

Role of Homocysteine Synthetase in an Alternate Route for Methionine Biosynthesis in *Saccharomyces cerevisiae*

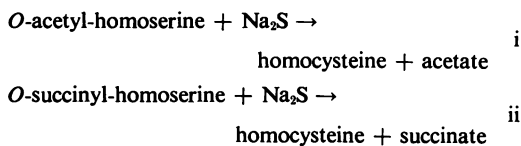
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Received for publication 4 February 1970

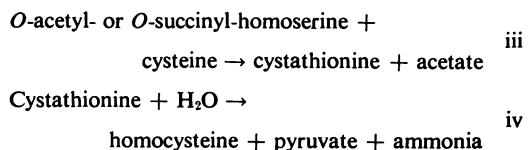
In vivo studies have shown that, in the absence of homoserine-*O*-transacetylase activity (locus *met*₂), the C₄-carbon moiety of ethionine is utilized (provided the ethionine resistance gene *eth-2r* is present) by methionine auxotrophs, except for *met*₃ mutants (homocysteine synthetase-deficient). Concomitant utilization of sulfur and methyl group from methylmercaptan or S-methylcysteine has been demonstrated. In the absence of added methylated intermediates, the methyl group of methionine formed from ethionine is derived from serine. In vitro studies with crude extracts of *Saccharomyces cerevisiae* have demonstrated that this synthesis of methionine occurs by the following reactions: CH₃-SH + ethionine ⇌ methionine + C₂H₅SH and S-methylcysteine + ethionine ⇌ methionine + S-ethylcysteine. In the forward direction, the second product of the second reaction was shown to be S-ethylcysteine; this reaction has also been found reversible, leading to ethionine formation. Genetic and kinetic data have shown that homocysteine synthetase catalyzes these two reactions, at 0.3% of the rate it catalyzes direct homocysteine synthesis: *O*-Ac-homoserine + Na₂S → homocysteine + acetate. The three reactions are lost together in a *met*₃ mutant and are recovered to the same extent in spontaneous prototrophic revertants from this strain. Methionine-mediated regulation of enzyme synthesis affects the three activities and is modified to the same extent by the presence of the recessive allele (*eth-2r*) of the regulatory gene *eth-2*. Affinities of the enzyme for substrates of both types of reactions are of the same order of magnitude. Moreover, ethionine, the substrate of the second reaction, inhibits the third reaction, whereas *O*-acetyl-homoserine, the substrate of the third reaction, inhibits the second reaction. An enzymatic cleavage of S-methylcysteine, leading to methylmercaptan production, has been shown to occur in crude yeast extracts. It is concluded that the enzyme homocysteine synthetase participates in the two alternate pathways leading to methionine biosynthesis in *S. cerevisiae*, one involving *O*-acetyl-homoserine and H₂S, the other involving the 4-carbon chain of ethionine and a mercaptyl donor. Participation of the two types of reactions catalyzed by homocysteine synthetase, in in vivo methionine synthesis, has been shown to occur in a *met*₂ partial revertant.

In vitro homocysteine biosynthesis has been shown to occur by either one of two distinct ways. The first path is by direct sulfhydrylation of *O*-acetyl or *O*-succinyl derivatives of homoserine, catalyzed by homocysteine synthetase (reactions i and ii):



Reaction i occurs in spinach (10), *Neurospora crassa* (13, 34), and yeast (1, 34). Reaction ii was demonstrated in *Escherichia coli* (34).

The second path is by intermediate synthesis of cystathionine (reaction iii) followed by its cleavage (reaction iv):



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Reaction iv, catalyzed by β-cystathionase, occurs

in bacteria, *N. crassa*, and yeast (4, 7). Reaction iii, catalyzed by cystathionine γ -synthetase, occurs in bacteria from *O*-succinyl-homoserine (4, 28) and also in spinach from *O*-acetyl- as well as *O*-succinyl-homoserine (9). In addition, purified cystathionine γ -synthetase from *Salmonella typhimurium* also catalyzes reaction ii (8). Recently, reaction iii has been demonstrated in *Neurospora* from *O*-acetyl-homoserine (13, 14). In yeast, no activity corresponding to this reaction has been detected so far (31; unpublished data).

Although enzymes for both pathways have been demonstrated in *Neurospora*, there are recent nutritional, enzymatic, and genetic data, showing that methionine is biologically synthesized via cystathionine (13, 23). In contrast, our previous findings with a methionine auxotroph of *Saccharomyces cerevisiae* devoid of homocysteine synthetase activity favors participation of reaction i in methionine biosynthesis and the absence of direct participation of cystathionine to this pathway. Moreover, we could show that synthesis and activity of homocysteine synthetase are controlled by the end product methionine (1). Additionally, we will show that revertants of homocysteine synthetase-deficient strains recover homocysteine synthetase activity together with prototrophy.

Regulation of synthesis of homocysteine synthetase as well as homoserine-*O*-transacetylase in *S. cerevisiae* has been shown to respond to a pleiotropic regulatory gene, *eth-2*. The presence of the recessive allele *eth-2r* in a haploid strain of *S. cerevisiae* results in a decreased repressibility of homocysteine synthetase and homoserine-*O*-transacetylase, together with the acquisition of ethionine resistance. The gene *eth-2* is unlinked to the two independent structural genes which correspond to these enzymes (*met₃* and *met₂*, respectively). The level of resistance to ethionine is greatly enhanced in the presence of another gene, *eth-1r* (dominant allele), which is independent of *eth-2* (2, 25). In *N. crassa*, a mutation (temperature conditional) leading to ethionine resistance also leads to loss of repressibility of several enzymes which are normally repressible by methionine. In this case, the enzymes involved are implicated in sulfate permeation and assimilation (17, 20).

A recombinant strain carrying a homoserine-*O*-transacetylase deficiency, *eth-1r* and *eth-2r* (CC 92-8D), was shown to exhibit peculiar growth characteristics by its ability to use ethionine, as well as methionine or *O*-acetyl-homoserine, for growth. In vivo studies, with differently labeled compounds indicated that, in such a strain, the carboxyl group of ethionine (and probably its entire 4-carbon skeleton) is used for methionine

biosynthesis, whereas sulfur and methyl groups do not originate from ethionine (3). Preliminary results using ³⁵S-CH₃ favored the participation of methylmercaptan to the synthesis of methionine from ethionine (3).

In this paper, biochemical and genetic evidence is presented for the participation of homocysteine synthetase in the alternate route of methionine synthesis in *S. cerevisiae* described above.

MATERIALS AND METHODS

Strains. The following haploid strains of *S. cerevisiae* were used: 4094-B— α , *ade₂*, *ura₁* (from the collection of F. Sherman); D6— α , *met₂*, *ura₁* (from the collection of M. Grenson); EY 9— α , *met₃* (from the collection of R. K. Mortimer); CH 82-7A— α , *ura₁*; CC 30-1D— α , *ade₂*, *ura₁*; CH 82-7D— α , *ade₂*, *ura₁*; CH 82-9C— α , *ade₂*, *ura₁*; CC 92-8D— α , *met₂*, *ade₂*, *ura₁*; CC 92-17A— α , *met₂*, *ade₂*, *ura₁*; and 113-2A— α , *ura₁*, *thr₂* (from our collection). Proceeding from information contained in Phillips and Kjellin-Straby (24), that strain D6 carried the *met₂* allele of Mortimer and Hawthorne (22), we then analyzed a *met₂* strain from the collection of R. K. Mortimer and found it homoserine-*O*-transacetylase-deficient. Consequently, our previous "met_a" gene has to be considered identical to *met₂*.

Appropriate diploids have been made by crosses between the haploid strains listed above. Spontaneous revertant strains have been isolated from EY 9 and CC 92-8D on the basis of methionine prototrophy.

Genetic analysis. Sporulation of diploids was induced by the method of McClary et al. (16). Ascospores were isolated according to Johnston and Mortimer (12).

Cultures. The synthetic medium (GO) was prepared by the procedure of de Robichon-Szulmajster and Magee (27), supplemented when necessary with uracil (10 mg/liter) and adenine (10 mg/liter). The concentration of amino acids added will be given below. Cultures (1 liter) of the appropriate medium (in 2-liter Fernbach flasks), inoculated from a 24-hr culture grown on YPGA medium (1), were shaken at 28 C and harvested between 2.0×10^7 and 3.0×10^7 cells/ml.

In vivo studies. The methods for cultivation in the presence of radioactive compounds (0.3 to 0.6 mc per 200 ml of culture), preparation of boiled extracts, protein hydrolysates, and amino acid chromatography have been previously described (3). Amounts of radioactive compounds formed have been calculated, accounting for isotopic dilution by exogenous unlabeled compounds present in the medium.

When ³⁵S-methylmercaptan was used as a methionine precursor, the culture was carried out in a special 300-ml flask (Fig. 1): an optical tube (no. 1) allowed optical density measurements without opening the flask, and a valve (no. 2) filled with mercury was used to release gas pressure; the exit valve was connected with two flasks containing 25% lead acetate and a third flask containing 50% KOH to trap radioactive volatile products; on the opposite side of the optical tube, a three-compartment side arm (no. 3) was attached. One compartment received 0.5 ml of 5 N

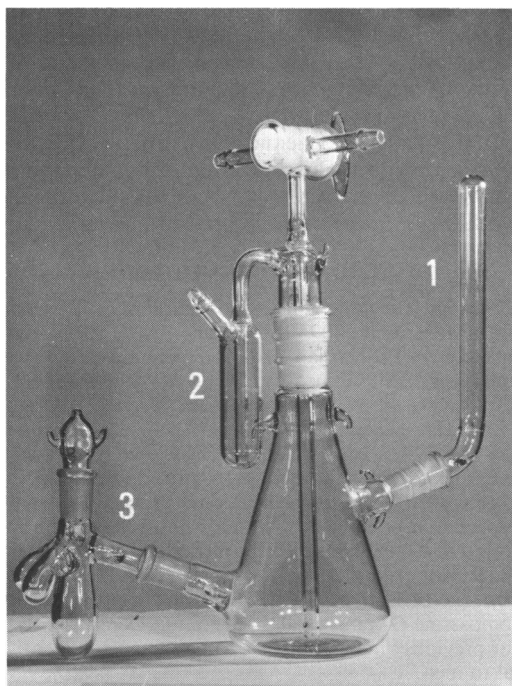


FIG. 1. Culture flask used for experiments with ^{35}S -methyl-isothiuronium sulfate. Repartition of various reagents and instructions for use are described in the text.

H_2SO_4 . The central compartment received ^{35}S -methyl-isothiuronium sulfate (424 mg) and the third compartment, 1 ml of 5 N NaOH. After closure, NaOH was mixed with ^{35}S -methyl-isothiuronium sulfate. The liberation of $^{35}\text{SH-CH}_3$ was accelerated by immersion of the side arm in a water bath at 60 C for a few minutes. Then complete liberation of $^{35}\text{S-CH}_3$ from its sodium salt, which could have been formed in the alkaline solution, was obtained by mixing the acid contained in the other compartment. All connections were made with ground-glass joints.

The culture (100 ml), inoculated at 1.0×10^7 cells/ml, was allowed to make one doubling of its mass and was stopped by immersing the whole flask into ground ice. Before opening the flask, the remaining gas was flushed out by bubbling air through for at least 2 hr. Cells were then harvested and treated as usual.

Enzyme assays. Extracts were prepared as already described (1). Homocysteine synthetase activity (reaction i) was estimated as described by Cherest et al. (1). The following reaction mixture used for determination of *in vitro* synthesis of methionine from ethionine (reaction vii) contained, in a final volume of 250 μl : tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8), 2.5 μmoles ; pyridoxal phosphate, 100 nmoles; dithiothreitol, 2.5 μmoles ; ^{12}C - or ^{14}C -L-ethionine (labeled either in the ethyl or in the carboxyl group), 2.5 μmoles ; S-methyl-L-

cysteine (^{32}S or ^{36}S , ^{12}C - or ^{14}C -methyl), 5 μmoles ; and crude extract, 80 μl (corresponding to approximately 1.0 mg of protein). Only one radioactive substrate was present at a time. Specific radioactivity used was 3.3×10^8 counts per min per nmole for S-methyl-L-cysteine and 2.5×10^8 counts per min per nmole for L-ethionine. Two types of controls have been carried out, one omitting extract, the other omitting the nonradioactive substrate from complete reaction mixtures. In some experiments, ^{35}S -methyl-L-cysteine was replaced by $^{35}\text{SH-CH}_3$. In this case, incubations were carried out in Warburg flasks in which the reaction mixture, without sulfur compounds, was introduced into the central compartment. The diverticule received 2.8 mg of S-methyl-iso-thiuronium sulfate and, just before closure, 50 μl of NaOH (0.4 N) which liberates SH-CH_3 . For demonstration of converse ethionine synthesis from methionine, incubations were made in the presence of ^{14}C -carboxyl-DL-methionine and S-ethyl-L-cysteine.

In all cases, after incubation for 1 hr at 28 C, the reaction was stopped by 2 min of boiling. The amount of radioactive products formed has been estimated in supernatant fluids by the same radiochromatographic techniques used for determination of homoserine-*O*-transacetylase activity (26). In addition, methionine, ethionine, S-methylcysteine, and S-ethyl-cysteine have been detected by the iodoplatinic reagent (33).

Protein estimation was carried out by the method of Lowry et al. (15) with bovine serum albumin as reference.

For practical reasons, specific activities will be expressed in milliunits per milligram of protein, i.e., nanomole per minute per milligram of protein.

Gas chromatography. The identification of the volatile product enzymatically formed from S-methylcysteine was obtained using two different columns: Chromasorb W (HMDS) containing 20% SE 30 and Chromasorb W (HMDS) containing 7% Craig polyester. Both columns were 300 cm long and 3 mm in diameter. Temperature was 80 C in all cases. Exclusion patterns were recorded on a flame ionization detector (Perkin Elmer F 11).

When gases were analyzed, incubations were carried out in tubes closed with special rubber covers. Gas samples were taken through the covers with a syringe, and needles and were immediately injected into the columns. The exclusion patterns were compared with pure compounds (methylmercaptan and dimethyl-disulfide).

Chemicals. *O*-acetyl-DL-homoserine was synthesized by the method of Sakami and Toennies (29). S-methyl-L-cysteine and S-ethyl-L-cysteine were purchased from Calbiochem, Los Angeles, Calif., and S-methyl-iso-thiuronium sulfate from E. Merck AG, Darmstadt, Germany. Methylmercaptan and dimethyl-disulfide were obtained from Fluka, Switzerland. $^{36}\text{SO}_4^{2-}$, $^{14}\text{C}_1$ -DL-homoserine, $^{14}\text{C}_1$ -(carboxyl)-DL-ethionine, $^{14}\text{C}_1$ -(ethyl₁₋₂)-L-ethionine, $^{14}\text{C}_1$ -(carboxyl)-DL-methionine, and S- ^{14}C -methyl-L-cysteine were obtained from C.E.A., France. ^{35}S -methyl-L-cysteine was purchased from Radiochemical Centre, Amersham, England, and $^{14}\text{C}_3$ -DL-serine from ICN Instrument Division, Oakland, Calif.

TABLE 2. Segregation of ethionine utilization among methionine auxotrophs^a

Diploid	Parental strain	Genes involved ^b	Addition to minimal medium ^c		
			DL-Methionine	O-acetyl-DL-homoserine	DL-Ethionine
CC 92	CH 82-7D × D6	$\frac{\text{eth-1r}}{\text{eth-1s}'} \frac{\text{eth-2r}}{\text{eth-2s}'} \frac{+}{\text{met}_2}$	192	192	79
CC 100	CH 82-7D × CC 92-8D	$\frac{\text{eth-1r}}{\text{eth-1r}'} \frac{\text{eth-2r}}{\text{eth-2r}'} \frac{+}{\text{met}_2}$	126	126	126
CC 109	CH 82-10D × D6	$\frac{\text{eth-1s}}{\text{eth-1s}'} \frac{\text{eth-2r}}{\text{eth-2s}'} \frac{+}{\text{met}_2}$	106	106	47
CC 123	4094-B × CC 92'-17A	$\frac{\text{eth-1s}}{\text{eth-1s}'} \frac{\text{eth-2s}}{\text{eth-2s}'} \frac{+}{\text{met}_2}$	80	80	0
CC 136	CH 82-7D × 113-2A	$\frac{\text{eth-1r}}{\text{eth-1s}'} \frac{\text{eth-2r}}{\text{eth-2s}'} \frac{+}{\text{thr}_3}$	60		22
CC 125	CC 92-8D × EY 9	$\frac{\text{eth-1r}}{\text{eth-1s}'} \frac{\text{eth-2r}}{\text{eth-2s}'} \frac{\text{met}_2}{+} \frac{+}{\text{met}_3}$	20 49	20 0	9 0

^a Final concentrations of amino acid were: 0.2 mM DL-methionine, 1 mM O-acetyl-DL-homoserine, 1 mM DL-ethionine. In addition, 2 mM DL-threonine was present in all media used for the study of segregation from CC 136.

^b Complete genotype of the haploid strains is given in Material and Methods.

^c Results indicate number of spores which were obtained by asci dissection. Segregation patterns reported concern only the methionine auxotroph population.

auxotroph segregants; the 2⁺/2⁻ ratio is compatible with segregation of only one character.

The absence of ethionine utilizers among segregants of diploid CC 123 (homozygous for eth-1s and eth-2s) shows that the presence of at least one of the ethionine resistance characters is required for ethionine utilization. This interpretation is reinforced by the finding that all the methionine auxotrophic segregants from diploid CC 100 (homozygous for eth-1r and eth-2r) are able to grow on ethionine. Furthermore, the comparison of data obtained from crosses CC 92 and CC 109 leads us to the conclusion that the presence of eth-2r alone is sufficient for ethionine utilization. However, careful comparison of the segregants from diploids CC 92 and CC 100 seems to indicate that the presence of eth-1r reinforces the ethionine utilization conferred by eth-2r in met₂ mutants. This effect is parallel to the increase in ethionine resistance observed previously in prototrophic strains carrying eth-1r in addition to eth-2r (2, 25).

Two possibilities were considered for reactions vi and vii which have never been described: first, an enzyme normally involved in the classical methionine biosynthetic pathway is able, in addition to its main reaction, to catalyze these two reactions; and second, an enzyme not involved in

the normal biosynthesis of methionine, is responsible for this catalysis. The participation of homoserine-O-transacetylase is excluded by the previous findings with met₂-deficient strains. Mutants blocked in other steps in methionine biosynthesis then had to be studied.

For this purpose, two diploids were constructed: one containing a deficiency for aspartokinase (locus thr₃) which confers a double auxotrophy (threonine + methionine), and the other, a deficiency for homocysteine synthetase (locus met₃). In the first cross, CC 136, 22 among 60 threonine + methionine auxotrophs were able to grow in the presence of threonine and ethionine instead of threonine and methionine (see Table 2). In the second cross, CC 125, two unlinked methionine genes were implicated (met₂ and met₃) leading to three types of methionine auxotrophic segregants. Among these auxotrophs, ethionine utilization occurs only in single met₂ auxotrophs and never in single met₃ or double met₂ met₃ segregants.

These results suggest that the gene met₃ which specifies homocysteine synthetase is implicated in ethionine metabolism.

In vitro experiments: relationship between homocysteine synthetase activity and direct synthesis of methionine from ethionine. All previous (3) and

present results pointed to the strain CC 92-8D (met_2 , "eth⁺") as the best candidate for in vitro study of methionine synthesis from ethionine. First, we attempted to demonstrate direct formation of methionine either from ethionine + methylmercaptan (reaction vi) or ethionine + S-methyl-cysteine (reaction vii). Reactions vi and vii were found to occur from extracts of CC 92-8D with specific activities of 2.1 and 1.05, respectively.

Reaction vi is not easy to control because of the volatile nature of methylmercaptan. Assuming, for the time being, that both reactions are catalyzed by the same enzymatic system, reaction vii was chosen as the standard assay system.

Although the in vivo capacity to utilize ethionine requires a peculiar genotype (e.g., CC 92-8D), it was conceivable that the ability to catalyze, in vitro, reaction vii is a more general feature of *S. cerevisiae*. Table 3 shows a comparative study of strains with different genotypes. It can be seen that direct methionine synthesis (reaction vii) can be demonstrated (in comparable amounts) by using met_2 mutants either able or unable to grow in the presence of ethionine. Moreover, similar activity was found in methio-

nine prototrophs, and this activity was not influenced by the presence or absence of ethionine resistance genes. On the other hand, as expected from the above genetic results, reaction vii could not be detected in extracts from a met_2 mutant which is devoid of homocysteine synthetase activity.

The absolute relationship between the activities of reactions i and vii is emphasized by enzymatic analysis of eight spontaneous revertants isolated from EY 9. It should be noticed (Table 3) that the recovery of both reactions remains strikingly constant and close to what is observed in the wild-type strains. Thus, it seems highly probable that homocysteine synthetase participates in reaction vii, with a specific activity which amounts to about 0.3% of the main reaction (i).

Origin of the sulfur, methyl, and carboxyl groups of methionine formed by reaction vii. In vitro studies were then undertaken to verify the in vivo findings and definitely establish the origin of different parts of the methionine synthesized, as well as the exact nature of the second product of reaction vii.

As reported above, all strains catalyzing reaction i are able to catalyze reaction vii. We have

TABLE 3. Relationship between homocysteine synthetase activity and direct synthesis of methionine from ethionine^a

Strain ^b	Enzymatic activity ^c		
	Reaction i	Reaction vii	Reaction vii/reaction i (%)
I. Methionine auxotrophs			
CC 92-8D (met_2 , eth-1r, eth-2r, eth ⁺)	190	0.980	0.51
D6 (met_2 , eth-1s, eth-2s, eth ⁻)	161	0.530	0.33
EY 9 (met_s , eth-1s, eth-2s, eth ⁻)	10	0	
II. Wild-type strains			
4094-B (eth-1s, eth-2s)	395	1.30	0.33
CH 82-7A (eth-1r, eth-2s)	420	1.21	0.29
CC 30-1D (eth-1s, eth-2r)	350	1.06	0.30
CH 82-7D (eth-1r, eth-2r)	342	1.12	0.30
III. Revertants from EY 9			
R 8	131	0.350	0.27
R 9	194	0.640	0.33
R 20	195	0.700	0.36
R 4	207	0.545	0.26
R 27	220	0.580	0.26
R 17	230	0.950	0.41
R 22	235	0.745	0.32
R 2	258	0.610	0.24

^a Cultures of wild-type or revertant strains were grown in minimal medium. Methionine auxotrophs were grown in the same medium complemented with 0.2 mM DL-homocysteine.

^b Abbreviations: eth⁺, ethionine utilizers; eth⁻, strains unable to grow on ethionine.

^c Expressed as nanomoles per minute per milligram of protein.

then chosen, for further investigation, the wild-type strain 4094-B, which was extensively used in other work from this laboratory. These studies have been carried out with radioactive substrates labeled in different positions. Results are summarized in Table 4.

In agreement with results obtained *in vivo*, it can be seen that the carboxyl group of methionine synthesized *in vitro* originates from the carboxyl group of ethionine. Moreover, comparable amounts of methionine were formed whenever S-methylcysteine or methylmercaptan were used as the second substrate.

In such incubations, the second product should be S-ethylcysteine and ethylmercaptan, respectively. To demonstrate S-ethylcysteine formation, we have used L-ethionine labeled in the ethyl group. Because of the close R_F values of methionine and S-ethylcysteine in any solvent system we tried, the supernatant fraction obtained after incubation was co-chromatographed with either methionine or S-ethylcysteine (unlabeled). Figure 2 shows the superimposing of the radioautogram and the iodoplatinate characterization of sulfur compounds for one typical chromatogram. It can be seen that, when co-chromatography was made with S-ethylcysteine, radioactive and iodoplatinate-positive spots perfectly coincide. On the contrary, when the supernatant fraction is co-chromatographed with methionine, the spot corresponding to methionine is slightly, but distinctly, lower than the radioactive spot. The results show that ethylcysteine is the radioactive product formed in these experiments. By analogy, it then seems probable that ethylmercaptan is formed as the second product of reaction vi. (It can be

pointed out that, in an experiment in which strain CC 92-8D was grown in the presence of ethionine ^{35}S , S-ethylcysteine, the second product of reaction vii, was found in boiled extract.)

Utilization of ^{35}S - or ^{14}C -methyl-labeled S-

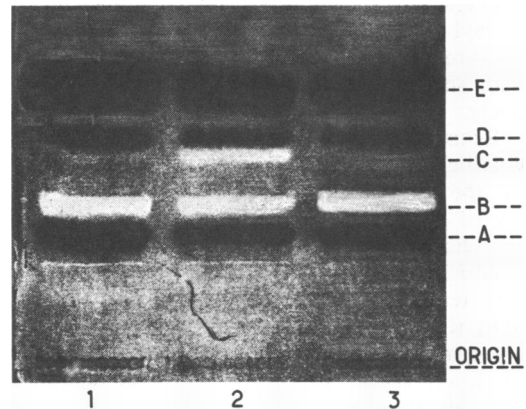


FIG. 2. Chromatographic identification of methionine and S-ethylcysteine. Thin-layer plates of cellulose powder (MN 300, Macherey, Nagel & Co.) have been submitted to ascending chromatography with *n*-butanol-acetic acid- H_2O (120:30:40) as solvent. Column 1, incubation mixture (10 μl) as described in text; column 2, incubation mixture (5 μl) + 0.1 μM DL-methionine (5 μl); column 3, incubation mixture (5 μl) + 0.1 μM S-ethyl-L-cysteine (5 μl). Sulfur-containing compounds are characterized by the iodoplatinate reagent (33). Black spots correspond to radioactive compounds, and white spots to nonradioactive compounds. A, Ethionine sulfoxide (R_F 0.32); B, S-methylcysteine (R_F 0.40); C, S-ethylcysteine (R_F 0.51); D, methionine (R_F 0.56); E, ethionine (R_F 0.69).

TABLE 4. Identification of products from reactions vi and vii^a

Radioactive substrates ^b	Nonradioactive substrates	No. of experiments	Radioactive products formed ^c		
			Methionine	Ethionine	Ethylcysteine
Crude extracts					
^{14}C -carboxyl-ethionine	SH- CH_3	4	2.01 (1.75-2.28)		
^{14}C -carboxyl-ethionine	S-methylcysteine	6	1.52 (1.1-2.1)		
^{14}C -ethyl-ethionine	S-methylcysteine	1			1.02
^{35}S -methylcysteine	Ethionine	2	1.31		
S- ^{14}C -methylcysteine	Ethionine	1	1.60		
^{14}C -carboxyl-methionine	S-ethylcysteine	3		1.3 (0.7-2.0)	
Dialyzed extracts					
^{14}C -carboxyl-ethionine	SH- CH_3	3	2.09 (1.42-2.9)		
^{14}C -carboxyl-ethionine	S-methylcysteine	3	1.43 (1.07-2.1)		

^a The wild-type strain 4094-B was used.

^b Final substrate concentrations were as follows: in L-forms, ethionine and methionine, 10 mM; S-methylcysteine and S-ethylcysteine, 20 mM. The exact concentration of methylmercaptan is unknown. Extreme values are given in parentheses when more than two experiments were carried out.

^c Results expressed as nanomoles per minute per milligram of protein.

methylcysteine permitted to show that both labels are recovered exclusively in the methionine formed. That amount of radioactive product formed was almost equivalent in each type of incubation is compatible with the proposed stoichiometry of reaction vii. In addition, Table 4 shows that utilization of carboxyl-labeled methionine leads to recovery of labeled ethionine. Reaction vii then appears to be catalyzed in both directions by homocysteine synthetase.

The finding that methionine is formed as well from S-methylcysteine or methylmercaptan could have been due to intermediate formation of S-methylcysteine from methylmercaptan and serine (or acetyl-serine) brought by crude extracts (reaction v). However, this explanation seemed unlikely as utilization of both substances was not modified by dialysis of crude extracts, as shown on the last part of Table 4.

Alternately, CH_3SH could be considered as the only substrate for methionine direct synthesis,

provided that S-methyl-cysteine could generate this compound. Cleavage of S-methyl-cysteine has been shown to occur in crude extracts which catalyze net methionine synthesis from methylmercaptan and ethionine. As shown in Table 5, mercaptan formation is probably due to enzymatic cleavage of S-methylcysteine, as it does not form in the absence of crude extract. Dithiothreitol is not necessary for this reaction to occur. In addition, it was found that S-ethylcysteine also undergoes an enzymatic cleavage, yielding a volatile product which reduces dithio-bis-nitrobenzoic acid (DTNB) and should then be ethylmercaptan (by analogy with the cleavage of S-methylcysteine; see below). In contrast, neither methionine nor ethionine yield any volatile reducing product under the same conditions (Table 5).

The volatile product formed from S-methylcysteine was identified by gas-chromatography as dimethyl disulfide. The DTNB reduction, observed when incubations were carried out in War-

TABLE 5. Mercaptan formation from different sulfur-containing amino acids^a

Reagents in culture flask ^b	Content of Warburg flasks (μ liters)								
	1	2	3	4	5	6	7	8	9
Main compartment ^c									
DTT, 10^{-1} M		25			25				
PLP, 10^{-2} M	10	10	10	10	10	10	10	10	10
SMC, 10^{-1} M				50	50	50			
SEC, 10^{-1} M							50		
Methionine, 10^{-1} M								25	
Ethionine, 10^{-1} M									25
Tris buffer pH 8, 1 M	25	25	25	25	25	25	25	25	25
Extract	80	80		80	80		80	80	80
Appendix 1									
SMITU	2.8 mg	2.8 mg	2.8 mg						
NaOH, 5×10^{-1} M	50	50	50						
Appendix 2									
DTNB, 10^{-2} M	100	100	100	100	100	100	100	100	100
Central well									
DTNB, 10^{-2} M	100	100	100	100	100	100	100	100	100
Mercaptan formed	+	+	+	+	+	-	+	-	-

^a Abbreviations: DTNB, dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; SMITU, S-methylisothio-urionium sulfate. The experiments were carried out in Warburg flasks as described under Materials and Methods. Volatile mercaptan formation was characterized by introducing DTNB (in phosphate buffer, 0.1 M, pH 7.5) into one of the side arms and into the central well. When SMITU was present, the yellow-orange color characteristic of SH compounds quickly appeared in the DTNB reagent after starting the reaction. When amino acids were present, the color gradually appeared during the 1-hr incubation period at 28 C (reported as + in the table). At the end of the experiment, the DTNB from the side arm was mixed with the incubated mixture in the main compartment and the color became intensively orange. When present, DTT very slightly reinforced the pale-yellow color of the DTNB reagent itself.

^b See Fig. 1 for illustration of flask.

^c Water was added to a final volume of 250 μ liters.

burg flasks, renders probable that methylmercaptan is first formed and then dimerized.

These results provide evidence for the existence in crude yeast extracts of a S-methylcysteine lyase. However, the formation of important amounts of S-ethylcysteine in reaction vii favors the interpretation that S-methylcysteine, as well as methylmercaptan, could be a true substrate for homocysteine synthetase.

Kinetic data for reactions catalyzed by homo-

cysteine synthetase. We will first consider reaction vii. The relationship between methionine formed and incubation time or protein concentration is given in parts A and B, respectively, of Fig. 3. The curves show the validity of the standard conditions used in previous parts of this study (see Materials and Methods). The K_m values have been estimated for both substrates of reaction vii, as shown in Fig. 3C and D. The data in Table 6 allow comparison of the affinities of homocysteine

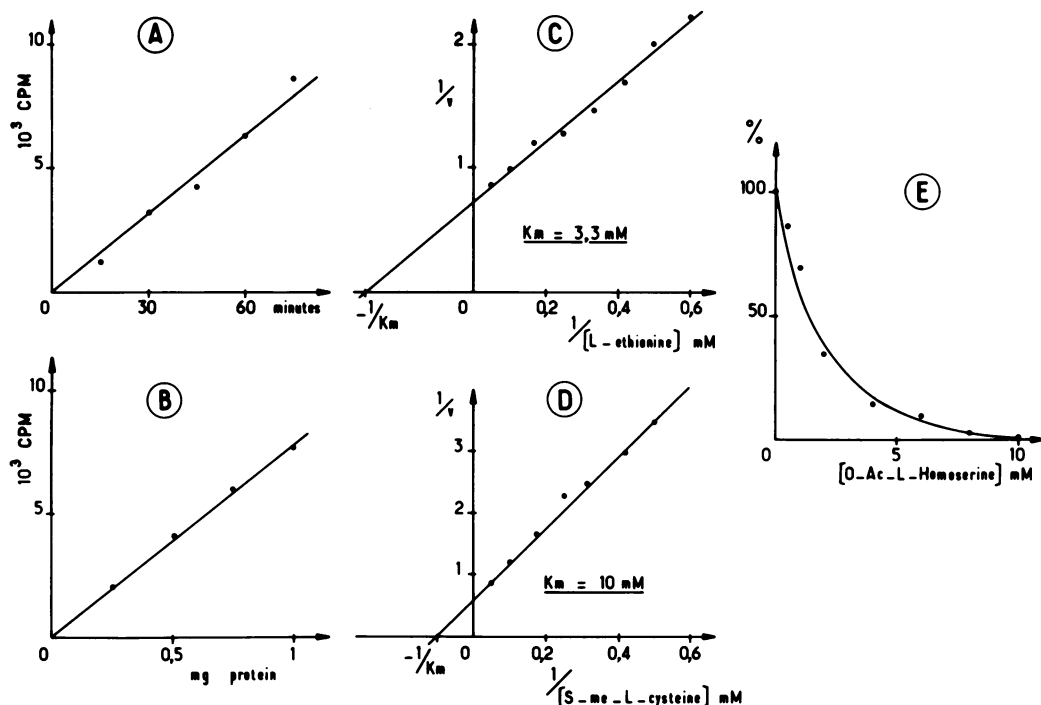


FIG. 3. Kinetic data for reaction vii. Crude extracts of 4094-B have been used. Measurements were carried out as described in the text. A, Time dependence of methionine formation from 14 carboxyl-L-ethionine; B, dependence of velocity on added crude extract (60-min incubation period); C, variation of activity with L-ethionine concentration (Lineweaver-Burk transformation); D, variation of activity with S-methyl-L-cysteine concentration (Lineweaver-Burk transformation); E, per cent activity in the presence of increasing concentrations of O-acetyl-homoserine.

TABLE 6. Comparative affinities of homocysteine synthetase towards substrates and inhibitors^a of reactions i and vii

Reaction	Substrate (L-form)	K_m (mM)	Inhibitor (L-form)	K_i (mM)
i: O-Ac-HS + Na ₂ S → HC + acetate	O-acetyl-homoserine	7.0 ^b	Methionine	1.9 ^b
	Na ₂ S	20.0	Ethionine	4.5
vii: Ethionine + SMC → methionine + SEC	Ethionine	3.3	S-methyl-cysteine	85.0
	S-methyl-cysteine	10.0	O-acetyl-homoserine	1.3 ^c

^a Crude extracts from strain 4094-B were used. Abbreviations: HC, homocysteine; O-Ac-HS, O-acetyl-homoserine; SMC, S-methyl-cysteine; SEC, S-ethyl-cysteine.

^b Values previously reported (1).

^c Apparent K_i value deduced from Fig. 3E. Other K_i values were obtained with two concentrations of substrate and calculated according to Dixon and Webb (5).

synthetase towards the substrates of reactions i and vii. Affinities for the substrates of reaction i (*O*-acetyl-homoserine and Na_2S) are twofold lower than affinities for the substrates of reaction vii (ethionine and *S*-methylcysteine).

We have previously reported that methionine inhibits direct homocysteine synthesis. This inhibition is competitive towards *O*-acetyl-homoserine (1). In addition, it can be seen in Table 6 that the methionine analogue, ethionine, inhibits *O*-acetyl-homoserine sulfhydrylation to the same extent as methionine. Ethionine inhibition can be explained by its structural analogy with methionine. However, in view of our findings about catalysis of reaction vi by homocysteine synthetase, it was conceivable that ethionine inhibition could be due to its activity as substrate. In turn, it seemed interesting to search for an inhibitory effect of *O*-acetyl-homoserine on reaction vii. Such an inhibition was effectively found (Fig. 3E and Table 6). On the contrary, *S*-methylcysteine has a very low inhibitory effect (see Table 6). Results in Table 6 show that there is a good agreement between K_m and K_i values for ethionine. On the contrary K_m and K_i values for *S*-methylcysteine and *O*-acetyl-homoserine are farther apart. Two kinds of explanations can be found to account for these discrepancies. First, sodium sulfide was found to exert an important inhibitory effect on reaction i at concentrations higher than the K_m value. In consequence, all measurements of reaction i were carried out by using a sulfide concentration which is half of the K_m value. Second, it must be pointed out that these determinations have been made with crude extracts in which other enzymatic activities can compete, under the incubating conditions used, for utilization of either substrates or inhibitors of homocysteine synthetase. For example, an *S*-methylcysteine lyase activity was shown to be present (see above). On another hand, it has been shown in *N. crassa* that *O*-acetyl-homoserine can directly react with methylmercaptan to yield methionine (21, 30). In previous experiments from this laboratory, extracts from *S. cerevisiae* were found able to catalyze, at a slow rate, methionine synthesis from *O*-acetyl-homoserine and *S*-methylcysteine. It is not yet possible to decide whether such a reaction implies previous splitting of *S*-methylcysteine.

In spite of the above restrictions, the hypothesis can be made that identical sites on homocysteine synthetase molecule are used by substrates of reaction i and vii.

Metabolic origin of the methyl group of methionine synthesized from ethionine and mercaptal compounds. It is well known that the β -carbon of serine is the precursor of the methyl group of methionine synthesized by any one of the classical pathways. It was then of interest to determine

whether the methyl group of methionine formed from the carbon skeleton of ethionine had the same metabolic origin, whatever the further intermediates might be.

An experiment was carried out with strain CC 92-8D (ethionine utilizer) grown in the presence of 0.3 mM DL-ethionine and 0.075 mM DL-serine-3- ^{14}C . Amounts of ^{14}C -methionine present in protein hydrolysate were estimated to be 2 μ moles and 0.36 μ moles per g (dry weight), respectively. A control experiment was run with a wild-type strain (4094-B) grown in minimal medium in the presence of only DL-serine-3- ^{14}C . In this case, 1.65 μ moles of ^{14}C -serine and 0.33 μ moles of ^{14}C -methionine were recovered. Neither of the strains used was serine-deficient, therefore isotopic dilution occurred. In both cases, the total amount of radioactive serine and methionine recovered are comparable, and the ratio between the two amino acids remains identical to what is expected from the yeast protein composition (6). These results permit us to conclude that serine provides the methyl group of methionine as efficiently when methionine arises from ethionine utilization as when methionine is formed in the classical pathway. Consequently, the methyl group of *S*-methylcysteine and methylmercaptan (intermediates in the alternate methionine pathway) must originate from serine.

Regulation of synthesis of homocysteine synthetase. We have previously reported that exogenous methionine extensively represses the synthesis of homocysteine synthetase in wild-type strains of *S. cerevisiae*. Furthermore, synthesis of this enzyme and homoserine-*O*-transacetylase synthesis were shown to be under the control of a regulatory gene, eth-2 (1). Since this study was concerned with the ability of homocysteine synthetase to catalyze two distinct reactions, one would predict that both activities should respond similarly to this gene-controlled repressibility. Results given in Table 7 fully agree with this hypothesis, i.e., in strains carrying the dominant allele eth-2s, repressibility is maximal for both reactions and is reduced to the same extent in a strain carrying the mutated recessive allele eth-2r.

Efficiency of ethionine utilization in vivo. We attempted to evaluate the respective contribution of both pathways to methionine biosynthesis in different strains. Results are summarized in Table 8.

The ethionine-sensitive strain (4094-B) grown in ^{35}S sulfate is taken as an internal reference. As previously reported, 10 to 12 μ moles of methionine per g (dry weight) are recovered in our standard conditions (3). Also in agreement with previous results, it can be seen that the ethionine utilizer, CC 92-8D, synthesizes as much methionine from ^{14}C -carboxyl-ethionine as is made by

the classical pathway in the wild-type strain. The ethionine-resistant strain CH 82-7D incorporates very little ^{14}C -carboxyl-ethionine into its protein. It can be pointed out that this labeling is equally divided into methionine and ethionine. Similar results have already been obtained in ^{35}S -ethionine experiments carried out either with sensitive or resistant strains (*unpublished data*). It seems then that some conversion of ethionine into methionine must occur. Preliminary results from this laboratory (M. Tingle) indicate that K_m values for activation of methionine and ethionine are 4×10^{-5} and 2×10^{-2} M, respectively, and are identical in strains carrying eth-1s, eth-2s or eth-1r, eth-2r characters. Affinities of the enzyme from both strains towards transfer ribonucleic acid (tRNA), as measured by the attachment assay with ^{14}C -methionine, also seem to be alike.

The low level of ethionine incorporation obtained in CH 82-7D is then compatible with the greater affinity of *S. cerevisiae* methionyl-tRNA synthetase for methionine than for ethionine. The above results show that, in a strain like CH 82-7D in which methionine can be synthesized in the presence of ethionine through the classical pathway, very little or no methionine arises from

ethionine. Identical results were obtained when a prototroph revertant from CC 92-8D was studied (revertant 6). Nevertheless a methionine bradytroph, also isolated from CC 92-8D (revertant 11), synthesizes 24% of its methionine from ethionine when grown in the presence of ^{14}C -carboxyl-ethionine. This result indicates that the alternate route for methionine biosynthesis can be expressed in such a strain. It would have been interesting to correlate expression of both pathways with the extent of homoserine-*O*-transacetylase recovery. Unfortunately, we have been unable to detect any homoserine transacetylase activity in the extracts of the two revertant strains examined so far. It might be possible that, in both cases, the reversion event has led to an unstable enzyme.

DISCUSSION

Previous *in vivo* experiments from this laboratory pointed towards the possibility that direct methionine synthesis could occur in *S. cerevisiae* by using methylmercaptan and the 4-carbon moiety of ethionine (3). In the present work, we have intended to demonstrate that methionine synthesis occurs *in vitro* from these two compounds by using crude extracts of *S. cerevisiae*.

Utilization of substrates labeled in different positions led to the following conclusions. (a) The carboxyl group of methionine originates from ethionine. (b) The sulfur atom of methionine originates either from methylmercaptan or from S-methylcysteine. (c) The methyl group was also provided by S-methylcysteine ($^{14}\text{CH}_3\text{SH}$ has not yet been tried). (d) During the reaction between S-methylcysteine and ethionine, S-ethylcysteine carrying the ethyl group from ethionine was recovered in the same amount as methionine. (e) Ethionine is synthesized from ^{14}C -carboxyl-methionine in amount comparable to the methionine synthesized in the converse reaction. All these results corroborate previous findings obtained from *in vivo* studies.

Are both methylmercaptan and S-methylcysteine true substrates of reactions v and vi. It

TABLE 7. Comparative repression of activities for two of the reactions catalyzed by homocysteine synthetase, in various strains

Strains	Genes involved	Repression by DL-methionine 2 mM	
		Reaction i ^a	Reaction vii ^b
		%	%
4094-B	eth-1s, eth-2s	86	89.5
CH 82-7A	eth-1r, eth-2s	82.5	86.0
CH 82-7D	eth-1r, eth-2r	35.0	24.0

^a Reaction i: *O*-acetyl-homoserine + H₂S → homocysteine + acetate.

^b Reaction vii: ethionine + S-methylcysteine → methionine + S-ethylcysteine.

TABLE 8. *In vivo* synthesis of methionine from labeled sulfate or ethionine in different strains of *S. cerevisiae*

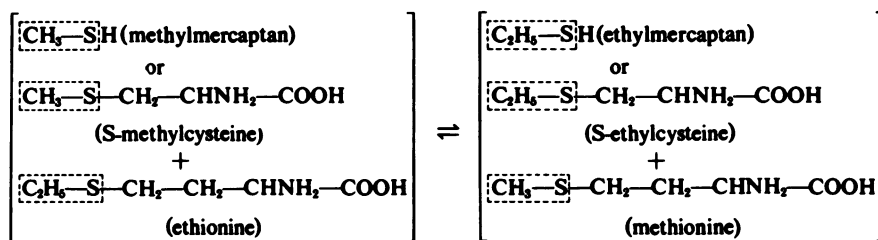
Strains	Ethionine resistance genes	Addition of radioactive compounds	Labeled amino acids into proteins ^a	
			Methionine	Ethionine
4094-B	eth-1s; eth-2s	$^{35}\text{SO}_4^{2-}$	10.9	0
CC 92-8D	eth-1r; eth-2r	^{14}C -carboxyl-ethionine	10.0	0.3
CH 82-7D	eth-1r; eth-2r	^{14}C -carboxyl-ethionine	0.4	0.4
CC 92-8D revertant 11	eth-1r; eth-2r	^{14}C -carboxyl-ethionine	2.4	0.4

^a Methionine and ethionine have been estimated in protein hydrolysates as previously described (3). Values expressed as micromoles per gram (dry weight).

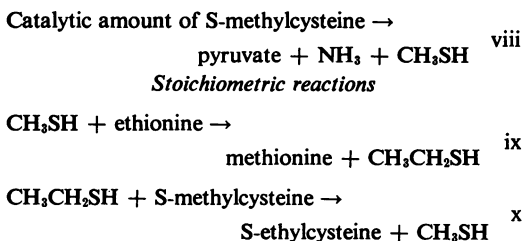
seemed unlikely that S-methylcysteine de novo synthesis occurring in the crude extract could be the origin of methylmercaptan incorporation, as dialysis does not modify incorporation of this compound. On the other hand, methylmercaptan was shown to arise from incubation of S-methylcysteine with crude yeast extract. A methylcysteine lyase of the kind recently described by Smith and Thompson (30) in *N. crassa* could be implicated. Although separation of the enzymatic fraction catalyzing methionine direct synthesis from the S-methylcysteine splitting activity can only definitely prove that S-methylcysteine is a true substrate for reaction vii, the in vitro formation of S-ethylcysteine from S-methylcysteine and ethionine indicates that it must be the case.

All the findings reported in the present paper favor the concept of an exchange of carboxyl groups between the two amino acids (ethionine and S-methylcysteine). Moreover, the transfer with an equivalent efficiency of sulfur and methyl groups from S-methylcysteine to methionine suggests that the entire thiomethyl group is transferred and exchanged with the thioethyl group of ethionine. Consequently, it seems conceivable that the entire 4-carbon chain of ethionine would be either transferred to methylmercaptan or exchanged with the 3-carbon chain of S-methylcysteine.

A direct exchange of mercaptyl groups can be postulated according to the following scheme:



The enzyme which reversibly catalyzes such a reaction could be named mercaptantransferase. Alternatively, a sequence of reactions involving transitory formation and reutilization of methylmercaptan cannot be excluded. A possible formulation could be:



In this case, homocysteine synthetase could

catalyze only one of the three reactions, most likely reaction ix.

To have a biological significance for the methionine auxotrophs able to utilize the 4-carbon moiety of ethionine (such as the met₂,eth-2r strains), all the above mechanisms (reactions v-x) imply the existence of a de novo synthesis of methylmercaptan which has not yet been proved. Nevertheless, methylmercaptan so far remains the only candidate for in vivo, as well as in vitro, alternate methionine biosynthesis from ethionine.

The theoretically possible formation of homocysteine and ethylmercaptan from ethionine and H₂S has been eliminated after finding that radioactive homocysteine could not be detected after incubation of ¹⁴COOH-ethionine and H₂S in conditions identical to those which allowed net methionine synthesis from ethionine and methylmercaptan or S-methylcysteine.

In vitro studies have shown that methionine auxotrophs unable to catalyze sulphydrylation of O-acetyl-homoserine have also lost the ability to utilize methylmercaptan or S-methylcysteine for an alternate biosynthesis of methionine (reactions vi and vii).

The relationship between the two activities has been established by genetic and enzymatic criteria. First, met₃ spontaneous revertants recover both activities with a constant ratio. In addition, regulation of both activities is placed under the con-

trol of the same regulatory gene, eth₂. Second complete inhibition of homocysteine direct synthesis from acetyl-homoserine, and H₂S is observed with ethionine as well as with methionine. In turn, O-acetylhomoserine is able to inhibit completely methionine direct synthesis from ethionine and S-methylcysteine. Affinities of the enzyme for each of these compounds, when considered as substrates or as inhibitors, are of the same order of magnitude. Mercaptan transferase activity described above and homocysteine synthetase activity thus seem to be catalyzed by the same protein. Although the presence of an active homocysteine synthetase appears to be essential for the two paths leading to methionine biosynthesis in *S. cerevisiae*, the normal path uses H₂S as the sulfur source and O-acetylhomoserine as

the activated 4-carbon amino acid, whereas the alternate path uses CH_3SH and the 4-carbon chain of ethionine.

It was found that ethionine utilization requires the presence of the allele *eth-2r* as well as the presence of homocysteine synthetase. This is supported by the fact that, among the methionine auxotrophs studied, *met*₂ (homoserine-*O*-transacetylase-deficient) and *thr*₃ (aspartokinase-deficient), only recombinants carrying the allele *eth-2r* are ethionine utilizers. The role of the allele *eth-2r* for in vivo expression of the alternate route leading to methionine biosynthesis could be explained if one assumes that ethionine, as well as methionine, can mediate repression of homocysteine synthetase. Unpublished experiments from this laboratory have shown that homocysteine synthetase activity remains unchanged in a resistant strain grown in the presence of ethionine and disappears in a sensitive strain grown in the same conditions. However, the disappearance of many other enzymes together with homocysteine synthetase in the latter strain does not permit to separate what, in this effect, is due to repression per se and to inactivation by incorporation of ethionine into protein molecules.

In vitro synthesis of methionine from ethionine (reactions vi and vii) is catalyzed by homocysteine synthetase with a specific activity of 1.5. Although this activity corresponds to less than 1% of the optimal activity observed for reaction i, it is comparable to the specific activity observed for the acetylation of homoserine (26), and, obviously, allows sufficient synthesis of methionine from ethionine in a methionine auxotroph other than a *met*₃ mutant. That a partial revertant at the *met*₂ locus synthesized considerably more methionine from ethionine than a methionine prototroph of convenient genotype shows that in vivo competition between both pathways might be expressed concomitantly.

Since ethionine is not a natural substrate, it was thought that *O*-acetyl-homoserine could be the natural acceptor of the mercaptal group. In vivo synthesis of methylmercaptan followed by mercaptylation of *O*-acetyl-homoserine has been recently proposed by Moore et al. (21) as an explanation for the leakiness of *N. crassa* mutant blocked after *O*-acetyl-homoserine. As already mentioned, in vitro experiments carried out by Smith and Thompson (30) in *N. crassa* and evidence reported in the present paper for *S. cerevisiae* have shown that methylmercaptan can arise from *S*-methylcysteine. Thus, the presence in yeast of an enzyme able to condense CH_3SH and serine (or acetyl-serine) would permit a cyclic recapture of methylmercaptan.

The findings that two distinct enzymes lead to

interconversion of methylmercaptan and *S*-methylcysteine, and that these two compounds are substrates for homocysteine synthetase, does not indicate their metabolic origin. So far, the only information concerns the methyl group which has been shown to originate from serine (with the same efficiency as in vivo methylation of homocysteine).

ACKNOWLEDGMENTS

The authors are thankful to F. de la Torre for her skillful assistance.

This work was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique (66.00.140) and from the C.E.A., France.

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