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A study of folic acid dependent reactions in bovine brain

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A STUDY OF FOLIC ACID DEPENDENT REACTIONS IN BOVINE BRAIN

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Dillard S. Broderick

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This thesis, by Dillard S. Broderick, is accepted in its present form by the Department of Chemistry of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.

To my sweet wife Marilyn

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I wish to express my sincere appreciation to Dr. John H. Mangum for his understanding, encouragement, and suggestions which he generously contributed during the course of this study.

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ABBREVIATIONS

NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PLP	pyridoxal 5-phosphate
FAD	flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FH_4	tetrahydrofolate
N^5-methyl FH_4	N^5-methyl tetrahydrofolate
DEAE-cellulose	diethylaminoethyl cellulose
ATP	adenosine triphosphate
DM-30	diethylaminoethyl polyacrylamide gel

INTRODUCTION

A. PREFACE

Workers have previously established conditions for the formation of methionine from homocysteine plus serine or formaldehyde (46). This process was later shown to be a three-step reaction with three different intermediate compounds involving derivatives of the coenzyme folic acid (20). In the first step, serine transhydroxymethylase (L-serine: tetrahydrofolate 5, 10 hydroyxmethyl-transferase E. C. 2.1.2.1.) catalyzes the interconversion of serine and glycine as shown in reaction 1 (Figure 1). N^5, N^{10} -methylene tetrahydrofolate is converted to N^5 -methyl tetrahydrofolate as shown in reaction 2 (Figure 2) by N^5, N^{10} -methylene tetrahydrofolate reductase (5-methyl tetrahydrofolate: NAD oxidoreductase E. C. 1.1.1.68). The N^5 -methyl group is then transferred to homocysteine to form methionine as shown in reaction 3 (Figure 3). This reaction is catalyzed by methionine synthetase.

The objectives of the present investigation were to study reactions 1 and 2 and partially purify serine transhydroxymethylase and N^5, N^{10} -methylene tetrahydrofolate reductase and characterize both of these enzymes.

The occurrence of serine transhydroxymethylase and N^5, N^{10}-methylene tetrahydrofolate reductase is widespread in nature, and both enzymes have been partially purified from several sources (31,33).

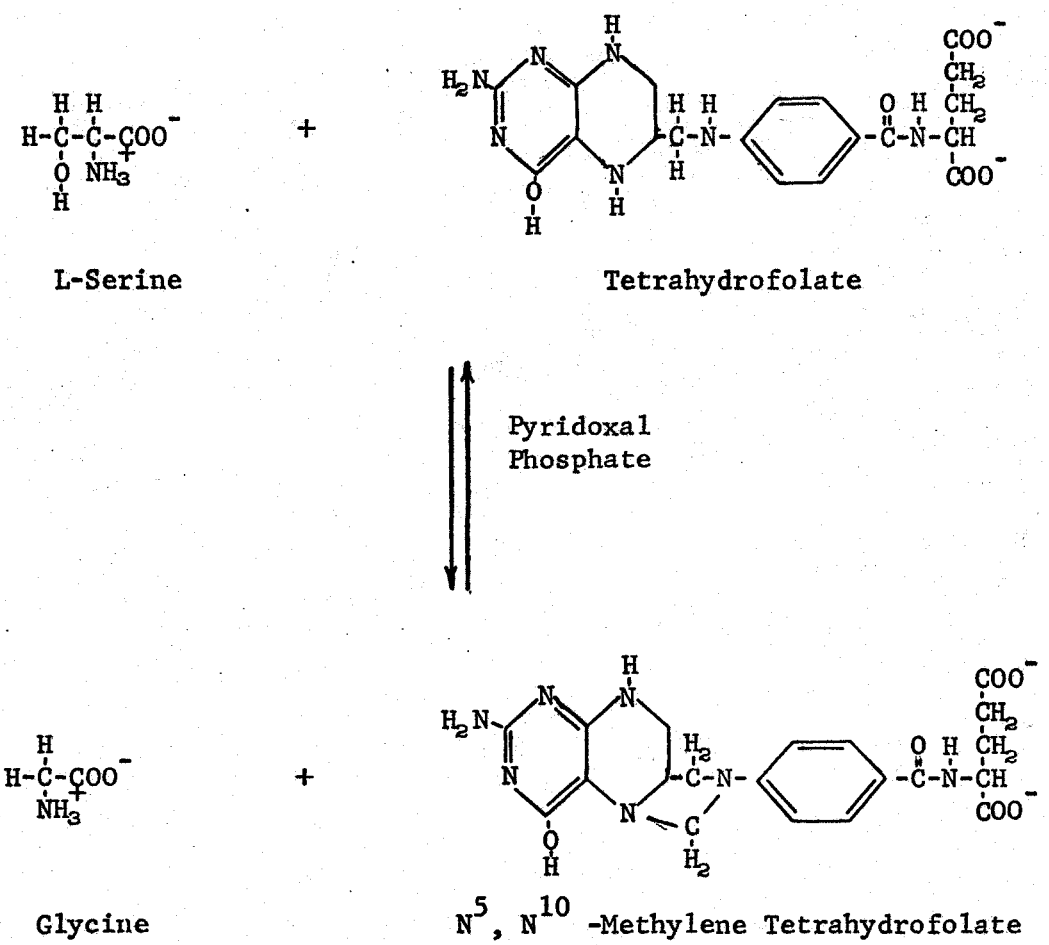
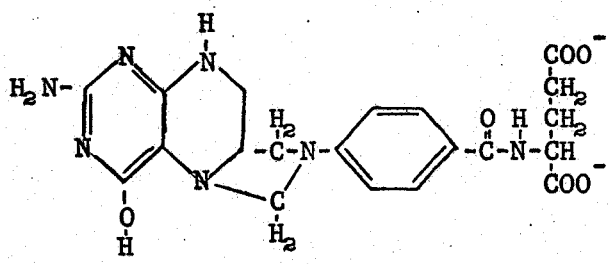


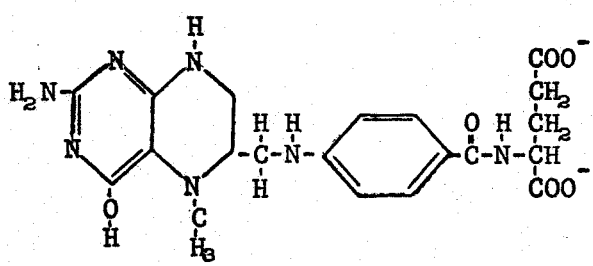
Figure 1. (Reaction 1) The Interconversion of Serine and Glycine

This reaction is catalyzed by serine transhydroxymethylase.



N^5, N^{10} -Methylene Tetrahydrofolate

+ $FADH_2$



N^5 -Methyl Tetrahydrofolate

+ FAD

Figure 2. (Reaction 2) The Conversion of N^5, N^{10} -Methylene Tetrahydrofolate to N^5 -Methyl Tetrahydrofolate

The reaction is catalyzed by N^5, N^{10} -methylene tetrahydrofolate reductase.

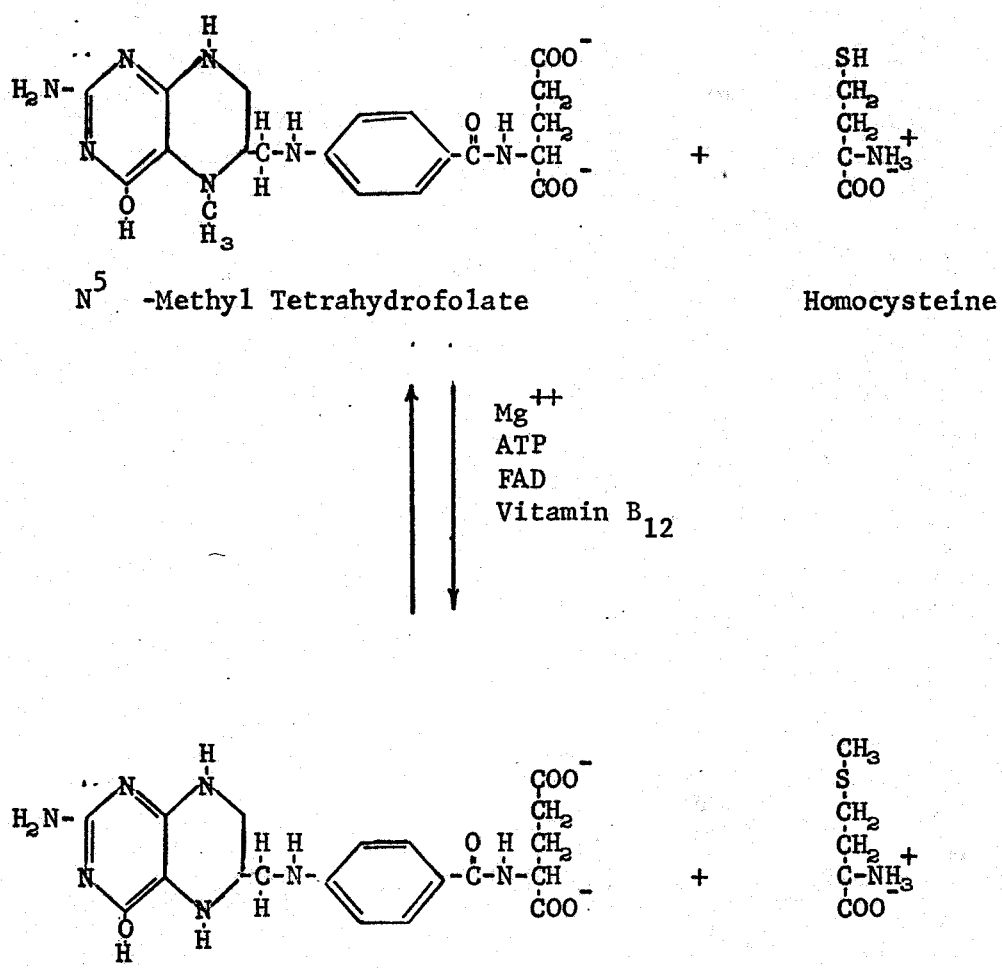


Figure 3. (Reaction 3) The Formation of Methionine from Homocysteine and N^5 -Methyl Tetrahydrofolate

The enzyme that catalyzed this reaction is methionine synthetase.

This thesis describes the partial purification and characterization of serine transhydroxymethylase and N^5, N^{10} -methylene tetrahydrofolate reductase from bovine brain. This represented the initiation of an investigation of folic acid dependent reactions in brain.

LITERATURE REVIEW OF SERINE TRANSHYDROXYMETHYLASE

The biological interconversion of serine and glycine was first demonstrated by Shemin in 1946 by using labeled serine (76). Later Wimnick et al. (22,85) showed that glycine was rapidly converted to serine in rat liver homogenates.

The condensation of glycine with a one-carbon compound was postulated by several investigators as the mechanism for the synthesis of serine. Sakami (63) demonstrated this by showing that C^{14} -formate was incorporated in serine at the beta carbon in the intact rat. He also showed that glycine labeled in the methyl carbon with C^{14} gave rise to serine labeled equally with C^{14} in the alpha and beta carbons (64). These results indicated that glycine could serve as a major source for the beta carbon of serine by breaking down to a one-carbon compound. Siekevitz and Greenberg (78) also verified these results by using rat liver slices. In addition to glycine and formate, other compounds were found to be the precursors of the beta carbon of serine. These included such compounds as the methyl groups of choline (65), acetone (66), and methionine (80).

Even though formate was shown to be an effective precursor of serine, most investigators felt that it was not the immediate precursor. Evidence was then provided that formaldehyde was the one-carbon

precursor (43). Siegel and Lafaye (77) provided support for this hypothesis by showing that the beta carbon of serine in rat liver homogenates came from formaldehyde and not formate. Formate was shown to be incorporated in serine at the beta position when ATP, alpha-ketoglutarate, citrate, and a reducing agent were included in the incubation with rat liver homogenates (57). In the conversion of the beta carbon of serine to the methyl group of choline or thymine, both the beta hydrogen atoms of serine were retained, which excluded formate as an intermediate (19).

While investigating the nature of the compound which condensed with glycine to form serine, it was found that folic acid was required in the reaction (28). Elwyn and Sprinson (18) found the conversion of serine to glycine to be only one-sixth as fast in folic acid deficient rats as in normal ones. It was also observed that the incorporation of formate in serine at the beta carbon was ten times greater in normal rats than in folic acid deficient rats (61).

It was demonstrated that the conversion of folic acid to tetrahydrofolate was necessary before it could participate in serine biosynthesis (4). Kisluik and Sakami (42) showed that folic acid stimulated serine biosynthesis maximally if ATP, NAD^+, and yeast extract were added to well dialyzed extracts of liver homogenates. Blakely (6) observed that if tetrahydrofolate was added to the incubation mixture, maximum serine biosynthesis occurred without these other co-factors.

It was subsequently shown that the biosynthesis of serine could utilize the following folic acid co-enzyme: N^5 -formyl tetrahydrofolate (35), N^{10} -formyl folate (62), and N^{10} -formyl tetrahydrofolate (32).

Both the N^5 - and N^{10} -positions of tetrahydrofolate appeared to be involved in binding formaldehyde. Kisluik (39) showed that formaldehyde reacted rapidly and non-enzymatically with tetrahydrofolate to form a compound which was a precursor of the beta carbon of serine. Blakley (8) demonstrated that the compound formed non-enzymatically from tetrahydrofolate and formaldehyde was the same as the compound formed from serine and tetrahydrofolate in the enzymatic conversion of serine to glycine. Osborn et al. (60) clearly showed the formaldehyde-tetrahydrofolate compound to be N^5, N^{10} -methylene tetrahydrofolic acid.

Pyridoxal 5-phosphate was a second co-factor, which was implicated in serine-glycine interconversion (45). Deodhar and Sakami (14) observed that liver extracts of pyridoxine-deficient chickens showed a reduced ability for the incorporation of formate into serine. Blakley (7) demonstrated the direct participation of pyridoxal phosphate with a partially purified enzyme from rabbit liver. The enzyme isolated from the livers of certain mammals also showed an absolute requirement for pyridoxal phosphate (1). It was then demonstrated that deoxypridoxine inhibited serine synthesis in partially purified rat liver preparations and that pyridoxal phosphate reversed the inhibition (2).

Pyridoxal 5-phosphate has long been considered to participate in the serine transhydroxymethylase reaction by reacting with the amino acid substrates to form activated Schiff base intermediates (73), but little or no direct evidence was published to support this mechanism until 1962 when Schirch and Mason (72) gave spectral evidence to support this conclusion.

The evidence thus far presented has demonstrated that a one-carbon fragment derived from formaldehyde condenses with glycine to form serine via a folic acid derivative i. e., N^5, N^{10} -methylene tetrahydrofolate. Pyridoxal phosphate is also required in the reaction and the amino acid is activated by the formation of a Schiff base in the presence of pyridoxal 5-phosphate.

Serine transhydroxymethylase has been reported to occur in a variety of mammalian, avian, and plant tissues as well as in microorganisms (31). Two serine transhydroxymethylases, which differ in physical properties, were reported to occur in rat liver (58) i. e., one in the soluble and the other in the mitochondrial fractions.

Serine transhydroxymethylase has been shown to form a complex with glycine (71); D-alanine (70); and glycine with either tetrahydrofolate (73) or N^5 -methyl tetrahydrofolate (71) or N^5 -formyltetrahydrofolate (74).

It has recently been demonstrated that the rabbit liver enzyme also catalyzed the cleavage of threonine to glycine and acetaldehyde (69). However, serine transhydroxymethylase from Clostridium cylindrosporum has an absolute specificity for serine (83).

C. LITERATURE REVIEW OF N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

An additional enzyme N^5, N^{10} -methylene tetrahydrofolate reductase has been implicated in the formation of methionine from homocysteine and N^5, N^{10} -methylene tetrahydrofolate (46). Initially this conversion was thought to involve only a single enzyme, methionine synthetase.

N^5, N^{10} -methylene tetrahydrofolate reductase was referred to earlier as the "205-2" enzyme because it represented a missing component of a methionine-less mutant strain of E. coli 205-2 (26). When this enzyme was incubated with NADH and C^{14} labeled N^5, N^{10} -methylene tetrahydrofolate, a product was formed that could be isolated by chromatography. It was assigned the structure N^5 -methyl tetrahydrofolate (46). This compound was also prepared chemically by the reduction of N^5, N^{10} -methylene tetrahydrofolate with sodium borohydride (36,67).

The source of the hydrogen for the methionine-methyl has been investigated using the N^5, N^{10} -methylene tetrahydrofolate reductase reaction. The fact it was shown that the hydrogen did not come from the pyrazine ring (40), supported the proposal of Larrabee et al. (46) that N^5 -methyl tetrahydrofolate acts as a donor in a transmethylation reaction during methionine synthesis, and that the question of de novo methyl synthesis resides in the N^5, N^{10} -methylene tetrahydrofolate reductase reaction. It was later found that chemically reduced FAD could substitute for NADH in the reaction (17), and Donaldson and Keresztesy (17) showed that the enzyme was a flavoprotein. Since it was shown that no loss of the hydrogen occurred during the transfer of the methyl group from tetrahydrofolate to homocysteine (36), the introduction of the one hydrogen into the methyl group of methionine must have occurred during the N^5, N^{10} -methylene tetrahydrofolate reductase reaction. Cathou (10) demonstrated that water contributed the hydrogen to the methyl of N^5 -methyl tetrahydrofolate. It was concluded that the reductase reaction does not involve a direct reduction of N^5, N^{10}-

methylene tetrahydrofolate by either NADH or by intramolecular reduction by the reduced pyrazine ring. It was shown that there was an exchange of hydrogens of reduced FAD with the hydrogen atoms of the solvent (10,36). The source of the three hydrogen atoms of the methyl group of N^5 -methyl tetrahydrofolate and of methionine were accounted for as the following: two hydrogen came from the beta-carbon of serine (31), and the third hydrogen came from a hydrogen of reduced FAD associated with N^5, N^{10} -methylene tetrahydrofolate reductase.

N^5, N^{10} -methylene tetrahydrofolate reductase was also known as prefolic A oxidase in hog liver (17). This activity was first detected by measuring the conversion of prefolic A to tetrahydrofolate in an incubation mixture containing a dye as an elector acceptor (16). Earlier it had been suggested that prefolic A was identical to N^5 -methyl tetrahydrofolate (16). This prompted a further investigation of the mammalian prefolic A oxidase, and its relationship to the bacterial N^5, N^{10} -methylene tetrahydrofolate reductase. The evidence which justified this investigation were the following: (1) the detection of the various mammalian tetrahydrofolate co-enzyme interconversion were accomplished by microbiological methods (3); (2) Donaldson and Keresztesy (17) reported that prefolic A synthesis catalyzed by hog liver enzyme (prefolic A oxidase) required tetrahydrofolate, NADH, and formaldehyde, and that this reaction was markedly activated by FAD; and (3) biosynthetic prefolic A was shown to be identical with prefolic A isolated from mammalian liver. It was therefore concluded that prefolic A oxidase and N^5, N^{10} -methylene tetrahydrofolate reductase activities were due to the same enzyme.

N^5, N^{10} -methylene tetrahydrofolate reductase is a flavoprotein and in the absence of menadione or an active electron acceptor, the over-all formation of N^5 -methyl tetrahydrofolate occurs in the following manner (46), (bacterial enzyme):

$$\text{NADH} + \text{Enzyme-FAD} + H^+ \rightleftharpoons \text{Enzyme-FADH}_2 + NAD^+$$

$$\text{Enzyme-FADH}_2 + N^5, N^{10}\text{-methylene tetrahydrofolate} \rightleftharpoons$$

$$N^5\text{-methyl tetrahydrofolate} + \text{Enzyme-FAD}$$

In the presence of menadione or an active electron acceptor, N^5 -methyl tetrahydrofolate is oxidized as shown below (46), (mammalian enzyme):

$$N^5\text{-methyl tetrahydrofolate} + \text{Enzyme-FAD} \rightleftharpoons$$

$$\text{Enzyme-FADH}_2 + N^5, N^{10}\text{-methylene tetrahydrofolate}$$

$$\text{Enzyme-FADH}_2 + \text{menadione} \rightleftharpoons \text{menadione } H_2 + \text{Enzyme-FAD}$$

Inhibition studies were consistent with the proposed schemes, and in the absence of tetrahydrofolate compounds, the enzyme possessed NADH-menadione reductase activity. N^5, N^{10} -methylene tetrahydrofolate reductase has previously been partially purified from bacteria (11), and hog liver (17).

MATERIALS AND EXPERIMENTAL METHODS

A. MATERIALS

1. Chemicals

Chemicals used in this study were obtained from the following sources: DL-serine-3-C^{14} (0.5 mc/mg), formaldehyde-C^{14} (0.5 mc/1.25 mg), PPO (2,5 diphenyloxazole), and POPOP (p-Bis[2-(5-phenyloxazolly)] benzene), New England Nuclear Corporation; folic acid, alcohol dehydrogenase, NADH, and sephadex G-200, Sigma Chemical Company; DM-30, FAD, pyridoxal phosphate, and hydroxlapatite, Calbiochem; DEAE-cellulose and mercaptoethanol, Matheson Coleman and Bell; dimedon and menadione, J. T. Baker Chemical Comapny; protamine sulfate, Nutritional Biochemical Corporation; and crystalline bovine albumin, Mann Research Laboratories, Incorporation. The highest purity compounds available were used without further purification.

2. Determination of pH

All pH determinations were made with a Corning Model 10 Glass Electrode pH Meter.

3. Protein Estimation

a. Biuret Method of Protein Estimation. Protein was determined by Biuret reaction according to the method of Gornall *et al.* (23). The Biuret solution was standardized against crystalline bovine albumin.

b. Protein Estimation by Ultraviolet Absorption. Protein estimation was based on the extinction coefficient of endolase given by Warburg and Christian (84).

c. Lowry Method of Protein Estimation. Proteins was also determined by the Lowry method (50). The Lowry solution was standardized against crystalline bovine albumin.

4. Preparation of Tetrahydrofolate

Tetrahydrofolate was prepared by reducing folic acid according to the method of O'Dell (59) as modified by Hatefi et al. (27). One gram of folic acid was placed in a pressure bottle with 0.5 gram of platinum oxide, and 75 ml of glacial acetic acid. The bottle was then placed in a Parr Pressure Reaction Apparatus connected to a hydrogen tank. The bottle was flushed 4 times with hydrogen using a pump to remove the gas. The solution of folic acid and platinum oxide was shaken in the Parr Pressure Reaction Apparatus under 35 pounds of hydrogen pressure for 3 hours. After hydrogenation the solution was filtered through a funnel under vacuum into a round-bottom flask suspended in liquid nitrogen. The round-bottom flask was swirled in the liquid nitrogen until the clear filtered solution was frozen. After freezing, the solution was lyophilized. The resulting while powder was sealed under a vacuum and stored at -20° in the dark.

When the material was to be used in the assay, an aliquot of the stock solution was diluted at 4.5 mg per ml in a 0.04 M percaptoethanol solution, and the pH was adjusted to 7.4. The solution was then stored in a Thunburg tube under an argon atmosphere at zero degrees. The tetrahydrofolate was stable from 2 to 3 weeks in the mercaptoethanol solution.

5. Preparation of N^5 -Methyl Tetrahydrofolate

N^5 -methyl tetrahydrofolate was prepared by the reduction of N^5, N^{10} -methylene tetrahydrofolate with borohydride (37). N^5, N^{10}-methylene tetrahydrofolate was prepared chemically by treating tetrahydrofolate with excess formaldehyde (60). Two hundred milligrams of tetrahydrofolate were dissolved in 8 ml of oxygen free 0.5 M phosphate buffer pH 7.0 containing 0.4 ml of 1:10 dilution of 37 per cent formaldehyde, and 1 millicurie of formaldehyde-C^{14} (0.5 mc/1.25 mg). The reaction mixture was flushed thoroughly with nitrogen and incubated for 10 minutes at 37°. Four hundred milligrams of potassium boroydride were added slowly to the incubation mixture, and the mixture was incubated for an additional 60 minutes at 37° under a nitrogen atmosphere. An additional 0.2 ml of 37 per cent formaldehyde was added slowly to the mixture which reacted with the excess borohydride. Eight milliliters of oxygen-free water was added to the reaction mixture, and the mixture was chromatographed immediately on DEAE-cellulose. The DEAE-cellulose column (3 X 10 cm) had previously been washed with 1 liter of water and with 400 ml of 0.133 M ammonium acetate. The above reaction mixture was carefully layered on the column, and the column was eluted by a linear gradient method in which the mixing chamber contained 400 ml of 0.133 M ammonium acetate, pH 6.9, and the reservoir contained 400 ml of 0.4 M ammonium acetate, pH 6.9. The ammonium acetate buffers contained 0.01 M mercaptoethanol. The effluent (10 ml fractions) was collected every 10 minutes with an automatic fraction collector, and N^5 -methyl tetrahydrofolate containing fractions were identified by their characteristic ultraviolet absorption maxima at

290 mμ and minima at 245 mμ. These fractions which appeared in the second peak were pooled and lyophilized. The residue was redissolved in sufficient 0.05 M potassium phosphate buffer pH 7.5, to give a solution which contained 2.5 μ moles of N^5 -methyl tetrahydrofolate per ml. The solution was then frozen and stored.

6. Preparation of DEAE-Cellulose

DEAE-cellulose was suspended in water, and the fines and smaller fibers were removed by repeated decantation. The remaining compacted mass was drained and washed with 1.0 M dipotassium phosphate until the washings were colorless as detected spectrometrically at 280 mμ. It was then washed with water until the pH of the suspension was the same as the water with which it was washed. The remaining mass was then drained and stored in water at a concentration of 25 mg per ml. The slurry was brought to a boil in vacuo to eliminate trapped air before the cellulose was packed into columns. The columns were fitted with clamped polyethylene tubing on their outlets. The cellulose slurry was added in portions until sufficient cellulose had been added to the column. The column was then drained dropwise until the cellulose was compacted. The columns were washed with a liter of water and with 500 ml of the desired buffer before using.

7. Preparation of Calcium Phosphate Gel

Calcium phosphate gel was prepared according to the method by Keilin and Hartree (34). One hundred fifty ml of calcium chloride solution (88.6 g $CaCl_2 \cdot 2H_2O$ per liter) was added to 160 ml of distilled water and shaken with 150 ml of trisodium phosphate solution (152 g $Na_3PO_4 \cdot 12H_2O$ per liter). The mixture was brought to pH 7.4 with 1.0 N

acetic acid, and the precipitate was washed 5 times by decantation with large volumes of water (6 liters). The precipitate was then centrifuged and suspended in 300 ml of water.

Hydroxylapatite columns were sometimes used instead of calcium phosphate gel. The hydroxylapatite columns were packed under 10 pounds of nitrogen pressure.

8. Preparation of G-200 Sephadex

Sephadex G-200 was swelled in a large excess of water in an 80° bath for 5 hours. After swelling, fines were removed by repeated washings and decantings. The sephadex slurry was then mixed with 2 times the volume of buffer and brought to a boil in vacuo to eliminate trapped air before packing the sephadex column. The excess buffer was siphoned off to give a thick sephadex slurry. The sephadex slurry was carefully poured down a glass rod into the sephadex column (3 X 90 cm) and reservoir. Enough slurry was added the first time so that no further additions were necessary. The slurry was allowed to settle in the column for about 1 hour. The column was then packed with a pressure head of 10 cm and care was exercised at all times to avoid a pressure head in excess of 15 cm. After the proper height in the column was reached, excess slurry was removed; the outlet was stopped, and the column was allowed to settle for a few hours. The upward adapter was then inserted, and the column was equilibrated with the desired buffer.

9. Preparation of DM-30 Column

DM-30 was allowed to swell in a large excess of 0.01 M potassium phosphate buffer pH 7.0 for 12 hours at 0°. After swelling, a column (2.5 X 30 cm) was packed by filling the column with 0.02 M potassium

phosphate buffer pH 7.0, and pouring the DM-30 slurry into a large funnel which had been placed above the column. The column was allowed to stand for 1 hour while the slurry settled to the bottom of the column. After settling, the column was washed with 0.02 M potassium phosphate buffer pH 7.0 for a few hours before using.

B. METHODS

1. Assay for Serine Transhydroxymethylase

The methods used to assay serine transhydroxymethylase have involved: (1) estimation of serine by periodate oxidation followed by chromotropic acid treatment (2,38); (2) determination of serine by manometric measurement of CO_2 release upon periodate oxidation (7); (3) manometric determination of O_2 uptake during oxidation of the C-1 unit to CO_2 (30); (4) colorimetric estimation of the disappearance of formaldehyde bound in N^5, N^{10} -methylene tetrahydrofolate with acetyl-acetone reagent (75); (5) spectrophotometric measurement of N^5, N^{10}-methylene tetrahydrofolate with N^5, N^{10} -methylene tetrahydrofolate dehydrogenase plus NADP in a two-step assay procedure (72); and (6) a radioactive assay using 3-C^{14}-serine (82).

The assay for serine transhydroxymethylase used in this study was a modification of the radioactive assay by Taylor and Weissbach (82). To a 12 ml test tube 0.1 ml of 0.3 M potassium phosphate buffer pH 7.4 (30 μ moles); 0.05 ml of 0.002 M pyridoxal phosphate (0.1 μ moles); 0.1 ml of enzyme (less than 1.0 mg); and 0.1 ml of 0.04 M mercapto-ethanol with 4.5 mg of tetrahydrofolate per ml (4 μ moles with 0.8 μ moles of tetrahydrofolate) were added. The mixture was incubated for 5 minutes at 37°. After incubation 0.1 ml of 3-C^{14}-L-serine 0.002 M

(0.2 μ mole) 6.0 X 10^6 cpm per μ mole was added and the mixture was again incubated for 15 minutes at 37^o.

The reactions were stopped by adding 0.3 ml of 0.4 M dimedon (5-5 dimethyl -1,3 cyclohexanedione) in 50 per cent ethanol. The mixture was heated for 5 minutes in boiling water and then cooled in an ice bath for 5 minutes. Five milliliters of toluene were added and the formaldehyde dimedon adduct was extracted by vigorous shaking. After 2 minutes of centrifugation, 3 ml of the upper toluene layer were added to 10 ml of Bray's solution (9) and counted in a Nuclear-Chicago Unilux I liquid scintillation counter for 1 minute. The control tube included everything except that 5 ml of water was added instead of 5 ml of toluene, and 3 ml of water wad added to the 10 ml of Bray's solution. This control tube gave the total count and the percentage of the reaction was taken from this count. A blank tube included everything except the enzyme, and the counts from this tube were substracted from the counts of the enzyme activity. One unit of enzyme is defined as the μ moles of L-serine converted per hour per mg of enzyme.

2. Assay for Threonine Aldolase

Threonine aldolase activity was estimated by measuring the rate of acetaldehyde formation by the method of Malkin and Greenberg (52). The acetaldehyde formed as shown in reaction 4 (Figure 4) was reduced by NADH and alcohol dehydrogenase to ethanol, reaction 5 (Figure 4). To a 3 ml cuvette of 1.0-cm light path were added 2.3 ml of 0.05 M potassium phosphate buffer pH 7.4 (115 μ moles), 0.1 ml of 0.005 M NADH (0.5 μ moles), 0.01 ml of alcohol dehydrogenase suspension (0.05 mg), 0.1 ml of enzyme (less than 1.0 mg), and 0.4 ml of 0.5 M L-threonine (200 μ moles)

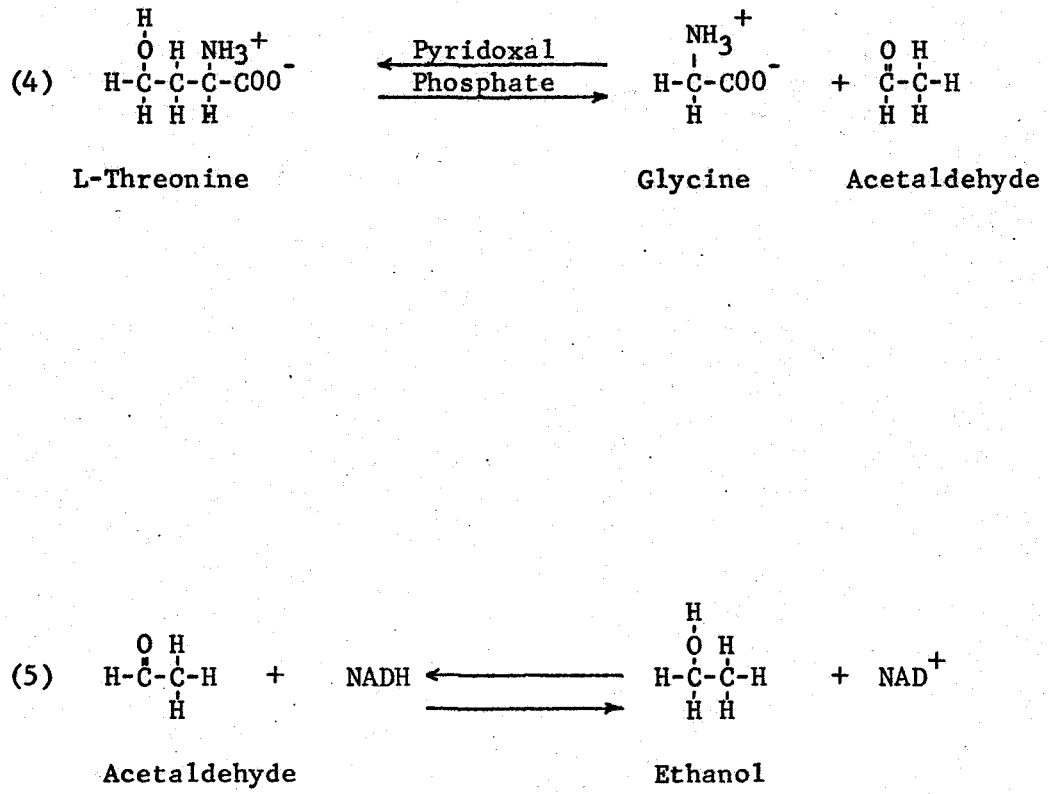


Figure 4. [Reactions 4 and 5] The Inconversion of L-Threonine to Glycine and Acetaldehyde and the Interconversion of Acetaldehyde to Ethanol

to bring the total volume to 3.0 ml. The phosphate buffer was preheated to 37° and the assay solution was kept at 37° during the assay using a jacketed cell compartment through which water at 39° was circulated. Readings were instigated immediately after addition of the substrate and the values were recorded for 15 minutes with a Beckman DB Spectrophotometer. The decrease in absorbancy at 340 mμ was used as a measurement of enzyme activity. An enzyme blank was unnecessary because there was no non-enzymatic conversion of threonine to glycine and acetaldehyde under standard assay conditions. The reference blank contained everything except the enzyme and NADH. The change in absorption was recorded on a Honeywell Electronik Recorder #19, and the slope of this line was used to determine the change in absorbancy units per minute. The specific activity is reported as μ moles of product formed per mg of protein per hour.

3. Assay for N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The standard assay for N^5, N^{10} -methylene tetrahydrofolate reductase was performed in the reverse direction of reaction 2 (Figure 2) where N^5 -methyl C^{14} tetrahydrofolate served as the substrate and menadione functioned as an artifical electron acceptor. The assay was adapted from a similar assay proposed by Kutzbach and Stockstad (44). The N^5, N^{10} -methylene tetrahydrofolate formed dissociated easily to yield labeled formaldehyde which was isolated as the dimedon adduct. The incubation mixture contained: 0.5 ml of potassium phosphate buffer pH 6.3 (250 μ moles), 0.05 ml of 0.0001 M of FAD (5 mμ moles), 0.1 ml of 0.02 M menadione (2 μ moles) (not completely dissolved), 0.05 ml of 0.1 M ascorbic acid (5 μ moles), 0.1 ml of enzyme (less than 1.5 mg),

and 0.05 ml of 0.0025 M (N^5-C^{14}) N^5 -methyl tetrahydrofolate (400 counts per minute per mμ mole) (125 mμ moles). The N^5 -methyl tetrahydrofolate was added after the other components of the mixture had incubated for 5 minutes at 37°. The entire mixture was then incubated for 15 minutes at 37°. The reaction was stopped by adding 0.3 ml of a solution of 0.4 M dimedon in 50 per cent ethanol and by heating for 5 minutes at 95°. The incubation mixture was cooled in ice, and the formaldehyde dimedon adduct was extracted into 5 ml of toluene by vigorous shaking for 15 seconds. The phases were then separated by centrifugation and 3 ml of the toluene phase was added to the Bray's solution (9) and counted in a Nuclear-Chicago Unlix I liquid scintillation counter. The control tube contained everything except that 5 ml of water was added instead of 5 ml of toluene, and 3 ml of water was added to 10 ml of Bray's solution. This control tube gave the total count and the percentage of the reaction was taken from this count. A blank tube included everything except the enzyme, and the counts from this tube were substracted from the counts of the complete assay mixture. The specific activity was defined as the μ moles of formaldehyde formed per hour per mg of enzyme.

4. Purification Scheme of Serine Transhydroxymethylase

Previous investigators have shown that serine transhydroxymethylase is widely distributed in nature. In this study, the enzyme was partially purified from fresh frozen bovine brain. Enzymatic activity decreases in brain frozen for more than a few weeks. The methods used in the purification scheme are a modification of the methods of Schirch (68) and Nakano et al. (58). All operations were carried out at 0°-5° unless otherwise indicated; centrifugations were performed

in the Servall Refrigerated RC-2 and RC-2B Centrifuges.

Step 1. Preparation of Crude Extract. One thousand grams of fresh frozen bovine brain were chopped into small pieces and homogenized for 2 minutes in a Waring blender with 2,000 ml of 0.03 M potassium phosphate buffer pH 6.5. The resulting homogenate was centrifuged for 15 minutes at 40,000 x g.

Step 2. Protamine Sulfate Precipitation. The supernatant fluid was then diluted with 0.03 M potassium phosphate buffer pH 6.5 to bring the protein concentration to 9 mg per ml. To this solution was added a sufficient volume of a 1.0 per cent protamine sulfate suspension, pH 6.5 such that the final protamine sulfate concentration was 6 mg per 100 ml. After 5 minutes had been allowed for precipitation, the mixture was centrifuged at 40,000 x g for 10 minutes. The precipitate was discarded. An alterant procedure involved a 2-step protamine sulfate precipitation: initially 2.5 mg of protamine sulfate was added per 100 ml and centrifuged (this precipitate was discarded); an additional 3.5 mg per 100 ml was added to the supernatant fraction and centrifuged (This precipitate was saved for the purification of N^5, N^{10} -methylene tetrahydrofolate reductase).

Step 3. Ammonium Sulfate Precipitation. Sufficient solid ammonium sulfate (500 gm) was added to the protamine sulfate supernatant fluid from Step 2 to produce a 33 per cent saturation. After stirring for 15 minutes, the inert protein was removed by centrifugation for 10 minutes at 40,000 x g. The supernatant solution was adjusted to 53 per cent saturation with solid ammonium sulfate (325 gm) and allowed to stand for 15 minutes with stirring prior to centrifugation for 10 minutes at 40,000 x g. The precipitate was dissolved in 100 ml of 0.03 M

potassium phosphate buffer pH 7.4, and dialyzed against 6 liters of 0.03 M potassium phosphate buffer pH 7.4 for 4 hours.

Step 4. Second Ammonium Sulfate Precipitation. The dialysate from step 3 was diluted with 0.03 M potassium phosphate buffer pH 7.4 to bring the protein concentration to 10 mg per ml. To this solution was added solid ammonium sulfate (35 gm) to produce a 30 per cent saturation. After standing for 15 minutes with stirring, the protein was removed by centrifugation at 40,000 x g for 10 minutes. The supernatant solution was then adjusted to 45 per cent saturation with solid ammonium sulfate (24 gm) and allowed to stand for 15 minutes with stirring prior to centrifugation for 10 minutes at 40,000 x g. The precipitate was dissolved in 50 ml of 0.005 M potassium phosphate buffer pH 7.4 and dialyzed against 6 liters of 0.005 M potassium phosphate buffer pH 7.4 for 6 hours.

Step 5. DEAE-Cellulose Column. The dialyzed solution from step 4 was added to a DEAE-Cellulose column (3 X 14 cm) which had been equilibrated with 0.005 M potassium phosphate buffer pH 7.4. The column was washed with the buffer until no protein could be detected in the eluate by its absorption at 280 mμ. Gradient elution was then applied to the column with 400 ml of 0.005 M potassium phosphate buffer pH 7.4 in the mixing vessel and 400 ml of 0.3 M potassium phosphate buffer pH 7.4 in the reservoir. The eluate was collected every 10 minutes and each tube containing protein was assayed. The fractions with the highest specific activity were pooled and precipitated by the addition of solid ammonium sulfate (50 per cent saturation). After standing for 15 minutes with stirring, the protein was collected by centrifugation at 40,000 x g for 10 minutes.

Step 6. Gel Filtration on Sephadex G-200. The protein from Step 5 was dissolved in 0.05 M potassium phosphate buffer pH 7.4. The concentrated solution (30 mg protein per ml) was carefully layered onto a sephadex G-200 column (3 X 90 cm) which had been equilibrated with 0.05 M potassium phosphate buffer pH 7.4. After washing the enzyme into the column with the same buffer, the buffer from the column was eluted at a rate of 0.3 ml per minute and the eluate collected every 20 minutes. The appearance of protein was followed by the absorbance at 280 mμ and peak tubes were assayed for enzyme activity. The tubes with the highest activity were then combined and solid ammonium sulfate was added to produce 50 per cent saturation. After stirring for 15 minutes, the protein was centrifuged for 10 minutes at 40,000 x g, and the precipitate was then frozen and stored.

5. Purification Scheme of N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The occurrence of N^5, N^{10} -methylene tetrahydrofolate reductase is widespread in nature, and the enzyme has been partially purified from several sources. In this investigation, N^5, N^{10} -methylene tetrahydrofolate reductase was partially purified from fresh frozen bovine brain. Enzymatic activity was found to decrease in brain frozen for more than a few weeks. The methods used in this purification scheme are modified from the purification schemes of Katzen and Buchanan (33) and Huennekens (29). All the operations in the procedure were carried out at 0-5° unless otherwise indicated; centrifugations were performed in either the Servall Refrigerated RC-2 or RC-2B Centrifuge and the Spinco Model L Ultracantrifuge.

Step 1. Preparation of Crude Extract. One thousand grams of fresh frozen bovine brain were chopped into small pieces and homogenized for 2 minutes in a Waring blender with 2,000 ml of 0.03 M potassium phosphate buffer pH 6.5. The resulting homogenate was centrifuged for 15 minutes at 40,000 x g.

Step 2. Protamine Sulfate Precipitation. The supernatant fluid from the crude extract was then diluted with 0.03 M potassium phosphate buffer pH 6.5 to bring the protein concentration to 9 mg per ml. To this solution was added with rapid stirring, a 1 per cent protamine sulfate suspension pH 6.5 to bring the final concentration to 2.5 mg per 100 ml. After 5 minutes had been allowed for precipitation, the mixture was centrifuged at 40,000 x g for 10 minutes. The precipitate was discarded, and to the supernatant fraction was added a further quantity of protamine sulfate to bring the total concentration of the latter to 6 mg per 100 ml. Again the precipitate was removed by centrifugation and the material precipitating between 2.5 and 6.0 mg per 100 ml of protamine sulfate concentration, which contained the enzyme activity, was dissolved in 300 ml of 0.5 M potassium phosphate buffer pH 7.4 using a glass homogenizer. The supernatant fluid was used for the purification scheme of serine transhydroxymethylase (see purification scheme of serine transhydroxymethylase).

Step 3. Ammonium Sulfate Precipitation. The suspension from step 2 was diluted with 0.5 M potassium phosphate buffer pH 7.4 to bring the final protein concentration to 10 mg per ml. To this solution was added with rapid stirring, solid ammonium sulfate (18 gm) to produce 12 per cent saturation. After standing for 15 minutes with stirring,

the inert protein was removed by centrifugation for 10 minutes at 40,000 x g. The supernatant solution was adjusted to 40 per cent saturation with solid ammonium sulfate (55 gm) and allowed to stand for 10 minutes at 40,000 x g. The precipitate was dissolved in 100 ml of 0.02 M potassium phosphate buffer pH 7.0 and dialyzed against 6 liters of 0.02 M potassium phosphate buffer pH 7.0 for 6 hours.

Step 4. High Speed Centrifugation. The dialysate from step 3 was centrifuged at 100,000 x g for 30 minutes in the Spinco (Model L) Untracentrifuge (No. 40 rotor). This step usually cleared the supernatant solution of the white precipitant material which occurred during dialyzing.

Step 5. Absorption and Elution from DM-30 Column. The enzyme solution from step 4 was added to a DM-30 column (2.5 X 30 cm) which had been equilibrated with 0.02 M potassium phosphate buffer pH 7.0. The column was washed with the buffer until no protein could be detected in the eluate by its absorption at 280 $m\mu$. A gradient elution was then applied to the column with 500 ml of 0.01 M potassium phosphate buffer pH 7.0 in the mixing vessel and 500 ml of 0.5 M potassium phosphate buffer pH 7.0 in the reservoir. The eluate was collected every 10 minutes and each tube containing protein was assayed. The fractions with the highest specific activity were pooled and precipitated by the addition of solid ammonium sulfate (50 per cent saturation). After standing for 15 minutes with stirring, the protein was collected by centrifugation at 40,000 x g for 10 minutes. The residue was suspended in 30 ml of 0.01 M potassium phosphate buffer pH 6.5 and dialyzed against 6 liters of the same buffer for 4 hours.

Step 6. Calcium Phosphate Gel. The dialysate from step 5 was carefully adjusted to pH 6.0 with 1 N acetic acid and 15 ml of the calcium phosphate gel (15 mg per ml) were added. The resulting mixture was allowed to stand for 5 minutes with stirring prior to centrifugation for 1 minute at 5,000 x g. The calcium phosphate gel residue was suspended in 15 ml of 0.01 M potassium phosphate buffer pH 6.5 with a glass homogenizer and recentrifugated at 5,000 x g for 1 minute. After 2 additional washings with 0.02 M potassium phosphate buffer pH 6.5 to remove contaminating proteins, the enzyme was eluted from the calcium phosphate gel with 20 ml of 0.05 M potassium phosphate buffer pH 7.4. Solid ammonium sulfate (8 gm) was added to the above eluate to bring the concentration to 55 per cent saturation, and the resulting mixture was allowed to stand for 15 minutes with stirring prior to centrifugation at 40,000 x g for 10 minutes. The resulting residue was collected and stored at this step by freezing.

Step 7. Gel Filtration on Sephadex G-200. The residue from step 6 was combined with other preparations carried to the same step and dissolved in 0.05 M potassium phosphate buffer pH 6.5. The protein concentration was adjusted to 30 mg per ml, and the concentrated solution was carefully layered onto a column (3 X 90 cm) of sephadex G-200 which had been equilibrated with 0.05 M potassium phosphate buffer pH 6.5. After washing the enzyme into the column with 0.05 M potassium phosphate buffer pH 6.5, the column was eluted with 0.05 M potassium phosphate buffer pH 6.5 at a rate of 0.3 ml per minute and the eluate was collected every 20 minutes. The appearance of protein was followed by the absorbance at 280 $m\mu$ and peak tubes were assayed for reductase activity.

The tubes with the highest activity were then combined and solid ammonium sulfate was added to produce a 50 per cent saturation. After stirring for 15 minutes, the protein was concentrated by centrifugation at 40,000 x g for 10 minutes and the precipitate was then frozen and stored.

EXPERIMENTAL RESULTS AND DISCUSSION

A. VALIDITY OF THE RADIOACTIVE ASSAY FOR SERINE TRANSHYDROXYMETHYLASE

Assay methods used by previous investigators (2,8,75) were cumbersome and somewhat lengthy. Several assay systems were investigated, and the best success was obtained with the radioactive assay by Taylor and Weissbach (82).

Serine and tetrahydrofolate were converted by serine transhydroxymethylase to glycine and N^5, N^{10} -methylene tetrahydrofolate according to reaction 1 (Figure 1) (1,5,30,41). The beta carbon of serine was transferred to N^5, N^{10} position of tetrahydrofolate in the reaction, and the N^5, N^{10} -methylene tetrahydrofolate formed dissociated easily to yield formaldehyde which could be isolated as the dimedon (5, 5-dimethyl 1,3 cyclohexadione) adduct (33,51). The assay was specific for the beta carbon of serine. This method is described in the Experimental Methods section.

The enzyme activity increased with enzyme concentration showing a first order relationship (Figure 5). With enzyme concentrations less than 1 mg, the reaction was linear and therefore concentrations at less than 1 mg per ml were used in the assay. The dependency of the assay on time is shown in Figure 6. The incubation period of 15 minutes gave a linear response and was chosen as the time used in the typical assay.

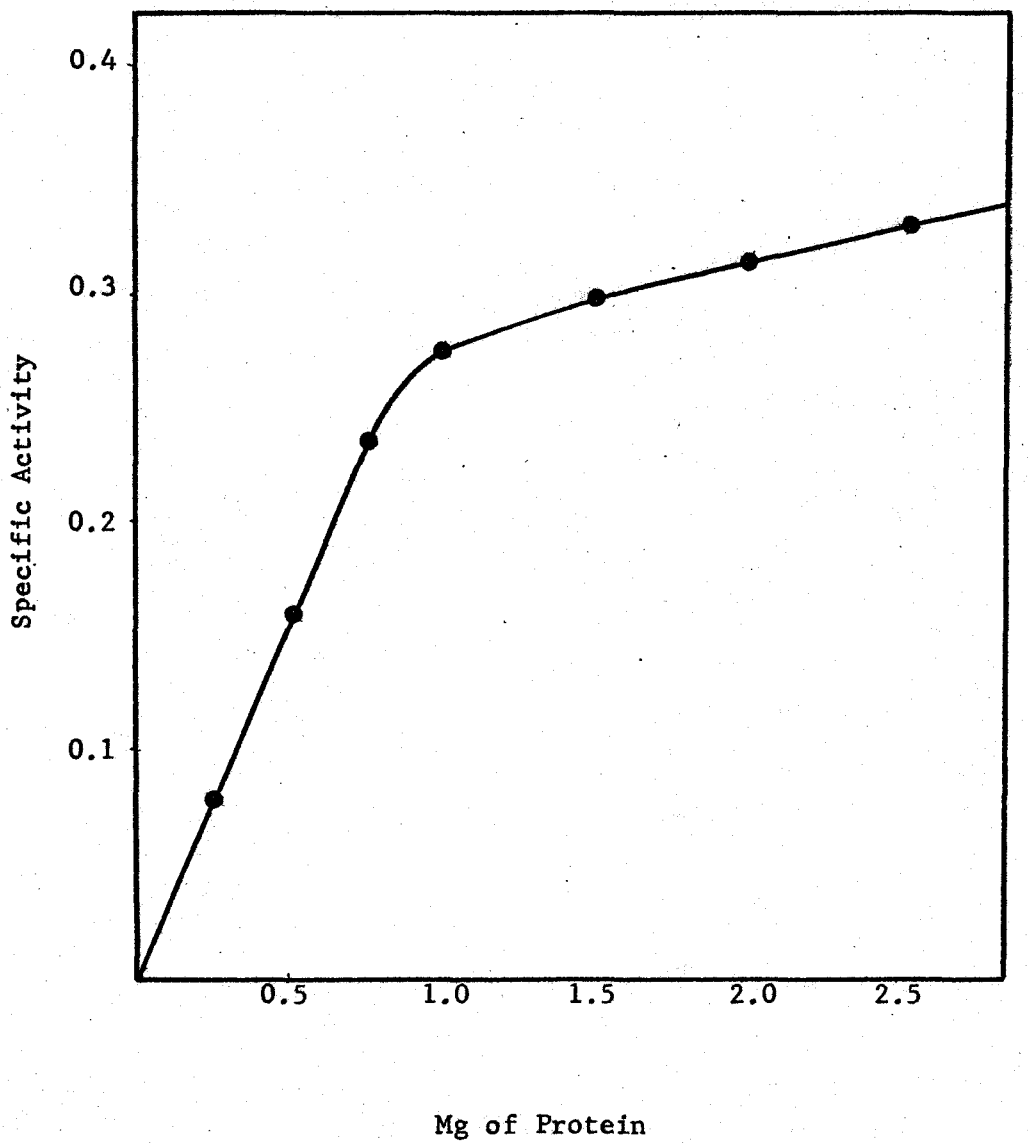


Figure 5. Dependence of the Extent of the Reaction on Enzyme Concentration for Serine Transhydroxymethylase.

The assay mixture contained 30 μ moles of potassium phosphate buffer 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta (C^{14})-serine and varying amounts of enzyme. The assay mixture was incubated at 37° for 15 minutes.

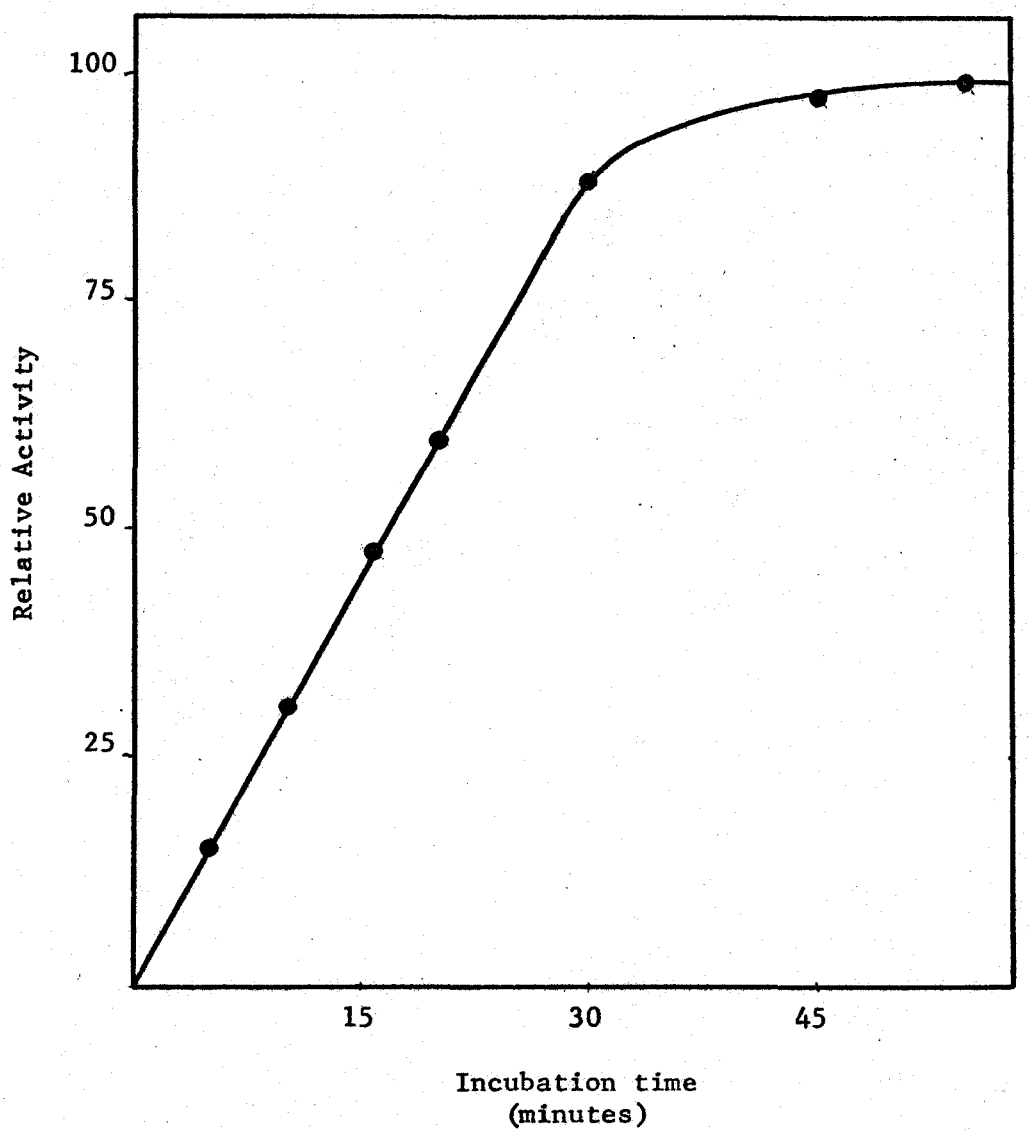


Figure 6. The Length of Incubation Time Versus the Activity of Serine Transhydroxymethylase.

The assay mixture contained 30 μ moles of potassium phosphate buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta (C^{14})-serine, and a mixture of enzyme. The assay mixture was incubated at 37° for varying lengths of time.

The dependence of the reaction rate of the assay on the complete system was also studied, and the results are listed in Table 1. There is an absolute dependency on the enzyme, tetrahydrofolate, pyridoxal phosphate, and L-serine. Mercaptoethanol prevents the oxidation of tetrahydrofolate, and thus with its omission, the specific activity dropped more than 40 per cent. Incubation at various temperatures and the effect this had on the rate of the reaction was also studied (Figure 7). The temperature range between 35°-40° was found to be optimal. However, when the temperature varied more than 5 degrees from this range, there was a rapid reduction in the rate. The assay mixture was always incubated 5 minutes before the substrate was added, and at higher temperatures the enzyme could have been partially or completely destroyed before the substrate was added.

The radioactive assay has proven to be a reliable assay, and the assay could be performed conveniently in less than 1 hour. Due to its sensitivity and specificity, the assay can also be used to measure crude extracts with low specificity activity.

B. VALIDITY OF THE ASSAY FOR THREONINE ALDOLASE

Braunstein *et al.* (48) reported the presence of an enzyme or enzyme system in various animal tissues which forms glycine from serine, threonine and certain other alpha-amino-beta-hydroxy acids, and they named this enzyme glycinogenase. Vilenkia (52) showed that the enzyme system yielded as much glycine from DL-threonine as it does from DL-serine and that it operated selectively on the L-amino acid isomer. The products of the breakdown of threonine were glycine and

TABLE 1

DEPENDENCE OF REACTION RATE ON COMPLETE ASSAY SYSTEM FOR SERINE TRANSHYDROXYMETHYLASE

Omission	Sp. Act. (μ moles)
None	.034
Enzyme	. .
Tetrahydrofolate	. .
Pyridoxal Phosphate	. .
Serine	. .
Mercaptoethanol	.020

The complete system contained 30 μ moles of potassium buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercapatoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta(C^{14})-serine, and a mixture of enzyme. All reactions were run at 37° C for 15 minutes in a total volume of 0.45 ml. Specific activity was defined as the amount of serine converted per hour per mg enzyme.

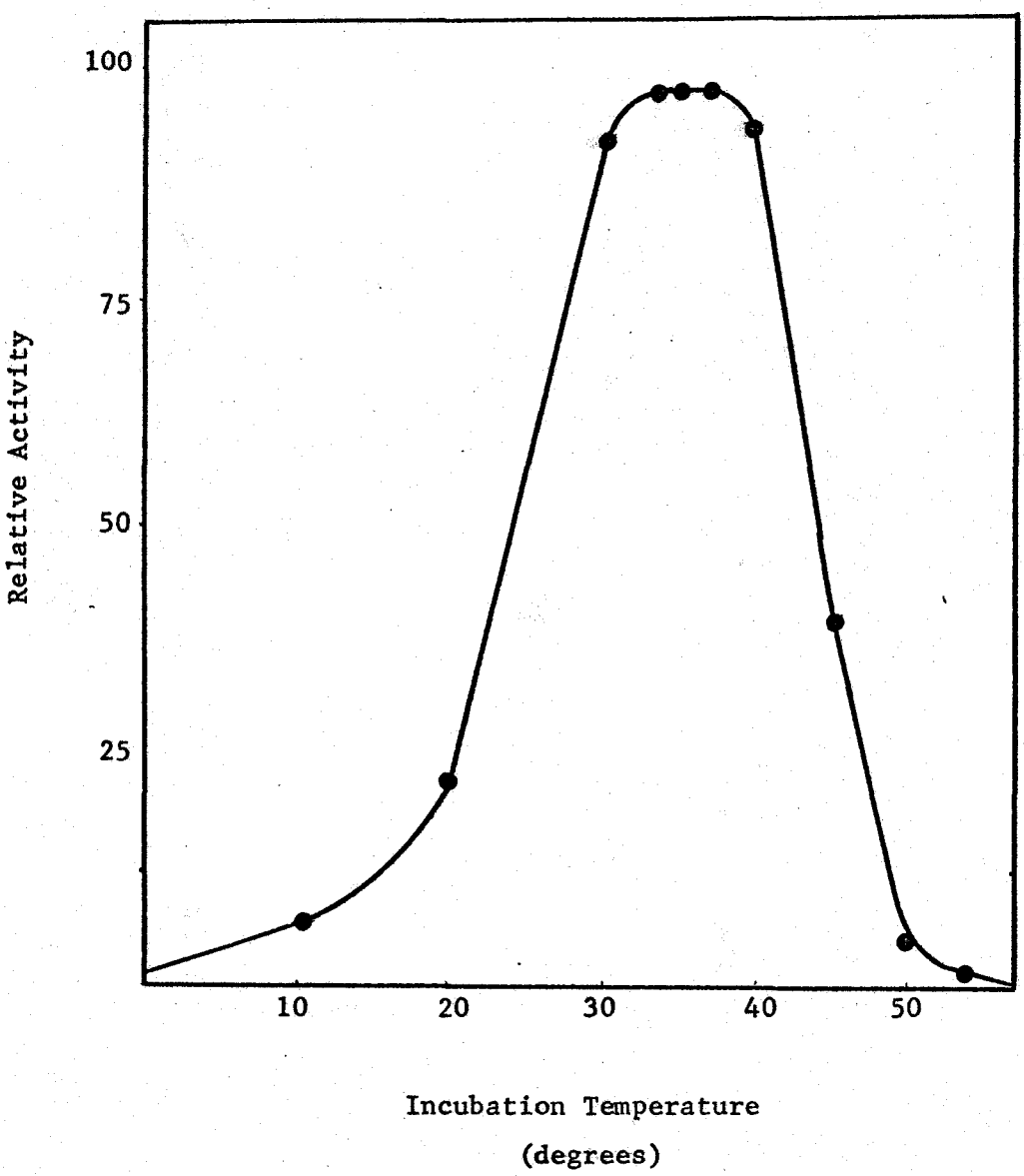


Figure 7. The Effect of the Incubation Temperature on the Radioactive Assay for Serine Transhydroxymethylase

The assay mixture contained 30 μ moles of potassium phosphate buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta-C^{14}-serine, and a mixture of enzyme. The assay mixture was incubated for 15 minutes at different temperatures.

acetaldehyde (52). The cleavage of threonine into glycine and acetaldehyde is similar to the reverse of an aldo condensation. Consequently, "threonine aldolase" appeared to be a more appropriate name and was thus designated so by Lin and Green (48). Schirch and Gross (69) just recently reported evidence in support of a single enzyme cleaving serine, threonine, and allothreonine to glycine and their respective compounds. In this study the partially purified serine transhydroxymethylase from hog liver and bovine brain was assayed for threonine aldolase.

Threonine aldolase activity was measured according to the assay of Malkin and Greenberg (52). This assay method is described in detail in the Experimental Methods section.

The enzyme activity was shown to increase with the enzyme concentration and there was an absolute dependency on the complete assay system (Table 2). It was also shown that the activity of threonine aldolase increased proportionally to the activity of serine transhydroxymethylase (Table 3) from hog liver. However, serine transhydroxymethylase from bovine brain would not cleave threonine to acetaldehyde and glycine. The partially purified enzymes from bovine brain showed no activity whatsoever with threonine (Table 3).

C. VALIDITY OF THE RADIOACTIVE ASSAY FOR N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

N^5, N^{10} -methylene tetrahydrofolate reductase catalyzes the interconversion of N^5, N^{10} -methylene tetrahydrofolate and N^5 -methyl tetrahydrofolate as shown in reaction 2 (Figure 2). This reaction can be detected by several means (46). The fact that N^5 -methyl tetrahydrofolate can serve as a growth factor for Lactobacillus casei but

TABLE 2

DEPENDENCE OF REACTION RATE ON COMPLETE ASSAY SYSTEM FOR THREONINE ALDOLASE

Omission	Sp. Act. (μ moles)
None	.040
Enzyme	. .
Pyridoxal phosphate	. .
NADH	. .
Alcohol dehydrogenase	. .
L-threonine	. .

The complete system contained 115 μ moles of potassium phosphate buffer pH 7, 0.2 μ moles of pyridoxal phosphate, 0.5 μ moles of NADH, 0.05 mg of alcohol dehydrogenase, 200 μ moles of L-threonine, and a mixture of enzyme from hog liver. All reactions were run at 37° for 15 minutes in a total volume of 3.0 ml. Specific activity was defined as μ moles of NAD^+ formed per mg enzyme per hour.

TABLE 3

THE ACTIVITY OF THREONINE ALDOLASE AS COMPARED WITH SERINE TRANSHYDROXYMETHYLASE ACTIVITY

Procedure	Sp. Act. for Threonine Aldolase	Sp. Act. for Serine Transhydroxy-methylase
Crude enzyme (hog liver)	.030	.035
Partially purified enzyme (hog liver)	.405	.400
Crude enzyme (bovine brain)	.020	.030
Partially purified enzyme (bovine brain)	. .	.570

The assay mixture of threonine aldolase contained 115 μ moles of potassium phosphate buffer pH 7.4, 0.2 μ moles of pyridoxal phosphate, 0.5 μ moles of NADH, 0.05 mg of alcohol dehydrogenase, 200 μ moles of L-threonine, and an enzyme from both bovine brain and hog liver. The assay mixture for serine transhydroxymethylase contained 30 μ moles of potassium phosphate buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta-C^{14}-serine, and an enzyme from both bovine brain and hog liver. All reactions were run at 37° for 15 minutes. Specific activity for threonine aldolase was defined as μ moles of NAD^{+} formed per hour per mg enzyme. Specific activity for serine transhydroxymethylase was defined as μ moles of serine converted per mg enzyme per hour.

not for S. faecalis or Leuconostoc citrovorum is the basis for a microbiological assay. N^5 -methyl tetrahydrofolate can also be assayed as a limiting component of methionine synthesis ([reaction 3] Figure 3). The enzyme could possibly be assayed by a direct spectrophotometric assay based on the decrease of absorbancy at 340 mμ since the absorption of the N^5, N^{10} -methylene tetrahydrofolate and N^5 -methyl tetrahydrofolate is negligible in this spectral region. Another method used in assaying the enzyme is by radioactive means. C^{14} labeled N^5, N^{10} -methylene tetrahydrofolate reacts with the aldehyde-binding reagent, dimedon, whereas N^5 -methyl tetrahydrofolate does not. C^{14} can also be recovered as C^{14} -labeled CH_3I upon treatment of N^5 -methyl tetrahydrofolate with HI (20).

In this investigation, N^5, N^{10} -methylene tetrahydrofolate reductase was assayed in the reverse direction of reaction 2 (Figure 2) by a modified method of the radioactive assay proposed by Kutzback and Stokstad (44). This procedure is described in detail in the Experimental Methods section. The enzyme activity as compared to enzyme concentration was shown to be linear up to a 1.5 mg of protein concentration (Figure 8).

The dependency of the enzyme activity in relationship to the incubation time is shown in Figure 9 and was found to be linear within the first 10 minutes of the incubation time. The dependency of the reaction rate of the assay on the complete system was also studied and the results are listed in Table 4. There was an absolute requirement for the enzyme, menadione, and N^5 -methyl (C^{14}) tetrahydrofolate. The omission of FAD decreased the activity by 20 per cent. This result could

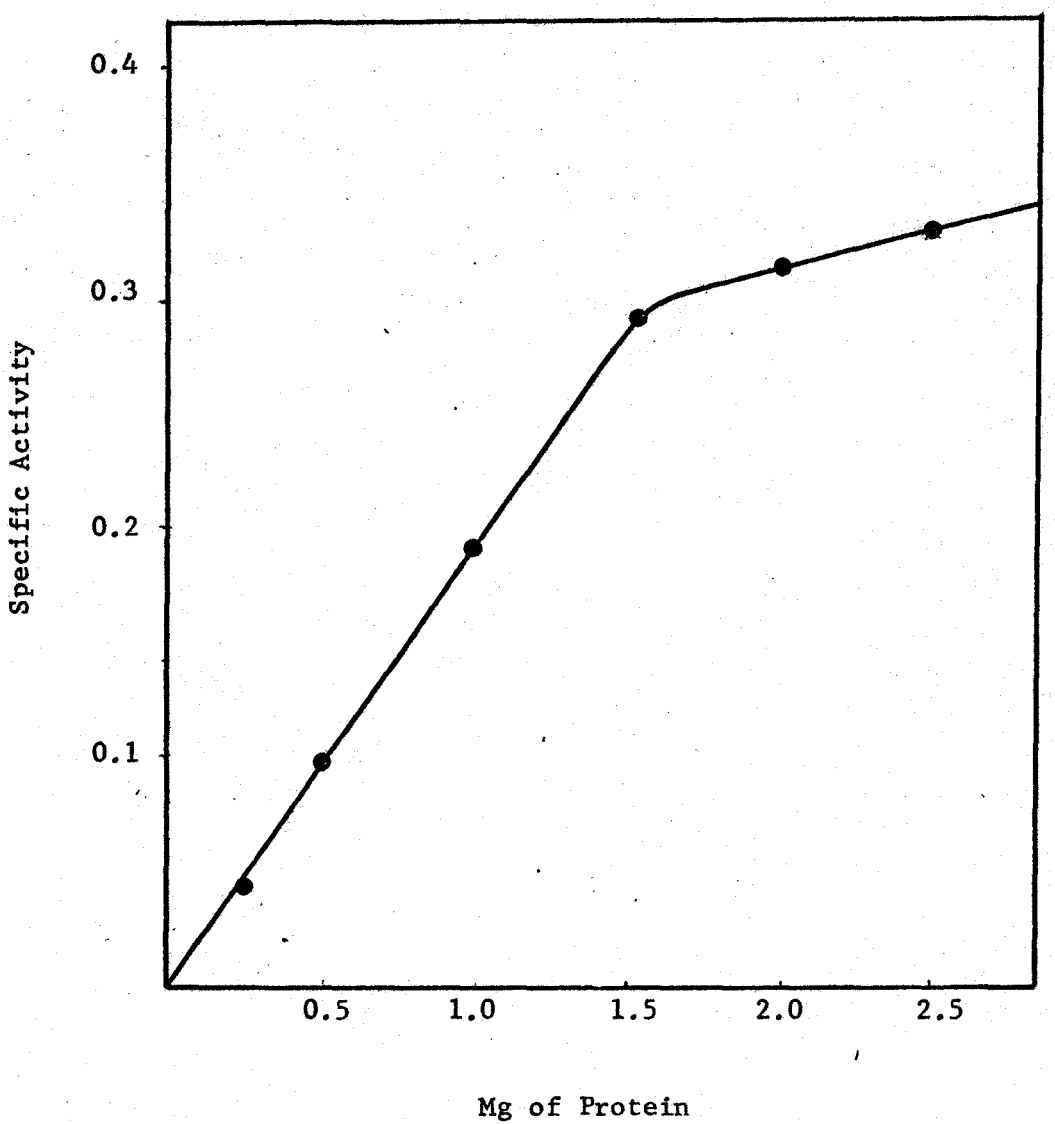


Figure 8. Dependence of the Extent of the Reaction of the Enzyme Concentration for N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The assay mixture contained 100 μ moles of potassium phosphate buffer pH 6.5, 5 mμ moles of FAD, 2 μ moles menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate and varying amounts of enzyme. The assay mixture was incubated at 37° for 10 minutes.

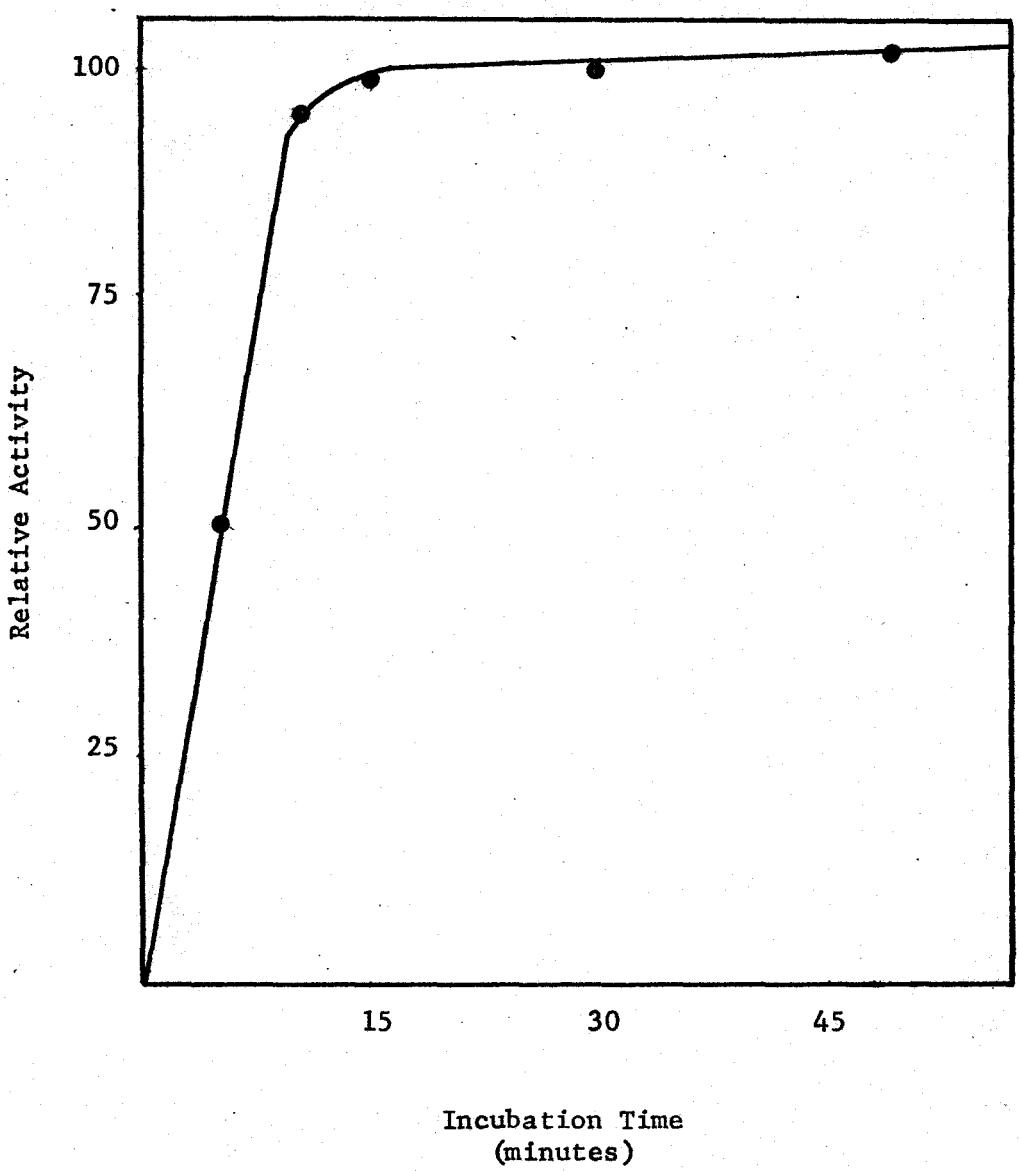


Figure 9. The Length of Incubation Time Versus the Activity of N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The assay mixture contained 100 μ moles of potassium phosphate buffer pH 6.5, 5 mμ moles of FAD, 2 μ moles of menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate and a mixture of enzyme. The assay mixture was incubated at 37° for varying lengths of time.

TABLE 4

DEPENDENCE OF REACTION RATE ON COMPLETE ASSAY SYSTEM FOR N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

Omission	Sp. Act. (μ moles)
None	.125
Enzyme	. .
FAD	.100
Ascorbic Acid	.165
Enzyme and Ascorbic Acid	.030
Menadione	.008
N^5 -Methyl Tetrahydrofolate	. .

The complete system contained 100 μ moles of potassium phosphate, pH 6.5, 5 mμ moles of FAD, 2 μ moles of menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate, and a mixture of enzyme. All reactions were run at 37° for 10 minutes in a total volume of 0.8 ml. The specific activity was defined as the μ moles of formaldehyde formed per hour per mg enzyme.

be explained by the presence of endogenous FAD in the enzyme preparation. N^5, N^{10} -methylene tetrahydrofolate reductase is a flavoprotein, and it is believed that FAD is bound to the enzyme (17). Ascorbic acid is a reducing agent and with its omission a non-enzymatic interconversion of N^5 -methyl tetrahydrofolate, to N^5, N^{10} -methylene tetrahydrofolate takes place. Ascorbic acid probably prevents the oxidation of the reaction mixture and thus prevents the non-enzymatic interconversion. The relative rate of the reaction and its dependency on the temperature of incubation was studied, and the maximum varied between 36° to 40° (Figure 10). A change in the incubation temperature of more than 5 degrees from this range decreased markedly the relative activity of the reaction. The assay mixture including the enzyme was always incubated 5 minutes before the addition of the substrate, and at higher temperatures the enzyme could have been partially or completely destroyed before the reaction took place.

The assay could be performed conveniently in less than 1 hour, and it proved to be a fairly reliable assay. Due to its sensitivity and specificity, the assay was used to measure crude extracts with low specificity activity.

D. PURIFICATION OF SERINE TRANSHYDROXYMETHYLASE

Serine transhydroxymethylase has been purified 150 to 200 fold from bovine brain. The purification scheme is described in the Experimental Methods section. Because of the nature of the enzyme from bovine brain, the purification schemes proposed by Schirch (68) and Nakano *et al.* (58) were extensively modified.

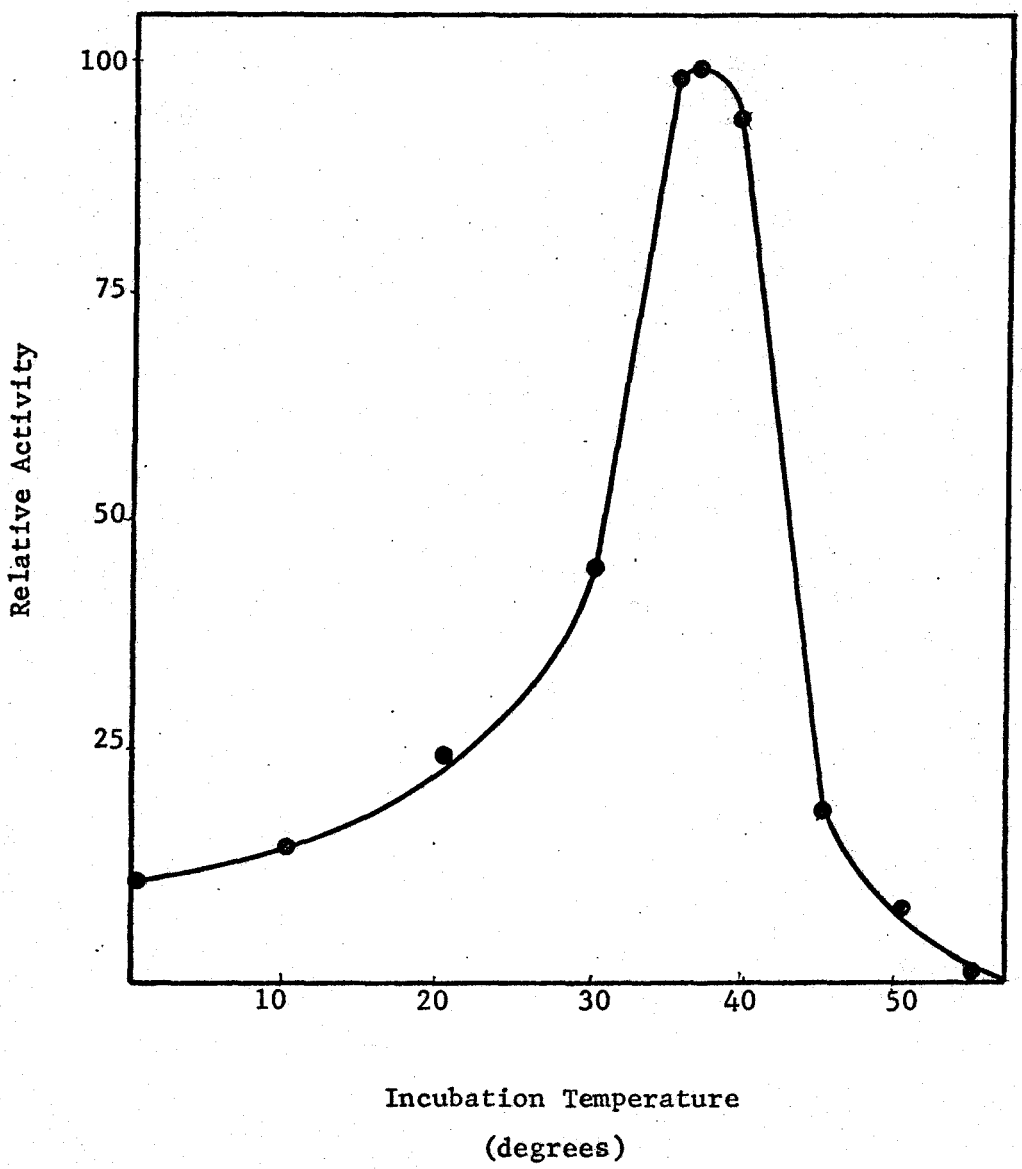


Figure 10. The Effect of the Incubation Temperature on the Radioactive Assay of N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The assay mixture contained 100 μ moles of potassium phosphate buffer pH 6.5, 5 mμ moles of FAD, 2 μ moles of menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate and a mixture of enzyme. The assay mixture was incubated for 10 minutes at different temperatures.

The crude extract was obtained by homogenizing and centrifuging, and it usually had a specific activity ranging from 0.025 to 0.040 μ moles of serine converted per mg enzyme per hour. The activity varied depending upon the condition of the frozen brain and upon the length of time the brain had been frozen. The crude extract could be frozen with little loss of activity, but it lost activity standing at 0° for any length of time.

The protamine sulfate precipitation step usually resulted in a 3-fold purification, and this step cleared the enzyme preparation of a milky white substance that contaminated the enzyme preparation. Protein concentration and ionic strength were essential factors in the success of this step. The first ammonium sulfate precipitation (between 33 and 53 per cent) increased the enzyme activity 4-fold over the previous step, while the second ammonium sulfate precipitation (30 to 45 per cent) brought the activity to a 20-fold purification over the initial crude extract activity. The success of these two steps was also very dependent upon ionic strength and protein concentration.

Column chromatography was next used in the enzyme purification. A DEAE-cellulose column resulted in a purification of about 5-fold over the second ammonium sulfate step. However, it was very essential that the ionic strength of the enzyme mixture be less than 0.01 M before it was added to the column. Caution was also used to prevent overloading of the column. Serine transhydroxymethylase was eluted from the column at an ionic strength at 0.09 to 0.15 M potassium phosphate buffer pH 7.4. The enzyme could be seen as a light yellow band moving down the column.

Gel filtration of sephadex G-200 was the last step in the procedure used for the purification of serine transhydroxymethylase. This step usually increased the enzyme purification above 150 fold of the initial crude enzyme activity. A summary of the purification scheme is given in Table 5. The enzyme was very stable when frozen at any step in the procedure and little or no loss of activity occurred. However, the enzyme lost activity after standing at 0° for any length of time and was very sensitive to heat.

E. PURIFICATION OF N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

N^5, N^{10} -methylene tetrahydrofolate reductase from bovine brain has been purified 100 fold by a modification of the procedures of Katzen and Buchanan (33) and Huennekens (29). The purification scheme is described in the Experimental Methods section.

The crude extract obtained by homogenization and centrifugation had a specific activity ranging from 0.020 to 0.040 μ moles of formaldehyde formed per mg enzyme per hour. The enzyme could be frozen overnight with little loss of activity. However, upon prolonged freezing, the enzyme lost activity. The enzyme was activated in solutions of high ionic strength and seemed to lose activity when dialyzed.

The protamine sulfate precipitate between 2.5 and 6.0 mg per 100 ml concentration of protamine sulfate usually resulted in a 4-fold purification when the protein concentration was maintained at 9 mg per ml. The ammonium sulfate precipitation (between 12 and 45 per cent) increased the enzyme activity 6 fold over the initial crude extract

TABLE 5

PURIFICATION OF SERINE TRANSHYDROXYMETHYLASE

Steps	Vol. (ml)	Prot. (mg/ml)	Tot. Prot. (mg)	Sp. Act.	Yield (%)	Fold
1. Crude Extract	1,850	13	24,000	0.030	100	1
2. Protamine Sulfate Precipitation	2,350	3.5	8,200	0.086	98	3
3. Ammonium Sulfate Precipitation	100	20	2,000	0.340	94	11
4. 2nd Ammonium Sulfate Precipitation	60	17	1,000	0.570	80	19
5. DEAE-Cellulose Column	28	4	112	2.850	49	95
6. G-200 Sephadex	28	2	58	4.530	36	151

Specific activity was defined as μ moles of serine converted per mg enzyme per hour.

activity. These two steps could be combined by skipping the protamine sulfate precipitation step and by taking the ammonium sulfate precipitate between 33 and 40 per cent saturation. This step resulted in a 4 to 5 fold purification with a 75 per cent yield. The protein concentration for this step was maintained at 14 mg per ml and the ionic strength was at 0.03 M.

The high speed centrifugation step was mainly used for clearing the enzyme solution and not for purification, even though it did result in a partial purification. The enzyme solution upon dialyzing became milky white and high speed centrifugation would usually clear it up.

The enzyme was further purified by a DM-30 column. This step resulted in an activity which represented a 5-fold purification. A DEAE-cellulose column was also used at this step of the procedure instead of the DM-30 column. However, the DM-30 column was more reliable and resulted in a higher yield. N^5, N^{10} -methylene tetrahydrofolate reductase was eluted from the column at an ionic strength of 0.2 to 0.3 M potassium phosphate buffer pH 7.0. The enzyme would not stick to the column unless the ionic strength was less than 0.05 M. The success of the calcium phosphate gel step was also dependent on ionic strength, and this step doubled the enzyme activity. Gel filtration using sephadex G-200 increased the activity again by one half. A summary of the purification scheme is given in Table 6.

The partially purified enzyme was very susceptible to oxidation and changed to a brown color when exposed to the air. Mercaptoethanol was used without success to prevent the oxidation of the enzyme. The purer the enzyme the more susceptible it was to oxidation and the less

TABLE 6

PURIFICATION OF N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

Steps	Vol. (ml)	Prot. (mg/ml)	Tot. Prot. (mg)	Sp. Act.	Yield (%)	Fold
1. Crude Extract	1,850	13	24,000	0.025	100	1
2. Protamine Sulfate Precipitation	310	15	4,750	0.100	79	4
3. Ammonium Sulfate Precipitation	100	27	2,700	0.155	70	6
4. High Speed Centrifugation	100	23	2,300	0.175	67	7
5. DM-30 Column	70	3	210	0.875	31	35
6. Calcium Phosphate Gel	20	3	60	1.750	18	70
7. G-200 Sephadex* (combined preparations)	5	4	20	2.650	9	106

Specific activity is defined as μ moles of formaldehyde formed per mg enzyme per hour.

* The G-200 Sephadex step was combined with 5 other preparations, and the results reported here have been divided by 5.

stable it was to freezing. The enzyme lost activity after standing at 0° for any length of time and was very sensitive to heat.

F. CHARACTERIZATION OF SERINE TRANSHYDROXYMETHYLASE

1. pH Optimum of the Reaction

Recorded in Figure 11 is the activity of serine transhydroxymethylase at several pH values. Although the pH optimum is at pH 7.6, the enzyme was routinely assayed at pH 7.4 due to the decreased stability of tetrahydrofolate at a higher pH. The pH optimum differed from serine transhydroxymethylase purified from rabbit liver (68). The enzyme from bovine brain was completely inactivated at a pH lower than 6.0, and it still had more than 60 per cent of its initial activity at pH 8.5. Once the enzyme had been inactivated at a lower pH, the activity could not be restored.

2. Temperature Optimum of the Reaction

The sensitivity of serine transhydroxymethylase to heating in a 0.3 M of potassium phosphate buffer pH 7.4 at various temperatures for 5 minutes is shown in Figure 12. The enzyme was rapidly denatured at 50°. At this temperature after 5 minutes, there was little or no activity remaining. Serine transhydroxymethylase from mammalian liver had been reported to be very heat stable (58,68), and a heat denaturing step has been used in the purification scheme. The enzyme from mammalian liver has also been reported to be more heat stable in the presence of serine (75). Nakano *et al.* (58) partially purified serine transhydroxymethylase from rat liver, and in their purification scheme, they incubated the enzyme for 3 minutes in the presence of 0.01 M serine

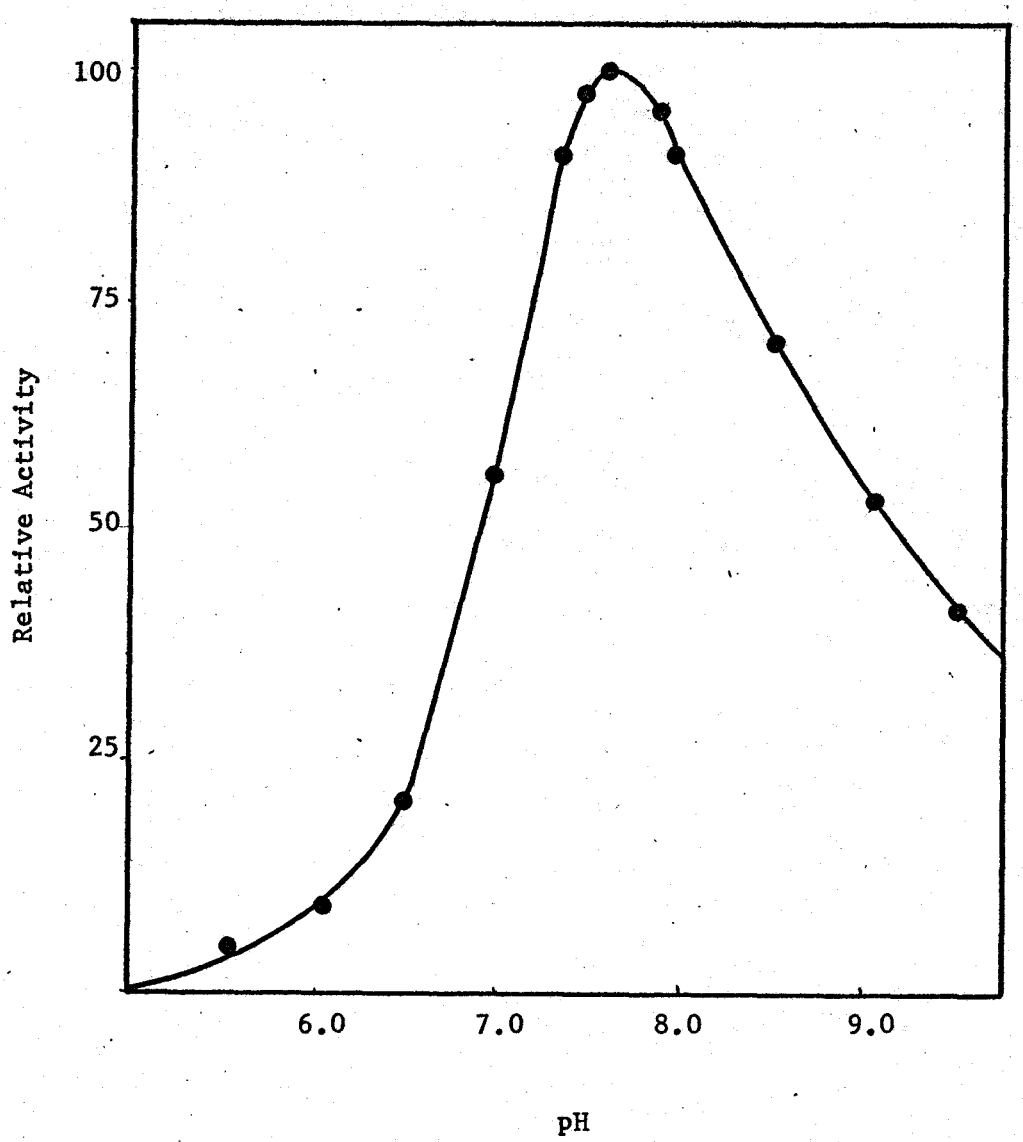


Figure 11. The Effect of pH on the Activity of Serine Transhydroxymethylase

The assays were performed in 0.5 M of potassium phosphate buffers. The assay mixtures contained: 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ moles of L-beta-(C^{14})-serine, and a mixture of enzyme. The assay mixtures were incubated for 15 minutes at 37°.

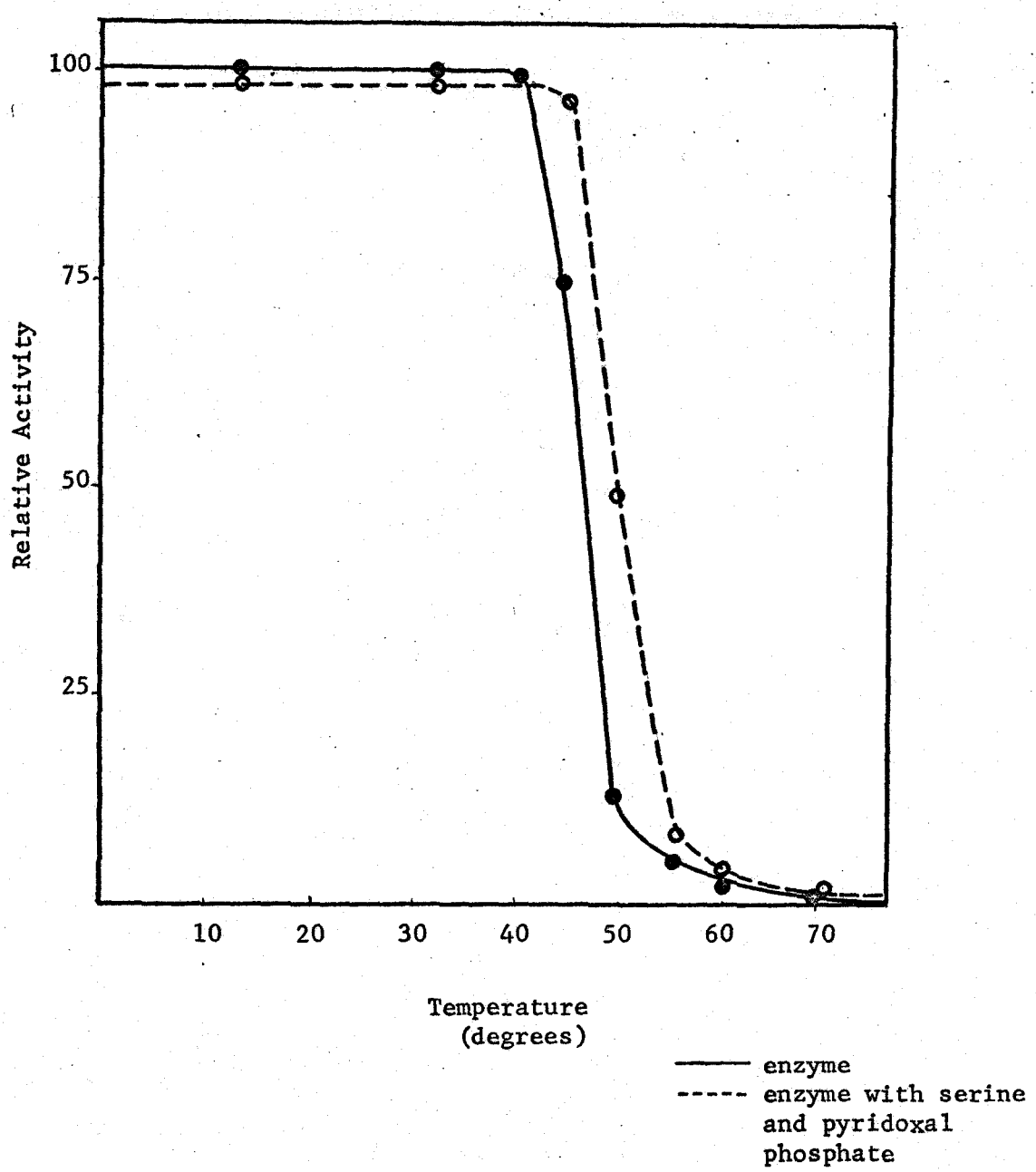


Figure 12. The Effect of Temperature on the Activity of Serine Transhydroxymethylase

The enzyme was incubated for 5 minutes at the indicated temperature and then assayed. The assay mixture contained: 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, 0.1 μ mole of L-beta-C^{14}-serine, 30 μ moles of potassium phosphate buffer pH 7.4 and a mixture of enzyme. The assay mixture was incubated at 37^{o} for 15 minutes.

at 70° and obtained an 18 fold purification at this step. The enzyme from bovine brain in the presence of 0.01 M serine was completely inactivated at 70° for 3 minutes. The enzyme maintained in the presence of 0.01 M serine at 55° for 5 minutes had essentially no activity remaining.

3. The Michaelis Constant of Serine

The principal function of enzymes is the catalysis of chemical reactions, and one of the prime factors influencing the rate of this catalytic reaction is substrate concentration. Michaelis and Menten (56) advanced a theory concerning this relationship between substrate and enzyme which states that the enzyme forms a complex with its substrate which subsequently breaks down to give the product of the reaction and releases the free enzyme. The initial rate of the reaction is dependent upon the concentration of the enzyme-substrate complex. Km (the Michaelis constant) is equal to the concentration (expressed in moles per liter) of the substrate which gives half the numerical maximal velocity. The value of the Km reflects the stability of the enzyme-substrate interaction and is of great practical value. A method of plotting this relationship which avoids some of the difficulties entailed with other methods of determining Km was described by Lineweaver and Burk (49). The method they used to determine Km was to plot 1/v against 1/s. A straight line can be obtained from the corresponding equation:

$$\frac{1}{v} = \frac{Km}{Vmax}\,\frac{1}{s} + \frac{1}{Vmax}\,.$$

This line cuts the base line at a point to give -1/Km.

The substrate used in the following determination of Km was L-beta-C^{14}-serine. The assay procedure is described in the Experimental

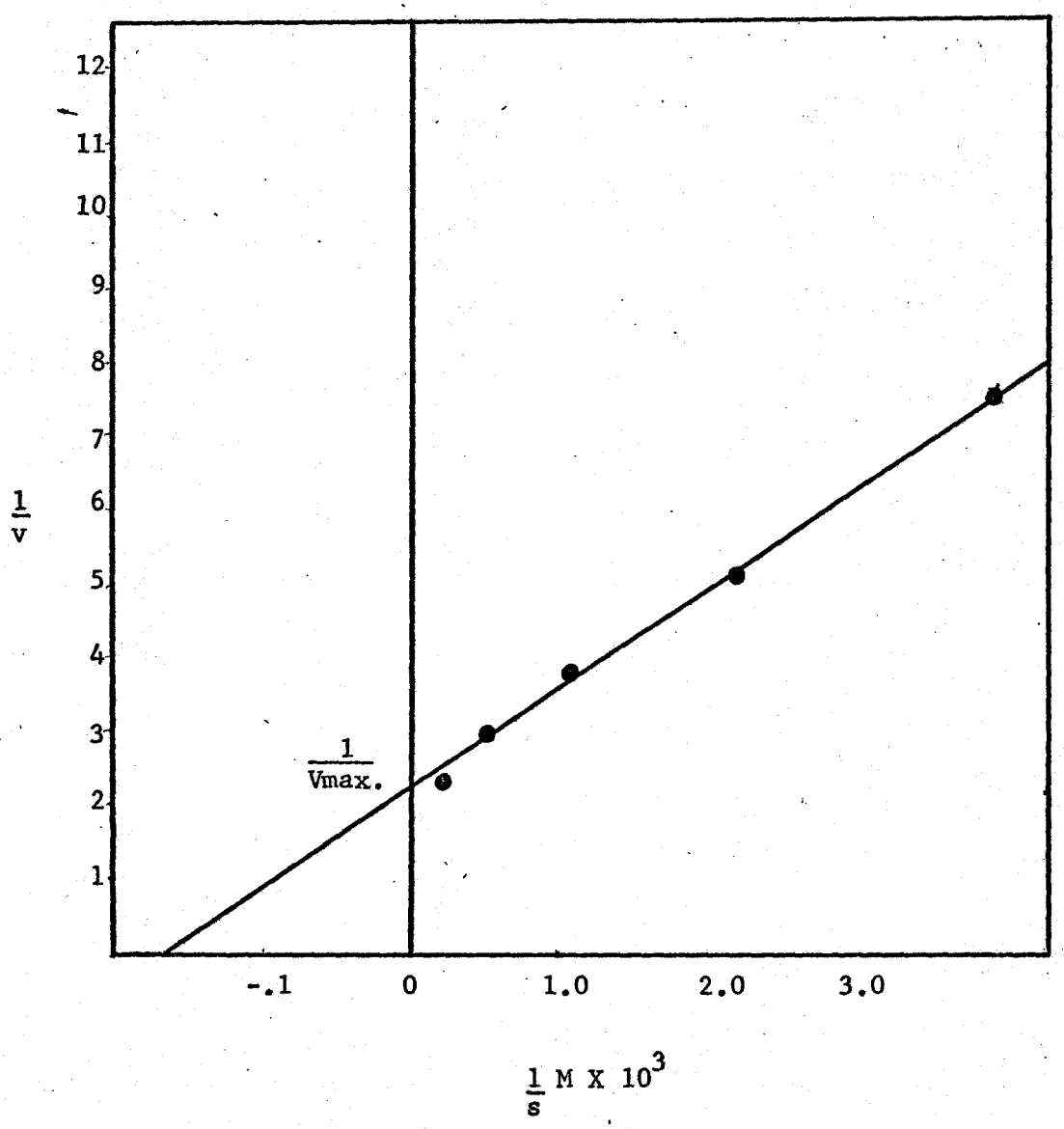


Figure 13. Lineweaver and Burk Method of Determination of the Michaelis Constant for Serine Transhydroxymethylase

The assay mixture contained 30 μ moles of potassium phosphate buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ moles of tetrahydrofolate, and a mixture of enzyme. The concentration of L-serine was varied from 0.11 mM to 3.51 mM. The assay mixture was incubated at 37° for 15 minutes.

Methods section. In Figure 13, 1/v is plotted against 1/s and the value for -1/Km was -1.55×10^{3} M. The Km for serine was calculated to be 6.7×10^{-4} M. The Km for serine transhydroxymethylase from rabbit liver was reported as 7.0×10^{-4} M (68). The extrapolated value of 1/Vmax was 2.2 which gave a value of 0.45 μ moles per hour per mg for the maximum velocity.

The enzyme was specific for L-serine. Schirch et al. (68,69) reported the enzyme from rabbit liver to catalyze alpha-methyl-DL-serine, hydroxymethyl serine, L-threonine, and DL-allothreonine. Serine transhydroxymethylase from bovine brain showed no catalytic activity for threonine (L-threonine was the only molecule the enzyme specificity was checked against -- see Table 3). However, serine transhydroxymethylase from bovine brain was inhibited in the presence of large quantities of threonine.

4. Absorption Spectrum of Serine Transhydroxymethylase

Serine transhydroxymethylase from bovine brain which had been purified 150 fold was yellow and exhibited an absorption peak at 415 mμ (Figure 14). Schirch (68) reported that serine transhydroxymethylase from rabbit liver which was 50 per cent pure exhibited a peak at 415 mμ and that this peak shifted to an absorption peak of 430 mμ when the enzyme was 95 to 100 per cent pure. The peak at 415 mμ from bovine brain enzyme could be observed after the first ammonium sulfate precipitate step and developed into a sharper peak the purer the enzyme became.

Serine transhydroxymethylase has been shown to require pyridoxal phosphate as a co-factor. The absorption peak at 415 mμ was attributed

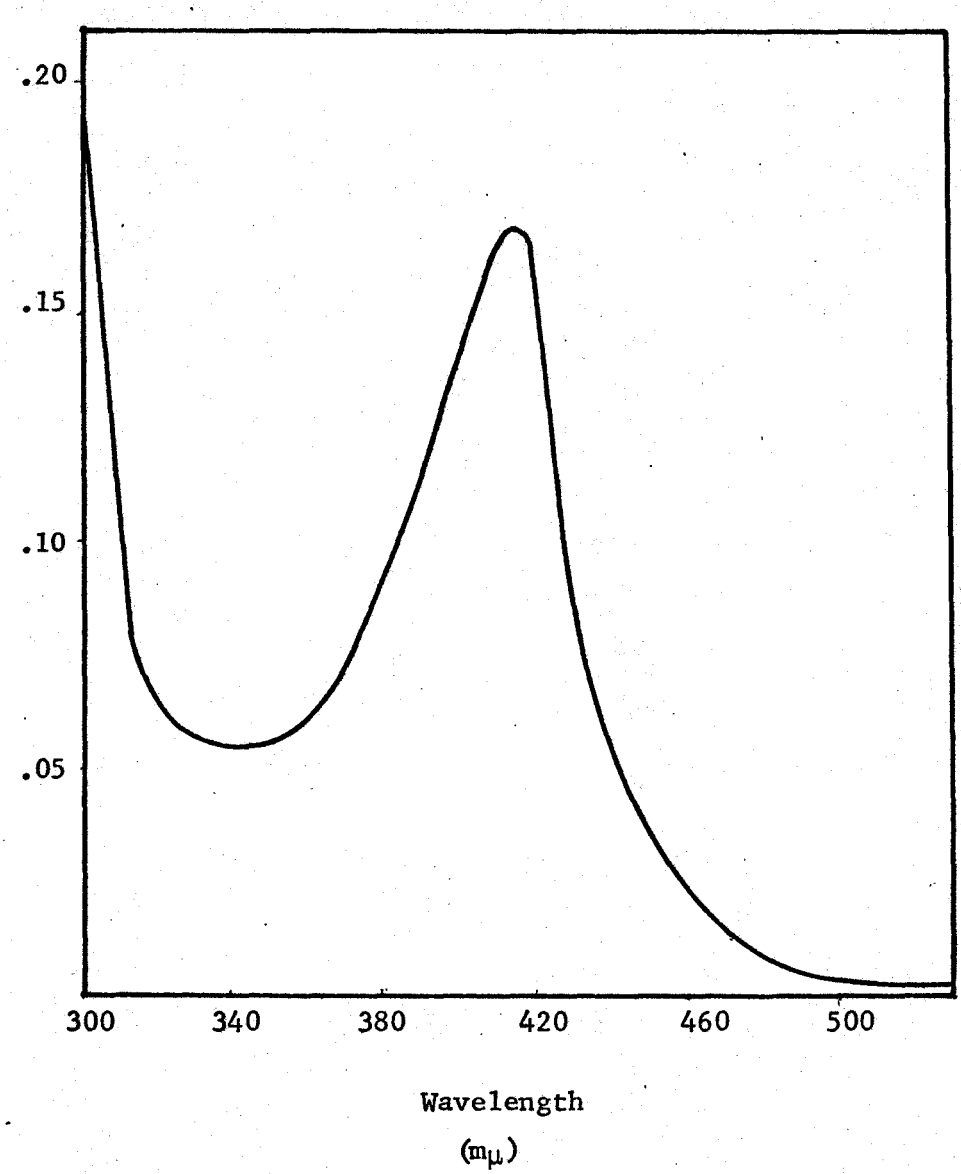


Figure 14. Absorption Spectrum of Serine Transhydroxymethylase at pH 7.4

Absorption spectrum of 3.0 mg per ml of enzyme was taken with the enzyme which was purified 150 fold.

to the enzyme-bound pyridoxal phosphate (72). Pyridoxal phosphate also formed complexes with amino acids (7,53), peptides, and proteins (13). These pyridoxal phosphate complexes also have absorption peaks above 400 mμ.

G. CHARACTERIZATION OF N^5, N^{10} -METHYLENE TETRAHYDROFOLATE REDUCTASE

1. pH Optimum of the Reaction

N^5, N^{10} -methylene tetrahydrofolate reductase activity was determined in a series of 0.5 M potassium phosphate buffer ranging in pH from 5.0 to 9.5. These results are recorded in Figure 15. The maximum catalytic activity was observed in the pH range of 6.3 and 6.8 with the optimum activity at pH 6.5. The enzyme had a relatively small pH range but reinactivation could be reversed from a low pH by raising the pH in a high ionic strength solution. However, the enzyme was denatured at a high pH.

2. Temperature Optimum of the Reaction

In Figure 16 the effects of the stability of N^5, N^{10} -methylene tetrahydrofolate reductase in a 0.5 M potassium phosphate buffer pH 6.5 maintained at various temperatures for 5 minutes is shown. At 55^o to 60^o the enzyme was rapidly denatured in the presence and absence of N^5 -methyl tetrahydrofolate. The purer forms of the enzyme were found to be more heat sensitive than the enzyme in the crude form. As the enzyme was purified, it was oxidized much easier, and it was not always known to what extent the enzyme was inactivated by oxidizing or by heating.

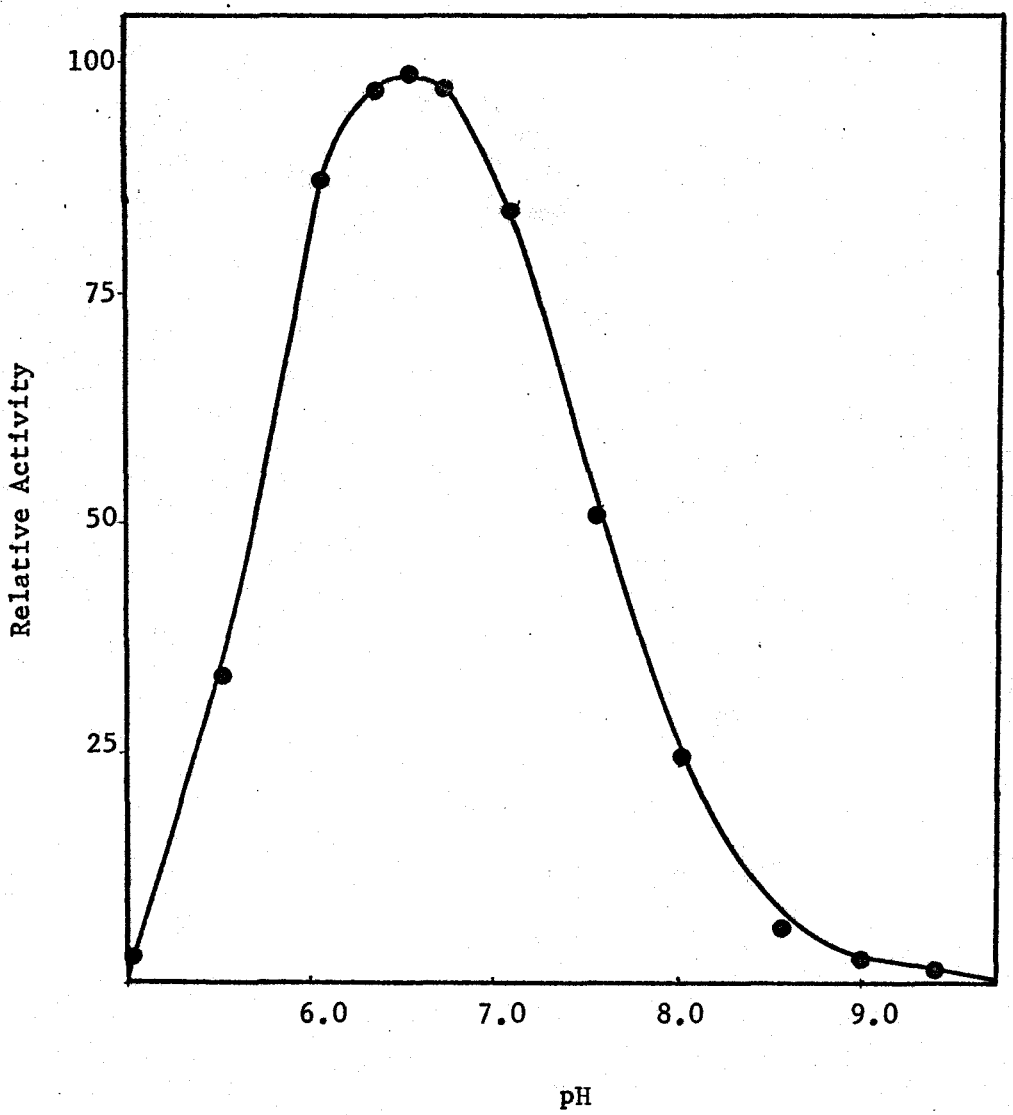


Figure 15. The Effect of pH on the Activity of N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The assay was performed in 0.5 M potassium phosphate buffers. The assay mixture contained 5 mμ moles of FAD, 2 μ moles of menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate, and a mixture of enzyme. The assay mixture was incubated at 37° for 10 minutes.

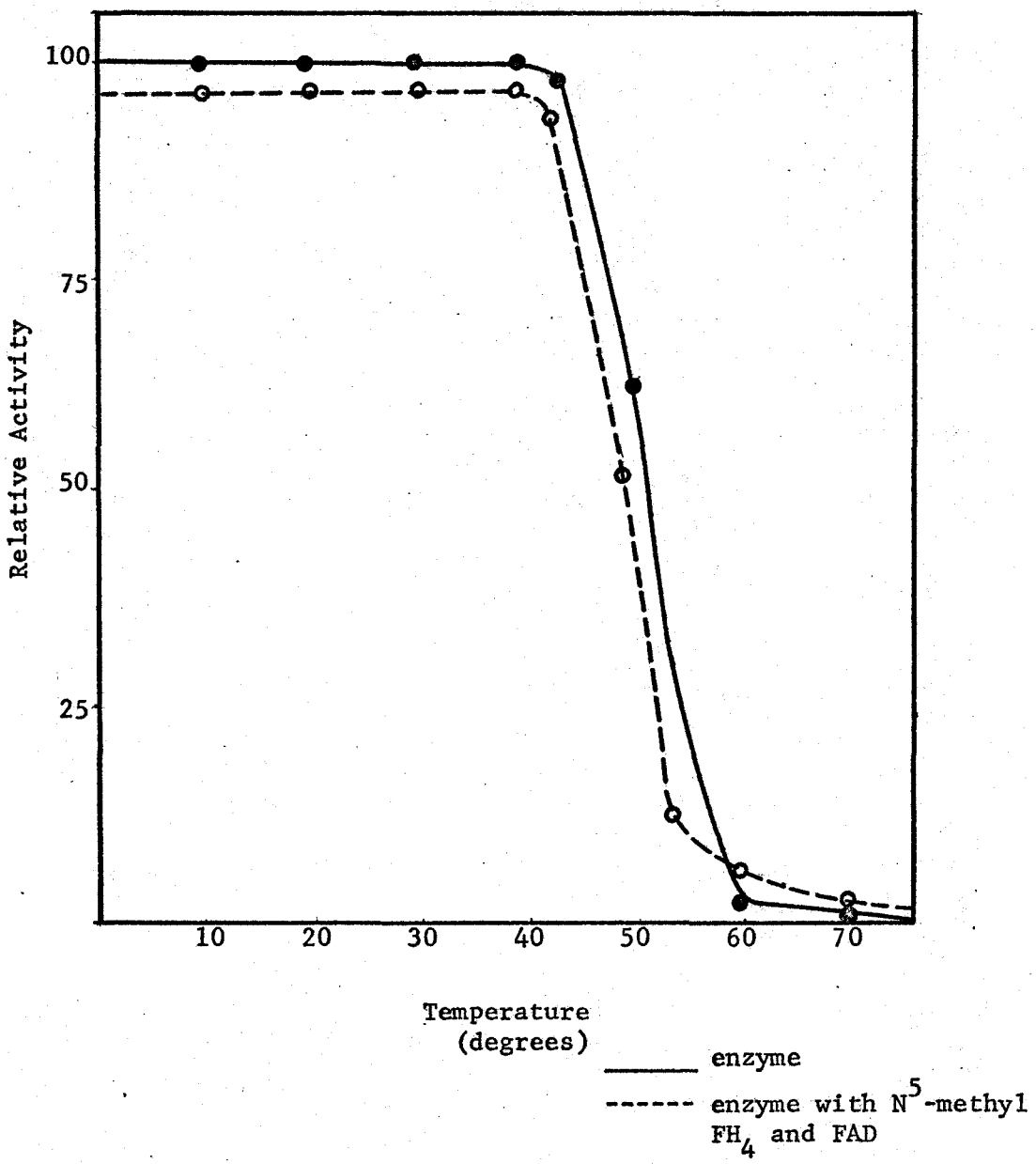


Figure 16. The Effect of Temperature on the Activity of N^5, N^{10} -Methylene Tetrahydrofolate Reductase.

The enzyme was incubated for 5 minutes at the indicated temperatures and then assayed. The assay mixture contained 5 mμ moles of FAD, 2 μ moles of menadione, 5 μ moles of ascorbic acid, 125 mμ moles of N^5 -methyl (C^{14}) tetrahydrofolate, 100 μ moles of potassium phosphate buffer pH 6.5, and a mixture of enzyme. The assay mixture was incubated at 37° for 10 minutes.

3. Substrate Concentration Effects on the Reaction

Enzymes which are biological catalysis influence the rate at which equilibrium is obtained, but they do not affect the over-all equilibrium of the reaction. It was recognized early in the history of enzymes that the rate of enzyme catalyzed reactions increased with increasing substrate until a concentration was reached beyond which further additions gave no increase in velocity. This phenomenon has been explained on the basis of catalytically active sites which react with the substrate (56). When all the active sites are occupied, a maximum velocity is reached. The effects of the substrate concentration on N^5, N^{10} -methylene tetrahydrofolate reductase are shown in Figure 17.

4. Absorption Spectrum of the Enzyme

The enzyme from bovine brain purified 100 fold was a light brownish yellow. The brown color was probably due to the oxidization of some of the enzyme. The enzyme exhibited an absorption peak at 405 $m\mu$ (Figure 18). The absorption peak at 405 $m\mu$ was first observed after the enzyme was eluted from the DM-30 column.

The N^5, N^{10} -methylene tetrahydrofolate reductase solution became cloudy after freezing or standing at 0^o, but cleared with high speed centrifugation. The absorption peak at 405 $m\mu$ disappeared with the cloudy solution, but it reappeared again with high speed centrifugation. However, the enzyme activity could be detected in both the supernatant fluid and the precipitate.

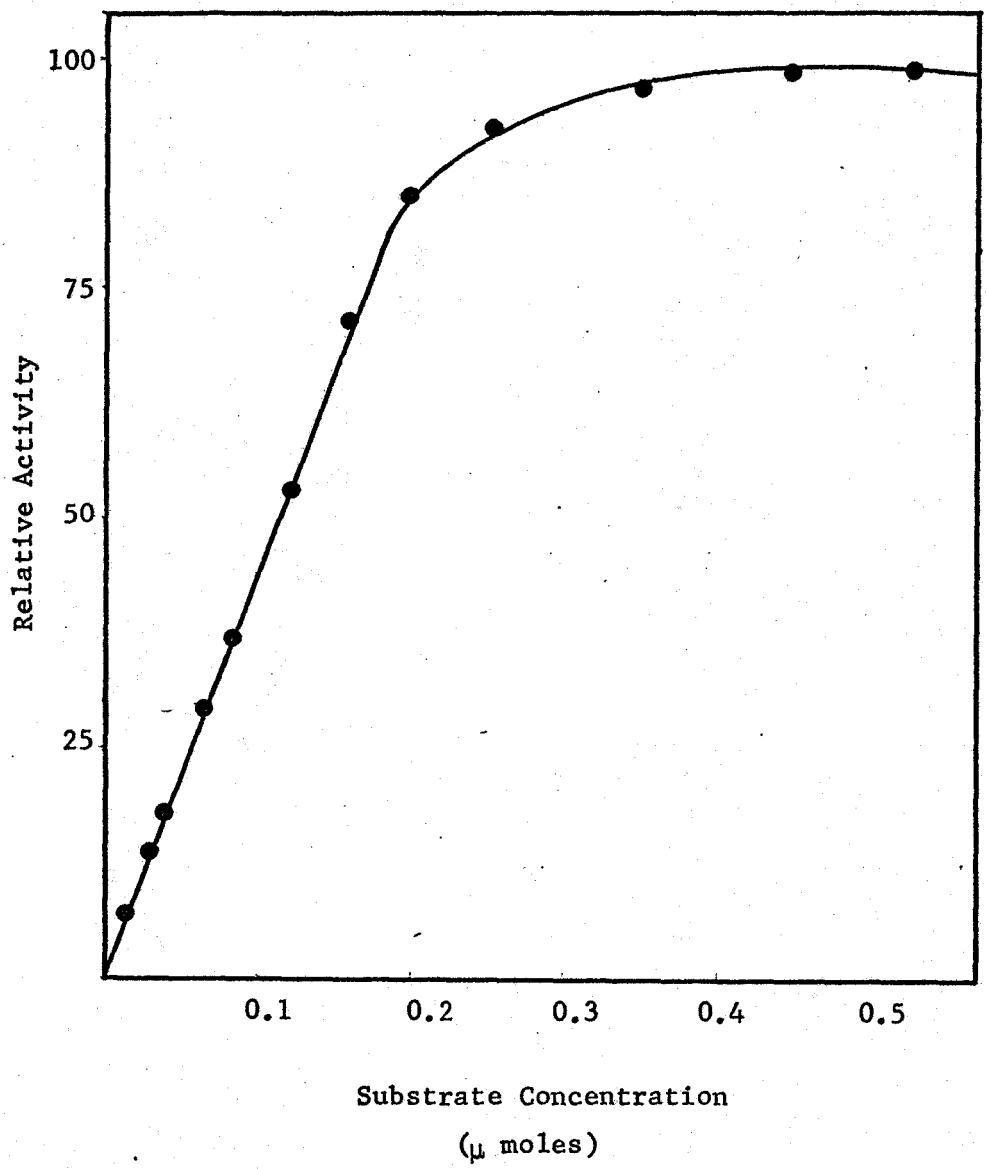


Figure 17. The Effect of Substrate Concentration on the N^5, N^{10} -Methylene Tetrahydrofolate Reductase Reaction

The assay mixture contained 30 μ moles of potassium phosphate buffer pH 7.4, 1 μ mole of pyridoxal phosphate, 4 μ moles of mercaptoethanol, 0.8 μ mole of tetrahydrofolate, and a mixture of enzyme. The concentration of N^5 -methyl (C^{14}) was varied from 0.01 to 0.5 μ mole. The assay was incubated at 37° for 10 minutes.

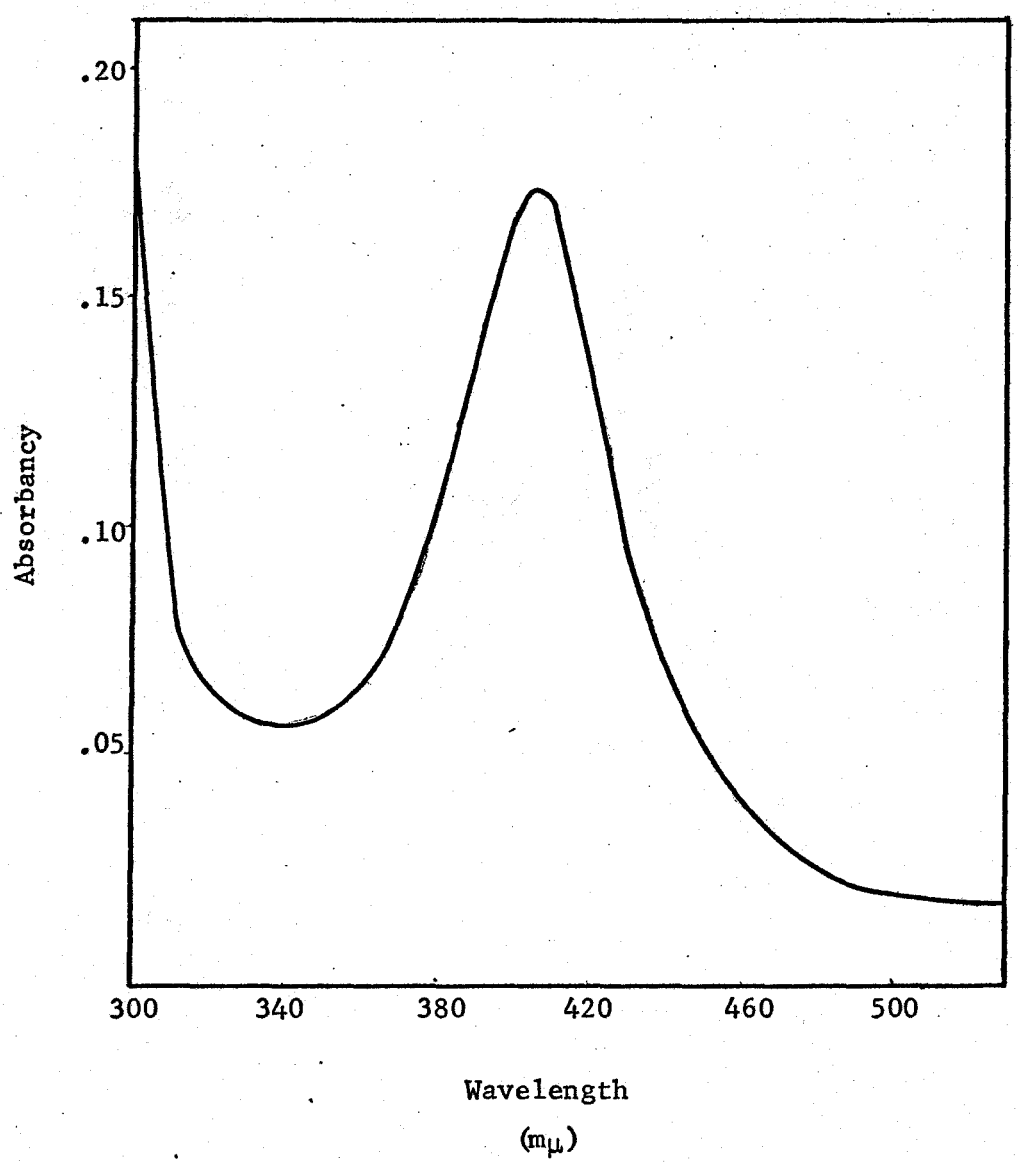


Figure 18. The Absorption Spectrum of N^5, N^{10} -Methylene Tetrahydrofolate Reductase

The absorption spectrum was taken with an enzyme which had been purified 100 fold.

SUMMARY

Serine transhydroxymethylase and N^5, N^{10} -methylene tetrahydrofolate reductase from bovine brain were purified 150 to 200 fold and 100 fold respectively by a combination of fractional precipitation and absorption procedures. Radioactive assays were employed in the detection of the product produced in the reactions.

Serine transhydroxymethylase from bovine brain required pyridoxal phosphate and tetrahydrofolate as co-enzymes in order for the reactions to proceed. The partially purified enzyme was yellow and exhibited an absorption peak at 415 mμ. The maximum catalytic activity occurred at pH 7.6, and the Michaelis constant for serine was 6.7×10^{-4} M. Serine transhydroxymethylase from bovine brain was heat sensitive and showed no catalytic reaction for threonine. The enzyme was also shown to exhibit different physical properties than serine transhydroxymethylase from mammalian liver and bacteria tissue.

N^5, N^{10} -methylene tetrahydrofolate reductase from bovine brain was shown to require FAD as a co-enzyme and menadione as an electron acceptor. The partially purified enzyme was a light brownish yellow with an absorption peak at 405 mμ. The maximum catalytic activity was determined at pH 6.5, and the enzyme was also shown to be heat sensitive. N^5, N^{10} -methylene tetrahydrofolate reductase oxidized readily in the air and would denature upon prolonged lengths of freezing.

Very little is known of the function of vitamins and co-enzymes in mammalian brain, and it is the hope of this study and other studies to follow that the function of folic acid as a co-enzyme and its dependent reactions in mammalian brain will be resolved.

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ABSTRACT

The enzymatic interconversion of serine to glycine and N^5, N^{10} -methylene tetrahydrofolate to N^5 -methyl tetrahydrofolate have been studied in a cell-free system derived from bovine brain. A partial purification of serine transhydroxymethylase and N^5, N^{10} -methylene tetrahydrofolate reductase has been achieved by ammonium sulfate fractionation, protamine sulfate fraction, and by various column chromatography procedures. The two enzymes studied catalyze sequentially the reductive transfer of the hydroxymethyl group of serine to the N^5 position of tetrahydrofolate forming N^5 -methyl tetrahydrofolate. The products of these reactions were assayed by the incorporation of C^{14} from a one-carbon precursor i.e., L-beta-(C^{14})-serine, or N^5 -methyl (C^{14}) tetrahydrofolate.

The partial purified serine transhydroxymethylase exhibited an absorption peak at 415 mμ due to the bound pyridoxal-5-phosphate and had a maximum pH optimum at 7.6. N^5, N^{10} -methylene tetrahydrofolate reductase is a flavoprotein with a maximum pH optimum at 6.5 and an absorption peak at 405 mμ.