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Title THE CYSTEINE TO HOMOCYSTEINE CONVERSION IN SACCHAROMYCES CEREVISIAE

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The mechanism of transsulfuration and the role of cystathionine in the biosynthesis of methionine in yeast were investigated.

Saccharomyces were shown to accumulate cystathionine by use of the ^{35}S labeled compound.

Cell-free extracts of the wildtype clone as well as methionine auxotrophs were shown to cleave cystathionine. The enzymatically produced compounds were shown to be pyruvic acid and homocysteine. Pyruvic acid was determined by the Rf value of the 2, 4-dinitrophenylhydrazone as compared to authentic. The absorption spectrum of the phenylhydrazone of authentic pyruvate was identical to that of the phenylhydrazone of the enzymatic reaction mixture. The sulfhydryl was shown to be homocysteine by comparing the Rf values of the radioactive cleavage product to a ninhydrin and sulfur positive reference spot of homocysteine, and by bioautographic procedures.

Transsulfuration and the role of cystathionine in methionine

biosynthesis in yeast were discussed in view of these results which varied somewhat from that which has been found for other organisms.

THE CYSTEINE TO HOMOCYSTEINE CONVERSION IN SACCHAROMYCES CEREVISIAE

by

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To Pat

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INTRODUCTION

Early observations that man and other mammals are unable to synthesize methionine stimulated interest in the elucidation of the biosynthetic pathway of this amino acid in organisms capable of methionine formation. From evidence accumulated in studies involving various microbial systems as well as tissue slices, a pathway was proposed. This scheme contained as one of its steps the conversion of the sulfur containing three carbon amino acid cysteine to homocysteine, its four carbon homologue. It was postulated that this conversion - termed transsulfuration - involved the enzymatic synthesis and cleavage of the seven carbon thioether cystathionine. Subsequent investigations revealed considerable variation in the predicted importance of cystathionine in different biosynthetic systems. Since this variation exists and since Saccharomyces differs from other organisms with regard to steps in the biosynthesis of methionine, this study was initiated in an attempt to clarify the mechanism of transsulfuration and the role of cystathionine in methionine biosynthesis in yeast.

LITERATURE SURVEY

An extensive and varied mass of evidence has been accumulated in the literature concerning the conversion of methionine to cystine in animals and the biosynthesis of methionine in microbial systems. The following scheme has been suggested for the conversion of methionine to cystine in the animal body and appears to be irreversible: methionine → homocysteine + serine → cystathionine → cysteine (du Vigneaud, 1952). A similar scheme has been proposed for the terminal reversible reactions in methionine biosynthesis in Neurospora crassa.

It has been shown (du Vigneaud, 1952) that homocysteine is converted to cysteine but that the reverse reaction does not take place in animals. Carboxyaminopropyl-S-cysteine (cystathionine) (Figure 1) was suggested as an intermediate in this conversion on the basis of the observation of the behavior of similar compounds (Brand et al., 1936). Brown and du Vigneaud (1941) were able to synthesize S-(β-amino-β-carboxyethyl) homocysteine (cystathionine) which was given its common name by Binkley and du Vigneaud (1942).

Feeding experiments have shown that cystathionine was able to replace cystine in the diet of the rat (du Vigneaud, Brown and Chandler, 1942) which suggested its involvement in the methionine pathway. Since it was known that homocysteine and choline replaced

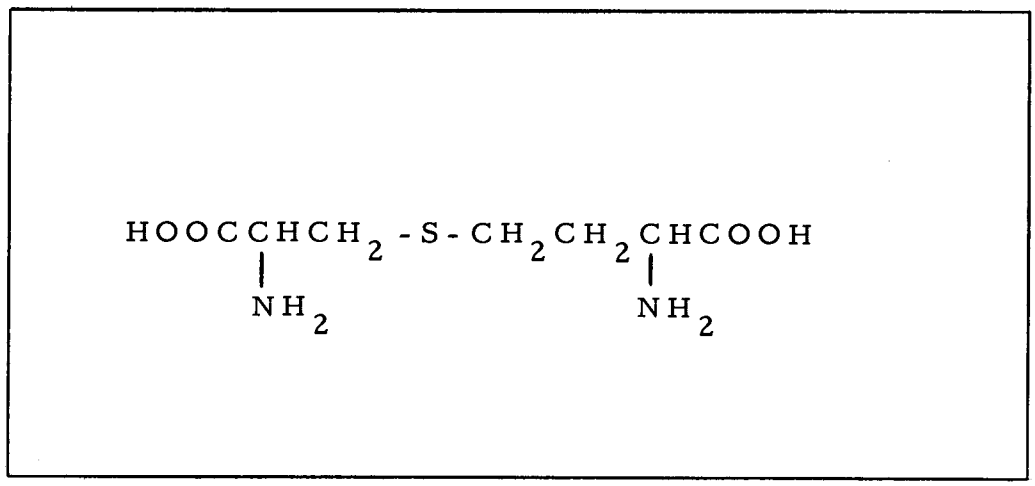

Figure 1. Structure of cystathionine.

the methionine requirement of rats (du Vigneaud et al., 1939), the finding that cystathionine and choline were unable to replace methionine suggested that cystathionine was cleaved to cysteine rather than to homocysteine (du Vigneaud, 1952). Later experiments employing rat liver slices and the saline extract of rat liver (Binkley, Anslow, and du Vigneaud, 1942) have shown an enzyme which cleaves cystathionine to cysteine. Matsuo and Greenberg (1958) were successful in crystallizing and characterizing an enzyme from rat liver which cleaved homoserine to α-ketobutyric acid and ammonia and cystathionine to α-ketobutyric acid, cysteine and ammonia. Additional evidence for the intermediate role of cystathionine was obtained by use of radioactive compounds. ^{35}S-cystathionine was fed rats and upon examination a new crop of hair was found to contain ^{35}S-cystine (Rachele et al., 1950).

The hypothesis that serine and homocysteine condensed to form cystathionine, with the carbon of serine becoming the carbon chain of cystine and homocysteine furnishing the sulfur, was suggested by the findings that when rats are fed ^{15}N-serine, the ^{15}N is found in the protein cystine (Stetten, 1942). Further evidence was furnished when it was observed that cysteine was synthesized by an enzyme which condensed serine and homocysteine (Binkley and du Vigneaud, 1942) and when cystathionine was found to be formed from the same materials (Selim and Greenberg, 1959). The cystathionine cleavage

and synthetic enzymes were separated and the cleavage enzyme used to assay the formation of cystathionine (Binkley, 1951).

Methionine doubly labeled with ^{34}S and ^{13}C was used in experiments to determine if methionine were degraded to a three carbon chain convertible to cystine. It was observed that the sulfur of methionine was indeed found in cystine but such was not the case with the carbon of methionine. These results left no doubt that the sulfur of methionine was transferred to some three carbon fragment, presumably serine (du Vigneaud et al., 1944). Thus it appears that in animals, cystathionine is formed from homocysteine and serine and is degraded to cysteine, α-ketobutyric acid and ammonia.

The study of methionine biosynthesis in microbial systems was greatly enhanced by the investigations of methionine auxotrophs of Neurospora by Horowitz (1947). Horowitz studied four classes of mutants. One of these was found to accumulate cystathionine in the medium, and another could not grow when supplied homocysteine and choline or betaine. From the observed pattern of nutritional requirements, Horowitz suggested that methionine biosynthesis proceeded through a series of gene controlled intermediates, illustrated by Figure 2. In addition, it was found that the sulfur atom of methionine was furnished by cysteine. Teas, Horowitz, and Fling (1948) observed that methionine and threonine auxotrophs of Neurospora had their methionine requirement satisfied by cystathionine or

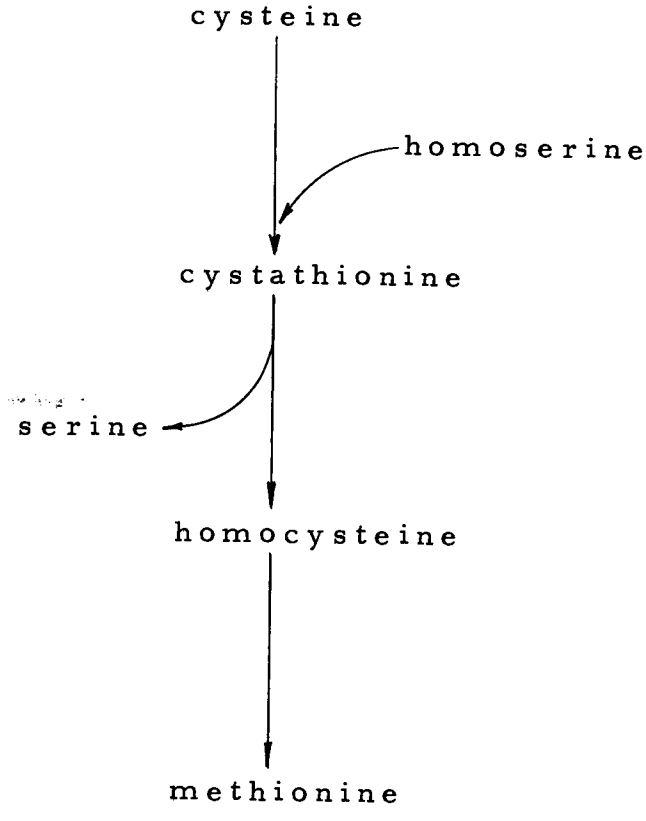

Figure 2. General scheme for methionine biosynthesis in microorganisms.

homocysteine but not by cysteine. Both requirements were satisfied by homoserine. These results suggested that homoserine was a precursor for the four carbon chain of methionine. Similar feeding studies carried on independently using methionine auxotrophs of Escherichia came to nearly the same conclusions (Lampen, Jones, and Perkins, 1947). Cystathionine was found to satisfy the cysteine requirement of certain mutants. No mutants requiring cystathionine were isolated at that time.

Binkley and Hudgins (1953) observed that partially purified extracts of Proteus morganni catalyzed the transfer of the sulfur of cysteine to the carbon chain of homoserine with the formation of the intermediate, L-cystathionine. L-Homocysteine, pyruvate and ammonia were the products of the cleavage. Serine was attacked but homoserine was not by this extract and both cysteine and homocysteine were desulfhydrated. This extract differed from the liver preparation observed (Binkley, 1951) in that the latter system produced homocysteine in place of cysteine, attacked serine but had little activity for homoserine.

Fischer (1957) found that extracts of Neurospora catalyzed the cleavage of cystathionine to homocysteine and cysteine. A mutant which required homocysteine for growth was found to lack an enzyme for the production of homocysteine from cystathionine. Furthermore, a cystathionine requiring mutant was found to be deficient in the

cysteine-producing enzyme. Fisher suggested that transsulfuration in Neurospora was a reversible process. Wijesundera and Woods (1953) observed similar results with cell-free extracts of a homocysteine requiring mutant of Escherichia and elaborated on their findings in a later paper (Wijesundera and Woods, 1962). These later results indicated that Escherichia had active enzymic mechanisms for the conversion of cystathionine to homocysteine, the precursor of methionine. This enzyme, as well as that of Proteus (Binkley and Hudgins, 1953) and mammalian liver cystathionases (Matsuo and Greenberg, 1959a), required pyridoxal phosphate. No evidence was found that cystathionine was cleaved to cysteine, α-ketobutyric acid and ammonia in the cell-free extracts of Escherichia used. This clearly indicated a difference in the transsulfuration mechanism in this microorganism as compared with the earlier reports. This view was supported by similar findings of Delavier-Klutchko (1964) which suggested that transsulfuration in Escherichia is irreversible. On the other hand, cystathionine was cleaved by two enzymes extracted from Neurospora (Flavin and Slaughter, 1964). The γ enzyme cleaved cystathionine to cysteine, α-ketobutyric acid and ammonia plus a small amount of homocysteine and pyruvate; while the β enzyme produces homocysteine, pyruvate, and ammonia.

The enzymatic synthesis of cystathionine furnished additional evidence for the intermediate role of that compound in methionine

biosynthesis. The thioether was formed from homoserine and cysteine by cell-free extracts of a homocysteine requiring mutant of Escherichia (Rowbury, 1961). Studies by Rowbury (1962a) and Rowbury and Woods (1961a, b, 1964a) indicated that cystathionase was repressed by the presence of methionine in the growth medium. An intermediate formed from succinate, coenzyme A, and homoserine by cell-free extracts of Escherichia and Salmonella typhimurium has been found which condenses with cysteine to form cystathionine. Feedback inhibition and repression by methionine has been demonstrated for some of the enzymes responsible for this synthesis (Rowbury, 1962b, 1964a; Rowbury and Woods, 1964b). Similar findings of a homoserine derivative of succinic acid has been demonstrated for Neurospora (Flavin, Delavier-Klutchko, and Slaughter, 1964). As further proof of the intermediate role of O-succinylhomoserine in methionine biosynthesis by Escherichia, the compound was found to accumulate in methionine auxotrophs which required cystathionine, homocysteine, or methionine for growth. Similar findings were demonstrated for Salmonella typhimurium (Rowbury, 1964b).

The bulk of the evidence accumulated seems to substantiate that animal systems and the protozoan Tetrahymena geleii (Genghof, 1949) irreversibly convert methionine to cystine proceeding according to the following pattern: methionine → homocysteine →

cystathionine → cysteine. The same pattern may occur in Saccharomyces and Neurospora except that it is a reversible process in these organisms. The pattern of methionine is substantially the same in Escherichia and Salmonella and proceeds irreversibly according to the following pattern: homoserine + succinyl CoA + cysteine → cystathionine → homocysteine → methionine.

Substantial evidence is available to question the validity of the pathways proposed. This evidence suggests that transsulfuration may proceed according to other mechanisms and pathways.

The addition of unlabeled cystathionine to cultures of Escherichia growing with ^{35}S as sole source of sulfur did not, as would be expected, decrease the extent of incorporation of radioactivity into methionine (Bolton, Cowie, and Sands, 1952). Roberts et al. (1955) in isotope competition studies of Escherichia coli B and mutant strains found no competitive effect of cystathionine when tested with ^{14}C or ^{35}S labeled compounds. They concluded that cystathionine was not an intermediate in carbon transformation nor was capable of furnishing sulfur to E. coli. However, Roberts et al. (1955) in isotope competition studies with Neurospora found that cystathionine had the competitive effect expected of an intermediate, that is, ^{32}S-cystathionine proved to be an effective competitor in reducing the incorporation of ^{35}S from ^{35}S-sulfate and ^{35}S-methionine. Wiebers and Garner (1960) showed that 65 percent of the radioactivity

incorporated into the cells from ^{35}S-cystathionine was present as methionine. This verified transsulfuration but did not confirm the obligate role of cystathionine in methionine biosynthesis of Neurospora. Additional findings by Wiebers and Garner (1960) found that radioactivity from ^{14}C-cystathionine was not incorporated into methionine. No permeability problem was possible as the mutant strain of Neurospora used could grow if furnished cystathionine. They concluded that the four carbon chain of cystathionine could be formed endogenously from homoserine and simultaneously from exogenously supplied methionine.

Ragland and Liverman (1956) extracted naturally occurring S-methyl-L-cysteine from Neurospora and demonstrated that it will serve as the sole sulfur source for certain strains. Roberts et al. (1955) reported that in Neurospora S-methylcysteine was as effective a competitor in reducing the incorporation of ^{35}S from $Na^{35}SO_4$ as cystathionine.

Dalal, Rege, and Sreenwasan (1961) observed that resting cells and cell-free extracts of Neurospora can synthesize methionine from homocysteine and formate or other methyl donors. Flavin (1962) found that Neurospora contains an enzyme capable of decomposing cystathionine to homocysteine, α-ketobutyrate, ammonia, and sulfur. An enzyme capable of forming homocysteine from homoserine and hydrogen sulfide as well as cysteine from serine and hydrogen

sulfide has been extracted from Neurospora (Wiebers and Garner, 1963). The same investigators suggested that neither cystathionine nor S-methylcysteine were precursors of methionine biosynthesis in Neurospora and that a possible pathway existed which included the sulhydration of homoserine followed by methylation of the resulting homocysteine to produce methionine.

The possibility remains that the true substrate for cystathionases is something other than the one proposed. Fromageot (1940), Smythe (1942), and Brinkley (1950) reported that liver cystathionases exhibited cysteine desulfurase activity. The purified crystalline cystathionase extracted from rat liver by Matsuo and Greenberg (1959b) also showed activity toward cysteine. Cavallini et al. (1962) reported that cystathionase and cysteine desulfhydrase were unseparable. They suggested that cystine was the true substrate. The same group confirmed that the two enzymes were identical (Mondovi, Scioscia-Santoro, and Cavallini, 1963). Loiselet and Chatagner (1964) concluded that either cystine or cysteine as well as cystathionine were substrates for cystathionases (cysteine desulfurases).

Finally, methionine auxotrophs of Saccharomyces have not been observed to grow when furnished cystathionine even though they respond to cysteine or homocysteine (Pomper, 1953). This suggested

a different methionine biosynthetic pathway in yeast which did not include cystathionine.

METHODS AND MATERIALS

Cultures

A single mutant strain of Neurospora crassa designated H98A which accumulated cystathionine was used in this investigation. The mutant was given to us by Dr. Joyce Wiebers. All other organisms used were strains of Saccharomyces cerevisiae. A wildtype diploid strain, (MCC), was obtained from the active culture collection of the Department of Microbiology, Oregon State University. A uracil requiring haploid (3701B) was obtained from Dr. D. C. Hawthorne. Methionine auxotroph (50M5) was isolated by Kemet D. Spence after ultraviolet irradiation of 3701B. Methionine auxotroph (S288-C18) was obtained from Dr. R. K. Mortimer. The organisms were maintained at 4^{o}C on agar slants of yeast complete medium (YCM) (Pigg, 1962).

Mutant Growth Requirements

Modified Wickerham's medium (Pigg, 1962) without methionine was used as the basic medium for testing the growth requirements of various methionine auxotrophs. Appropriate additions were made to the medium to give a final concentration of 1 mM of the additive. The mutants, grown 24 hours in yeast complete

medium, were washed three times in sterile distilled water, resuspended in water and streaked onto the surface of prepoured agar. The plates were observed for growth at 24, 48, and 72 hours. Since it was contemplated that one or more of the auxotrophs might be used as a microbiological assay of the products of the enzymatic cleavage of cystathionine, all possible cleavage products, serine, homoserine, alanine, α-amino-n-butyric acid, pyruvic acid, α-ketobutyric acid, in addition to cysteine, homocysteine, cystathionine, and methionine, were tested for their ability to sustain growth of the mutants.

Preparation of ^{35}S-cystathionine

In order to obtain ^{35}S-cystathionine of high purity and with high specific activity, a biological synthesis was chosen. ^{35}S-cystathionine was prepared according to the method of Wiebers and Garner (1960). This involved the extraction of the mycelial pads of Neurospora strain H98A which had been grown in the presence of $Na_2{}^{35}SO_4$. The alcoholic extract was streaked on large sheets of Whatman No. 4 paper and chromatographed in phenol-water (80:20 w/v). The cystathionine band was located by finding the radioactive band which corresponded to a reference spot of authentic cystathionine run alongside. The radioactive band was located by use of the Vanguard Autoscanner model 880 automatic chromatogram scanner. Following location the band was excised and eluted in

water. The eluant was restreaked and chromatographed in butanol-acetic acid-water (2:1:1). After elution, the radioactive compound was determined as pure by comparison to the Rf value of authentic cystathionine, and the absence of other labeled materials or contaminating ninhydrin positive material. The radioactivity of the prepared compound was determined by adding an appropriate amount of the solution to a scintillation vial containing 52 percent toluene and 48 percent ethanol mixture containing 0.4 percent PPO (2, 5-diphenyl oxazole) and 0.02 percent POPOP (1,4-bis-2-(5-phenyl oxazolyl)-benzene) (Pigg, Spence, and Parks, 1962). The vial was then counted using the Packard Tri-Carb Automatic Liquid Scintillation Spectrometer, model 3000. Determination of the amino acid content of the cystathionine solution followed the procedure of Moore and Stein (1954) using a modified ninhydrin solution for a colorimetric determination at 540 mμ using the Coleman Nepho-Colorimeter, model 9.

Accumulation Studies

The organisms to be used in the investigation of amino acid accumulation were grown in yeast extract supplemented medium at 30°C with agitation. The cells were harvested, washed three times with distilled water, and resuspended in synthetic medium containing the amino acid at a final concentration of 1 mM and approximately

1 μc or 5 μc of either ^{14}C or ^{35}S labeled amino acid. The reaction mixture was incubated at 30°C on a rotary shaker; samples were taken at 0, 0.5, 1, and 2 hours. These samples were immediately cooled in an ice bath to retard further reaction. The cells were washed three times in ice-cold 0.67 M phosphate buffer (pH6.6), and were resuspended in the same buffer to a volume of 6 ml. A 5 ml portion of the suspension was boiled for 20 minutes and centrifuged; 0.5 ml of the supernatant fluid was used to assay the radioactivity using liquid scintillation counting as previously described. Control reaction mixtures were treated similarly with the exception that they were maintained at 0°C during the incubation period.

Randomly ^{14}C labeled algal hydrolysate was used to measure the accumulation of the amino acids contained therein. The same procedure as previously described was used, with the exception that 149 mg per liter of Vitamin Free Casamino Acids (Difco) were used as carrier with 5 μc of ^{14}C algal hydrolysate.

Cell quantities were measured nephelometrically with a Coleman model 9 Nepho-Colorimeter. Direct microscopic cell counts and samples of aqueous cell suspensions dried to constant weight were used for preparing calibration curves.

Cell-Free Extracts

The various strains of Saccharomyces to be used were grown in yeast complete medium for 24-48 hours at 30°C, harvested, and resuspended in synthetic minimal medium overnight. The cells were then reharvested, washed, and suspended in a minimum of 0.1 M citric acid - NaOH buffer pH 7.5 so that they could barely be pipetted. After freezing in dry ice, the cultures were disrupted by subjecting them to 10,000 pounds pressure in the Eaton press (Eaton, 1962). An equal volume of citric acid buffer was added to the disrupted cells to extract the soluble protein and to thaw the mixture. Cells and debris were removed by centrifugation in the Servall RC-2 Refrigerated Centrifuge at -4°C at approximately 20,000 x G. for 30 minutes. The cell-free extract was frozen in 5 ml aliquots and retained activity for several weeks. It was observed that the preparation lost activity upon thawing and refreezing.

In order to eliminate small molecular weight constituents from the crude extract, 1 ml was placed directly onto the bed of a 6 x volume of Sephadex G-50 fine gel in a column of internal diameter of 1 cm. The protein was eluted with 0.1 M phosphate buffer pH 7.5 and the first 1.3 ml of protein solution was collected.

For cleavage of cystathionine the complete reaction mixture consisted of 1 ml of the protein solution, 10 μg pyridoxal phosphate,

1.6 μg magnesium sulfate, and 50 μ moles of DL-allo-cystathionine in a final volume of 3 ml. The reaction proceeded for 30 minutes at 37°C and was terminated by addition of 0.5 ml of freshly prepared 25 percent trichloroacetic acid.

Enzyme activity was followed by use of 5, 5'-dithiobis-(2-nitrobenzoic acid) which develops a yellow color in combination with compounds which contain an SH group (Ellman, 1959). The yellow color was easily discernible, developed instantaneously, showed no fading during a ten minute period and had an absorption maximum at 412 mμ. No attempts were made to quantitate, by use of this reagent, the cleavage products formed. However, color production is proportional to concentration, is linear, and could be used for this purpose. Enzymatic activity was judged as occurring if yellow color was produced which was visibly darker than the controls. Controls were found to have an absorbancy at 412 mμ of approximately null.

Identification of the Products of the Enzymatic Cleavage of Cystathionine

Keto acids were determined to be present by the formation of the 2, 4-dinitrophenylhydrazone (Cavallini, Frontali, and Toschi, 1949b) which were first extracted with ethyl acetate followed by extraction with sodium carbonate (Friedeman and Haugen, 1943).

The keto acid phenylhydrazone was identified by descending chromatography on one inch wide strips of Whatman No. 3 mm paper 35 cm long using a solvent system of butanol-ethanol-water (50:10:40 v/ v) (Cavallini, Frontali, and Toschi,1949a). In addition, absorption spectra of the red or brown color which develops when strong base was added to the phenylhydrazones of the cleavage products of cystathionine were made using the Cary model II Recording Spectrophotometer. The 2, 4-dinitrophenylhydrazones of authentic pyruvic acid and ketobutyric acid were prepared and treated in the same manner.

Identification of the SH Containing Moiety

Strip Scanning

A two fold stoichiometric amount of N-ethylmaleimide (NEM) was added to the complete reaction mixture (Hanes, Hird, and Isherwood, 1950). The purpose of the addition was to stabilize the sulfhydryl containing compound to prevent streaking during chromatography and to act as a trap for any sulfhydryl formed. The NEM derivatives of L-cysteine and L-homocysteine were also prepared as references.

The complete reaction mixture consisted of 10 μg pyridoxal phosphate, 1.6 μg of magnesium sulfate, 100 μ moles of

N-ethylmaleimide, 50 μ moles of DL-allo-cystathionine and approximately 2 μc of ^{35}S-cystathionine. Following a reaction period of approximately one hour, the reaction was terminated by the addition of trichloroacetic acid. The mixture was filtered and the filtrate spotted on sheets of Whatman No. 1 paper. Descending chromatography was used to separate the compounds in a solvent system of butanol-acetic acid-water (2:1:1 v/v). Two spray reagents were used to locate the amino acids. Chloroplatinate reagent (Toennies and Kolb, 1951) was used to locate the sulfur containing compounds and was followed by 0.5 percent ninhydrin in butanol. The latter reagent's color development was hastened by heating.

One or 1.25 inch wide strips were made of the paper by cutting in the direction of the separation so that the strip contained all of the material originally spotted. The strip was assayed using the Vanguard chromatogram scanner for radioactive areas to be compared to the ninhydrin and sulfur positive reference spots of the NEM derivatives of cysteine and homocysteine.

Microbiological Assay

Following an incubation period of 30 minutes at 37°C, two volumes of the complete reaction mixture with no added NEM or ^{35}S-cystathionine was terminated using trichloroacetic acid. The mixture was filtered, made up to 12 ml with 0.1 M phosphate buffer

(pH 7.5) and sterilized by passage through a filter using a Millipore Micro-syringe holder. Two and five-tenths ml of the sterile material was added to four tubes each containing 5 ml of double strength modified Wickerham's medium without methionine. Each tube was made up to a final volume of 10 ml by the addition of sterile phosphate buffer. In addition to the reaction mixture, four tubes containing each of the following additions were made using the same technique: 25 μ moles L-cysteine, 25 μ moles L-homocysteine thiolactone, 25 μ moles DL-allo-cystathionine, phosphate buffer, and the product of a substateless reaction mixture. Twenty-four hour cultures of S288-C18 (me-3) and 50M5 (me-4) grown in yeast complete medium were harvested, washed and resuspended in sterile distilled water. Each organism was inoculated into two tubes of each type. The tubes were incubated at 30°C with agitation. Growth was determined nephelometrically at 12, 24, and 48 hours.

RESULTS OF METHODOLOGICAL STUDIES

Cystathionine Concentration

The concentration of radioactive cystathionine in the preparation can be seen from Figure 3. Calculations of four samples using the standard curve showed the 1 ml of the preparation contained between 160 and 171 μg of cystathionine. The average amount was 164 μg per ml.

The specific activity of the preparation as determined using the counting efficiency of our fluor system was 1.95 μc per 164 μg of the amino acid cystathionine.

Sephadex Treatment

Table 1 shows the necessity for partial purification of the enzyme using gel filtration. It can be seen that untreated enzyme, at this concentration of protein, produces a sulfhydryl color reaction equal to approximately 10 μg cysteine. On the basis of these experiments, all enzyme preparations were Sephadex treated before incubation. These results also show that the zero time control sample produced essentially no color and that the difference between the yellow color produced when enzyme activity is present and absent is clearly discernible.

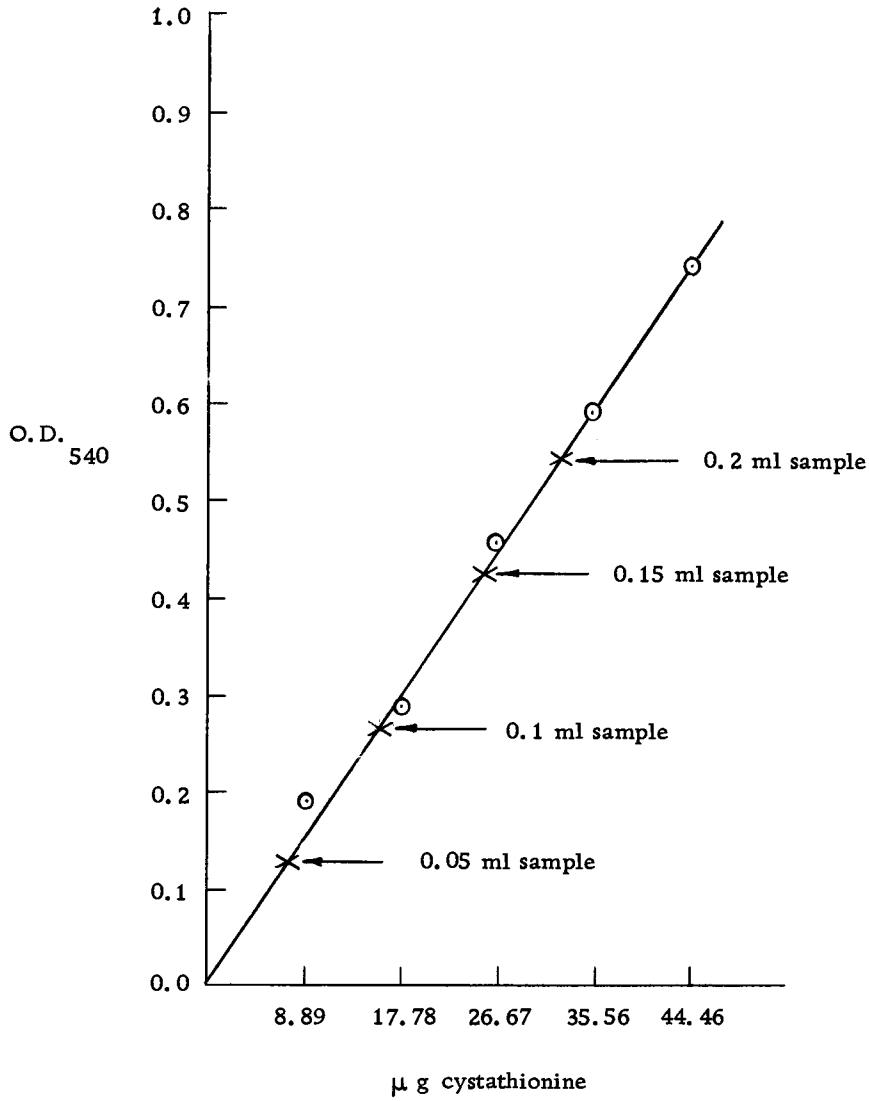

Figure 3. Concentration of prepared ^{35}S-cystathionine.

Table 1. The Effect of Sephadex Treatment on Control Level of Color Development

	$O.D._{412}$
15 μg cysteine	0.153
0 time control	0.007
Sephadex treated reaction mixture	0.253
Untreated reaction mixture	0.102

EXPERIMENTAL RESULTS

Mutant Growth Requirements

The growth requirements of selected methionine auxotrophs may be seen in Table 2. Mutant 50M5 required cysteine, homocysteine, or methionine for growth on a methionine-less medium and was designated as an me-4 or cysteine mutant. Clone S288-C18, on the other hand, required homocysteine or methionine for growth and was designated an me-3 or homocysteine mutant. Mutant types are defined in Figure 4. It was of interest to note that neither mutant could grow when inoculated onto a medium without methionine but containing cystathionine as a sole organic sulfur source. Neither strain responded to the other additions which were the possible products of the cleavage of cystathionine.

According to the method used, Saccharomyces strains 5015D, 5036D, 5011B, 8090, 8082, 80BM30, and 50XM2 were designated me-4 mutants.

Accumulation Studies

The rapid accumulation of randomly ^{14}C labeled algal hydrolysate and methionine may be seen in Figure 5. The rate of accumulation was most rapid during the first hour. The accumulation process

Table 2. Growth Requirements of Selected Methionine Auxotrophs of Saccharomyces cerevisiae

Addition to Growth Medium	Auxotroph	
	50M5	S288-C18
None	-	-
L-cysteine	+	-
DL + allo-cystathionine	-	-
L-homocysteine thiolactone	+	+
L-methionine	+	+

None of the following compounds was able to sustain growth of the organisms: DL-serine, DL-homoserine, DL-alanine, α-amino-n-butyric acid, pyruvic acid, α-ketobutyric acid.

The following organisms had the same growth requirements as 50M5: 8090, 8082, 5015D, 5011B, 5036D, 80BM30, and 50XM2.

Growth medium employed was modified Wickerham's complete medium without methionine.

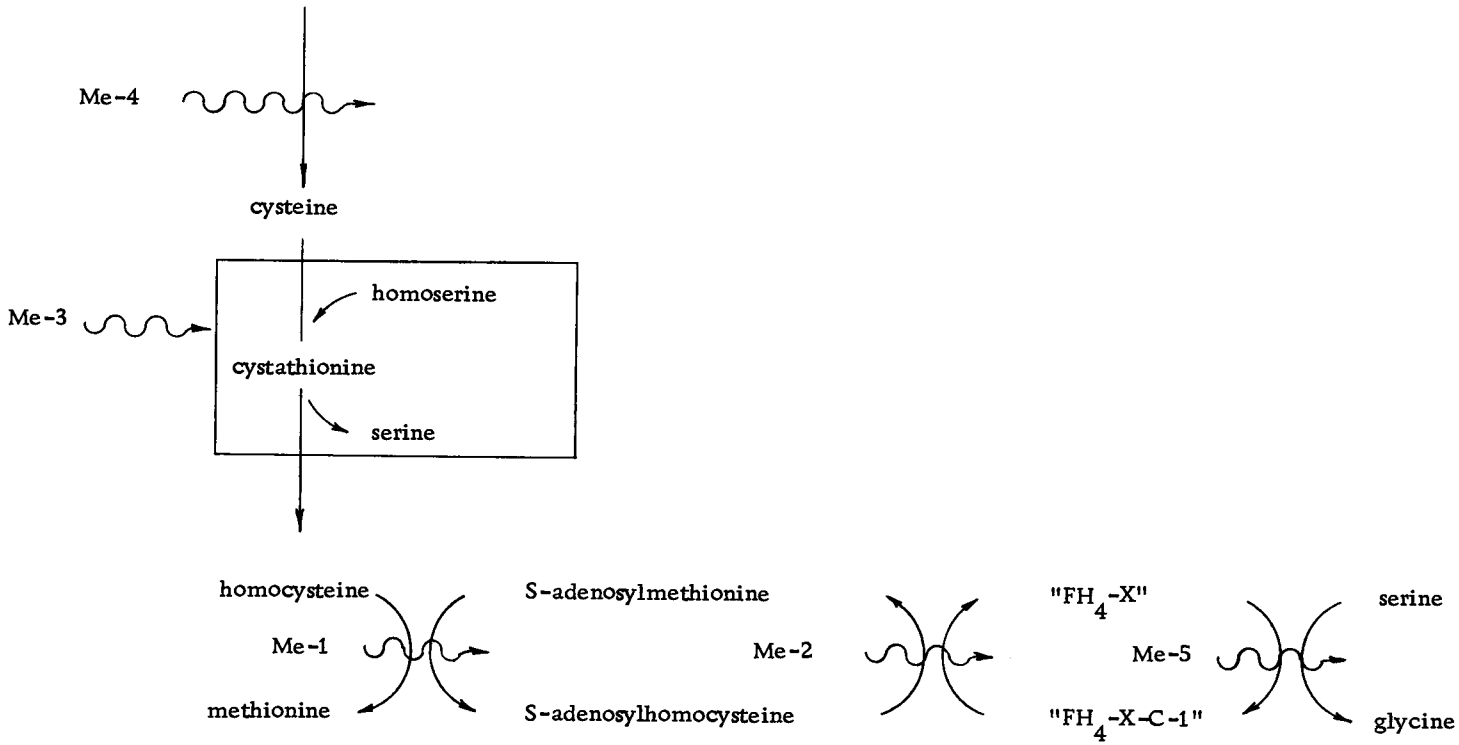

Figure 4. Terminal reactions in methionine biosynthesis in Saccharomyces cerevisiae. Mutant groups are located at the point of their enzymic deficiencies with a wavy arrow.

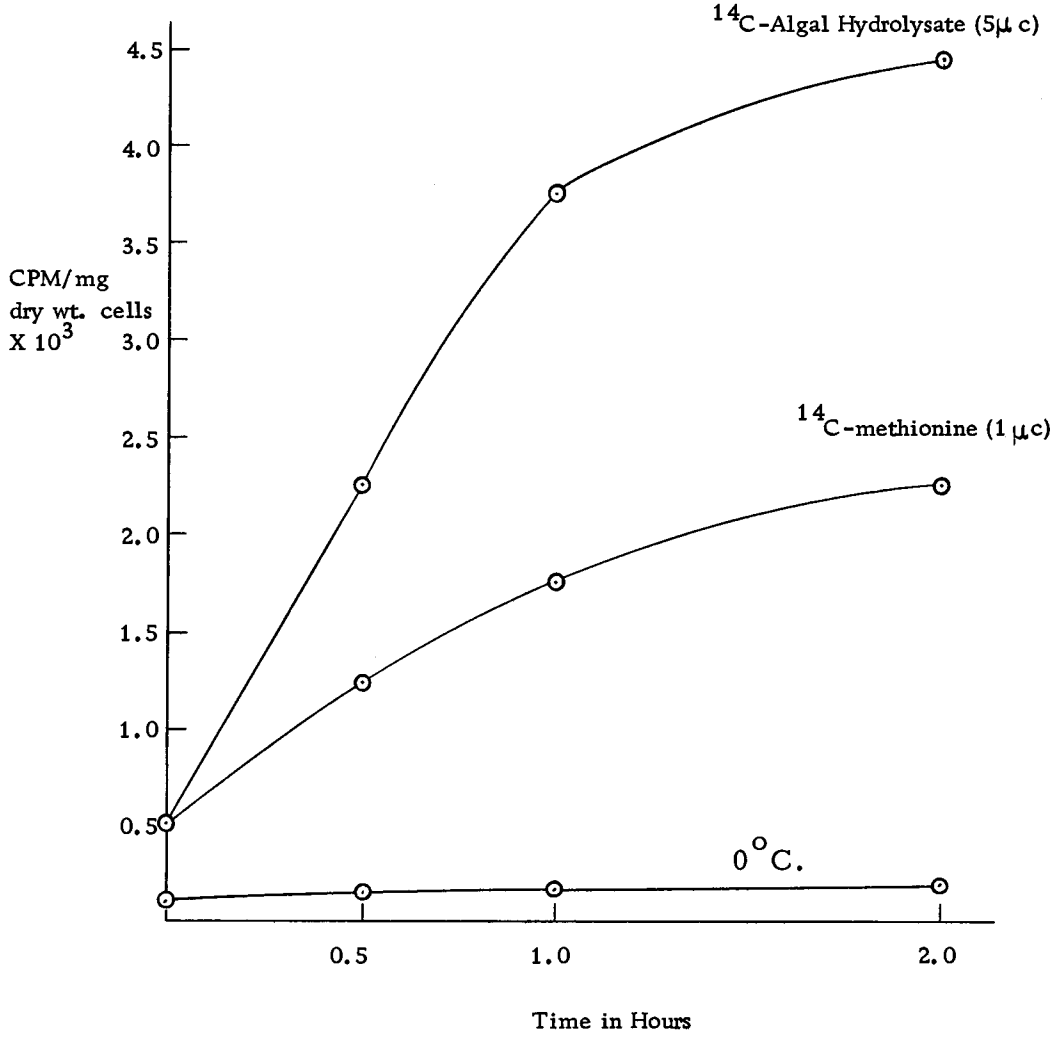

Figure 5. Accumulation of ^{14}C-methionine and ^{14}C-algal hydrolysate by 3701B.

has been shown to be under the control of a single gene and relatively nonspecific (Sorsoli, Spence, and Parks, 1964). The relative nonspecificity of the accumulation mechanism was reflected by the relatively high concentration of radioactive algal hydrolysate in the boiled extract of the cells.

Since the accumulation mechanism appeared nonspecific, it was anticipated that most amino acids would be accumulated by the organism. Figure 6 shows that ^{35}S-cysteine, ^{35}S-cystathionine, as well as ^{14}C-methionine were actively transported by these cells. Methionine appeared to be accumulated to a greater extent than the other amino acids, but different counting efficiencies for the two isotopes could account for this difference. The accumulation studies clearly revealed that cystathionine was accumulated in yeast cells under the conditions used.

Identification of Pyruvate

The Rf values of the chromatographed keto acid 2, 4-dinitrophenylhydrazones may be seen in Table 3. Some variance in the observed Rf values occurred. This was attributed to differences in sodium carbonate concentration in the samples. However, cochromatography of the unknown phenylhydrazone with that of pyruvate resulted in a single spot. A control mixture employing added α-ketobutyric acid phenylhydrazone revealed two distinct

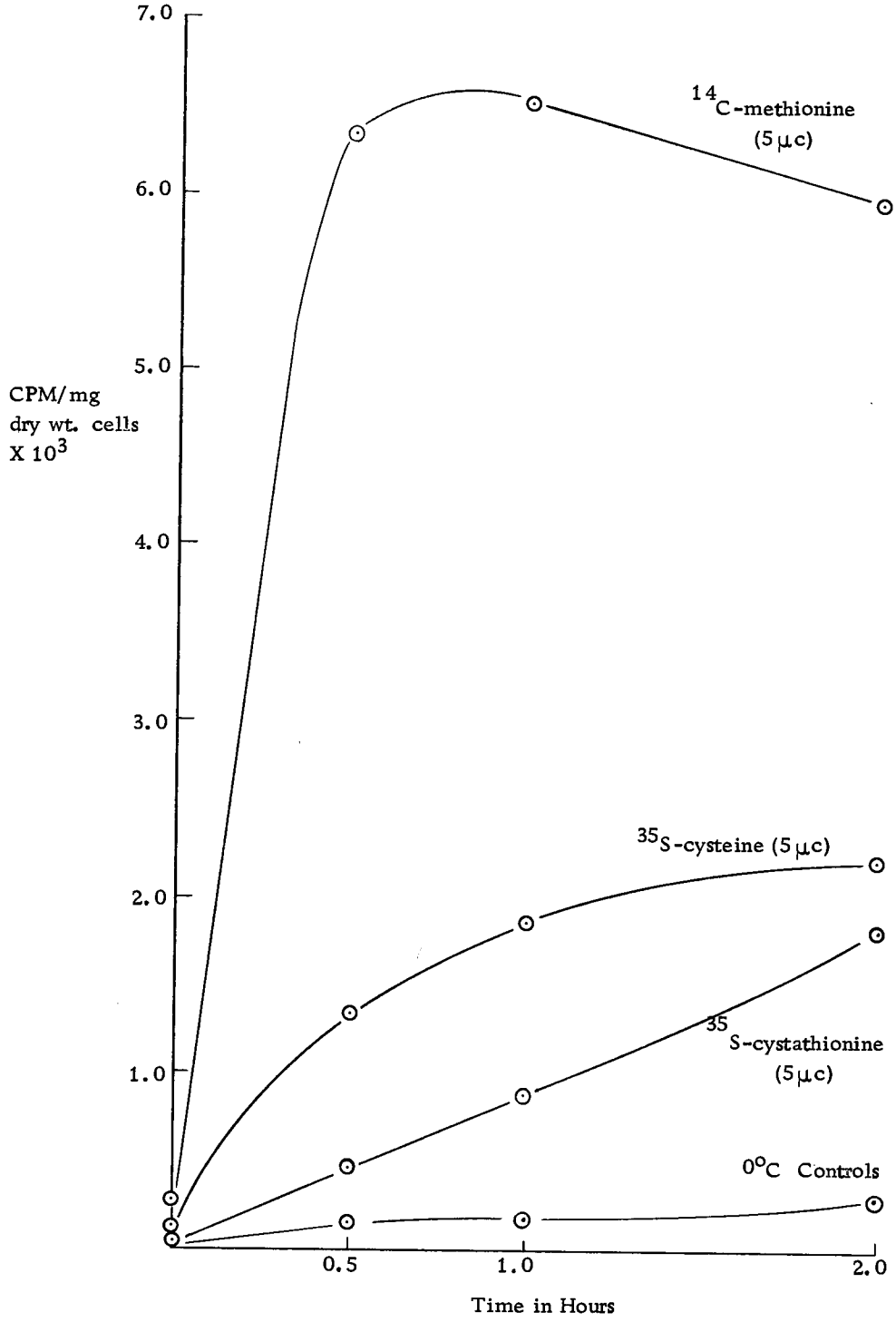

Figure 6. Accumulation of ^{14}C-methionine, ^{35}S-cysteine, and ^{35}S-cystathionine by 3701B.

Table 3. The Rf Values of Keto Acid 2, 4-dinitrophenyl-hydrazones

	Rf		
Phenylhydrazone	S288-C18	50M5	WT
Unknown	0.39	0.37	0.38
Pyruvic acid	0.41	0.40	0.42
α-Ketobutyric acid	0.71	0.63	0.60
Unknown + pyruvic acid	0.37	0.37	0.36
Unknown + α-Ketobutyric acid	0.38 0.66	0.37 0.67	0.37 0.58

migrating materials. These corresponded to predicted Rf values for the α-ketobutyrate and pyruvate conjugates. These results showed clearly that within the limits of detection of the procedure, no α-ketobutyrate was produced enzymatically from cystathionine. Pyruvate was the only keto acid observed in these experiments.

As a check on this procedure and to confirm the results obtained, an absorption spectrum of each phenylhydrazone was made. Figure 7 shows the absorption spectra of the NaOH developed color of the phenylhydrazones of the keto acids formed by enzymatic cleavage of cystathionine. The spectrum of the α-ketobutyric acid derivative shows an absorbance maximum at 385 mμ. The derivative of pyruvate as well as the phenylhydrazones of the enzymatic reaction mixtures prepared from 3701B and S288-C18 showed identical peak absorbancy at 435 mμ. In addition, the derivative of the keto acid formed by the cleavage enzymes of MCC and 50M5 had absorption spectra identical to that of pyruvate. These data confirm the chromatographic evidence that the keto acid formed from cystathionine by the enzymes of 3701B, S288-C18, MCC, and 50M5 is pyruvic acid.

Identification of Homocysteine

Sulfur containing amino acid separation with paper

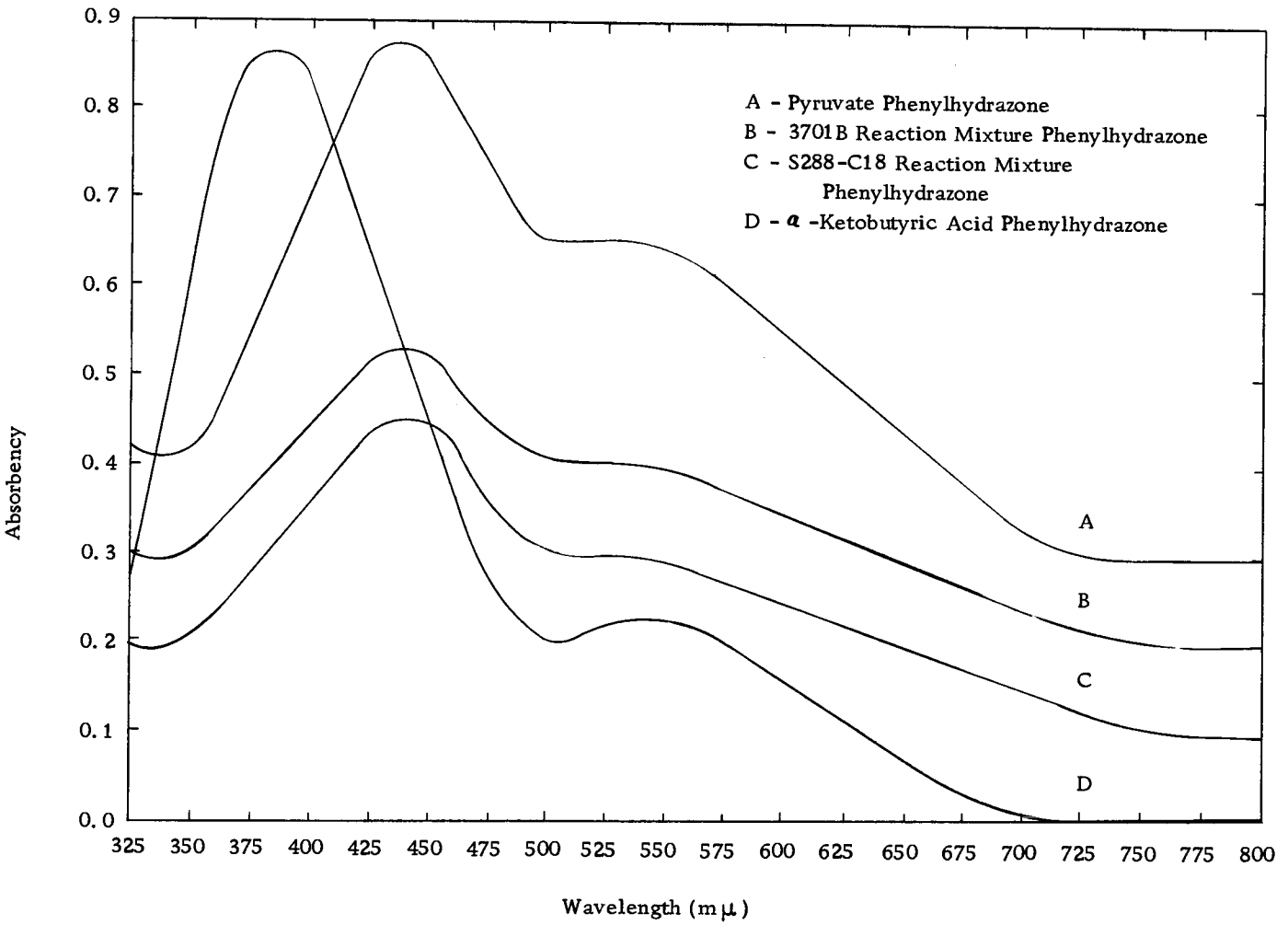

Figure 7. Absorption spectra of authentic keto acid phenylhydrazones and the phenylhydrazones of the enzymic reaction products.

chromatography presents a serious problem of streaking. In addition, cysteine and homocysteine are unstable compounds rapidly oxidizing, thus, the problem of separating a three carbon from its four carbon homologue was compounded. The results of the paper chromatographic separation of the N-ethylmaleimide (NEM) derivatives of the products of the enzymatic cleavage of ^{35}S-cystathionine by an enzyme from 3701B (WT) may be seen in Figure 8. The reference spots of the NEM derivatives of homocysteine and cysteine may be seen to be overlapping yet discreet. The tracing of fluctuations in the radioactivity on the paper strip corresponded to the cystathionine reference spot which, as expected, was the major peak; a second unknown peak and a third discreet peak which corresponded to the homocysteine reference spot also were observed. Although the cysteine and homocysteine reference spots overlapped, the radioactivity appeared wholely associated with the homocysteine spot. The radioactivity did not extend far enough to include all of the area which would be associated with cysteine. The area on the strip corresponding to the homocysteine derivative was found to contain sulfur, ninhydrin color and radioactivity. The control tracing, trichloroacetic acid at zero time, showed only a single radioactive peak. The area on the strip was sulfur and ninhydrin positive and corresponded to the cystathionine reference spot. It was interesting to note that when NEM was not added until the end of the reaction

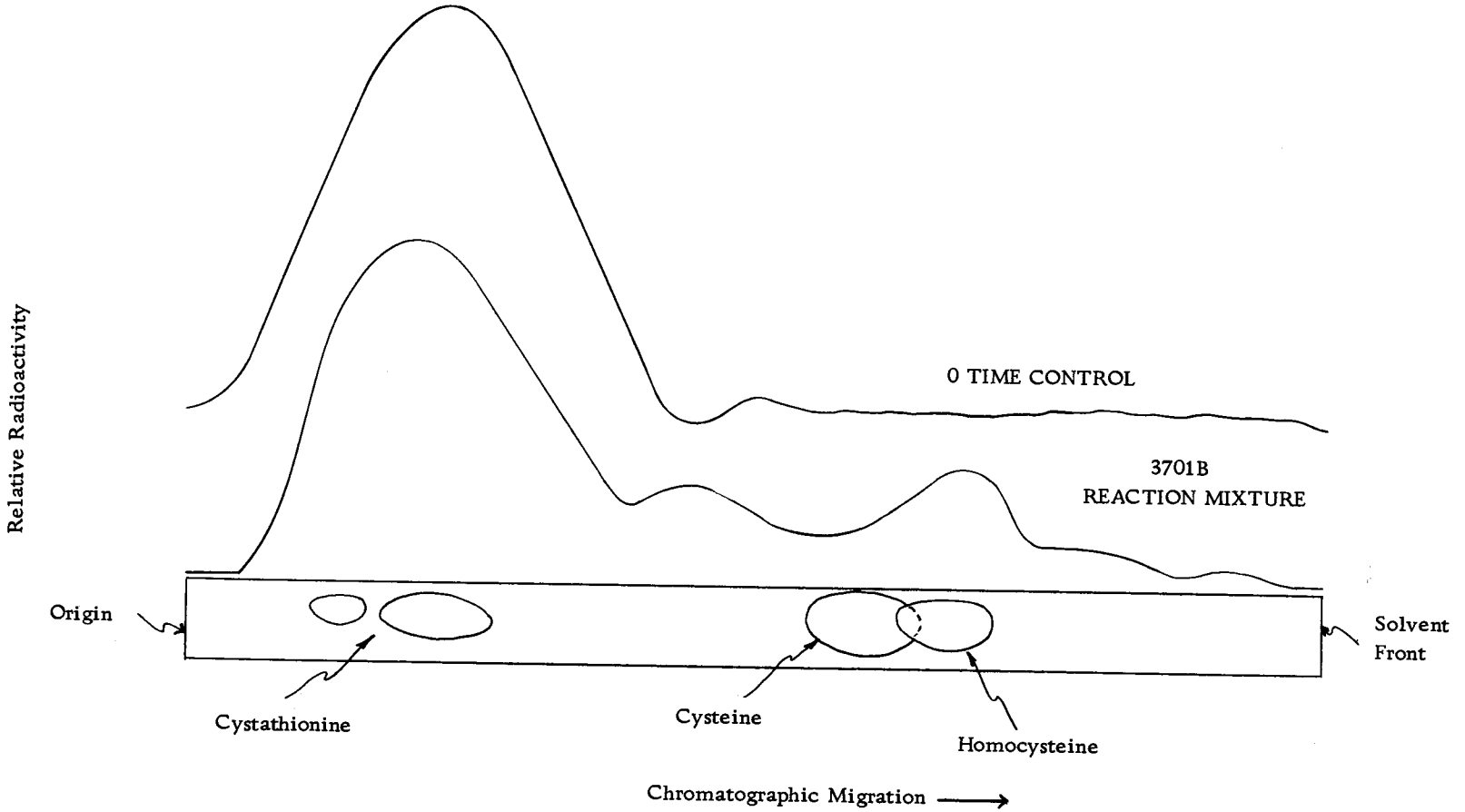

Figure 8. Radioactivity profile of chromatographed reaction products of wildtype Saccharomyces.

period, the sulfur and ninhydrin positive area corresponding to homocysteine was lacking.

The enzyme extracted from S288-C18, a homocysteine requiring mutant, cleaved cystathionine according to the pattern illustrated in Figure 9. It may be clearly seen that the degradative products of cystathionine formed in this reaction were identical to those produced by the wildtype organism. Homocysteine was one of these products.

As a further check on the above studies, a microbiological assay of the products of the cleavage of cystathionine was attempted employing S288-C18, the single homocysteine requiring mutant available. If homocysteine were produced by the enzymic extracts, it should be available to satisfy the organic sulfur requirement for this organism. Cysteine would not do so. The results of the assay may be seen in Table 4. The mutant responded to some compound in the reaction mixture as shown by an increase in growth. Homocysteine was indicated as the growth promoting compound, since the organism previously had been shown not to respond to the other possible cleavage products of cystathionine.

Although the growth response of 50M5, a cysteine requiring mutant, was not as clearly demonstrated; growth did occur to an extent greater than that of the controls. This response was expected, if one of the cleavage products was indeed homocysteine.

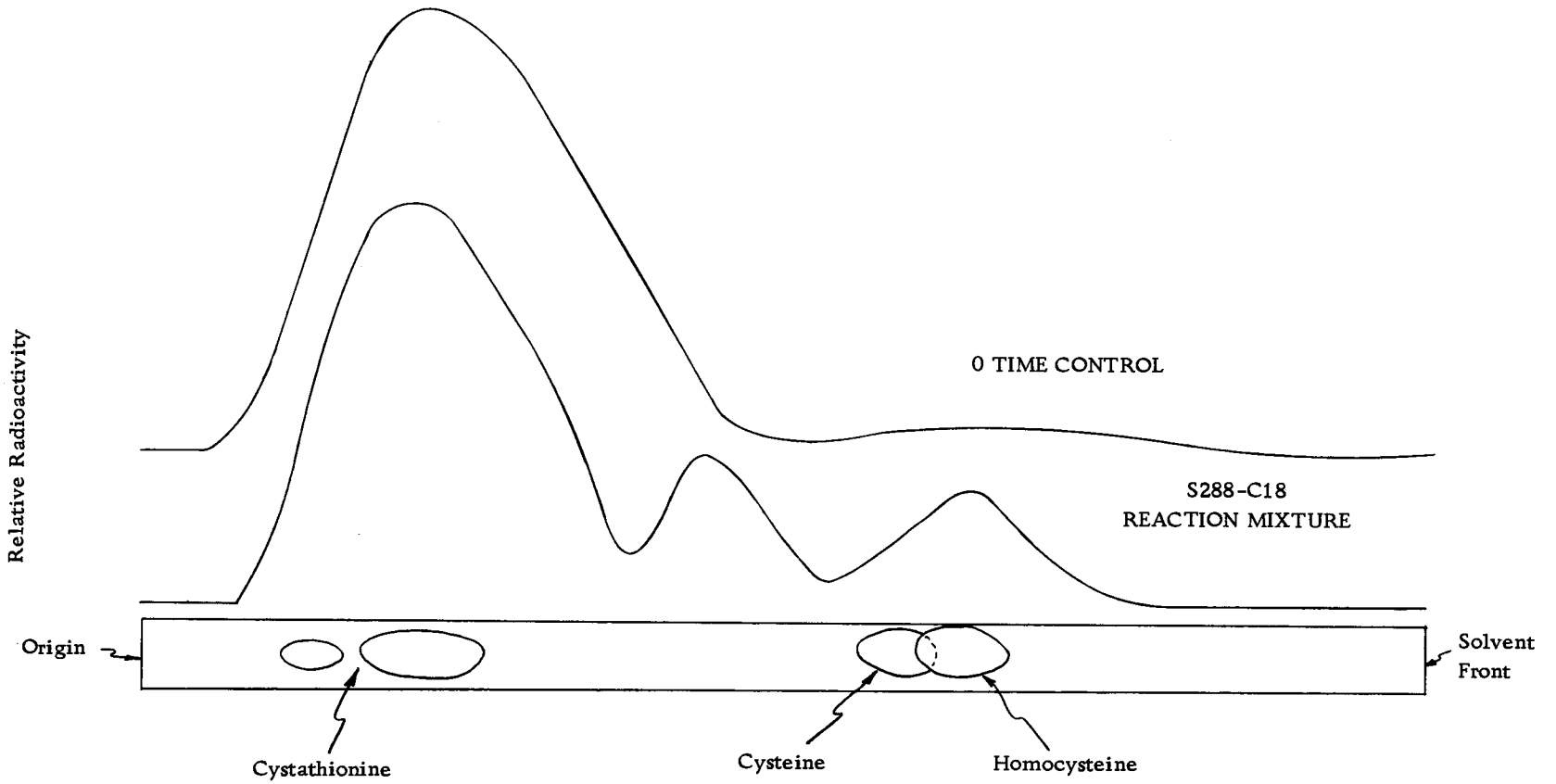

Figure 9. Radioactivity profile of chromatographed reaction products of a homocysteine requiring mutant of Saccharomyces.

Table 4. The Microbiological Assay of Enzymatic Cleavage Products of Cystathionine

Growth medium addition		Auxotroph					
		S288-C18			50M5		
		Hours of Incubation					
		12	24	48	12	24	48
Buffer	a	11	10	10	35	36	59
	b	12	10	13	30	35	52
Cysteine	a	10	9	12	37	49	93
	b	11	7	11	40	53	93
Homocysteine thiolactone	a	24	68	100+	51	100+	100+
	b	23	67	100+	48	100+	100+
Cystathionine	a	10	8	15	29	32	50
	b	10	9	17	28	35	65
Substrateless reaction products	a	12	13	15	20	18	31
	b	15	15	13			
Reaction products	a	51	61	59	52	65	95
	b	45	62	63	52	65	92

Growth as expressed by nephelos units.

Growth medium used was Wickerham's complete without methionine.

a, b represent duplicate incubations.

DISCUSSION

Early observations that cystathionine was unable to dilute the incorporation into methionine of ^{35}S from radioactive inorganic sulfur sources (Roberts et al., 1955) and that cystathionine was unable to furnish an organic sulfur source for mutants blocked early in the proposed pathway (Pomper, 1953) led to speculation that exogenously supplied cystathionine was unavailable for metabolism to intact microorganisms. Our experimental results clearly indicated that cystathionine was accumulated by Saccharomyces.

Homocysteine requiring mutants of Neurospora (Fischer, 1957) and Escherichia (Wijesundera and Woods, 1953) lack an enzyme for the production of homocysteine from cystathionine. Contrary to the data supporting those findings, our observations show that both the wildtype Saccharomyces and the homocysteine requiring clone S288-C18 contain an enzyme capable of producing pyruvate and homocysteine from cystathionine. It becomes difficult to understand why S288-C18 is unable to utilize cystathionine as a sole organic sulfur source.

Various alternatives may be offered to explain these results. One speculation, of course, would be that free cystathionine is not an intermediate in the methionine biosynthetic pathway in yeast. This is not to exclude, however, the existence of a cystathionine

complex as the actual biosynthetic precursor of homocysteine in the methionine pathway. Yeast would be unable to utilize free cystathionine if they were unable to activate cystathionine for biosynthetic purposes. Such a situation has been reported in histidine biosynthesis in Neurospora (Ames, 1955). In that organism, some histidine auxotrophs accumulated imidazoles which were not excreted into the medium by the wildtype or several other histidine auxotrophs. These accumulated compounds were inactive in replacing in any of the auxotrophs the histidine required by those organisms. It was later learned that, if extracts were made of the mycelium of the mutants, phosphorylated derivatives of the imidazoles existed. The phosphorylated substances were not permeable to the intact organisms, hence were neither excreted by mutants nor utilized when fed to the intact organisms. Since there is no enzyme for the activation of the imidazole derivatives, they are biosynthetically inactive. It is interesting to note that cystathionine has been shown to accumulate only in Neurospora cultures. Although Neurospora can metabolize cystathionine it is probably used only as a source of sulfur (Wiebers and Garner, 1964).

It has been found that in tryptophan biosynthesis certain auxotrophs metabolize indole even though the physiologically important constituent is indoleglycerol phosphate. Thus it can be seen that this enzyme catalyzes a reaction which is not the usual

physiological one in the intact organism and a compound is acted upon which probably does not exist *per se* in the whole cell (Yanofsky, Helinski, and Maling, 1961).

The possibility remains that the degradation of cystathionine observed was due to a reaction of no physiological importance. Evidence exists that the enzyme involved is, in reality, cysteine desulfhydrase (Cavallini *et al.*, 1962). This is an enzyme which is believed to produce thiocystine from cystine (Szczepkowski and Wood, 1965) in addition to pyruvate, H_2S and ammonia (Binkley, 1943) from cysteine. Thus what we may be observing is an enzyme acting *in vitro* on a substate that is physiologically atypical. It is also possible that the disruption of the cell causes conformational changes in cysteine desulfhydrase which makes it available to catalyze the cleavage of cystathionine. Such activity may not be possible in the enzyme's native state.

In as much as enzymes are ordered in such a manner to facilitate transfer of substrate, it is possible that the enzyme catalyzing the reaction observed is producing a compound which would be compartmentalized in the whole cell. Such localization may make the homocysteine formed unavailable for methionine biosynthesis but yet subject to degradative reactions. Thus the disruption of the integrity of the cell would be producing an observed phenomena which would exist only *in vitro*. It can be envisioned that in the

whole cell, homocysteine would be desulfhydrated to become a source of homoserine or desulfhydrated and deaminated for an additional source of α-ketobutyrate. These predictions presuppose that cystathionine is indeed cleaved in the intact organism. We have not demonstrated this. We have shown only that it is accumulated.

It is possible that in the whole cell there exists an inhibitor which prevents the action of cystathionase. In our system the inhibitor could have been removed by Sephadex treatment. If a specific inhibitor does exist, its structure would be genetically determined. Hence mutants lacking this inhibitor should be obtainable. The presence of an inhibitor could be investigated by preparing a mutant which required either homocysteine or cystathionine for growth starting with a known homocysteine mutant. Thus it could be postulated that this new ability to utilize cystathionine in the new mutant was due to a genetic inability in the organism to synthesize the physiological inhibitor.

It cannot be absolutely ascertained that cysteine and α-ketobutyrate are not produced by these enzymic preparations. It is possible that our isolation procedure was more specific for the products obtained. Cysteine, if produced, could be rapidly converted to pyruvate by the same enzyme. However, this does not detract from the finding of the production of homocysteine by a homocysteine requiring mutant.

SUMMARY

This is a report of the results of experiments undertaken in an attempt to clarify the mechanism of transsulfuration and the role of cystathionine in methionine biosynthesis in yeast.

Methionine auxotrophs with genetically determined blocks at specific intermediates in the proposed pathway of methionine biosynthesis were characterized. Homocysteine and cysteine requiring mutants were selected for further study.

Radioactive carbon and sulfur were used to determine that cystathionine was accumulated by the intact organism.

Cell-free extracts of the wildtype, homocysteine requiring, and cysteine requiring mutants were shown to cleave cystathionine. Pyruvic acid was identified as one of the cleavage products. Confirmation was obtained by comparing the absorption spectrum of authentic pyruvate phenylhydrazone with the phenylhydrazones obtained from the enzymatic reaction mixture.

A sulfhydryl was shown to be the other enzymatic cleavage product of cystathionine. This has been identified as homocysteine by a variety of procedures.

Some speculation on the differences in yeast methionine biosynthesis as compared with other organisms as suggested by these results is made. The possible significance of results showing a

mutant containing an enzyme which can cleave cystathionine in cell-free experiments but is unable to use the cystathionine in the intact organism is also discussed.

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