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Title: ASPECTS OF THE REGULATION OF METHYL GROUP FORMATION IN METHIONINE BIOSYNTHESIS BY SACCHAROMYCES CEREVISIAE

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Abstract approved: Dr. Leo W. Parks

Serine transhydroxymethylase from Saccharomyces cerevisiae has been purified to a specific activity representing a 180-fold increase over the crude extract. The enzyme has been partially characterized and appears to be similar to those reported in bacterial and animal systems. Spectral data indicate that the pyridoxal phosphate cofactor is bound to the enzyme, presumably as the Schiff base, but can be resolved by normal purification procedures. Stability of the enzyme has been achieved in 30% glycerol. Molecular weight estimates, determined by sucrose density sedimentation, give values ranging from 180,000-200,000. Enzyme activity is stimulated by divalent cations. While serine transhydroxymethylase is both inhibited and repressed to a limited degree by methionine, the other presumed end products of pathways involving the enzyme, specifically

the purines and thymine, show no repressive or inhibitory effects on the enzyme. No evidence has been obtained for isoenzymes of serine transhydroxymethylase.

Effect of methionine on sulfate uptake and incorporation into protein indicate a strong repressive control of methionine biosynthesis by exogenous methionine.

Aspects of the Regulation of Methyl Group Formation in Methionine Biosynthesis by *Saccharomyces cerevisiae*

by

Kenji Dennis Nakamura

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Professor of Microbiology
in charge of major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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INTRODUCTION

Methionine's role as a constituent of protein is only one part of its involvement in the cell. In procaryotic organisms the synthesis of most, if not all, proteins begins with methionine. It is N-formyl-methionyl-tRNA which serves as the initiating residue for protein synthesis. It now appears that this form of methionine also initiates peptide-chain synthesis in the mitochondria of eucaryotic organisms. Through its conversion to the high energy sulfonium compound, S-adenosylmethionine, methionine serves as the major methyl group donor in the many biological transmethylation reactions of the cell. Participation of S-adenosylmethionine as the methyl group donor has been established in the methylation of DNA, RNA, phospholipids, sterols, and polysaccharides. In view of methionine's involvement in such a wide variety of cellular processes, a precise control on its synthesis would seem advantageous to the cell.

The majority of work on the control of methionine biosynthesis previously centered on the enzymes involved in the pathway leading to the four carbon moiety of the amino acid. Relatively little work had been done in the important pathway providing the methyl group of methionine. Recent investigations have provided evidence that serine is the ultimate methyl donor for methionine biosynthesis. The pathway leading from the beta-carbon of serine to methionine has been

postulated to be a three step process mediated by a polyglutamyl derivative of tetrahydrofolic acid. The major portion of this dissertation is concerned with the enzyme involved in the first step of this pathway, serine transhydroxymethylase. This enzyme has been extensively studied with respect to serine-glycine interconversion and pyridoxal phosphate metabolism, but little work has been done on its involvement in methionine biosynthesis. An investigation of the possible controls operating at this step was pursued to determine the role that serine transhydroxymethylase plays in the overall control of methionine biosynthesis. To that end, a purification procedure has been developed to study the properties of the enzyme and to determine whether isoenzymic forms of serine transhydroxymethylase exist.

REVIEW OF LITERATURE

Methionine biosynthesis in Saccharomyces cerevisiae can be considered to result from the convergence of three pathways shown in Figure 1. One pathway provides the four carbon skeleton of methionine and is derived from aspartic acid. A second provides for the assimilation of the sulfur group and the third is responsible for generation and attachment of the methyl group of methionine.

The methionine biosynthetic pathway of yeast is unique in that cystathionine is not an obligatory intermediate. Early studies in Neurospora crassa (Horowitz, 1947) and Escherichia coli (Lampen et al., 1947) have demonstrated that in these organisms, homocysteine, the immediate precursor of methionine, was formed by the cleavage of a seven-carbon compound, cystathionine. Though yeast contain cystathionine synthetase activity, the levels are low when compared to Neurospora crassa (Delavier, Klutchko and Flavin, 1965) and Escherichia coli (Rowbury and Woods, 1964). Furthermore, methionine auxotrophs of yeast have not been found in which cystathionine is able to satisfy the methionine requirement (Pigg et al., 1962).

Recent studies have provided evidence for the role of an enzyme catalyzing the direct formation of homocysteine from sulfide and O-acetyl-homoserine. This enzyme, homocysteine

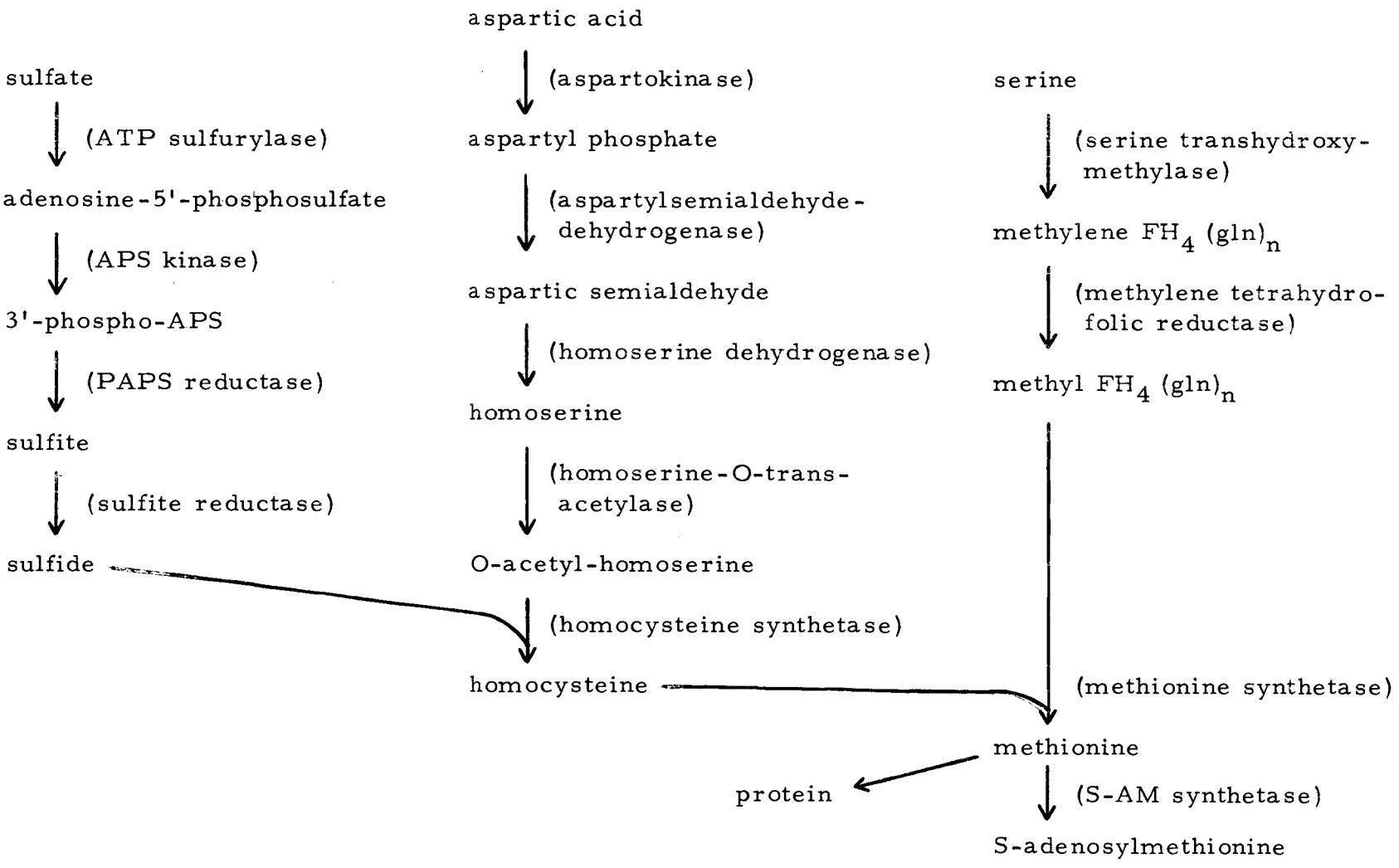

Figure 1. Proposed biosynthetic pathway of methionine in yeast.

synthetase, has been shown to be missing in a mutant unable to synthesize methionine (Cherest et al., 1969). Homoserine-O-transacetylase, the enzyme catalyzing the formation of O-acetyl-homoserine, has also been found to be lacking in a mutant unable to synthesize methionine (de Robichon-Szulmajster and Cherest, 1967). The portion of the pathway leading from aspartate to homoserine is shared with the biosynthetic pathway of threonine. Mutants lacking any one of the three enzymes involved in this portion of the pathway, aspartokinase, aspartic semi-aldehyde dehydrogenase or homoserine dehydrogenase, exhibit a requirement for both threonine and methionine (de Robichon-Szulmajster et al., 1971).

Studies on the enzymatic synthesis of the methyl group of methionine have been largely confined to bacterial systems. Early studies on Escherichia coli indicated that serine could serve as the source for the methyl group of methionine (Cohn et al., 1953; Rowbury and Woods, 1961). Folic acid was also shown to play a role in the methylation of homocysteine in avian liver (Doctor et al., 1957) and in bacteria (Kisluik and Woods, 1960). Isolation of methionine mutants of E. coli lacking either serine transhydroxymethylase or methylene tetrahydrofolate reductase, implicated these two enzymes in the methionine biosynthetic pathway (Hatch et al., 1961). Development of a cell-free system for methionine synthesis from Saccharomyces cerevisiae demonstrated that the beta-carbon of serine could

be converted to the methyl group of methionine. Requirements for the cell-free methylation of homocysteine to give methionine included a reducing system, a pyridine nucleotide, FAD, pyridoxal phosphate, a source for a folate derivative, serine, homocysteine, and cell-free extract (Botsford and Parks, 1967). Serine transhydroxymethylase (E.C. 2.1.2.1.) was postulated as the first enzyme of this pathway. This enzyme catalyzes the conversion of serine and tetrahydrofolic acid (FH_4) to glycine and a one-carbon derivative of folic acid.

$$\text{serine} + FH_4 \rightleftharpoons \text{glycine} + N^5, N^{10}\text{methylene-}FH_4$$

A methionine mutant of S. cerevisiae lacking serine transhydroxymethylase was reported, thus suggesting the enzyme is involved in methionine biosynthesis in yeast (Pigg et al., 1962).

Evidence also indicated that S-adenosylmethionine might serve as the methyl donor for methionine synthesis. S-adenosylmethionine: homocysteine methyltransferase was demonstrated in yeast to be able to synthesize methionine by a direct transmethylation of homocysteine (Shapiro, 1958). Methionine mutants deficient in this enzyme were reported, suggesting a direct role for S-adenosylmethionine in the biosynthesis of methionine (Pigg et al., 1962). However, further studies on the cell-free system for methionine synthesis demonstrated no requirement for S-adenosylmethionine (Botsford and Parks, 1967).

Regulation of biosynthetic pathways by their end products can be achieved by two mechanisms, (1) repressing the synthesis of enzymes involved in that pathway (Cohn et al., 1953; Vogel, 1957), or (2) specific inhibition of enzymatic activity, generally in the first step of that metabolic pathway (Umbarger, 1961). The control of the enzymes involved in the synthesis of homocysteine have been studied in great detail with respect to both of these mechanisms. The first enzyme of the methionine specific portion of that pathway, homoserine-O-transacetylase, is strongly repressed by methionine (de Robichon-Szulmajster and Cherest, 1967). This enzyme was also reported to be inhibited by S-adenosylmethionine, but this has been attributed to impurities interfering in the particular assay system (de Robichon-Szulmajster et al., 1971). The second enzyme in this portion of the pathway, homocysteine synthetase, is effectively controlled by methionine both at the level of synthesis (Cherest et al., 1969) and enzymatic activity (Wiebers and Garner, 1967). Homoserine dehydrogenase, the last common enzyme in the portion of the pathway shared with threonine biosynthesis, is repressed by methionine and inhibited by both end products (Karassevitch and de Robichon-Szulmajster, 1963). The repression of aspartokinase by methionine has recently been reported, though the degree of repression is small (Cherest et al., 1971).

The controls operating specifically in the synthesis of the methyl group of methionine have been studied in less detail. Studies with cell-free extracts of E. coli demonstrated the repression of the enzymes which function in the overall synthesis of methionine from homocysteine and serine (Cohn et al., 1953). The repression of methylene tetrahydrofolate reductase by methionine has been described in bacteria (Katzen and Buchanan, 1965). Feedback inhibition of this enzyme by methionine has also been reported in a mammalian liver system (Kutzbach and Stokstad, 1967). In S. cerevisiae, culturing cells in the presence of excess methionine did not affect the capacity of cell-free extracts to methylate homocysteine (Botsford and Parks, 1969). This same study showed that serine transhydroxymethylase was only slightly repressed by methionine. However, the enzyme was inhibited by methionine and to a greater extent by S-adenosyl-methionine. Studies on the control of serine transhydroxymethylase are complicated by its involvement in biosynthetic pathways leading to end products other than methionine. The reaction serves as a source of one carbon folate derivatives involving serine transhydroxymethylase in the biosynthesis of purines and thymine (Mudd and Cantoni, 1964). Yeast have also been shown to synthesize serine from glycine though there is still some question as to whether they utilize glyoxalate or the alpha-carbon of glycine as a source for the one carbon fragment (DeBorso and Stoppani, 1967).

Control of an enzyme or enzymes involved in a pathway leading to several end products encounters an obvious problem. In the absence of independent controls, an excess of one product could result in a deficiency in the others. Several mechanisms have been described which can provide this independent control. Multivalent repression was first discovered in the isoleucine-valine-leucine pathway in E. coli (Freundlich et al., 1962). In this control system, no repression occurs unless all the end products are in excess. A multivalent repression system has since been reported in isoleucine-valine biosynthesis in S. cerevisiae (Magee and Hereford, 1969). Control of enzymatic activity by multiple end products has also been demonstrated. Three patterns of control of activity, collectively known as multivalent end-product inhibition, have been described in various bacterial systems. In one type known as concerted feedback inhibition, none of the end products exert any effect on enzyme activity unless all are present. Such a pattern of inhibition has been demonstrated for aspartokinase in Bacillus polymyxa (Paulus and Gray, 1964). In a second type, known as cooperative end-product inhibition, each end product is weakly inhibitory whereas together, they are more than additive in their inhibitory effect (Caskey et al., 1964). In cumulative end-product inhibition, demonstrated for glutamine synthetase in E. coli, partial inhibition by each end product occurs with neither cooperation nor antagonism existing between the inhibitors (Woolfolk and Stadtman,

1967). An alternative to such controls is the mode of regulation in which several enzymes, termed isoenzymes, catalyze the same reaction and are independently controlled by the end products. A number of examples have been demonstrated in bacterial systems, including the isoenzymes of aspartokinase in the threonine-methionine pathway in E. coli (Stadtman et al., 1961). Evidence for isoenzymic forms of methylenetetrahydrofolic acid dehydrogenase, an enzyme involved in purine biosynthesis, has also been reported in S. cerevisiae (Lazowska and Luzzati, 1970).

MATERIALS AND METHODS

Cultures

Most experiments utilized Saccharomyces cerevisiae 3701B, a haploid yeast auxotroph requiring uracil. This strain was originally obtained from H. L. Roman. Two methionine mutants were also used. Saccharomyces cerevisiae 22B, a haploid auxotroph requiring methionine was obtained from R. K. Mortimer. Saccharomyces cerevisiae 80BM1, a haploid auxotroph requiring methionine and uracil, was obtained from K. D. Spence.

Growth Conditions

For some experiments dealing with the purification of serine transhydroxymethylase, cultures were grown aerobically at 30 C on a rotary shaker in 2 liter flasks containing 1 liter of growth medium. The growth medium consisted of 1% tryptone, 2% dextrose, and .5% yeast extract (TDY). Cells were growh from a 1% inoculum (v/v) and harvested at 18 to 24 hours. The cells were washed three times with 0.1 M potassium phosphate buffer, pH 7.3 and resuspended in an equal volume of Wickerham's defined complete medium less methionine (WCLM), containing 0.1 g of uracil per liter (Wickerham, 1946) and aerated for an additional five to seven hours before harvesting.

For large scale purification of serine transhydroxymethylase, cells were grown in a New Brunswick Fermacell Fermentor, Model CF-50. Thirty liters of TDY medium were inoculated with 4 liters of a 24 hour culture of 3701B. Air flow was maintained at two cubic feet per min. The cells were grown for 18 to 20 hours at 30 C before being harvested with a Sharples continuous flow centrifuge. After being washed three times, the cells were resuspended in 35 liters of WCLM medium and aerated for an additional six hours before harvesting.

In experiments dealing with the effect of various growth supplements on serine transhydroxymethylase, cells were grown in WCLM plus the appropriate supplement for 18 to 24 hours. The cells were aseptically harvested and resuspended in twice the volume of the same growth medium and grown for an additional six to eight hours before harvesting.

For the sulfate uptake experiments, a low sulfate modification of WCLM was used which included replacement of $MgCl_2$ for $MgSO_4$ and NH_4Cl for $(NH_4)_2SO_4$. Five mg of Na_2SO_4 was added per liter. Cells were grown aerobically at 30 C from a 1% inoculum (v/v) in 100 ml of medium in 300 ml Bellco side arm flasks. Growth was monitored turbidimetrically on a Klett-Summerson colorimeter equipped with a green (#54) filter.

Isotopic Measurements

All determinations were made on a Packard Model 3214 Tri-Carb Liquid Scintillation Spectrometer.

The scintillation fluid employed in the Taylor and Weissbach assay for serine transhydroxymethylase, consisted of 0.3% PPO, 0.01% POPOP in toluene. For the sulfate uptake experiments, the scintillation fluid consisted of 0.4% PPO in a 1:1 mixture of toluene and 95% ethanol.

Determination of $^{35}SO_4^=$ Uptake and Incorporation

At regular intervals of the culture cycle, 2 ml samples were removed and immediately cooled in an ice bath. The cells were removed by centrifugation (3000 x g) at 4 C. A 0.1 ml sample was removed from the supernatant and counted in 10 ml of scintillation fluid. Counts were routinely corrected for quenching by use of a radium-226 external standard. A sample was removed from the supernatant fraction to determine methionine concentration.

For determination of sulfate incorporation into protein, cultures grown in 100 ml of medium were harvested in early stationary phase (220 Klett units) and cells were washed three times in cold 0.1 M potassium phosphate buffer, pH 7.3. Cells were resuspended in 5 ml of 5% trichloroacetic acid (TCA), boiled for five min and immediately

centrifuged (15000 x g). The TCA treatment was performed twice. The precipitate was washed twice in 5 ml of 95% ethanol and centrifuged. The remaining precipitate was dried, weighed, and dissolved in 1 ml of 0.1N NaOH. A 0.01 ml sample was removed for counting.

Microbiological Assay for Methionine

Methionine was determined using the methionine requiring strain of Streptococcus faecalis #9790. The commercially prepared Methionine Assay Medium of Difco was used and standard Difco procedure was followed. Growth was determined on a Coleman Model 9 Nephelo-Colorimeter.

Protein Determination

Protein concentrations were determined according to the method of Lowry et al. (1951), using crystalline bovine serum albumin as the standard.

Polyacrylamide Disc Gel Electrophoresis

Disc gel electrophoresis was performed by the method of Davis (1964). All experiments were performed using the standard 7% gel. Protein was stained using a 1% amido black solution in 7% acetic acid. Destaining was carried out overnight in 5% acetic acid. Proteins were also stained with Coomassie blue using the method of Chrambach (1967).

Absorption Measurements

All spectral observations were made on 1 ml solutions in 1 cm quartz microcells. Measurements were made on a Zeiss PMQ II Spectrophotometer.

Serine Transhydroxymethylase Assay

Serine transhydroxymethylase was assayed by the method of Taylor and Weissbach (1965) with minor modifications. These included using a sodium bicine buffer, pH 8.5 and terminating the reaction with 0.5 ml of 1.0 M sodium acetate buffer, pH 4.5. The activity as a function of enzyme concentration and time have previously been reported (Botsford and Parks, 1969) using potassium phosphate buffer. The bicine buffer does not alter either of these parameters. The assay involves the use of a radioactive label in the beta carbon of serine and measures the amount of radioactive product formed. Under the conditions of the assay, the radioactive C-1 unit of methylene tetrahydrofolic acid, undergoes rapid equilibration with carrier formaldehyde and is trapped as the dimedon derivative. This derivative is extractable into toluene and radioactivity assayed by liquid scintillation. Recovery and counting efficiency of the assay is determined by running a control with 0.1 μmole formaldehyde-C-14 in place of serine-3-C-14. A background control was also run without enzyme, since a small percentage of serine-3-C-14 is extractable in the toluene. Incubation was

carried out for 15 min at 37 C. One unit of activity is defined as the formation of 10^{-2} μ moles of formaldehyde per minute. When DL-serine-3-C-14 with a specific activity of 10^6 dpm/μ mole is used, this results in a net production of 10^4 x dpm per minute.

Molecular Weight Estimates

Molecular weight estimates were made by sucrose density centrifugation according to the method of Martin and Ames (1961). A sample of purified serine transhydroxymethylase and a suitable standard enzyme of known molecular weight was layered on a 5-20% linear sucrose gradient. Runs extended for 10 to 12 hours at 120,000 x g. The standards of known molecular weight were yeast alcohol dehydrogenase, MW 150,000 (Hayes and Velick, 1954) and beef liver catalase, MW 250,000 (Sumner and Gralen, 1938). All centrifugations were made on a Beckman L2-65 preparative ultracentrifuge using the SW-50 swinging bucket rotor.

Purification of Serine Transhydroxymethylase

The temperature throughout the purification was maintained at 0-4 C except where noted. All buffers unless otherwise stated were supplemented with 5 x 10^{-8}M pyridoxal phosphate and 5 x 10^{-6}M dithiothreitol.

Step 1. Preparation of crude extract: Washed cells were resuspended in 0.1M potassium phosphate buffer, pH 7.3 using a 1:1 ratio of wet weight of cells to volume of buffer. The cells were disrupted in a Bronwill MSK cell homogenizer for 50-60 sec using a bead size of 0.25 mm. The cells were separated from the extract by centrifugation for 30 min at 25,000 x g. Glycerol was added to the supernatant to make it 30% (v/v) with respect to glycerol.

Step 2. Heat treatment: The 30% glycerol solution of crude extract was heated at 55 C in a rotary shaker water bath for 15 min. It was then cooled to 4 C and centrifuged for 30 min at 25,000 x g.

Step 3. Protamine sulfate: A 1% solution of protamine sulfate was added dropwise to the heated extract to give a final ratio of 0.1 mg protamine sulfate to 1.0 mg protein and centrifuged immediately at 20,000 x g for 30 min. The supernatant was dialyzed against 10-15 volumes of 0.1M potassium phosphate buffer for six hours with four changes of buffer.

Step 4. Ammonium sulfate fractionation: Solid ammonium sulfate was added to the supernatant bringing it to 35% saturation and the resultant solution centrifuged after two hours. Additional solid ammonium sulfate was added to the supernatant to bring the final concentration to 55% saturation. After two hours the solution was centrifuged and the precipitate was dissolved in 0.05 M potassium phosphate buffer. This solution was then dialyzed for 14-18 hours against 200

volumes of the same buffer with five changes of buffer.

Step 5. DEAE-Sephadex: The dialyzed solution from step 4 was added to a 2.5 x 90 cm column of DEAE-Sephadex A-50 which had been equilibrated in 0.05 M potassium phosphate buffer, pH 7.3. The column was washed with this buffer until the eluate showed an absorbancy of less than 0.1 at 280 nm. The remaining protein was eluted with a linear KCl gradient (0-0.4M). Five ml fraction were collected and the tubes containing high specific activity were pooled. The pooled sample was concentrated with a Diaflo ultrafiltration unit equipped with an XM-50 membrane.

Step 6. Sephadex G-200: The protein from step 5 was added to an upward flow Sephadex G-200 (2.5 x 90 cm) which had been equilibrated with 0.1 M potassium phosphate buffer, pH 7.3. Three ml fractions were collected and the tubes containing highest specific activity were pooled and concentrated as above. The enzyme was stored at -10 C in 30% glycerol (v/v).

Amino Acid Analysis

The amino acid analysis of yeast protein was initially performed on a Beckman Spinco Model 120B Amino Acid Analyzer. For the simultaneous determination of amount of amino acids and their radioactive specific activities, studies utilized a Phoenix model K-800 Amino Acid Analyzer equipped with an anthracene, flow-through cell

scintillation spectrometer. The procedure of Moore and Stein (1963) was used to prepare the protein hydrolysate.

Materials

All chemicals were obtained from commercial sources and were of the highest purity available.

DL-serine-3-C-14 was obtained from Amersham/Searle Corporation. Sufficient cold DL-serine was added to give a final specific activity of 10^6 dpm/μmole.

Sulfate-S-35 was obtained from Amersham/Searle Corporation.

S-adenosylmethionine was obtained from Calbiochem and was converted from the iodide salt to the chloride salt by passage through a short column of Dowex-1 Cl^-. The concentration was determined spectrophotometrically (Schlenk and DePalma, 1957).

L-tetrahydrofolic acid was obtained from the Sigma Chemical Company. It was dissolved in 0.05 M sodium bicine buffer containing 1% mercaptoethanol. The pH was adjusted to 8.3 with NaOH and the solution was distributed in 2 ml portions in small screw capped vials and stored frozen. For determination of apparent Michaelis constants, the tetrahydrofolic acid was partially purified on a DEAE-cellulose column using the method described by Schirch and Jenkins (1964). The concentration was determined spectrophotometrically.

Pyridoxal-5'-phosphate was obtained from the Sigma Chemical Company.

Yeast alcohol dehydrogenase and beef liver catalase were obtained from the Sigma Chemical Company.

RESULTS

Uptake of radioactive sulfate by yeast growing in a defined medium in the absence of methionine is shown in Figure 2. Sulfate continued to be taken up throughout the log phase of the culture cycle. Sulfate utilization by cells grown in a medium supplemented with methionine is shown in Figure 3. The methionine in the medium was exhausted midway through the culture cycle. No sulfate uptake occurred until the methionine content of the medium was depleted. If an additional 40 μg/ml of methionine was added just prior to exhaustion of methionine, or if the initial concentration of methionine was high enough (80 μg/ml) so that methionine was not totally consumed, no sulfate uptake was observed throughout the entire growth cycle. The effect on sulfate utilization by the addition of methionine to a previously unsupplemented medium is shown in Figure 4. Sulfate accumulation is halted almost immediately on addition of methionine. Radioactive sulfate incorporation into protein isolated from yeast cells grown in the presence and absence of methionine is shown in Table 1. The incorporation of exogenous sulfate by cells grown in the presence of methionine is less than 1% of that of cells grown in the absence of methionine. The radioactive specific activities of methionine and cysteine plus cystine were comparable when cells were grown in the absence of methionine. The sulfate incorporation in the presence of

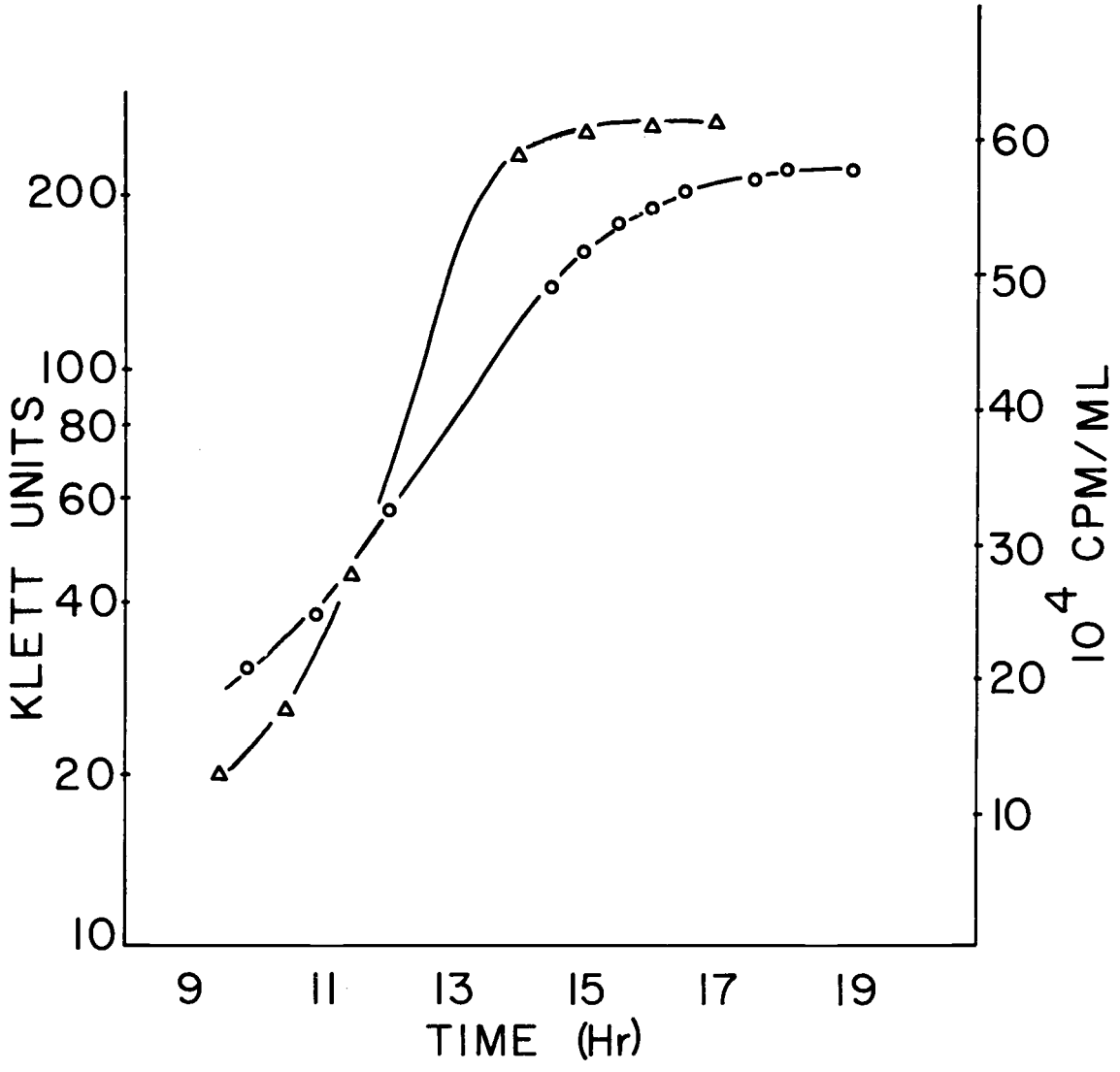

Figure 2. Sulfate uptake by S. cerevisiae in the absence of methionine. The cpm at the beginning of the experiment was 65 x 10^4 cpm/ml. △-△ $^{35}SO_4^=$ uptake; ○-○ growth.

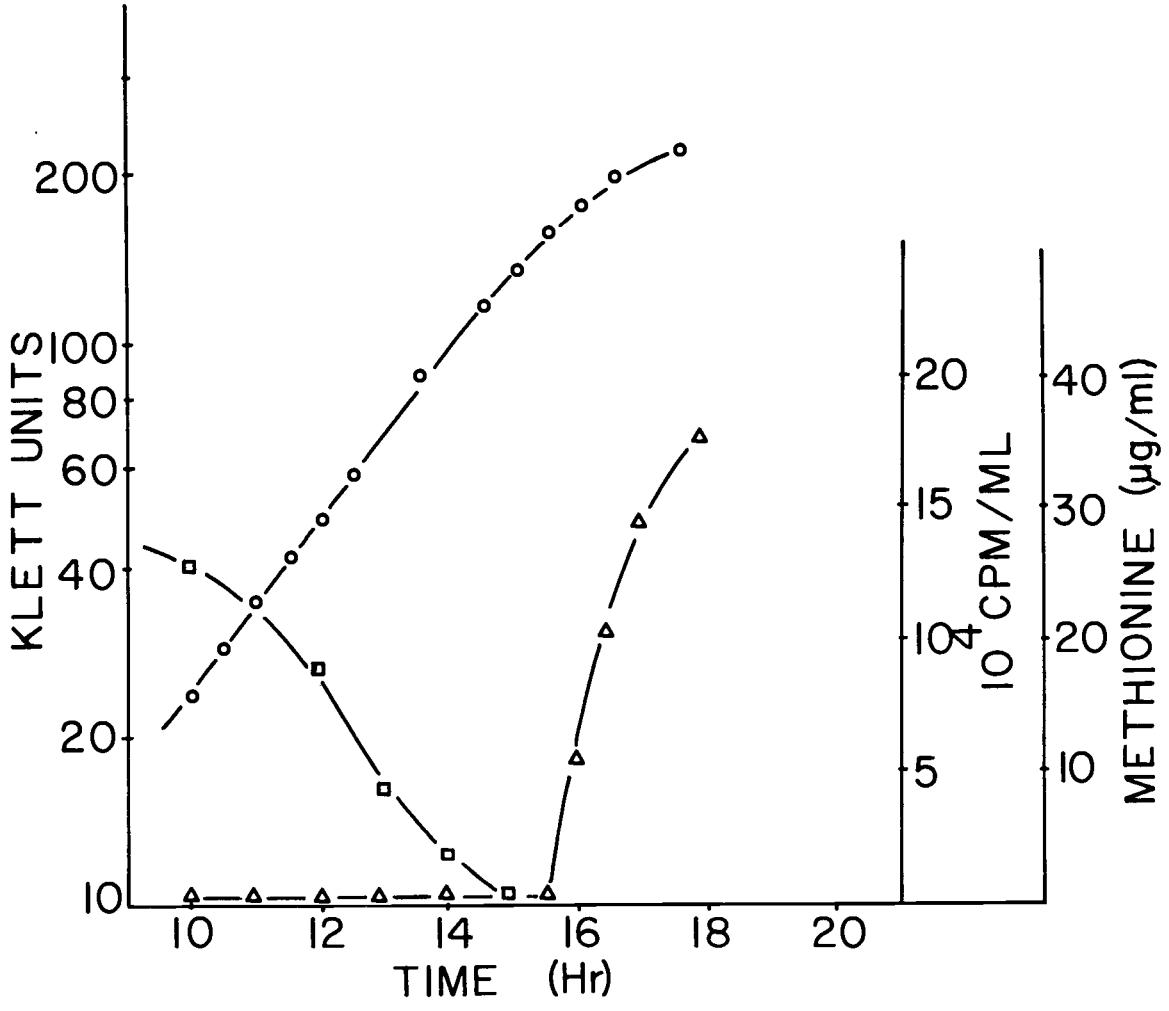

Figure 3. Sulfate uptake by S. cerevisiae in the presence of methionine. The cpm at the beginning of the experiment was 44 x 10^4 cpm/ml. Methionine concentration at the beginning of the experiment was 40 g/ml. Δ-Δ $^{35}SO_4^=$ uptake; o-o growth; □-□ methionine concentration.

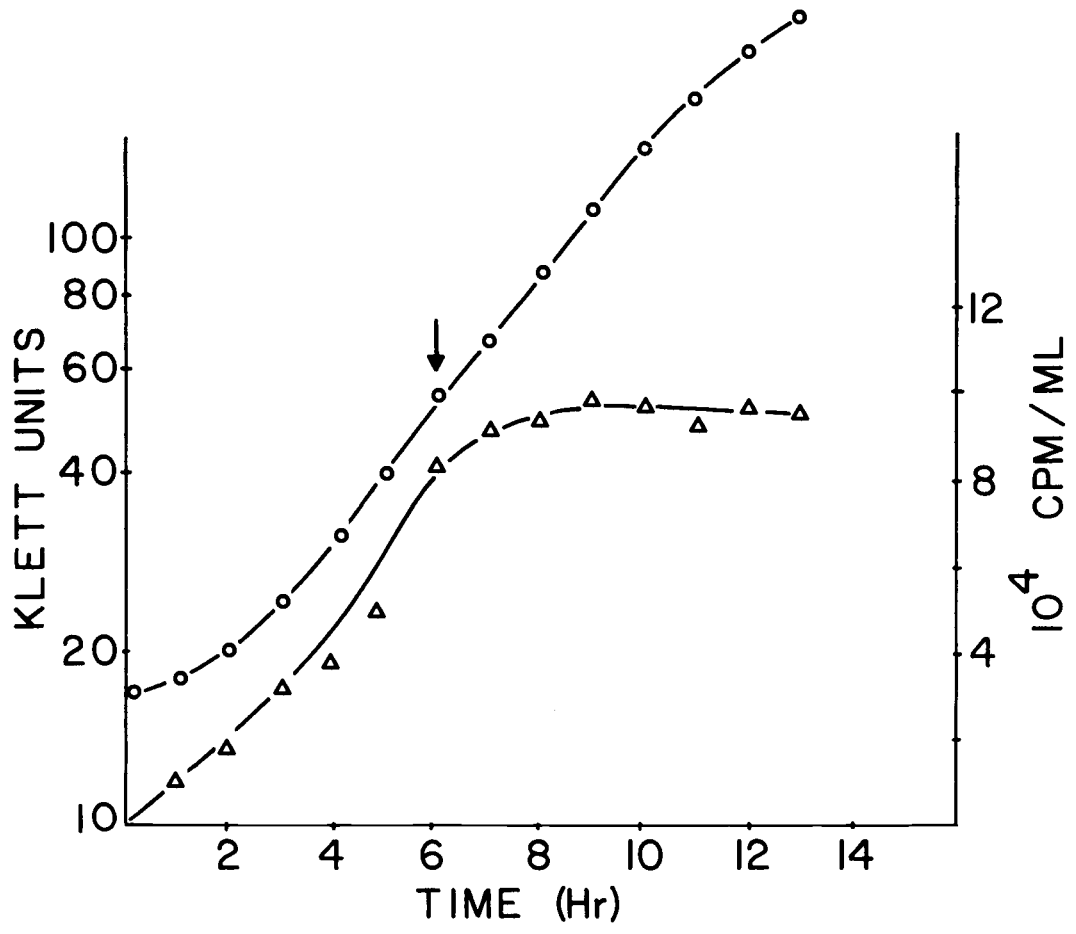

Figure 4. Effect of methionine addition on sulfate uptake by S. cerevisiae. The cpm at the beginning of the experiment was 36×10^4 cpm/ml. At the time indicated by the arrow, methionine was added to a concentration of 30 g/ml. Δ-Δ $^{35}SO_4^{=}$ uptake; o-o growth.

methionine was too low for specific activity determinations of the amino acids.

Table 1. Effect of exogenous methionine on $^{35}SO_4^=$ incorporation into protein.

Supplement	Initial cpm/ml of medium x 10^4	Final cpm/ml of medium x 10^4	cpm/mg of protein x 10^4
none	22.1	.86	16.0
methionine[1]	22.6	21.7	.14

[1]Initial methionine concentration was 80 μg/ml

Amino acid analysis of yeast protein shows the methionine content to be between five and ten times greater than that of cysteine plus cystine. The cysteine plus cystine value varied greatly due to the very low percentage of the two amino acids found in yeast protein. Methionine constituted between 1 and 3% of the amino acid content of yeast protein (g/100g protein).

The standard reaction mixture for the Taylor and Weissbach (1965) assay for serine transhydroxymethylase is shown in Table 2. A slight modification of the assay previously described has been made (Botsford and Parks, 1969). Sodium bicine buffer, 0.1M, pH 8.5 was used instead of 0.6M potassium phosphate. Bicine buffer has a much greater buffering capacity at the higher pH range than does phosphate buffer and eliminated the need for the high ionic strengths previously used. The final pH of the reaction mixture, when pH 8.5 buffer was

used, was 8.4. The pH at which the enzyme has highest activity exhibits a much broader range, as shown in Figure 5, than had previously been demonstrated in phosphate buffer (Botsford and Parks, 1969). The other parameters of the assay such as dependence of activity on enzyme concentration and time as well as the saturating levels of the reaction components were the same as reported for phosphate buffer.

Table 2. The standard reaction mixture for serine transhydroxymethylase.

Component	Amount (μmoles)	Concentration (mM)	Volume[2] (ml)
Sodium bicine, pH 8.5	20	40	.20
Pyridoxal phosphate	.20	.40	.10
Tetrahydrofolic acid	.40	.80	.05
DL-serine-3-C-14	.20	.40	.05
Mercaptoethanol	.40	.80	.05
Enzyme	5-300μg[1]		.05

[1]Amount of enzyme varied greatly depending on the degree of purification.

[2]Final volumes greater than 0.5 ml were sometimes used to accommodate further additions.

A summary of a typical purification procedure of serine transhydroxymethylase is presented in Table 3. Such a procedure generally results in a 150-200 fold increase in specific activity. Enzyme purifications were sometimes performed on a much larger scale than is outlined in the table, but the same procedure was used.

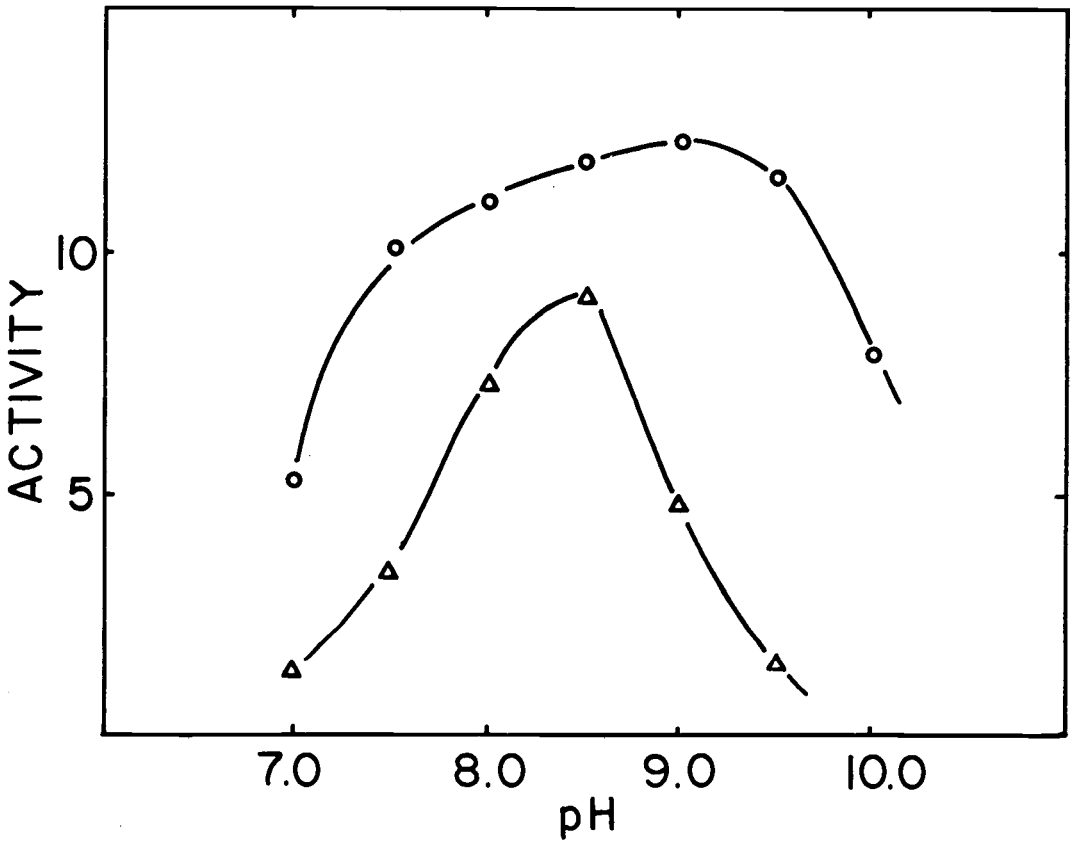

Figure 5. Effect of pH on serine transhydroxymethylase activity. o-o sodium bicine buffer (40 mM); Δ-Δ potassium phosphate buffer (120 mM).

Table 3. Purification of serine transhydroxymethylase from Saccharomyces cerevisiae.

Fraction	Volume ml	Protein mg	Specific Activity units/mg	Yield %
Crude extract	250	7150	0.36	100
Heat treatment	228	3920	0.59	90
Protamine sulfate	220	2200	0.83	71
Ammonium sulfate	10	400	3.91	60
DEAE-Sephadex chromatography	6	52	20.6	42
G-200 Sephadex chromatography	5	4.5	66.3	12

The elution profile of serine transhydroxymethylase from a DEAE-Sephadex column is shown in Figure 6. The enzyme normally was eluted at KCl concentrations between .10-.15 M. Fractions 59-69 were pooled and concentrated. The elution pattern from G-200 Sephadex is shown in Figure 7. Fractions 32-45 were pooled and concentrated. A decrease in specific activity in the latter portion of the protein peak suggests the presence of a contaminating protein. A second chromatographic run in G-200 in the presence of 10^{-4}M methionine, an inhibitor of the enzyme, caused no observable shift in the elution characteristics. When this purified preparation was run on polyacrylamide disc gel electrophoresis, it exhibited one major and one minor band with relative mobilites of .113 and .321, respectively, when stained with amido black. A third and much lighter band was observed with a relative mobility of .292 when gels were stained with Coomassie blue. Attempts to elute serine transhydroxymethylase

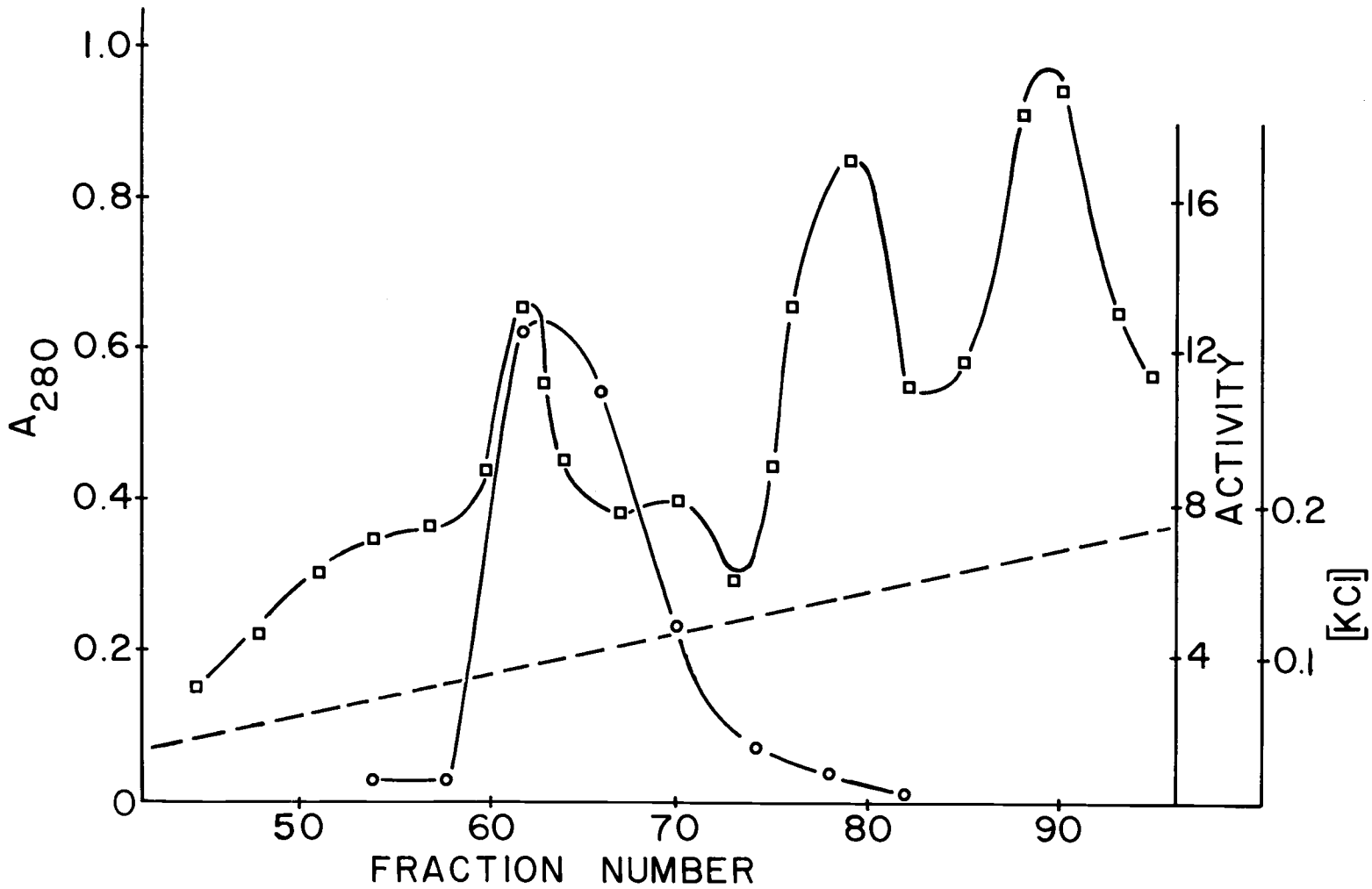

Figure 6. Elution profile of serine transhydroxymethylase from DEAE-Sephadex A-50. o-o activity; □-□ absorbance; ---- KCl concentration.

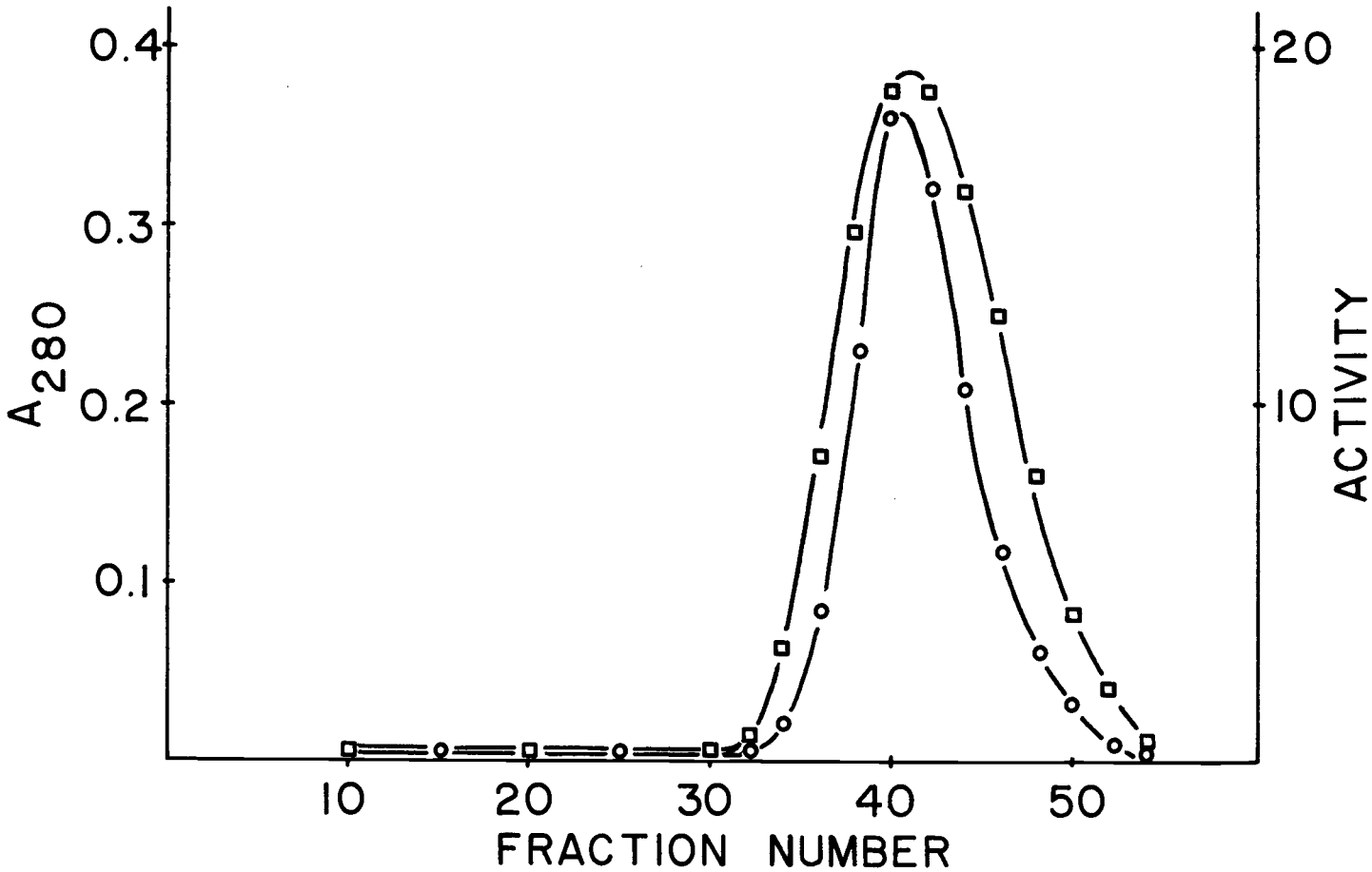

Figure 7. Elution profile of serine transhydroxymethylase from G-200 Sephadex. o-o enzyme activity; □-□ absorbance.

activity from the disc gels were unsuccessful. On one occasion some activity was detected in the area of the .113 band, but amounted to less than one half of an activity unit.

When pyridoxal phosphate was not used as a supplement in the buffer during purification, the purified enzyme showed an absolute requirement for pyridoxal phosphate as well as tetrahydrofolic acid. This resolution of pyridoxal phosphate from the enzyme does not occur in the crude extract by simple passage through a G-25 Sephadex column. The crude enzyme retains 30-40% of the activity of the control when no additional pyridoxal phosphate is added to the reaction mixture. However, crude extracts also demonstrate an absolute requirement for tetrahydrofolic acid.

Apparent Michaelis constant for the various reaction components are shown in Table 4. The K_m with respect to serine is an order of magnitude greater than had previously been reported (Botsford and Parks, 1969). The value obtained for the tetrahydrofolic acid is a rough estimate at best, due to its extremely rapid oxidation on exposure to air. All values represent an average of several determinations.

Table 4. Apparent Michaelis constants for the reaction components for serine transhydroxymethylase.

Compound	K_m x 10^{-4}M
L-serine[1]	7.0
Pyridoxal phosphate	1.1
Tetrahydrofolic acid	2.5

The reaction mixture was as noted in Table 2. The enzyme source was purified serine transhydroxymethylase.

[1]For K_m determinations, L-serine was used. For general activity determinations DL-serine was used. The D-form has been found not to affect the activity.

[2]Tetrahydrofolic acid from commercial source was purified on a DEAE-Sephadex column, as described in Methods section, just prior to use.

The absorption spectrum of the purified serine transhydroxymethylase is shown in Figure 8. The purified enzyme exhibited a single absorption maximum at 280 nm. On addition of pyridoxal phosphate, an additional absorption peak appeared at 440 nm. Addition of cysteine to the holoenzyme caused a rapid absorbance decrease in the 440 nm peak with the concomitant appearance of a new peak at 330 nm. When this cysteine-treated enzyme was passed through a G-25 column, it exhibited no activity in the absence of added pyridoxal phosphate. Addition of pyridoxal phosphate to the reaction mixture restored 83% of the original activity.

The effect of S-adenosylmethionine on the absorption spectrum of the holoenzyme is shown in Figure 9. A decrease and shifting of

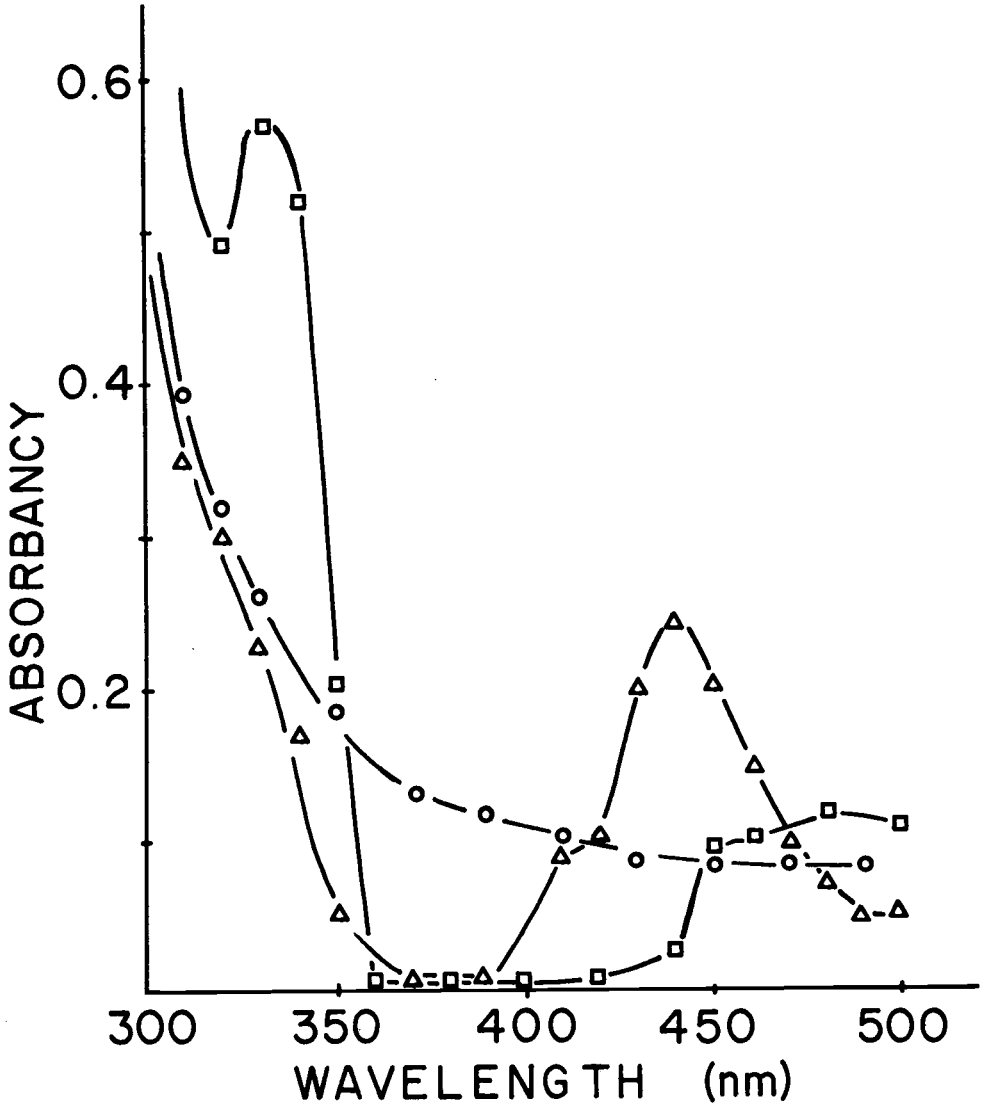

Figure 8. Absorption spectrum of purified serine transhydroxymethylase. o-o spectrum of purified enzyme, 2.3 mg/ml; Δ-Δ spectrum after 15 min incubation with 4.0×10^{-4}M pyridoxal phosphate; □-□ spectrum of holoenzyme after incubation with 5.0×10^{-3}M L-cysteine.

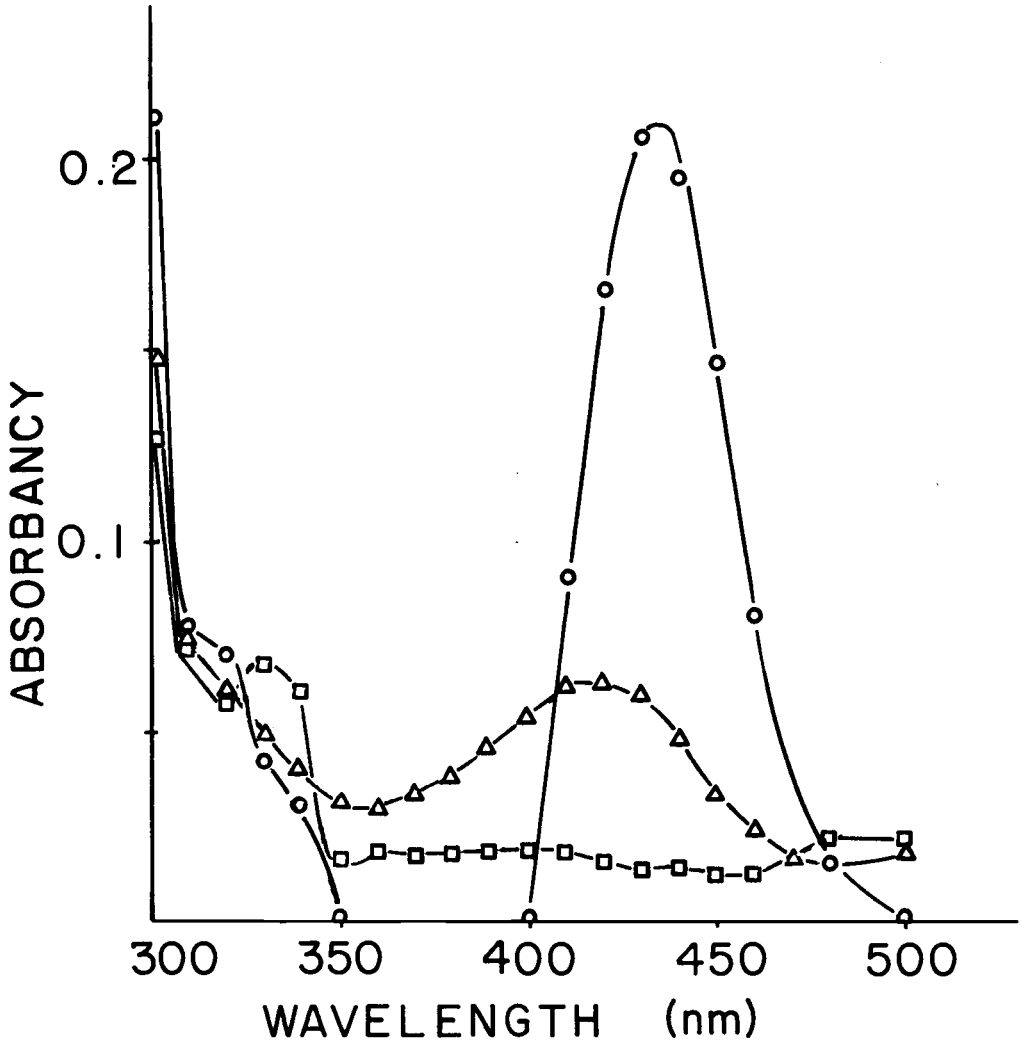

Figure 9. Effect of S-adenosylmethionine on the absorption spectrum of serine transhydroxymethylase, o-o spectrum of the holoenzyme; Δ-Δ spectrum after incubation with .5mM S-adenosylmethionine; □-□ spectrum after further addition of .5mM cysteine.

the 440 nm peaked to 450 nm was observed. A further addition of cysteine caused a complete loss of the 450 nm peak and the appearance of a small peak at 330 nm. The addition of 10 μM methionine had no observable effect on the absorption spectrum of the holoenzyme. The effect of S-adenosylmethionine and cysteine on the absorption spectrum of free pyridoxal phosphate is shown in Figure 10. Reaction of cysteine with pyridoxal phosphate to form the stable thiozolidine compound is indicated by loss of the 390 nm peak and formation of a new peak at 330 nm. A reaction between S-adenosylmethionine and pyridoxal phosphate is also indicated by the decrease and shifting of the 390 nm peak to 400 nm, though the nature of the interaction is not known.

The effect of metal cations on serine transhydroxymethylase activity is shown in Table 5. Divalent cations are much more effective than the monovalent cations tested. The effect of Mg^{++} concentration on serine transhydroxymethylase activity is shown in Figure 11. Cation stimulation was found to be somewhat variable in crude extracts. The maximum stimulation ever obtained was about three-fold. Cation stimulation of purified enzyme was also variable, but no more than a 1.5 to 2-fold increase was ever observed.

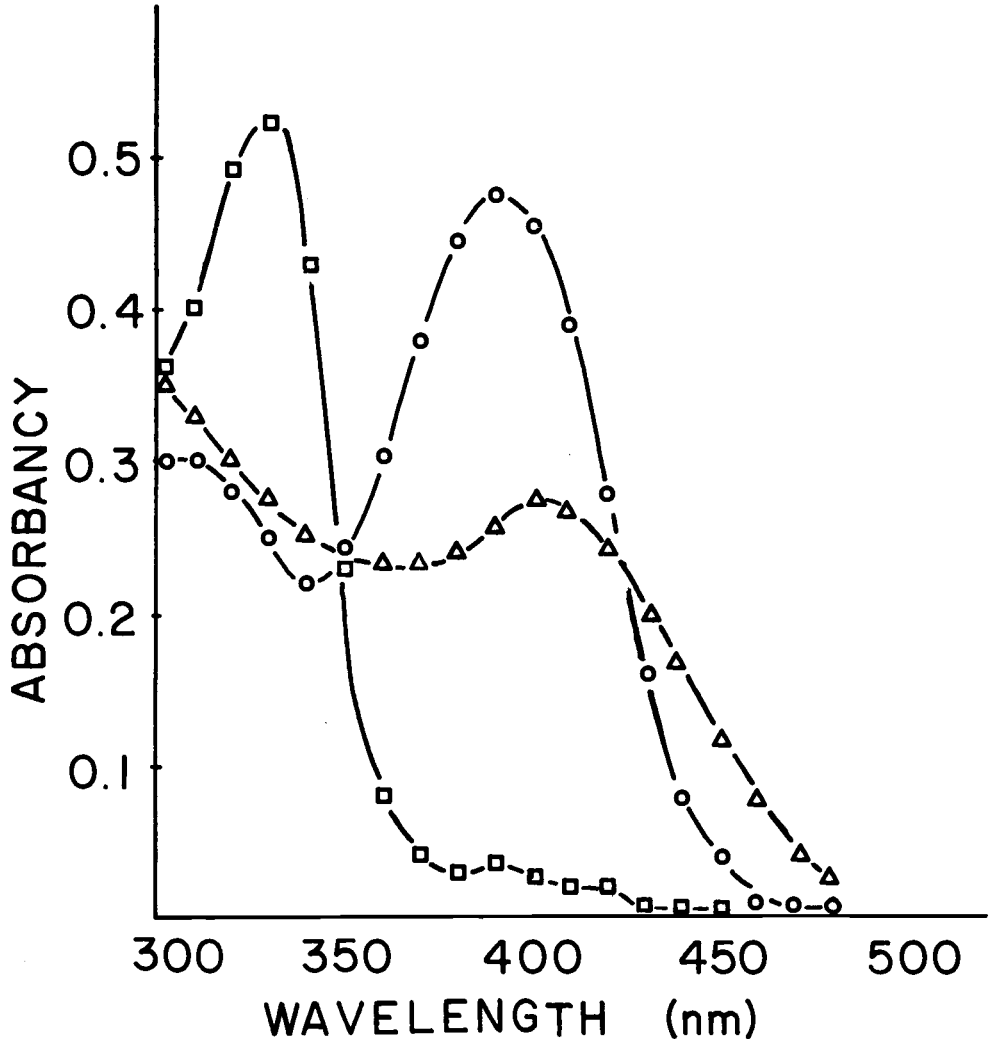

Figure 10. Effect of S-adenosylmethionine and cysteine on the absorption spectrum of free pyridoxal phosphate.
o-o spectrum of 2.0×10^{-4}M pyridoxal phosphate;
Δ-Δ spectrum of 2.0×10^{-4}M pyridoxal phosphate after addition of 1.0×10^{-3}M S-adenosylmethionine;
□-□ spectrum of 2.0×10^{-4}M pyridoxal phosphate after addition of 1.0×10^{-3}M L-cysteine.

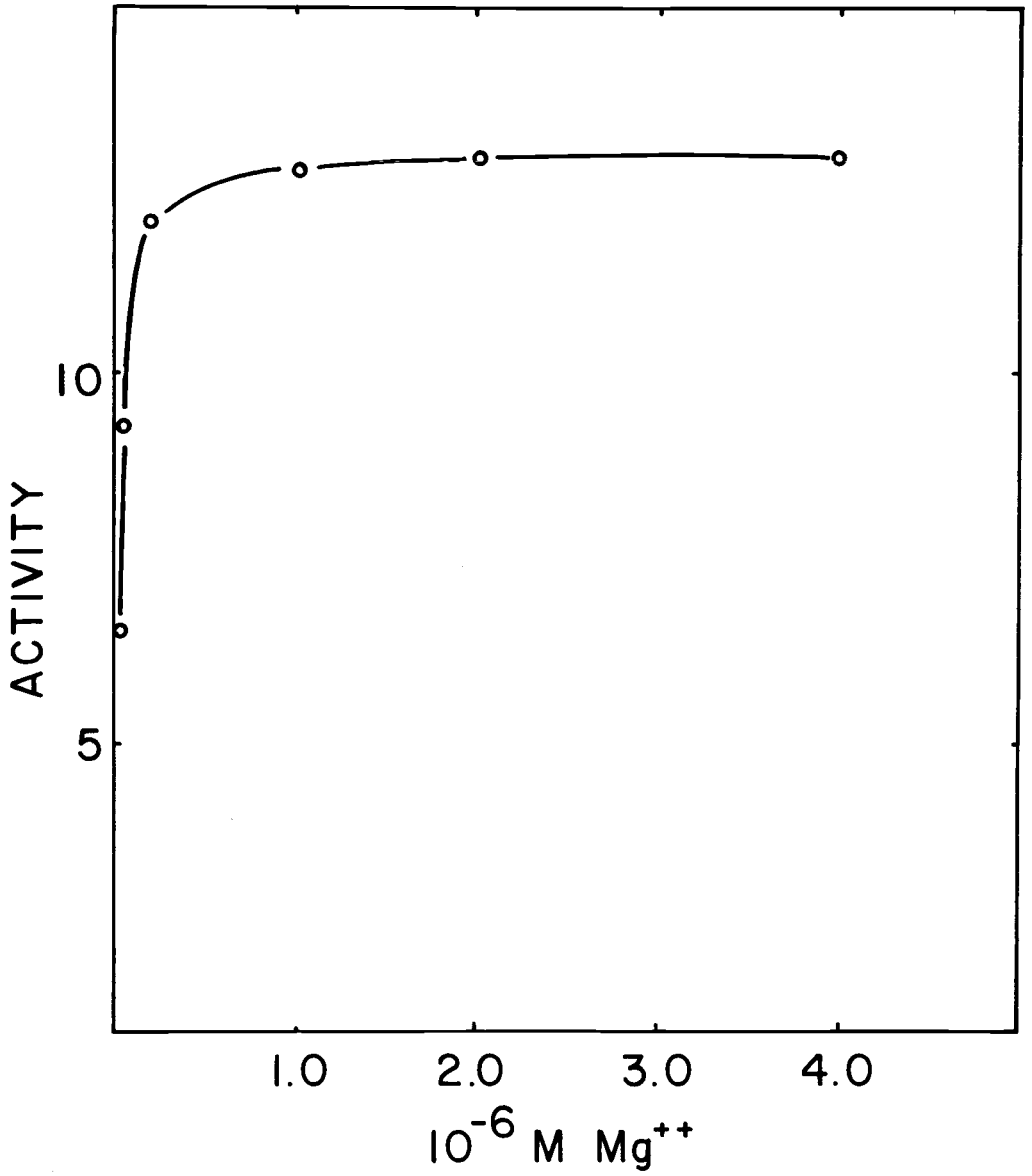

Figure 11. Effect of Mg^{++} concentration on serine transhydroxymethylase activity.

Table 5. Effect of metal cations on serine transhydroxymethylase activity.

Cation	Concentration (mM)	Activity units	% Control
none	-	4.1	100
Mg^{++}	1	8.0	195
Mn^{++}	1	7.0	171
Ca^{++}	1	7.7	188
Fe^{++}	1	4.5	110
Zn^{++}	1	2.4	58
K^{+}	10	6.3	153
Na^{+}	10	6.0	146
Na^{+}	20	7.6	185

The enzyme used was purified through the ammonium sulfate step of purification. All cations used were in the chloride form.

Molecular weight estimates were determined by sucrose density sedimentation. A crude estimation of molecular weight (MW) was obtained from the following equation:

$$\frac{\text{distance traveled from meniscus by unknown}}{\text{distance traveled from meniscus by standard}} = \left(\frac{MW_{unknown}}{MW_{standard}}\right)^{2/3}$$

When alcohol dehydrogenase (MW 150,000) was used as the standard, calculated molecular weight of serine transhydroxymethylase ranged from 180,000 to 190,000. When catalase (MW 250,000) was used, the molecular weight values for serine transhydroxymethylase ranged from 190,000 to 200,000.

Serine transhydroxymethylase is quite unstable, especially in the purified state. The purified enzyme will lose more than 50% of its activity in a ten hour period and serine, pyridoxal phosphate, or dithiotreitol provided only a small degree of protection. The stability

is greatly increased in 30% (v/v) glycerol as the purified enzyme is stable for up to one month in 30% glycerol when stored at -10 C. The protective effect of glycerol on a crude enzyme solution heated at 55 C is demonstrated in Figure 12. In the presence of glycerol, no loss of activity occurred even after 20 min at 55 C. When glycerol was not present, a large loss in activity occurred within the first two min. However, as seen from the same figure, the crude preparation heated in the absence of glycerol exhibited a heat stable serine transhydroxymethylase activity. When this stable enzyme activity was concentrated and again heated at 55 C, it continued to demonstrate a greater stability to heat inactivation as can be seen in Figure 13. However, this "stable" serine transhydroxymethylase showed comparable properties to unheated enzyme with respect to K_m, sensitivity to inhibition, and pH optimum. Molecular weight estimates showed no noticeable change in size of the enzyme. Purified serine transhydroxymethylase did not exhibit such a heat stable fraction, when heated in the absence of glycerol.

Attempts to demonstrate complete separation of the regulatory control from the active unit of serine transhydroxymethylase have been unsuccessful. The loss of sensitivity to inhibition by methionine and S-adenosylmethionine during aging of crude preparations has been reported (Botsford and Parks, 1969) and are confirmed in Table 6. However, preferential inactivation of the regulatory property over

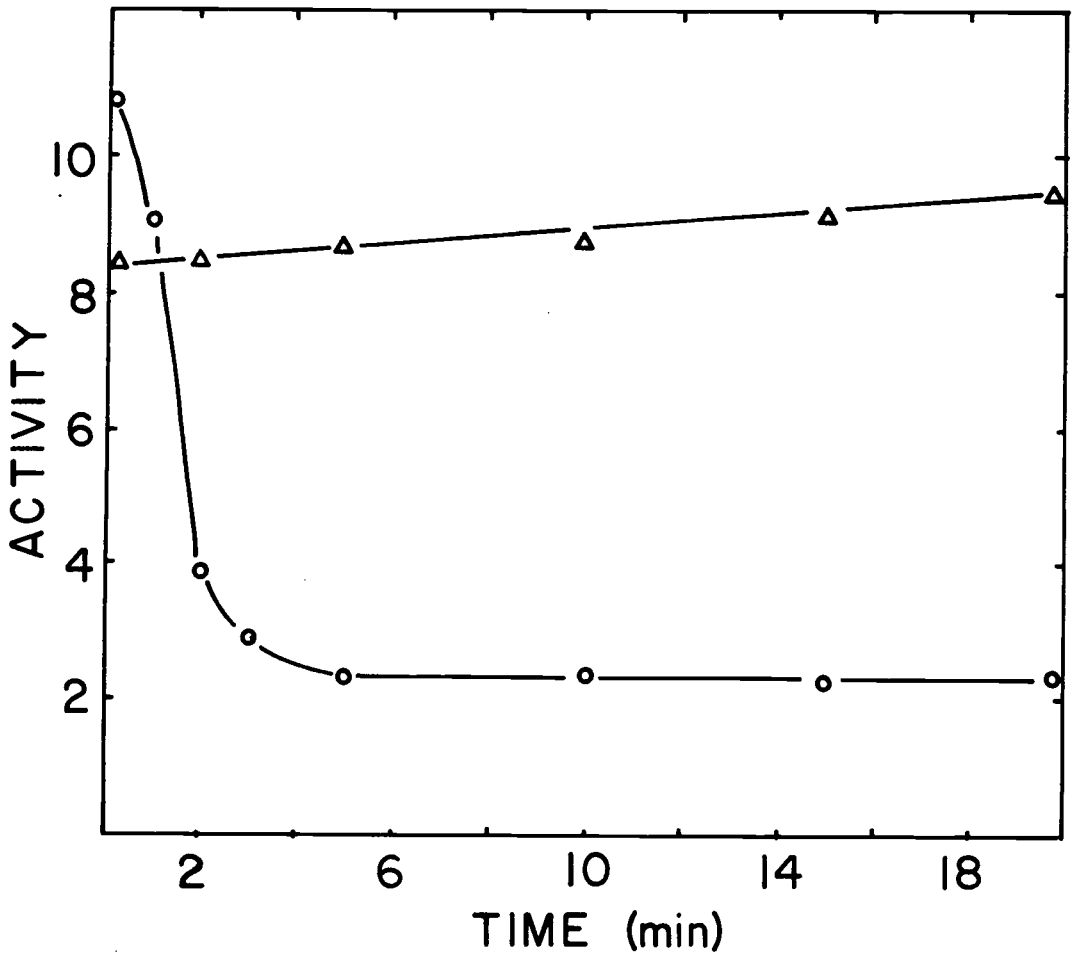

Figure 12. Effect of glycerol on heat inactivation of serine transhydroxymethylase. Enzyme source was dialyzed crude extract. Heating was done in a 55 C water bath using 10 ml of extract. One ml samples were removed. o - o no glycerol; Δ - Δ 30% glycerol.

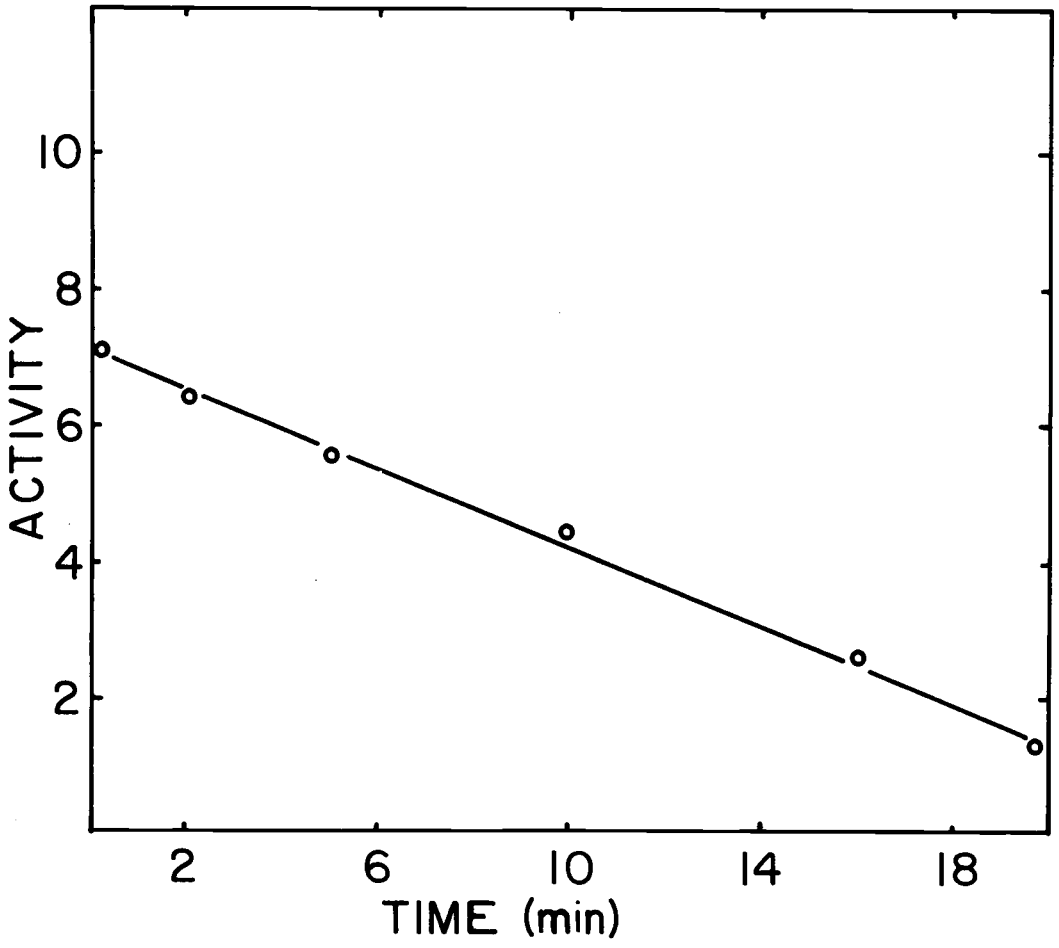

Figure 13. Heat inactivation of "heat stable" serine transhydroxymethylase. Heating was done in a 55 C water bath using 10 ml of extract. No glycerol was present.

the activity does not occur with the purified preparation of the enzyme. Attempts to separate the regulatory portion from the active portion by use of urea, pH extremes, as well as heating, have failed.

Table 6. Effect of aging on feedback inhibition properties of crude and purified serine transhydroxymethylase.

		Activity and % Inhibition					
		initial[2]		after 10 hr		after 24 hr	
Enzyme	Inhibitor[1]	act.	inhib.	act.	inhib.	act.	inhib.
crude	none	7.94	-	6.83	-	3.68	-
	L-met	6.44	19	5.60	18	3.28	11
	S-AM	4.52	43	4.16	39	2.94	20
purified	none	5.62	-	2.04	-	0	-
	L-met	5.05	10	1.87	8	0	-
	S-AM	3.86	31	1.31	27	0	-

[1]Both S-adenosylmethionine and methionine were present in 1 mM concentrations.

[2]The crude and purified preparations which had been stored in 30% (v/v) glycerol, were passed through a small G-25 Sephadex column just prior to the initial activity determinations.

Various forms of the purines and thymine were tested to determine whether they had any effect on serine transhydroxymethylase activity. No significant inhibition of activity was shown by guanine, guanosine, thymine, thymidine, adenine, adenosine, or adenosine di- or tri- phosphates.

Serine transhydroxymethylase was purified from cultures grown in various supplemented media to determine if any qualitative changes had occurred in the properties of the enzyme. The supplements included (1) 10 mM methionine, (2) 10 mM glycine, (3) 10 mM serine,

(4) 2 mM each of adenosine, guanosine, and thymidine, and (5) 2 mM each of methionine, adenosine, guanosine and thymidine. In each experiment, cells were grown as described in the Methods section. The purified enzymes did not differ in K_ms with respect to the reaction components, sensitivity to methionine and S-adenosylmethionine, pH optimum, or cation stimulation. As had been previously reported (Botsford and Parks, 1969), culturing of cells in glycine resulted in a three to four fold increase in the level of serine transhydroxymethylase.

The effects of methionine and glycine on the levels of serine transhydroxymethylase in two methionine auxotrophs are shown in Table 7. Both mutants, 80BM1 and 22B are defective at some step in the methyl transfer pathway of methionine biosynthesis, though the actual blocked step has not been elucidated. Cells were grown in tryptone broth (TDY) for 18 hours, harvested, and then aerated for seven hours in Wickerham's complete medium less methionine (WCLM) with the indicated supplements. The levels of the enzyme were significantly higher in the two auxotrophs as compared to the prototrophic strain. Serine transhydroxymethylase was also purified from one of these mutants, 80BM1. The enzyme exhibited the same properties as the enzyme purified from the prototrophic strain, 3701B.

Table 7. Effect of glycine and methionine on the levels of serine transhydroxymethylase in methionine auxotrophs.

Strain	Specific Activity, units/mg		
	none	methionine	glycine
3701B (met^+)	.38	.23	1.04
22B (met^-)	1.22	1.12	1.31
80BM1 (met^-)	.89	.52	1.32

The glycine and methionine supplements were present in 10 mM concentrations.

Activity was determined in crude extracts which had been desalted on a G-25 column prior to assaying.

DISCUSSION

Results of the sulfate uptake experiments and the incorporation of sulfate into protein indicate that controls on the synthesis of methionine by Saccharomyces cerevisiae are quite rigorous. Previous work on sulfate incorporation into S-adenosylmethionine indicated that methionine synthesis was only slightly repressed in the presence of exogenous methionine (Schlenk and Zydek, 1967). The degree of repression was, in fact, comparable to the repression reported for serine transhydroxymethylase by Botsford and Parks (1969). Since over 95% of a cell's sulfur content is located in methionine and cysteine, the degree of uptake was taken as an indication of in vivo synthesis of these amino acids. The results suggested that in the presence of an exogenous supply of methionine, virtually no biosynthesis of methionine occurred and this was also reflected in the degree of sulfate incorporation into protein, which was decreased by more than 99% when cells were grown in the presence of methionine. Since the cysteine content of yeast protein is 10-20% that of methionine, the low degree of incorporation of sulfate into protein in the presence of methionine indicate that methionine also decreases sulfate incorporation into cysteine, though the low degree of incorporation prevented a precise determination of the extent of this decrease. Two biosynthetic pathways for cysteine have been proposed, though in both

pathways, serine provides the three carbon moiety of cysteine. One route involves the direct synthesis of cysteine from serine and sulfide catalyzed by serine sulfhydrase, an enzyme which has been isolated from yeast (Schlossman and Lynen, 1957). An alternative route derives the sulfur group from methionine through the intermediates, homocysteine and cystathionine. This pathway, referred to as reverse transsulfurization, has also been demonstrated in yeast (Delavier-Kluchko and Flavin, 1965). The results of the sulfate incorporation into protein in the presence of exogenous methionine when coupled with the fact that all cysteine mutants of yeast thus far isolated can satisfy their requirement with methionine indicate a direct role for methionine in the synthesis of cysteine. The results do not give any indication as to the pathway of sulfur from methionine to cysteine nor do they rule out the direct synthesis of cysteine from serine and sulfide as a physiologically important route.

The apparent strict control on methionine biosynthesis is consistent with controls previously reported for several of the enzymes involved in two of the pathways of methionine biosynthesis (sulfur assimilation and homocysteine synthesis). The first enzyme in the sulfur pathway, ATP sulfurylase, is strongly repressed by exogenous methionine (de Vito and Dreyfuss, 1964). Several enzymes involved in the homocysteine pathway, in particular homoserine dehydrogenase and homocysteine synthetase are also repressed by methionine with

the latter enzyme being sensitive to methionine inhibition as well. (Cherest et al., 1969).

Less is known of the controls in the pathway leading to the methyl group of methionine. One obvious point of departure into this pathway was the first enzyme, serine transhydroxymethylase. Several possible assays were available for the enzyme, providing for the measurement of the reaction in both directions. The assay of Taylor and Weissbach (1965) was chosen for its sensitivity, relative ease of operation, and because it measured the reaction in the direction providing the methyl group for methionine synthesis. Preliminary investigation indicated that the enzyme was significantly repressed by methionine (Spence, 1965). Subsequent studies by Botsford and Parks (1969), which have been corroborated in this thesis, showed that the maximum degree of repression was only 10-20%. This is indeed small when compared to the 100% repression by methionine of ATP sulfurylase (de Vito and Dreyfuss, 1964) or the 70% repression of homoserine-O-transacetylase (de Robichon-Szulmajster and Cherest, 1967). A more obvious comparison seemed to be with aspartokinase, the first enzyme in the methionine-threonine portion of the pathway leading to homoserine. This enzyme is only slightly repressed by methionine while showing strong repression by the second end product, threonine (de Robichon-Szulmajster et al., 1971; Stadtman et al., 1961). Serine transhydroxymethylase is also involved in multiple pathways.

Serine, through the serine transhydroxymethylase reaction, appears to be a principal source of one carbon folate derivatives (Mudd and Cantoni, 1964). Thus the enzyme becomes involved in the biosynthesis of the purines and thymine. The purines and thymine when present as supplements in the medium demonstrated no repressive effects on the synthesis of serine transhydroxymethylase. The possibility of a coordinate control on the enzyme by its various end products was also investigated. The purines and thymine, even in combination with methionine, showed no strong repressive effect on the enzyme.

Serine transhydroxymethylase activity is inhibited by methionine, but the maximal inhibition appears to be less than 35%. Purines and thymine do not affect the reaction, either alone or in combination with methionine. S-adenosylmethionine inhibits the reaction to a greater extent than methionine, but spectral data, shown in Figure 9, suggest that this may be partly due to interference with the pyridoxal phosphate binding with the enzyme. These data do not preclude a function of S-adenosylmethionine as a regulatory inhibitor since the decrease in the 440 nm peak does not account for all the inhibition observed in the in vitro situation. In serine transhydroxymethylase isolated from rabbit liver, it has been demonstrated that the decrease in the enzyme-bound pyridoxal phosphate peak caused by cysteine shows a direct relationship with loss of activity (Schirch and Mason, 1962). Inhibition of several pyridoxal phosphate enzymes by

homocysteine has been shown to be due to reaction of pyridoxal phosphate with homocysteine to form a compound devoid of coenzyme activity and that the inhibition is non-competitive with respect to the substrate (Pestaña et al., 1971). On the other hand, the inhibition of serine transhydroxymethylase by S-adenosylmethionine has been previously shown to be competitive with respect to the substrate, serine (Botsford and Parks, 1967). Methionine had no observable effect on the absorption spectrum of the enzyme.

A third approach to the question of control at the step of serine transhydroxymethylase was studied. Since the enzyme is involved in methionine, purine and thymine biosynthesis there was the possibility of isoenzymic forms of serine transhydroxymethylase existing in the cell, each controlled by its respective end product. To this end, a purification scheme for the enzyme was developed. If multiple forms of the enzyme were present, the possibility existed that they could be separated by normal enzyme purification procedures. In the event that this could not be demonstrated, the purification would also enable study of any qualitative changes in the properties of the enzyme from cells grown in various supplements. However, numerous purification techniques failed to show multiple forms of the enzymes.

Several properties of the purified enzyme were determined. Apparent Michaelis constants were determined for the various components of the reaction, including serine, pyridoxal phosphate and

tetrahydrofolic acid. The alkaline pH for optimal activity was found to have a much broader range than had previously been reported (Botsford and Parks, 1969). Molecular weight studies on both crude and purified enzyme show it to be large, in the area of 200,000. Molecular weights reported for this enzyme in rabbit liver vary from 185,000 (Fujioka, 1969) to 331,000 (Schirch and Mason, 1963). A previously unreported cation activation of the enzyme was demonstrated. Metal cation requirements are generally associated with non-enzymatic pyridoxal-dependent reactions. However, since the cations showed no activity in the reaction mixture in the absence of serine transhydroxymethylase, the stimulatory effect is presumed to be enzymatic.

The absorption spectrum of serine transhydroxymethylase purified in the absence of pyridoxal phosphate exhibits an absorption maximum only at 280 nm and the enzyme shows no activity if pyridoxal phosphate is not added to the reaction mixture. Incubation of the enzyme with pyridoxal phosphate produces a second peak at 440 nm indicative of an enzyme-bound pyridoxal phosphate. Purified serine transhydroxymethylase from rabbit liver exhibits absorption maxima at 280 and 430 nm. The 430 nm peak has been demonstrated to be due to the attachment of pyridoxal phosphate to a lysine residue of the enzyme, presumably through a Schiff base formation between the cofactor and amino acid (Schirch and Mason, 1963). The cofactor is dissociable from yeast serine transhydroxymethylase by normal purification

procedures, while the enzyme from rabbit liver requires incubation with cysteine followed by dialysis to remove the pyridoxal phosphate (Schirch and Mason, 1962). The cofactor is even more easily dissociable from the enzyme from Clostridium cylindrosporum, since even crude extracts require added pyridoxal phosphate for significant activity (Uyeda and Rabinowitz, 1968).

Serine transhydroxymethylase was purified from cells grown in minimal media and minimal media supplemented with (1) methionine, (2) serine, (3) glycine, (4) adenosine, guanosine and thymidine, or (6) methionine, adenosine, guanosine, and thymidine. As had been previously reported by Botsford and Parks (1969), glycine supplemented media resulted in a 2-3 fold increase in the levels of serine transhydroxymethylase. Serine caused a slight increase in the level of enzyme while the purines and thymidine had no effect. Properties of the purified enzyme isolated from the cultures grown in the various media were found not to vary. The K_m values for serine, pyridoxal phosphate, and tetrahydrofolic acid were comparable. Sensitivity to feedback inhibition was identical, including the insensitivity to the purines and thymine. There was no observable differences in pH optima nor cation stimulation.

The heat inactivation profile for partially purified serine transhydroxymethylase in the absence of glycerol presented the only indications for the possibility of more than one form of the enzyme.

However, since the heat stable activity exhibited the same properties with respect to K_ms, sensitivity to feedback inhibition, pH optima, and molecular weight, it is questionable as to whether or not this represents a second isoenzyme of serine transhydroxymethylase. Purified enzyme did not exhibit this heat stable fraction. Some component of the crude preparation, not necessarily protein, may be exerting a partial protective effect. Furthermore the ratio of heat stable to heat unstable activity did not vary in cells grown in the variously supplemented media described above.

The results of the levels of serine transhydroxymethylase in methionine auxotrophs, when compared with the levels in the prototroph, suggest that even in the absence of methionine, the capacity for the production of the enzyme is not fully expressed by the prototrophic strain. Since the enzyme purified from the methionine mutant 80BM1 exhibits the same properties as the enzyme from the prototroph, the increased levels of activity in these mutants are not due to an altered serine transhydroxymethylase. Furthermore, since both presumably different mutants showed the increased levels of the enzyme, this suggests that the higher levels are not due to a defective control on synthesis. It has previously been suggested that the increase levels caused by glycine may be due to an inducible biosynthetic system, since serine may be derived from glycine by the serine transhydroxymethylase reaction (Botsford and Parks, 1969). Such inducible

biosynthetic pathways where the immediate precursor induces the synthesis of the subsequent enzyme, have been reported in the uracil biosynthesis in yeast (Lacroute, 1968) and in leucine biosynthesis in Neurospora (Gross, 1965). However, the levels of serine transhydroxymethylase in the methionine mutants present the possibility that the increased levels may be due to a derepression mechanism.

CONCLUSIONS

Evidence presented in this work suggest a strict control of methionine biosynthesis in yeast by exogenous methionine. This conclusion seems consistent with the repressive controls previously demonstrated on key enzymes involved in the synthesis of the four-carbon moiety of methionine as well as the first enzyme in the pathway of sulfur assimilation.

The controls operating in the pathway of methyl group synthesis have proven more difficult to elucidate. The absence of strong repressive controls on serine transhydroxymethylase by methionine may be reflective of its involvement in several biosynthetic pathways. Any control system on the first enzyme of a pathway encounters an obvious problem when this pathway leads to several end products. Without some form of independent control, the high level of one end product could cause a deficiency in the others. Several possible means of such a control have been investigated at the step of serine transhydroxymethylase.

The enzyme has been previously shown to be inhibited by methionine but the maximum degree of inhibition appears to be less than 35%. None of the other presumed end products which share the serine transhydroxymethylase step, have been shown to inhibit the enzyme, nor do they enhance the inhibition demonstrated by methionine.

Cells have been cultured in various media containing the different end products and their effects on the enzyme determined with respect to both quantity of enzyme produced and properties of the enzyme. Special attention was given to any changes which might have taken place in the sensitivity to inhibition by the various end products. The properties of the enzyme, as well as their levels, were found not to vary to any significant degree. Furthermore, no evidence for isoenzymes has been provided by the isolation procedure for serine transhydroxymethylase.

Thus far all evidence indicated that serine transhydroxymethylase does not play a major role in the control of methionine biosynthesis. It has been shown that the controls on the synthesis of methionine appear quite rigid and that while methionine has a limited effect on activity of serine transhydroxymethylase, its effect on synthesis of that enzyme is minimal. A methionine mutant which had previously been reported to be devoid of serine transhydroxymethylase has been found to contain activity. Screening of a number of mutants presumably defective in the methyl transfer pathway, have also failed to produce a mutant lacking serine transhydroxymethylase. These results may be accountable if one assumes that a single serine transhydroxymethylase provides the methyl group not only to methionine, but to the purines and thymine as well. The apparent lack of independent controls at this step implies that if such controls do exist, they would be

required at subsequent steps in the respective pathways. A prime candidate for control in the pathway of methyl group synthesis for methionine, might be the second enzyme in the pathway. This enzyme, methylene-tetrahydrofolate reductase, is the first enzyme of the methionine specific portion of the pathway and catalyzes the irreversible conversion of methylenetetrahydrofolic acid to methyltetrahydrofolic acid. Studies on this enzyme will have to await development of a suitable assay.

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