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The terminal reactions in the biosynthetic pathway of methionine in Saccharomyces cerevisiae were investigated in this study. Analyses of a number of methionine auxotrophs for biochemical deficiences established four mutant groups. It was observed from qualitative feeding experiments that some auxotrophs were able to utilize S-adenosylmethionine (AM) and S-adenosylhomocysteine (AH) to satisfy their methionine requirement indicating a possible role of these compounds in the biosynthetic pathway. Enzymatic analyses indicated further differences in the mutant groups. One group, designated as Me-1, was unable to substitute any suspected intermediate tested for the methionine requirement. These organisms also lacked any S-adenosylmethionine-homocysteine transmethylase activity. The mutant group designated as Me-2 included organisms which were able to utilize S-adenosylmethionine or methionine for growth; this group was further divided following completion of serine hydroxymethylase assays with those organisms

showing no activity of this enzyme now noted as the Me-5 group.

Methionine synthesis was accomplished in cell-free preparations of Saccharomyces cerevisiae utilizing serine as the one-carbon donor. Synthesis was obtained using a boiled enzymatic extract or a Sephadex column eluate of an enzymatic extract. To determine the natural cofactor involved a number of suspected cofactors were tested in the system. Methyl tetrahydrofolate, methyl B_{12}, tetrahydrofolate, and dihydrofolate inhibited methionine synthesis. However, Teropterin stimulated activity indicating that Teropterin may be the cofactor involved or may be converted to the natural cofactor by our cell-free preparation. The synthesis of methionine was dependent upon the availability of S-adenosylhomocysteine indicating that this compound may be methylated to S-adenosylmethionine in this pathway.

It seems evident that S-adenosylmethionine can be formed in yeast in two ways: by the direct methylation of S-adenosylhomocysteine or by the reaction of methionine and adenosine triphosphate catalyzed by AM synthetase. A study of AM synthetase was also performed, and it was shown that AM synthetase is inducible in the presence of large concentrations of methionine.

Recent reports indicate that in Escherichia coli two independent systems for methionine biosynthesis are functional, one being dependent upon vitamin B_{12} and the other independent of vitamin B_{12}. Comparative studies indicate that in mammalian systems vitamin B_{12} and folic acid derivatives are also involved.

The data obtained in this study suggest that although the terminal reactions of methionine biosynthesis in Saccharomyces cerevisiae have characteristics of both the vitamin B_{12}-dependent and vitamin B_{12} independent systems, the yeast pathway is unique. A scheme which is compatible with the data is suggested; the terminal reactions would involve at least three enzymes including serine hydroxymethylase, S-adenosylhomocysteine methylase and S-adenosylmethionine-homocysteine transmethylase with Teropterin or a product of Teropterin, S-adenosylhomocysteine and S-adenosylmethionine serving as intermediates.

THE TERMINAL REACTIONS IN THE BIOSYNTHESIS OF METHIONINE IN *SACCHAROMYCES CEREVISIAE*

by

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THE TERMINAL REACTIONS IN THE BIOSYNTHESIS OF METHIONINE IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

Elucidation of the biosynthetic pathway of methionine has been the subject of many research projects within recent years. Early comparative studies indicated that the pathway was not a universal one, but rather some very distinguishing characteristics were noted among various species. Extensive work with mammalian systems, particularly rabbit liver and pig liver systems, led researchers to believe that vitamin B_{12} and folic acid derivatives were involved. Studies involving *Escherichia coli* cell-free preparations led investigators to believe that two independent systems for methionine biosynthesis were operating in this organism. This study was initiated to determine whether any similarities or differences could be noted between the biosynthetic pathway of *Saccharomyces cerevisiae* and other organisms. The study was concentrated on the terminal reactions involved in the biosynthetic sequence.

REVIEW OF LITERATURE

Early work with Neurospora crassa (Horowitz, 1947; Teas, Horowitz, and Fling, 1948) and Escherichia coli (Lampen, Roepke, and Jones, 1947) methionine auxotrophs indicated that the biosynthesis of methionine from cysteine occurred in a series of steps with a number of intermediate compounds. As indicated in Figure 1, the intermediates included the formation of cystathionine via a condensation of cysteine with homoserine. A cleavage of this intermediate then was predicted with the formation of serine and homocysteine. The final reaction in the biosynthetic pathway was the methylation of homocysteine to yield methionine.

An "active" form of methionine, now known as S-adenosylmethionine (AM), was first isolated by Cantoni (1953). Cantoni also demonstrated that S-adenosylmethionine is formed by the reaction of methionine and adenosine triphosphate (ATP) in the presence of AM synthetase. The enzyme, AM synthetase, was earlier designated as the methionine activating enzyme (Cantoni and Durell, 1957). However, this nomenclature was often confused with the activation of the amino acid in protein synthesis to form a methionine-adenosine monophosphate complex. The AM synthetase reaction is an unusual one in that the ATP completely undergoes dephosphorylation to yield orthophosphate and pyrophosphate, and the sulfur of methionine is bound to the adenosine moiety, as seen in Figure 2. The sulfonium compound has a strong positive charge and is active in methyl group transfer (Shapiro and Schlenk, 1960). However, all evidence indicates that AM is not enzymatically degraded

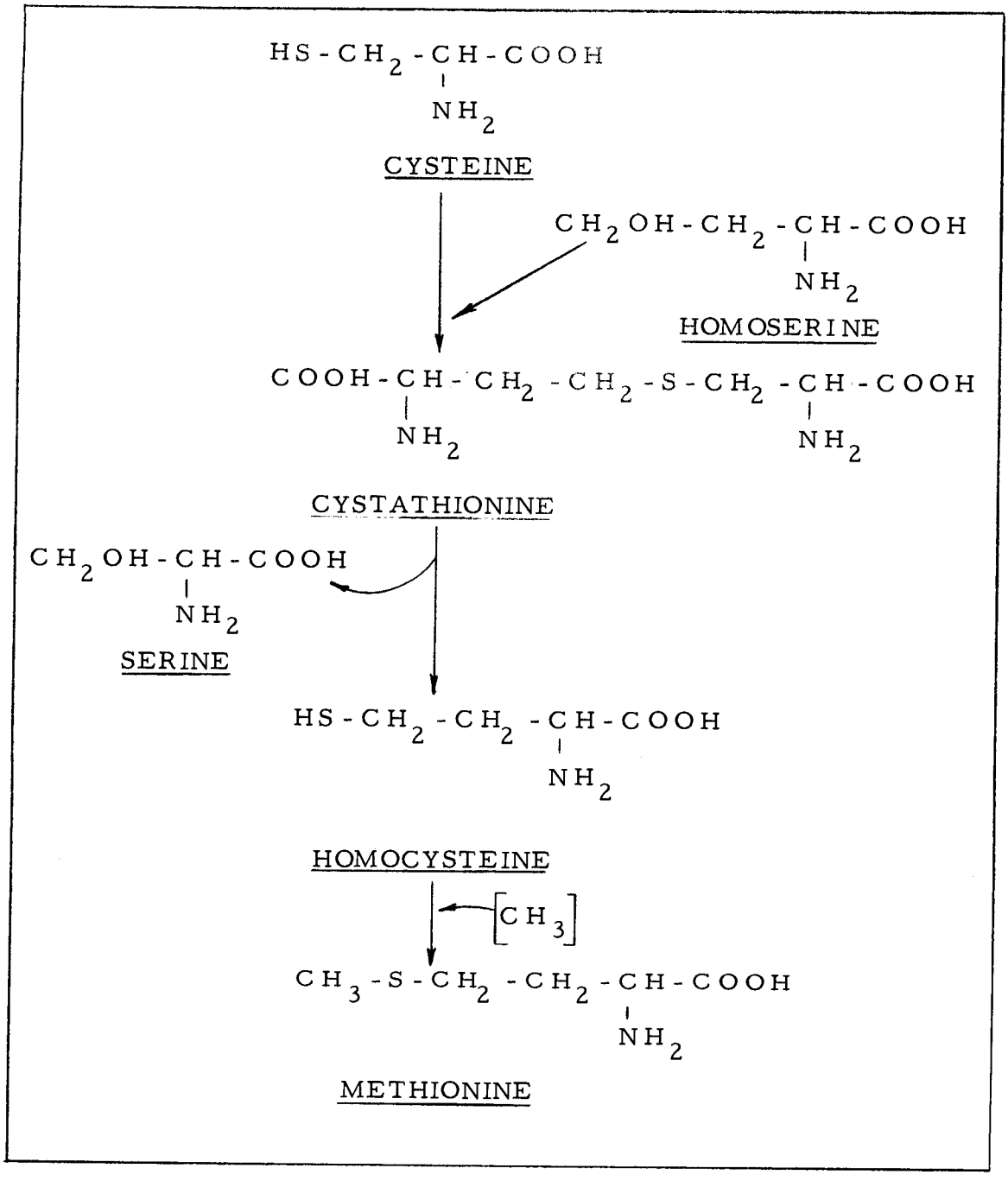


Figure 1. General scheme for methionine biosynthesis in microorganisms.

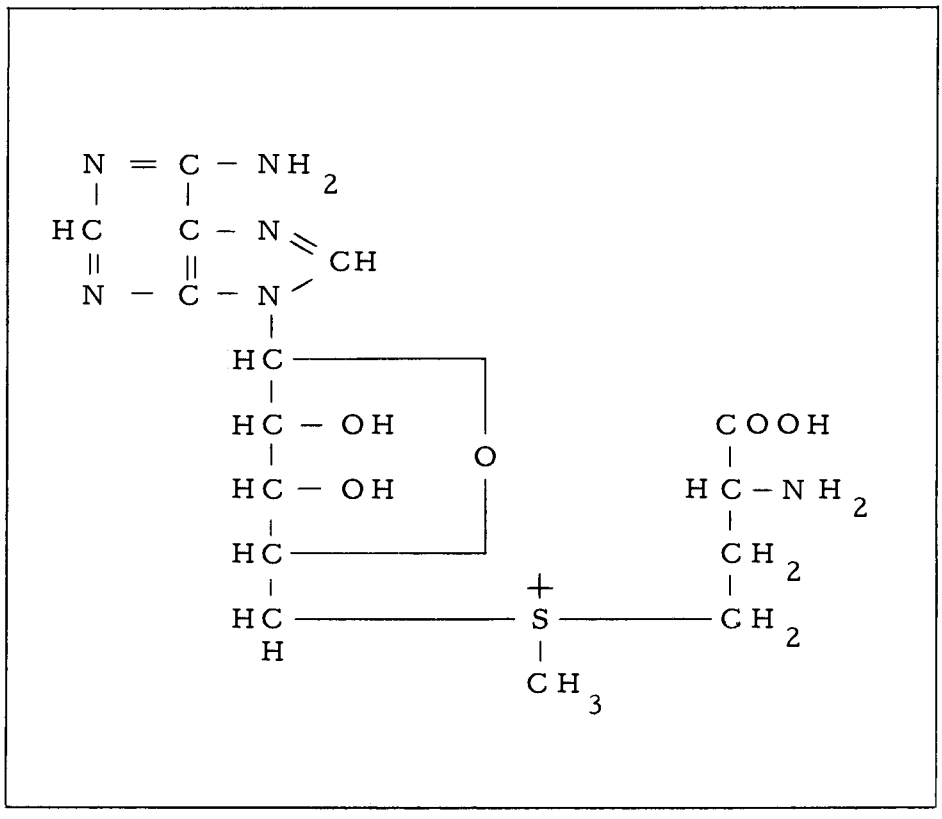


Figure 2. Structure of S-adenosylmethionine (AM).

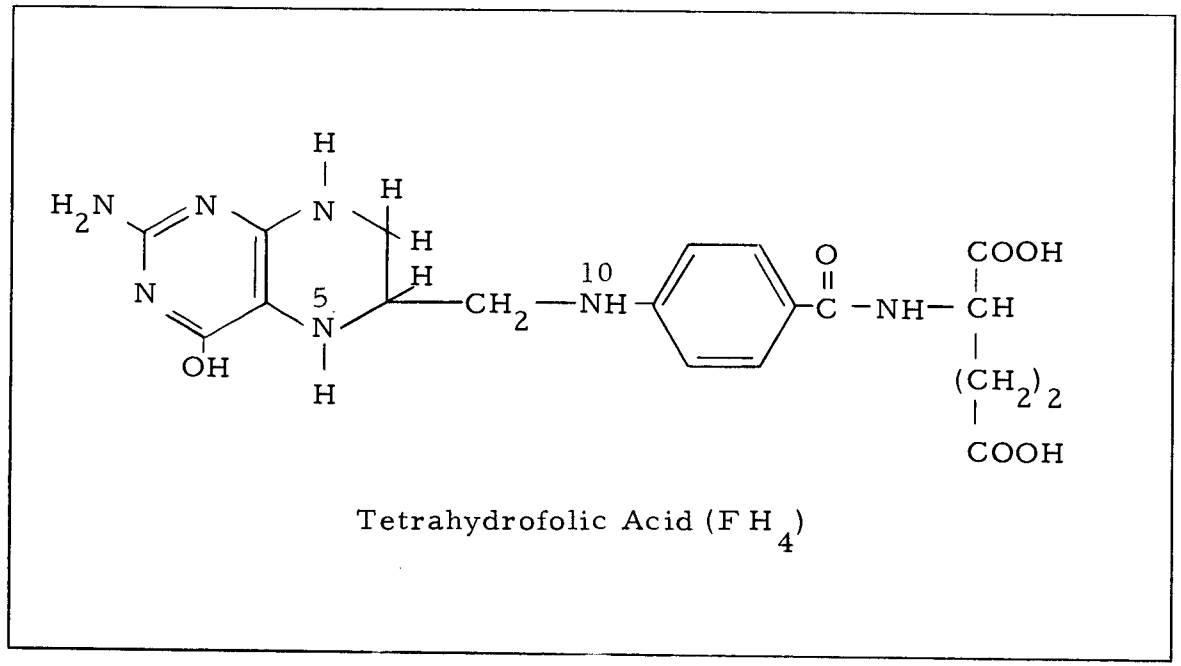
to methionine under any conditions (Shapiro and Mather, 1958). S-adenosylhomocysteine (AH), the demethylated product of AM, may be synthesized in two ways: (1) by the reaction of homocysteine and adenosine in the presence of AH condensing enzyme (Duerre and Schlenk, 1962); and (2) by the removal of the methyl group of AM (Shapiro and Schlenk, 1960).

Previous work on the biosynthesis of methionine in Saccharomyces cerevisiae (Pigg, Spence and Parks, 1962) led us to conclude that although the yeast system was similar in some respects to others studied, obvious differences had been observed. From the study of a number of methionine auxotrophs four mutant groups were described; their separation was dependent upon the clone's growth response to suspected biosynthetic intermediates. Organisms designated as Me-1 were able to grow only when methionine was supplied; Me-2 and Me-5 groups responded to both S-adenosylmethionine and methionine; Me-3 clones were able to utilize homocysteine, AH, and AM in place of the methionine requirement; and Me-4 mutants were able to grow on cysteine and the intermediates on which the Me-3 group could grow. Enzymatic analyses allowed further division of the mutants into a fifth group with Me-5 differing from Me-2 in that Me-5 had no serine hydroxymethylase activity. None of our auxotrophs utilized cystathionine as a substitute for methionine even though cysteine and homocysteine mutants were isolated. It appeared to us that either cystathionine was unable to penetrate the cell or that cystathionine was not involved in the biosynthetic sequence in yeast. Research on this problem is in

progress and is the subject of a thesis to be presented by W. A. Sorsoli, Oregon State University, Department of Microbiology.

The mechanism of the terminal reaction in the biosynthesis of methionine, the methylation of homocysteine, is another aspect of methionine biosynthesis that is of particular interest. Early studies made it apparent that the conversion of homocysteine to methionine was not a single step process, but that actually a number of enzymatic reactions were involved. In the various systems studied homocysteine was the final acceptor of the methyl group, and either serine or formaldehyde served as the donors of the one-carbon fragment which appeared ultimately as the methyl group of methionine. Work by Woods and co-workers (Woods, Foster, and Guest, 1965) and Buchanan and his colleagues (Elford et al., 1965) indicated that two independent systems for methionine biosynthesis were available in Escherichia coli. One system required vitamin B_{12} derivatives while the other was independent of vitamin B_{12}. Both systems appear to require derivatives of tetrahydrofolic acid (FH_4), Figure 3.

Buchanan's group (Larrabee et al., 1963) studied the vitamin B_{12}-dependent system with two mutant strains of E. coli: #113-3 which required either methionine or vitamin B_{12}, and #205-2 which required both methionine and p-aminobenzoic acid. They observed that three enzymatic fractions were essential for methionine synthesis from homocysteine and serine: (1) serine hydroxymethylase which catalyzed the reaction of serine and tetrahydrofolic acid to yield glycine and N^5N^{10}-methylene FH_4; (2) an enzymatic fraction designated as



Tetrahydrofolic Acid (FH_4)

Figure 3. Structure of tetrahydrofolic acid (FH_4).

"205-2" enzyme since it was lacking in strain #205 studied; and (3) "B_{12}" enzyme. The last enzyme contained vitamin B_{12} or a derivative of vitamin B_{12} associated with the protein as a tightly bound prosthetic group. The requirements for overall methionine synthesis from serine indicated that all three enzymatic fractions were necessary. To determine the order of action of each constituent, double incubation studies were performed. It was observed that serine hydroxymethylase first acted on serine to form an "active" one carbon unit which was carried by FH_4 derivatives. The "205-2" enzyme then preceeded the B_{12} enzymatic action. These workers (Larrabee et al., 1961) were also able to isolate a tetrahydrofolic acid compound which appeared to be an intermediate in the methylation of homocysteine series of reactions. The intermediate was identified as 5-methyl tetrahydrofolic acid (m-FH_4). The "205-2" enzyme is now designated as N^5N^{10}-methylene tetrahydrofolate reductase, a flavoprotein.

With the discovery of the m-FH_4 intermediate, workers in the United States and England immediately attempted to determine the action of 5-methyl tetrahydrofolate in the biosynthesis of methionine. Buchanan's group (Elford et al., 1965) has predicted that a tetrahydrofolate cycle is involved in which N^5N^{10}-methylene tetrahydrofolate is reduced to the 5-methyl tetrahydrofolate derivative. They suggested that the methyl derivative may then transfer its methyl group becoming tetrahydrofolic acid. The FH_4 may then be converted to N^5N^{10}-methylene tetrahydrofolate.

Another laboratory (Weisbach et al., 1963) also studying cell-free extracts of E. coli strains found that the transfer of the methyl group from 5-methyl tetrahydrofolate is dependent upon S-adenosyl-methionine and a vitamin B_{12} compound. ATP and reduced triphospho-pyridine nucleotide stimulated the system slightly. An interesting finding was that a number of vitamin B_{12} derivatives (Weissbach, Redfield, and Dickerman, 1964) could be used including the vitamin B_{12} coenzyme of Barker, (Smith et al., 1962), methyl-B_{12} and vitamin B_{12r}. Other vitamin B_{12} derivatives were utilized if they were first reduced to vitamin B_{12r}. The terminal reaction, the methylation of homocysteine by 5-methyl tetrahydrofolate is unique in that three methylated compounds are necessary - 5-methyl tetrahydrofolate, S-adenosylmethionine and methyl-B_{12}. These data suggest that a methyl transport pathway is operating. It is proposed that the reduced B_{12} protein accepts the methyl group of 5-methyl tetrahydrofolate and thus forms a m-B_{12} protein. The authors further suggest that AM functions in this reaction. If AM-C^{14} is applied to the reaction mixture, no C^{14} appears in methionine even though catalytic amounts of AM are necessary for the reaction to proceed. The last step of the methyl transport pathway may be described as a transmethylation reaction, for the methyl group from m-B_{12} protein is transferred to homocysteine forming methionine.

Investigators in Huenneken's laboratory (Kerwar et al., 1964) found recently that the pig liver extracts have the same requirements for methionine biosynthesis as the vitamin B_{12}-dependent system.

They also observed that AM replaced the ATP and magnesium requirements. This would be expected since the formation of AM proceeds from methionine and ATP in the presence of magnesium ions and AM synthetase. Methyl-B_{12} and also AM participate catalytically in the methylation of homocysteine by 5-methyl FH_4. However, methyl-B_{12} is not the primary methyl donor in the system, and they propose that it functions in the labilization of the methyl group from m-FH_4.

Woods and coworkers (Woods, Foster, and Guest, 1965) disagree with the interpretation of Weissbach, Redfield, and Dickerman (1964), since their studies with an auxotroph indicate that the role of S-adenosylmethionine is different. In their system m-B_{12} is involved but AM action is prior to the formation of m-FH_4. They observe an absolute requirement for AM in methionine synthesis from m-FH_4 using a complete system of B_{12} enzyme, homocysteine, phosphate buffer, mercaptoethanol and a reducing system of NAD, FAD, ethanol, and ethanol dehydrogenase. However, if m-B_{12} is employed in the system, the addition of AM has no effect. This indicates to them that AM acts prior to the formation of m-B_{12}. They propose that the B_{12} enzyme has two active sites; one is reduced to the B_{12} state and the other site is the point of attachment of AM. The reduced B_{12} is methylated by a transfer of the methyl group of AM. The AH which results remains tightly bound and is remethylated by 5-methyl tetrahydrofolate. The methylated B_{12} then undergoes a transmethylation with homocysteine to yield methionine.

Although the research on the vitamin B_{12}-independent system

has not been as extensive as the vitamin B_{12}-dependent system, some important differences in the two have been reported. Woods and his co-workers (1965) observed that the synthesis of methionine in this system is specific for N^5-methyl tetrahydrofolate triglutamate as the methyl donor. The monogluamate tetrahydrofolate derivative is completely inactive in this system. The enzyme has been designated as N_5-methyl tetrahydropteroltriglutamate-homocysteine methyltransferase but earlier noted as Enzyme B. Extensive work by these workers has shown that the enzyme fraction contains no B_{12} component. Also an important distinction from the vitamin B_{12}-dependent system is that there is no requirement for AM or $FADH_2$ in the vitamin B_{12}-independent system.

Work by Kisliuk (Woods, Foster, and Guest, 1965) has indicated that Aerobacter aerogenes may also have the ability to utilize both mechanisms for methionine synthesis. In this work it was observed that when A. aerogenes is confined to a vitamin B_{12}-independent system only the N^5-methyl tetrahydrofolate triglutamate served as the methyl donor. However, it was also noted that upon the addition of high concentrations of AM, the organism then synthesized the B_{12}-enzyme, and also showed methyl B_{12}-homocysteine methyltransferase activity.

This study with yeast was prompted by the availability of AM and AH utilizing mutants. Since vitamin B_{12} has never been reported in Saccharomyces, an interesting mechanism for methyl group synthesis could be predicted in which S-adenosylhomocysteine could act as an

acceptor for preformed methyl groups. Isotopic data in support of this possibility have been reported (Duerre and Schlenk, 1962). It was the intent of this study to determine if the terminal reactions in the biosynthetic pathway of methionine in Saccharomyces cerevisiae have any properties like that of the vitamin B_{12}-dependent or vitamin B_{12}-independent pathways of other organisms.

METHODS AND MATERIALS

Experimental Materials

Organisms

Strains of Saccharomyces cerevisiae were utilized in this study. A diploid, wild type strain designated as MCC was obtained from the culture collection of the Department of Microbiology, Oregon State University. A uracil auxotroph, noted as 3701B, was obtained from Dr. D. C. Hawthorne. Methionine auxotrophs of 3701B were prepared by ultraviolet irradiation. Additional mutant clones were obtained from Dr. H. Roman, Dr. C. C. Lindegren, Dr. R. K. Mortimer, and Dr. S. Pomper.

Culture Media

Stock cultures were maintained on a yeast complete medium, Table 1. Cultures were also grown in a medium designated as TCA or a modified Wickerham's medium (1946), Table 2 and Table 3.

S-Adenosylmethionine

S-Adenosylmethionine (AM) was synthesized from ATP and L-methionine using the method of Cantoni (1957) and incorporating the modification of Bremer and Greenberg (1961). The reaction was catalyzed by the methionine activating enzyme isolated from rabbit liver. AM was isolated as the crystalline trireineckate and then extracted with organic solvents. The purity of the AM was determined

TABLE 1

Composition of yeast complete medium

All amounts are per liter of medium	
Glucose	20.0 g
Tryptone	20.0 g
Yeast Extract	10.0 g
Agar*	15.0 g

*If solid medium is desired

TABLE 2

Composition of TCA medium

All amounts are per liter of medium	
Glucose	20.0 g
Tryptone	10.0 g
Yeast Extract	5.0 g
Agar*	15.0 g

*If solid medium is desired

TABLE 3

Composition of modified Wickerham's medium

All amounts are per liter of medium			
H_3BO_3	0.010 mg	KH_2PO_4	0.875 g
$CuSO_4 \cdot 5H_2O$	0.010 mg	K_2HPO_4	0.125 g
KI	0.010 mg	$MgSO_4 \cdot 7H_2O$	0.500 g
$FeCl_3 \cdot 6H_2O$	0.050 mg	NaCl	0.100 g
$ZnSO_4 \cdot 7H_2O$	0.070 mg	$CaCl_2 \cdot 2H_2O$	0.100 g
Biotin	0.002 mg	Glucose	20.000 g
Thiamin·HCl	0.400 mg	Ammonium sulfate	1.000 g
Pyridoxine·HCl	0.400 mg	Adenine sulfate	0.010 g
Uracil	10.000 mg		
Inositol	2.000 mg	L-methionine	0.005 g
Ca Pantothenate	0.400 mg	Agar*	15.000 g

*If solid medium is desired

chromatographically and spectrophotometrically. The concentration of AM at pH 7.0 was measured spectrophotometrically at 260 mμ with the Beckman DU spectrophotometer using 15,400 as the extinction coefficient (Schlenk and DePalma, 1957b). Radioactive AM was prepared by using L-methionine-methyl-C^{14} in the reaction mixture.

S-Adenosylhomocysteine

S-Adenosylhomocysteine (AH) was prepared using the rat liver enzyme method of de la Haba and Cantoni (1959) as modified by Duerre (1962).

5'-Methylthioadenosine

5'-Methylthioadenosine (MTA) was prepared by boiling S-adenosylmethionine, pH 7.0, for ten minutes.

L-Homocysteine

L-Homocysteine was prepared daily from its thiolactone by treating the desired concentration with 1M tris(hydroxymethyl)aminomethane until pH 8.5 was reached. The solution was left at room temperature for 10 minutes, the pH then adjusted to 7.0 and the solution immediately used.

Tetrahydrofolic acid

Tetrahydrofolic acid (FH_4) was prepared by a modification of the method of Silverman and Noronha (1961) which involved the

reduction of folic acid by sodium hydrosulfite in the presence of ascorbate. This was followed by purification and separation of the folic acid components by gradient elution in phosphate buffer on columns of DEAE-cellulose and Hyflo Super Cel. The purity of the FH_4 was determined spectrophotometrically with FH_4 having a maximum absorption at 297 mμ, (Huennekins, 1959). The FH_4 was stored at -20°C in 0.1 M phosphate buffer, pH 7.5, plus one percent cysteine.

5-Methyl Tetrahydrofolic Acid

5-Methyl tetrahydrofolic acid was prepared according to the method of Keresztesy and Donaldson (1961).

Methyl-B_{12} and Teropterin

Methyl-B_{12} was obtained as a gift from Dr. S. S. Kerwar. The Teropterin used was a gift of Dr. H. P. Broquist.

Preparation of Cell-free Extracts

Active cultures were prepared in a growth medium at 30°C on a rotary shaker for 40 to 48 hours. The organisms were then harvested and washed with buffer. If desired, the organisms were then resuspended in phosphate buffer plus one per cent glucose, and vigorously aerated for two to three hours at 30°C. Immediately following aeration the organisms were again harvested and washed. Cell disintegration was accomplished in two ways. Some of the extracts

were prepared by sonic treatment for twenty minutes in a Raytheon 10-kc model DF-101 sonic oscillator. In the later part of this study most of the disintegration was accomplished by passing the organism in a frozen state through an Eaton press (Eaton, 1962). The sonic and press extracts were centrifuged at approximately 20,000 x G for twenty minutes in the cold to remove most of the cell debris and unbroken cells. Further centrifugation was performed if desired by the Spinco model L refrigerated ultracentrifuge at 100,000-104,000 x G for thirty minutes. The supernatant fraction after removal of the lipid layer was treated as the enzymic extract. All preparations were stored at -20°C until immediately prior to use. If desired the enzymatic extracts were treated with Sephadex G-50, immediately prior to use, using ten volumes of Sephadex to one volume of extract.

Boiled Extracts

A fresh enzymatic preparation was placed in a boiling water bath for ten minutes. The solution was then cooled and centrifuged at approximately 5,000 x G to remove the denatured protein. The resulting supernatant fraction was treated as the boiled extract and kept at -20°C until used.

Protein Determinations

The concentration of protein in the enzymic extracts was measured spectrophotometrically with the Folin-Ciocalteu phenol reagent

according to the method of Lowry et al., (1951). Crystalline bovine albumin was used as the protein standard.

Experimental Methods

AM Synthetase Assay

The enzymatic reaction mixture included 20 μmoles L-methionine containing 0.8 μc of C^{14}-H_3-methionine, 100 μmoles $MgCl_2$, 30 μmoles adenosine triphosphate, 15 μmoles reduced glutathione, 60 μmoles tris buffer, pH 7.5, and 0.2 ml of cell-free extract. The total volume of the reaction mixture was 1.7 ml. Reactions were stopped by the addition of 0.2 ml of 1.5 N perchloric acid. The precipitated protein was removed by centrifugation.

The AM synthetase reaction involves the formation of AM from methionine and ATP. To determine the concentration of AM formed in the reaction, a short Dowex-50 column procedure was utilized. The method of Shapiro and Yphantis was modified so that the labeled AM was recovered in 7N acid. Radioactivity was measured with a Tri-Carb liquid scintillation spectrometer. The specific activity of the enzyme was designated as counts per minute/mg protein/hour incubation period.

Cell-free Methionine Synthesis Assay

The reaction mixture included 20 μmoles ATP, 20 μmoles AH, 40 μmoles homocysteine, 40 μmoles serine, 60 μmoles tris buffer, pH 7.5,

10 μmoles $MgCl_2$, 10 μmoles KCl, 10 μmoles $TPNH_2$ cofactor, and cell-free extract which was prepared by sonic treatment or treatment with an Eaton press. The enzymatic extract was treated with Sephadex G-50 immediately before use. The reaction mixture was incubated at 35°C for 3 hours. Following the incubation the reaction was stopped by boiling. The quantity of methionine synthesized was assayed microbiologically with Streptococcus faecalis strain 9790 or Streptococcus faecalis strain 8042. The specific activity of the enzyme was designated as mμmoles methionine synthesized/mg protein/3 hour incubation period.

Determination of Methionine Concentration

The concentration of methionine was assayed microbiologically using Streptococcus faecalis strain 9790 or strain 8042 obtained from the American Type Culture Collection (Steele et al., 1949). The assay was performed in methionine assay medium obtained from Difco Laboratories. The organisms respond to methionine but are not able to utilize homocysteine or serine to satisfy the methionine requirement. The reaction mixtures were boiled before assaying, so that any AM formed would be converted to 5'MTA which the assay organisms are unable to utilize for growth. The assay materials were incubated for 16-18 hours at 35°C, and growth was measured nephelometrically with a Coleman Model 9 Nepho-Colorimeter.

Determination of Cell Concentration

The concentration of yeast cells was determined nephelometrically using a Coleman Model 9 Nepho-Colorimeter and related to a standard curve which gave the determination in mg dry weight of yeast cells.

Paper Chromatography

Ascending paper chromatography was employed to separate and identify reaction components. Chromatograms on Whatman No. 1 filter paper sheets were developed in a n-butanol-glacial acetic acid-water system (60:25:15, v/v). After the developing solvent was allowed to migrate for 18-20 hours, the paper was dried in an oven. Every chromatogram was then scanned with a short wave ultraviolet light (Mineralite, model SL 2537), and the quenching areas indicative of adenine-containing compounds were marked. Two spray reagents were employed to further locate and identify the separated compounds. Ninhydrin in butanol was sprayed on the chromatogram, heated, and the separated amino acid spots observed. Chloroplatinate reagent (Tonennies and Kolb, 1951) was used to detect sulfur-containing compounds.

RESULTS

Saccharomyces cerevisiae strain MCC was cultured in Wickerham's synthetic medium and either supplemented with L-methionine or unsupplemented with methionine. At various time intervals samples were removed, and the concentration of cells determined nephelometrically. Figure 4 shows that organisms incubated in the presence of methionine display a slight stimulation of growth in the late exponential phase of growth while organisms cultured in the absence of methionine do not show the stimulation.

The MCC strain was then grown in Wickerham's minimal medium, harvested, washed, and resuspended in Wickerham's minimal medium plus methionine. To determine if the yeast was accumulating S-adenosylmethionine under these conditions, the concentration of AM within the yeast cell was analyzed. The AM was extracted from the cells with perchloric acid; separation of the sulfonium compound was achieved on a Dowex column, and the concentration of AM determined spectrophotometrically. Figure 5 indicates that the concentration of AM increased up to three hours and then leveled off. To test the activity of AM synthetase under these same conditions, cell-free preparations of AM synthetase were prepared. Figure 6 shows that the activity of the AM synthetase sharply increased within the first hour of growth in the methionine-supplemented medium. However, after three hours of incubation in the medium the activity of the enzyme started to decrease. This correlated with a decrease in the concentration of methionine in the medium as shown in Figure 7.

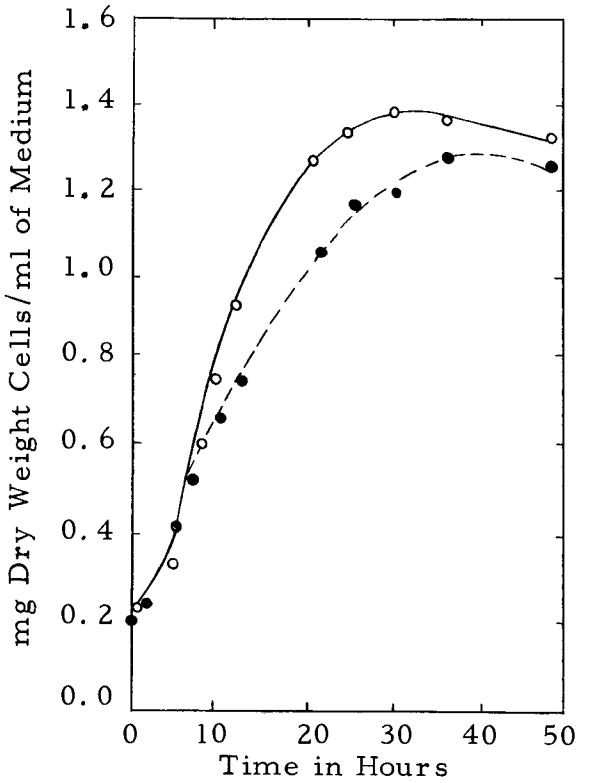


Figure 4. Growth of Saccharomyces cerevisiae in liquid synthetic media supplemented (o) and unsupplemented (•) with L-methionine.

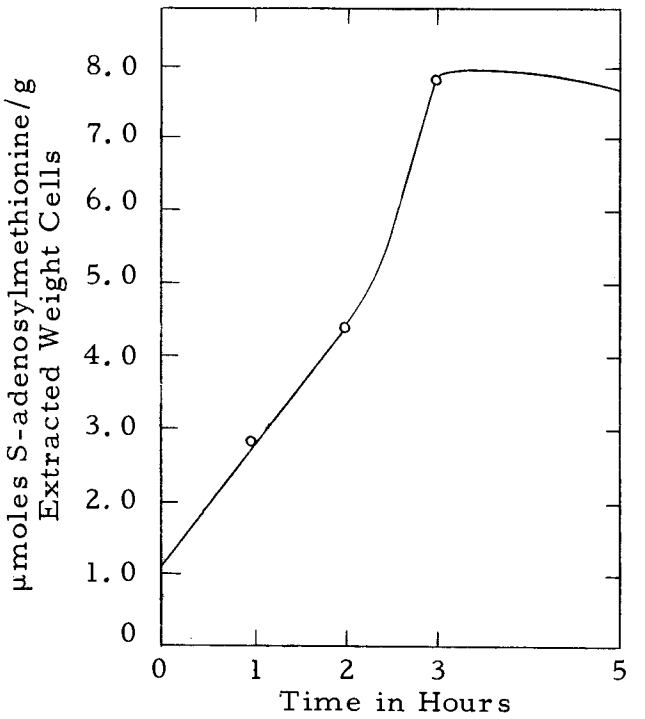


Figure 5. Concentration of S-adenosylmethionine within yeast cells during incubation on methionine-supplemented synthetic medium.

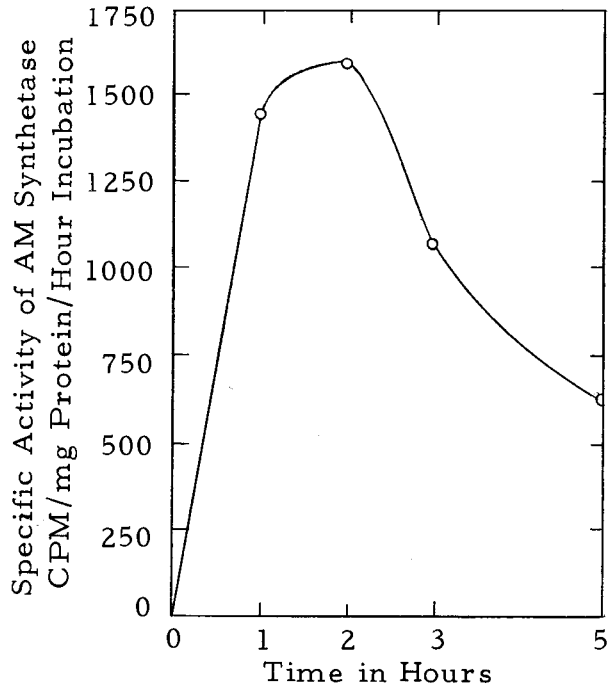


Figure 6. Specific activity of the methionine-activating enzyme during accumulation of methionine by growing cells.

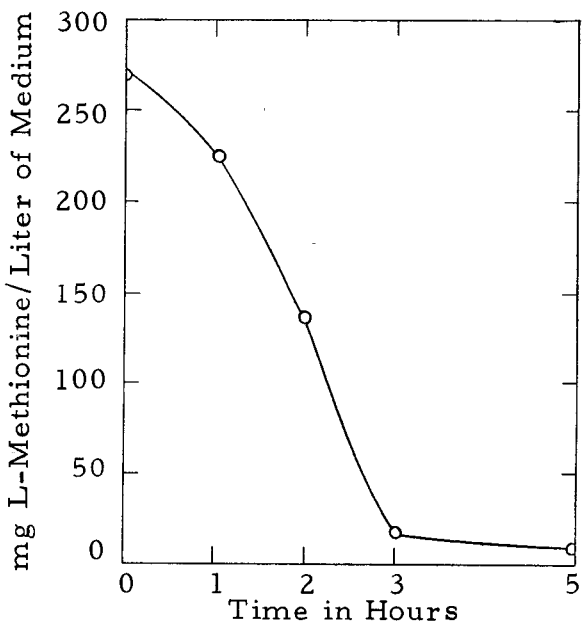


Figure 7. Disappearance of L-methionine from synthetic growth medium inoculated with Saccharomyces cerevisiae.

The concentration of methionine in the medium was determined microbiologically using Streptococcus faecalis strain 9790.

Cell-free synthesis of methionine was studied in preparations of both strain MCC and strain 3701B. The results are shown in Tables 4 through 12, utilizing various cofactors for synthesis. The results of various cofactors are included in individual tables because variation in total methionine synthesis was observed in different enzymatic preparations.

It can be seen in Table 4 that upon treatment of enzymatic preparations of both strain MCC and strain 3701B with Sephadex all activity is removed from the cell-free extract. Activity may be regained when the fraction removed by the Sephadex is again admixed with the enzyme source or upon the addition of a boiled extract of the enzymatic preparation.

Table 5 shows that methionine synthesis in Sephadex-treated cell-free extract of MCC is stimulated upon the addition of TPNH or DPNH in contrast to FAD additions. A complete reaction mixture included homocysteine, AH, ATP, Mg^{++}, serine, buffer, boiled extract, and cell-free extract.

Using a different enzymatic preparation of MCC, it may be seen again in Table 6 that cell-free synthesis of methionine has been accomplished using Sephadex-treated enzymatic preparation. Utilizing the boiled extract as a cofactor, synthesis was accomplished. Since no reducing agents were employed in the preparation of the boiled extract, no FH_4 should be present in the supplement. When

TABLE 4

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations

Organism	mμmoles methionine synthesized /mg protein/three hour incubation
MCC	
Complete less cofactor	0.
Complete plus boiled extract	35.
Complete plus column eluate	25.
3701B	
Complete less cofactor	0.
Complete plus column eluate	15.

TABLE 5

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete	235.
Complete plus TPNH	300.
Complete plus DPNH	310.
Complete plus FAD	210.

TABLE 6

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete reaction mixture	80.
Complete less ATP	95.
Complete less AH	55.
Complete less boiled extract, ATP, AH, serine	40.
Zero time control	0.

ATP was omitted from the reaction mixture, greater synthesis was observed. The stimulation of methionine biosynthesis when ATP is omitted was a curious finding. Table 6 also indicates that the cell-free synthesis of methionine is dependent upon the availability of AH.

To determine the natural cofactor involved in this cell-free synthesis, a number of defined cofactors were tested in the yeast system. Tables 7 and 8 indicate that the addition of m-FH_4 did not stimulate the synthetic reactions and instead inhibited the synthesis. Even in reaction mixtures without ATP, in which normally there is increased methionine accumulation by the measurements used, there was no synthesis of methionine. The addition of oxidized m-FH_4 likewise inhibited methionine synthesis.

The addition of Teropterin stimulated methionine synthesis in the cell-free synthesis. Table 9 indicates that the methionine synthesized is dependent upon the availability of the cofactors and is also dependent upon the concentration of S-adenosylhomocysteine.

Table 10 shows that the addition of m-B_{12} and the joint addition of m-B_{12} and m-FH_4 did not stimulate methionine synthesis in this system. As seen in Table 11 the addition of FH_4 and FH_2 significantly inhibit the synthesis of methionine.

Cell-free preparations of strain 3701B further substantiate the results obtained with strain MCC, as seen in Table 12. An eluate from the Sephadex column stimulates methionine synthesis while m-FH_4 and oxidized m-FH_4 inhibit the synthesis.

TABLE 7

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC utilizing methyl tetrahydrofolate as the cofactor

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete with m-FH_4	73.
Complete without m-FH_4	94.
Complete without ATP	89.
Zero time control	0.

TABLE 8

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC utilizing m-FH_4 and m-FH_4 derivatives as cofactors

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete with m-FH_4	137.
Complete with oxidized m-FH_4	95.
Complete without cofactor	168.
Complete with Sephadex eluate	316.
Zero time control	0.

TABLE 9

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC and utilizing Teroperin as cofactor

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete with Teropterin	30.
Complete without Teropterin	25.
Complete without ATP	40.
Complete without AH, ATP	15.
Zero time control	0.

TABLE 10

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC and utilizing m-B_{12} and m-FH_4 as cofactors

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete	74.
Complete with m-B_{12}	71.
Complete with m-FH_4 and m-B_{12}	60.

TABLE 11

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain MCC and utilizing FH_4 and FH_2 as cofactors

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete	145.
Complete with FH_4	62.
Complete with FH_2	54.

TABLE 12

Cell-free synthesis of methionine using Sephadex-treated enzymatic preparations of S. cerevisiae strain 3701B

	mμmoles methionine synthesized /mg protein/three hour incubation
Complete with Sephadex eluate	31.6
Complete without cofactor	16.8
Complete with m-FH_4	13.7
Complete with oxidized m-FH_4	9.5
Zero time control	0.0

DISCUSSION

A number of investigations (Schlenk and DePalma, 1957a; Svihla and Schlenk, 1959) demonstrated that the yeast cell accumulates large quantities of S-adenosylmethionine when grown in the presence of large quantities of methionine. Gawel, Turner and Parks (1962) analyzed a number of bacterial species and found that none of these accumulated significant quantities of AM. However, Saccharomyces cerevisiae did concentrate AM under the same conditions. Svihla and Schlenk (1959; 1960) observed that the AM that is accumulated by the yeast cell is stored and concentrated in the vacuole. This S-adenosylmethionine is then passed to daughter cells during budding. All growth studies indicate that the AM is not utilized as a metabolite under these conditions.

S-Adenosylmethionine may be synthesized in two ways. The AM synthetase enzyme which catalyzes the formation of AM from methionine and ATP has been studied extensively. The other mechanism of AM formation involves a direct methylation of S-adenosylhomocysteine. This mechanism has been supported by the work of Duerre and Schlenk (1962) involving growing cells of Candida utilis and Saccharomyces cerevisiae. They observed that upon the addition of labeled C^{14}-AH to the whole cells, AM could be recovered which contained a specific activity of 79 to 83 per cent of the AH supplied. If the endogenous supply of AM was subtracted a quantitative recovery of the supplement was obtained, indicating to the authors that the attachment of a one carbon unit was directly to S-adenosylhomocysteine. They

reasoned that a circuitous route would lead to dilution with non-labeled adenine compounds from the metabolic pool. They also obtained further evidence in the whole cell preparations by adding labeled adenosine and nonlabeled AH. Very little activity resided in the AM recovered indicating that a direct methylation of A H was operating.

Although it seems likely that two mechanism of AM formation are possible in the yeast system, the earlier observations led us to suspect that the formation of AM under these conditions was accomplished by means of AM synthetase. As seen in Figures 5 and 6, upon the cultivation of strain MCC in the presence of excess methionine, the synthesis of the AM synthetase is induced. This then allows for the increased synthesis and accumulation of AM within the yeast cell.

The maintenance of high levels of the AM synthesizing enzyme is dependent upon a supply of methionine. It can also be seen from Figure 5 that even at zero time of incubation with methionine there is a significant quantity of AM available in the yeast cell. At this time there appears to be no or very little AM synthetase activity. This could be a reflection on the assay system, since very small amounts of activity could be overlooked in the procedure. However, it also seems likely that this may give further proof to a mechanism of AM formation which has been proposed by Duerre and Schlenk (1962). It seems possible that AM synthesis could occur by a combination of the homocysteine-adenosine condensing enzyme catalyzing the formation of S-adenosylhomocysteine. The AH is then directly methylated to form S-adenosylmethionine.

Cell-free preparations utilized in the synthesis of methionine in our Saccharomyces cerevisiae system give further support to this hypothesis. Utilizing a variety of cofactors it was observed, as seen in Tables 4 through 12, that methionine synthesis could be accomplished. A boiled extract and a Sephadex eluate served as cofactors in this reaction. This was definitely dependent upon the availability of S-adenosylhomocysteine in the system, indicating that AM could be formed by a direct methylation. In an attempt to determine the natural cofactor involved, a number of defined cofactors were tested in this same system. It was seen that FH_4, FH_2, m-FH_4, m-FH_2, and m-B_{12} did not stimulate methionine synthesis and in fact, seemed to inhibit the reactions. In an earlier part of this study (Pigg, Spence and Parks, 1962) it was observed that FH_4 inhibited the serine hydroxymethylase reaction, giving further substantiation that FH_4 is not the natural cofactor involved.

Teropterin was the only chemically defined cofactor which served to stimulate the overall synthesis of methionine from serine. Since it was utilized in the synthesis it appears that either Teropterin is the natural cofactor involved or can be converted to the natural cofactor in our system.

In our study attempts were made to isolate any AM which was formed from AH; however, none was isolated. It seems possible that only catalytic amounts of AM would be necessary for the methionine biosynthetic pathway to operate. With our system we would be limited in our ability to detect small amounts of AM. In addition, extensive

studies on the metabolism of AM have indicated that this sulfonium compound is extremely labile and is rapidly degraded to a number of products (Shapiro and Mather, 1958).

The finding that overall methionine biosynthesis was stimulated when adenosine triphosphate was omitted was interesting. It may indicate that ATP does not decrease the amount of methionine formed under these conditions but effects a removal of methionine via the AM synthetase reaction. Therefore a great portion of the methionine synthesized during the incubation period when ATP is included may rapidly be converted to AM. Our microbiological assay procedure precludes detection of the methionine trapped in the form of AM since the sulfonium compound is degraded to 5'-methylthioadenosine by boiling.

From this study it seems likely that the biosynthesis of methionine in Saccharomyces cerevisiae is similar to both the vitamin B_{12}-dependent and the vitamin B_{12}-independent systems observed in Escherichia coli, as indicated in Table 13. All studies indicate that vitamin B_{12} is not found in yeast. In this respect the organism is similar to the vitamin B_{12}-independent mechanism. Neither of these systems is able to utilize m-FH_4; however they are able to utilize the triglutamate derivative of tetrahydrofolic acid. The yeast system does differ in that S-adenosylhomocysteine is required for synthesis, and it seems likely that S-adenosylmethionine is involved in the pathway. The vitamin B_{12}-dependent system does require AM in the synthetic pathway but again differs in that m-FH_4 may be utilized as the methyl donor. Although investigators in different laboratories

TABLE 13

A comparison of the reported methionine biosynthetic schemes

	Escherichia coli		Saccharomyces cerevisiae
	B_{12}-dependent	B_{12}-independent	
Involvement of AM	+	-	+
Involvement of m-FH_4	+	-	-
Involvement of vitamin B_{12}	+	-	-
Involvement of Teropterin	+	+	+
Involvement of $FADH_2$	+	-	-
Involvement of AH	-	-	+
Anaerobic conditions	+	-	-
S-Adenosylmethionine-homocysteine transmethylase	?	no	yes

differ on the mode of action of m-B_{12}, it seems quite clear that in the B_{12}-dependent systems studied, m-B_{12} may be involved in the terminal reaction of methionine biosynthesis.

From our data it is clear that the terminal reaction of methionine biosynthesis in Saccharomyces cerevisiae is unique in nature. It appears that the methylation of homocysteine to yield methionine is certainly not a one-step reaction but involves at least three enzymes and a number of cofactors, as shown in Figure 8. Utilizing serine as the ultimate methyl group donor it seems that the first reaction involves serine hydroxymethylase yielding the one carbon unit to a cofactor. From our work it appears that either the cofactor is Teropterin or that Teropterin is able to be converted to the natural cofactor involved. The Teropterin cofactor then may be utilized to methylate AH which can be formed from homocysteine and adenosine. The methylated product is AM. The terminal reaction could be a transmethylation of AM to homocysteine yielding AH and methionine. In this scheme it seems very possible that AM and AH could react in catalytic manner. If the system were operating to form methionine faster than methionine could be utilized in other reactions, it seems quite likely that the AM synthetase could be induced and used to trap methionine by catalyzing the formation of S-adenosylmethionine.

From our previous work (Pigg, Spence and Parks, 1962) methionine auxotrophs of S. cerevisiae were separated into four distinct categories. This grouping was established as the result of qualitative feeding experiments in which mutant clones were tested for the ability

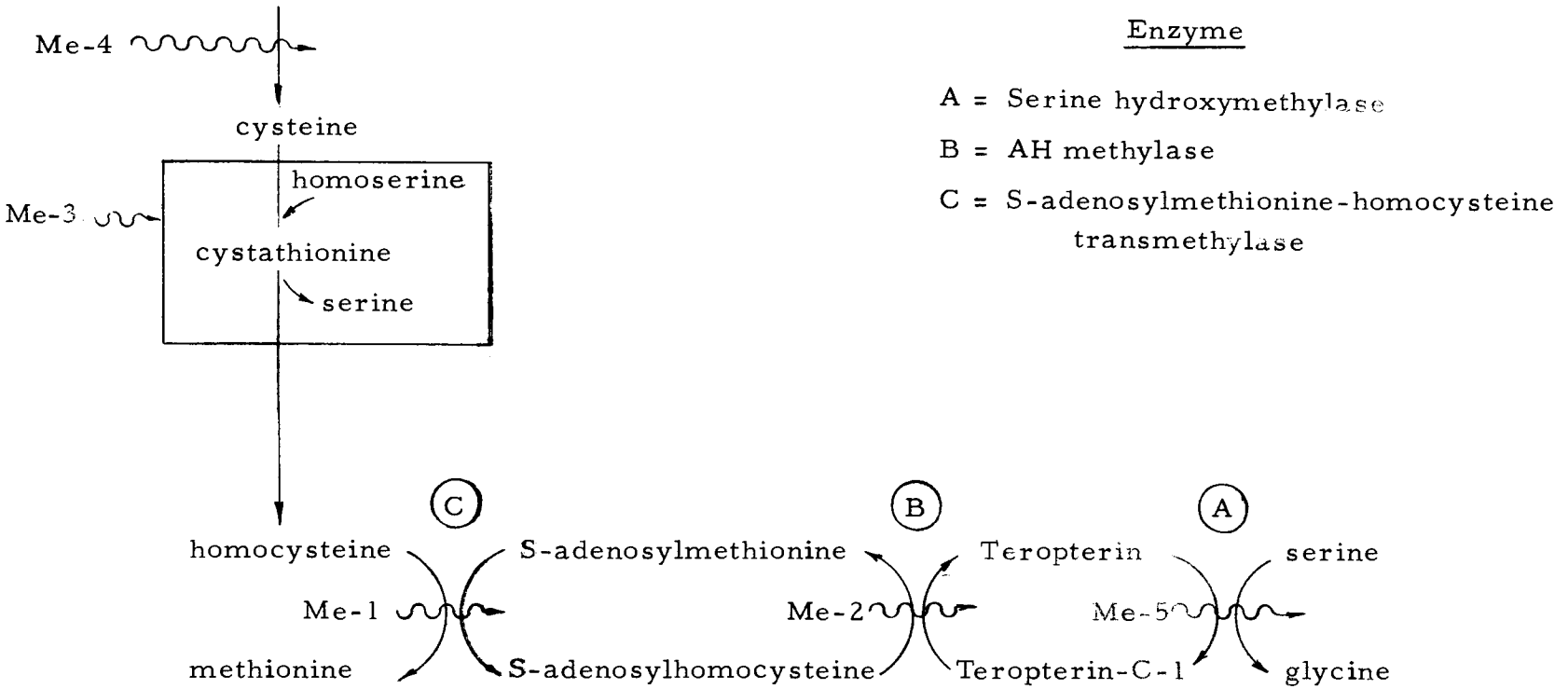


Figure 8. Terminal reactions in methionine biosynthesis in Saccharomyces cerevisiae. Mutant groups are located at the point of their enzymatic deficiencies with a wavy arrow.

to substitute suspected intermediates in methionine biosynthesis for their methionine requirement. Enzymatic analysis on these auxotrophs further divided the organisms designated as Me-2 mutants into two groups. The organisms which were found to be lacking in serine hydroxymethylase activity were classified as Me-5 mutants. The Me-1 group which includes organisms which were unable to utilize any supplement to replace their methionine requirement were found to be lacking in the S-adenosylmethionine-homocysteine transmethylase enzyme. These genetic and enzymatic data seem consistent with the biosynthetic scheme proposed. Although more definitive studies of the methyl group transfer are needed, it is quite clear that the Saccharomyces cerevisiae system is unique in nature. The results obtained in this study provide information in designing experiments for further extensive studies in the yeast system. Further studies in Saccharomyces cerevisiae system are warranted and will be of comparative biochemical significance in studies of amino acid biosynthesis.

SUMMARY

The terminal reactions of the biosynthetic pathway of methionine in Saccharomyces cerevisiae were studied in this project. Our results clearly indicate that the methylation of homocysteine to yield methionine is not a one-step process but that a number of enzymes and cofactors are involved. Utilizing serine as the ultimate carbon donor in a cell-free system, overall methionine synthesis was accomplished. A boiled extract and an eluate from a Sephadex column served as natural cofactors for methionine synthesis. A number of chemically defined cofactors were tested to determine if they could substitute for the natural cofactor involved. Methyl-tetrahydrofolate, methyl-dihydrofolate, methyl-B_{12}, tetrahydrofolic acid, and dihydrofolic acid all failed to stimulate the synthetic reactions and even appeared to inhibit synthesis. Teropterin served to stimulate methionine biosynthesis indicating that either Teropterin is the natural cofactor involved or that it may be converted to the natural cofactor in our cell-free system. The synthesis of methionine was strictly dependent upon the availability of S-adenosylhomocysteine, indicating that perhaps one means of S-adenosylmethionine formation was involved. AM which may be synthesized in two ways, may be formed by a direct methylation of AH. The other means of AM formation is catalyzed by AM synthetase and involves the reaction of methionine and adenosine triphosphate.

A study of the action of the AM synthetase enzyme was also performed. It was clearly shown that the AM synthetase is an inducible

enzyme in the presence of methionine.

In our earlier work enzymatic and nutritional studies on a number of methionine auxotrophs clearly indicated that five mutant groups could be designated. Some mutants which are unable to synthesize methionine are lacking the serine hydroxymethylase enzyme while another group lacks the S-adenosylmethionine-homocysteine transmethylase enzyme.

From our results it is clear that the mechanism of methionine biosynthesis in yeast possesses characteristics of both the vitamin B_{12}-dependent and the vitamin B_{12}-independent systems of Escherichia coli. Our data suggest that a predicted scheme including serine hydroxymethylase, Teropterin, S-adenosylhomocysteine, S-adenosylmethionine, and S-adenosylmethionine-homocysteine transmethylase may be operating.

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