ENZYMATIC STUDIES ON SOME METHIONINE AUXOTROPHS OF YEAST

by

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INTRODUCTION

Methionine is ubiquitously distributed in the proteins of all living systems. Since early studies showed that man is unable to synthesize methionine, it was classified as an essential amino acid. However, some microorganisms are capable of methionine biosynthesis, and much research in recent years has been devoted to elucidating the pathway involved. Work with Neurospora by a number of individuals has established the general sequence for methionine biosynthesis. Recent experiments on Saccharomyces suggest a somewhat altered mechanism of methionine formation in this organism.

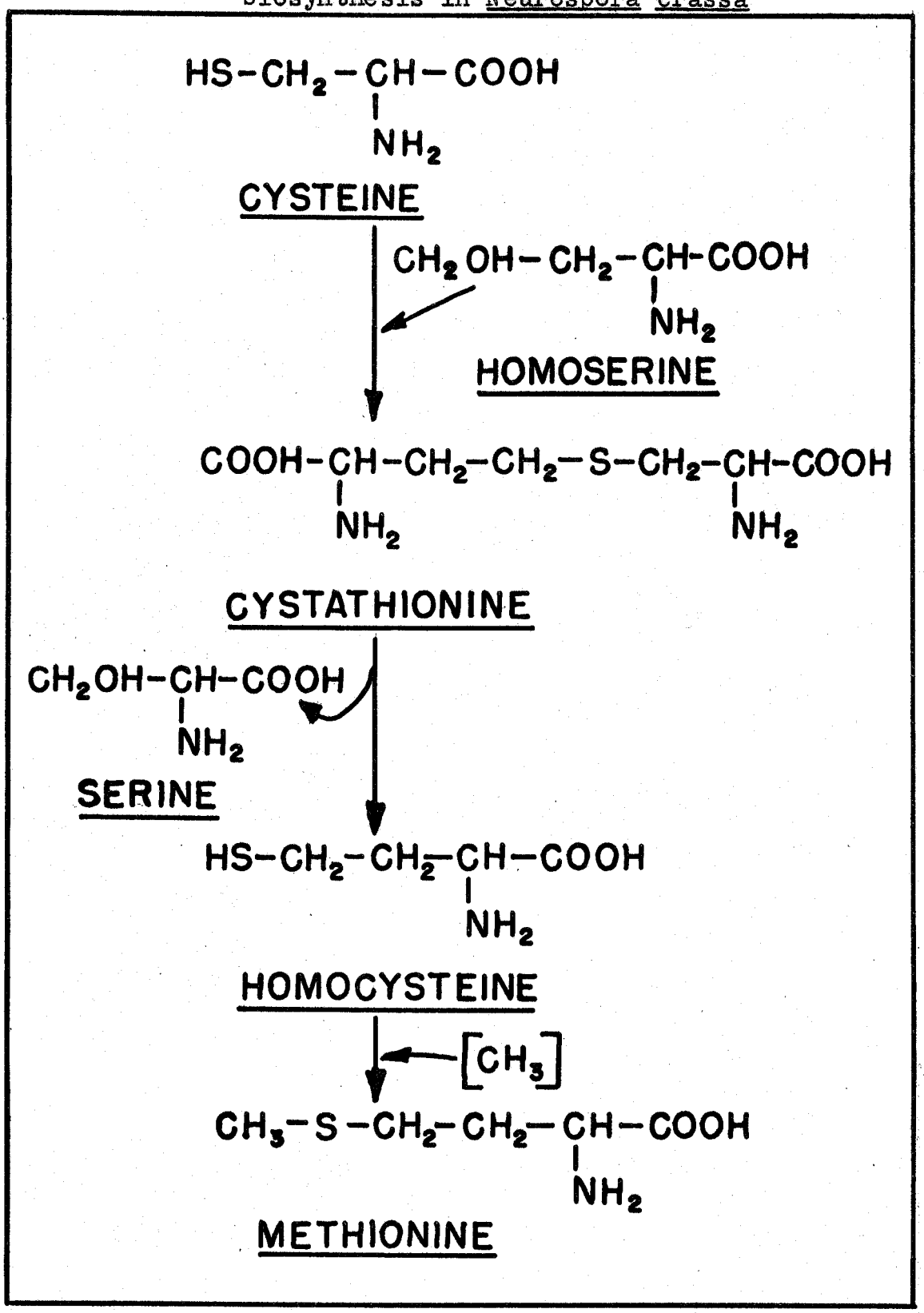
These experiments were initiated as a part of a broad program to investigate the biosynthesis of the sulfur-containing amino acids in yeast. They were designed to study the possibility that S-adenosylhomocysteine accepts methyl groups from reduced folic acid derivatives to form S-adenosylmethionine. The final reaction in methionine biosynthesis would then be a transfer of the "active" methyl group from S-adenosylmethionine to homocysteine to yield methionine.

REVIEW OF LITERATURE

The present state of knowledge on methionine biosynthesis has largely been contributed by studies with Neurospora crassa and Escherichia coli. In 1947, Horowitz observed that a large percentage of Neurospora mutants could not synthesize methionine, and these were classed as methionine auxotrophs (16, p. 255-264). He studied four strains which were unable to carry out the terminal steps of the biosynthesis. One mutant which could grow on homocysteine but not on cysteine, accumulated cystathionine in the medium. Another was unable to utilize homocysteine even with common methyl donors, such as choline or betaine, being supplied. However, since some of the other strains were able to use homocysteine, Horowitz suggested that methionine biosynthesis proceeded through a series of gene-controlled reactions involving intermediates including cysteine, cystathionine, and homocysteine. The pathway is shown in Figure 1.

Workers from the same laboratory (42, p. 651-658) obtained evidence the following year that in Neurospora, the sulfur atom of methionine is derived from cysteine. Homoserine was predicted to be the precursor of the four-carbon chain of cystathionine and therefore of the carbon-skeleton of methionine.

Figure 1. Terminal reactions in methionine biosynthesis in Neurospora crassa



During this same time, studies by Lampen et al. (20, p. 55-66) on Escherichia coli observed similar results on methionine biosynthesis. E. coli wild type organisms are normally able to synthesize their entire requirements of sulfur-containing substances from inorganic sulfur at any oxidation level. These workers obtained mutants which were blocked at various points along the proposed pathway, with the exception that no cystathionine-requiring mutants were obtained. Their work with syntrophism suggested that the pathway proceeded via cysteine ----> cystathionine ----> homocysteine ----> methionine. The authors were unable to suggest any mechanism for the conversion of cysteine to homocysteine except via cystathionine and offered the possibility that the cysteine to cystathionine path could be reversed. However, Davis (8, p. 271-275) points out that it is very possible that cystathionine is unable to penetrate the cell in growth experiments such as those done by Lampen's group.

Some recent evidence by Rowbury and Woods (30, 36 p.) supports the idea that cystathionine may be the immediate precursor of homocysteine. Cell-free preparations of E. coli contained cystathionase which was repressed during cell culture on methionine. Growth in the presence of 10 mmoles methionine repressed cystathionase activity by 80 per cent, while growth in homocysteine did not appear to

affect cystathionase production. This appears to be another example of repression of enzyme formation by the product of a sequence of reactions and offers presumptive evidence of a role for cystathionine in methionine biosynthesis in E. coli.

Although the pathway of methionine biosynthesis in microorganisms still was not completely defined, it was realized that methionine played an important role in the metabolism of all organisms. Borsook and Dubnoff (2, p. 363-375) showed that methionine could act as a methyl donor to guanidinoacetic acid in liver tissue slices, but that glucose, adenosine triphosphate (ATP), and aerobic conditions were necessary. The process of transferring the methyl group, known as transmethylation, appeared to be more complex than early nutritional experiments had indicated (11, p. 59-85).

Cantoni, in 1951 (4, p. 745-754), working on the biosynthesis of N-methyl nicotinamide from methionine and nicotinamide also realized that the process of transmethylation was not a simple one. He believed that the process consisted of a minimum of two steps, one of which involved the activation of methionine to form "active methionine". Soon afterwards Cantoni demonstrated that ATP could activate methionine in the presence of rat liver enzyme to yield a compound now commonly known as

S-adenosylmethionine (AM). He postulated the structure of the compound, and in 1953 succeeded in isolating AM (5, p. 403-416). The structure was confirmed by total synthesis (1, p. 4280-4284) and is shown in Figure 2. The synthesis of AM is a unique reaction in that ATP completely undergoes dephosphorylation to orthophosphate and pyrophosphate, while the sulfur of methionine becomes bound to adenosine.

The possible involvement of AM in methionine biosynthesis has been predicted but not extensively studied up to the present time. Schlenk and DePalma (32, p. 1051-1057) reported that yeast accumulate large amounts of AM. This has been used as an isolation source for AM, and has made it possible to obtain adequate quantities for study of AM metabolism in greater detail. Research on the metabolism of AM (37, p. 631-633; 27, p. 295-305) gives no evidence that it is enzymatically degradated to methionine.

Shapiro, in 1956 (35, p. 730-735), used cell-free extracts of Aerobacter aerogenes and Escherichia coli to synthesize methionine from homocysteine and methylmethionine. He also found that S-adenosylmethionine was able to replace methylmethionine to a limited extent in these systems. During these studies he used a modification of the nitroprusside test (22, p. 871-876) along with chromatography to identify the methionine produced.

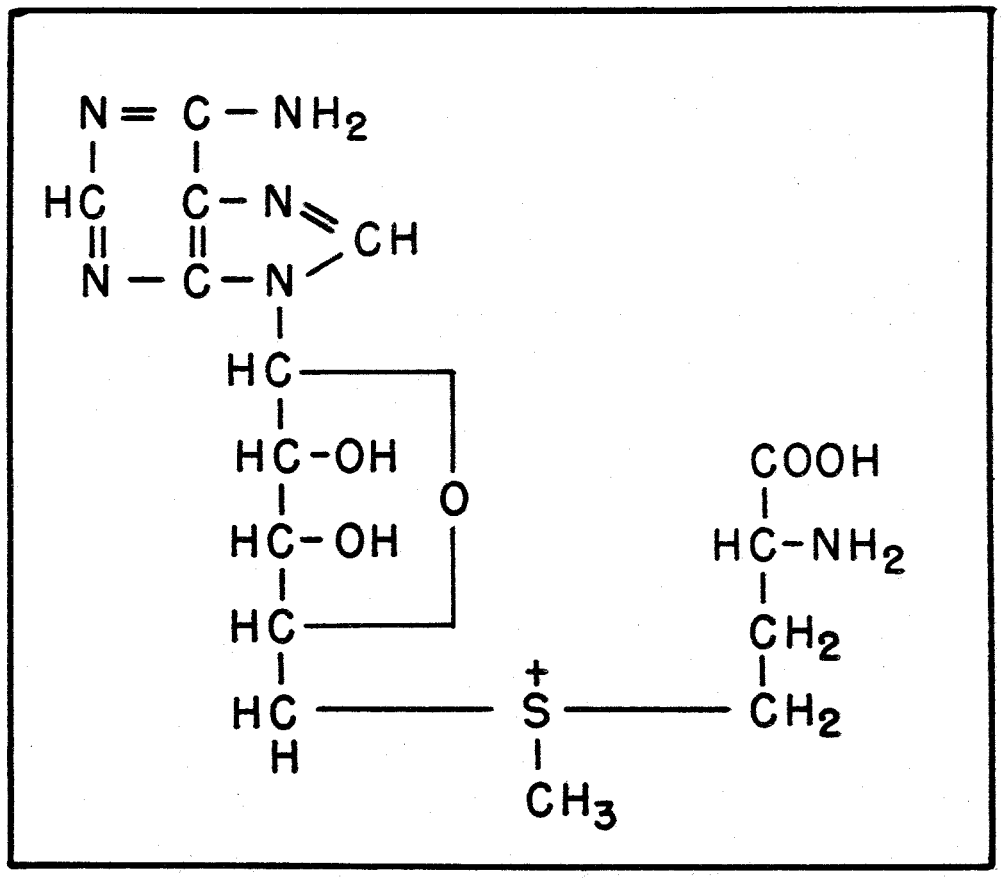


Figure 2. Structure of S-adenosylmethionine (AM)

Shapiro and Yphantis, in 1958 (38, p. 241-244), reported a new method to detect small amounts of methionine. The procedure combined a tracer assay with column chromatography to greatly increase the sensitivity. They observed methylation of homocysteine with AM in cell-free extracts of Aerobacter aerogenes, Saccharomyces cerevisiae, Candida utilis, and some strains of E. coli. The transmethylation reactions of AM are striking in that there appears to be an easy removal of the methyl group enzymatically, but it is very difficult to remove the group chemically (33, p. 65). Also, a large variety of methyl acceptors have been reported, forming C-C, C-N, C-S, and C-O bonds. This subject has been reviewed by Schlenk and Shapiro (34, p. 237-280).

From studies on the mechanism of the biosynthesis of the methyl group of methionine, it appears that folic acid derivatives and vitamin B_{12} are involved. Cross and Woods, in 1954 (7, p. xvi-xvii), studied the synthesis of methionine by cell-free extracts of an Escherichia coli mutant which required either glycine or serine for growth. They suggested that the β-carbon of serine was converted to methionine. Further work (14, 26 p.) indicated that optimum synthesis of methionine from homocysteine and serine required a heated extract of E. coli (HEC) plus vitamin B_{12}, and that tetrahydrofolic acid (FH_4) under

anaerobic conditions could replace the heated extract. Glucose, adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), and vitamin B_{12} still were required for optimum synthesis. Early work with Clostridium sticklandii showed that a heated extract of this organism had greater activity in the serine to glycine reaction; the stimulatory component was called "Co C." Woods and coworkers (40, p. 3-12) found that "Co C" was active in methionine synthesis with E. coli, and that the HEC was active for the serine to glycine reaction of the Clostridium. However, all they were able to determine at this time was that the heated extract of E. coli contained a folic acid derivative.

In a recent article, Jones et al. (18, p. 566-574) found that there were definite differences in the FH_4 and the heated extract of E. coli. The serine to methionine reaction is competitively inhibited by FH_4, and it was observed that FH_4-triglutamates resemble the heated extract. They suggested that the N^5-formyl group of reduced folic acid, Figure 3, is concerned with the attachment of the cofactor to the apoenzyme, and that it is the N^{10}-position which is active in the transfer of the one-carbon unit.

Wijesundera and Woods first reported (47, p. 229-241) repression of methionine synthesis in E. coli by growth in methionine. Further studies by Rowbury and Woods in

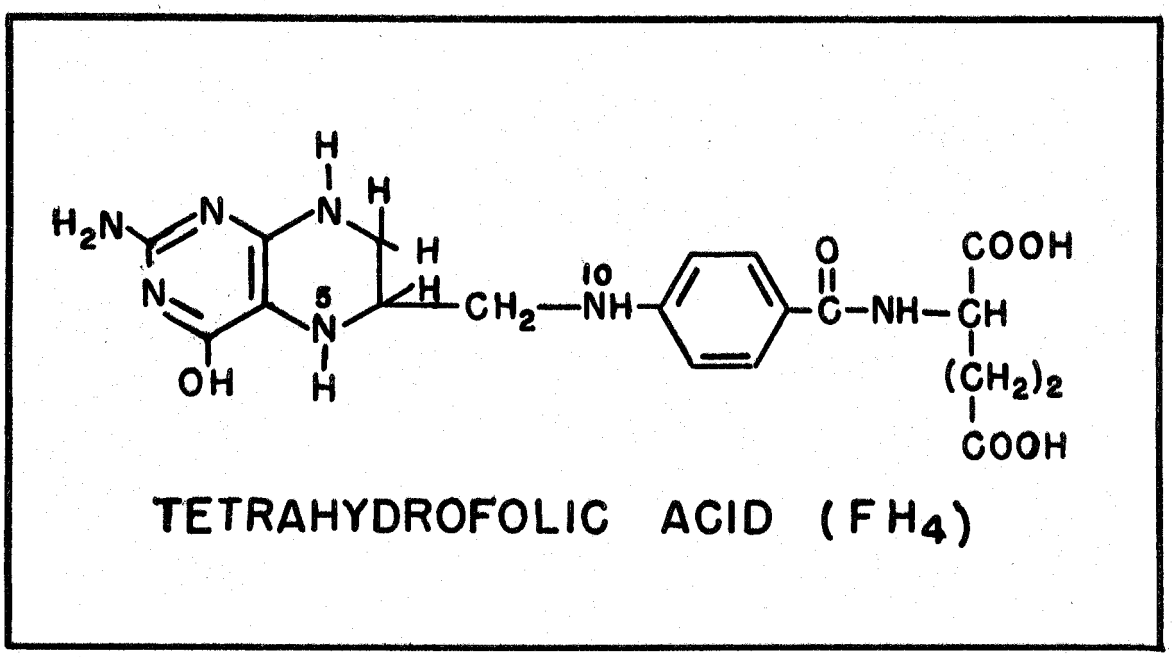


Figure 3. Structure of tetrahydrofolic acid (FH_4)

1961 (29, p. 129-144) determined that an "inactive" organism, or an organism grown in the presence of methionine, could regain activity when grown without methionine. They established that the formation of the enzyme required de novo synthesis since the synthesis was inhibited by chloramphenicol and was also greatly diminished when the supply of amino acids was restricted. A mixture of serine, homocysteine, and vitamin B_{12} also repressed enzyme formation after a short lag.

Nakao and Greenberg (23, p. 603-620) found that labeled serine or formaldehyde is converted to the methyl group of methionine in cell-free enzyme systems from sheep liver. They showed that the presence of methionine exerted an activating effect on this conversion which was not due to a simple isotopic exchange reaction. When S-adenosylmethionine was added to the system, the synthesis of methionine was inhibited. This suggested to the authors that there was a possibility that AM is the initial product formed.

The complexity of the cofactors required predicts that the methylation of homocysteine involves several steps. Work by the investigators at Oxford has been unable to detect any individual steps by the fractionation of the enzymatic extract (40, p. 3-12). However, Hatch et al.

(13, p. 1095-1101) reported a system of three partially purified enzyme fractions which are essential for methionine biosynthesis in a mutant of Escherichia coli requiring either methionine or vitamin B_{12} for growth. They report that one of the required enzymes, which was identified as serine hydroxymethylase, catalyzes the initial step in the synthesis of the methyl group. A one-carbon unit is transferred to FH_4 at the hydroxymethyl level of oxidation. It is then reduced to the methyl level of oxidation and transferred to homocysteine. The other enzymatic fractions obtained were tentatively designated as "B_{12} enzyme" and "205-2 enzyme". A combination of the three enzymatic fractions carried out methionine synthesis if serine, homocysteine, pyridoxal phosphate, FH_4, ATP, reduced DPN, and flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) were added. They observed that S-adenosylmethionine increased methionine synthesis slightly when added to the complete reaction mixture, but it was unable to completely replace the normal substrates.

At the present time, it appears that two aspects of methionine biosynthesis in microorganisms need further study. (1) The details of the formation of homocysteine have not been completely studied and (2) knowledge of the actual conversion of homocysteine to methionine is still

not understood. Shapiro and Schlenk (34, p. 237-280) in their recent review on sulfonium compounds point out that resolution of the actual source of the methyl group of methionine must wait until purified enzyme systems permit complete investigations. It is toward this end that the present study is directed.

METHODS AND MATERIALS

Cultures and Cultural Media

Active cultures

All organisms used in this study were strains of Saccharomyces cerevisiae. MCC, a wild type diploid strain, was obtained from the active culture collection of the Department of Microbiology, Oregon State University. A uracil requiring strain, #3701B, was obtained from Dr. D. C. Hawthorne. Some methionine auxotrophs were prepared by K. Spence from #3701B by ultraviolet irradiation and were designated as #80BM30, #50XM2, #50M5, #80BM1, #S288C18, #45M92, and #820M10. Other methionine requiring yeasts were obtained from the following individuals: #5036D, #5015D, #5011B, #5812, #5815, and #4987B from Dr. H. Roman; #8090 and #8082 from Dr. C. C. Lindegren; #22B, Dr. R. K. Mortimer; and #62-28, Dr. S. Pomper. The organisms were maintained at 4°C on agar slants of yeast complete medium (Table 1).

Commercial yeast

Fleischman's commercial active dry Baker's yeast was obtained from Standard Brands, Inc. and stored under vacuum at 2-5°C until ready for use.

TABLE 1

Composition of Yeast Complete Medium (YCM)

Component	Per Cent
Glucose	2.0
Tryptone	2.0
Yeast extract	1.0
*Agar	1.5

* If solid medium is desired

Cultural media

Yeast complete medium, Table 1, and modified Wickerham's medium (45, p. 294-295), Table 2, were used in this study.

Preparation of Cell-free Extracts

Active cultures

Active cultures were grown in yeast complete medium at 30°C on a rotary shaker for 40 to 48 hours. The organisms were then harvested and washed two times with 0.01 M phosphate buffer. The buffers used throughout the preparation were either at pH 6.7 or pH 7.5, depending upon the enzymatic assay to be performed. Recovered cells were then resuspended in 0.1 M KH_2PO_4 plus one per cent glucose, and vigorously aerated for two to three hours at 30°C. Immediately following aeration the organisms were again harvested, washed, and resuspended in an equal volume of buffer. Cell disintegration was accomplished by sonic treatment for thirty minutes in a Raytheon 10-kc model DF-101 sonic oscillator. In order to remove most of the cell debris, the sonic extracts were first centrifuged at approximately 20,000 x G for twenty minutes in the cold. Further centrifugation was performed in the Spinco model L refrigerated ultracentrifuge at 100,000-104,000 x G for

TABLE 2

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

* Agar added if solid medium is desired.

thirty minutes. The supernatant fraction exclusive of the lipid layer was treated as the enzymatic extract. All enzyme preparations were stored at -20°C until immediately prior to use.

Dialysis treatment was performed by enclosing the extract in Dexstar casing and dialyzing against twenty to thirty volumes of 0.02 M phosphate buffer overnight with stirring at 2-5°C.

Commercial yeast

Commercial active dry yeast, suspended in 0.1 M phosphate buffer, was sonically disintegrated and treated as described above.

Boiled extracts

A fresh enzymatic preparation was placed in a boiling water bath for ten minutes. The solution was then cooled immediately 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 enzymatic extracts was measured spectrophotometrically with the Folin-Ciocalteu phenol reagent according to the method of Lowry

et al. (21, p. 265-275). Crystalline bovine albumin (The Armour Laboratories) was used as the protein standard.

Chromatographic Procedures

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 at approximately 50°C. 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. Five-tenths per cent ninhydrin in butanol was sprayed on the chromatograms; these were heated to hasten color development, and the separated amino acid spots observed. Chloroplatinate reagent (43, p. 824) was used to detect sulfur-containing compounds.

Ion-exchange resin chromatography

Columns were prepared by adding 0.1 g of Dowex 50W, in the H^+ form, to a sintered glass funnel of one cm

internal diameter. To prepare columns in the Li^+ form, five ml of a 25 per cent solution of LiCl was washed through the column, followed by a ten ml distilled water rinse.

Liquid Scintillation Counting

The automatic Packard Tri-Carb Liquid Scintillation Spectrometer, model 314-DC, was used for carbon-14 assays. Two scintillation fluid mixtures were used. One consisted of 0.4 per cent 2,5-diphenyl oxazole (PPO) in 48 per cent ethanol and 52 per cent reagent grade toluene. A second counting fluid was identical to the first except it was supplemented with 0.02 per cent POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene). POPOP, PPO, and counting vials were obtained from the Packard Instrument Company, LaGrange, Illinois.

Preparation of Substrates

S-Adenosylmethionine

S-Adenosylmethionine (AM) was synthesized from ATP and l-methionine using the method of Cantoni (6, p. 58-61), but incorporating the modification of Bremer and Greenberg (3, p. 206). The reaction was catalyzed by the methionine activating enzyme isolated from rabbit liver. AM was isolated as the crystalline trireineckate and then extracted

with various organic solvents. The purity of the AM was determined 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 (32, p. 1055) as the extinction coefficient. Radioactive AM was prepared by using l-methionine methyl-C-14 (Volk Radio-Chemical Company, Chicago, Illinois) in the reaction mixture.

5'-Methylthioadenosine

5'-Methylthioadenosine (MTA) was prepared by boiling S-adenosylmethionine, pH 7.0, for ten minutes (27, p. 302).

L-Homocysteine

L-Homocysteine was prepared daily. 30.8 mg l-homocysteine-thiolactone was dissolved in a small amount of distilled water, adjusted to pH 8.5 with 1 M tris-hydroxymethylamino methane, and then made to 1.0 ml volume with distilled water. The treated homocysteine was allowed to stand at room temperature for ten minutes before use.

S-Adenosylhomocysteine

S-Adenosylhomocysteine (AH) was prepared using the rat liver enzyme method of de la Haba and Cantoni (9, p. 603-608) as modified by Duerre (10).

Tetrahydrofolic acid

Tetrahydrofolic acid (FH_4) was prepared by a modification of the method of Silverman and Noronha (39, p. 180-182) 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 (N, N-Diethylaminoethylcellulose) and Hyflo Super Cel (Fisher Scientific Company). Silverman and Noronha protected the FH_4 from air oxidation with one per cent mercaptoethanol; however, results obtained in this investigation showed the mercaptoethanol was not suitable in the system studied. Better yields of FH_4 were obtained by using cysteine in place of mercaptoethanol. The purity of the FH_4 was determined spectrophotometrically in a Beckman DU and a Cary No. 11 Recording Spectrophotometer. FH_4 has a maximum absorption at 297 mμ (17, p. 379). FH_4 was stored at -20°C in 0.1 M phosphate buffer, pH 7.5, plus one per cent cysteine. Immediately prior to use, the solution was allowed to thaw and then placed in ice until used. The tetrahydrofolic acid was always used within one week of preparation.

Reagents

All solutions were made from reagent grade chemicals

and dissolved in distilled water. The glassware used was cleaned with dichromate solution and rinsed with distilled water.

Experimental Procedures

Mutant mapping

Feeding techniques were employed to determine the specific blocks of the methionine auxotrophs. Mutants were grown in either YCM broth or modified Wickerham's complete medium for 40-48 hours at 30°C to obtain good cell growth. The organisms were then harvested, washed carefully twice with sterile distilled water, and resuspended in the appropriate dilutions of sterile distilled water. Five-tenths of a ml of the diluted suspension was pipetted into a sterile petri dish, fifteen to twenty ml of modified Wickerham's complete less methionine medium plus agar added, and after sufficient mixing, the agar was allowed to solidify. Solutions of homocysteine, S-methyl-cysteine, cysteic acid, cysteine, ergothionine, cystathionine, methionine, ethionine, serine, and homoserine were prepared from reagent grade chemicals. Using sterile technique, sterile filter tabs were charged with the appropriate feeding supplement and then placed on the surface of the agar plate. Cultures were incubated at 30°C and checked for growth at 24, 48, and 72 hours.

Additional growth tests were performed to see if S-adenosylmethionine, S-adenosylhomocysteine, and 5'-methylthioadenosine would support growth. The solutions of AM, AH, and MTA were chromatographed as previously described, the quenching-spots were observed with the ultraviolet lamp, marked, and excised. The filter strips were placed on the inoculated testing agar plates, and the organisms incubated as previously described.

Transmethylase assay

The assay procedure is based on the fact that S-adenosylmethionine having a strong basic charge (32, p. 1057) is easily retained on a strongly-acidic cation exchange resin, while methionine is not. The reaction mixture of the methyl acceptor, AM ($C^{14}H_3$), enzyme, and buffer is incubated under the desired conditions. After the designated time, a small aliquot of the reaction mixture is carefully placed onto the column, the column rinsed with the desired eluant, and the eluate collected for liquid scintillation counting.

The complete reaction mixture included 20 μmoles treated homocysteine, 5 μmoles radioactive S-adenosylmethionine, 50 μmoles phosphate buffer, pH 6.7, and the enzymatic preparation in a total volume of 1.0 ml. The enzyme was prepared in phosphate buffer at pH 6.7. After

the reaction mixture was incubated at 35°C for 45 minutes, a 0.2 ml aliquot was layered carefully onto a Dowex 50 (Li^+ form) column followed by 0.3 ml of a 0.1 N H_2SO_4 rinse. The total eluate was collected in a scintillation vial, and 19.5 ml. of scintillation fluid added. The counting solution was placed in the deep-freeze for dark adaptation and then transferred to the turntable of the automatic Packard Tri-Carb Liquid Scintillation Spectometer. Each sample was counted at least three times, and an average value obtained.

The specific activity of the enzyme was expressed as the number of counts per minute per mg protein per 45 minutes.

Serine hydroxymethylase assay

Serine hydroxymethylase activity was observed using the method of Whiteley (44, p. 233) with a slight modification. The extent of the reaction was determined spectrophotometrically following formaldehyde disappearance under conditions such that the amount of formaldehyde consumed in the reaction equaled the amount of serine synthesized. Formaldehyde was determined by the method of Nash (24, p. 416-421) using monomethylol dimethyl hydantoin as the formaldehyde standard. The standard was obtained from Dr. H. R. Whiteley.

In this assay, FH_4 and formaldehyde react to form N^5, N^{10}-methylene FH_4, which donates the one carbon unit to glycine in the presence of serine hydroxymethylase. At the end of the incubation period, perchloric acid is added to break down any remaining N^5, N^{10}-methylene FH_4 to FH_4 plus formaldehyde so that the formaldehyde disappearing is exactly the amount used in the synthesis of serine.

The complete reaction mixture included 0.2 ml FH_4 solution, 5 μmoles formaldehyde, and 80 μmoles phosphate buffer, pH 7.5, which is preincubated at 30°C for ten minutes. Fifty μmoles glycine, 10 μgrams pyridoxal phosphate, and the enzymatic preparation were then added to give a total volume of 1.2 ml, and the reaction mixture was reincubated at 30°C for an additional ten minutes. Three-tenths ml of 3.5 per cent perchloric acid was added to stop the reaction. The mixture was centrifuged at approximately 2000 x G for five minutes to remove the denatured protein. Two-tenths ml of the supernatant was assayed for formaldehyde using the Nash reagent, and absorption of the colorimetric product was determined at 412 mμ in the Beckman DU spectrophotometer. Formaldehyde standards were run concurrently, and the amount of formaldehyde present calculated from a standard curve. Control tubes were also run less glycine, less FH_4, and less

formaldehyde. Corrections were made to account for the disappearance of formaldehyde in the absence of a suitable one-carbon acceptor. Corrections were also made for the small amount of absorbance observed in tubes without formaldehyde. An additional control was included which contained all the components but in which the reaction was stopped before enzyme was added by the addition of perchloric acid.

The specific activity of the enzyme was expressed as the number of mμmoles serine synthesized per mg of protein per ten minutes.

METHODOLOGICAL STUDIES

Transmethylase Assay

The sensitivity of the assay for the methylation of homocysteine with S-adenosylmethionine as the methyl donor is dependent upon the analytical techniques employed to detect the final product, methionine. Until recently, the nitroprusside colorimetric test (22, p. 871-876) was used to assay transmethylase activity. However, the maximum sensitivity of the method is only about 0.2 µmoles methionine per ml. Shapiro and Yphantis (38, p. 241) report that the test is difficult to adapt to the particular enzyme system under study. These workers at Argonne National Laboratory have developed a tracer assay combined with the aid of column chromatography which increased the sensitivity 1000-fold (33, p. 64). Modifications of this procedure were devised in this study, and it was determined that 50 to 60 per cent of the methionine formed in the reaction could be detected by the modified procedure. Shapiro and Yphantis (38, p. 242) claimed a 40 per cent recovery; however, only 15 per cent recovery was routinely obtained in this laboratory without the modifications described below.

It was necessary to determine the proper conditions

which must be met so that the radioactive methyl donor would be retained on the column, while the radioactive product could be washed through. In order to determine the size and type of the column to be used, known radioactive substrates were assayed, and the per cent recovery in the eluate calculated. Two types of Dowex 50W (Li^+ form) resins were checked in this study: 100-200 mesh, eight per cent cross linked, and 50-100 mesh, eight per cent cross linked. The amount of resin used was carefully weighed, and the influence of different eluants on the amount of methionine recovered was analyzed. Distilled water, 0.1 N H_2SO_4, and 0.1 M methionine were used to rinse the columns. From the results of these experiments which are shown in Table 3, 0.1 g Dowex 50W, 50-100 mesh, with sulfuric acid as the eluant was most suitable for the remainder of the assays.

Shapiro and Yphantis reported that the columns were washed with three aliquots of 0.25 ml distilled water, to give a total of 0.95 ml aqueous solution for counting. It was observed in this study that the large amount of water in the scintillation fluid resulted in considerable quenching. The scintillation fluid mixtures used herein could have a maximum of five per cent water in solution (25, p. III-7). The counting solutions used by Shapiro and Yphantis included 0.4 per cent PPO in an equal volume of

TABLE 3

Recovery of Radioactive Samples
From Ion Exchange Resin Columns

Resin size in g*	Mesh size	Eluant	Per cent recovery	
			Methionine	S-adenosyl-methionine
0.3	50-100	0.1 M methionine	5	
0.3	50-100	0.1 N H_2SO_4	6	
0.3	100-200	0.1 M methionine	1	
0.3	100-200	0.1 N H_2SO_4	1	
0.2	50-100	H_2O	15	4
0.2	50-100	0.1 N H_2SO_4	25	3
0.1	50-100	H_2O	40	8
0.1	50-100	0.1 N H_2SO_4	55	5

* Dowex 50W prepared in Li^+ form.

toluene and alcohol. Results obtained in this study indicated that the addition of a small quantity of POPOP, a "secondary fluor", increased the counting efficiency by about 25 per cent as shown on Table 4.

Serine Hydroxymethylase Assay

Tetrahydrofolic acid, prepared by the method of Silverman and Noronha (39, p. 180-182), was used in the early work on serine hydroxymethylase. This procedure involved the use of one per cent mercaptoethanol to protect FH_4 from air oxidation. However, difficulties were observed with this preparation in that an inhibition of the reaction by this concentration of mercaptoethanol was observed as shown in Table 5. This finding instigated a study of the effect of other reducing agents upon the activity of active dry yeast enzymatic preparations. Cysteine, adjusted to pH 7.0, at 0.1 per cent concentration was found to give sufficient protection to the FH_4 preparation and at this concentration did not interfere with the serine hydroxymethylase assay (Table 5).

TABLE 4

Effect of Fluor on Scintillation Counter Response

Radioactive Sample*	Scintillation Fluid Mixture**	Counts per Minute	Per cent Increase
methionine	0.4% PPO	333	22
methionine	0.4% PPO + 0.02% POPOP	433	
S-adenosyl-methionine	0.4% PPO	7,780	33
S-adenosyl-methionine	0.4% PPO + 0.02% POPOP	11,570	

* 0.5 ml aqueous solution in 19.5 ml scintillation fluid.

** In 48 per cent ethanol and 52 per cent toluene.

TABLE 5

Effect of Reducing Agents on Serine Hydroxymethylase Activity of Saccharomyces

Supplement Added*	Specific Activity**
FH_4 in 1 per cent mercaptoethanol	10
1.6 mg mercaptoethanol	10
0.16 mg mercaptoethanol	40
FH_4 in 0.1 per cent cysteine	40
0.1 per cent cysteine	60
none	60

* Each reaction vessel contained formaldehyde, glycine, pyridoxal phosphate, buffer, and active dry yeast cell-free preparation.

** Expressed as mμmoles serine synthesized/mg protein/ 10 minute incubation period.

RESULTS

As shown in Table 6, the growth requirements of the methionine auxotrophs varied but seemed to group into four distinct categories: (1) those organisms which were able to utilize cysteine, homocysteine, S-adenosylmethionine, and S-adenosylhomocysteine to fulfill their methionine requirements, (2) those which were able to utilize only homocysteine, AM, and AH in place of methionine, (3) those able to replace methionine with AM only, and (4) one organism which appears to be able to grow only in the presence of methionine. For convenience, the organisms in group 1 were designated as "cysteine" mutants; those in group two as "homocysteine" auxotrophs; and those in groups three and four as "methionine" mutants. It is interesting to note that none of the organisms studied was able to grow on cystathionine or ethionine.

Cell-free preparations of strain MCC were studied to determine the best conditions for transmethylase activity. The effect of dialysis treatment, as shown on Table 7, shows about a forty per cent increase in specific activity after dialysis. The dialysed treated preparations were used throughout the remainder of the assays.

The specific activities of the methionine auxotrophs tested, shown in Table 8, appear to vary over a wide range.

TABLE 6

Growth Requirements of Methionine Auxotrophs of *Saccharomyces cerevisiae*

Solutions*	Auxotrophs			
	#62-28	#22B, #5813, #5812, #4987B, #45M91, #820M10, #80BM1	#8090, #5015D, #5011B, #5036D, #8082, #5288-C18	#50M5, #50XM2, #80BM30
l-methionine	+	+	+	+
l-homocysteine-thiolactone	-	-	+	+
dl + allo-cystathionine	-	-	-	-
l-cysteine	-	-	-	+
S-adenosylmethionine	-	+	+	+
S-adenosylhomocysteine	-	-	+	+

* Also tested were l-cysteic acid, dl homoserine, dl serine, S-methylcysteine, l-ergothionine, ethionine, and 5'methylthioadenosine; however, none of the mutants studied could utilize any of these components to satisfy the methionine requirement.

TABLE 7

Effect of Dialysis on Transmethylase Activity of *Saccharomyces cerevisiae* Strain MCC

Preparation	Specific Activity*
non-dialyzed, pH 6.7	780
dialyzed, pH 6.7	2000

* Expressed as the number of counts per minute/mg protein/45 minute incubation period.

TABLE 8

Transmethylase Activity of *Saccharomyces cerevisiae* Strains

Organism	Mutant Designation*	Specific Activity**
80BM30	cysteine	1770
50XM2	cysteine	590
50M5	cysteine	2470
5011B	homocysteine	1370
8082	homocysteine	1920
5015D	homocysteine	1230
80BM1	methionine	2160
22B	methionine	1570
5812	methionine	1170
62-28	methionine	0
MCC	wild type	2000
3701B	wild type	1230

* As determined by feeding techniques.

** Expressed as the number of counts per minute/mg protein /45 minute incubation period.

Only the mutant that has a specific requirement for methionine was without any activity, that is, was completely incapable of methionine synthesis from homocysteine.

Dialysis treatment completely eliminated serine hydroxymethylase activity with dry yeast preparations. Partial recovery was obtained by adding FH_4, a boiled extract of MCC or of active dry yeast, and Mn^{++} as shown in Table 9. The enzyme appears to be very sensitive to freezing and thawing, since several repetitions of this procedure significantly decreased activity. All assays on mutants were made within ten days of preparation, and the cell-free preparations were frozen and thawed only one time.

One interesting point is the MCC preparations appeared to show more activity when FH_4 was omitted from the reaction mixture (Table 10). The type of reducing agent present had no effect on this observation. Strain #3701B and all mutants tested failed to show this effect.

Mutants were tested using non-dialyzed preparations, and supplemented with either FH_4 prepared in 0.1 per cent cysteine or with a boiled extract of an MCC preparation. A boiled extract of a dialyzed MCC preparation was used as a control. The specific activities of the mutants are also shown in Table 10.

TABLE 9

Effect of Dialysis on Serine Hydroxymethylase Activity of Saccharomyces

Supplement Added*	Specific Activity**
none	0
FH_4 in 0.1% cysteine	0
FH_4 in 0.1% cysteine + 1 µmole Mn++	20
FH_4 in 0.1% cysteine + 1 µmole Mn++ + boiled extract***	30
boiled extract + 1 µmole Mn++	40

* Each reaction vessel contained formaldehyde, glycine, pyridoxal phosphate, buffer, and active dry yeast cell-free preparation.

** Expressed as mµmoles serine synthesized/mg protein/10 minute incubation period.

*** Boiled extract prepared from dry yeast cell-free extracts.

TABLE 10

Serine Hydroxymethylase Activity of *Saccharomyces cerevisiae* Strains

Organism	Mutant designation*	Specific Activity[x]		
		Complete mixture**	Less FH_4	Supplemented with boiled extract***
62-28	methionine	0	0	40
22B	methionine	0	0	0
5812	methionine	0	0	130
80BM1	methionine	0	0	40
8082	homocysteine	30	0	80
5015D	homocysteine	90	0	110
5011B	homocysteine	0	0	60
50XM2	cysteine	100	0	80
80BM30	cysteine	0	0	30
50M5	cysteine	0	0	230
MCC	wild type	20	30	30
3701B	wild type	0	0	60

* As determined by feeding experiments.

** Contains formaldehyde, glycine, pyridoxal phosphate, enzyme, buffer, and FH_4 prepared in 0.1 per cent cysteine.

*** Boiled extract prepared from MCC cell-free extracts.

x Expressed as mumoles serine synthesized/mg protein/10 minute incubation period.

DISCUSSION

Predictions of the actual blocks incurred by the auxotrophs on the proposed pathway of methionine biosynthesis were made from the feeding experimental data (Figure 4). From these data the possible intermediates in the pathway can be visualized. However, there are several limiting factors involved. It is conceivable that the compound utilized is not actually metabolized as such, but is converted to another form which is the true intermediate. That is, from the data observed, it is impossible to determine if homocysteine is used as such or if it is first converted to another compound, such as S-adenosylhomocysteine, before it satisfies the methionine requirement. Cell permeability may also limit the utilization of exogenously supplied intermediates. If a compound is not utilized, it may be possible that the cell is unable to get the compound inside in the form that it is supplied. In spite of these two factors, the data offer suggestive evidence that the compounds which satisfy the methionine requirement are closely allied to the actual pathway.

As noted, none of the yeast mutants grew on cystathionine. This effect has been observed in Escherichia coli (20, p. 55-66) and Saccharomyces cerevisiae (28, p. 666-670). Davis (8, p. 271-275) speculates that this may

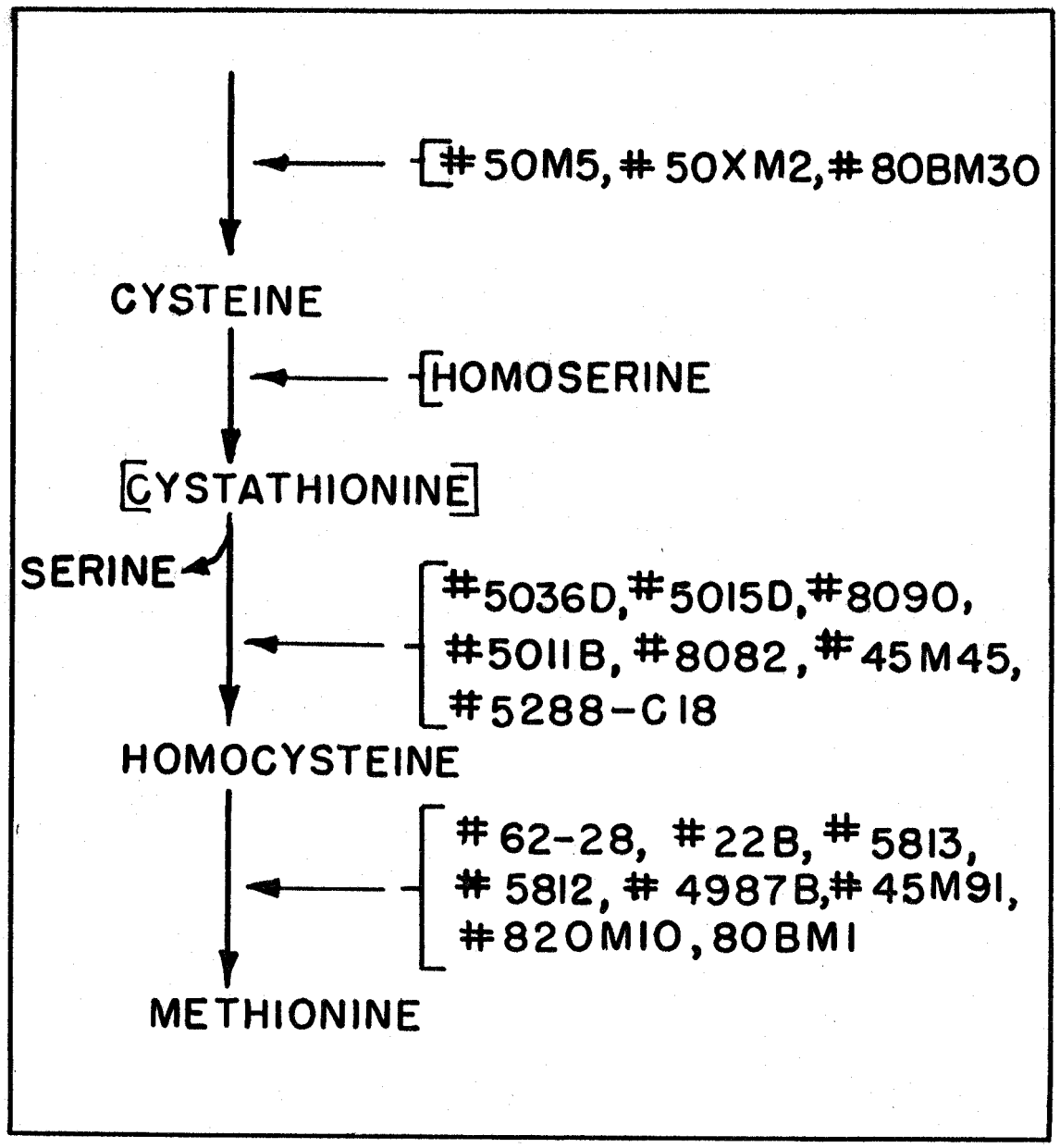


Figure 4. Predicted block of yeast methionine auxotrophs in the methionine biosynthetic pathway

be due to a cell permeability barrier. This appears to be true in the E. coli system now, especially in view of the recent finding of repressed cystathionase formation (30, 36 p.). Although it is reasonable to assume that cystathionine is unable to get into the cell, the possibility that cystathionine is not involved in the methionine biosynthetic pathway of yeast can not be ruled out. In Figure 4, genetic lesions were mapped as occurring after cystathionine assuming cystathionine to be on the pathway. This was done only because the organisms did not utilize homoserine. From the data observed, the actual block incurred is not able to be mapped until more information is obtained on cystathionine. Since the main interest of this study was the actual mechanism of the methylation of homocysteine, the conversion of cysteine to homocysteine was not studied intensively.

The suppression of yeast growth by ethionine has been observed earlier in yeast cultures (26, p. 169-176). Parks observed that no one specific reaction is responsible for the inhibition.

Presumptive evidence obtained in the mapping of the auxotrophs offered the possibility that the terminal reaction in methionine biosynthesis may be a transmethylation of homocysteine by S-adenosylmethionine. Auxotroph #62-28, which was unique among the group of mutants in this study

in that it was able to utilize only methionine, offered a chance to test this hypothesis. From the enzymatic data it is clear that this organism is unable to accomplish the transmethylase reaction. This further suggests that the final step in methionine biosynthesis in yeast involves the methylation of homocysteine by AM.

All of the other mutants tested showed transmethylase activity. This agrees with the earlier observation that the other mutants were able to utilize AM to satisfy their methionine requirement.

A careful analysis of the feeding experimental data reveals another possibility for support of the predicted mechanism for methionine synthesis. Mutants #22B, #5812, and #80BM1, which can utilize only methionine and AM, should be incapable of the methylation of S-adenosylhomocysteine. One reaction involved in the conversion of the β-carbon of serine to AH would involve the serine hydroxymethylase assay. Analyses of cell-free preparations of the auxotrophs showed that one organism was deficient in this reaction. Auxotroph #22B lacks serine hydroxymethylase and as such is incapable of the methylation of AH. Other reactions effecting the conversion of AH to AM will be the subject of further investigations.

Serine hydroxymethylase of MCC has less activity with tetrahydrofolic acid present, than when the enzyme is

unsupplemented with the folic acid cofactor. That MCC has a cofactor other than FH_4 which is involved in the serine hydroxymethylase reaction is a logical conclusion. Added FH_4 may compete with the natural cofactor at the reaction site. It is also noted that there is more activity restored to the dialyzed preparation upon the addition of boiled extract than upon the addition of FH_4. The Oxford group (29, p. 129-144) recently reported that FH_4 inhibits the serine to methionine reaction studied in E. coli, and it was clearly evident that the heated extract of E. coli was supplying something other than FH_4. Strain MCC does appear to be different from the other yeast strains observed in this study as the data in Table 10 indicated. None of the methionine auxotrophs tested had any serine hydroxymethylase activity unless some form of FH_4 was added.

The data obtained on the methionine auxotrophs also seem to indicate the possibility that FH_4 is not the natural cofactor involved. A number of organisms showed no activity with FH_4 but did have activity with a boiled extract of MCC. Others showed an increase in activity upon the addition of BE. Due to the fact that no reducing agents were employed in the preparation of the boiled extract, no FH_4 should be present in the supplement. The organisms which were able to utilize FH_4 may have a mechanism to convert it to the natural cofactor involved.

It is suggested from the data observed in this study that the methylation of homocysteine in yeast involves a number of reactions. The initial reaction is proposed to involve serine hydroxymethylase to yield an active one-carbon unit as a reduced folic acid derivative. This unit is utilized in the methylation of S-adenosylhomocysteine to yield S-adenosylmethionine which then converts homocysteine to methionine by the transmethylase enzyme. Figure 5 diagrammatically shows the proposed scheme and the location of the key mutants.

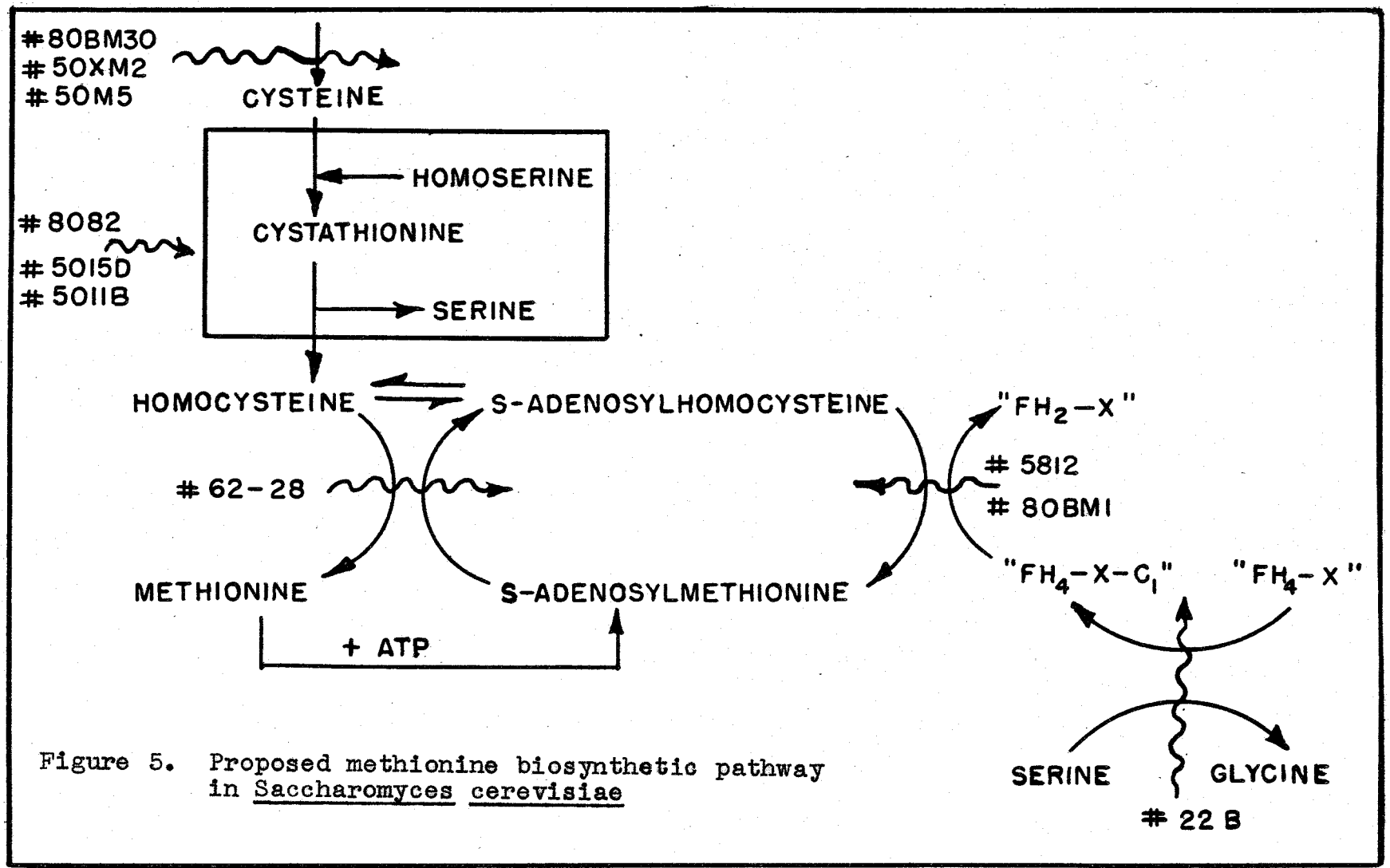


Figure 5. Proposed methionine biosynthetic pathway in Saccharomyces cerevisiae

SUMMARY

The specific blocks in the proposed methionine biosynthetic pathway of seventeen methionine auxotrophs of yeast were mapped using feeding techniques. The mutants appeared to group into four distinct categories: (1) those organisms which were able to grow on cysteine and the other proposed intermediates, (2) organisms which were able to utilize homocysteine, S-adenosylhomocysteine, and S-adenosylmethionine in place of methionine, (3) those able to grow on AM and methionine, and (4) those able to grow only when methionine was supplied. No auxotrophs grew on cystathionine, and the possibility that cystathionine does not exist in the pathway in yeast must be considered.

Ten of the auxotrophs were selected for enzymatic study of two systems believed to participate in the methylation of homocysteine. One organism lacked serine hydroxymethylase and another the transmethylase enzyme. They were strategically located on the mutant classification scheme to give support to the predicted biosynthetic mechanism.

A biosynthetic pathway of the conversion of homocysteine to methionine in yeast was presented. Data observed from the mapping of the methionine auxotrophs, the

transmethylase assay, and the determination of serine hydroxymethylase activity all lend support to the pathway suggested.

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