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Title: METHIONINE BIOSYNTHESIS IN SACCHAROMYCES CEREVISIAE: THE ONE-CARBON TRANSFER PORTION OF THE PATHWAY

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The folate mediated one-carbon transfer is responsible for the generation of the methyl group of methionine, and is also involved in thymidylic acid and purine biosynthesis. The understanding of the mechanisms and controls involved in one-carbon metabolism is incomplete, and is complicated by the existence of conjugated folate cofactors containing up to seven glutamic acid residues. The information that is available about the one-carbon transfer has been obtained using mainly the monoglutamyl tetrahydrofolate forms. Since the physiological balance of the conjugated derivatives could be critical, we preferred to utilize boiled cell extracts as a source of the cofactors.

In this investigation, we have examined the one-carbon transfer in the unicellular eucaryote Saccharomyces cerevisiae. The

generation of the methyl group of methionine (requiring the involvement of a polyglutamyl cofactor) was analyzed, and its relationship to thymidylic acid biosynthesis was explored. It appears that thymidylate synthetase may compete for polyglutamyl cofactors, and that the cellular dUMP concentration could influence methionine biosynthesis. It was also established that an interaction between S-adenosylmethionine and pyridoxal phosphate which can inhibit serine transhydroxymethylase, may cause growth inhibition of yeast when the cells are cultured in excess methionine.

Methionine Biosynthesis in *Saccharomyces cerevisiae*: The One-Carbon Transfer Portion of the Pathway

by

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Typed by Velda D. Mullins for Ronny William Trewyn

To my father, Harold W. Trewyn.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

METHIONINE BIOSYNTHESIS IN SACCHAROMYCES CEREVISIAE: THE ONE-CARBON TRANSFER PORTION OF THE PATHWAY

INTRODUCTION

Methionine is unique among the amino acids in the variety of biological reactions in which it is involved. Like the other amino acids, methionine has a role in protein synthesis. However, it serves not only a typical amino acid function within proteins, it is also the N-terminal amino acid in protein synthesis. In procaryotes and the mitochondria of eucaryotes, this chain-initiation function is fulfilled by formylated methionyl transfer RNA, while in the cytoplasm of eucaryotes, an unformylated species is utilized.

Methionine is the methyl group donor (via S-adenosylmethionine) for numerous cellular transmethylation reactions. These reactions can occur at the macromolecular level, e.g., transmethylations of DNA, RNA, protein, and polysaccharides, or they can occur at the micromolecular level, e.g., transmethylations of fatty acids, sterols, biogenic amines, and other biosynthetic intermediates. The physiological significance of the transmethylation is often obscure, but it has been implicated in such diverse phenomena as DNA replication, control of translation, and respiratory competency.

Since the cell is dependent upon a continuing supply of methyl groups, it is important to understand those factors affecting methyl group synthesis and transfer. Significantly, S-adenosylmethionine inhibits the enzyme serine transhydroxymethylase which is the reputed source of methyl groups for methionine biosynthesis. Other cellular functions may compete with methionine formation for one-carbon units generated by serine transhydroxymethylase. These include thymidylic acid and purine biosyntheses. In the latter case, two one-carbon units are required per purine molecule formed.

The importance of regulating the flow of one-carbon units is obvious, since nucleotide and methionine biosyntheses are vital cellular processes. In addition, the necessity for strict control of methionine biosynthesis is indicated by the detrimental effects an excess of the amino acid can have on biological systems. Partial retardation of growth is a well-known phenomenon with yeast cultured in high levels of methionine, and toxic effects have been reported for methionine when fed in high levels to various higher organisms.

Investigating the one-carbon transfer in the unicellular eucaryote Saccharomyces cerevisiae offers many experimental advantages, since this organism has been studied genetically and physiologically more than perhaps any other eucaryote. Thus, extensive information is available for comparative purposes.

Defined cultural conditions can be utilized and maintained. Nutritionally deficient mutants are obtained readily, and specific hereditary aberrations can be combined in individual cells by simple genetic manipulations.

REVIEW OF LITERATURE

Methionine is one of the 20 amino acids commonly observed as a structural unit of proteins. After aminoacylation of the appropriate transfer RNA (tRNA), methionine also acts in initiating protein synthesis. This occurs as N-formyl-methionyl-tRNA in procaryotes and the mitochondria of eucaryotes (Smith and Marcher, 1968; Takeda and Webster, 1968), and as methionyl-tRNA in the cytoplasm of eucaryotes (Prichard et al., 1970; Smith and Marcher, 1970).

Methionine is converted to S-adenosylmethionine (S-AM) by the enzyme methionine adenosyltransferase (EC 2.5.1.6) as depicted in Figure 1 (Cantoni and Durell, 1957). The sulfonium compound can then donate the methyl group in numerous cellular transmethylation reactions. These include transmethylations of DNA (Gold et al., 1963), RNA (Borek and Srinivasan, 1965), fatty acids (O'Leary, 1962), and sterols (Parks, 1958). At least 40 distinct enzymatic transmethylations involving S-AM were identified almost 10 years ago (Cantoni, 1965), and the list has grown substantially since that time.

With the variety of reactions for which methionine is required, the necessity for a sizable cellular supply seems likely. However, excess methionine can have detrimental effects on biological

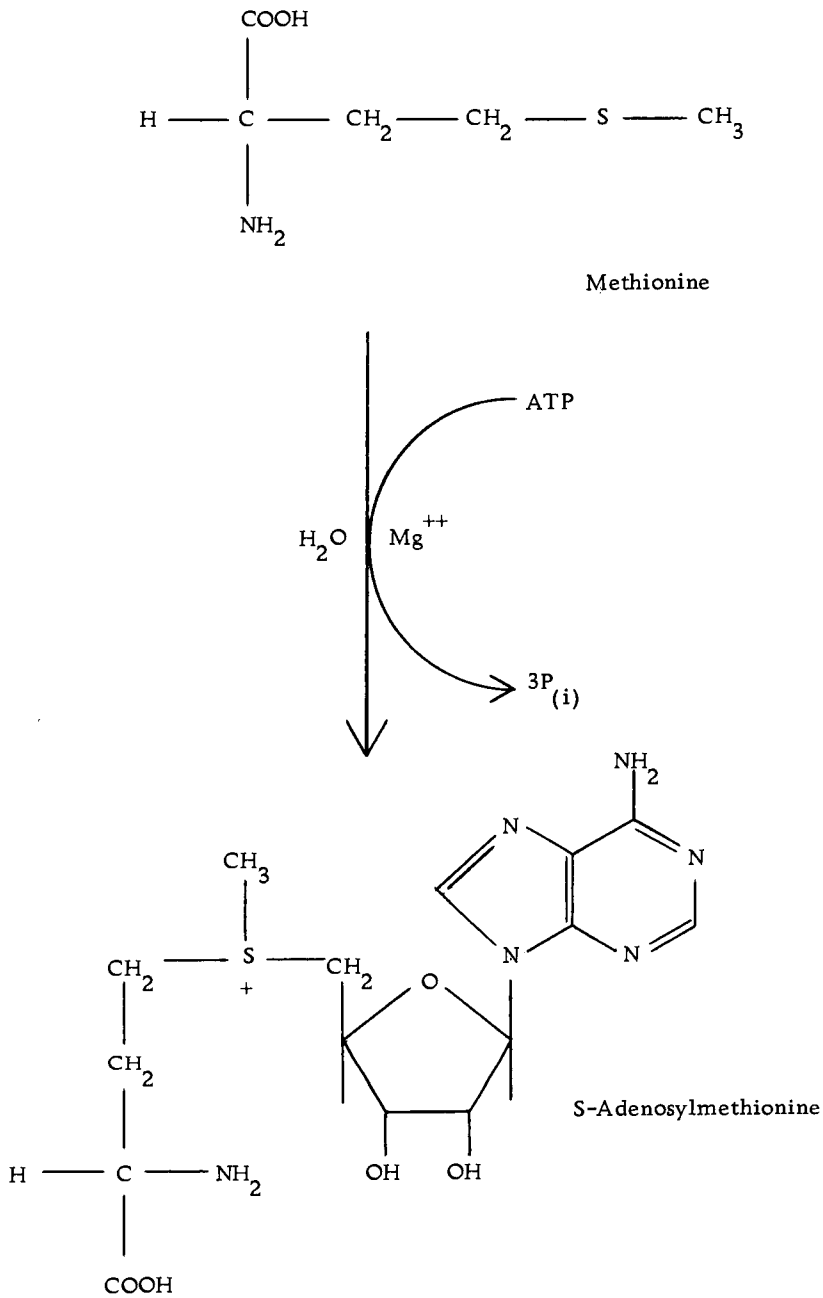
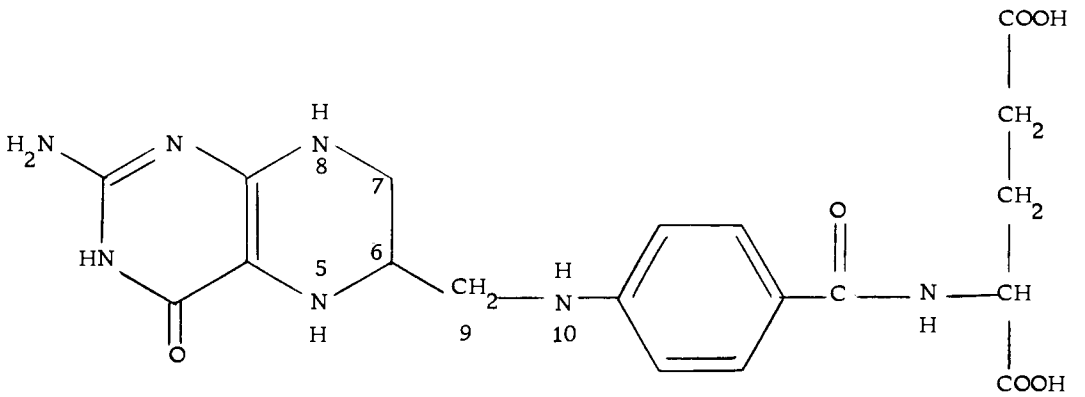


Figure 1. Conversion of methionine to S-adenosylmethionine.

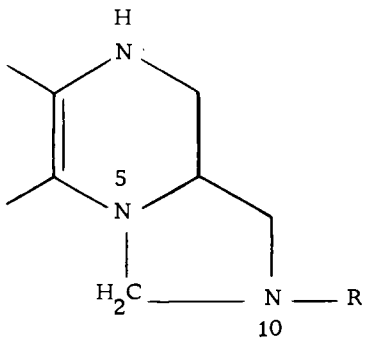
systems. The growth of yeasts is retarded if they are cultured in high levels of methionine (Schlenk and DePalma, 1957a and b), and toxic effects on rats have been reported with excess dietary methionine (Girard-Globa et al., 1972). Therefore, it is recognizable that the uncontrolled, overproduction of methionine by cells could be potentially harmful.

The multiple cellular requirements for methionine and the potential detrimental effects if it were overproduced, suggest that strict regulation must be exerted in the formation of this amino acid. The controls over the biosynthesis of the four-carbon and sulfur moieties of methionine have been examined extensively, and many of the enzymes in these two converging pathways have been shown to be subject to feedback inhibition and repression (Cherest et al., 1969; de Robichon-Szulmajster and Cherest, 1967; Wiebers and Garner, 1967). These individual controls appear to be relatively strict when examined in vitro.

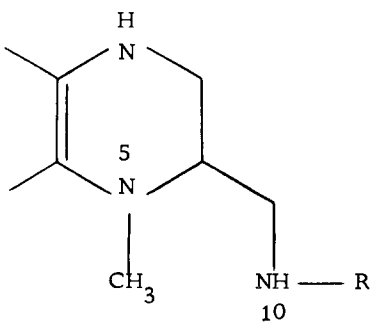
The information available about the mechanism and control of the one-carbon transfer portion of the methionine biosynthetic pathway has come from a variety of procaryotic and eucaryotic systems. In all cases, the pathway appears to utilize derivatives of tetrahydrofolic acid as carriers of the one-carbon unit, and the cofactor and intermediates are depicted in Figure 2. It has also been established that the beta-carbon of serine can be utilized for



Tetrahydrofolic acid (FH_4)



N^5, N^{10} - methylene FH_4



N^5 - methyl FH_4

Figure 2. Tetrahydrofolic acid (FH_4) and the one-carbon transfer derivatives.

the de novo synthesis of the methyl group of methionine (Botsford and Parks, 1967; Cohn et al., 1953; Rowbury and Woods, 1961). The relationships among the various one-carbon units as related to serine are shown in Figure 3. It should be emphasized, however, that the mechanism and control of the one-carbon transfer are not identical in all systems, and that the understanding of the pathway is far from complete.

Methylene tetrahydrofolate can be generated by serine transhydroxymethylase as depicted in Figure 3, and it can be obtained from formate and formaldehyde (Mudd and Cantoni, 1964) through reduction of the one-carbon unit on tetrahydrofolate coenzymes. It has been observed with Saccharomyces cerevisiae that feeding (^{14}C)-formate to methionine-grown cells results in 99% of the radioactivity in S-AM being in the adenosyl moiety, whereas when the cells are grown without methionine, 23% is in the adenosyl moiety and 77% is in the methyl moiety (Lor and Cossins, 1972). These results suggest that the formate carbon can be utilized as the one-carbon unit in methionine and purine biosyntheses, but the results for the cells grown without exogenous methionine indicate that there may be unequal utilization of formate by the two pathways. Of course, these results do not establish the in vivo contribution of formate or serine in the absence of an exogenous source, and the mechanism is further complicated by the fact that methylene

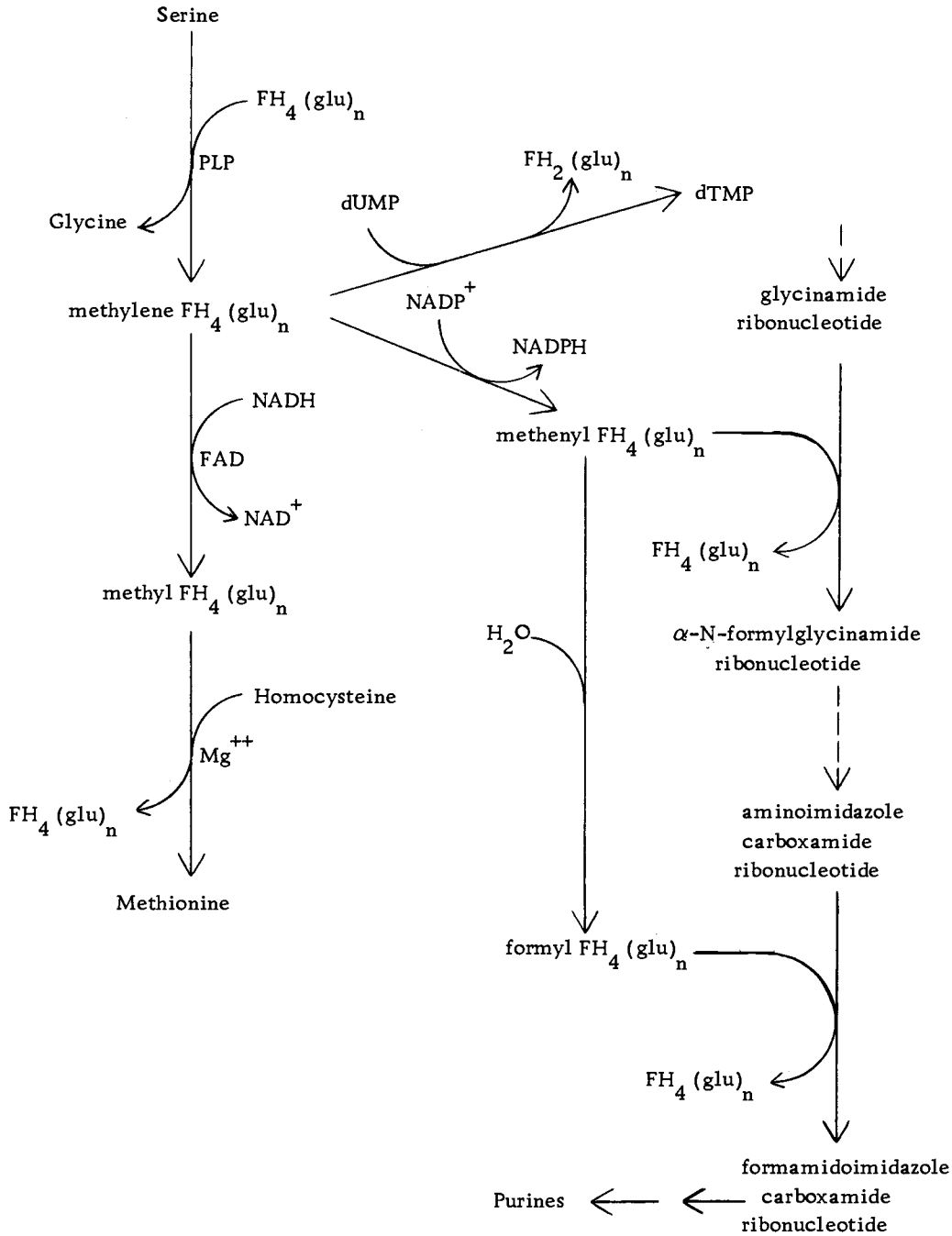
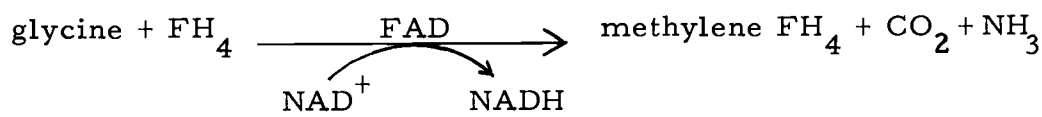


Figure 3. Generation of one-carbon units from the β-carbon of serine for methionine, thymidylic acid, and purine biosynthesis. Tetrahydrofolic acid is abbreviated $FH_4(glu)_n$ because the number of glutamic acid residues attached in γ-linkages may be variable.

tetrahydrofolate can be generated in some organisms by the following reaction (Robinson et al., 1973):



This reaction, catalyzed by glycine decarboxylase, has not been shown to have a role in methionine or nucleotide biosyntheses, but the possibility cannot be ruled out.

Much information is available about individual enzymes in the one-carbon transfer for methionine synthesis. In its simplest form, a mechanism encompassing three enzymatic reactions has been proposed (Mansouri et al., 1972). These enzymes are serine transhydroxymethylase (EC 2.1.2.1), methylene tetrahydrofolate reductase (EC 1.1.1.68), and methyl tetrahydrofolate: homocysteine methyltransferase.

Serine transhydroxymethylase has been purified and characterized from a variety of sources. The enzyme from Escherichia coli has a molecular weight of 170,000, and it exhibits little or no susceptibility to feedback inhibition when tested with methionine or S-AM (Mansouri et al., 1972). However the enzyme's synthesis can be repressed as much as 18-fold with methionine in the growth medium. The enzyme from S. cerevisiae is slightly larger with a molecular weight of 180,000 to 200,000 (Nakamura

et al., 1973), and it can be feedback inhibited by methionine and S-AM (Botsford and Parks, 1969). However, the inhibition by S-AM is somewhat unusual, in that it appears to be caused by an interaction with the enzyme's cofactor, pyridoxal phosphate (PLP) (Nakamura et al., 1973). No repression has been observed by methionine, thymine, or the purines, but a substantial derepression (induction) of enzyme synthesis occurs when the growth medium is supplemented with glycine (Botsford and Parks, 1969).

A cytoplasmic and a mitochondrial serine transhydroxymethylase have been purified from rabbit liver. They were found to be different immunochemically and in molecular weight (Fujioka, 1969). Most of the work with the rabbit liver system was done without distinguishing between the two enzymes, and under these circumstances, serine transhydroxymethylase was shown to be feedback inhibited by tetrahydrofolate coenzymes (Schirch and Mason, 1963), thymidine, and guanosine (Taylor et al., 1966). No repression has been observed. An additional finding in rabbit liver is that the cytoplasmic enzyme may also catalyze the following reaction (Akhtar and El-Obeid, 1972):

threonine (and/or allothreonine) $\rightleftharpoons$ glycine + acetaldehyde

A similar observation has been made with rat liver, where a cytoplasmic serine transhydroxymethylase was reported to be identical with allothreonine aldolase and aminomalonate

decarboxylase (Palekar et al., 1973). A mitochondrial serine transhydroxymethylase was also found in rat liver, but it did not contain the aldolase or decarboxylase activity. The significance with regard to the one-carbon transfer of two serine transhydroxymethylase enzymes with different subcellular locations and substrate specificities has not been established. It is noteworthy, however, that glycine is one of the end-products of each of the known reactions, and considering the derepressive effect of glycine in S. cerevisiae (Botsford and Parks, 1969), it could be that the reverse reaction can serve an important biosynthetic role.

Methylene tetrahydrofolate reductase may be a key control point in the one-carbon transfer. Although no repression can be demonstrated for this enzyme from rat liver, it can be inhibited in vitro as much as 75% by S-AM (Kutzbach and Stokstad, 1967 and 1971). Methionine does not inhibit the enzyme in vitro, but excess dietary methionine causes a large reduction in the in vivo concentration of the reaction's end-product methyl tetrahydrofolate (Noronha and Silverman, 1962). Since the S-AM concentration in liver increases substantially when excess methionine is fed to rats (Baldessarini, 1966), it seems likely that S-AM can feedback inhibit methylene tetrahydrofolate reductase in vivo as well as in vitro.

Methylene tetrahydrofolate reductase from yeast may be regulated in a manner similar to that in rabbit liver. In this case,

however, the enzyme has been reported to be feedback inhibited by methionine and not S-AM (Combepine et al., 1971; Lor and Cossins, 1972). As with the rabbit liver enzyme, the maximum inhibition appeared to be approximately 75%, and no repression was observed.

The reductase in E. coli is not subject to feedback inhibition, but it is subject to almost complete repression, when high levels of methionine are present in the growth medium (Dickerman and Weissbach, 1964). However, the enzyme can exhibit an unusual response to low levels of the amino acid. With a methionine-vitamin B_{12} auxotroph of strain K_{12}, it was observed that low levels of methionine (in a medium containing sufficient cyano-B_{12} for growth) actually stimulated (derepressed) methylene tetrahydrofolate reductase synthesis to levels vastly higher than if no methionine were present (Dickerman and Weissbach, 1964). The increase in enzyme activity was associated with growth inhibition, but the inhibition could be overcome by adding guanosine and uridine to the medium prior to the second cell division. Therefore, if proper controls are not exerted at this critical juncture, the ensuing disruption of the normal flow of one-carbon units can have a detrimental effect on cell growth.

The cofactor requirements for the terminal enzyme in the pathway, methyl tetrahydrofolate: homocysteine methyltransferase, can vary between organisms and within the same organism. E. coli

has two enzymes that can catalyze the transfer of the methyl group to homocysteine. One of these enzymes requires vitamin B_{12}, a monoglutamyl form of methyl tetrahydrofolate, and catalytic amounts of S-AM, while the other enzyme requires only a triglutamyl form of methyl tetrahydrofolate (Taylor and Weissbach, 1969; Whitfield and Weissbach, 1968). The synthesis of both enzymes can be repressed by methionine, as can the other enzymes in the pathway in E. coli (Kung et al., 1972). In addition, vitamin B_{12} can repress the synthesis of the non-B_{12} transmethylase and methylene tetrahydrofolate reductase by a mechanism distinct from that of methionine repression (Dawes and Foster, 1971; Kung et al., 1972; Milner et al., 1969). The total repressive controls over the transmethylases do not appear to be of the degree, however, of those exerted over methylene tetrahydrofolate reductase.

In eucaryotic systems, only single enzymes have been reported that catalyze the terminal step in the one-carbon transfer, although the enzyme requirements can vary between systems. The transmethylase in hog liver contains a derivative of vitamin B_{12} as a cofactor or prosthetic group, requires catalytic amounts of S-AM, and utilizes the monoglutamyl form of methyl tetrahydrofolate (Buchanan, 1971). The enzyme in yeast is apparently of the non-B_{12} variety, and there is no requirement for S-AM (Botsford and Parks, 1967). In addition, this enzyme requires a

polyglutamyl form of the cofactor to methylate homocysteine efficiently (Botsford and Parks, 1967; Burton et al., 1969).

Whereas feedback inhibition and no repression have been reported for serine transhydroxymethylase and methylene tetrahydrofolate reductase from eucaryotes, the opposite is true for regulation of the transmethylase. The transmethylase in S. cerevisiae has been reported to be subject to repression of up to 65% when the growth medium is supplemented with methionine (Lor and Cossins, 1972). A similar degree of derepression has been reported for cultured baby hamster kidney cells when homocysteine was substituted for methionine in a growth medium containing B_{12} and folic acid (Kamely et al., 1973).

The requirement for a polyglutamyl cofactor by many of the methyl tetrahydrofolate: homocysteine methytransferases has been established by in vitro enzyme assays. However, in vivo evidence has been obtained with Neurospora crassa, where a methionine mutant was found to contain only monoglutamyl derivatives of tetrahydrofolate (Selhub et al., 1971). This polyglutamyl cofactor requirement for the terminal step in methionine biosynthesis introduces the question of how the one-carbon unit is added to the cofactor. The fungus Coprinus lagopus requires a triglutamyl form of the cofactor to methylate homocysteine, and it has been reported that the organism's serine transhydroxymethylase and methylene tetrahydrofolate reductase can utilize either

monoglutamyl or triglutamyl cofactors (Salem and Foster, 1970). Therefore, the generation of the one-carbon unit in C. lagopus appears to occur by similar means with both cofactors. In other organisms requiring a polyglutamyl cofactor, it has not been demonstrated that the first two enzymes in the pathway can utilize multiple forms of the cofactors, so other mechanisms cannot be discounted. It is possible that isozymes could exist, or the additional glutamyl residues could be added subsequent to the first two reactions.

Regulating the availability of the polyglutamyl forms of tetrahydrofolate could offer a means of controlling methionine biosynthesis, but very limited information is available about enzymes involved in the synthesis and degradation of these cofactors. An enzyme catalyzing the addition of γ-glutamyl residues to tetrahydrofolate has been reported in E. coli (Griffin and Brown, 1964). The reaction requires ATP, Mg^{++}, L-glutamic acid, and reduced folic acid, but little else is known about the mechanism or control of this reaction. Enzymes catalyzing the excision of glutamyl residues, γ-glutamyl carboxypeptidases, have been located in a variety of systems (Bird et al., 1946; Prescott and Affronti, 1968; Wolff et al., 1949), but as with the enzyme adding residues, little direct evidence is available about the control of these enzymes.

Evidence suggesting there is substantial control over the

synthesis and degradation of the polyglutamyl forms of the tetrahydrofolate coenzymes has been obtained. Growth of C. lagopus in minimal medium supplemented with methionine results in a large reduction in the cellular pool of conjugated forms of tetrahydrofolate, when compared to cells grown in an unsupplemented medium (Salem and Foster, 1971). The control of at least the γ-glutamyl carboxypeptidase must be by a mechanism other than repression in C. lagopus, since the cells grown with and without methionine both contain the active enzyme.

Little information is available about the relationship of methionine biosynthesis to thymidylic acid and purine biosynthesis, especially in cells requiring a polyglutamyl cofactor for methionine biosynthesis. In cells such as S. cerevisiae where a mixture of monoglutamyl and polyglutamyl forms of tetrahydrofolate are found (Lor and Cossins, 1972), it is possible that only the monoglutamyl derivatives are utilized in nucleotide biosynthesis. This would separate the two pathways, and it would not be necessary to expect regulatory elements in common in the one-carbon transfer to control the flow of one-carbon units to methionine and the nucleotides. However, it has been reported with C. lagopus that only polyglutamyl cofactors are found in fresh homogenates of cells grown in minimal medium without methionine (Salem and Foster, 1971). Unless thymidylic acid and the purines are synthesized by

some unknown mechanism (not requiring folate), this would indicate that their biosynthesis must involve polyglutamyl cofactors. However, the nucleotide biosynthetic enzymes have not been shown to utilize these cofactors. It would also be necessary for these enzymes in C. lagopus to use either monoglutamyl or triglutamyl cofactors (as with the methionine biosynthetic enzymes) or for isozymes to exist, since there is almost a complete loss of the triglutamyl forms when methionine is included in the growth medium (Salem and Foster, 1971).

An additional, potentially important aspect of the one-carbon transfer has been reported recently for mammalian and avian tissues (Banerjee and Snyder, 1973). It was observed that methyl tetrahydrofolate can mediate N-methylation and O-methylation of a variety of biogenic amines more efficiently than S-AM. It had been thought that S-AM was the universal methyl group donor in all such methylation reactions, but that thinking may have to be revised. Whether methyl tetrahydrofolate ultimately will be found to have a role in transmethylations in all systems remains to be seen, but the possibility must be considered when examining the mechanism and control of the one-carbon transfer. The findings (mentioned previously) of a maximum of 75% inhibition of methylene tetrahydrofolate reductase from rat liver and S. cerevisiae could indicate an in vivo requirement for methyl tetrahydrofolate to

carry out N- and O-methylations. This possibility has merit, since rat liver is one of the tissues reported to carry out such reactions with methyl tetrahydrofolate in vitro.

MATERIALS AND METHODS

Organisms

Haploid strains of Saccharomyces cerevisiae were utilized throughout the course of this investigation. These strains are described in Table 1. For most of the studies, strain 3701-B was utilized as the parental control. Strains 234-5.1 and 234-15.1 were obtained by ethyl methanesulfonate (EMS) mutagenesis of 3701-B, and they were found to be blocked in the one-carbon transfer.

Strain C 390-2B is a pleiotropic methionine mutant which became petite at the same time the methionine requirement arose. These markers segregate together.

When the effects of high intracellular S-adenosylmethionine (S-AM) concentrations were analyzed, strain 5036-D was utilized.

Growth Conditions

In the early portion of this investigation and when the methionine mutants were utilized, cells to be used for enzyme extracts were grown in yeast extract-peptone (YEP) broth. This medium contained 1% yeast extract, 2% peptone, and 2% dextrose. A 1% inoculum into 1 liter of YEP in a 2 liter flask was utilized, and

Table 1. Description of Saccharomyces cerevisiae strains utilized.

Strain Number	Mating Type	Nutritional Requirements	Respiratory Competency	Source of Strain
3701-B	a	uracil	grande	O. S. U. [a]
234-5.1	a	uracil, methionine	grande	EMS [b]
234-15.1	a	uracil, methionine	grande	EMS [b]
C390-2B	α	uracil, methionine adenine, leucine	petite	H. L. Roman [c]
5036-D	a	adenine, methionine histidine, tryptophan	grande	H. L. Roman [c]

[a] Strain 3701-B is from the Oregon State University active culture collection.

[b] Strains 234-5.1 and 234-15.1 were obtained by ethyl methanesulfonate (EMS) mutagenesis of strain 3701-B.

[c] Strains C390-2B and 5036-D were obtained from Dr. H. L. Roman, Department of Genetics, University of Washington.

growth was carried out at 30 C on a rotary shaker for 18-24 hours. Cells were then harvested by centrifugation, washed twice with distilled water, and resuspended in Wickerham's minimal (WM) medium plus uracil (Wickerham, 1946). The cells were then aerated an additional 4-6 hours. Later in the investigation, 3701-B was grown directly in WM + uracil (from a 1% inoculum in YEP) for 18-24 hours.

When respiratory growth was required, growth was carried out in YEP with subsequent aeration in WM + uracil as before, but with 3% ethanol replacing the dextrose in both media.

When high intracellular S-AM concentrations were desired, strain 5036-D was grown in Wickerham's complete (WC) medium (Wickerham, 1946) minus tyrosine and pyridoxine, and containing methionine at a concentration of 5.0 mg/ml. Cultures grown in the same medium, but containing methionine at a concentration of 0.03 mg/ml, were used as controls. Inoculations were made from stationary phase starter cultures that had been grown in the medium containing 0.03 mg methionine per ml. A 1% inoculum (into 1 liter of medium in a 2 liter flask) was used in all cases except when lag phase cells were to be harvested and then a 5% inoculum was used. Cells were grown at 30 C on a rotary shaker. For the determination of tyrosine aminotransferase levels throughout the culture cycle, cells were grown in a 10 liter carboy at 30 C with vigorous

aeration.

Growth was monitored by direct cell counts with a Model F Coulter Counter. Alternatively, cells were grown in 50 or 100 ml of WM or WC medium plus or minus appropriate supplements in 300 ml Bellco side-arm flasks, and growth was monitored with a Klett-Summerson Colorimeter.

Feeding experiments were run using WC agar (1.7% Noble agar) minus methionine. Cells were grown overnight in 10 ml of YEP broth, after which they were washed, and resuspended in sterile distilled water. A small volume of these cells was added to 20 ml of liquid WC agar maintained at 50 C, and the agar was poured into a sterile petri dish. After the agar solidified, sterile filter paper discs saturated with 0.01 M L-homocysteine, L-methionine, or S-adenosyl-L-methionine were placed on the agar. The plate was then incubated at 30 C for 1-4 days, and halos of growth appeared around the discs where feeding occurred.

Mutagenic Techniques

When 3701-B was to be treated with mutagen, it was grown on a shaker at 30 C in 10 ml of WM + uracil from a heavy inoculum. After 4-6 hours, 0.5 ml of ethyl methanesulfonate (EMS) was added, the tube was shaken vigorously to disperse the EMS, and the tube was placed on the shaker for 1 hour. The cells were then

harvested by centrifugation, and resuspended in 10 ml of WM + uracil and methionine. After an additional 12-18 hours on the shaker, serial dilutions were made, and cells were plated on YEP agar. The plates were incubated at 30 C for 3-4 days, after which they were replica plated to WC, WC - methionine, and YEP agar. Suspected methionine mutants were picked after incubating the plates for 2-3 days. Verification of the methionine requirement was made by restreaking the cells on WC and WC - methionine agar.

Genetic Complementation Analysis

Methionine mutants found to be blocked in the one-carbon transfer by the feeding experiments previously described were characterized further by genetic complementation analysis. Mutants of opposite mating type were grown overnight in YEP broth, and were streaked along two sides of a thin rectangle (15 x 40 cm) of YEP agar on a glass cover slip (30 x 50). The cover slip was placed on a dissecting chamber, and cells of opposite mating type were placed together with a Brower Micromanipulator. After positioning a number of pairs, the chamber was incubated at 30 C, and the pairs were examined periodically to determine if cell fusions resulted. The agar section was then placed on a YEP agar plate, and incubated at 30 C for 2-4 days. Zygotic progeny were then transferred to WC and WC - methionine agar to determine if

the mutations were complementary. Aseptic techniques were utilized with all procedures.

Preparation of Extracts

Washed cells were resuspended in 0.1 M phosphate buffer, pH 7.0, (1 ml/gram wet weight of cells), and broken in a Bronwill Tissue Homogenizer. The broken cells were centrifuged at 25,000 x g for 20 minutes. For crude cell extracts, the supernatant was dialyzed overnight against 2 liters of 0.01 M phosphate buffer, pH 7.0, with 3 changes of buffer. However, when tyrosine aminotransferase was to be assayed, a 4 to 5 ml portion of the 25,000 x g supernatant was removed and stored frozen for at least 48 hours before dialysis. Denatured protein was removed by centrifugation at 25,000 x g for 20 minutes.

Partial fractionation of the one-carbon transfer enzymes was accomplished using solid ammonium sulfate. The initial 25,000 x g supernatant was utilized, and the 0-35% and 35-60% ammonium sulfate precipitates were resuspended in 0.01 M phosphate buffer, pH 7.0. These fractions and the 60% supernatant were dialyzed as before.

Methyl tetrahydrofolate: homocysteine methyltransferase was partially purified by modification of the procedure of Burton and Sakami (1970). Instead of using commercial bakers' yeast,

3701-B was grown in WM + uracil, harvested, and broken as described above. The extract was then centrifuged at 8,000 x g for 20 minutes, and the precipitate was discarded. The ammonium sulfate precipitation (535 g/l), heat denaturation (55 C for 5 minutes), acid treatment (pH 4.5), and reprecipitation with ammonium sulfate (535 g/l) described by Burton and Sakami were then utilized. The final precipitate was resuspended, dialyzed overnight against 2 liters of 0.02 M phosphate buffer, pH 7.4, with two changes of buffer, and centrifuged at 25,000 x g for 20 minutes to remove denatured protein. The supernatant was utilized in the cell-free system.

For the preparation of boiled cell extracts, the cells were broken in 0.1 M phosphate buffer, pH 7.0, containing 0.001 M 2-mercaptoethanol, and the initial 25,000 x g supernatant was placed in a boiling water bath for 2 minutes. Denatured protein was removed by centrifugation, and the supernatant was replaced in the boiling water bath for an additional 2 minutes. After centrifuging again, the boiled extract was distributed in small vials and stored frozen.

Mitochondria were isolated from cells grown in the ethanol containing media described under Growth Conditions. Harvested cells were resuspended in 0.5 M sucrose in 0.1 M phosphate buffer, pH 7.0, and were broken as described above. Mitochondria

were obtained by centrifuging extracts at 2, 500 x g for 20 minutes, discarding the pellet, and centrifuging the supernatant at 25, 000 x g for 20 minutes. The mitochondria (pellet) were washed 2-3 times with the sucrose-phosphate buffer. The supernatant was centrifuged at 105, 000 x g for 60 minutes to insure removal of promitochondria, and the supernatant (S_{105}) was utilized for comparison with the mitochondria. Both procedures have been described previously (Thompson et al., 1974).

Protein was determined by the method of Lowry et al., (1951), using bovine serum albumin as the standard.

Spectrophotometric Analysis

Complex formation between PLP and S-AM was monitored with a Model 11 Cary recording spectrophotometer. To eliminate any pH change upon addition of S-AM, the system was buffered with 0.1 M phosphate buffer (pH 7.0).

Cell-Free Methionine Synthesis

The cell-free system for the synthesis of methionine from homocysteine and serine has been described (Botsford and Parks, 1967). The same components were utilized in this investigation, but the boiled extract was prepared as described above. In addition, controls minus homocysteine were used instead of "zero time"

controls, and the homocysteine was prepared from the thiolactone with 1 N NaOH as previously described (Killick, 1971). The methionine bioassay with Streptococcus faecalis 9790 was utilized to quantitate the methionine produced in 3 hours in the cell-free system.

Enzyme Assays

Serine transhydroxymethylase (EC 2.1.2.1) was assayed by the method of Taylor and Weissbach (1965) with the modifications of Nakamura et al. (1973).

Tyrosine aminotransferase (EC 2.5.1.5) was assayed by the method of Granner and Tomkins (1970) with the following modifications. A 2-3 minute preincubation period was utilized, and for the inhibition studies, the S-AM was not added to the "zero time" controls until immediately after a 12 minute reaction time. For the usual assay procedure, 0.06 ml of the 5 mM pyridoxal phosphate (PLP) solution was utilized instead of 0.01 ml. In all cases, the reaction was started by the addition of 0.05 ml of a diluted dialyzed crude extract. Spectrophotometric measurements were made with a PMQ II Zeiss spectrophotometer.

Ornithine aminotransferase (EC 2.6.1.13) was assayed by the method of Jenkins and Tsai (1970) with only two minor modifications. In the assay mixture, 30 mM phosphate buffer, pH 7.0,

was substituted for sodium pyrophosphate, and the reaction time was 45 minutes instead of 20. For ornithine aminotransferase from S. cerevisiae, the reaction rate was linear beyond 45 minutes.

Pyridoxal phosphokinase (EC 2.7.1.35) was assayed by the method of McCormick et al. (1961). The subsequent assay for pyridoxal phosphate (PLP) produced in the reaction mixture was carried out with tyrosine decarboxylase by the method of Chabner and Livingston (1970) with minor modifications. Folded filter paper squares saturated with phenethylamine (0.2 ml) were utilized in the well to collect CO_2. After complete evolution of CO_2, the filter paper was placed into 10 ml of Bray's scintillation cocktail (Bray, 1960) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. D, L-tyrosine-1-^{14}C was utilized in place of L-tyrosine-1-^{14}C, but it was diluted to the same specific activity with respect to L-tyrosine as recommended by Chabner and Livingston.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed by the method of Kudy and Noltmann (1966) with only minor modifications. The glycylglycine buffer was replaced with 0.1 M Tris-HCl buffer, pH 7.5, and the enzyme dilutions were made with the Tris buffer instead of EDTA.

S-Adenosylmethionine and Pyridoxal Phosphate Determinations

Cells were harvested by filtration after which they were extracted with perchloric acid by the method of Schlenk et al. (1965). In general, 5 ml of 1.5 N perchloric acid were added per gram (wet weight) of cells. Extraction was carried out at 25 C in a water bath shaker with constant agitation for 1 hour. This was followed by centrifugation at 25, 000 x g for 10 minutes.

S-AM concentrations were determined by the chromatographic procedure of Schlenk et al.(1965), and PLP concentrations were determined by the method of Chabner and Livingston (1970) already described. For the determination of PLP, dilutions of the perchloric acid extracts were made with 0. 1 M sodium acetate buffer, pH 5. 5.

Materials

S-AM (HSO_4) and S-adenosyl-L-homocysteine were obtained from Boehringer Mannheim. Tyrosine decarboxylase apoenzyme was purchased from Sigma Chemical Co. L-Ornithine·HCL was obtained from Calbiochem, and o-aminobenzaldehyde was obtained from K and K Laboratories. D, L-1-^{14}C Tyrosine was purchased from International Chemical and Nuclear Co. All other substrates and cofactors were obtained from Sigma Chemical Co.

RESULTS

Requirements for Cell-Free Methionine Synthesis

Verification of the requirements necessary for the transfer of the β-carbon of serine to homocysteine was undertaken. The effect on methionine biosynthesis of leaving any of the components out of the reaction mixture is shown in Table 2. That the monoglutamyl form of tetrahydrofolate (FH_4) will not substitute for the boiled cell extract was also verified, and more significantly, it is shown in Table 2 that the monoglutamyl derivative greatly inhibits the complete system. In addition, the possibility of glycine decarboxylase generating the one-carbon unit was tested by substituting glycine for serine. As can be seen, addition of glycine to the system resulted in no more methionine biosynthesis than if glycine and serine were left out.

Storing the Crude Cell Extract

It was reported by Nakamura *et al.* (1973) that storing serine transhydroxymethylase in 30% glycerol (v/v) greatly stabilizes this labile enzyme. To determine if this treatment would be effective with the enzymes involved in the complete one-carbon transfer system, dialyzed crude cell extracts were stored frozen with or

Table 2. Requirements for cell-free methionine synthesis.

Reaction Mixture	% Yield [a]
Complete [b]	100
- pyridoxal phosphate	53
- NAD	13
- FAD	10
- glucose-6-phosphate	36
- Mg^{++}	80
- serine	18
- serine + glycine [c]	16
- homocysteine	7
- boiled cell extract	34
- boiled cell extract + FH_4 [d]	16
- dialyzed crude cell extract	0
+ FH_4 [d]	46

[a] The % yield is with regard to the complete control, less the minus dialyzed crude cell extract value. In a typical complete system, 25-40 nmoles of methionine were synthesized.

[b] The complete reaction mixture was as described in the Materials and Methods.

[c] Glycine, when added, was present at a concentration of 10 mM.

[d] Tetrahydrofolic acid (FH_4), when added, was present at a concentration of 0.8 mM.

without 30% glycerol. As can be seen in Figure 4, storing the extract in glycerol resulted in almost total retention of activity for the duration tested, whereas a rapid loss of activity was observed initially for the extract without glycerol.

Methionine Mutant Analysis

S. cerevisiae methionine auxotrophs were analyzed to select those blocked in the one-carbon transfer portion of the pathway. Feeding experiments established a number of the mutants as having their nutritional requirement fulfilled by methionine or S-adenosylmethionine (S-AM), but not by homocysteine. Because homocysteine would not substitute for methionine, these mutants were presumed to be blocked in the one-carbon transfer.

The auxotrophs were characterized further by genetic complementation analysis. Using this technique, the mutants not utilizing homocysteine to satisfy their methionine requirement were found to fall into only two complementing groups. A limited number with low reversion frequencies were selected from each group for analysis in the cell-free system.

Crude cell extracts were prepared with each mutant, and methionine synthesis in the cell-free system with each extract was compared to that of 3701-B. Mutants 234-5.1 and 234-15.1 (one from each genetic complementary group) were selected for further

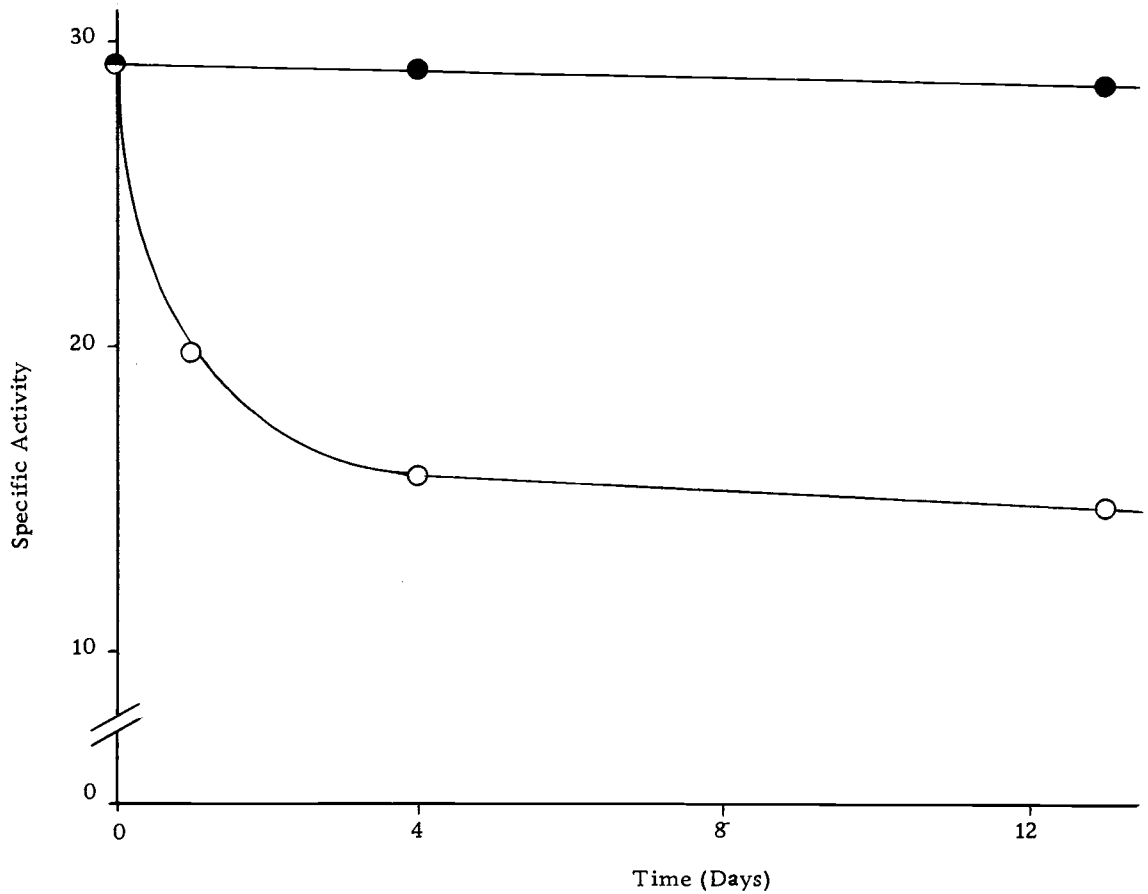


Figure 4. Storing dialyzed crude cell extracts at -10° C. ● With 30% (v/v) glycerol. ○ Without glycerol. Specific activity is expressed as nmoles methionine/mg protein produced in 3 hours.

study based on their low synthesis of methionine in the cell-free system, their low reversion frequencies, and their complete cessation of growth when transferred to medium not containing methionine. The mutants' synthesis of methionine in the cell-free system is compared to 3701-B in Table 3, and their growth when transferred to WC - methionine is compared to 3701-B in Figure 5. That the 234-5.1 and 234-15.1 mutations are complementary was verified by combining the mutants' crude extracts in the cell-free system, and this can also be seen in Table 3.

The pleiotropic methionine mutant C390-2B received from Dr. Roman was also examined. Although the mutant appeared to be blocked in the one-carbon transfer when the feeding experiment was carried out, extensive methionine synthesis was observed in the cell-free system (Table 3) when a C390-2B crude extract was used. That the in vitro synthesis was not caused because C390-2B is a leaky mutant was suggested by the complete termination of growth after transferring to WC - methionine (Figure 5).

Serine Transhydroxymethylase in 234-5.1 and 234-15.1

Serine transhydroxymethylase activity was measured with crude cell extracts of 234-5.1, 234-15.1, and 3701-B to determine if either of the methionine mutants was defective at this step in

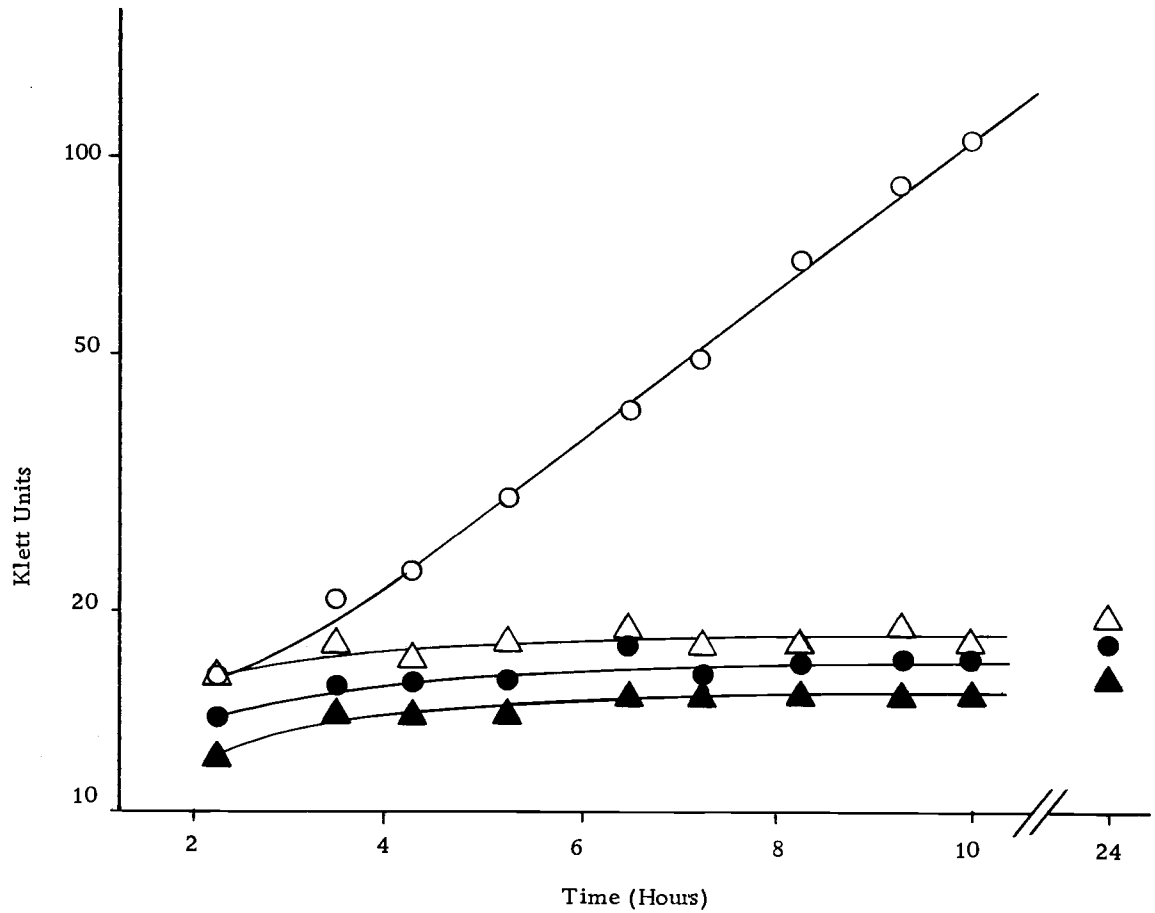


Figure 5. Growth of S. cerevisiae strains in WC - methionine. ○ 3701-B; ● C390-2B; ▲ 234.5.1; △ 245-15.1. A 5% inoculum from early stationary phase cultures in WC medium was utilized in each case. Growth was monitored with a Klett-Summerson Colorimeter.

Table 3. Methionine biosynthesis in the cell-free system with crude cell extracts of 3701-B, 234-5.1, 234-15.1, and C390-2B.

Enzyme Source	Enzyme Activity [a]	% of Control
3701-B (Control)	29	100
234-5.1	4	14
234-15.1	2	7
234-5.1 + 234-15.1	25	86
C390-2B	19	66

[a] Enzyme activity is expressed as nmoles methionine/mg protein produced in 3 hours.

Table 4. Serine transhydroxymethylase activity in crude cell extracts of 3701-B, 234-5.1, and 234-15.1.

Enzyme Source	Enzyme Activity [a]	% of Control
3701-B (Control)	5.0	100
234-5.1	4.3	86
234-15.1	2.3	45

[a] Enzyme activity is expressed as nmoles/min/mg protein.

the one-carbon transfer. As can be seen in Table 4, there was a reduction in serine transhydroxymethylase activity with both mutants, but even the 55% reduction with 234-15.1 is not of sufficient magnitude to account totally for the decrease in methionine biosynthesis in that strain.

Mutants' Boiled Extracts

The possibility of an accumulation of one-carbon intermediates in the mutants was tested by making boiled cell extracts of the mutant strains, and combining these with crude cell extracts in the cell-free system. As shown in Table 5, the 3701-B enzymes can utilize the boiled extracts from each of the strains, whereas the enzymes from the mutants 234-5.1 and 234-15.1 cannot utilize any of the boiled extracts.

The enzymes from the methionine mutant C390-2B can utilize the 3701-B and 234-5.1 boiled extracts to some extent, but cannot utilize its own (C390-2B) boiled extract at all. For some unknown reason, the C390-2B enzyme activity with 234-15.1 boiled extract was very low.

Table 5. Methionine biosynthesis in the cell-free system with boiled extracts from 3701-B, 234-5.1, 234-15.1, and C390-2B.

Enzyme Source	Boiled Extract Source [a]			
	3701-B (%)	234-5.1 (%)	234-15.1 (%)	C390-2B (%)
3701-B	100 [b]	116	69	73
234-5.1	3	0	2	1
234-15.1	2	0	0	0
C390-2B	31	42	8	0

[a] Boiled cell extracts were prepared from undialyzed crude cell extracts as described in the Materials and Methods.

[b] The 3701-B enzymes with the 3701-B boiled extract in the cell-free system was utilized as the control. The percentages are calculated on the nmoles methionine/mg protein produced in 3 hours in the cell-free system as compared to this control.

Synthesis of Polyglutamyl Cofactors

The most likely function of the boiled extract is to provide polyglutamyl forms of tetrahydrofolate for methionine synthesis in the cell-free system (Botsford and Parks, 1967). The *in vitro* synthesis of polyglutamyl cofactors with *Escherichia coli* extracts has been reported to occur in an ATP-dependent reaction (Griffin and Brown, 1964), so comparable conditions were employed in our experiments. However, including ATP, glutamic acid, and the monoglutamyl form of tetrahydrofolic acid would not replace

the boiled cell extract requirement in the cell-free system as can be seen in Table 6. Additional possibilities for glutamate addition were also tested. The glutamyl moiety of glutathione has been reported to be attached in a γ-linkage to numerous amino acids in mammalian systems (Tate et al., 1973), so glutathione and tetrahydrofolate were utilized in the methionine synthesizing reaction. It was also feasible that the polyglutamyl moiety could be synthesized independently, and then attached to tetrahydrofolate. This possibility was tested by including γ-glutamyl-glutamic acid in the system with tetrahydrofolate and ATP or glutathione. However, none of the combinations utilized would replace the boiled extract requirement as shown in Table 6.

Subcellular Location of the Methionine Biosynthetic Enzymes

The discovery of a cytoplasmic and mitochondrial serine transhydroxymethylase in mammalian systems (Fujioka, 1969) led to the possibility of two such enzymes existing in S. cerevisiae. The complete one-carbon transfer system was analyzed concurrently with serine transhydroxymethylase and the results are shown in Table 7. As can be seen, the methionine biosynthetic enzymes and serine transhydroxymethylase appear to be confined to the S_{105} fraction with no significant activity associated with the mitochondria.

Table 6. Synthesis of polyglutamyl cofactors in the methionine cell-free system.

Components added to the cell-free system [a]						
Boiled Extract (Control)	FH_4 (0.8 mM)	ATP (5 mM)	G-SH[b] (5 mM)	Glu [c] (5 mM)	γ-glu-glu[d] (1.5 mM)	% Yield
+						100
	+					16
	+	+		+		19
	+	+			+	16
	+		+	+		20
	+		+		+	18

[a] The complete system minus the boiled cell extract was utilized in all cases except with the control where the boiled extract was included.

[b] Glutathione is abbreviated G-SH.

[c] Glutamic acid is abbreviated glu.

[d] γ-glutamyl-glutamic acid is abbreviated γ-glu-glu.

Table 7. Subcellular location of serine transhydroxymethylase and the one-carbon transfer enzymes.

Enzyme(s) Assayed	Activity [a]	
	S_{105} [b]	Mitochondria
Serine transhydroxymethylase	0.95	0.04
One-carbon transfer	31	1

[a] Serine transhydroxymethylase activity is expressed as nmoles/min/mg protein. One-carbon transfer activity is expressed as nmoles methionine/mg protein produced in 3 hours.

[b] S_{105} is the supernatant fraction after centrifugation at 105, 000 x g.

Ammonium Sulfate Fractionation

Treatment of undialyzed crude cell extracts with ammonium sulfate was utilized to fractionate the one-carbon transfer enzymes. The fractions were dialyzed after treatment with ammonium sulfate as described in the Materials and Methods. As can be seen in Table 8, the one-carbon transfer enzymes were separated into two fractions, i.e., the 0-35% precipitate and 60% supernatant fractions. However, the 60% supernatant exhibited high activity when present in the cell-free system alone. The requirement for the two active fractions is demonstrated further in Figure 6, where increasing amounts of the individual fractions were added to the cell-free

Table 8. Separation of the one-carbon transfer enzymes from 3701-B by ammonium sulfate fractionation.

Crude Cell Extract (Control)	$(NH_4)_2SO_4$ Fractions [a]			Enzyme Activity [b]	% of Control
	0-35%	35-60%	60%+		
+				30	100
	+	+	+	26	87
	+	+		24	80
	+		+	45	150
		+	+	8	27
	+			19	63
		+		15	50
			+	28	93

[a] The $(NH_4)_2SO_4$ fractions were prepared as described in the Materials and Methods. The resuspended 0-35% and 35-60% precipitates and 60% supernatant (60%+) were utilized.

[b] Enzyme activity is expressed as nmoles methionine/mg protein produced in 3 hours.

system with or without the other fraction. When a constant amount of the 60% supernatant (0.33 mg protein/ml) was utilized, the specific activity of the one-carbon transfer enzymes remained constant if the 0-35% ammonium sulfate fraction was present at a concentration of 1 to 4 mg protein/ml. However, in the absence of the 60% supernatant, the specific activity did not reach a plateau with the 0-35% concentrations utilized. A completely different

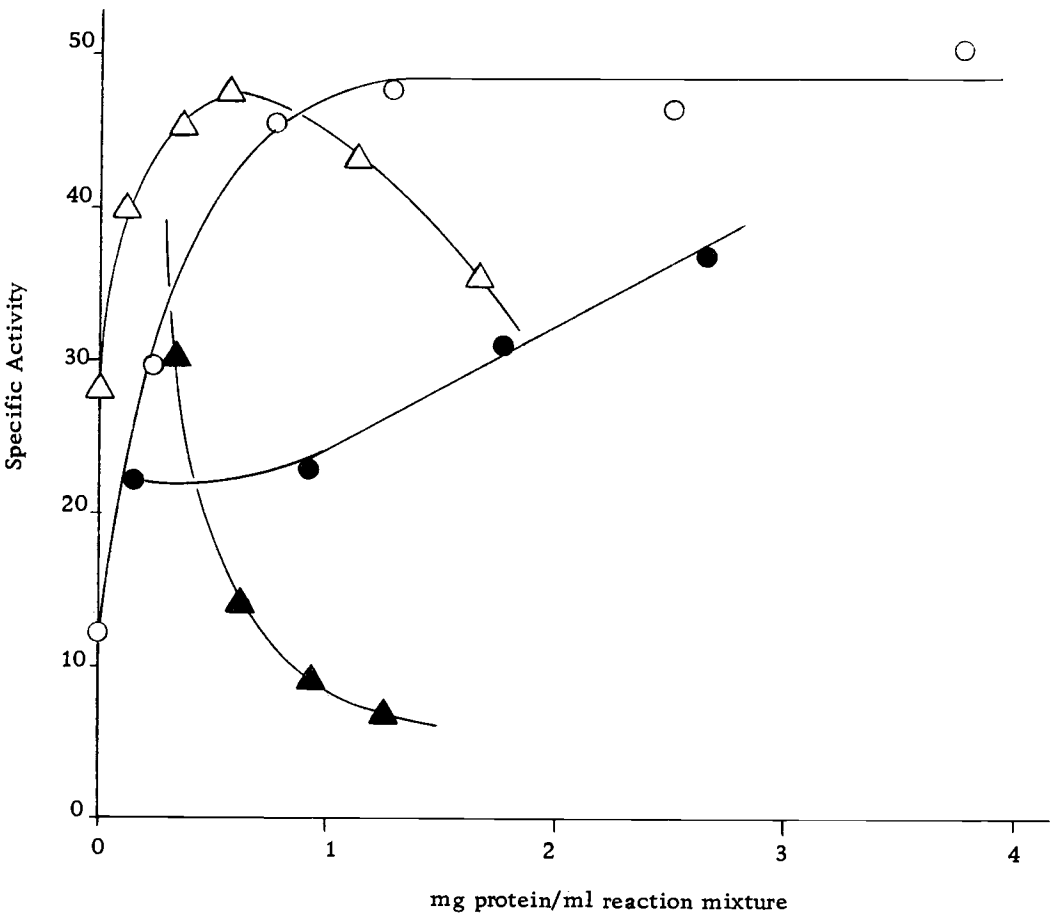


Figure 6. Methionine biosynthesis in the cell-free system with increasing amounts of the ammonium sulfate fractions. ○ Increasing the 0-35% fraction with a constant amount (0.33 mg protein/ml) of the 60% + fraction. ● Increasing the 0-35% fraction without the 60%+ fraction. △ Increasing the 60%+ fraction with a constant amount (0.76 mg protein/ml) of the 0-35% fraction. ▲ Increasing the 60%+ fraction without the 0-35% fraction. Specific activity is expressed as n moles methionine/mg protein produced in 3 hours.

pattern was observed when increasing concentrations of the 60% supernatant were utilized. In the absence of the 0-35% fraction, the specific activity decreased with increasing protein content, suggesting that the high specific activity of the 60% supernatant observed in Table 8 was due to the low protein content. It is also likely that the rate-limiting enzyme in the one-carbon transfer was located in the 0-35% fraction, since increasing the 60% supernatant concentration in the presence of a constant amount of the 0-35% fraction (0.76 mg/ml) did not result in a plateau in specific activity.

Analysis of serine transhydroxymethylase activity in the ammonium sulfate fractions was undertaken, and the results are shown in Table 9. As can be seen, no activity was observed in the 60% supernatant, while there was a substantial amount in the 0-35% and 35-60% fractions. To determine if serine transhydroxymethylase was the rate limiting reaction in the one-carbon transfer, the purified enzyme (Nakamura et al., 1973) was added to the cell-free system containing the 0-35% fraction (1.6 mg protein/ml) and 60% supernatant (0.4 mg protein/ml). No increase in total methionine production was observed by adding the purified enzyme.

Ammonium sulfate fractionation of the 234-5.1 and 234-15.1 extracts allowed determining in which fraction the mutant enzymes

Table 9. Serine transhydroxymethylase activity in the $(NH_4)_2SO_4$ fractions.

$(NH_4)_2SO_4$ Fraction [a]	Enzyme Activity [b]
0-35%	2.8
35-60%	2.3
60%+	0

[a] The $(NH_4)_2SO_4$ fractions were prepared as described in the Materials and Methods. The resuspended 0-35% and 35-60% precipitates and 60% supernatant (60%+) were utilized.

[b] Enzyme activity is expressed as nmoles/min/mg protein.

were located. As shown in Table 10, combining the fractions in the cell-free system demonstrated that the altered enzyme in 234-15.1 was in the 0-35% fraction, and the one in 234-5.1 was in the 60% supernatant.

Methyl Tetrahydrofolate:Homocysteine Methyltransferase

Partially purified methyl tetrahydrofolate: homocysteine methyltransferase from S. cerevisiae 3701-B was utilized to characterize the methionine mutants further. This enzyme preparation was combined with extracts of 234-5.1 and 234-15.1 in the cell-free system, and the results are presented in Table 11. As can be seen, the methyltransferase complemented the 234-5.1

Table 10. $(NH_4)_2SO_4$ fractionation of the methionine mutants one-carbon transfer enzymes.

234-5.1[a]			234-15.1[a]			Enzyme Activity[b]
Crude	0-35%	60%+	Crude	0-35%	60%+	
+						2
			+			1
+			+			22
	+	+				4
				+	+	5
	+				+	24
		+		+		5

[a] The mutant crude cell extracts, 0-35% ammonium sulfate fractions, and 60% supernatants were utilized.

[b] Enzyme activity is expressed as nmoles methionine/mg protein produced in 3 hours.

extract, but not the 234-15.1 extract.

Effect of Glycine in the Growth Medium

It was reported that supplementing the growth medium of S. cerevisiae with glycine resulted in a substantial derepression of serine transhydroxymethylase (Botsford and Parks, 1969). The effect of this treatment on the complete one-carbon transfer system was analyzed and compared to the effect on serine transhydroxymethylase. It was found that the specific activity of the methionine

Table 11. Methyl tetrahydrofolate: homocysteine methyltransferase in the cell-free system with extracts of 234-5.1 and 234-15.1.

Methyltransferase [a]	234-5.1 [b]	234.15.1 [b]	Enzyme [c] Activity
+			13
	+		9
		+	6
+	+		30
+		+	5
	+	+	19

[a] Methyl tetrahydrofolate: homocysteine methyltransferase was partially purified as described in the Materials and Methods.

[b] Dialyzed crude cell extracts of the mutants 234-5.1 and 234-15.1 were utilized.

[c] Enzyme activity is expressed as nmoles methionine/mg protein produced in 3 hours.

biosynthetic enzymes in the cell-free system was increased 2.8 times by growing the cells in glycine, compared to 3.8 times for serine transhydroxymethylase.

Glycine Inhibition

Since glycine is an end-product of the serine transhydroxymethylase reaction, it was likely that glycine could inhibit the reaction if only by mass action. The inhibition of serine

transhydroxymethylase by glycine is shown in Figure 7, and it can be compared to the inhibition of the complete one-carbon transfer system which is also shown in Figure 7. The arrows indicate the points where the serine and glycine concentrations were equal in each system. Glycine exhibits an inhibitory effect in both cases, but the dynamics of the inhibition appear to differ.

Spectrophotometric Analysis

Analysis of the serine transhydroxymethylase-PLP holoenzyme purified from S. cerevisiae indicated that the inhibition by S-AM was caused by an interaction with the PLP cofactor (Nakamura et al., 1973). Free PLP was examined spectrophotometrically in this investigation to determine if any alterations could be observed upon addition of S-AM. At neutral pH, the aldehyde function of PLP has a characteristic absorption peak at 388 nm (Peterson and Sober, 1954). A 5×10^{-4} M solution of PLP gave the typical pattern shown in Figure 8 (Curve No. 1). It can be seen in Curve No. 2 and Curve No. 3 that the PLP peak was depressed and red shifted by increased concentrations of S-AM. The absorption spectrum obtained with the higher S-AM concentration (Curve No. 3) exhibits one peak near 410 nm and another peak near 335 nm. A similar alteration was not observed when S-adenosylhomocysteine was substituted for S-AM, or when the S-AM was converted to 5'-methylthioadenosine

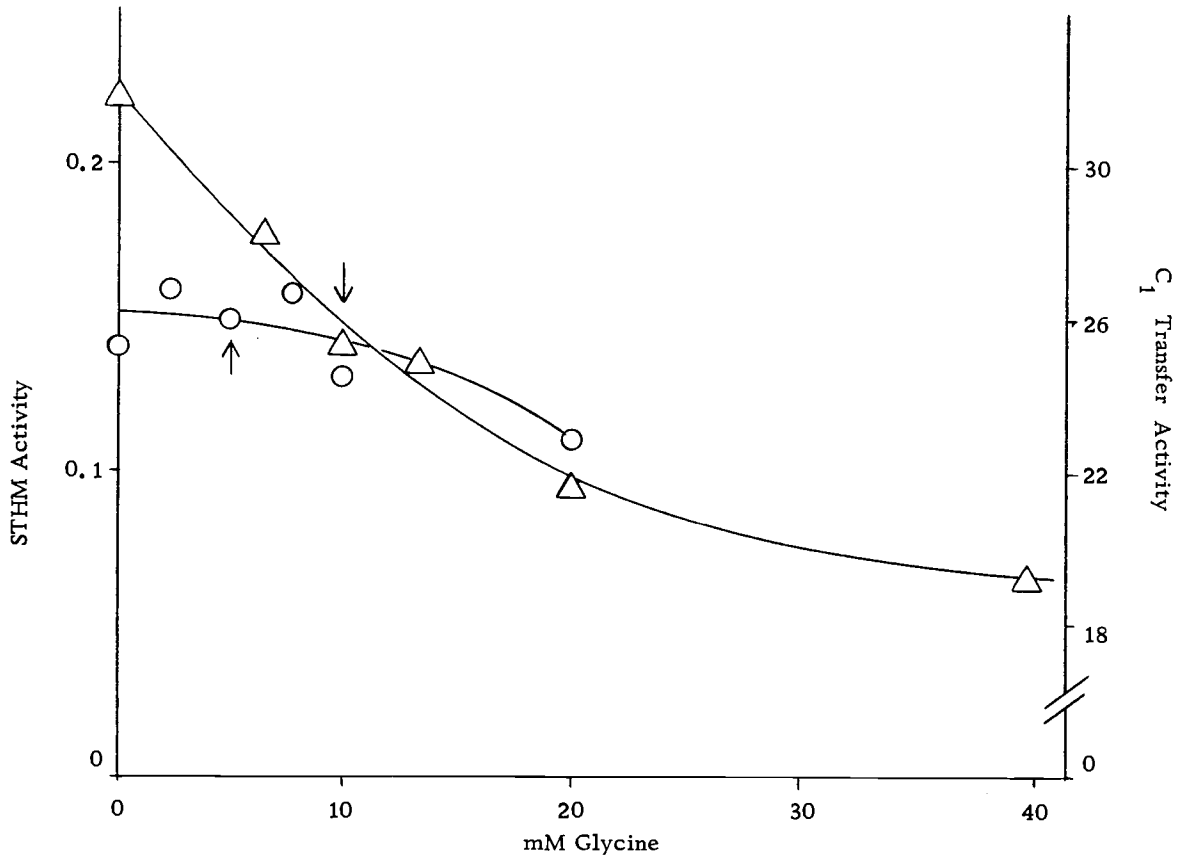


Figure 7. Effect of glycine on serine transhydroxymethylase (STHM) and one-carbon transfer activity. At 5 mM (↑), the glycine concentration is equal to the serine concentration in the STHM system (O). At 10 mM (↓), the glycine concentration is equal to the serine concentration in the C_1 transfer system (△). In both systems, the glycine concentration was increased to a maximum of 4 times the serine concentration. STHM activity is expressed as nmoles/min/mg protein. C_1 transfer activity is expressed as nmoles methionine/mg protein produced in 3 hours.

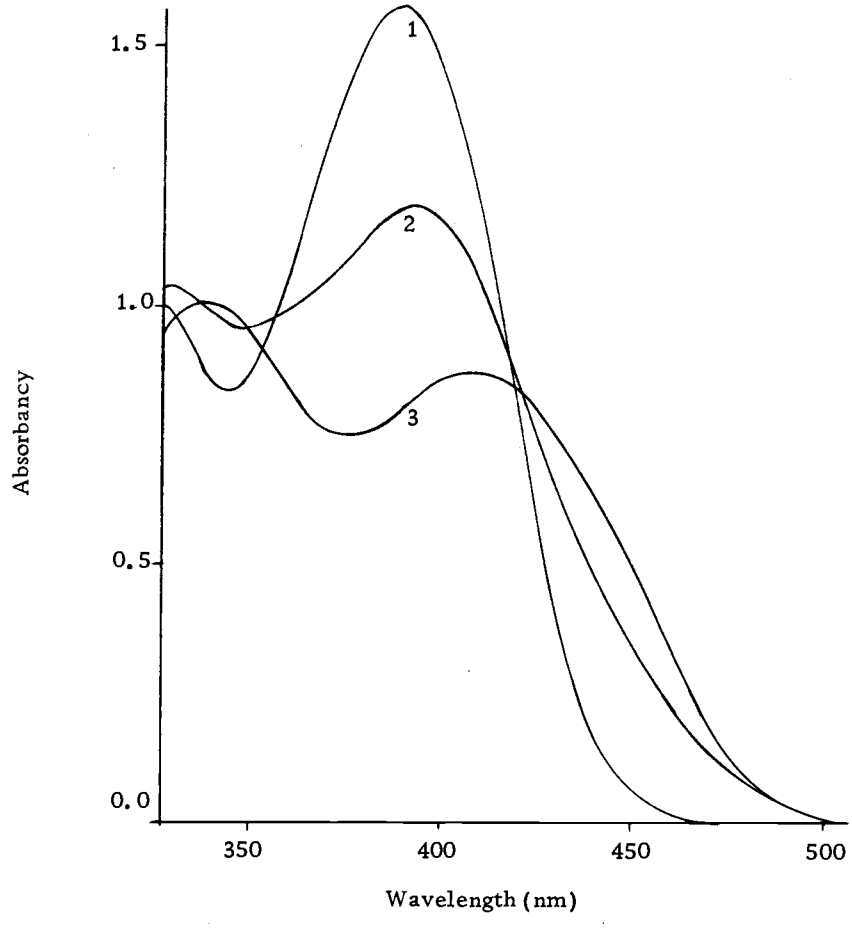


Figure 8. Absorption spectrum of PLP with or without S-AM. 5×10^{-4}M PLP with: (1) no S-AM, (2) 9×10^{-4}M S-AM, and (3) 5×10^{-3}M S-AM.

by boiling (Schlenk and DePalma, 1955). In addition, pyridoxine exhibited no reaction with S-AM.

The reaction between S-AM and PLP, as measured by the change in absorbance at 388 nm, was complete within 30 seconds when incubated at 37 C. Neither methionine nor adenosine (at concentrations equivalent to those used for S-AM, i.e., 5×10^{-3} M), had any effect on the PLP 388 nm peak, even when incubated with PLP at 37 C for up to 1 hour.

Tyrosine Aminotransferase Inhibition by S-AM

The spectrophotometric evidence for an interaction between S-AM and PLP did not prove that such a union caused the inhibition of serine transhydroxymethylase, or establish the effect of the interaction on other PLP-requiring enzymes. To determine if the sulfonium compound could act as a generalized inhibitor of PLP-dependent enzymatic reactions, the effect of S-AM on tyrosine aminotransferase was investigated.

Tyrosine aminotransferase was inhibited *in vitro* by S-AM as shown in Figure 9. The maximum inhibition attained with the S-AM concentrations utilized was approximately 60%. Using the method of Lineweaver and Burk (1934), a K_M of 1.2×10^{-5} M was established for PLP. As can be seen in Figure 10, the inhibition by

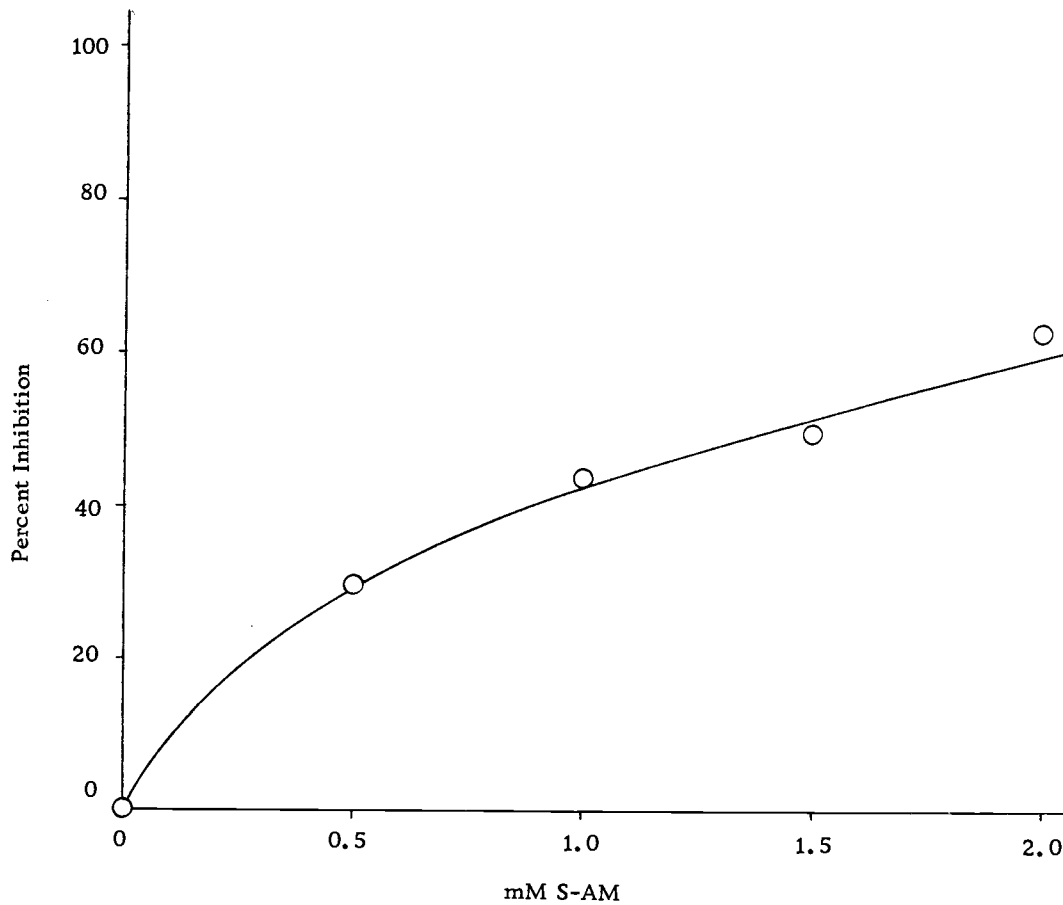


Figure 9. Inhibition of tyrosine aminotransferase in vitro by S-AM.

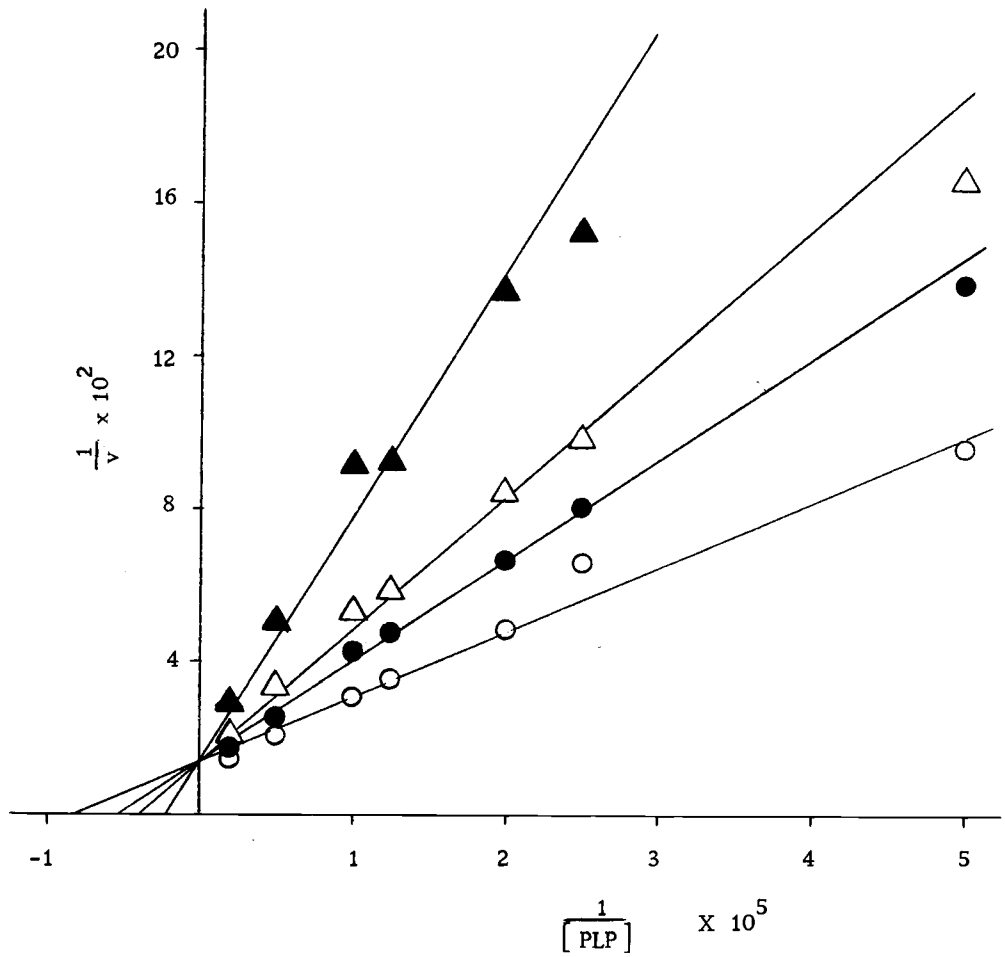


Figure 10. Lineweaver-Burk plots of tyrosine aminotransferase at different S-AM concentrations. ○ No S-AM; ● 5×10^{-4} M S-AM; △ 1×10^{-3} M S-AM; ▲ 2×10^{-3} M S-AM.

S-AM was of a competitive nature with regard to PLP. A K_I for S-AM was established using the method of Dixon (1953), and as can be seen in Figure 11, the inhibition constant was approximately 1×10^{-3} M.

Effect of High Intracellular S-AM Content

Even if the inhibition of serine transhydroxymethlase by S-AM is caused by a generalized interaction with PLP, this inhibition could be of significance in the one-carbon transfer. Since growth of yeast in excess methionine has been reported to cause a substantial increase in the cellular S-AM content (Schlenk and DePalma, 1957a and b), determining the significance of S-AM inhibition might be possible by growing the cells in excess methionine. The levels of S-AM at different periods during the growth cycle were determined for cells grown in high levels of methionine and compared to cells grown in low levels. The results are presented in Table 12. The levels observed during exponential growth appeared to be relatively independent of the time of harvest, i.e., the S-AM level appeared to be at a relatively steady-state concentration throughout exponential phase. Elevated levels were observed in cells grown in high methionine concentrations, and the higher steady-state level was apparently attained quite early as indicated by the lag phase

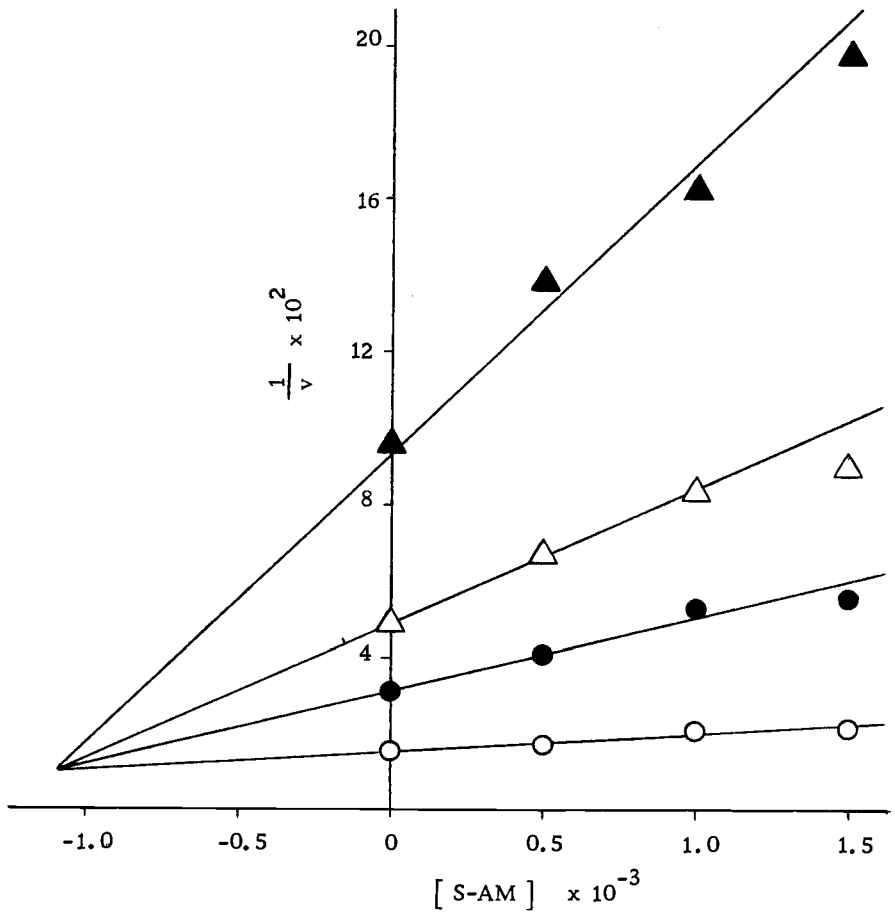


Figure 11. Dixon plots of tyrosine aminotransferase at different PLP concentrations. ○ 5×10^{-5} M PLP; ● 1×10^{-5} M PLP; △ 5×10^{-6} M PLP; ▲ 2×10^{-6} M PLP.

value. It can also be seen in Table 12 that the characteristic accumulation of S-AM did not occur until stationary phase.

The specific activities of serine transhydroxymethylase and the complete one-carbon transfer system from late exponential phase cells are shown in Table 13. These activities are compared for cells grown in low and high levels of methionine, and it appears that the growth of 5036-D in high levels of methionine resulted in an increase in the specific activity of both systems.

Additional enzymes were examined to determine if the increase in specific activity observed for the methionine biosynthetic enzymes from cells grown in excess methionine was atypical. The activity of tyrosine aminotransferase throughout the culture cycle is shown in Figure 12 for cells grown in low and high methionine concentrations. As can be seen, the specific activity was increased greatly by growing the cells in excess methionine. The effect of this cultural condition on glucose-6-phosphate dehydrogenase, ornithine aminotransferase, and pyridoxal phosphokinase was also investigated. As can be seen in Table 14, both glucose-6-phosphate-dehydrogenase and ornithine aminotransferase exhibited an increase in specific activity, while pyridoxal phosphokinase exhibited a decrease.

PLP levels at different periods during the growth cycle are shown in Table 15. As with S-AM, the PLP levels appear to be

Table 12. S-AM levels in cells grown in medium containing low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations.

Methionine in Growth Medium (mg/ml)	Growth Phase at Time of Harvest	S-AM Content (nmoles/g)
0.03	Lag	1, 300
	Exponential	1, 100
	Stationary	1, 600
5.0	Lag	2, 100
	Exponential	1, 900
	Stationary	8, 600

Table 13. Activities of serine transhydroxymethylase and the complete one-carbon transfer system in cells grown in low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations.

Enzyme(s) Assayed	Methionine Concentration in the Growth Medium	Enzyme Activity [a/]
Serine transhydroxy-methylase	0.03	2.1
	5.0	3.0
One-carbon transfer	0.03	34
	5.0	45

[a/] Serine transhydroxymethylase activity is expressed as nmoles/min/mg protein.
One-carbon transfer activity is expressed as nmoles methionine/mg protein produced in 3 hours.

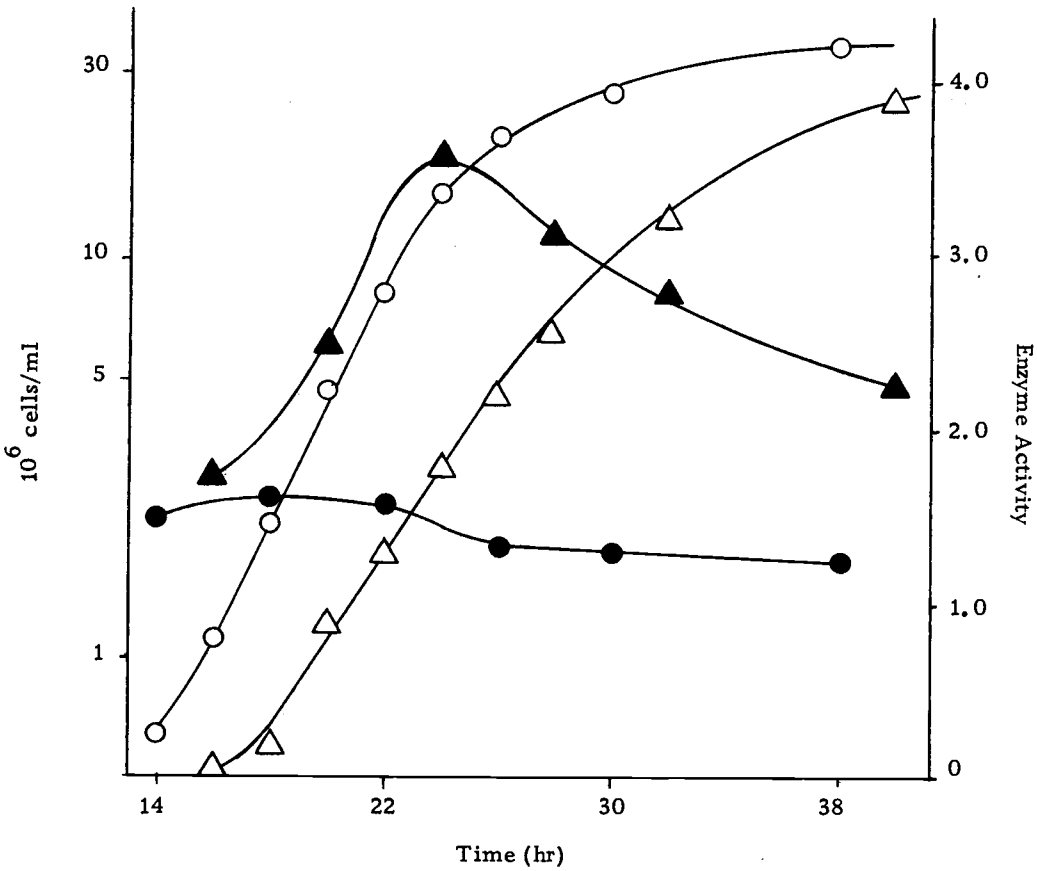


Figure 12. Tyrosine aminotransferase activity in cells grown in medium containing low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations. ○ (cells/ml) x 10^6 in low methionine; △ (cells/ml) x 10^6 in high methionine; ● tyrosine aminotransferase activity in cells grown in low methionine; ▲ tyrosine aminotransferase activity in cells grown in high methionine. Enzyme activity is expressed as μmoles/min/mg protein.

Table 14. Enzyme activities in cells grown in medium containing low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations.

Enzyme	Growth Phase at Time of Harvest [a]	Methionine in Growth Medium		
		0.03 mg/ml (Control)	5.0 mg/ml	
		Enzyme Activity [b]	Enzyme Activity [b]	% of Control
Ornithine Aminotransferase	Exponential	4.63×10^{-4}	9.54×10^{-4}	206
	Stationary	4.61×10^{-4}	6.19×10^{-4}	134
Pyridoxal Phosphokinase	Exponential	11.80×10^{-5}	9.75×10^{-5}	82
	Stationary	6.33×10^{-5}	4.95×10^{-5}	78
Glucose 6-Phosphate Dehydrogenase	Exponential	1.51×10^{-1}	2.58×10^{-1}	171
	Stationary	1.99×10^{-1}	2.06×10^{-1}	103

[a] Exponential represents late exponential phase and stationary represents early stationary phase. Approximately two doublings of a culture separate the two times.

[b] Enzyme activities are all expressed as μmoles/min/mg protein.

Table 15. PLP levels in cells grown in medium containing low (0.03 mg/ml) and high (5.0 mg/ml) methionine concentrations.

Methionine in Growth Medium (mg/ml)	Growth Phase at Time of Harvest	PLP Content (nmoles/g)
0.03	Lag	13.3
	Exponential	6.5
	Stationary	25.0
5.0	Lag	17.7
	Exponential	18.9
	Stationary	24.0

at a relatively steady-state concentration during exponential phase, and the level is higher in the cells grown in high levels of methionine.

Adenosine Inhibition

It has been reported that adenosine does not inhibit serine transhydroxymethylase *in vitro* (Nakamura *et al.*, 1973). However, adenosine was found to inhibit the complete one-carbon transfer system in the present investigation. As can be seen in Figure 13, substantial inhibition by adenosine was observed in the *in vitro* system, but a typical hyperbolic inhibition curve with increasing adenosine concentration was not followed. This unusual inhibition pattern suggested that the mode of inhibition was by some means

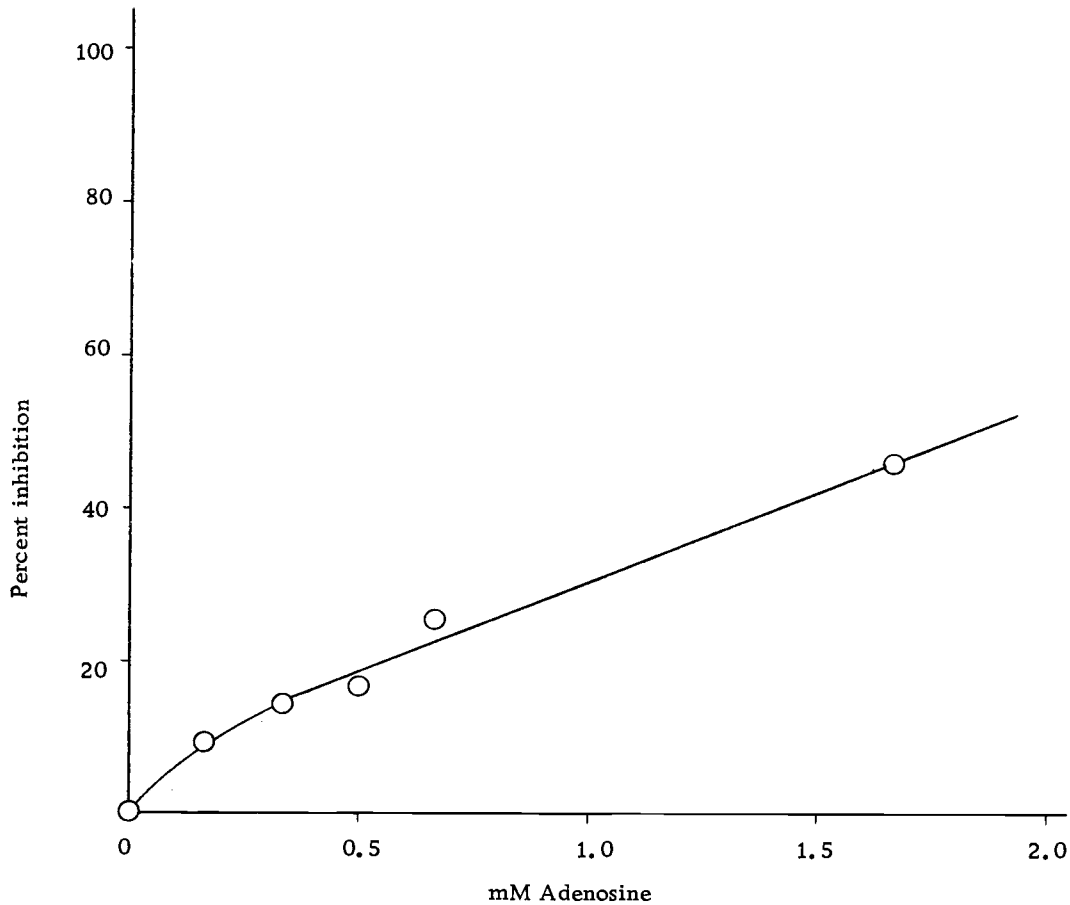


Figure 13. Inhibition of methionine biosynthesis in the cell-free system by adenosine.

other than feedback inhibition. It was possible that competition for homocysteine by the following reaction could cause the inhibition (Duerre and Schlenk, 1962):

L-homocysteine + adenosine $\rightleftharpoons$ S-adenosyl-L-homocysteine

To determine the significance of this reaction in the inhibition of methionine biosynthesis *in vitro*, additional homocysteine was added to the reaction mixtures. That homocysteine was able to reverse the inhibition by adenosine is shown in Table 16.

Table 16. Reversal by homocysteine of adenosine inhibition of cell-free methionine synthesis.

Homocysteine Concentration (mM)	Adenosine Concentration (mM)	Enzyme Activity[a/]	% Inhibition
10	0	22.0	0
10	1.0	14.9	32
15	1.0	17.1	22
20	1.0	19.4	12

a/ Enzyme activity is expressed as nmoles methionine/mg protein produced in 3 hours.

Inhibition by dUMP

A potential means of regulating the flow of one-carbon units to methionine is to have additional reactions competing for intermediates in the pathway. Although the nucleotide biosynthetic

enzymes utilizing one-carbon units have not been reported to carry out their reactions with polyglutamyl cofactors, such a possibility cannot be overlooked. The effect of dUMP on methionine biosynthesis in the cell-free system was examined to determine if thymidylate synthetase could compete with the reductase for the polyglutamyl form of methylene tetrahydrofolate. As can be seen in Figure 14, substantial inhibition was observed by adding dUMP to the reaction mixtures. The inhibition was not reversed by increasing the serine concentration, indicating that the inhibition was not caused by substrate depletion. It was also established that adding thymine, thymidine, dTMP, or dTTP to the reaction mixtures did not cause significant inhibition or reverse the dUMP inhibition of methionine synthesis. These results are shown in Table 17.

The effect of dUMP on serine transhydroxmethylase is shown in Table 18. As can be seen, dUMP did not have an inhibitory effect even though it was present at a concentration that would cause almost maximum inhibition in the complete one-carbon transfer system.

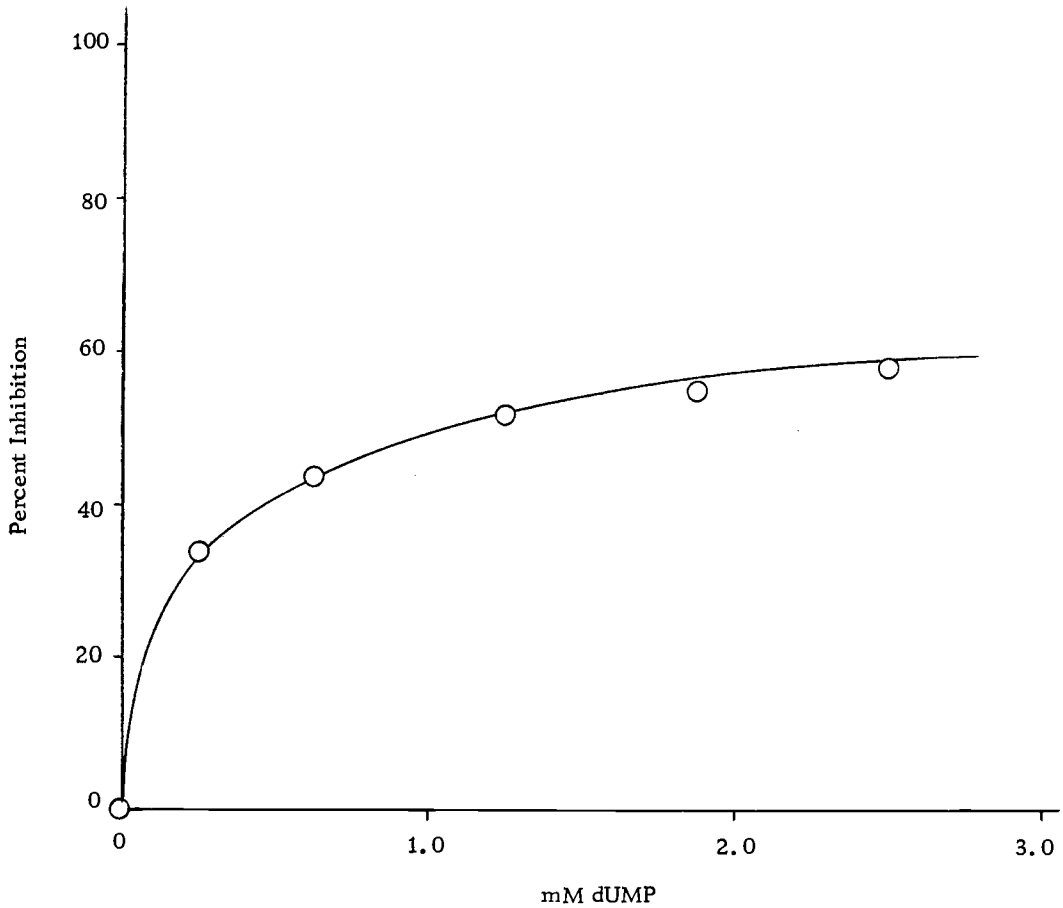


Figure 14. Inhibition of methionine biosynthesis in the cell-free system by dUMP.

Table 17. Effect of thymine, thymidine, dTMP, and dTTP on methionine biosynthesis in the cell-free system ± dUMP.

Concentration in the Cell-Free System (mM)					% Yield
dUMP	Thymine	Thymidine	dTMP	dTTP	
-	-	-	-	-	100
1.0	-	-	-	-	34
-	1.0	-	-	-	98
-	-	1.0	-	-	88
-	-	-	1.0	-	84
-	-	-	-	1.0	85
1.0	1.0	-	-	-	36
1.0	-	1.0	-	-	32
1.0	-	-	1.0	-	34
1.0	-	-	-	1.0	34

Table 18. Effect of dUMP on serine transhydroxymethylase.

mM dUMP	Enzyme Activity [a/]
None	3.7
2.5	3.6

[a/] Enzyme activity is expressed as nmoles/min/mg protein.

DISCUSSION

The diverse biological roles of methionine have dictated the need for a more clear understanding of the mechanisms and controls involved in methionine biosynthesis. The information available concerning the generation of the methyl moiety of methionine has come from a variety of organisms, and no definitive study has been reported for a single species. A significant problem associated with such a study is the variety and complexity of the folate cofactors involved. The observation in eucaryotic systems of mixtures of cofactors having from one to seven glutamyl residues (attached in γ-linkages) has complicated the investigation, since these cofactors are not readily isolated or synthesized. Therefore, the analysis of the over-all one-carbon transfer was severely limited because the means were not available to mimic the *in vivo* situation *in vitro*.

The results obtained in this investigation establish many parameters necessary to allow analysis of the one-carbon transfer. It was determined that storing cell extracts in 30% glycerol (v/v) increased the stability of the enzymes involved. This likely represents a stabilization of serine transhydroxymethylase, since comparable results were obtained with 30% glycerol when storing this enzyme (Nakamura *et al.*, 1973).

It was also established during the course of the investigation (results not presented) that failure to treat the boiled extract for a total of four minutes in the boiling water bath gave ambiguous results. A shorter period of time often resulted in at least one of the enzymes involved in the one-carbon transfer not being inactivated completely. It was then possible for the boiled extract to complement the crude extract from mutant 234-5.1, and based on the cell-free synthesis of methionine, 234-5.1 would not appear to be blocked in the one-carbon transfer. Using the boiled cell extract in the one-carbon transfer system in vitro results in the mixture being chemically undefined, but it offers the great advantage of allowing the actual physiological conditions to be paralleled more closely.

The ammonium sulfate fractionation procedure offered a rapid means of separating the one-carbon transfer enzymes into two fractions. Serine transhydroxymethylase was located in the 0-35% fraction (Table 9), as was one of the other methionine biosynthetic enzymes. This second observation is based on the fact that both of the one-carbon transfer mutants have a functional serine transhydroxymethylase (Table 4), but the mutant enzyme in 234-15.1 was in the 0-35% fraction (Table 10).

The genetic analysis of mutants 234-5.1 and 234-15.1 suggested that the mutations were complementary, and this was

verified in vitro by combining extracts from the two strains in the cell-free methionine synthesizing system (Table 3). It was possible to establish that 234-5.1 was blocked at methyl tetrahydrofolate: homocysteine methyltransferase by combining the mutant crude extract with the purified methyltransferase in the cell-free system (Table 11). Complementation was observed. As mentioned earlier, the enzyme complementing the 234-5.1 mutation was observed to be quite heat stable, and it is noteworthy that the optimal temperature for the methyltransferase from yeast has been reported to be 57 C (Burton and Sakami, 1970).

The pathway is usually depicted as in Figure 3, with three enzymes involved in the conversion of the β-carbon of serine to methionine. Based on the observations with the methyltransferase, it is then likely that mutant 234-15.1 has a defective reductase. It also follows that methylene tetrahydrofolate reductase is the rate limiting enzyme in the pathway. It was established that the limiting enzyme was in the 0-35% ammonium sulfate fraction (Figure 6), and that it was not serine transhydroxymethylase or methyl tetrahydrofolate: homocysteine methyltransferase. Therefore, by elimination it should be the reductase. Considering its location at the branch point of one-carbon generation for methionine and nucleotide biosynthesis, this enzyme would be a likely candidate for limiting the distribution. Consistent with this

view are the observations that the reductase is the site of control via feedback inhibition by methionine and S-adenosylmethionine (S-AM) in certain systems (Lor and Cossins, 1972; Kutzbach and Stokstad, 1971).

Methionine mutant C390-2B appears to be blocked at the polyglutamyl synthesizing enzyme. The one-carbon transfer enzymes were functional in the cell-free system when wild-type boiled extract was utilized (Table 3), but feeding experiments suggested C390-2B was blocked in the one-carbon transfer. These apparently conflicting results would be expected, however, if the strain could not synthesize the polyglutamyl forms of the tetrahydrofolate cofactors. This is because the 3701-B boiled extract would supply the polyglutamyl cofactors. It was also possible, however, that C390-2B was mutant in the synthesis of the four-carbon moiety, but could not utilize exogenous homocysteine in the feeding experiments. To distinguish between these possibilities, a boiled extract of C390-2B was used in the cell-free system with a C390-2B enzyme extract. No methionine was produced under these circumstances (Table 5), indicating the alteration is in cofactor synthesis.

It is known that serine can be synthesized in yeast from glycine and a one-carbon unit derived from glycine (DeBorso and Stoppani, 1967). A probable candidate for the enzyme generating the

one-carbon unit from glycine is glycine decarboxylase. Methylene tetrahydrofolate generated in this reaction could then be linked to glycine by serine transhydroxymethylase. This would explain induction of serine transhydroxymethylase observed by Botsford and Parks (1969), when glycine was included in the aeration medium. However, it was observed in the present investigation that aeration in glycine also increased the activity of the complete one-carbon transfer system, so it was possible that the methylene tetrahydrofolate was available for methionine biosynthesis as well. The possibility of glycine decarboxylase generating one-carbon units for methionine biosynthesis was tested by substituting glycine for serine in the cell-free system. There was no methionine biosynthesis (Table 2), suggesting the enzyme does not have a role in this aspect of one-carbon metabolism.

The differential in vitro inhibition of serine transhydroxymethylase and the complete one-carbon transfer by glycine (Figure 7) could be explained by a limited role for glycine decarboxylase. If it utilized only the monoglutamyl cofactor, greater inhibition of the complete one-carbon transfer system than of serine transhydroxymethylase could be observed. The glycine would inhibit methionine biosynthesis not only by mass action as with the serine transhydroxymethylase assay, it would also inhibit by supplying additional one-carbon units utilized in the reverse

reaction. If only a single serine transhydroxymethylase utilizes both monoglutamyl and polyglutamyl cofactors as in C. lagopus (Salem and Foster, 1970) using the monoglutmayl form in the reverse reaction would limit enzyme availability for the polyglutamyl one-carbon transfer required for methionine biosynthesis. The existence of only a single serine transhydroxymethylase in S. cerevisiae appeared likely during purification (Nakamura et al., 1973), and was indicated further in the present investigation by the absence of the mitochondrial enzyme (Table 7) found in higher eucaryotic systems (Fujioka, 1969).

The interaction between S-AM and PLP shown by the analysis of purified serine transhydroxymethylase (Nakamura et al., 1973) was verified in the present investigation. Spectrophotometric examination established the probable involvement of certain functional groups in the interaction. The fact that the interaction altered the PLP 388 nm peak (Figure 8) and that pyridoxine was unreactive with S-AM indicates that the aldehyde group on PLP is involved. The red shift of the 388 nm peak when S-AM was added is characteristic of the formation of a Schiff base (Matsuo, 1957), so this would tend to implicate the amino group on the methionine moiety of S-AM as being the group that interacts with the aldehyde on PLP. However, methionine did not cause a red shift of the PLP peak suggesting that more than simple nonenzymatic Schiff base formation is

involved. The fact that the depression of the 388 nm peak and the subsequent increase in the 330 nm range observed when S-AM was added is characteristic of the formation of a ring compound (Matsuo, 1957) adds further evidence for the complexity of the interaction. This complexity was partially resolved by the failure of adenosine, 5'-methylthioadenosine, and S-adenosylhomocysteine to elicit a spectral alteration of the PLP peak, thereby implicating the sulfonium function of S-AM. It seems likely that the sulfonium function interacts with PLP at some site other than the aldehyde group, and in doing so, it allows or accelerates the formation of the Schiff base.

The competitive inhibition of tyrosine aminotransferase with regard to PLP (Figures 10 and 11) is as expected for an S-AM:PLP complex. The fact that this enzyme which is unrelated to S-AM biosynthesis is inhibited in vitro, establishes that S-AM may act as a generalized inhibitor of PLP-dependent enzymatic reactions in vivo. Such inhibition has been reported for other PLP-binding compounds. Tyrosine aminotransferase has been studied quite extensively in mammalian systems, and along with numerous other PLP-dependent enzymes, has been shown to be inhibited by a wide variety of compounds which interact with PLP (DuVigneaud et al., 1957; Pestana et al., 1971; Black and Axelrod, 1969; Braunstein, 1960). Most of the work has involved in vitro studies, but in vivo

evidence of inhibition has also been obtained (DuVigneaud et al., 1957; Pestana et al., 1971).

The accumulation of S-AM in yeast reported to occur during culturing in high levels of exogenous methionine (Schlenk and DePalma, 1957a and b) offered a possible means of establishing the effect in vivo of the S-AM interaction with PLP. The S-AM level during exponential growth was found to be increased approximately 70% in the cells grown in excess methionine, and during stationary phase the increase was over 500% (Table 12).

To determine the effect of the increased intracellular S-AM concentration on the one-carbon transfer, serine transhydroxymethylase and the complete one-carbon transfer system were assayed from cells grown in high levels of methionine (Table 13). Somewhat unexpectedly, the activity of both assays was increased 30-40% when compared to cells grown in low levels of methionine. In the case of serine transhydroxymethylase alone, this could represent an increased requirement for one-carbon units for adenylic acid biosynthesis. The increased synthesis of S-AM would utilize a sizable quantity of ATP, so additional adenylic acid may be required for ATP biosynthesis. This is quite likely, since the cellular ATP pool is not depleted (Bailey and Parks, 1972).

The increased activity of the complete one-carbon transfer should be indicative of an increase in the activity of methylene

tetrahydrofolate reductase, since this appears to be the rate determining enzyme. The reason for an increase in activity of an enzyme which should be inhibited by methionine under the cultural conditions utilized (Lor and Cossins, 1972) is unclear.

The increase in specific activity of the one-carbon transfer was relatively minor when compared to some other enzymes. Tyrosine aminotransferase (Figure 12) and ornithine aminotransferase (Table 14) exhibited a two-fold increase in activity at a comparable time during the culture cycle. A somewhat lower increase was observed for glucose-6-phosphate dehydrogenase, while a decrease in activity was exhibited by pyridoxal phosphokinase (Table 14). The increase in activity of the two PLP-requiring aminotransferase enzymes suggested that a shortage of PLP might be limiting enzyme end-products. Consistent with this view is the report of a substantial increase in the formation of PLP-dependent apoenzymes when pyridoxine-deficient mutants of E. coli are starved for pyridoxine (Wasmuth et al., 1973). An additional indication of a shortage of PLP in the present investigation was demonstrated by the lower activity of pyridoxal phosphokinase.

The levels of PLP in cells grown in excess methionine were measured during the growth cycle, and compared to cells grown in a low concentration of methionine (Table 15). The higher level

during exponential growth in cells grown in excess methionine could represent PLP not bound to enzymes, since enzyme-bound cofactor is usually removed with denatured protein (Storvick et al., 1964; Storvick and Peters, 1964). If the PLP were complexed to S-AM, it could be metabolically unavailable while being measurably at a higher concentration. Since S-AM is concentrated in the vacuoles in yeast when they are grown in excess methionine (Svihla and Schlenk, 1959), PLP complexed to S-AM could be concentrated there also. Such an occurrence would impose a state of PLP-starvation on the cell, and would offer an explanation for the growth inhibition of yeast associated with culturing in excess methionine.

Competition for substrates and one-carbon intermediates offers a possible means of controlling the flow of one-carbon units. It was demonstrated that adenosine can compete for homocysteine with methyl tetrahydrofolate in vitro, and thereby inhibit methionine biosynthesis (Figure 13 and Table 16).

The utilization of polyglutamyl cofactors in nucleotide biosynthesis has not been reported, although they have been linked indirectly to purine biosynthesis in clostridial systems. It was reported by Uyeda and Rabinowitz (1967) that methylene tetrahydrofolate dehydrogenase from Clostridium cylindrosporum can utilize the triglutamyl form in addition to the monoglutamyl. Significantly,

the Michaelis constants indicated that the enzyme had a three-fold greater affinity for the triglutamyl cofactor.

The polyglutamyl derivative of methylene tetrahydrofolate was linked to thymidylic acid biosynthesis in this investigation. Methionine synthesis in the cell-free system was inhibited substantially by dUMP (Figure 14), but the serine transhydroxymethylase assay alone was not (Table 18). This indicates that thymidylate synthetase may utilize the polyglutamyl cofactor preferentially. The magnitude of the inhibition suggests that thymidylate synthetase can compete effectively with the reductase for methylene tetrahydrofolate when dUMP is available. These findings demonstrate that the cellular level of dUMP may influence the flow of one-carbon units for methionine biosynthesis, and they establish a possible function for the polyglutamyl cofactors in higher organisms.

SUMMARY

The results presented in this work provide fundamental information on the one-carbon transfer process. These include isolation and characterization of methionine mutants blocked in the one-carbon transfer, preliminary enzyme fractionation techniques, and folate cofactor isolation procedures.

There appears to be only a single serine transhydroxymethylase in S. cerevisiae, so it must utilize the monoglutamyl and polyglutamyl forms of tetrahydrofolic acid. This enzyme is subject to only limited regulation, and the inhibition by S-AM apparently is caused by a generalized interaction with PLP.

The formation of the S-AM: PLP complex inhibits tyrosine aminotransferase, an enzyme unrelated to S-AM biosynthesis. The generalized inhibition of PLP-dependent enzymatic reactions by S-AM could be the cause of the growth inhibition of yeast associated with culturing in excess methionine. The enzyme and cofactor levels under these cultural conditions are indicative of S-AM inducing PLP starvation.

The apparent lack of strict enzyme controls in the one-carbon transfer has been confusing. However, it appears that an intricate

balance of reactions competing for one-carbon units may be involved. It seems that thymidylate synthetase may utilize polyglutamyl cofactors preferentially, and that the dUMP level may influence the flow of one-carbon units to methionine.

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