 0.36 N HCl after 2 to 10 min of incubation. The mixture was left at room temperature for 10 min prior to measurement of the absorbance at 350 nm. The production of 5,10-methenylTHF was calculated using $E_{350} = 24\ 900$.[127]

FormylTHF synthetase was assayed at 30°C in an incubation mixture containing 100 mM triethanolamine-HCl, pH 8, 20 mM sodium formate, 1 mM ATP and $MgCl_2$, 50 mM potassium chloride, 0.63 mM (±)-THF, and 200 mM 2-mercaptoethanol in a volume of 1 ml. The 10-formylTHF produced was measured as the methenyl derivative after acidification as described for the dehydrogenase assay.

Cyclohydrolase was assayed by following the decrease in absorbance at 355 nm of an incubation mixture containing 100 mM triethanolamine hydrochloride, pH 7.3, 200 mM 2-mercaptoethanol, and 56 μM 5,10-methenylTHF at room temperature.

Hexokinase, catalase, alcohol dehydrogenase, aldolase and β-amylase activities were assayed using established procedures.[128-132]

(B) PREPARATION OF SUBSTITUTED SEPHAROSE 4B

Adipate dihydrazide was prepared by refluxing 50 ml of diethyladipate with 100 ml of hydrazine and 100 ml of ethanol for 3 h.[133] After cooling, the hydrazide was recovered by filtration and recrystallized twice from ethanol/water.

Sepharose 4B was activated with cyanogen bromide by the method of March et al.[134] A slurry of washed Sepharose (50% gel by volume) was suspended with 1 volume of 2 M Na_2CO_3 in an ice bath, and 0.05 volume of cyanogen bromide (2 g/ml in acetonitrile) was added and stirred for 1 to 2 min. The gel was washed with 10 volumes each of cold 0.1 M $NaHCO_3$, pH 9.5; water; and 0.1 M $NaHCO_3$, pH 9.5. The activated Sepharose 4B was resuspended in 1 volume of a saturated solution of adipate dihydrazide in 0.1 M $NaHCO_3$, pH 9.5, and stirred overnight at 4^o. The Sepharose-adipate dihydrazide derivative was washed with 10 volumes each of 0.2 M NaCl, water and 0.1 M sodium acetate, pH 5.

Ligand ($NADP^+$) was attached to the Sepharose-adipate dihydrazide as described by Lamed et al.[133] For our purposes, 0.02 M $NADP^+$ was oxidized at 4^o for 1 h in the dark with 0.02 M sodium metaperiodate in 0.02 M sodium phosphate, pH 7.0. The reaction mixture was subsequently diluted with 4 volumes of 0.125 M sodium acetate, pH 5.0. For small columns, 1.25 volumes of the oxidized $NADP^+$ solution were added to 1 volume of Sepharose-adipate dihydrazide and stirred gently for 3 h in the dark at 4^o. After addition of 3.75 volumes of 2 M NaCl, stirring was continued for another 30 min, and the gel was washed with 10 volumes each of 0.2 M NaCl, water, and the buffer to be used for chromatography.

For preparative columes, 2 volumes of $NADP^+$ solution were used in the coupling reaction. The amount of $NADP^+$ in the wash was determined spectrophotometrically at 259 nm using the extinction coefficient[135] of 17 800 $cm^{-1}M^{-1}$. $NADP^+$ bound to the resin was estimated by difference to be 2.4 to 4.5 μmole/ml of packed gel. The loading capacity of the substituted Sepharose was estimated at about 120 μg of Sephadex A-25 purified enzyme/ml of packed gel by applying excess enzyme so that activity appeared in the eluate. The enzyme units bound were obtained by difference between the activity added to the column and that found in the eluate. The $NADP^+$-Sepharose lost approximately 3% bound ligand/day (calculated from the loss of A_{260}) while the binding capacity was estimated to decrease by about 5%/day. Greater than 80% of the bound enzyme could be eluted from the column with 2 mM $NADP^+$ in the starting buffer.

Sepharose to which ATP was bound was prepared in the same manner, substituting ATP for $NADP^+$.

(C) PURIFICATION OF TRIFUNCTIONAL PROTEIN

MethyleneTHF dehydrogenase, methenylTHF cyclohydrolase, and formylTHF synthetase were partially purified by the

method of Drury and MacKenzie[136] which included ammonium sulfate and polyethylene glycol fractionation followed by chromatography utilizing Sephadex A-50 and phosphocellulose columns in phosphate-glycerol buffer and a Sephadex A-25 column in phosphate buffer containing dimethylsulfoxide. The three enzyme activities were finally purified to homogeneity by affinity chromatography on $NADP^+$-Sepharose by the following procedure: enzyme after the DEAE-Sephadex A-25 chromatography was exchanged into 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3, by desalting on columns of Sephadex G-25 (0.7 X 13 cm) immediately prior to application to the $NADP^+$-Sepharose column. For analytical experiments, freshly prepared $NADP^+$-Sepharose was packed in 1-cm diameter columns ranging in height from 3.4 to 5.4 cm and equilibrated with 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3. Enzyme in the same buffer was applied to the column, washed with 10 to 30 ml of 0.06 M or 0.08 M KCl in the same buffer, and eluted by a linear gradient of $NADP^+$, KCl, or NAD^+ in the starting buffer.

Similarly, chromatography on ATP-Sepharose was done with enzyme in 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3. Following adsorption of the enzyme, the column was washed with 3 column volumes of the same buffer and eluted with a linear gradient of either ATP, CTP or KCl.

A larger scale column (2.5 X 5 cm) was used with the same buffers to prepare purified enzyme. About 1.5 mg of protein from the Sephadex A-25 purification step was solvent exchanged and applied to the column, and washed with 50 ml of 0.05 M triethanolamine-HCl, 0.08 M KCl, 20% glycerol, pH 7.3. Activity was eluted with 30 ml of the wash buffer containing 5 mM $NADP^+$.

When the affinity gel 2',5'-ADP-Sepharose, an analogue of $NADP^+$-Sepharose, became commercially available, it was used instead of the $NADP^+$-Sepharose. In this case, enzyme was diluted with 20% glycerol to reduce the potassium phosphate concentration from 0.1 to 0.04 M and was applied to the 2',5'-ADP-Sepharose column equilibrated with 0.06 M potassium phosphate, 20% glycerol, pH 7.3. The column was washed with five column volumes of this buffer and the enzyme was eluted with the buffer containing 2 mM $NADP^+$ and then concentrated with dry Sephadex G-25.

(D) AMINO ACID ANALYSIS

Amino acid composition of the trifunctional protein was determined on a Beckman Amino Acid Analyser, model 120C, modified to accommodate the single column method. Protein was hydrolysed in 6 N HCl in evacuated tubes at 110°C for

various lengths of time.Cysteine analysis by performic oxidation was by the method of Hirs[137] while tryptophan was determined spectrophotometrically by the method of Bencze and Schmid.[138]

(E) ESTIMATION AND VERIFICATION OF THE SUBUNIT MOLECULAR WEIGHT

Dodecyl sulfate polyacrylamide gel electrophoresis was carried out by the general procedure of Weber and Osborn[139] using ovalbumin, bovine serum albumin, and phosphorylase a as standards. Samples were either used directly or precipitated with 7% trichloroacetic acid and redissolved with 20μl of 0.1 N NaOH, 3% Na_2CO_3. Sucrose, 2-mercaptoethanol and dodecyl sulfate were then added to concentrations of 20%, 1% and 1% respectively. The samples were heated in boiling water for 2 min and 4μl of 0.04% bromophenol blue was added after cooling in ice. The reliability of the molecular weight determined on dodecyl sulfate polyacrylamide gel electrophoresis was verified by the method of Banker and Cotman.[140]

(F) MEASUREMENT OF NATIVE MOLECULAR WEIGHT

(a) Sedimentation in Sucrose Density Gradients.

The procedure of Martin and Ames[141] was used except the sucrose solutions were made in 0.05 M potassium phosphate, pH 7.3 containing 0.5 mM $NADP^+$ instead of 0.05 M Tris-HCl, pH 7.5. The $NADP^+$-phosphate buffer was used to stabilize the trifunctional enzyme. In addition, a SW 50.1 rotor was substituted for the SW 39 rotor.

Results from Martin and Ames showed that molecular weights determined and calculated by their method could differ by as much as 27% depending on the protein standard used. The calculation was based on the assumption that proteins are perfect spheres. With this assumption, an approximate molecular weight for proteins was obtained from equation 17;

$$S_1/S_2 = (MW_1/MW_2)^{2/3} \qquad (17)$$

where S and MW denote sedimentation constant and molecular weight respectively. The discrepancies of up to 27% in the molecular weight of an unknown protein determined with different molecular weight standards were attributed to

variations in shape among proteins, both standards and unknown, and to inaccuracies in reported molecular weights of the former. In an attempt to average out the shape factor as well as the inaccuracies of reported molecular weights for the protein standards, a standard curve was constructed from which molecular weights could be extrapolated. This was done based on the logarithmic transformation of equation 17 into a linear relationship according to equation 18.

$$\log S_1 - \log S_2 = 2/3 \log (MW_1) - 2/3 \log (MW_2) \quad (18)$$

Since S_1/S_2 is equal to (distance migrated by protein$_1$)/(distance migrated by protein$_2$)[141], which is equivalent to R_{f1}/R_{f2} where R_f is the ratio of the distance migrated by a protein to that of the total distance available for migration in the gradient. By substitution of R_f for S, equation 18 becomes:

$$\log R_{f1} - \log R_{f2} = 2/3 \log (MW_1) - 2/3 \log (MW_2) \quad (19)$$

By setting $R_{f2} = 1$, thus fixing MW_2, equation 19 becomes:

$$\log R_{f1} = 2/3 \log (MW_1) + C \quad (20)$$

where C is a constant equal to -2/3 log (MW_2). Equation 20 then becomes a typical linear equation: $y = ax + b$, where $y = \log R_{f1}$; $a = 2/3$; $x = \log (MW_1)$; $b = c$. By plotting $\log R_f$ against log (MW) for a number of known molecular weight standards, a linear relationship should theoretically be generated. However, because the geometry of the SW 50.1 rotor is slightly different from the SW 39 rotor and that proteins are not perfect spheres, the calibration curve may deviate from a straight line.

When the trifunctional folate enzyme was applied to the same centrifugation run as the standards, an apparent molecular weight could be extrapolated from the standard curve. The accuracy of the determination depends on the assumption that the shape and partial specific volume of the unknown protein are similar to those of the standards.

Protein standards for the sucrose density gradients were prepared either by dissolving in, or dialyzing against 0.05 M potassium phosphate, pH 7.3. The trifunctional enzyme was changed into the same buffer by gel filtration in Sephadex G-25. Protein samples of 0.1 ml were applied per gradient containing one or more of the following proteins: horse liver alcohol dehydrogenase (MW = 80 000[142]), yeast hexokinase (MW = 102 000[142]), yeast alcohol dehydrogenase

(MW = 141 000[142]), rabbit muscle aldolase (MW = 160 000[142]), sweet potato β-amylase (MW = 201 000[142]), bovine liver catalase (MW = 232 000[142]), or trifunctional folate enzyme (20 μg). Centrifugation was at 3°C, 37 800 rpm for 11 h and the gradients were fractionated using an Auto Densi-Flow obtained from Buchler Instruments. The proteins were located by enzymatic assays (see enzyme assays).

(b) Polyacrylamide Gel Electrophoresis.

The method was that of Hedrick and Smith[143] based on the observation that proteins differing in molecular weights when electrophoresed in polyacrylamide gels of various concentrations, the plots of logarithm of electrophoretic mobilities vs gel concentrations for each protein gave straight lines. The slopes of the lines were found to be proportional to the molecular weights of the proteins.

The procedure of Hedrick and Smith was followed except electrophoresis was at 4°C and no sample diluent buffer was used. The proteins (13 μg) in 0.06 M potassium phosphate, 20% glycerol, pH 7.3 containing 2 mM $NADP^+$ were applied directly onto the stacking gels. Molecular weight standards of bovine serum albumin (MW = 67 000[144]), bovine liver catalase (MW = 232 000[142]), horse spleen apoferritin

(MW = 443 000[142]), and bovine thyroglobulin (MW = 669 000[142]) were used.

To ensure that the folate-dependent enzyme was not inactivated by the procedure, gels containing this protein were extruded from their tubes after electrophoresis and immersed in 5 ml of methyleneTHF dehydrogenase assay mix at 30°C for 30 min. Subsequently, the gels were transferred into 5 ml of 0.18 N HCl at room temperature for at least 10 min prior to gel scanning at 350 nm to locate dehydrogenase-active protein species. The trifunctional protein was found to be active by this method but was inactive if sample diluent buffer (0.06 M imidazole-HCl, 50% glycerol, pH 5.7) was used in the electrophoretic procedure.

(c) Cross-Linking with Bifunctional Reagents.

Two bifunctional reagents, dimethyl suberimidate and dithiobis(succinimidyl propionate) have been used for cross-linking through lysyl residues. Dimethyl suberimidate, which is a popular water soluble reagent, requires pH 8.5 and reaction time of at least 3 h at room temperature for cross-linking[145], while dithiobis(succinimidyl propionate) is more reactive, effecting good cross-linking of hemoglobin in 10

min at 0^{o} and neutral pH[146]. This reagent has an intramolecular disulfide bond cleavable by sulfhydryl reagents, making the cross-linking in effect, reversible. The dimethyl suberimidate cross-link on the contrary, is stable to sulfhydryl reagents.

In cross-linking proteins, one has to contend with two events; intermolecular and intramolecular cross-linking. While intermolecular cross-links are very sensitive to protein concentrations due to the bimolecular nature of the reaction, intramolecular cross-links are insensitive. Alexander et al[147] while experimenting with bovine serum albumin, concluded that cross-linking at protein concentrations of 1 mg/ml or less resulted in almost total intramolecular modifications. Since subunit determination requires only intramolecular cross-links, coupling with dimethyl suberimidate was carried out at low protein concentrations of 10 μg/ml in 10% glycerol, 0.05 M potassium phosphate, 0.25 M triethanolamine-HCl, pH 8.5 containing 1 mM $NADP^{+}$ and 3 mg/ml dimethyl suberimidate. The reaction mixture was left overnight at room temperature prior to precipitation in 7% trichloroacetic acid. The samples were electrophoresed in dodecyl sulfate polyacrylamide gel by the method of Weber and Osborn.[139]

Cross-linking with dithiobis(succinimidyl propionate) was at 200 μg/ml protein concentration in 0.1 M triethanolamine hydrochloride, pH 7.3 containing 100 μg/ml of dithiobis(succinimidyl propionate). Reactions were at room temperature for 0.5 to 1 h and terminated with 0.05 volume of 1 M ethanolamine. Solutions corresponding to approximately 5 μg protein were made 40% glycerol, 2% dodecyl sulfate, with or without 2% 2-mercaptoethanol and boiled for 2 min. The samples were electrophoresed in Weber and Osborn dodecyl sulfate polyacrylamide gels.

Non-cross-linked proteins were treated under the same conditions as described above except deleting the cross-linkers.

To ensure that the specified conditions yielded cross-linking that denoted subunit compositions, proteins with known quaternary structures such as bovine liver catalase, rabbit muscle aldolase, porcine lactate dehydrogenase, yeast alcohol dehydrogenase, bovine hemoglobin, and ovalbumin (all tetrameric except ovalbumin which is monomeric[142]) were cross-linked under identical protein concentrations and reaction conditions as those used for the trifunctional enzyme.

Molecular weight standards were prepared by the method of Carpenter and Harrington[148] by cross-linking bovine serum albumin intermolecularly.

(G) PREPARATION AND PURIFICATION OF A METHYLENETHF DEHYDROGENASE-METHENYLTHF CYCLOHYDROLASE FRAGMENT

A methyleneTHF dehydrogenase-methenylTHF cyclohydrolase fragment was prepared and purified as follows: trifunctional enzyme was first solvent exchanged into 0.1 M triethanolamine-HCl, pH 7.3, on a Sephadex G-25 column resulting in final protein concentrations between 62 and 250 μg/ml. Proteolytic cleavage was carried out at 20°C for 1 h in 0.1 M triethanolamine-HCl, pH 7.3 containing 0.08 mM $NADP^+$ using 500μg trypsin per unit of dehydrogenase activity. When purified enzyme is used, these conditions are approximately 10:1 trypsin: enzyme on a molar basis. After digestion, two equivalents of soybean trypsin inhibitor and an equal volume of cold 40% glycerol were added and the digest was applied to an $NADP^+$-Sepharose column (1 X 5 cm) equilibrated with 0.05 M triethanolamine-HCl, 20% glycerol, pH 7.3. The column was washed with five column volumes of the starting buffer and eluted with (a) 20 ml of 0.3 M potassium phosphate, 20% glycerol, pH 7.3; (b) 10 ml of 2 mM

$NADP^+$ in the starting buffer; or (c) a linear gradient of 13 ml each of buffer and buffer containing 1.5 mM $NADP^+$. The dehydrogenase-cyclohydrolase fragment could also be purified on 2',5'-ADP-Sepharose columns using the same elution conditions as for the $NADP^+$-Sepharose column but substituting 0.04 M potassium phosphate, 20% glycerol, pH 7.3 for the starting buffer.

The concentration of $NADP^+$ was determined spectrophotometrically at 259 nm.[135]

(H) PREPARATION AND PURIFICATION OF A SYNTHETASE FRAGMENT

Native trifunctional enzyme was prepared and stored following chromatography on phosphocellulose.[136] The enzyme was further purified on a column of DEAE-Sephadex A-25[136] and dialyzed overnight against 0.1 M potassium phosphate, 20% glycerol, pH 7.3, just prior to proteolysis. Three units of freshly prepared trifunctional enzyme (4 units dehydrogenase mg^{-1} protein) was exchanged to 0.1 M triethanolamine-HCl, 0.1 M ammonium sulfate, 5 mM $MgCl_2$, pH 7.7 by filtration on a 1 X 22 cm column of Sephadex G-25. Enzyme was located by the cyclohydrolase activity and fractions immediately behind the activity peak were checked for phosphate

contamination with $CaCl_2$. Only enzyme fractions free of phosphate were used for subsequent chymotryptic cleavage.

To the solvent-exchanged trifunctional enzyme was added $MgCl_2$, ATP and chymotrypsin to final concentrations of 5.4 mM, 0.45 mM and 90 μg per ml respectively and digestion was allowed to proceed for 45 min at 30°C. The total volume was 2.2 ml containing approximately 2 units of dehydrogenase activity (approx. 0.5 mg protein). During digestion, 0.1 ml aliquots were diluted at 15 min intervals into 4 volumes of cold 0.1 M potassium phosphate, 20% glycerol, pH 7.3 containing 0.25 mM phenylmethylsulfonylfluoride (PMSF) to stop further proteolysis. These aliquots were later assayed for dehydrogenase, cyclohydrolase and synthetase activities to assess the degree of digestion. Aliquots were also electrophoresed on dodecyl sulfate polyacrylamide gels. After 45 min of digestion, 0.02 volumes of 10 mM PMSF in 30% isopropanol was mixed into the remaining digest and incubated at room temperature for 10 min. The sample was chilled, diluted 4-fold and made to 20% glycerol prior to application to a 0.8 X 10.5 cm column of 2',5'-ADP-Sepharose in 0.04 M potassium phosphate, 20% glycerol, pH 7.3. After washing with 3 column volumes of this buffer, dehydrogenase activity was eluted with either 2 mM $NADP^+$ in 0.04 M potassium phosphate, 20% glycerol, pH 7.3, or 0.5 M potassium

phosphate, 20% glycerol, pH 7.3.

The enzyme which did not bind to the 2',5'-ADP-Sepharose column contained only synthetase activity. Fractions containing this activity were pooled and applied directly onto a 0.8 X 12 cm column of phosphocellulose equilibrated with 0.04 M potassium phosphate, 20% glycerol, pH 7.3. This column was washed with 3 column volumes of the buffer and eluted with this buffer containing 10 mM MgATP readjusted to pH 7.3 with potassium hydroxide. Fractions containing synthetase activity were pooled and concentrated 5-fold by vacuum dialysis against 0.04 M potassium phosphate, 20% glycerol, pH 7.3 at 4°C, and stored in the presence of 5 to 10 mM ATP.

The synthetase fragment was separated from uncleaved enzyme and identified using two dimensional polyacrylamide gel electrophoresis. The first dimension was carried out under non-denaturing conditions while the second used a dodecyl sulfate slab gel. Discontinuous cylindrical polyacrylamide gels were prepared according to the method of Hedrick and Smith[143] using 8% gels, but contained 20% glycerol in both stacking and separation gels and were electrophoresed at 4°C. Approximately 20 μg protein was applied per gel. Upon completion of electrophoresis, the gels were

extruded, immersed in 5 ml of synthetase assay mix for 20 min at 30°C and transferred to 5 ml of 0.18 N HCl for at least 10 min prior to gel scanning at 350 nm to locate synthetase-active proteins. Subsequently, the cylindrical gels were sliced in half longitudinally with one half being stained with Coomassie Brilliant Blue for protein while the other half was electrophoresed in the second dimension on a 9% polyacrylamide slab gel in dodecyl sulfate. The dodecyl sulfate gel electrophoresis was essentially that described by Laemmli[149] except that the sample gels were equilibrated against 5 ml of the Laemmli sample buffer with the addition of NaOH for neutralization and were fixed on top of the stacking gel with 1% agar in the stacking buffer. The synthetase-active segments of a second cylindrical gel were excised and electrophoresed on the same slab gel. Molecular weight protein standards were photopolymerized in 8% gels of Hedrick and Smith[143] using riboflavin instead of ammonium persulfate, processed and electrophoresed in parallel with the sample gels. Dodecyl sulfate polyacrylamide gels of other protein samples followed the procedure of Laemmli.[149]

(I) IMMUNOCHEMISTRY

Antibodies to the trifunctional protein were prepared following the injection scheme of Harboe and Ingild.[150] New Zealand White Albino rabbits weighing about 2.5 Kg each were injected with 100 to 200 μg purified trifunctional enzyme, emulsified in a volume of Freund incomplete adjuvant, at 2 week intervals for the initial 6 weeks. Subsequently, booster shots were given every 6 weeks. Bleeding was from an ear artery and antiserum was obtained through centrifugation of the clotted blood. The globulin fraction was partially purified by ammonium sulfate precipitation by the method of Campbell et al.[151] The pellet was dissolved in 25% of the original volume and dialyzed exhaustively against borate-saline. The antibody preparation containing 0.01% merthiolate was stored frozen at -20°C. Normal serum was similarly prepared from rabbits that did not receive injections. A quantitative precipitin test using the method of Campbell et al[151] gave an antibody titer of at least 0.1 mg/ml. The accuracy was limited by the amount and concentration of pure antigen that can be obtained from pig liver. Immunodiffusion was done on ready to use Hyland immunodiffusion plates. Undiluted antibody preparation was placed in the center well and dehydrogenase-cyclohydrolase fragment; synthetase fragment, purified to the stage of the

phosphocellulose column; and the uncleaved enzyme were added to peripheral wells. Normal serum was added as a control. The plates were developed at room temperature in a humid chamber.

(J) AMINO-TERMINAL SEQUENCE

The methods of Gros and Labouesse[152] and Weiner et al[153] were used for N-terminal analysis based on the dansylation of the N-terminal amino acid. The former method was slightly modified such that following dansylation, an ether extraction was used to reduce the amount of dansic acid which interferred with dansyl amino acid identification. The Weiner method was also used for sequence determination using Edman degradation to expose new N-terminals. Approximately 5 nmole polypeptide were used per analysis. In all cases, dansyl amino acids were identified using the the method of Bertrand et al[154] by their position on two-dimensional thin layer chromatography relative to standard dansyl amino acids. The identity of each dansyl amino acid was confirmed by demonstrating that it co-chromatographed with only one of the standards.

The N-terminal analysis of the synthetase fragment

was modified since following chromatography on the phosphocellulose column, there remained a residual amount of uncleaved protein in the preparation. To separate the fragment from the uncleaved protein, approximately 100 μg of synthetase fragment preparation, purified on the phosphocellulose column was concentrated 2-fold with Sephadex G-25 and precipitated with 7% trichloroacetic acid. The precipitate was dissolved in 0.11 ml of 1% dodecyl sulfate, 0.2 M $NaHCO_3$, pH 9.8 and boiled for 2 min. Dansylation was accomplished by incubating for 20 min at 37°C with 0.5 volumes of 0.5% W/V dansyl chloride in acetone.[153] Dansylated protein was made 1% in 2-mercaptoethanol and incubated at 37°C for an additional 20 min. Bromophenol blue and a drop of glycerol was added prior to electrophoresis in a 0.6 X 8 cm cylindrical 9% Laemmli gel at 4 milliamps per gel.

Following electrophoresis, the gel was extruded and viewed unstained under ultraviolet light. Degradative products of dansyl chloride, which were the major fluorescent species, were stacked with the bromophenol blue thus eliminating interference from locating the fluorescent protein bands. The band corresponding to the synthetase fragment was removed, homogenized and extracted in 5 volumes of 50 mM $NaHCO_3$, 0.1% dodecyl sulfate in a 37°C shaker for 4 hours followed by an overnight extraction with a second aliquot

of 5 volumes. The extracts were combined and dried under a stream of nitrogen aided by a 250 W heat lamp. The residue was washed with 0.5 ml of 10% trichloroacetic acid followed by 0.2 ml of 1 N HCl prior to hydrolysis in 0.1 ml of 6 N HCl in an evacuated tube at 110°C for 4 to 5 h for dansyl amino acid identification.

CHAPTER III

RESULTS

(A) PURIFICATION OF ENZYMES

Completion of the purification of methyleneTHF dehydrogenase, methenylTHF cyclohydrolase and 10-formylTHF synthetase was accomplished by the addition of an affinity chromatographic step to previously established procedures.[136] At no stage of the 535-fold purification was there a separation of these three activities.

Affinity chromatography utilizing the affinity ligand, $NADP^+$, was based on the rationale that $NADP^+$ is a substrate only of the dehydrogenase. The choice of buffer for this column was important. When the enzyme in 0.05 M potassium phosphate, 20% glycerol, pH 7.3, was applied to an $NADP^+$-Sepharose column equilibrated with the same buffer, the activities appeared almost entirely in the wash. However, if the sample was first solvent exchanged into 0.05 M triethanolamine-HCl, 20% glycerol, pH 7.3, on a Sephadex G-25 column, and applied to an $NADP^+$-Sepharose column equilibrated with this buffer, the enzyme was adsorbed. Washing with 10-column volumes of 0.06 M KCl in starting buffer resulted in no leakage of enzyme, but the addition of 2 mM $NADP^+$ to this buffer

Table 1

PURIFICATION OF TETRAHYDROFOLATE ENZYMES

Fraction	Volume	Protein	Enzyme Activity			Purification	(yield[a])
			Dehydrogenase	Synthetase	Cyclohydrolase		
	ml	*mg*		μmol *min*$^{-1}$			
Supernatant solution	1,100	41,400	560	1,028	1,056	1	(100)
Ammonium sulfate	500	16,200	379	948	827	1.7	(68)
Polyethylene glycol 6000	84	6,400	234		659	2.7	(42)
Sephadex A-50	95	536	147	289	500	20	(26)
Phosphocellulose	11	78	95	194	305	90	(20)
Sephadex A-25[b]	8	7.6	30	58	89	291	(5.5)
NADP-Sepharose	9	2.8	21	35[c]	61	535	(3.8)

[a] Calculated for the dehydrogenase.

[b] Corrected values presented for fractionation steps normally performed on a smaller scale (see Experimental Procedures).

[c] Variable due to instability of the synthetase activity.

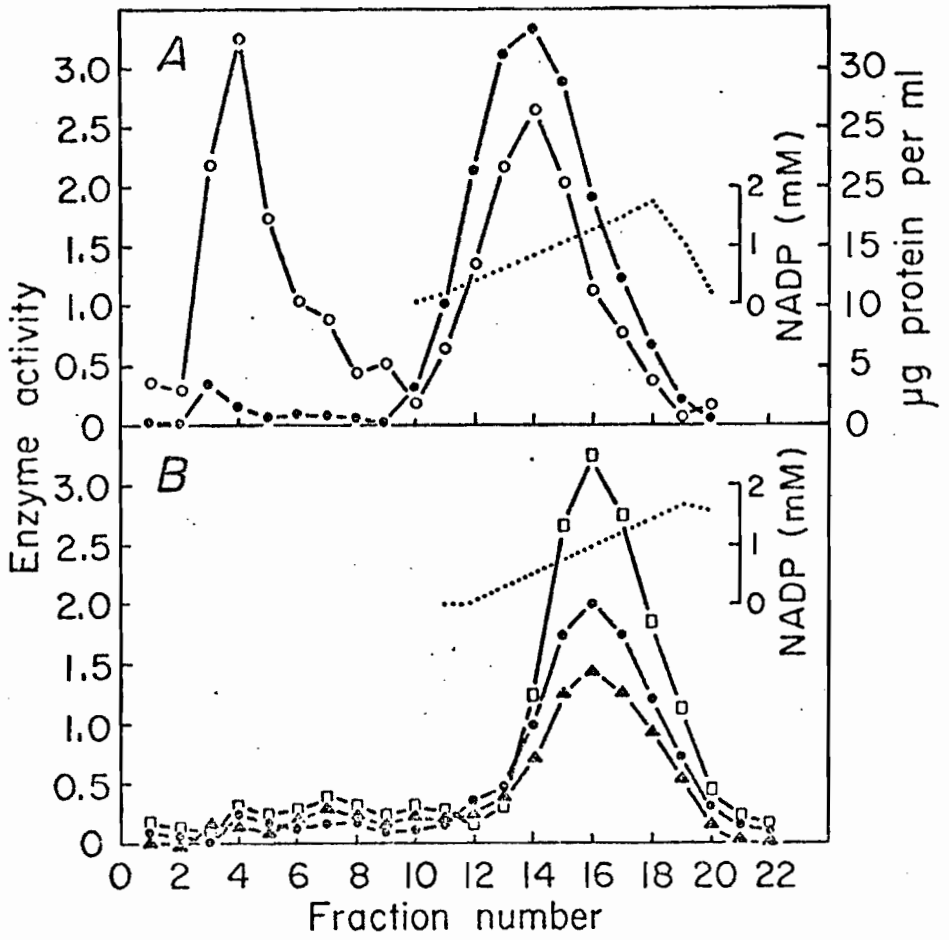


Figure 3: Chromatography of DEAE-Sephadex A-25-purified enzyme on $NADP^+$-Sepharose. A. Approximately 0.5 mg of protein in 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3, was applied to a column (0.7 X 13 cm) and washed with 10 ml of the buffer containing 60 mM KCl. A linear gradient of 10 ml each of starting buffer and buffer containing 2 mM $NADP^+$ was applied and followed with another 10 ml of buffer. Dehydrogenase activity is expressed as 25 X μmol $min^{-1}ml^{-1}$. B. A similar experiment using a column (1 X 5.4 cm) of $NADP^+$-Sepharose washed with 10 ml of 80 mM KCl in starting buffer and eluted with $NADP^+$ as above. Enzyme activities are expressed as μmol $min^{-1}ml^{-1}$ adjusted by the following factors: ●, dehydrogenase (20X); □, synthetase (15X); and ▲, cyclohydrolase (6X); o, Lowry protein; ...., $NADP^+$.

eluted the majority of the enzyme in a single, well defined peak. Because the enzymes are not particularly stable under these conditions, affinity chromatography must be completed as quickly as possible. While the dehydrogenase and cyclohydrolase decrease in activity by about 10% in 4 h, the synthetase loses 50% of its activity during the same time. This instability accounts for the variable synthetase acitvity of the purified enzyme (Table 1).

The elution of the three enzyme activities from the $NADP^+$-Sepharose column is presented in figure 3. A gradient of $NADP^+$ resulted in elution of all three enzyme activities with the same profile where the peak fraction eluted at 1 mM $NADP^+$. Attempts to elute the enzymes (figure 4) using a KCl gradient resulted in a very broad and ill defined elution profile where about half the enzyme was eluted at 0.25 M KCl. Further evidence for the specificity of the $NADP^+$-Sepharose column was provided by an attempt to elute the enzyme with NAD^+ which was unsuccessful even with concentrations as high as 12 mM.

Preparative affinity chromatography using a step elution with $NADP^+$ yielded the protein with dehydrogenase activity of 7.5 μmole $min^{-1}mg^{-1}$ representing a 535-fold purification. Substituting 2',5'-ADP-Sepharose for $NADP^+$-

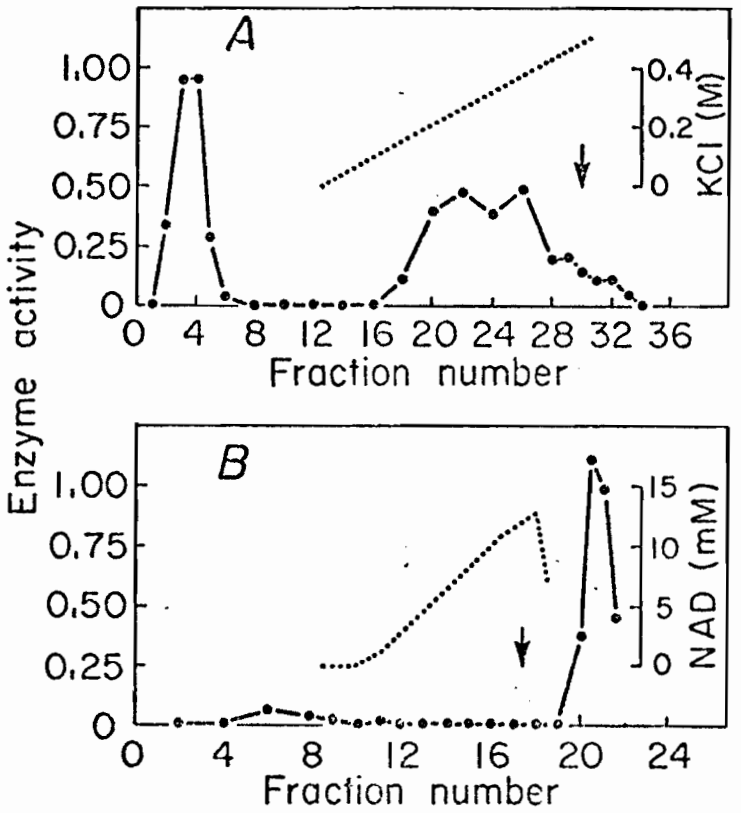


Figure 4: KCl and NAD^+ elution profile of trifunctional protein on $NADP^+$-Sepharose columns. A. Trifunctional protein was applied onto an $NADP^+$-Sepharose column (0.7 X 9 cm), equilibrated with 0.05 M triethanolamine-HCl, 20% glycerol, pH 7.3. The column was washed with 20 ml of the same buffer and eluted with a linear gradient of 20 ml each of the same buffer and buffer containing 0.5 M KCl. B. Trifunctional protein was adsorbed onto an $NADP^+$-Sepharose column (1 X 6 cm), washed with 10 ml of 80 mM KCl in the above buffer, followed by a linear gradient of 10 ml each of the starting buffer and the buffer containing 15 mM NAD^+. The gradient was followed by 5 ml of the starting buffer. Arrows mark the application of 10 ml of 0.5 M potassium phosphate, pH 7.3. Enzyme activities are in units per ml. Symbols are: •, dehydrogenase activity; ...., elution gradient.

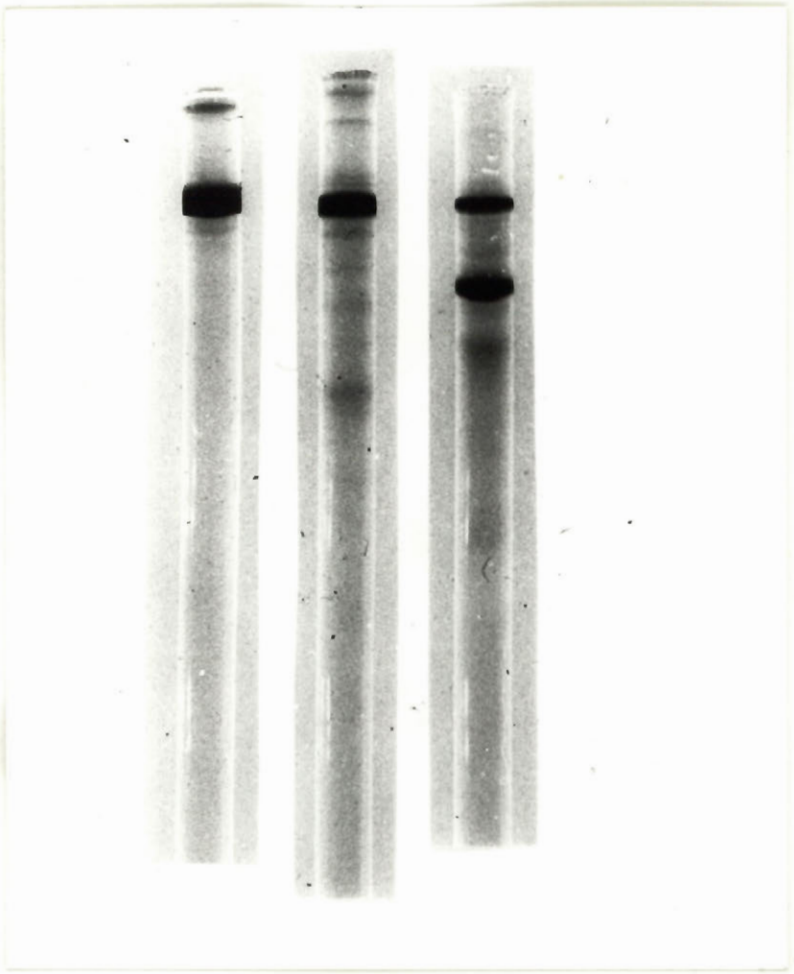


Figure 5: Equivalent amounts of protein (10 μg) from $NADP^+$-Sepharose, Sephadex A-25 and phosphocellulose steps of purification electrophoresed on 9.5% polyacrylamide gels.

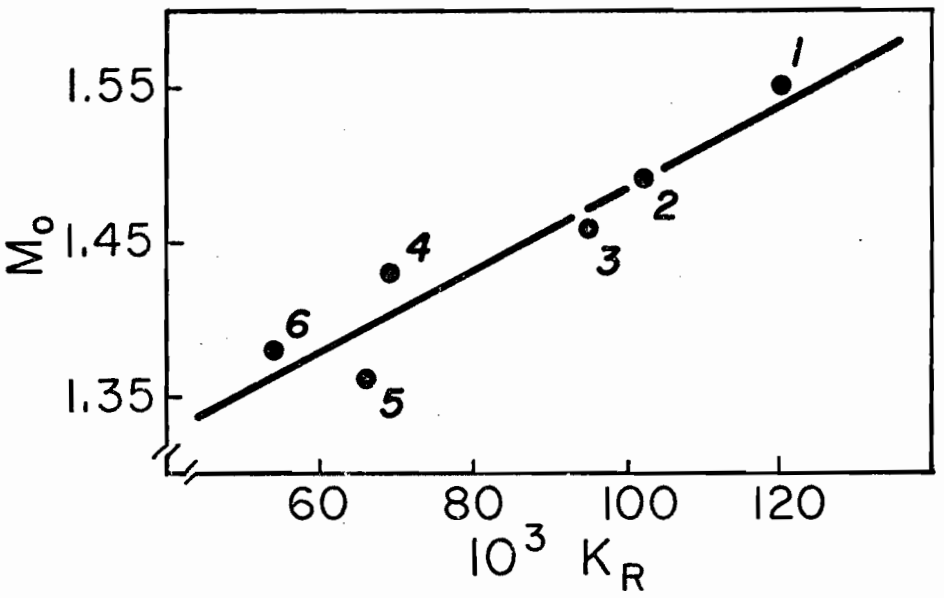


Figure 6: Plot of the free electrophoretic mobility (M_o) against retardation coefficient (K_R), as described by Banker and Cotman[140] for the trifunctional protein (2), and the standard proteins: 1, β-galactosidase; 3, phosphorylase a; 4, catalase; 5, pyruvate kinase; 6, ovalbumin.

Sepharose resulted in equal or better purification. Purification of the dehydrogenase, cyclohydrolase and synthetase activities is summarized in Table 1.[136]

A comparison of the dodecyl sulfate gel electrophoresis patterns of the last three purification steps is shown in figure 5. The $NADP^+$-Sepharose eluate is represented by one major band of molecular weight 100 000 daltons.

The validation experiment for the dodecyl sulfate gel molecular weight determination indicates that the protein does not have anomalous mobility since its position in a plot of free electrophoretic mobility (Mo) versus retardation coefficient (K_R) falls on a straight line generated by five standard proteins (figure 6).

A second type of affinity chromatography was carried out using ATP-Sepharose which had 3.6 μmole of ATP/ml of packed gel. This chromatography, designed to utilize binding at the synthetase site, was not as specific as the $NADP^+$ column (figure 7). However, a linear gradient of ATP eluted the three activities together, with the peak fraction appearing at 1.1 mM ATP. Although the activities could be eluted with a gradient of CTP, a 4-fold higher concentration was required, indicating some degree of specificity. When

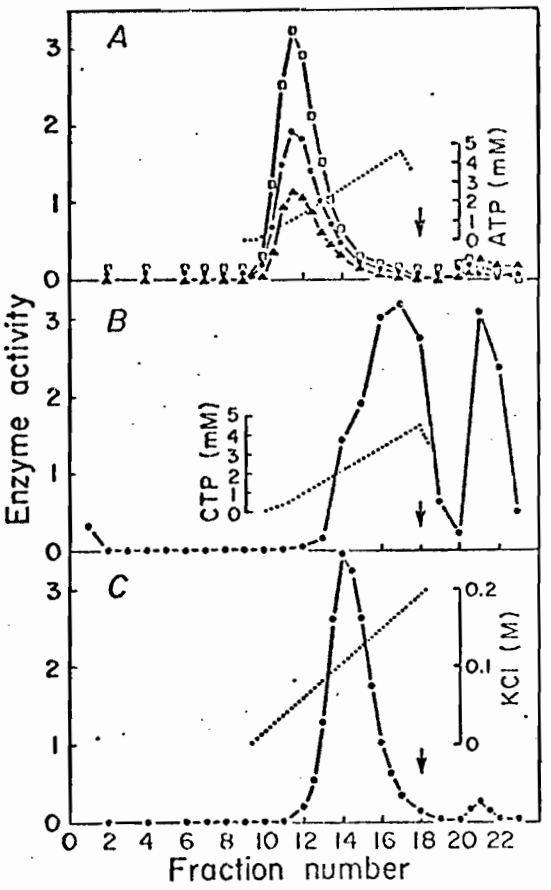


Figure 7: Chromatography of DEAE-Sephadex A-25-purified enzyme on columns (1 X 4.4 cm) of ATP-Sepharose. Aliquots of protein (0.5 mg) were solvent exchanged to 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3, on a Sephadex G-25 column prior to application to the ATP columns. After washing with 10 ml of this buffer, linear gradients composed of 10 ml each of the following in starting buffer (readjusted to pH 7.3 where necessary) were applied: A, 0 and 5 mM ATP; B, 0 and 5 mM CTP; C, 0 and 0.2 M KCl. The gradients were followed with 5 ml of buffer and the application (arrow) of 10 ml of 0.5 M potassium phosphate, pH 7.3. Enzyme activities are expressed as μmol $min^{-1}ml^{-1}$, adjusted by the following factors: A, dehydrogenase (15X), synthetase (12X), and cyclohydrolase (2X); B, dehydrogenase (50X); C, dehydrogenase (25X). ●, dehydrogenase; □, synthetase; ▲, cyclohydrolase.

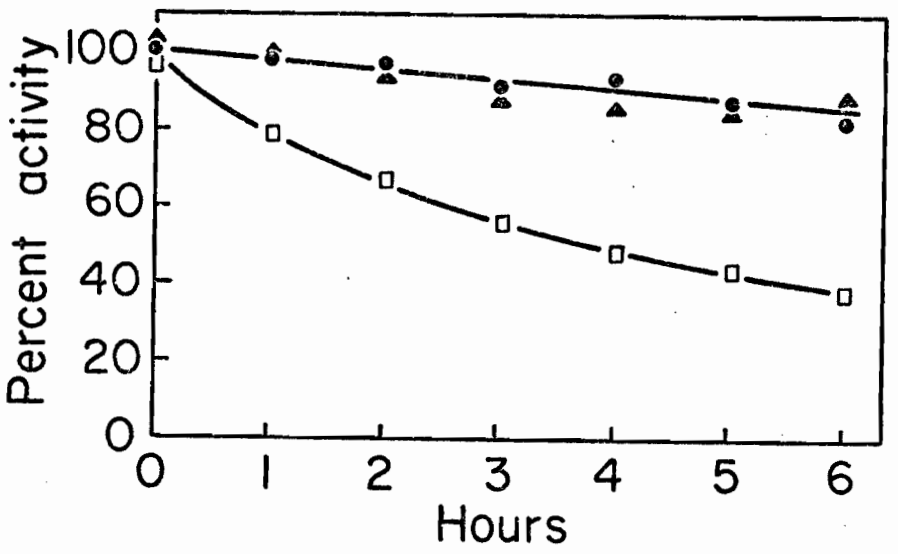


Figure 8: Trifunctional protein purified from the Sephadex A-25 column was stored in 0.05 M triethanolamine hydrochloride, 20% glycerol, pH 7.3 at 4°C. Protein concentration was 0.2 mg/ml. Symbols are: ●, dehydrogenase; ▲, cyclohydrolase; □, synthetase.

compared with ATP, a relatively high concentration of KCl (100 mM) was required to elute the activities, although this was not as high as that required in the case of the $NADP^+$-Sepharose.

(B) STABILITIES OF THE THREE ENZYME ACTIVITIES

Trifunctional protein purified from the Sephadex A-25 column was solvent exchanged to 0.05 M triehtanolamine-HCl, 20% glycerol, pH 7.3 on a Sephadex G-25 column. The enzyme was stored at 4^oC at a protein concentration of 0.2 mg/ml and enzyme activities were determined with time (figure 8). The dehydrogenase and cyclohydrolase were found to inactivate at the same rate but slower than the synthetase activity.

(C) NATIVE MOLECULAR WEIGHT OF THE TRIFUNCTIONAL ENZYME

The molecular weight determined by the method of Martin and Ames on sucrose density gradients (figure 9) was 170 000 daltons.

Gel scanning of the purified protein electrophoresed in Hedrick and Smith polyacrylamide gels under non-denaturing

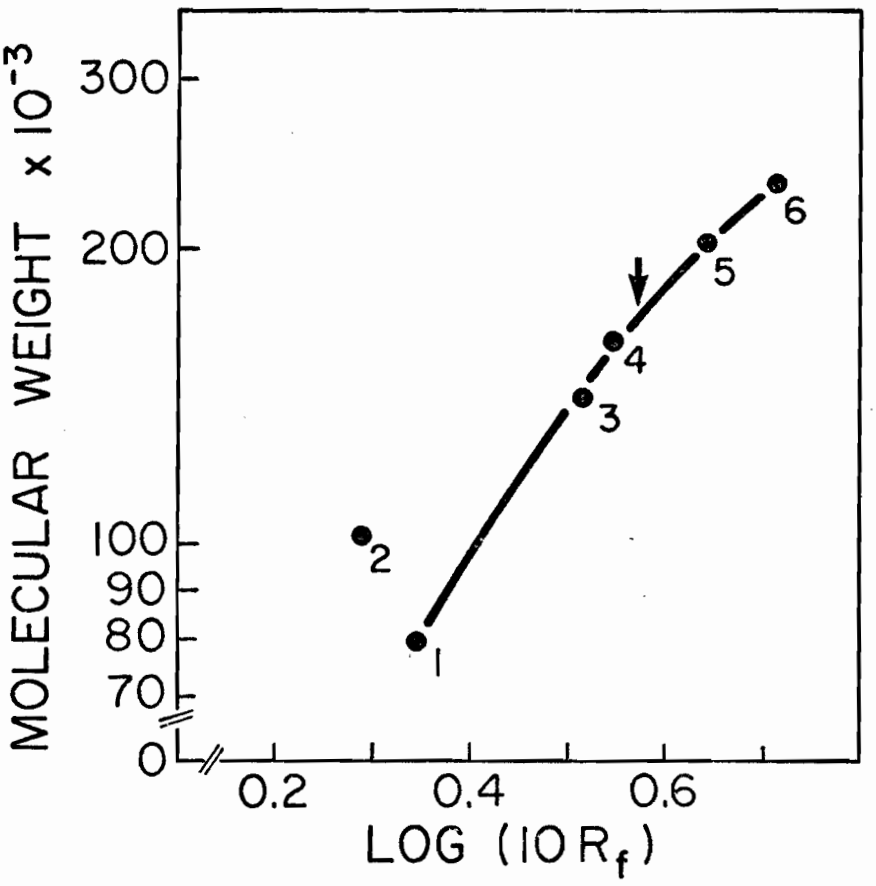


Figure 9: Native molecular weight determination by the method of Martin and Ames.[141] Protein standards are: 1, horse liver alcohol dehydrogenase, 80 000; 2, yeast hexokinase, 102 000; 3, yeast alcohol dehydrogenase, 141 000; 4, rabbit muscle aldolase, 160 000; 5, β-amylase, 201 000; 6, bovine liver catalase, 232 000. The arrow marks the apparent molecular weight of the trifunctional folate-dependent enzyme.

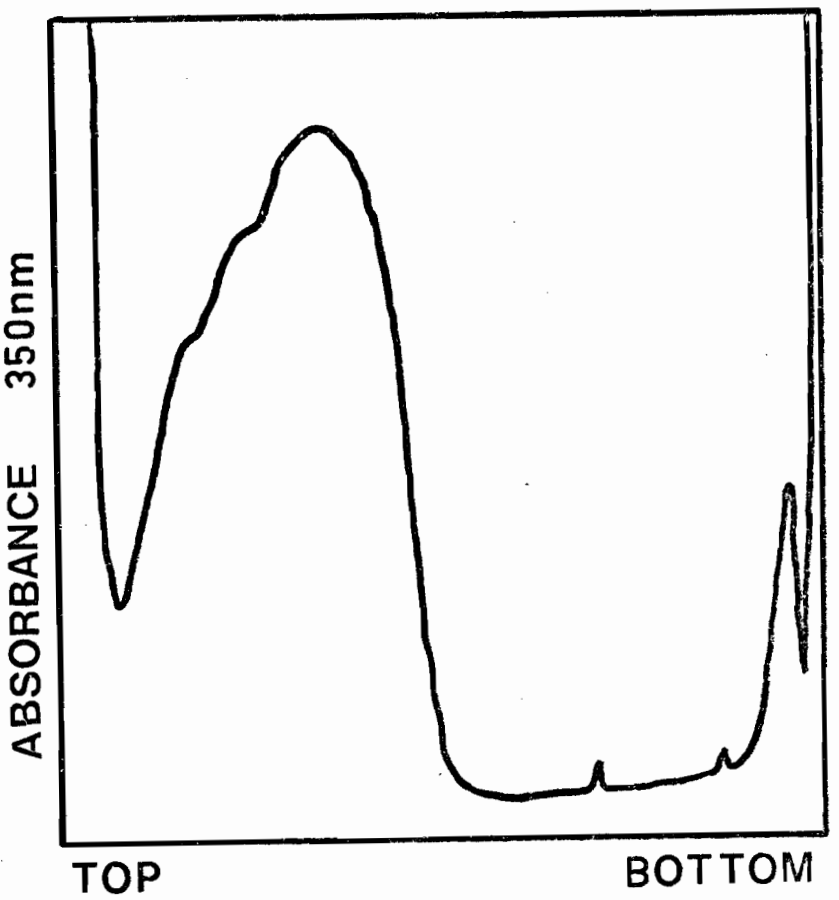


Figure 10: Trifunctional protein (13μg) is electrophoresed in non-denaturing 8% Hedrick and Smith polyacrylamide gels.[143] Following electrophoresis, the gel was immersed in methylene-THF dehydrogenase assay mix, acidified, and gel scanned at 350 nm. Absorbance at 350 nm corresponds to dehydrogenase activity.

conditions is shown in figure 10. The absorbance at 350 nm represents methyleneTHF dehydrogenase activity under the assay conditions. Three dehydrogenase-active species were found corresponding to the two shoulders and a peak at 350 nm. The actual protein bands in the gel were discrete and well separated although the activity peaks appear diffuse. This is because detection at 350 nm is the absorbance of a small molecular weight product, methenylTHF, generated by the dehydrogenase activity.

The arrows in the calibration curve for the Hedrick and Smith gels (figure 11) mark the position of two of the dehydrogenase-active species observed in figure 10. From extrapolation, molecular weights of 230 000 and 325 000 daltons, corresponding to dimers and trimers were estimated, with the majority being the dimer. The third and most retarded dehydrogenase-active species was probably a tetramer.

Quaternary structure determined by cross-linking proteins with dimethyl suberimidate and dithiobis(succinimidyl propionate) is shown in figure 12. The control, lactate dehydrogenase, when cross-linked with dimethyl suberimidate showed four discrete bands of about equal intensity on dodecyl sulfate gels, illustrating its tetrameric structure. Ovalbumin, a monomer, exhibited no difference between the

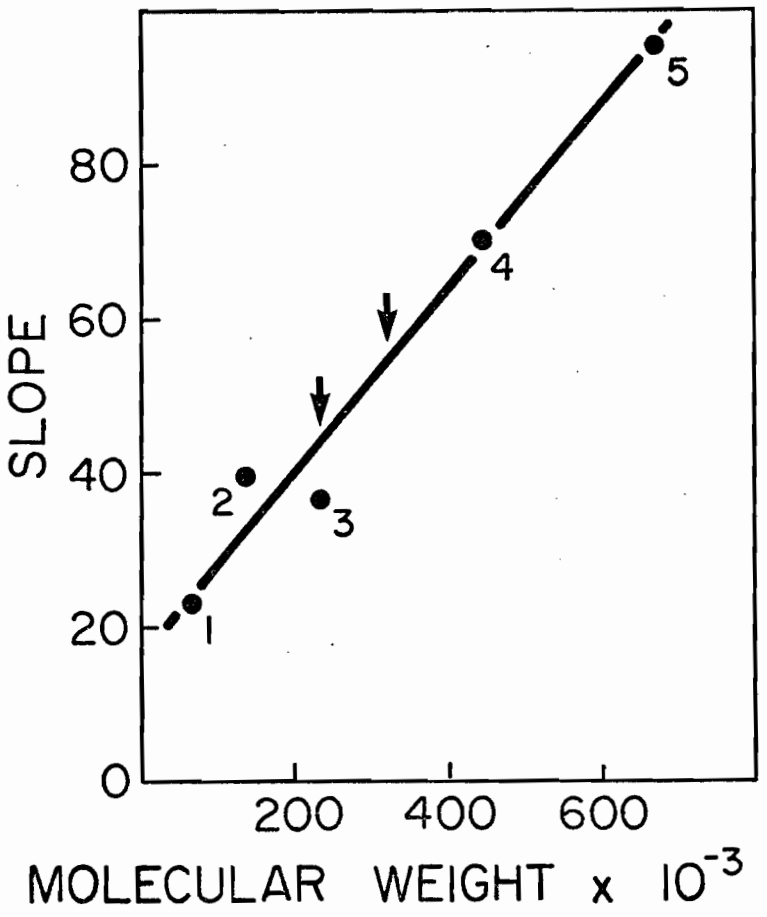


Figure 11: Calibration curve for native molecular weight determination by the method of Hedrick and Smith.[143] Protein standards are: 1, bovine serum albumin monomer, 67 000; 2, bovine serum albumin dimer, 134 000; 3, bovine liver catalase, 232 000; 4, horse spleen apoferritin, 443 000; 5, thyroglobulin, 669 000. The two arrows indicate the apparent molecular weights of the trifunctional protein with the majority being of the lower molecular weight species.

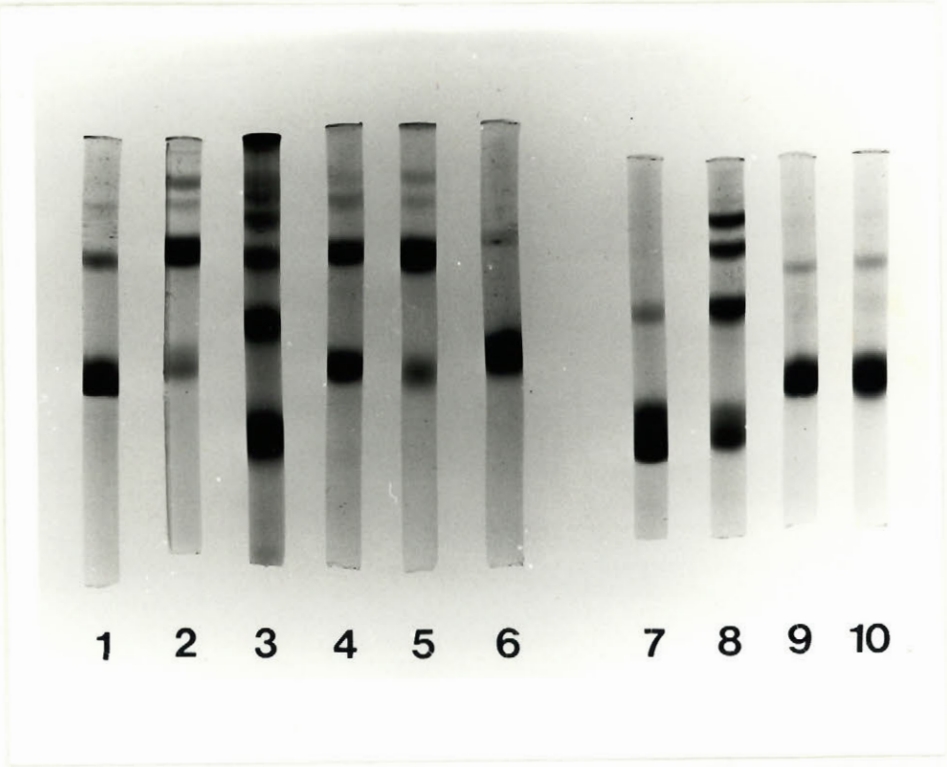


Figure 12: Cross-linking of the trifunctional protein followed by dodecyl sulfate polyacrylamide gel electrophoresis on 4% and 6% Weber and Osborn gels grouped on the left and right respectively. From left: gels 1 and 2 represent trifunctional enzyme which was non-cross-linked and cross-linked with dimethyl suberimidate respectively; gel 3 is a molecular weight standard with bands corresponding to 67 000, 134 000, 201 000, 268 000, 335 000 and 402 000 daltons; gels 4 and 5 contained trifunctional enzyme which was not reduced prior to electrophoresis. Gel 4 was not cross-linked while gel 5 was cross-linked with dithiobis(succinimidyl propionate); gel 6 was the duplicate of gel 5 except it was reduced with 2-mercaptoethanol prior to electrophoresis; gels 7 to 10 are controls run under the same conditions as gels 1 and 2 containing, respectively, non-cross-linked and cross-linked lactate dehydrogenase (tetrameric) and non-cross-linked and cross-linked ovalbumin (monomeric). Aliquots of 10 μg protein were applied per gel.

cross-linked and non-cross-linked sample, demonstrating insignificant amounts of intermolecular cross-linking. The same results were obtained when the cross-linker dithiobis-(succinimidyl propionate) was used. Cross-linking of other tetrameric proteins (catalase, aldolase, yeast alcohol dehydrogenase, hemoglobin) using dimethyl suberimidate, all resulted in four bands on dodecyl sulfate gels. However, the effect of dithiobis(succinimidyl propionate) on the same proteins was less pronounced, and dodecyl sulfate gels showed either single (no cross-linking) or four bands depending on the protein being cross-linked.

Cross-linking of trifunctional protein with dimethyl suberimidate results in four bands of various intensities on dodecyl sulfate polyacrylamide gels. From a calibration curve constructed from molecular weight standards of intermolecularly cross-linked bovine serum albumin, a molecular weight of 100 000, 200 000, 300 000 and 400 000 daltons were assigned to the bands corresponding to monomer, dimer, trimer and tetramer. The major cross-linked species was the dimer with small amounts of trimer and tetramer. The trimer and tetramer were probably derived from aggregates of the trifunctional protein that were observed in the Hedrick and Smith gels.

Table 2

Amino Acid Composition of Trifunctional Protein

Amino Acids	Moles per subunit			
	24 hrs	48 hrs	72 hrs	Nearest Integer
Lysine	61.5	61.8	62.8	62
Histidine	20.8	21.2	21.6	21
Arginine	36.2	38.3	37.3	37
Aspartic acid	81.4	79.7	80.1	80
Threonine	55.4	54.0	53.2	57
Serine	46.1	43.0	42.0	48
Glutamic Acid	96.5	94.3	92.5	94
Proline	59.6	55.4	53.7	56
Glycine	93.7	105.5	98.3	99
Alanine	81.9	81.2	79.6	81
½ Cystine	22.1[b]	-	-	22
Valine	61.0	64.2	63.3	63
Methionine	16.4	17.1	17.1	17
Isoleucine	55.8	56.4	58.5	58
Leucine	89.4	88.5	88.7	89
Tyrosine	12.5	11.9	12.9	12
Phenylalanine	29.2	29.8	31.4	30
Tryptophan	-	-	-	10[a]

(a) determined by the method of Bencze and Schmid[140]
(b) determined by the method of Hirs[139]

Cross-linking of the trifunctional protein with dithiobis(succinimidyl propionate) gave results which were similar to those observed for dimethyl suberimidate. In the absence of 2-mercaptoethanol, the non-cross-linked sample showed significant amounts of dimer. Addition of the bifunctional reagent increases the amount of dimers and higher molecular weight species. However, if the cross-linked protein was treated with 2-mercaptoethanol prior to electrophoresis, the amount of dimer was significantly decreased, demonstrating cleavage of disulfide bonds.

(D) AMINO ACID COMPOSITION OF THE TRIFUNCTIONAL PROTEIN

The amino acid composition is shown in Table 2. The values for serine and threonine were extrapolated to zero time of hydrolysis. Isoleucine and leucine levels were obtained from the 72 hours hydrolysis. All other amino acids were the average of the three determinations.

(E) PREPARATION, PURIFICATION AND PROPERTIES OF A DEHYDROGENASE-CYCLOHYDROLASE FRAGMENT

A dehydrogenase-cyclohydrolase fragment could be

Table 3

EFFECT OF TRYPSIN ON THE SYNTHETASE AND DEHYDROGENASE ACTIVITIES

Enzyme was purified through DEAE-Sephadex A-25 and was digested with trypsin for 1 h as described in Materials and Methods. The mixture contained 160 μg protein and 0.26 units dehydrogenase activity per ml.

μg trypsin/unit dehydrogenase	Activity (%)	
	Dehydrogenase	Synthetase
0	100	100
5	95	66
50	85	14
500	83	0.7

Table 4

RECOVERIES OF ENZYME ACTIVITIES FOLLOWING TRYPSIN CLEAVAGE
Native enzyme preparations from different purification steps were used for trypsin digestion. The ratio of trypsin to enzyme activity was kept at the approximate ratio of 500 μg per unit of dehydrogenase activity. The specific activities of the DEAE-Sephadex A-25, $NADP^+$-Sepharose and 2',5'-ADP-Sepharose enzyme preparations were 1.6, 4.7, and 5.1 units per mg, respectively.

Purification step	Dehydrogenase (%)	Cyclohydrolase (%)	Synthetase (%)
DEAE-Sephadex A-25	109	92	2
$NADP^+$-Sepharose	110	138	5
2',5'-ADP-Sepharose	79	119	3

prepared by proteolytic digestion of the multifunctional protein only under conditions where the protein structure is not stabilized. Consequently, potassium phosphate buffers or solutions containing glycerol could not be used. Digestion was carried out in triethanolamine-HCl, pH 7.3, where all the activities, but particularly the synthetase, are somewhat labile. $NADP^+$ was added to the buffer to stabilize the dehydrogenase activity of both the native protein and the fragment produced during proteolysis.

Digestion of the trifunctional enzyme with trypsin results in loss of synthetase activity, with optimal results being obtained with rather high amounts of trypsin, 500 μg per unit of dehydrogenase activity, as shown in Table 3. Using these conditions for proteolytic digestion of enzyme at different stages of purification, it was observed that the synthetase could be almost completely inactivated, with little change in the dehydrogenase and cyclohydrolase activities (Table 4). If $NADP^+$ is excluded, trypsin treatment reduces the dehydrogenase to less than 40% of the original activity after digestion for 1 h. It is not clear if the loss of dehydrogenase is due to proteolytic cleavage, since the greater instability of the fragment must certainly be a contributing factor. $NADP^+$ did not protect the synthetase from proteolysis, but 2 mM MgATP was somewhat effective in

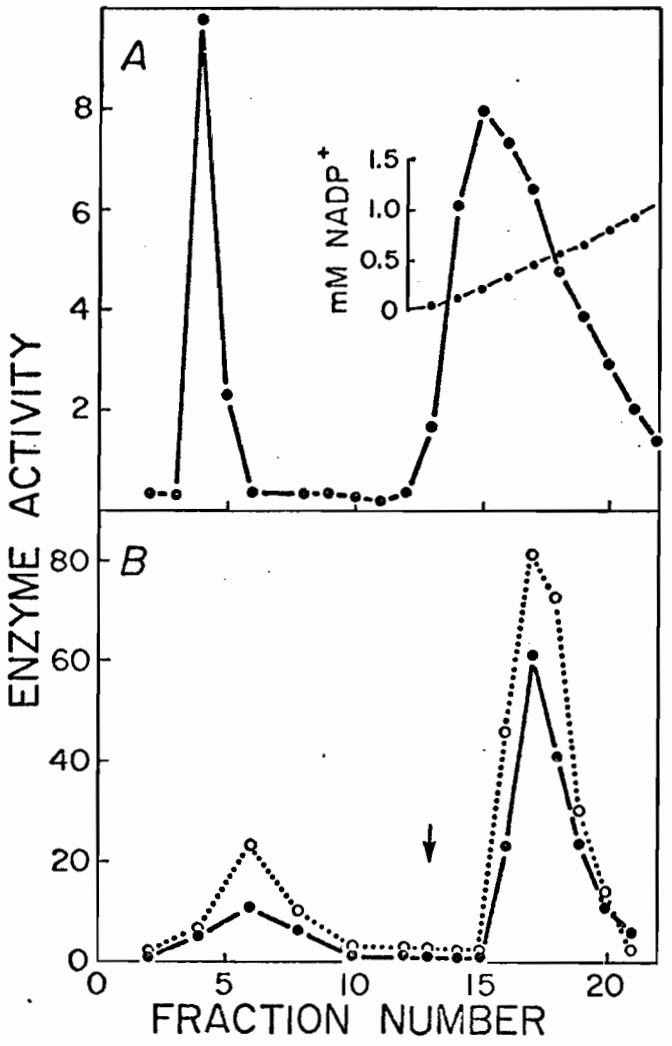


Figure 13: (A) Enzyme (100 μg) purified through chromatography on DEAE-Sephadex A-25, was treated with trypsin and applied to an $NADP^+$-Sepharose column. The dehydrogenase activity was eluted with a linear gradient of $NADP^+$. (B) Enzyme (200 μg) previously purified on an $NADP^+$-Sepharose column, was digested with trypsin and applied to a second $NADP^+$-Sepharose column. The fragment was eluted with 2 mM $NADP^+$ as in Materials and Methods. Symbols are: ●, dehydrogenase; and o, cyclohydrolase. Activities are expressed as units/ml X 10^3.

this regard.

Attempts at purifying an active fragment with dehydrogenase activity on an $NADP^+$-Sepharose column were successful and the results are shown in figure 13. The adsorbed enzyme fragment was eluted with a gradient of $NADP^+$ and the enzyme activity peak corresponded to an $NADP^+$ concentration of 0.2 mM (figure 13a). In figure 13b, a step elution using the starting buffer containing 2 mM $NADP^+$ showed that the dehydrogenase and the cyclohydrolase activities copurified. The synthetase activity was undetectable throughout the column.

It appears that there is not a significant change in the dehydrogenase and cyclohydrolase activities after proteolytic cleavage, indicating no major change in the turnover rates of the remaining active sites. Attempts to test this more directly by measuring specific activities of the fragment were only partially successful. For example, a fragment isolated from the 2',5'-ADP-Sepharose column had a dehydrogenase specific activity of 8.7 compared with 5.1 units/mg of the purified native enzyme. Based only on the loss of protein, the predicted activity would have been approximately 15 units/mg. While the increase supports the observation that the dehydrogenase activity is now a property

Figure 14: Dodecyl sulfate gel electrophoresis of tryptic digest on 10% polyacrylamide gels. From the left, the gels represent: undigested protein (10μg); tryptic digest (70μg); purified fragment (2.5μg); trypsin (10μg) and trypsin inhibitor (10μg).

Table 5

STABILITY OF THE TRYPTIC FRAGMENT

Tryptic fragment was prepared by digestion of DEAE-Sephadex purified enzyme, and isolated on $NADP^+$-Sepharose as described in Materials and Methods, by elution with 0.3 M phosphate buffer, 20% glycerol, pH 7.3. Aliquots were stored for 24 h at 4°C under the conditions outlined.

Additions	Activity (%)	
	Dehydrogenase	Cyclohydrolase
None	4	9
2 mM $NADP^+$	37	30
2 mM $NADP^+$, 5 mM dithiothreitol	79	61
2 mM $NADP^+$, 5 mM dithiothreitol (sealed flask containing 2-mercaptoethanol)	102	67

of a smaller protein fragment, the absolute value must be interpreted cautiously in view of the errors associated with the lability of the protein and the small quantities obtainable.

Dodecyl sulfate polyacrylamide gel electrophoresis of the trypsin-digested enzyme is shown in figure 14. The cleavage by trypsin produced an active fragment as well as some low molecular weight peptides. When the digest was purified on the $NADP^+$-Sepharose column, the enzymatically active fragment obtained appears to be homogeneous having a subunit molecular weight of 33 300 $\pm$ 1200. Both the native enzyme and particularly the purified tryptic fragment are rather labile proteins; this problem is compounded because of the low amounts of protein we can obtain, and thus a requirement to work with rather dilute protein solutions. Table 5 shows the results of storing the tryptic fragment for 24 h under different conditions; it appears that it is extremely easily oxidized and is protected by reducing agents. Storage in a test tube held within a suction flask containing 10 ml of 2-mercaptoethanol and sealed under reduced pressure provided the best conditions to maintain enzymic activity.

Certain properties of the cyclohydrolase activity were

Table 6

EFFECT OF INHIBITORS ON THE CYCLOHYDROLASE ACTIVITY

An aliquot of $NADP^+$-Sepharose-purified native enzyme was cleaved with trypsin and the active fragment was isolated from a second $NADP^+$-Sepharose column by step elution using 0.3 M phosphate, 20% glycerol, pH 7.3. The substrate (±)-methenyltetrahydrofolate, was 4.6×10^{-5} M in all assays, and results are expressed with standard deviations.

Inhibitor concentration	Inhibition (%)	
	Native enzyme	Tryptic fragment
1 mM $NADP^+$	47 ± 5	30 ± 3
6 mM MgATP	37 ± 5	35 ± 5
6 mM ATP	66 ± 4	75 ± 3

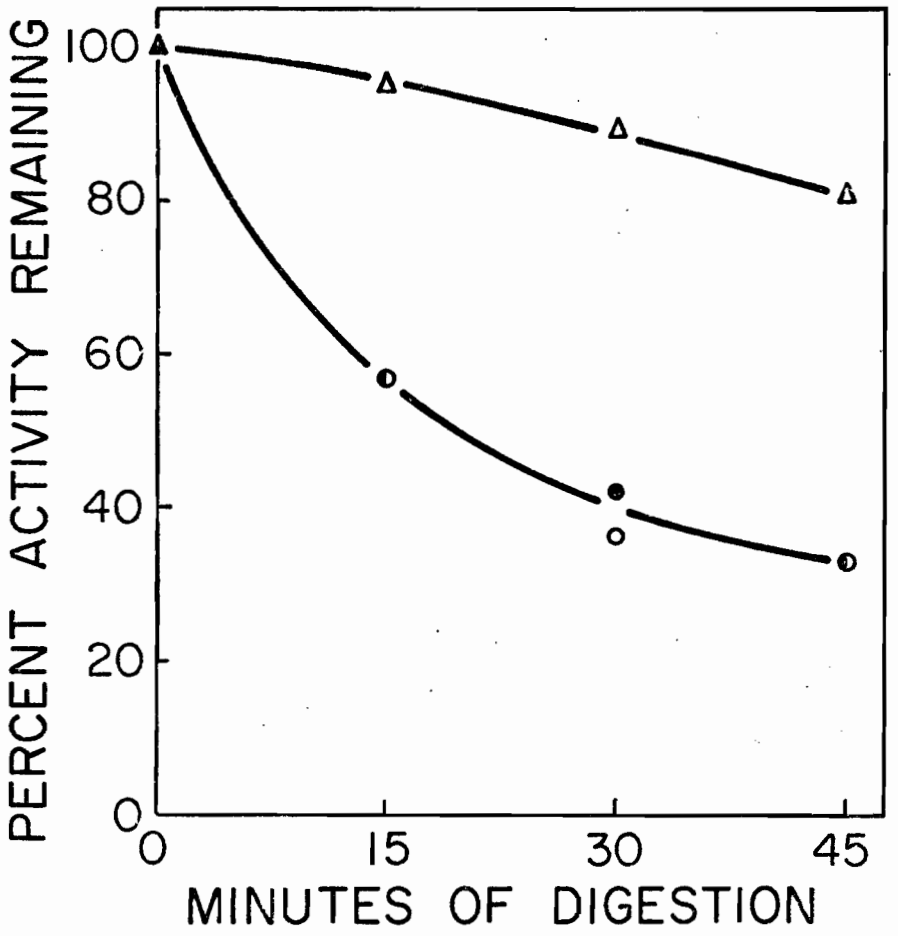


Figure 15: Effect of chymotryptic digestion on the activities of the trifunctional protein. Symbols are: Δ, synthetase activity; o, cyclohydrolase activity; •, dehydrogenase activity.

compared in the native enzyme and the tryptic fragment and are shown in Table 6. It appears that trypsin treatment does not abolish the sensitivity of the cyclohydrolase to inhibition by these three compounds.

(F) PREPARATION AND PURIFICATION OF A SYNTHETASE FRAGMENT

The effect of chymotryptic digestion on the enzyme activities of the trifunctional protein are shown in figure 15. The rates of chymotryptic inactivation of the dehydrogenase and cyclohydrolase were equal and greater than that for the synthetase. On dodecyl sulfate polyacrylamide gels (figure 16), the proteolytic fragments electrophoresed with apparent molecular weights of 67 000 and 65 000 daltons for the major and minor bands. Increasing the digestion time resulted in a decrease in the intensity of the uncleaved 100 000 dalton protein band with a concomitant increase in the 67 000 and 65 000 dalton fragments. The proteolysis must be carried out on enzyme freshly prepared from the DEAE-Sephadex A-25 column since early experiments correlated the decline in the extent of digestion with the length of storage of the enzyme purified from this column. The digest could not be carried to completion since the synthetase activity is not very stable and a maximal yield of fragment was

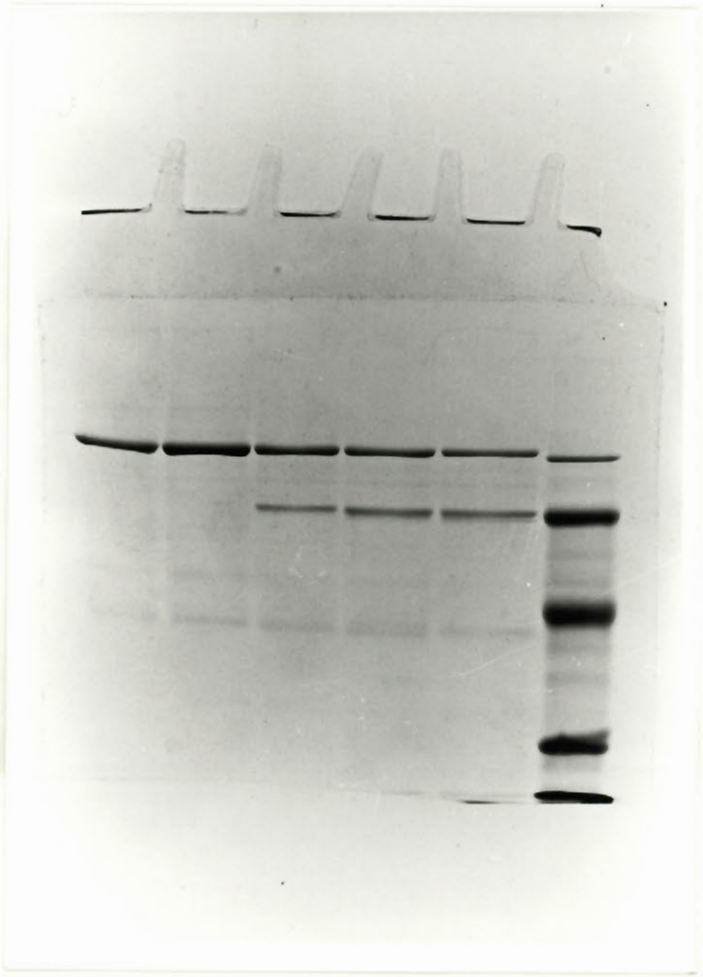


Figure 16: Dodecyl sulfate polyacrylamide gel electrophoresis of the chymotryptic digest. From the left, samples are: trifunctional enzyme, digest at 0, 15, 30, 45 minutes. The slot at the right contains Pharmacia molecular weight standards of 94 000, 67 000, 43 000, 30 000 and 20 000 daltons. Except for the standards, 10 μg of protein was applied per well.

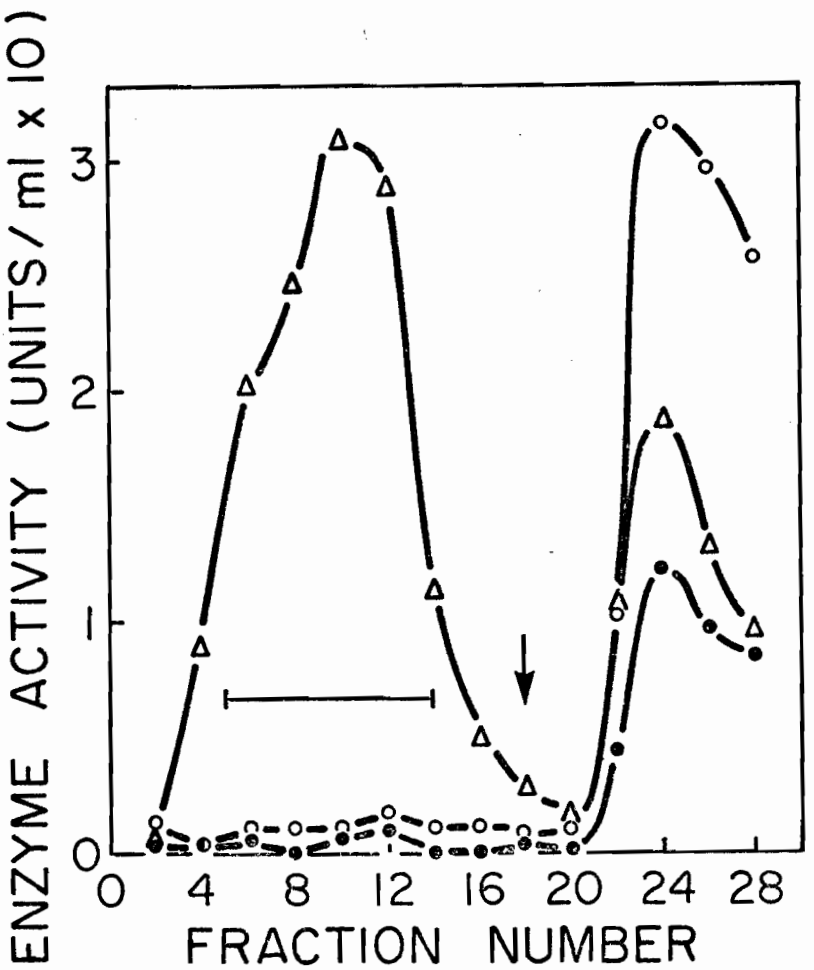


Figure 17: Chromatography of chymotryptic digest on 2',5'-ADP-Sepharose. The bar indicates the fraction pooled and subsequently applied to the cellulose phosphate column. The arrow denotes change of buffer to 0.5 M potassium phosphate, 20% glycerol, pH 7.3. Symbols represent Δ, synthetase activity; o, cyclohydrolase activity, •, dehydrogenase activity.

obtained after about 45 min digestion. To separate the majority of uncleaved protein from the active synthetase fragment, the diluted digest was passed through a 2',5'-ADP-Sepharose column (figure 17). The trifunctional protein was adsorbed while the wash contained only the synthetase, free from either dehydrogenase or cyclohydrolase activities. On dodecyl sulfate gels (figure 18), it was observed that the protein that binds to the column and was eluted with high phosphate consisted only of undigested 100 000 dalton species. The wash (slot 4) contained both fragments with some uncleaved polypeptide, which was denatured in dehydrogenase-cyclohydrolase activities since these two activities were not obtained in that fraction.

The phosphocellulose column (figure 19) was used in changing the synthetase fragment into a more suitable buffer, concentrating it 2 to 3-fold, as well as to purify the synthetase free of low molecular weight proteins, including chymotrypsin. The preparation at this stage of purification consisted mainly of a polypeptide of 67 000 with small amounts of 100 000 and 65 000 species.

A problem existed in determining whether the fragments contained synthetase activity since it was possible that

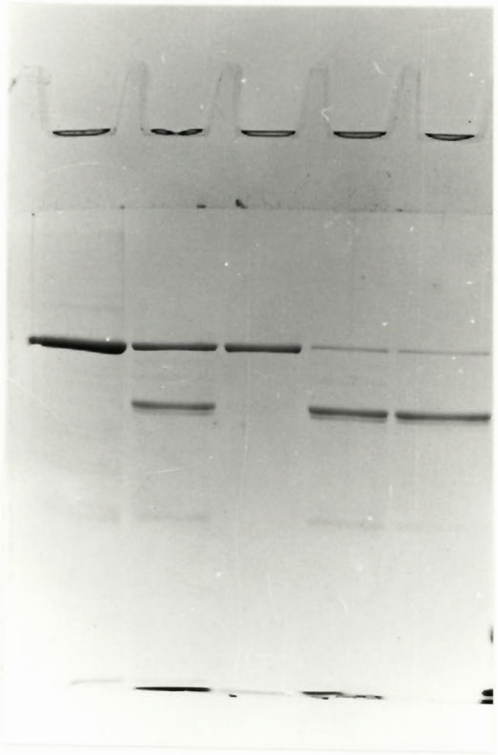


Figure 18: Dodecyl sulfate polyacrylamide gel electrophoresis of the chymotryptic digest following various manipulations. The sample wells contain from the left: untreated trifunctional enzyme; crude chymotryptic digest; digest adsorbed to 2',5'-ADP-Sepharose column; digest unadsorbed by the same column; and enzyme eluted from the cellulose phosphate column. Samples of 10μg were electrophoresed.

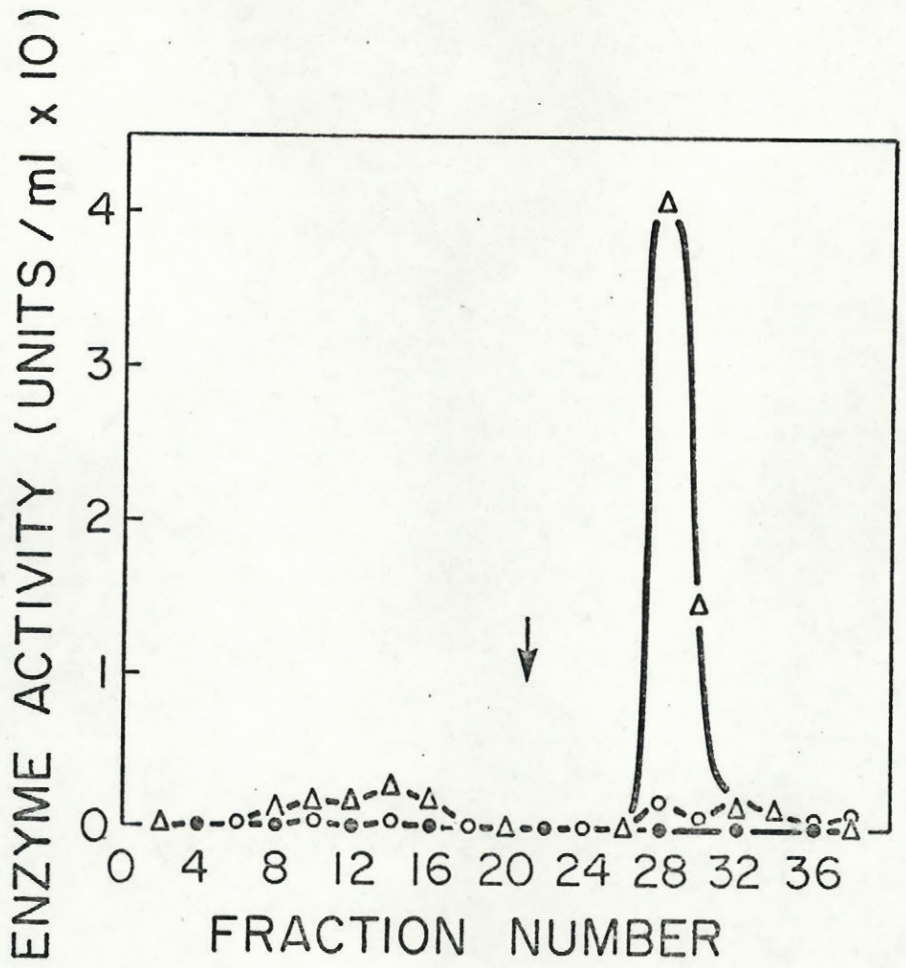


Figure 19: Chromatography of the synthetase fragment on phosphocellulose. Symbols are: Δ, synthetase activity; o, cyclohydrolase activity; •, dehydrogenase activity. The arrow indicates the application of buffer containing 10 mM MgATP.

this activity could arise solely from contamination by the uncleaved enzyme. This possibility was highly improbable since when equal synthetase units were applied to dodecyl sulfate polyacrylamide gels (figure 18), the protein band corresponding to the uncleaved enzyme in the digest was too faint, as compared with native enzyme, to account for all the synthetase activity. For confirmation, the protein mixture from the phosphocellulose column was electrophoresed on a cylindrical non-denaturing polyacrylamide disc gel which, when assayed for synthetase activity, gave a double synthetase peak (figure 20). When the sections of gel corresponding to each of these peaks were sliced out and analysed on dodecyl sulfate polyacrylamide gels, peak A was observed to contain several molecular weight species while peak B, which contained a majority of synthetase activity, consisted only of the 67 000 species and possibly some 65 000 species, thus demonstrating that this fragment has synthetase activity.

The synthetase fragment purified by phosphocellulose chromatography was not very stable, losing 50% activity in 6 days when stored at $4^{o}C$ in 0.04 M potassium phosphate, 20% glycerol, pH 7.3 in the presence of 6 mM ATP.

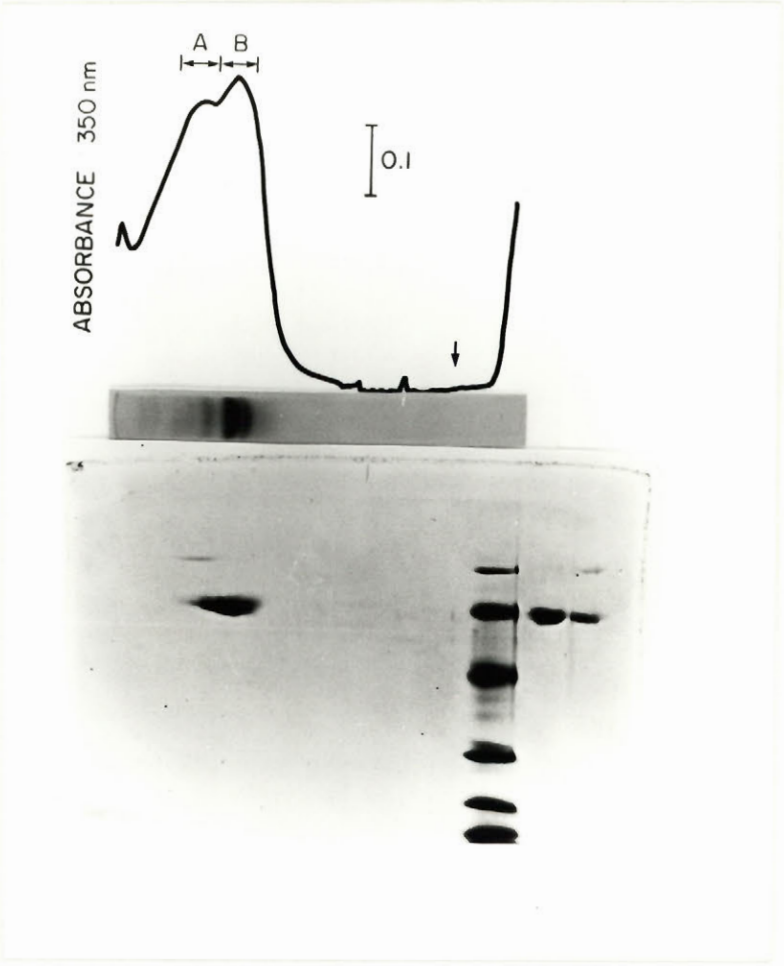


Figure 20: Two dimensional polyacrylamide gel electrophoresis (see Methods) where the first dimension was run under non-denaturing conditions in a cylindrical gel followed by a slab gel containing dodecyl sulfate in the second dimension. After the first electrophoresis, the gel was immersed in synthetase assay mix, then acidified and scanned at 350 nm for synthetase-active protein bands. The gel was equilibrated with dodecyl sulfate buffer and electrophoresed in the second dimension. The portion of the cylindrical gel to the right of the arrow was removed prior to applying it to the slab gel. The tract of 6 protein bands on the slab gel is Pharmacia molecular weight standards of 94 000, 67 000, 43 000, 30 000, 20 000, 14 000 daltons. Slices of a second cylindrical gel corresponding to sections B and A, respectively, were applied separately to the slab gel to the right of the molecular weight standards.

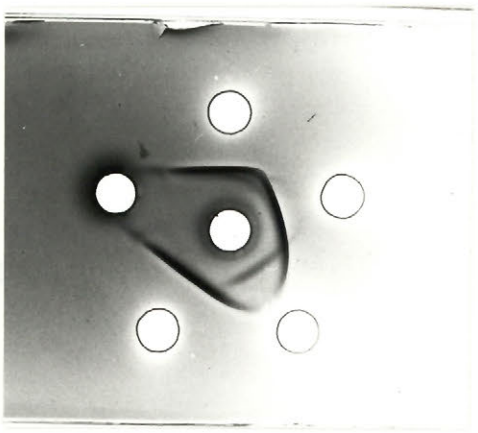


Figure 21: Double immunodiffusion of fragments obtained from the trifunctional protein against antibodies prepared against the latter. Clockwise, from the top, the wells contained: uncleaved trifunctional enzyme of 100 000 daltons (0.5μg); dehydrogenase-cyclohydrolase fragment of 33 000 daltons (0.8μg); synthetase fragment of 67 000 daltons (3μg); uncleaved trifunctional enzyme (0.5μg); and normal rabbit serum. The center well contained rabbit antibodies prepared against the uncleaved enzyme. The plate was washed, dried, and stained with Coomassie Brilliant Blue.

(G) IMMUNOLOGICAL STUDIES

On immunodiffusion, the antibodies prepared as in Methods formed a single precipitation line against the purified uncleaved enzyme of 100 000 daltons and cross-reacted with both the dehydrogenase-cyclohydrolase fragment of 33 000 daltons and the synthetase fragment of 67 000 daltons (figure 21). The interaction between the precipitation lines generated by the dehydrogenase-cyclohydrolase and synthetase fragments showed a pattern of non-identity indicating little or no overlap of antigenic sites, thus suggesting that the two fragments were obtained from different regions of the trifunctional polypeptide. From the intensity of the precipitation lines observed in several diffusion plates using different concentrations of antigens, it appears that the major portion of the antibodies produced in the rabbit are directed against the much smaller dehydrogenase-cyclohydrolase segment and not the larger synthetase portion of the trifunctional protein.

(H) AMINO-TERMINAL ANALYSIS

The amino-terminal sequence analysis of the uncleaved trifunctional enzyme and the dehydrogenase-cyclohydrolase

Table 7

AMINO-TERMINAL ANALYSIS

Proteins	Amino Acid Residues		
	First	Second	Third
Trifunctional	ala -	pro -	ala
Dehydrogenase-Cyclo-hydrolase fragment	ala -	pro -	ala
Synthetase fragment	phe leu (ala) -	? -	?

fragment showed the first three amino acids to be alanine-proline-alanine in each case (Table 7). On the other hand, the amino-terminal residue of the synthetase fragment was heterogeneous, consisting of at least three amino acids, the most prominent being phenylalanine, leucine, and some alanine.

CHAPTER IV

CONCLUSION

(A) PURIFICATION OF THE TRIFUNCTIONAL PROTEIN

MethyleneTHF dehydrogenase, methenylTHF cyclohydrolase, and formylTHF synthetase have been shown to co-purify more than 500-fold to apparent homogeneity. The inability of three sequential chromatographic steps,[136] and two affinity columns which should preferentially bind to the dehydrogenase or synthetase sites to resolve the three activities demonstrates that the enzymic activities are physically associated in some manner. The elution of the three activities from the $NADP^+$-Sepharose column is quite specific as illustrated by comparison of the effectiveness of $NADP^+$, NAD^+ and KCl. The ATP-Sepharose column was found to be less specific.

The final purified preparation yields a single protein band on dodecyl sulfate gels. This band is the only one seen to increase in intensity during the final steps of the purification and demonstrates that the protein is composed of only one size of polypeptide chain. Amino-terminal analysis of the preparation gave a single amino acid sequence of ALA-PRO-ALA, confirming homogeneity. Estimates of the subunit molecular

weight on dodecyl sulfate gel electrophoresis was 100 000 daltons.

The existence of the trifunctional polypeptide in porcine liver suggests that similar proteins could be observed in other species. At approximately the same time, Paukert et al[155] purified the same three enzymes from sheep liver and demonstrated that the activities were found in a single type of polypeptide. MethyleneTHF dehydrogenase has been purified to homogeneity from Clostridium cylindrosporum[47] and partially purified from several sources including yeast[156], calf thymus[53], and beef liver[1]. The enzyme from mammalian sources in particular was reported to be quite unstable; the beef liver enzyme, for example, could be purified 40 to 50-fold but lost all activity after 96 h of storage[1]. The yeast enzyme is more stable and has been purified 125-fold. Lazowska and Luzzati[157,158] have shown that Saccharomyces cerevisiae contains two molecular forms of the dehydrogenase, one of which is missing in ade-3 mutants. It is of interest that these same mutants have only 10% of the normal cyclohydrolase and synthetase activities. Later, Zelikson and Luzzati[159] discovered that yeast contains two sets of folate enzymes, one cytoplasmic, while the other is mitochondrial. In ade-3 mutants, it is the cytoplasmic dehydrogenase-cyclohydrolase-synthetase activities which are missing. In the same year,

Paukert et al[160] purified these three activities from yeast and showed that they are covalently linked.

The best characterized bacterial system of folate dependent enzymes is that of C. cylindrosporum where Rabinowitz and co-workers purified and characterized the dehydrogenase, cyclohydrolase and synthetase which are separate enzyme species.[47,126,45]

The situation with the bovine liver enzymes is as yet not conclusive. Results of Rowe and Lewis[1] differ from our findings in pig liver. These authors reported a 40-fold purification of synthetase that was free of dehydrogenase and cyclohydrolase activities. The preparation was unstable and lost all activity within 72 h. In addition, they reported two forms of cyclohydrolase of about 30 000 and 60 000 daltons, with the latter being considerably more stable. It thus appears that the dehydrogenase, cyclohydrolase, and synthetase are separable species in beef liver. However, it is not clear that the dehydrogenase and cyclohydrolase preparations were free of contaminating folate dependent enzyme activities. It is possible that instability of the enzymes and the use of different purification schemes for each of the activities combined to give preferential stability to the activity sought. Some of the instability may be due to proteolysis

in the crude extracts. Recently, the trifunctional protein containing the same three activities has been purified from rabbit[161] and chicken livers[67].

(B) NATIVE STRUCTURE OF THE TRIFUNCTIONAL PROTEIN

The molecular weight of the trifunctional protein determined by gel filtration[136] is 136 000 to 150 000 daltons. Since a single band corresponding to a subunit molecular weight of 100 000 daltons was obtained from dodecyl sulfate gels, the possibility that the native structure contains two peptides (100 000 and 50 000) can be eliminated. The properties of the trifunctional protein on dodecyl sulfate gels determined by the method of Banker and Cotman (figure 6) were normal, so the discrepancies in molecular weights were probably attributable to anomalous behaviour in gel filtration. The native molecular weights determined by three independent methods; values obtained by sedimentation (figure 9), retardation in polyacrylamide gels (figure 11) and cross-linking (figure 12) were 170 000, 230 000 and 200 000 daltons respectively corresponding to a dimer of identical subunits of 100 000 daltons. Some polyaggregates were also observed in the Hedrick and Smith gels (figure 11) and cross-linked samples (figure 12).

When the non-cross-linked trifunctional protein, untreated by 2-mercaptoethanol, was electrophoresed in dodecyl sulfate gels (figure 12), a significant amount of dimer was observed. The ability to greatly reduce the quantity of dimer by pre-treatment with 2-mercaptoethanol suggests inter-subunit disulfide bonds. Whether this is the "natural" structure, an artifact due to non-specific dithiothreitol-induced disulfide interchange in the Sephadex A-25 chromatographic step, or an artifact due to spontaneous oxidation is not known.

(C) AMINO ACID ANALYSIS

The amino acid composition of the protein is not unusual. Calculations for hydrophobicity by the methods of Waugh[162] and Bigelow[163] gave values for nonpolar side chain (NPS) frequency of 0.34 and an average hydrophobicity (HØave) of 1103 cal/residue. These approximate the averages of 0.33 and 1000 to 1100 cal/residue respectively.

(D) DEHYDROGENASE-CYCLOHYDROLASE FRAGMENT

The bifunctional dehydrogenase-cyclohydrolase fragment

could be prepared by the tryptic digestion of the trifunctional protein, followed by purification on an $NADP^+$-Sepharose column. The preparation was homogeneous with a single band on dodecyl sulfate polyacrylamide gels corresponding to a subunit molecular weight of only 33 000 daltons, thus implying closely interacting active centres.

The dehydrogenase-cyclohydrolase activities function to interconvert methyleneTHF and formylTHF through the intermediate methenylTHF. MethyleneTHF, if not further reduced and used for methionine synthesis, is proposed to be converted to formylTHF which is then oxidized to yield CO_2 and regenerate THF.[20,164] The three activities of the multifunctional protein could be viewed as reactions designed to produce formylTHF for this "overflow" pathway, and may be more advantageous for this purpose if associated as a multifunctional protein. The location of both dehydrogenase and cyclohydrolase activities within a relatively small portion of the polypeptide is consistent with their integrated metabolic function. Cohen and MacKenzie[165] observed the product of the dehydrogenase (methenylTHF), without having to equilibrate with the buffer, could be preferentially channelled through the cyclohydrolase to form the product of the cyclohydrolase (10-formylTHF), thus eliminating the need to accumulate intermediates. Since both methenylTHF and formylTHF are

required for purine synthesis, it is probable that some methenylTHF intermediate is made available for this purpose.

A dehydrogenase-cyclohydrolase complex composed of five non-identical polypeptides has recently been obtained from E. coli.[166] The presence of another protein with closely associating dehydrogenase-cyclohydrolase activities obtained from organisms widely separated on the evolutionary scale supports the notion that the close interaction between these two activities confers some advantage to the organism.

(E) PREPARATION AND PURIFICATION OF A SYNTHETASE FRAGMENT

Proteolysis of the trifunctional protein using chymotrypsin under conditions designed to protect the synthetase activity results in the concomitant loss of dehydrogenase and cyclohydrolase activities with retention of much of the synthetase activity in a fragment of about two-thirds the original polypeptide. Paukert et al,[160] using trypsin cleavage of the enzyme from yeast, isolated a synthetase fragment of similar size, and also observed concomitant loss of dehydrogenase and cyclohydrolase during digestion. Although the yeast and pig liver enzymes are similar in their organization of the three activities within the same molecule, there

appear to be some structural differences. I have been unable to obtain an active synthetase fragment from the pig liver enzyme using trypsin under the conditions outlined by Paukert et al.[160] While the synthetase activity of the yeast enzyme is stable for 3 h at 37°, the pig liver synthetase is inactivated at least as quickly as the dehydrogenase-cyclohydrolase even at 30°. Dodecyl sulfate gels indicated multiple sites of proteolysis under these conditions.

(F) ORGANIZATION OF THE DEHYDROGENASE-CYCLOHYDROLASE-SYNTHETASE ACTIVITIES WITHIN THE POLYPEPTIDE

(a) Interrelationship of Dehydrogenase-Cyclohydrolase Activities

From stability studies of the trifunctional protein stored in triethanolamine-HCl buffer (figure 8), the dehydrogenase and cyclohydrolase appear to be closely associated in that both inactivate at the same rate. Other evidence for the close association include the same rate of inactivation by chymotryptic digestion (figure 15) and the physical location of the two activities in a relatively small polypeptide of 33 000 daltons. Also, Cohen and MacKenzie[165] have shown that the product of the dehydrogenase, without having to

equilibrate with the buffer, could be channelled through the cyclohydrolase to form the product of the latter. Furthermore, the dehydrogenase and cyclohydrolase from rabbit liver were reported by Schirch[167] to inactivate at the same rate using the sulfhydryl reagent, 5,5'-dithiobis(nitrobenzoic acid). In addition, the Ki's of the inhibitors: THF, 5-formyl-THF, 5-methylTHF and 2-mercaptoethanol for both activities from rabbit liver[161] are essentially the same with the exception of 10-formylTHF which is a 4-fold better inhibitor for the cyclohydrolase than the dehydrogenase. From similarities in response of the dehydrogenase and cyclohydrolase activities to denaturation, proteolysis, chemical modification, their location in a rather small proteolytic fragment, as well as their kinetic properties, including channelling of the methenyl intermediate, argue for the existence of the dehydrogenase and cyclohydrolase activities in a composite site within a single domain.

(b) Relative Positions of the Activities within the Polypeptide

The sum of the molecular weights of the tryptic and chymotryptic fragments closely approximates the molecular weight of the native trifunctional polypeptide and the apparent lack of overlap between the two fragments, as

deduced from immunodiffusion studies (figure 21), supports the hypothesis that the native molecule is composed of two domains; a smaller dehydrogenase-cyclohydrolase domain and a larger synthetase domain.

The location of these domains in the polypeptide could be deduced from the amino-terminal analysis (Table 7). The first three residues of the amino-terminal sequence of the dehydrogenase-cyclohydrolase fragment and of the uncleaved enzyme are the same, demonstrating that the dehydrogenase-cyclohydrolase activities comprise the amino-terminal portion of the polypeptide. Conversely, the synthetase fragment, having different amino-terminals, should then be derived from the carboxyl-terminal region. The heterogeneous amino-terminals of the synthetase fragment(s) are compatible with this two-domain structure if one envisions chymotrypsin to cleave at different sites on the polypeptide giving a population of synthetase fragments varying slightly in molecular weight and having different amino-terminal residues, as were observed in our experiments.

CHAPTER V

GENERAL DISCUSSION OF ONE-CARBON METABOLISM

MethyleneTHF dehydrogenase, methenylTHF cyclohydrolase and formylTHF synthetase have been purified from livers of sheep[155], pig[136], rabbit[161], and chicken[67], as well as from yeast[160] and human chronic lymphocytic leukemic blast cells.[168] In each case, the activities were found to be contained within a single polypeptide. The ubiquity of the trifunctional protein implies added advantages over having the different activities on separate proteins.

In E. coli[166], a complex of only dehydrogenase-cyclohydrolase was found and synthetase was not detectable at any stage of the purification. The three activities from Clostridia, however, are separable,[45,47,126,169-171] but the cyclohydrolase co-purified with the formiminoTHF cyclodeaminase,[126] an enzyme catalysing a reaction immediately prior to cyclohydrolase in the degradation pathway for purines in this organism.

The linkage of the dehydrogenase, cyclohydrolase and synthetase on the evolutionary scale probably started from a primodial cell which carried the activities on separate proteins. From this early beginning, two types of complexes

evolved; one, where cyclohydrolase was attached to cyclodeaminase (Clostridium) while the other have the former linked to dehydrogenase (E. coli). The dehydrogenase-cyclohydrolase complex was later to incorporate the synthetase to form the trifunctional protein found in eukaryotes. A similar scheme of evolution from independent proteins, to enzyme complex, to multifunctional protein was proposed for tryptophan synthetase from Bacillus subtilis, to E. coli, to Neurospora crassa.[113]

Advantages for the existence of a dehydrogenase-cyclohydrolase domain appear to be the ability to channel the intermediate, methenylTHF, from the dehydrogenase to the cyclohydrolase,[165] thus eliminating the need to accumulate the unstable intermediate. The presence of folate enzyme clusters could result in the reduction of transit times for metabolites to diffuse among catalytic sites, as well as in localization of substrates.[100] The latter is especially important in view of the low concentration (2×10^{-5} M) of THF and its derivatives found in the liver.[172]

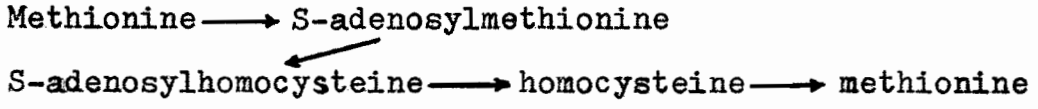
The distribution of folate derivatives varies depending on the tissue examined. For example, the percent of the total found in the forms of 10-formylTHF and 5-methylTHF are: rat liver, 20% and 60%; monkey liver, 20%-40% and

60%-80%; yeast, 77% and 20%, respectively.[173] The remaining 20% of the folates in rat liver is free THF. Because of the very low intracellular concentrations of methyleneTHF and methenylTHF, substrates of the dehydrogenase and cyclohydrolase respectively, the localization of these compounds should offer a definite advantage over uniform dispersion; especially in view of the dehydrogenase apparent Km of 3×10^{-6} M for the methylenetetrahydropteroylpentaglutamate, the most probable *in vivo* substrate (MacKenzie unpublished data). The ultimate advantage would be to eliminate the need to accumulate intermediates by channelling intermediates into the next enzyme in the metabolic chain as was observed for the dehydrogenase-cyclohydrolase activities.[165]

The possible advantage of enzyme stabilization by conservation of the trifunctional structure is highly unlikely since the three activities, which are separate in *C. cylindrosporum*, are very stable, while the trifunctional protein is labile. Nor is the regulation of these three activities by a single effector probable. However, the localization of minute quantities of labile substrates may have merits. It had been observed that the trifunctional protein partially co-purified through multiple purification steps with serine transhydroxymethylase in rabbit liver[161] and glycineamide ribonucleotide transformylase from chicken liver,[67] both

of which are folate dependent enzymes. It seems possible that the whole folate metabolic pathway may be clumped into a giant multicomponent enzyme cluster, localizing the various forms of one-carbon units.

Krebs[20] has proposed a model for the regulation of one-carbon metabolism whereby the modulating factor is the essential amino acid, methionine. In this model, the key enzymes are 10-formylTHF dehydrogenase which is presumed to be activated by methionine[20], and methyleneTHF reductase, inhibited by S-adenosylmethionine.[174,175] Methionine is partially degraded and regenerated cyclically by the following reactions:



The final step requires a methyl transfer from 5-methylTHF catalyzed by homocysteine: 5-methylTHF methyltransferase.

In the <u>in vivo</u> situation, the vast majority of folate derivatives in the liver are one-carbon linked.[173] However, degradation of histidine and synthesis of glycine from serine require free THF so this form of the coenzyme must also be made available. The main outlets for one-carbon units are

proposed by Krebs[20] to be homocysteine: 5-methylTHF methyltransferase and 10-formylTHF dehydrogenase. Although biosynthetic steps such as the synthesis of purines also release free THF, they are inflexible and rigorously controlled by other factors.

The Krebs' model functions as follows: at high intracellular concentration of methionine, the concentration of S-adenosylmethionine coordinately increases. As a result, the excessive S-adenosylmethionine blocks (inhibits) methyleneTHF reductase while the methionine opens (activates) the 10-formylTHF dehydrogenase outlet, thus directing the excess flow of one-carbon units towards 10-formylTHF for oxidation with concomitant release of free THF. The drop in 5-methylTHF concentration reduces methionine regeneration from homocysteine which then condenses with serine and is degraded via cystathionine. On the other hand, when the supply of methionine is limited, maximum methionine regeneration occurs. This is because the low concentration of methionine no longer activates formylTHF dehydrogenase. At the same time, the scarcity of S-adenosylmethionine reduces inhibition of methyleneTHF reductase thus opening the pathway for maximum methionine recovery by siphoning one-carbon units into 5-methylTHF which is used in the methyltransferase reaction, producing methionine and THF. Although it explains the

regulation of methionine metabolism, this model appears to be deficient in explaining the regulation of one-carbon metabolism in that it is insensitive to the relative concentrations of one-carbon-linked and free THF, and assumes that methionine activates 10-formylTHF dehydrogenase.

A modified version of Krebs' model is proposed here where 10-formylTHF dehydrogenase is still maintained as the regulator of one-carbon metabolism. This model need not assume methionine to be an activator of 10-formylTHF dehydrogenase. Kutzbach and Stokstad[60] observed that the pig liver 10-formylTHF dehydrogenase is insensitive to 5-methylTHF and 5-formylTHF but could be inhibited by low concentrations of THF (Ki approx. 1 μM), so much so that assay conditions require the addition of 10-formylTHF synthetase to couple the dehydrogenase activity so as to eliminate accumulation of free THF. To examine the regulation of one-carbon metabolism, consider that the total amount of free THF plus one-carbon linked THF are relatively constant so that a flux in the one-carbon linked THF pool would result in an equal but opposite flux in the free THF pool. Because of this interrelationship, a regulation controlling either pool would in effect control both.

The biosynthetic steps requiring one-carbon units

such as methionine recovery, synthesis of serine and purines generate free THF. If the rate of generation of one-carbon units from free THF is insufficient to compensate for its utilization in biosynthetic steps, an accumulation of free THF (concomitant depletion of one-carbon units) results. The increase in THF concentration would inhibit 10-formylTHF dehydrogenase so as to conserve one-carbon units. However, if the rate of formation of active one-carbon units is faster than their utilization, there will be a depletion of free THF (concomitant accumulation of one-carbon units). As a result, the reduction in THF inhibition of 10-formylTHF dehydrogenase results in an increase in the rate of one-carbon unit degradation to THF.

The observations by Krebs and Hems[20] and Waydhas[176] that formate and formiminoglutamate (histidine) oxidations by perfused liver are stimulated by methionine could be explained by the leaching of methionine from the liver preparations. As a result, the lack of methionine also reduces the concentration of its derivatives, homocysteine and S-adenosylmethionine. The concentrations of these 2 products in the liver preparations may be at such low levels that methyl transfer from 5-methylTHF to homocysteine is essentially blocked. Meanwhile, the near absence of S-adenosylmethionine allows the methyleneTHF reductase to operate uninhibited thus siphoning

one-carbon units into 5-methylTHF. The leaching of methionine, then, may be analogous to vitamin B_{12} deficiency where 5-methylTHF is accumulated at the expense of other forms of folates. The addition of methionine to the perfusate of liver preparations raises the concentration of its degradative products, homocysteine and S-adenosylmethionine. The availability of these two metabolites stimulates the methyl transfer from 5-methylTHF to homocysteine, with a concomitant release of THF. Meanwhile, S-adenosylmethionine inhibits further reduction of one-carbon units to 5-methylTHF. THF, which is released in the methyl transfer reaction, is a necessary cofactor for the degradation of formiminoglutamate and for the folate-dependent pathway for formate oxidation. The availability of THF results in an increase in formiminoglutamate and formate oxidation.

To examine the fine structures of one-carbon regulation, attention is focused on the substrates, the one-carbon linked THF derivatives, as well as the folate-dependent enzymes. It has been observed that the vast majority of the liver folate is in the form of one-carbon linked THF.[173] The apparent storage forms observed are 5-methylTHF and 10-formylTHF as these are relatively stable forms under physiological conditions. MethyleneTHF and methenylTHF would not constitute good storage forms since the former degrades

spontaneously into THF and formaldehyde[177] while the latter spontaneously hydrolyses to 10-formylTHF at physiological pH.[54] At equilibrium, the ratio of methenylTHF to 10-formyl-THF is about 0.04 to 0.05. Of the two one-carbon units found in relative abundance, 5-methylTHF is analogous to a one-carbon sink in that it can not be reoxidized to the other forms of one-carbon derivatives.[2] Its only recourse is to be demethylated in methionine regeneration. In terms of the synthesis of serine, thymidylate and purines, the only major active one-carbon pool available for interconversion is 10-formylTHF.

The main pathway for the influx of one-carbon units is serine via serine transhydroxymethylase resulting in methyleneTHF.[2] The methyleneTHF, if not used directly, is presumed to be reduced by the reductase to 5-methylTHF or oxidized to 10-formylTHF by the combined action of methylene-THF dehydrogenase and methenylTHF cyclohydrolase.

The cyclohydrolase from pig liver has been shown to be inhibited by $NADP^+$ and ATP at physiological concentrations,[136] thus implying some regulatory function. Since under physiological pH, the formation of 10-formylTHF from methenyl-THF, catalysed by cyclohydrolase also occurs spontaneously, it seems regulation at the cyclohydrolase activity is

ineffectual. Furthermore, if the presence of the 10-formyl-THF synthetase is for the fixation and subsequent oxidation of formate, a Km of 5.4 mM and 16 mM for the sheep[155] and pig[136] liver enzymes, respectively, appears to be high in view of the minor metabolic role of formate. Several sources of formate are known, one of which is bacterial action on carbohydrate in the gut[20]. Formate can also be generated by the degradation of tryptophan and glycine[29].

The proposed model for the regulation of one-carbon metabolism is shown in figure 22. The one-carbon pool is represented by a series of pipes joined to a 10-formylTHF reservoir. One-carbon units entering the system from serine and histidine, if not immediately used are channelled into the 10-formylTHF pool by means of methyleneTHF dehydrogenase and methenylTHF cyclohydrolase. Associated with the 10-formyl-THF reservoir are the enzymes 10-formylTHF dehydrogenase and 10-formylTHF synthetase. The role of 10-formylTHF dehydrogenase has already been discussed. The synthetase may be present for the equilibration of 10-formylTHF with THF and formate. The concentrations of these three substrates may be such that the synthetase <u>in situ</u> may be operating at near chemical equilibrium. Should this situation exist, it may have regulatory implications since an excess increase in one component would automatically shift the equilibrium in

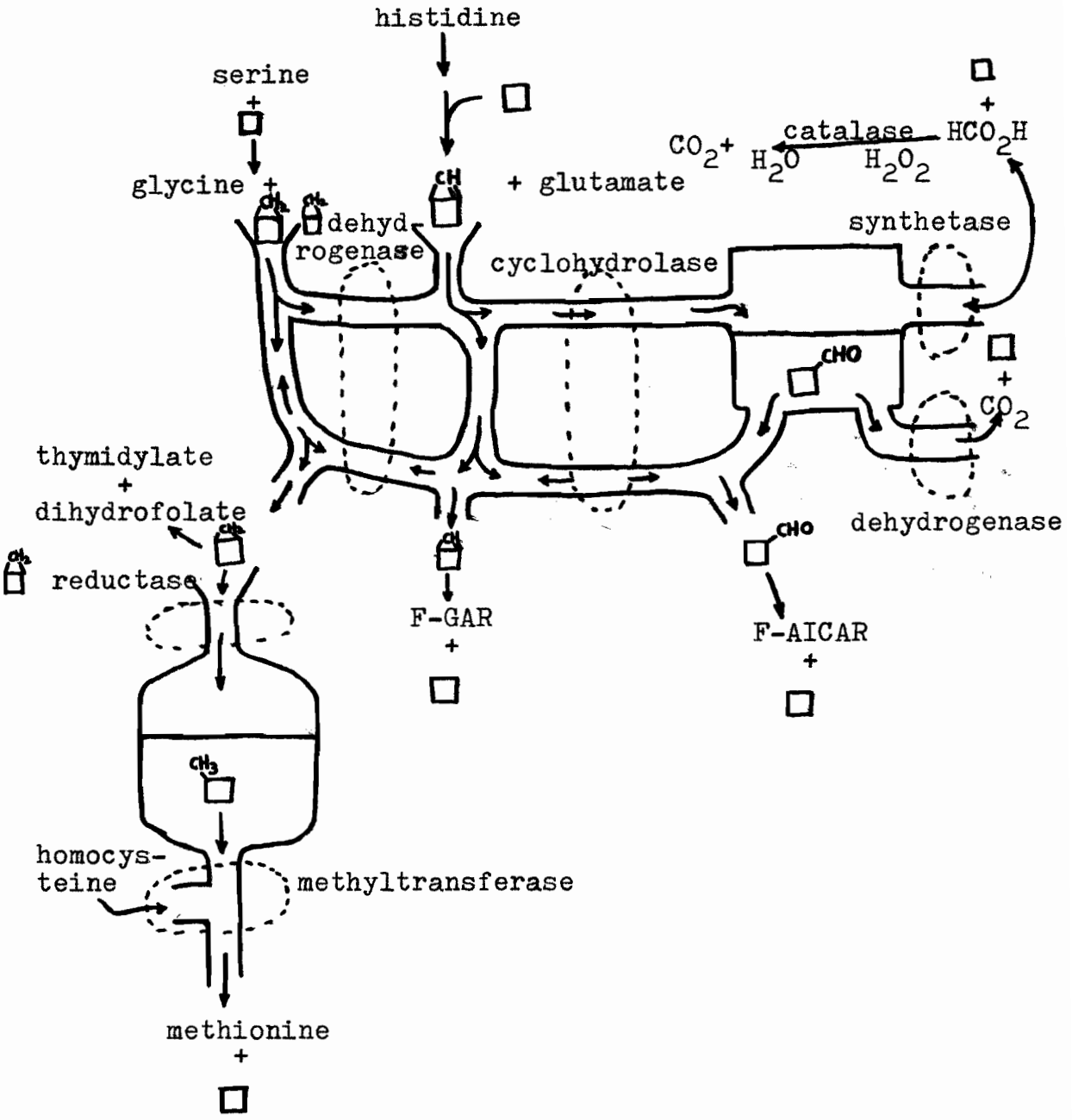


Figure 22: Proposed model for the regulation of one-carbon metabolism. Symbols are: □, tetrahydrofolate with the top left and right corners representing N^5 and N^{10} positions, respectively; ⬭, enzymes; F-GAR, N-formylglycinamide ribonucleotide; F-AICAR, 5-formamido-imidazole-4-carboxamide ribonucleotide.

the direction of its utilization. Formate which may accumulate, could be oxidized by the peroxidatic activity of catalase by the following reaction:[178,179]

$$HCO_2H + H_2O_2 \xrightarrow{\text{catalase}} CO_2 + 2H_2O$$

The near equilibrium condition at which the synthetase is operating *in situ* is supported by the reversibility of the synthetase reaction. For example, Krebs[20] has observed that rat liver when perfused with formate solutions results in oxidation of formate via the THF pathway. On the other hand, *Clostridia* generates formate from one-carbon linked THF in the reverse direction.[45]

In support of the formate oxidation pathway via catalase, Keilin[178] observed that of the three proteins, catalase, metmyoglobin, and peroxidase that have peroxidatic activity, catalase was the only one which uses formate. Krebs[20], using methionine as stimulant, explained the lower percentage increase of formate oxidation as compared to histidine oxidation by a second degradative pathway via catalase. Furthermore, Nicholls[180] suggested that the presence of catalase in liver may not just be for the breakdown of H_2O_2 into H_2O and O_2 but rather for the peroxidatic breakdown of H_2O_2 (i.e. requiring a hydrogen donor for the catabolism of H_2O_2). Waydhas et al[176] using perfused rat liver, concluded that

the pathway for the peroxidatic oxidation of formate is present although operating at a low level, while Chiao and Stokstad[181] reported that approximately 25% of normal formate oxidation proceeds by the non-folate-dependent pathway.

According to the model presented, there is little methyleneTHF and methenylTHF present at any one time which is consistent with Scott's findings.[173] Because of the low concentrations of these substrates, reactions requiring these cofactors, such as 5-methylTHF, thymidylate and F-GAR synthesis, quickly deplete existing supplies and require quick regeneration from the readily available 10-formylTHF pool. Efficient regeneration of methyleneTHF and methenylTHF requires the use of the cyclohydrolase activity as well as the channelling that occurs between the dehydrogenase and cyclohydrolase activities.

There are several observed properties compatible with this proposed sequence of events. First, the production of methyleneTHF from 10-formylTHF has to go through the intermediate, methenylTHF which is unstable and readily reverts to 10-formylTHF spontaneously. In addition, this intermediate is available only in low concentrations. The ability to channel the methenylTHF from the cyclohydrolase to the dehydrogenase to form methyleneTHF would avoid the problems

associated with low concentrations and labile substrates for the methyleneTHF dehydrogenase activity.

On the other hand, if the flow was directed from the methyleneTHF to 10-formylTHF via the intermediate, methenyl-THF, there is no desperate need for channelling of the methenylTHF through the cyclohydrolase activity since this intermediate if released into the solution would spontaneously convert to 10-formylTHF non-enzymatically.

Finally, if the controlled flow is from the 10-formyl-THF to methyleneTHF as postulated, the cyclohydrolase would be a good choice for regulation of purine and thymidylate synthesis. The cyclohydrolase from pig liver is inhibited by ATP and $NADP^+$ at physiological concentrations.[136]

From the arguments presented, it appears that one-carbon units entering from serine, histidine, and glycine, if not immediately used, are directed into the 10-formylTHF pool, the active pool for 5-methylTHF, thymidylate, purine and serine synthesis. Upon demand, flow of one-carbon units are in the reverse direction for biosynthetic steps, subject to local regulations at such enzymes as the cyclohydrolase, methyleneTHF dehydrogenase and the methyleneTHF reductase.

The main regulator for the relative amounts of free THF to one-carbon linked THF may be the 10-formylTHF dehydrogenase, while the synthetase may be present as a fine adjustment to this regulation.

In view of the proposed model, there arises a need to modify certain experiments. For example, channelling experiments between the dehydrogenase and cyclohydrolase were in the direction of methyleneTHF to 10-formylTHF.[161,165] It is not certain whether channelling occurs in the reverse direction. The cyclohydrolase activity as well as its inhibition studies were largely assayed in the direction of methenylTHF to 10-formylTHF. Whether there are differences in the reverse direction is not known. Furthermore, to determine whether the synthetase plays a part in regulation, its kinetic properties as well as the intracellular concentrations of formate must be known to elucidate possible effectors as well as to verify the equilibrium state at which it operates. Finally, it would be interesting to know whether the rate of non-enzymatic conversion of 10-formylTHF to methenylTHF under physiological conditions would be at a sufficient rate for methenylTHF-requiring enzymes which include methyleneTHF dehydrogenase and glycinamide ribonucleotide transformylase.

Addendum - Following the completion of this thesis, I have learned that a recent report by Scrutton and Beis[182] shows that the 10-formylTHF dehydrogenase can be purified to homogeneity from rat liver. The enzyme was found to be insensitive to methionine and its degradative products, but inhibited by tetrahydrofolate.

REFERENCES

1. Rowe, P.B., and Lewis, G.P. (1973) Biochemistry 12, 1962-1969
2. Blakley, R.L.: The Biochemistry of Folic Acid and Related Pteridines, in: Frontiers of Biology. vol. 13. Amsterdam, North-Holland Publishing Co., 1969
3. Hoffbrand, A.V. (1975) in: Progress in Haematology, vol. 9, pg. 85-105. E.B. Brown, ed., Grune and Stratton Inc., N.Y.
4. Houlihan, C.M. and Scott, J.M. (1972) Biochem. Biophys. Res. Commun. 48, 1675-1681
5. Brown, J.P., Davidson, G.E., and Scott, J.M. (1974) Biochim. Biophys. Acta 343, 78-88
6. Curthoys, N.P., Scott, J.M., and Rabinowitz, J.C. (1972) J. Biol. Chem. 247, 1959-1964
7. Pfiffner, J.J., Calkins, D.G., Bloom, E.S., and O'Dell, B.L. (1946) J. Am. Chem. Soc. 68, 1392
8. Blakley, R.L. (1957) Biochem. J. 65, 342-348
9. Coward, J.K., Chello, P.L., Cashmere, A.R., Parameswaran, K.N., DeAngelis, L.M. and Bertino, J.R. (1975) Biochem. 14, 1548-1552
10. Noronha, J.M. and Aboobaker, V.S. (1963) Arch. Biochem. Biophys. 101, 445-447

11. Butterworth, C.E.,Jr., Baugh, C.M., Krumdieck, C. (1969) J. Clin. Invest. 48, 1131-1142

12. Buyze, H.G., and Engel, C. (1948) Biochim. Biophys. Acta 2, 217-222

13. Tamura, T., Buehring, K.U. and Stokstad, E.L.R. (1972) Proc. Soc. Exp. Biol. Med. 141, 1022-1025

14. Nixon, P.F., and Bertino, J.R. (1972) N. Engl. J. Med. 286, 175-179

15. Griffin, M.J. and Brown, G.M. (1964) J. Biol. Chem. 239, 310-316

16. Thenen, S.W. and Stokstad, E.L.R. (1973) J. Nutr. 103, 363-370

17. Lavoie, A., Tripp, E. and Hoffbrand, A.V. (1974) Clin. Sci. Mol. Med. 47, 617-630

18. Taylor, R.T., Hanna, M.L. and Hutton, J.J. (1974) Arch. Biochem. Biophys. 165, 787-795

19. Gawthorne, J.M. and Smith, R.M. (1973) Biochem. J. 136, 295-304

20. Krebs, H.A. and Hems, R. (1976) Adv. Enzyme Regul. 14, 493-513

21. Kisliuk, R.L. and Sakami, W. (1955) J. Biol. Chem. 214, 47-57

22. Grillo, M.A., Fossa, T. and Coghe, M. (1966) Comp. Biochem. Physiol. 19, 589-596

23. Grignani, F., Martelli, M.F., Tonato, M. and Finzi, A.F. (1967) Arch. Dermatol. 96, 577-585

24. Whiteley, H.R. (1960) Comp. Biochem. Physiol. 1, 227-247

25. Alexander, N. and Greenberg, D.M. (1956) J. Biol. Chem. 220, 775-785

26. Huennekens, F.M., Hatefi, Y. and Kay, L.D. (1957) J. Biol. Chem. 224, 435-444

27. Schirch, L.G. and Mason, M. (1963) J. Biol. Chem. 238, 1032-1037

28. Schirch, L. and Ropp, M. (1967) Biochem. 6, 253-257

29. Bender, D.A. (1975) in: Amino Acid Metabolism, published by William Clowes and Sons Ltd., London

30. Sagers, R.D. and Gunsalus, I.C. (1961) J. Bacteriol. 81, 541-549

31. Klein, S.M. and Sagers, R.D. (1962) J. Bacteriol. 83, 121-126

32. Klein, S.M. and Sagers, R.D. (1966) Bacteriol. Proc. pg. 103

33. Baginsky, M.L. and Huennekens, F.M. (1966) Biochem. Biophys. Res. Commun. 23, 600-605

34. Klein, S.M. and Sagers, R.D. (1965) Bacteriol. Proc. pg. 89

35. Klein, S.M. and Sagers, R.D. (1967) J. Biol. Chem. 242, 297-300

36. Baginsky, M.L. and Huennekens, F.M. (1967) Arch. Biochem. Biophys. 120, 703-711

37. Tabor, H. and Wyngarden, L. (1959) J. Biol. Chem. 234, 1830-1846

38. Stifel, F.B., Taunton, O.D., Greene, H.L., Lufkin, E.G., Hagler, L. and Herman, R.H. (1974) Biochem. Biophys. Acta 354, 194-205

39. Bertino, J.R., Simmons, B. and Donohue, D.M. (1962) J. Biol. Chem. 237, 1314-1318

40. Bertino, J.R., Simmons, B. and Donohue, D.M. (1964) Biochem. Pharmacol. 13, 225-233

41. Himes, R.H. and Wilder, T. (1965) Biochim. Biophys. Acta 99, 464-475

42. Himes, R.H. and Cohn, M. (1967) J. Biol. Chem. 242, 3628-3635

43. Whiteley, H.R. and Huennekens, F.M. (1962) J. Biol. Chem. 237, 1290-1297

44. Himes, R.H. and Rabinowitz, J.C. (1962) J. Biol. Chem. 237, 2903-2914

45. Rabinowitz, J.C. and Pricer, W.E.,Jr. (1962) J.Biol. Chem. 237, 2898-2902

46. Jaenicke, L. (1955) Biochim. Biophys. Acta 17, 588-589

47. Uyeda, K., and Rabinowitz, J.C. (1967) J. Biol. Chem. 242, 4378-4385

48. Tonato, M., Martelli, M.F., Morettini, D. and Grignani, F. (1967) Enzymol. Biol. Clin. 8, 209-214

49. Scrimgeour, K.G. and Huennekens, F.M. (1960) Biochem. Biophys. Res. Commun. 2, 230-233

50. Dalal, F.R. and Gots, J.S. (1967) J. Biol. Chem. 242, 3636-3640

51. Dalal, R. and Gots, J.S. (1966) Biochem. Biophys. Res. Commun. 22, 340-345

52. Taylor, R.T., Dickerman, H. and Weissbach, H. (1966) Arch. Biochem. Biophys. 117, 405-412

53. Yeh, Y.-C., and Greenberg, D.M. (1965) Biochim. Biophys. Acta 105, 279-291

54. Kay, L.D., Osborn, M.J., Hatefi, Y. and Huennekens, F.M. (1960) J. Biol. Chem. 235, 195-201

55. Lombrozo, L. and Greenberg, D.M. (1967) Arch Biochem. Biophys. 118, 297-304

56. MacKenzie, R.E. (1973) Biochem. Biophys. Res. Commun. 53, 1088-1095

57. Greenberg, D.M., Wynston, L.K. and Nagabhushanam, A. (1965) Biochemistry 4, 1872-1878

58. Osborn, M.J., Hatefi, Y., Kay, L.D. and Huennekens, F.M. (1957) Biochim. Biophys. Acta 26, 208-210

59. Huennekens, F.M. and Scrimgeour, K.G. (1963) in: Methods in Enzymology, vol. 6, pg. 373, S.P. Colowick and N.O. Kaplan (eds.), Academic Press, N.Y.

60. Kutzbach, C. and Stokstad, E.L.R. (1971) Methods in Enzymology 18b, 793-798

61. Kutzbach, C. and Stokstad, E.L.R. (1968) Biochem. Biophys. Res. Commun. 30, 111-117

62. Goldthwait, D.A., Peabody, R.A. and Greenberg, G.R. (1956) J. Biol. Chem. 221, 555-567

63. Hartman, S.C., Levenberg, B. and Buchanan, J.M. (1956) J. Biol. Chem. 221, 1057-1070

64. Goldthwait, D.A., Peabody, R.A. and Greenberg, G.R. (1956) J. Biol. Chem. 221, 569-577

65. Hartman, S.C. and Buchanan, J.M. (1959) J. Biol. Chem. 234, 1812-1816

66. Warren, L. and Buchanan, J.M. (1957) J. Biol. Chem. 229, 613-626

67. Caperelli, C.A., Chettur, G., Lin, L.Y. and Benkovic, S.J. (1978) Biochem. Biophys. Res. Commun. 82, 403-410

68. Buchanan, J.M. and Schulman, M.P. (1953) J. Biol. Chem. 202, 241-252

69. Greenberg, G.R. (1954) Fed. Proc. 13, 745-759

70. Flaks, J.G. and Buchanan, J.M. (1954) J. Am. Chem. Soc. 76, 2275-2276

71. Flaks, J.G., Erwin, M.J. and Buchanan, J.M. (1957) J. Biol. Chem. 229, 603-612

72. Levin, A.P. and Magasanik, B. (1961) J. Biol. Chem. 236, 184-188

73. Humphreys, G.K. and Greenberg, D.M. (1958) Arch. Biochem. Biophys. 78, 275-287

74. Lorenson, M.Y., Maley, G.F. and Maley, F. (1967) J. Biol. Chem. 242, 3332-3344

75. Phear, E.A. and Greenberg, D.M. (1957) J. Am Chem. Soc. 79, 3737-3741

76. Brown, S.S., Neal, G.E. and Williams, D.C. (1965) Biochem. J. 97, 34c-36c

77. Maley, F. and Maley, G.F. (1961) Cancer Res. 21, 1421-1426

78. Silber, R., Gabrio, B.W. and Huennekens, F.M. (1963) J. Clin. Invest. 42, 1913-1921

79. Wahba, A.J. and Friedkin, M. (1962) J. Biol. Chem. 237, 3794-3801

80. Greenberg, D.M., Nath, R. and Humphreys, G.K. (1961) J. Biol. Chem. 236, 2271-2276

81. Cohen, S.S., Flaks, J.G., Barner, H.D., Loeb, M.R. and Lichtenstein, J. (1958) Proc. Natl. Acad. Sci. U.S. 44, 1004-1012

82. Heidelberger, C., Griesbach, L., Cruz, O., Schnitzer, R.J. and Grunberg, E. (1958) Proc. Soc. Exptl. Biol. Med. 97, 470-475

83. Kline, I., Venditti, J.M., Mead, J.A.R., Tyrer, D.D. and Goldin, A. (1966) Cancer Res. 26, 848-852

84. Heidelberger, C. (1965) Progr. Nucl. Acid. Res. Mol. Biol. 4, 1-50

85. Silber, R., Huennekens, F.M. and Gabrio, B.W. (1962) Arch. Biochem. Biophys. 99, 328-332

86. Nath, R. and Greenberg, D.M. (1962) Biochemistry 1, 435-441

87. Bertino, J.R., Iannotti, A.T., Perkins, J.P. and Johns, D.G. (1966) Biochem. Pharmacol. 15, 563-571

88. Kaufman, B.T. and Gardiner, R. (1965) Federation Proc. 24, 541

89. Donaldson, K.O. and Keresztesy, J.C. (1962) J. Biol. Chem. 237, 1298-1304

90. Kutzbach, C. and Stokstad, E.L.R. (1967) Federation Proc. 26, 559

91. Kutzbach, C.A. and Stokstad, E.L.R. (1967) Federation Proc. 25, 721

92. Katzen, H.M., Buchanan, J.M. (1965) J. Biol. Chem. 240, 825-835

93. Buchanan, J.M., Elford, H.L., Loughlin, R.E., MacDougall, B.M. and Rosenthal, S. (1964) Annals N.Y. Acad. Sci. 112, 756-773

94. Dickerman, H., Redfield, B.G., Bieri, J.G. and Weissbach, H. (1964) J. Biol. Chem. 239, 2545-2552

95. Loughlin, R.E., Elford, H.L. and Buchanan, J.M. (1964) J. Biol. Chem. 239, 2888-2895

96. Chello, P.L. and Bertino,J.R. (1973) Cancer Res. 33, 1898-1904

97. Arakawa, T. (1970) Amer. J. Med. 48, 594-598

98. Herbert, V. (1962) Transactions of the Association of American Physicians 75, 307-320

99. Kirschner, K. and Bisswanger, H. (1976) Annu. Rev. Biochem. 45, 143-166

100. Welch, G.R. (1977) Prog. Biophys. Molec. Biol. 32, 103-191

101. Drury, E.J., Bazar, L.S. and MacKenzie, R.E. (1975) Arch. Biochem. Biophys. 169, 662-668

102. Beaudet, R. and MacKenzie, R.E. (1975) Biochim. Biophys. Acta 410, 252-261

103. Kornberg, T., Kornberg, A. (1974) The Enzymes 10, 119-144

104. Setlow, P., Brutlag, D., Kornberg, A. (1972) J. Biol. Chem. 247, 224-230

105. Klenow, H., Henningsen, I. (1970) Proc. Natl. Acad. Sci. USA 65, 168-175

106. Setlow, P., Kornberg, A. (1972) J. Biol. Chem. 247, 232-240

107. Jacobsen, H., Klenow, H., Overgaard-Hansen, K. (1974) Eur. J. Biochem. 45, 623-627

108. Zalkin, H. (1973) Adv. Enzym. 38, 1-39

109. Jackson, E.N. and Yanofsky, C. (1974) J. Bacteriol. 117, 502-508

110. Hwang, L.H. and Zalkin, H. (1971) J. Biol. Chem. 246, 2338-2345

111. Li, S.-L., Hanlon, J., Yanofsky, C. (1974) Nature 248, 48-50

112. Davis, R.H. (1967) In: Organizational Biosynthesis, eds. H.J. Vogel, J.O. Lampen, V. Bryson, pg. 303-322, New York: Academic

113. Bonner, D.M., DeMoss, J.A., Mills, S.E. (1965) In: Evolving Genes and Proteins, eds. V. Bryson, H.J. Vogel, pg. 305-318, New York: Academic

114. Manney, T.R. (1970) J. Bacteriol. 102, 483

115. Truffa-Bachi, P., Veron, M., Cohen, G.N. (1974) CRC Crit. Rev. Biochem. 2, 379-415

116. Theze, J., Saint-Girons, I. (1974) J. Bacteriol. 118, 990-998

117. Veron, M., Falcoz-Keily, F., Cohen, G.N. (1972) Eur. J. Biochem. 28, 520-527

118. Zipkas, D. and Riley, M. (1975) Proc. Natl. Acad. Sci., USA 72, 1354-1358

119. Yourno, J., Kohno, T., Roth, J.R. (1970) Nature 228, 820-824

120. Rechler, M.M., Bruni, C.B., Martin, R.G. and Terry, W. (1972) J. Mol. Biol. 69, 427-452

121. Muller-Hill, B., Kania, J. (1974) Nature 249, 561-563

122. Apte, B.N., Zipser, D. (1973) Proc. Natl. Acad. Sci. USA 70, 2969-2973

123. Perrin, D. (1963) Cold Spring Harbour Symp. Quant. Biol. 28, 529-532

124. Ullman, A. and Perrin, D. (1970) in: The Lactose Operon, eds. J.R. Beckwith and D. Zipser (Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.), pg. 143-172

125. Hendrix, R.W. and Casjens, S.R. (1974) Proc. Natl. Acad. Sci. USA 71, 1451-1455

126. Uyeda, K. and Rabinowitz, J.C. (1967) J. Biol. Chem. 242, 24-31

127. Rabinowitz, J.C., and Pricer, W.E.,Jr. (1957) J. Biol. Chem. 229, 321-328

128. Darrow, R.A. and Colowick, S.P. (1962) Methods in Enzymology 5, 226-235

129. Beers, R.F.,Jr., and Sizer, I.W. (1952) J. Biol. Chem. 195, 133

130. Vallee, B.L., and Hoch, F.L. (1955) Proc. Natl. Acad. Sci. 41, 327

131. Jagannathan, V., Singh, K., and Damodaran, M. (1956) Biochem. J. 63, 94

132. Bernfeld, P. (1955) Methods in Enzymology 1 (Colowick, S.P., and Kaplan, N.O., eds.) pg. 149, Academic Press, Inc. N.Y.

133. Lamed, R., Levin, Y., and Wilchek, M. (1973) Biochim. Biophys. Acta 304, 231-235

134. March, S.C., Parikh, I., and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152

135. Siegel, J.M., Montgomery, G.A. and Bock, R.M., (1959) Arch. Biochem. Biophys. 82, 288-299

136. Tan, L.U.L., Drury, E.J. and MacKenzie, R.E. (1977) J. Biol. Chem. 252, 1117-1122

137. Hirs, C.H.W. (1956) J. Biol. Chem. 219, 611-621

138. Bencze, W.L. and Schmid, K. (1957) Anal. Chem. 29, 1193-1196

139. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412

140. Banker, G.A. and Cotman, C.W. (1972) J. Biol. Chem. 247, 5856-5861

141. Martin, R.G. and Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379

142. Darnall, D.W. and Klotz, I.M. (1975) Arch. Biochem. Biophys. 166, 651-682

143. Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155-164

144. Squire, P.G., Moser, P. and O'Konski, C.T. (1968) Biochem. 7, 4261-4272

145. Davies, G.E. and Stark, G.R. (1970) Proc. Nat. Acad. Sci. 66, 651-656

146. Lomant, A.J. and Fairbanks, G. (1976) J. Mol. Biol. 104, 243-261

147. Alexander, P., Fox, M., Stacey, K.A. and Smith, L.F. (1952) Biochem. J. 52, 177-184

148. Carpenter, F.H. and Harrington, K.T. (1972) J. Biol. Chem. 247, 5580-5586

149. Laemmli, U.K. (1970) Nature 227, 680-685

150. Harboe, N., and Ingild, A. (1973) In: A Manual of Quantitative Immunoelectrophoresis. Methods and Applications. Axelson, N.H., Kroll, J., and Weeke, B. (eds.) Universitetforlaget, Oslo

151. Campbell, D.H., Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. (1970) Methods in Immunology, 2nd edition, W.A. Benjamin, Inc. N.Y.

152. Gros, C. and Labouesse, B. (1969) Eur. J. Biochem. 7, 463-470

153. Weiner, A.M., Platt, T., and Weber, K. (1972) J. Biol. Chem. 247, 3242-3251

154. Bertrand, O., Kroviarski, Y., and Boivin, P. (1978) J. Chromat. 147, 435-437

155. Paukert, J.L., D'Ari-Strauss, L., and Rabinowitz, J.C. (1976) J. Biol. Chem. 251, 5104-5111

156. Ramasastri, B.V., and Blakely, R.L. (1962) J. Biol. Chem. 237, 1982-1988

157. Lazowska, J., and Luzzati, M. (1970) Biochem. Biophys. Res Commun. 39, 34-39

158. Lazowska, J., and Luzzati, M. (1970) Biochem. Biophys. Res. Commun. 39, 40-45

159. Zelikson, R. and Luzzati, M. (1977) Eur. J. Biochem. 79, 285-292

160. Paukert, J.L., Williams, G.R. and Rabinowitz, J.C. (1977) Biochem. Biophys. Res. Commun. 77, 147-154

161. Schirch, L. (1978) Arch. Biochem. Biophys. 189, 283-290

162. Waugh, D.F. (1954) Adv. Prot. Chem. 9, 326-437

163. Bigelow, C.C. (1967) J. Theoret. Biol. 16, 187-211

164. Krebs, H.A., Hems, R. and Tyler, B. (1976) Biochem. J. 158, 341-353

165. Cohen, L. and MacKenzie, R.E. (1978) Biochim. Biophys. Acta 522, 311-317

166. Dev, I.K. and Harvey, R.J. (1978) J. Biol. Chem. 253, 4245-4253

167. Schirch, L., Mooz, E.D., and Peterson, D. (1978) In: Chemistry and Biology of Pteridines, eds. R.L. Kisliuk and G.M. Brown, pg. 495-500, Elsevier North Holland, Inc.

168. Conrad, D.R. and Cheng, Y.-C. (1978) Fed. Proc. 37, 493

169. Moore, M.R., O'Brien, W.E. and Ljungdahl, L.G. (1974) J. Biol. Chem. 249, 5250-5253

170. O'Brien, W.E., Brewer, J.M. and Ljungdahl, L.G. (1973) J. Biol. Chem. 248, 403-408

171. Ljungdahl, L., Brewer, J.M., Neece, S.H. and Fairwell, F. (1970) J. Biol. Chem. 245, 4791-4797

172. Williams, D.L. and Spray, G.H. (1970) Brit. J. Haematol. 19, 353-360

173. Scott, J.M. and Weir, D.G. (1976) Clinics in Haematol. 5, 547-568

174. Kutzbach, C.A. and Stokstad, E.L.R. (1967) Biochim. Biophys. Acta 139, 217-220

175. Kutzbach, C. and Stokstad, E.L.R. (1971) Biochim. Biophys. Acta 250, 459-477

176. Waydhas, C., Weigl, K. and Sies, H. (1978) Eur. J. Biochem. 89, 143-150

177. Blakley, R.L. (1960) Biochem. J. 74, 71-82

178. Keilin, D. and Hartree, E.F. (1955) Biochem. J. 60, 310-325

179. Chance, B. (1952) J. Biol. Chem. 194, 471-481

180. Nicholls, P. (1965) Biochim. Biophys. Acta 99, 286-297

181. Chiao, F. and Stokstad, E.L. (1977) Biochim. Biophys. Acta 497, 225-233

182. Scrutton, M.C. and Beis, I. (1979) Biochem. J. 177, 833-846

CONTRIBUTION TO KNOWLEDGE

1. The porcine liver enzymes; methyleneTHF dehydrogenase, methenylTHF cyclohydrolase and 10-formylTHF synthetase were purified to apparent homogeneity by the addition of an affinity chromatographic step to previously established procedures. The protein was shown to be a dimer of identical subunits of 100 000 daltons and its amino acid composition was determined.

   Rabinowitz and his colleagues investigating the enzymes from sheep liver[155], and our work on pig liver, independently demonstrated for the first time that these folate-dependent enzymic activities form a trifunctional protein.

2. A bifunctional fragment containing dehydrogenase and cyclohydrolase activities was prepared from the trifunctional protein by tryptic digestion. The fragment was purified by $NADP^+$-Sepharose chromatography to an extent that a single band was observed on dodecyl sulfate gels. The subunit molecular weight was estimated to be 33 000 daltons. The cyclohydrolase in the fragment retained the ability to be inhibited by ATP and $NADP^+$.

3. A monofunctional synthetase fragment(s) was prepared by the chymotryptic digestion of the trifunctional protein.

The fragment(s) was partially purified by chromatography on 2',5'-ADP-Sepharose and phosphocellulose. The synthetase activity was shown to be associated with a fragment(s) of subunit molecular weight of 65 000 to 67 000 daltons.

4. From immunodiffusion studies, the synthetase and dehydrogenase-cyclohydrolase fragments appear to be derived from different regions of the polypeptide. Similarities in properties between the dehydrogenase and cyclohydrolase, and their physical location within a relatively small polypeptide indicate the catalytic sites of these activities may be physically integrated. From N-terminal sequence analysis of the two fragments as well as the trifunctional protein, the dehydrogenase-cyclohydrolase activities are deduced to be at the N-terminal region of the polypeptide.

5. A modified version of the Krebs' model for the regulation of one-carbon metabolism is proposed. One-carbon units entering via methyleneTHF and methenylTHF, if not immediately used, are proposed to be siphoned into the 5-methylTHF or 10-formylTHF pools. Upon demand, flow of one-carbon units is from the 10-formylTHF pool. The flow is probably subject to local regulations at such enzymes as the cyclohydrolase, methyleneTHF dehydrogenase

and methyleneTHF reductase.

10-FormylTHF dehydrogenase is still maintained to be the main regulator for the relative concentrations of free THF to one-carbon linked THF except the effector is proposed to be free THF, not methionine.