# A GENETIC AND BIOCHEMICAL ANALYSIS OF SELECTED YEAST METHIONINE AUXOTROPHS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

# A GENETIC AND BIOCHEMICAL ANALYSIS OF SELECTED YEAST METHIONINE AUXOTROPHS

## INTRODUCTION

The evolution of regulatory control processes for a large variety of physiological activities in microorganisms provided for the cells great versatility in adjusting to environmental changes. Many thousands of years of ecological competition have forced the selection of those living systems expressing a high degree of metabolic efficiency and parsimony in energy utilization. Since the macromolecular constituents of cells account for approximately eighty percent of the cell's dry weight, an effective control limiting the formation of these components to the minimum amounts dictated by the environment would be of paramount importance for survival.

Control of protein synthesis is accomplished by limiting the availability of amino acid precursors as well as by regulating the condensation of the preformed amino acids into the protein structure. Sites for these phenomena are at the level of enzyme function (feedback inhibition) and at the synthesis of new enzyme protein (repression and induction).

A large amount of data has been accumulated showing the repressive and feedback effects of biosynthetic end-products in bacterial systems. In yeast, however,

investigations have not been extensive. Because of their great metabolic versatility, ease of culture, and well defined genetic mechanism, yeasts were used in this study of metabolic regulation. Studies were made in this laboratory in an attempt to ascertain the effect of methionine amd certain methionine analogs on the biosynthesis of this amino acid in Saccharomyces cervisiae.

# REVIEW OF LITERATURE

Repression was first discovered in 1953 when Monod and Cohen-Bazire found that the formation of the enzyme tryptophan synthetase was selectively inhibited by tryptophan and certain trytophan analogs in Escherichia coli (30, p. 530-532). Mutants of this organism have been isolated which are insensitive to 5-methyltryptophan in the presence of which the normal wild type will not grow (6, p. 3490-3492). When cultured with the natural amino acid, cellular synthesis of at least two of the tryptophan biosynthetic enzymes was repressed in the wild type; however, the 5-methyltryptophan insensitive mutants produced high levels of these enzymes regardless of the presence of tryptophan. The genes conferring non-repressibility have been mapped and shown to be some distance from the cluster of genes responsible for the synthesis of tryptophan (52, p. 425-447).

Many other isolated cases of enzyme repression have been studied; however, for the most part, the genotypic-phenotypic relation of the control mechanisms has not yet been determined. These include repression of l-threonine deaminase by l-isoleucine (46, p. 415-420), aspartokinase by l-threonine (41, p. 2033-2038), methylation of l-homocysteine by l-methionine (38, p. 129-144; 49, p. 229-241), seven enzymes of the arginine biosynthetic

sequence by arginine (11, p. 469-478; 47, p. 491-496), alkaline phosphatase by orthophosphate (44, p. 460-469), four enzymes of the histidine biosynthetic pathway by histidine (1, p. 1453-1461), and of enzymes involved in pyrimidine biosynthesis (53, p. 677-692).

At the present time the mechanism of genetic control of enzyme synthesis in anabolic systems is limited, and is based almost entirely on studies involving Escherichia coli and Salmonella typhimurium. The catabolic, or inducible regulatory controls of the lactose system in Escherichia coli have been explained by the hypothesis of Jacob and Monod (16, p. 315-356). Significant parallels have been reported between induction and repression within the limits of this hypothesis, to indicate that repression was probably the physiological antithesis of induction in bacteria.

In order to evaluate the data from repressible systems on the basis of the Jacob-Monod hypothesis, it is necessary to consider in detail its major conclusions. These are: (1) all regulatory mechanisms act by inhibition rather than activation of protein synthesis; (2) in addition to the classical structural genes, there are regulatory types, the operator and the repressor genes; and (3) the control mechanisms operate at the genetic level. In the lactose system of Escherichia coli all of

these considerations are fulfilled. The four genes involved have been mapped and three were found to be directly linked on the chromosome. These were the "operator", β-galactosidase and galactoside permease, in that order of sequence. The fourth gene, the "regulator", was located some distance down the chromosome. Investigations of this system have been extensive and indicate that the regulator gene sends out a cytoplasmic messenger which acts directly on the operator. This union disallows the formation of both enzymes involved until the repressor substance is intercepted, presumeably by the inducer galactose or some other compound that would replace the natural inducer. The operator, when no longer repressed, allows the formation of enzymes to proceed (in this case β-galactosidase and its corresponding permease). This gene was distinctly different from the repressor gene in that it operated only at the genetic level. Mutations at either site were found to leave the system non-inducible or constitutive, but mating experiments showed that the genes responsible for these phenomena were different.

In order to make repressible systems conform to the Jacob-Monod hypothesis it is necessary that one envision the repressor metabolite as being an integral part of the cytoplasmic inhibitor formed by the regulator gene. Absence of the biosynthetic product would then yield the

messenger ineffectual, releasing the operator, which would in turn allow enzyme formation.

Recent evidence has shown that certain analogies do exist between the inducible systems and the synthetic systems. The tryptophan studies were mentioned earlier in this thesis. These experiments and the two following investigations indicated that genes corresponding to the regulator genes of the inducible system did exist for anabolic control.

At least seven enzymes of the arginine biosynthetic pathway were simultaneously repressed when the organism, Escherichia coli, was grown in the presence of this amino acid (11, p. 469-478; 47, p. 491-496). Mutants have been obtained that are resistant to the arginine analog, canavanine. Several and probably all the enzymes of arginine biosynthesis became constitutive in these organisms. As in the inducible systems, the mutations responsible for this phenomenon were located at a considerable intergenic distance from the structural genes responsible for determining the protein structure of the individual enzymes.

Constitutive mutants for alkaline phosphatase, normally repressible by orthophosphate in the wild type organisms have been isolated (44, p. 460-469). These mutations were found to be located some distance from the gene responsible for the enzyme, and were also shown to be recessive.

Mutations similar to those of the operator gene in the inducible system have been found in Salmonella typhimurium. Four enzymes of the histidine biosynthetic sequence have been shown to be coordinately repressed in the presence of histidine (1, p. 1453-1461). Mutations were found that were not complemented for by any of the individual mutations. These specific mutations were known to be linked closely to the cluster of the nine genes responsible for the enzymes of the biosynthetic sequence and affected complete cessation of formation of all the enzymes in the pathway (2, p. 369-378; 14, p. 323-353).

Inconsistencies have been found, however, when comparing the repressible systems to those of induction. In Escherichia coli, the genes responsible for the synthesis of arginine were found not to be adjacently linked, but were scattered throughout the chromosome (10, p. 173-182). Difficult to explain was the discovery that in some strains of Escherichia coli B, arginine actually stimulated the formation of at least two of its own biosynthetic enzymes instead of repressing them (9, p. 961-971). Sufficient evidence has not yet been obtained to explain the full significance of these observations. This phenomenon has been termed "modulation" and is assumed at present to involve repression by a non-end-product.

As mentioned earlier, studies of this nature in

organisms other than bacteria have not been extensive. Initial studies on the regulation of tryptophan biosynthesis in Neurospora crassa indicated that the formation of the terminal enzyme in the sequence, tryptophan synthetase, was not markedly affected by the presence of tryptophan as in bacteria (21, p. 964-973). Further investigations showed that there was slight repression of this enzyme by this amino acid and its analog, 6-methyltryptophan. It appeared that the strongest repression occurred on enzymes prior to the tryptophan synthetase reaction. The indole-synthesizing ability of the organism was reduced to non-detectable levels under these conditions while the loss of tryptophan synthetase activity was relatively much less.

In other cases with Neurospora no comparable end-product repressive effects have been reported. Slight repression was observed on the formation of pyrroline-5-carboxylate reductase by proline (54, p. 335-338). As yet, no repressive effect has been detected on the biosynthetic enzymes for histidine (1, p. 1453-1461), nor has repression been found of shikimic acid synthesis (12, p. 885-904).

A single case of repression in yeast, that of aspartokinase repression by l-threonine, was reported by Stadtman and coworkers (41, p. 2033-2038). They found that aspartokinase synthesis by Saccharomyces cerevisiae

was reduced by 40 to 50 percent when the organism was grown in the presence of 20 millimolar l-threonine. Preliminary studies in this laboratory indicated that the ability of yeast to convert l-homocysteine to l-methionine was repressed when the cells had been cultured in l-methionine (34).

The phenomenon referred to as "negative feedback" has been found to occur in many microbial systems (45, p. 301-312). In Escherichia coli three apparently different aspartokinases were found to exist (41, p. 2033-2038). One was specifically and non-competitively inhibited by l-lysine. A second was specifically and competitively inhibited by l-threonine. The existence of a third aspartokinase, inhibited by l-homoserine, was suggested by limited evidence. The condensation of phosphoribosyl-pyrophosphate with adenosinetriphosphate has been shown to be strongly inhibited by l-histidine, the endproduct of this pathway (3, p. 2019-2026) in Salmonella typhimurium. In Neurospora, it has been shown that preformed indole-synthesizing activity was inhibited by l-tryptophan, 4-methyltryptophan, and 6-methyltryptophan. The inhibition was at least partially competitive since the effects were somewhat reversed by the presence of the indole precursor, anthranilic acid (22, p. 215-223).

The use of amino acid analogs has been of great value in the study of control mechanisms. Many mutants

resistant to the effects of normally inhibiting concentrations of certain analogs were found to be non-repressible by the corresponding amino acid. Mutants of Escherichia coli have been isolated and studied which were insensitive to the presence of 5-methyltryptophan and were found to be non-repressible by the naturally occurring amino acid (6, p. 3490-3492). Other instances of amino acid analogs exerting repressive effects have been widely reported. It was found that in addition to l-tryptophan, 6-methyltryptophan repressed the formation of tryptophan synthetase in Escherichia coli (23, p. 81-90). Repression by methionine analogs has been shown to occur in this organism (38, p. 129-144). Although varying in their repressing ability, those shown to be effective repressors include dl-methionine sulfoxide, dl-methionine sulfone, dl-ethionine, S-methyl-l-cysteine, S-methyl-dl-methionine, dl-norvaline and dl-norleucine. Repression of indole-synthesizing activity not only occurred in the presence of l-tryptophan in Neurospora, but was also affected by 5-methyltryptophan, 6-methyltryptophan, and d-tryptophan (22, p. 215-233).

If the information obtained from repressive and inductive studies on bacteria is to be applied to Neurospora and yeasts, one must make several concessions. First, the fact must be considered that the genes responsible for the synthesis of given endproducts are not

found to be linked, and are found not infrequently on different chromosomes (13, p. 217-226; 24, p. 161-170). The multi-chromosome system of these organisms makes it necessary to envision an arrangement of multiple operators and regulators.

To determine the genetic implications of the control mechanisms one must determine the linkage relationships of the genes as well as the synthesis of certain specific enzymes. Yeast are especially suited for such studies, since indefinitely stable haploid and diploid clones are easily obtained and maintained. In addition, the ease of genetic manipulation made possible gene dosage studies.

The first successful isolations of ascospores were carried out in 1937 on the yeast Saccharomyces cerevisiae var. ellipsoideus (50, p. 99-120). They were able to separate the four spores of the spore-tetrad and found that upon germination, four different kinds of colonies resulted. It was later shown by these workers that Mendelian segregation occurred for certain morphological characters in Saccharomycodes Ludwigii Hansen (51, p. 357-370). Study of morphological characters gave way for the most part to fermentative characters in the 1940's (29, p. 346-352), and finally incorporated virtually every readily studied phenotypic character observed including requirements for vitamins (37, p. 381-395), amino acids (26, p. 314-318) and purines and pyrimidines (36, p. 456-464).

The first chromosome map constructed involved four chromosomes of Saccharomyces and depicted nine genetic markers (27, p. 675-676; 28, p. 800-802). Linkage was determined by genetic analysis and revealed pantothenate, adenine, thiamin, pyridoxine and inositol markers to be on one chromosome, galactozymase and melibiase on a second, p-aminobenzoic acid on another, and the "a" mating type on a fourth (25, p. 24.1-24.7).

The dispersion of genes concerned with a single biochemical pathway throughout a number of different chromosomes has been shown by Hawthorne and Mortimer in Saccharomyces cerevisiae (15, p. 1085-1110):

| | |
|---|---|
| Chromosome I | (adenine) |
| Chromosome II | (galactose, lysine) |
| Chromosome III | (histidine, threonine) |
| Chromosome IV | (galactose, tryptophan) |
| Chromosome V | (histidine, tryptophan, threonine) |
| Chromosome VI | (histidine) |
| Chromosome VII | (adenine, tryptophan) |
| Chromosome VIII | (threonine) |
| Chromosome IX | (histidine, lysine). |

# MATERIALS AND METHODS

## Cultures

Wild type and mutant strains of Saccharomyces cerevisiae were used in this study. Cultures of strain #3701B were obtained from Dr. H. L. Roman, Department of Genetics, University of Washington. Strain #3701B, a haploid auxotroph requiring uracil, served as the parent strain for all analog insensitive and biosynthetically deficient mutants isolated for this work. The strains isolated and their genetic deficiences will be presented later.

Other mutant organisms used include strains #JB4, #DK30, and #JB100 (serine); #EY9, #AH3, #EW14 and #JA41 (cysteine); #S288-C18 (homocysteine); #ET48, #Y16 and #C4 (S-adenosylmethionine) obtained from Dr. R. K. Mortimer; and #62-28, a pH sensitive multiple mutant requiring, under normal conditions, l-methionine, adenine, histidine, pantothenic acid and p-aminobenzoic acid (35, p. 353-361) was obtained from Dr. S. Pomper.

The methionine requiring bacterium, Streptococcus faecalis, strain #9790, was employed as the test organism in the microbiological assays for methionine.

## Media

Stock cultures of all yeast cultures were maintained on yeast complete (YC) medium (42). Analog insensitive

strains were also maintained on modified Wickerham's minimal medium (48, p. 293-301) plus the normally inhibiting analog.

Chemicals

When available, all chemicals were obtained from commercial sources and used without further purification. The S-adenosyl-l-methionine and S-adenosyl-l-homocysteine were prepared enzymatically from rabbit (4, p. 205-216; 5, p. 58-61) and rat livers (7, p. 603-608), respectively.

Irradiation and Selection of Mutants

Mutant colonies of 3701B were prepared by ultraviolet irradiation of the culture suspended in minimal medium or minimal medium–10 percent YC medium. A 30 watt germicidal lamp with an energy dosage of 12 ergs/$mm^2$/sec at the surface of the medium was used. A variety of exposure times were employed, the optimum appearing to be from 40-50 seconds in the simpler medium and from 70-90 seconds in the other.

After irradiation the cells were spread over prepoured plates of YC medium to yield from 50-100 colonies per plate. Suspected mutants were determined by the velveteen replica plate method (20, p. 399-406) using minimal media supplemented with the desired amino acid.

Analog insensitive mutants were plated at high concentrations ($10^9$-$10^{10}$ cells per plate) and selected for

their ability to grow on normally inhibiting concentrations of the methionine analog.

Mutant Characterization

The specific block of each mutant was determined by feeding techniques. Six millimeter filter paper disks soaked in one percent solutions of the suspected methionine biosynthetic intermediates were placed on the surface of pour plates containing each of the auxotrophs tested. A halo of growth around the pads of supplementing compounds usually occurred in from 48 to 72 hours of incubation at 30° C. Chemicals tested in this fashion included: l-cysteic acid, l-serine, l-cysteine, dl-allocystathionine, l-homocysteine, l-homoserine, l-methionine, S-adenosyl-l-methionine and S-adenosyl-l-homocysteine.

Dominance and recessiveness of dl-ethionine auxotrophs was determined by mating the AE90 strains with ET48 and streaking the test diploid on the defined minimal medium containing 25 milligrams dl-ethionine per liter. The plates were checked for growth after incubation at 30°c for 40 and 68 hours.

Genetic Manipulation

Exponentially growing haploids in YC medium were mated for desired crosses by reinoculation with the complementary mating type in a fresh tube of the same medium and incubated at 30°C for 24 hours. Selection of diploid

(and subsequent separation of haploid) was accomplished by streaking resulting growth on a medium which lacked growth factors complemented for in the diploid.

To sporulate, the yeast diploids were inoculated into the non-synthetic complete medium broth and incubated at 30°C for 24 hours (caps were loosened to insure the entry of oxygen). The cultures were then centrifuged, washed, and placed (as a mass cell droplet) on prepoured Fowell's medium (8, p. 149-160). Sporulation was usually in evidence at the end of 48 hours incubation (31). Free spore tetrads were obtained using the technique of Johnston and Mortimer (18) with only slight modifications of procedure. The ascospore tetrads were separated on a two percent agar-distilled water slab using a Brower Micromanipulator. After dissection the slab was placed in toto on a prepoured plate of non-synthetic complete medium and incubated until maximum germination was obtained. Tetrads which gave rise to four germinating spores were transferred to another plate of complete medium and characterization carried out by the replica plate method.

Selection of Antagonistic Analogs

The ability of the methionine analogs to inhibit growth was tested by a method analogous to the feeding experiments. Sterile filter pads soaked in 1 percent

solutions of the analog were placed on a pre-poured plate containing 3701B in minimal medium. Incubation was carried out for 48 hours at 30°C and the inhibition reported as the width of the ring from the outer edge of the pad to the first perceptible growth. The methionine analogs which showed inhibition were then tested for their ability to replace methionine as a growth supplement. This was determined by plating methionine and methionine precursor requiring mutants of 3701B in the presence of 10 milligrams per liter analog in the minimal medium. The analogs tested included dl-methionine sulfone, dl-methionine-dl-sulfoxide, dl-methionine methyl sulfonium, S-methyl-l-cysteine, N-acetyl-dl-methionine, dl-ethionine and dl-methionine-dl-sulfoximine.

Derepression Procedure

Yeast strains tested were inoculated into YC medium and incubated for 20 hours on a shaker at 30°c. The cells were then centrifuged and washed twice in distilled water. After obtaining a total cell count the suspension was inoculated into derepression media maintained at 30°C. Derepression was carried out in Wickerham's minimal medium plus the desired amino acid or analog. All amino acids and analogs added were present at a final concentration of 1 millimolar. After the desired incubation period each sample taken was immediately placed in an ice bath

to stop the process. After the zero-time sample all flasks were placed on a rotary shaker and kept at 30°C for the remainder of the experiment. Samples were removed at intervals and assayed for their ability to methylate homocysteine.

Formation of Methionine

Samples, after the derepression interval, were centrifuged and washed twice in cold 0.067M phosphate buffer plus 0.005M l-serine (buffer at pH 6.6) and diluted to 8 ml per tube. One milliliter of a solution containing 18 mM l-homocysteine, 0.011M $MgCl_2$ and 0.075M glucose was then added to each tube bringing the final concentration of these compounds in the final reaction mixture to 2 mM l-homocysteine, 0.0012M $MgCl_2$ and 0.008M glucose. A duplicate of each tube was heated for 15 minutes at 100°C before addition of the homocysteine solution and used as a control. When derepression was carried out in methionine, a second control (non-heated) was run without homocysteine.

Microbiological Assay

Methionine was determined using the methionine requiring strain of Streptococcus faecalis, #9790. Growth response was measured on the Colman Model 9 Nephelo-Colorimeter. The final results were expressed as net

millimicromoles methionine formed per milligram dry weight of cells.

# RESULTS

Specific genetic block determinations established five groups of methionine mutants. These have been arranged in Table 1 according to their ability to be supplemented with serine, cysteine, homocysteine, S-adenosylmethionine, S-adenosylhomocysteine and methionine, and will be designated as mutants of the specific block, (i.e., according to the earliest predictable biochemical deficiency).

Linkage data from selected crosses indicate that no gene-gene linkage exists between the markers of the methionine biosynthetic pathway. The results from arbitrary crosses are shown in Table 2. These include linkage ratios between S-adenosylmethionine and cysteine markers, S-adenosylmethionine and serine markers, cysteine and cysteine mutants, and cysteine and serine markers. Due to poor mating of strain #S288-C18 and lack of complete ascospore germination of strain #62-28, satisfactory studies of the homocysteine and methionine relationships have not been successful.

Twelve ethionine insensitive mutants and four methionine sulfoximine resistant mutants were isolated. Methionine analogs for mutant selection were screened for their non-metabolizability and their inhibiting powers. Ethionine and methionine sulfoximine were chosen for these

TABLE 1

Growth Requirements of Methionine Auxotrophs

| Supplement* | Mutant Site** | | | | |
|---|---|---|---|---|---|
| | me-1 | me-2 me-5 | me-3 | me-4 | me-6 |
| Serine | - | - | - | - | + |
| Cysteine | - | - | - | + | + |
| Homocysteine | - | - | + | + | + |
| S-AM | - | + | + | + | + |
| S-AH | - | - | + | + | + |
| Methionine | + | + | + | + | + |

*l-isomers were used in all cases. Abbreviations S-AM and S-AH are substituted for S-adenosylmethionine and S-adenosylhomocysteine, respectively.

**me-1 mutant (62-28); me-2 and me-5 mutants (45M29, 45M45, 45M91, 45M92, 80BM1, 100M1, 502M1, 820M1, 820M3, 820M4, 820M10, 820M20, 820M30, ET48, Y16 and C4); me-3 mutant (S288-C18); me-4 mutant (50M1, 50M2, 50M4,50M5, 50M6, 50M7, 50XM1, 50XM2, 50XM3, 80BM2, 80BM3,80BM5, 80BM6, 80BM7, 80BM30, 80BM50, M137, M237, YMM2, YMM3, EY9, P65, AH3, EW14, JA41); me-6 mutant (80BS40, JB4, DK30, JB100).

TABLE 2

Linkage Relationships of Methionine Auxotrophs

| Diploid Number | Haploid Cross | Marker Cross* | Segregation** | | |
|---|---|---|---|---|---|
| | | | P.D. | R.D. | T.T. |
| 4033 | 45M29xET48$ | S-AM x S-AM | 0 | 1 | 3 |
| 4037 | 50M1xET48$ | Cys. x S-AM | 1 | 3 | 6 |
| 4041 | 50M6xET48$ | Cys. x S-AM | 1 | 0 | 7 |
| 4043 | 50XM1xET48$ | Cys. x S-AM | 4 | 3 | 11 |
| 4044 | 50XM2xET48$ | Cys. x S-AM | 1 | 4 | 4 |
| 4099 | 45M29xJB4 | S-AM x Ser. | 10 | 11 | 29 |
| 4100 | 45M45xJB4 | S-AM x Ser. | 6 | 6 | 17 |
| 4104 | 50M2xJB4 | Cys. x Ser. | 0 | 2 | 2 |
| 4105 | 50M4xJB4 | Cys. x Ser. | 1 | 4 | 5 |
| 4106 | 50M5xJB4 | Cys. x Ser. | 1 | 3 | 8 |
| 4107 | 50M6xJB4 | Cys. x Ser. | 0 | 2 | 4 |
| 4108 | 50M7xJB4 | Cys. x Ser. | 1 | 0 | 4 |
| 4109 | 50XM1xJB4 | Cys. x Ser. | 8 | 10 | 17 |
| 4111 | 50XM3xJB4 | Cys. x Ser. | 0 | 5 | 1 |
| 4112 | 80BM1xJB4 | S-AM x Ser. | 1 | 1 | 3 |
| 4121 | 502M1xJB4 | S-AM x Ser. | 1 | 1 | 4 |
| 4268 | 50M1xEW14 | Cys. x Cys. | 0 | 2 | 4 |

*Cys. = Cysteine; S-AM = S-adenosylmethionine;
Ser. = Serine.

**P.D. = Parental ditype; R.D. = Recombinant ditype;
T.T. = Tetratype.

experiments since they exhibited both of these characters. Compounds omitted from the experiments were either not inhibitory under the conditions employed, or they could be used by some mutants to satisfy the methionine requirement. The effect of these analogs are shown in Table 3.

Both genetically dominant and recessive ethionine resistant mutants have been obtained. These relationships were determined by mating the appropriate analog resistant haploid with a known sensitive haploid of complementary mating type. Resistance of the resulting zygotic clone was tested in the same fashion as were the parental strains. The results are shown in Table 4. Attempts to mate the methionine sulfoximine mutants have proven unsuccessful. For ease of discussion the coding numbers shown in Table 5 have been assigned to the mutants used in these experiments. The relationship of the me-3 and me-1 mutants to the methionine biosynthetic pathway is shown in Figure 1.

Derepression data have been obtained for a variety of yeast mutants as well as the wild-type strain. The employment of the methionine auxotrophs facilitated the validification of the procedure used for derepression assay; the first because it could not produce homocysteine, (me-3), and the second because it cannot form S-adenosylmethionine-homocysteine transmethylase (me-1), the enzyme

TABLE 3

Analog Inhibition of Strain #3701B

| Methionine Analog | Zone of Inhibition (mm) |
|---|---|
| dl-Ethionine | 22 |
| dl-Methionine-dl-sulfoxide** | 0 |
| dl-Methionine sulfone | 0 |
| dl-Methionine-dl-sulfoximine | 10 |
| dl-Methionine methyl sulfonium | 0 |
| S-Methyl-l-cysteine | 11* |
| N-acetyl-dl-methionine | 0 |

*Inhibition was completely relieved by the end of the fifth day.

**Methionine sulfoxide could be used as a methionine source by the me 2, 3, and 4 mutants tested.

TABLE 4

Genetic Characterization of Ethionine Insensitive Mutants

| a parent | α parent | Zygote Number | Growth | |
|---|---|---|---|---|
| | | | 48 Hours Incubation* | 68 Hours Incubation |
| A2E90 | ET48 | 6000 | + | ++ |
| A3E90 | ET48 | 6001 | - | - |
| A4E90 | ET48 | 6002 | + | +++ |
| A5E90 | ET48 | 6003 | - | - |
| A6E90 | ET48 | 6004 | + | ++ |
| A7E90 | ET48 | 6005 | - | - |
| A8E90 | ET48 | 6006 | + | ++++ |
| A9E90 | ET48 | 6007 | + | ++++ |
| A10E90 | ET48 | 6008 | - | - |
| 3701B | ET48 | 7000 | - | - |

*Concentration of ethionine used was the same as that for isolation of the haploids (25 mg per liter).

TABLE 5

Coding For Organisms Used in Derepression Studies

| Haploid Number | Marker | Code Number |
|---|---|---|
| 3701B | Wild Type | WT |
| S28$-C18$ | Homocysteine | Me-3 |
| 62-28$ | Methionine | Me-1 |
| A$E90 | Ethionine Resistance | $E^{r8+}$ |
| A3E90 | Ethionine Resistance | $E^{r3-}$ |
| $SON3 | Methionine-sulfoximine Resistance | $SN^{r3}$ |

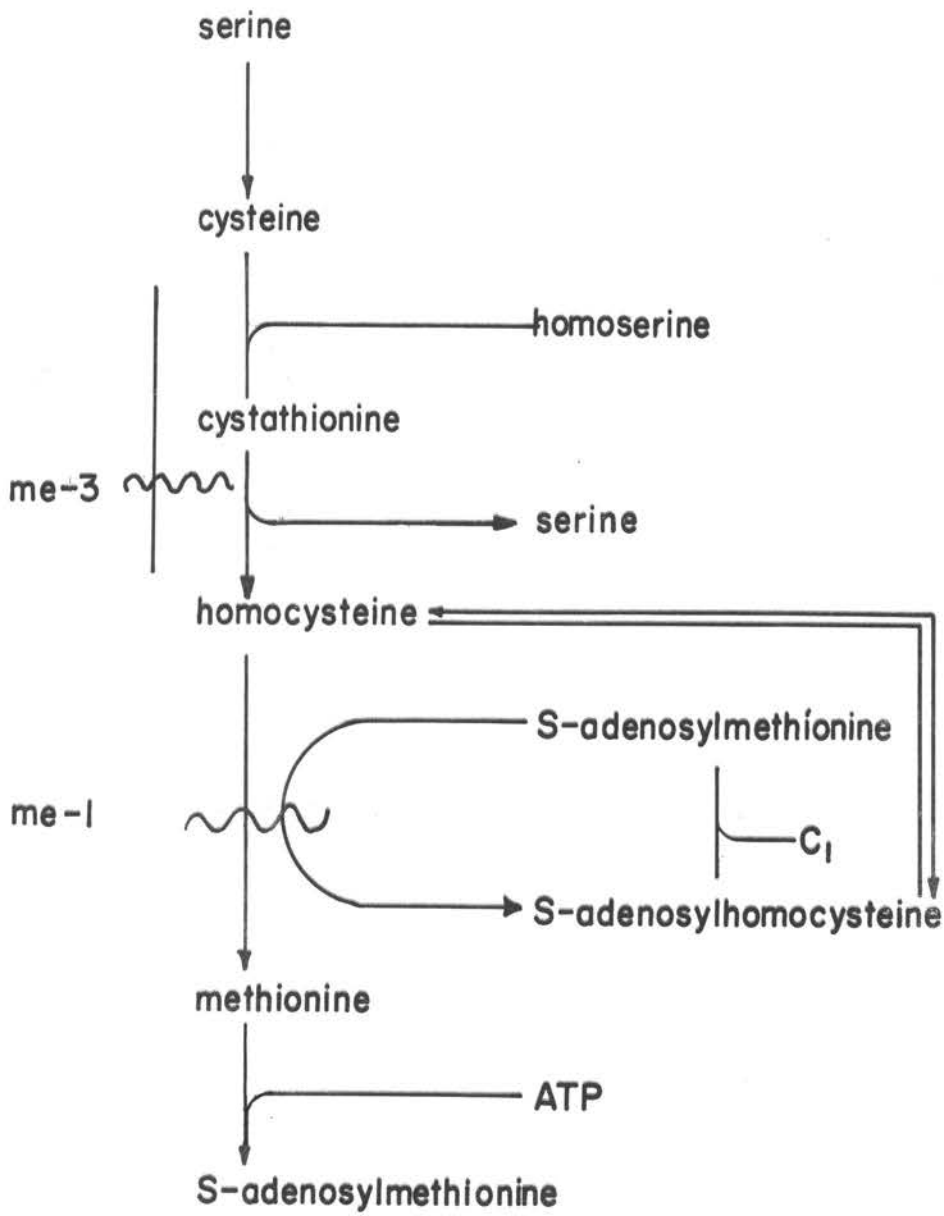



Figure 1. Proposed methionine biosynthetic pathway in Saccharomyces cerevisiae (34)

required for the conversion of homocysteine to methionine (34).

The comparative increase in the capacity for conversion of homocysteine to methionine during derepression in the absence of analogs and amino acids is shown in Figure 2. The greatest increase in enzyme can be seen to occur in me-3, the mutant which apparently cannot synthesize the carbon chain for methionine under these conditions. The absence of methionine-synthesizing activity in me-1 is expected since this organism lacks the transmethylase enzyme. The wild type organism shows a typical form of derepression with no detectable lag, while the dominant ethionine insensitive mutant reaches fully derepressed wild type levels in less than half the time; ultimately attaining a level of about twice that found in the non-mutant. Detectable lags are present in the remaining two organisms. Derepression is completely retarded in the recessive ethionine mutant during the first half hour and then only attains about half the wild type level at the end of the two hour incubation. The methionine sulfoximine resistant mutant, on the other hand, after an initial lag surpasses the level of methionine-synthesizing ability found in the parental wild type.

The effect of ethionine and methionine sulfoximine on the capacity for methionine synthesis in the wild-type organism is shown in Figure 3. The ability of the

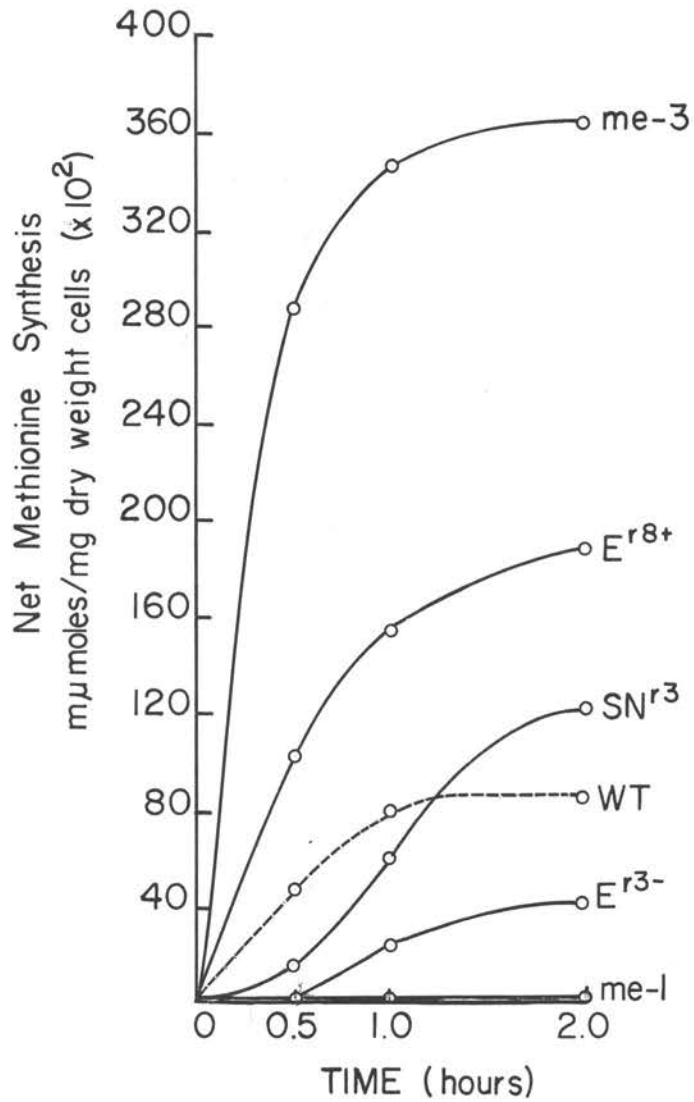



Figure 2. Kinetics of formation of methionine-synthesizing ability during derepression in the absence of amino acids or analogs

parental strain to convert homocysteine to methionine is significantly reduced during derepression in the presence of these analogs.

The dominant $E^{r8+}$ (Figure 4) reaches approximately the same level of methionine-synthesizing ability in the presence of ethionine and methionine sulfoximine as that attained under maximally derepressed conditions in the absence of the analogs. As shown in Figure 5, the recessive $E^{r3-}$ reacts quite differently to the presence of these analogs. Methionine sulfoximine shows the same inhibitory effects on this mutant as in the parental strain. Ethionine interferes with derepression at one point, but has a stimulatory effect, both initially and on the final methionine-synthesizing ability level. A similar effect was noticed in the wild-type organism when derepression was permitted in the presence of only small amounts of ethionine. The analogous reaction of $SN^{r3}$ when derepressed in the presence of these analogs is shown in Figure 6. Again the insensitivity of the mutants to their parent analog is shown. The response by this mutant to ethionine, however, is similar to the wild-type clone. It is important to mention that methionine sulfoximine caused the accumulation of methionine during derepression in all organisms except $SN^{r3}$. This effect was unique with this analog; in all other instances the initial endogenous level of methionine dropped during

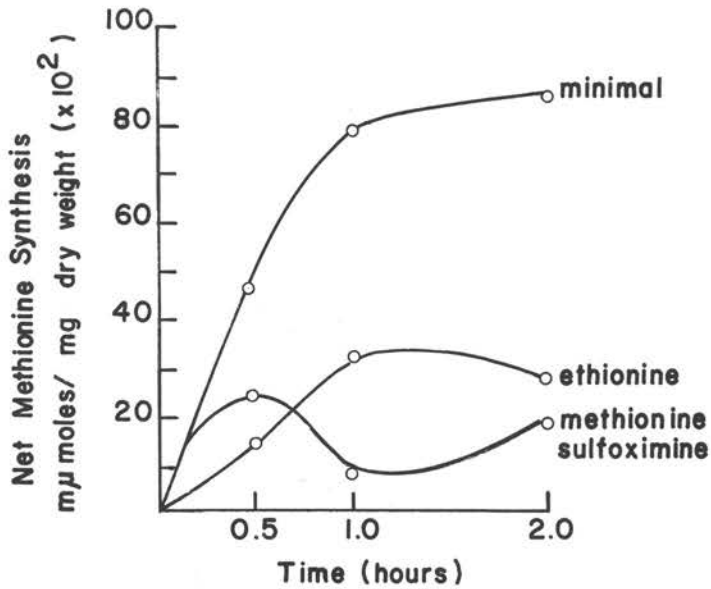



Figure 3. Kinetics of formation of methionine-synthesizing ability in strain #3701B

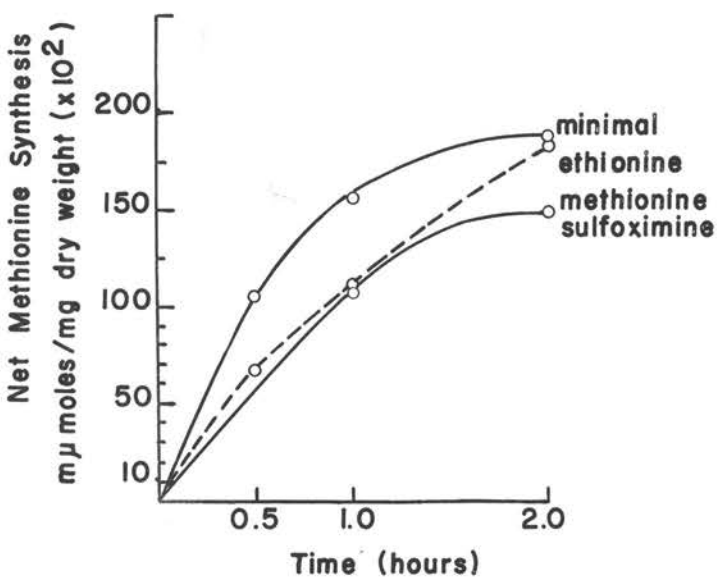



Figure 4. Kinetics of formation of methionine-synthesizing ability in strain #A8E90

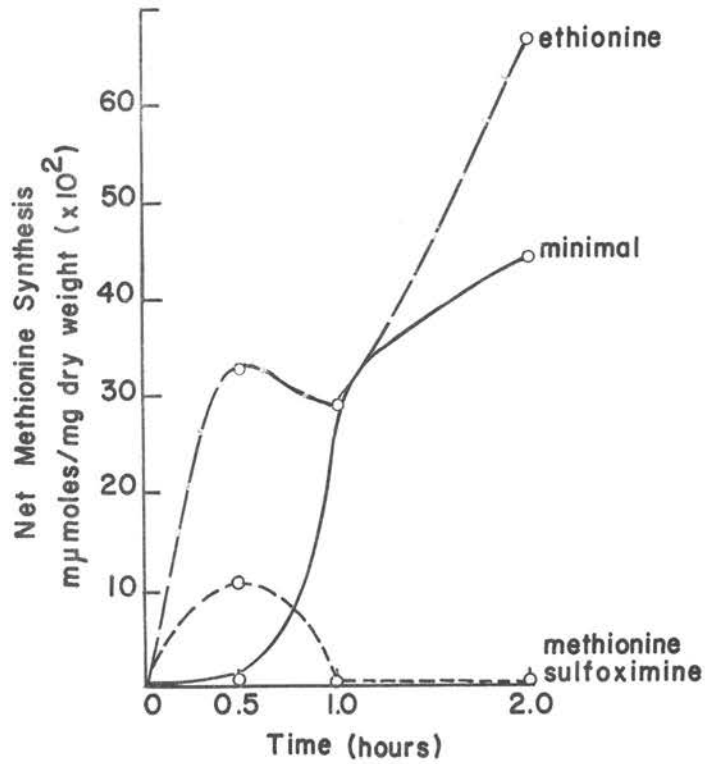



Figure 5. Kinetics of formation of methionine-synthesizing ability in strain #A3E90

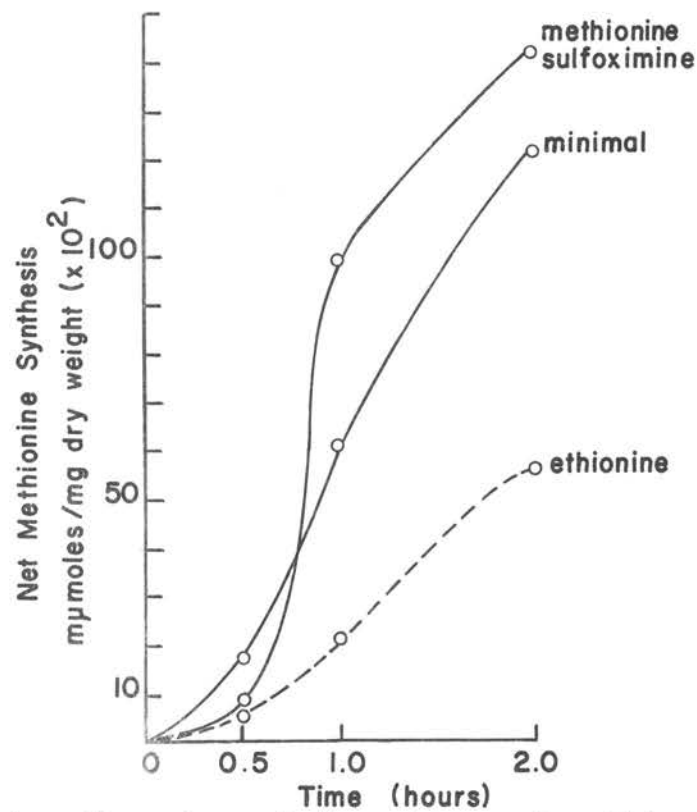



Figure 6. Kinetics of formation of methionine-synthesizing ability in strain #8SON3

derepression in the presence and absence of the analogs.

The greatest increase in enzyme activity occurred in me-3, the organism which can synthesize no methionine (since it cannot form homocysteine) under the conditions employed in derepression. The final level of methionine-synthesizing ability is seen to be four times as high as those of the wild-type strain, #3701B, and twice those reached by $E^{r8+}$. The effects of the analogs are characteristic of the wild-type organism. The results for this organism are shown in Figure 7.

When derepression was permitted in the presence of l-methionine, large quantities of this amino acid were rapidly accumulated by all the organisms except the recessive ethionine mutant (Figure 8). The rapid drop in intracellular methionine levels after the first hour is probably due to the stimulation of some factor resulting in the rapid loss of the free amino acid. The presence of such a mechanism is reflected in net methionine lost during this same interval and is shown in Figure 9. Derepression of methionine-synthesizing ability is shown to occur even in the presence of high intracellular concentrations of free methionine in Figure 10. The significance of the apparent lack of repression under these conditions will be discussed in detail in the next section.

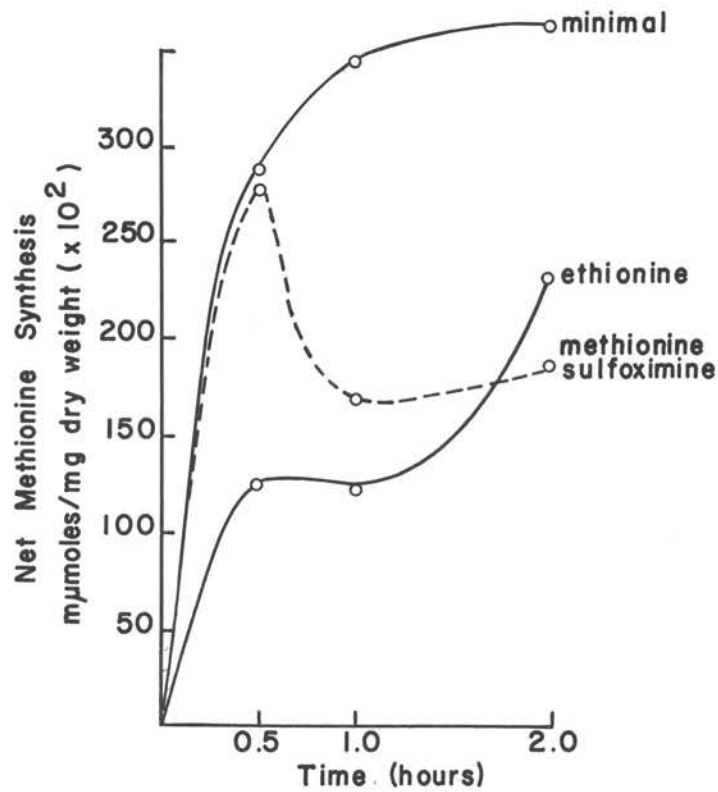



Figure 7. Kinetics of formation of methionine-synthesizing ability in strain #S288-C18

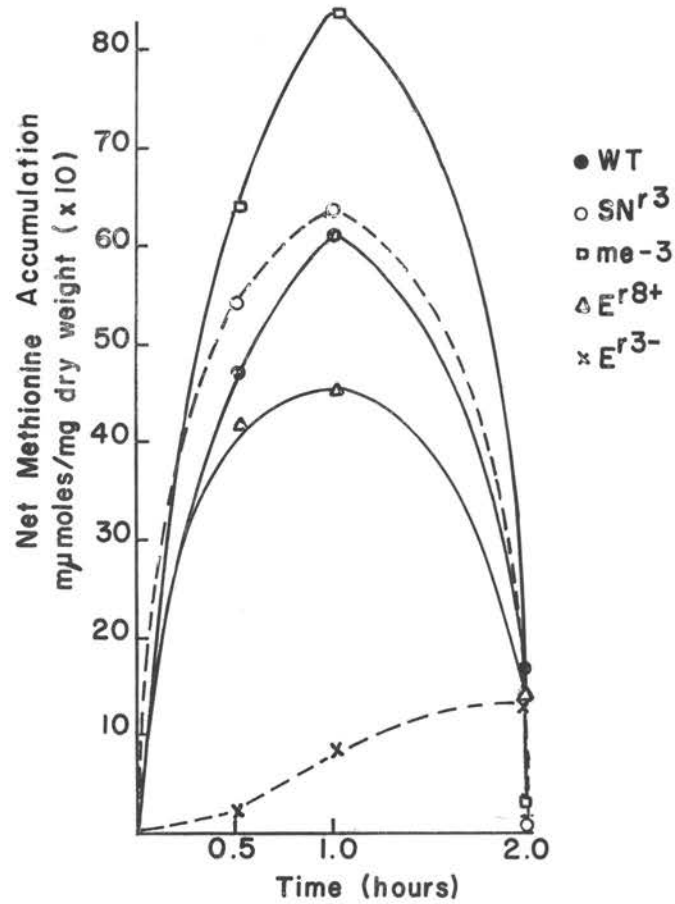



Figure 8. The accumulation of methionine during derepression in the presence of 1 millimolar l-methionine

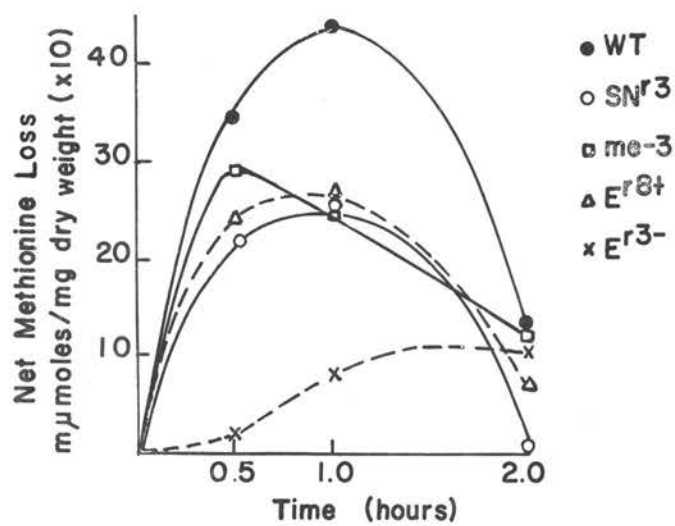



Figure 9. Disappearance of endogenous methionine during derepression in the presence of 1 millimolar l-methionine

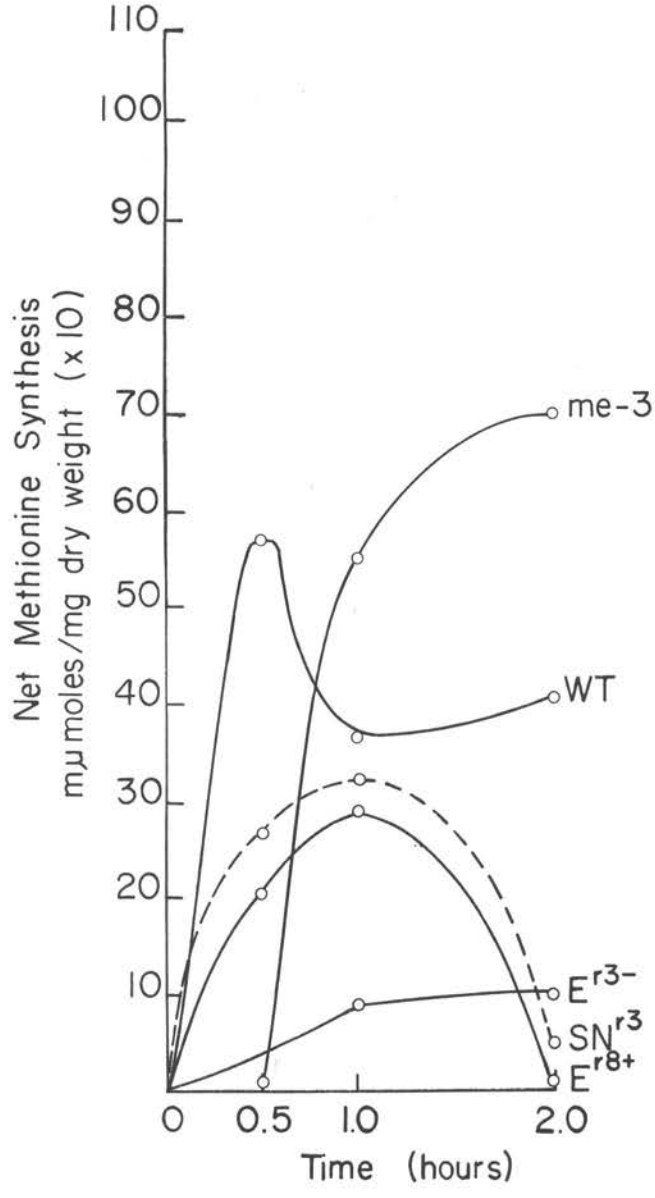



Figure 10. Kinetics of formation of methionine-synthesizing ability during derepression in the presence of 1 millimolar l-methionine

# DISCUSSION

Examination of the methionine mutants isolated in these studies verified the probable biosynthetic sequence reported by Pigg et al (34). Preliminary genetic investigations on these mutants showed no indication of linkage between the structural genes responsible for methionine biosynthesis. Random distribution of the different genetic loci for enzymes of a biosynthetic sequence has been shown to occur in Saccharomyces without exception (15, p. 1085-1110; 24, p. 161).

Derepression in strain #3701B was found to occur both in the presence and absence of l-methionine. When derepression is permitted in the presence of l-methionine, large amounts of this amino acid are accumulated from the medium, reaching a peak concentration in about one hour. Induction of some enzyme, not pertinent to methionine formation (possibly the methionine activating enzyme) results, upon further incubation, in the rapid disappearance of accumulated methionine.

It might appear on cursory examination of the data that methionine exerts a role affecting modulation similar to arginine control in Escherichia coli B (10, p. 173-182). If methionine were responsible for the induction of methionine-synthesizing ability, it would be expected that the ability to methylate homocysteine would increase as

the endogenous methionine concentration increases. This happens when derepression is carried out in the presence of exogenously supplied l-methionine; however, this is not observed during derepression in the methionineless medium. The endogenous concentration of methionine decreases during the interval of maximum derepression abrogating any modulation effect.

It now becomes apparent that free methionine does not repress nor inhibit by feedback inhibition the enzyme (s) responsible for its own formation. The next most logical compound that should be considered is S-adenosylmethionine.

It is known that large amounts of intracellularly stable S-adenosylmethionine are rapidly formed in yeast in the presence of excess l-methionine (39, p. 1037-1050; 40, p. 1051-1057). It has been pointed out that the concentration of methionine decreases during derepression in minimal medium. It is safe to assume from these facts that the methionine lost is at least partially converted to S-adenosylmethionine, which could then act as the repressor. This possibility is clouded by the fact that derepression occurs in the presence of large amounts of S-adenosylmethionine (shown by the disappearance of l-methionine during the incubation in the methionine test medium). It may be concluded (as with l-methionine) that uncombined S-adenosylmethionine, does not repress

methionine synthesizing ability.

The lack of repression by the free metabolite is, however, not an unusual observation. It appears that the effector (repressing metabolite) cannot act alone, but achieves its effects as a component part of a repressor substance. No positive evidence has yet been obtained concerning the chemical identity of repressors, but since they are presumably primary products of the regulator gene, the assumption that they are polyribonucleotides appears the most plausible conclusion (17, p. 193-211). All available evidence indicates that the cytoplasmic product of the regulator gene is the repressor of structural gene expression rather than the metabolic product alone being the repressor. Our data support these concepts.

It should be pointed out that the construction of these experiments limits the ability to establish methionine or S-adenosylmethionine as the effector. S-adenosylmethionine more favorably qualifies than methionine as the logical repressive metabolite for two reasons already mentioned; (1) methionine is a precursor to S-adenosylmethionine, and; (2) the concentration of S-adenosylmethionine increases during both derepression in the presence and absence of exogenously supplied l-methionine. If repression had been carried out in a medium which differed from the derepression medium only by the presence of

methionine, the effector could be more accurately determined; however, the media differed greatly. Repression was carried out in a very rich medium containing all the stable components of normal yeast cells, while derepression was permitted in a physiologically minimal medium containing only carbon, nitrogen and mineral supplements. Under these circumstances the effector remains obscure; however, it is positively shown that repression of methionine-synthesizing ability can be accomplished.

Failure to find a direct correlation between the presence of the assumed effector and the repression of enzyme synthesis makes it necessary to offer an alternate explanation. Current investigations with Escherichia coli and Salmonella by Stent and Brenner (43, p. 2005-2014) and Kurkland and Maaløe (19, p. 193-210) offer evidence in those systems that explains the phenomena observed in these experiments with Saccharomyces. It was found by these investigators that in a rich amino acid supplemented medium, the soluble RNA fraction accounted for only one-fourth of the total RNA fraction. When these cells were transferred to a minimal medium lacking one or more amino acid supplements the soluble RNA (s-RNA) then accounted for one-half of the total RNA fraction. Evidence suggested that the s-RNA was the inhibitor of ribosomal RNA synthesis (the synthesis of both abruptly halts after transfer from complete to minimal medium

lacking amino acid supplements (32, p. 63-74). The assumption that the repressor for protein synthesis is probably a polyribonucleotide (17, p. 193-211) and the later observation that s-RNA is initially halted, finally attaining a greater proportion of total RNA after transfer to a minimal medium lacking amino acid supplements (19, p. 193-210) may explain the lack of repression existent in the derepression experiments performed in this laboratory. It would be expected that during derepression in minimal medium there would be a great demand placed on the available s-RNA for transfer of amino acids to the protein forming mechanisms. The relaxation of the demand after sufficient protein synthesis would leave increasingly larger amounts of s-RNA free for other purposes. It is apparent that the increasing availability of s-RNA would parallel increased repression of a system. This indicates that the s-RNA fraction could possibly function as the repressor molecule and when in combination with S-adenosylmethionine, would become the active repressor of methionine-synthesizing activity. One may even speculate on the possibility that S-adenosylmethionine may compete with methionine for the free s-RNA repressor molecules; an inactive combination when methionine-sRNA, an active repressor when S-adenosylmethionine-sRNA. Though the involvement of s-RNA in this mechanism is allowed only on a speculative basis, it would account for the

results obtained in these investigations and correlates with the evidence now existent concerning the roles of RNA and amino acids in the repression of protein synthesis.

The information obtained in the studies involving the methionine analogs and their respective mutants tend to support several aspects of the preceding conclusions. With the exception of the methionine sulfoximine mutant, all the organisms derepressed in the presence of this analog accumulated methionine. Since earlier observations have not shown methionine to repress the methionine-synthesizing activity, it appears that the effect of methionine sulfoximine is primarily in the limitation of available S-adenosylmethionine for the methylation of homocysteine.

The effect of ethionine appears to be quite different. Net methionine synthesis is reduced in all clones except the ethionine resistant mutants. No methionine is accumulated under these conditions. From these observations and the data to follow, it appears that ethionine has a two-fold effect: (1) it interferes with the methylation of homocysteine by the formation of S-adenosylethionine, an effect which has been shown to occur in yeast (33, p. 169-176) and; (2) the S-adenosylethionine formed can replace S-adenosylmethionine as a component part of the active repressor.

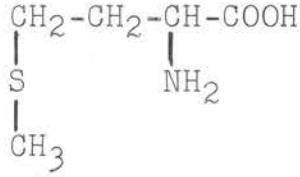
A mutation which would yield the repressor inactive when combined with the ethionine derivitive, S-adenosylethionine, could account for the immunity of this recessively resistant organism to the parent analog. In the diploid form, the natural repressor would be present and cell resistance would then become identical with the wild type. If then ethionine were added to the derepression medium it should stimulate derepression by more actively competing with available S-adenosylmethionine for repressor sites resulting in inactive repressor formation. The results show that ethionine does increase the levels of derepression reached by this organism.

The analog resistance of the dominant ethionine mutant appears to involve increased selectivity for the normal substrate by the methionine activating enzyme. This assumption is supported by the observation that methionine sulfoximine does not cause as great an accumulation of methionine in this organism as in the other clones. The dominance of this organism can not be explained on the basis of a constitutive operator gene mutation since derepression is evident. The greater selectivity of this enzyme, however, may supply the diploid with enough protection from S-adenosylethionine to allow its growth; and therefore remain dominant in the diploid state. Furthermore, if the methionine activating enzyme is selective for methionine, the S-adenosylmethionine

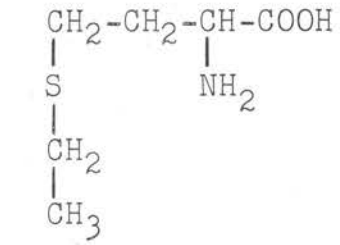
produced may competitively overcome the effects of S-adenosylethionine in the transmethylation reactions.

A similar protection (against both ethionine and methionine sulfoximine) is not evident in the methionine sulfoximine mutant. Since the parent analog causes an accumulation of methionine in the other organisms it can be safely concluded that methionine sulfoximine does not effect the methionine-synthesizing ability, but interferes in some way with the methionine activating enzyme. It follows that a mutation increasing selectivity of this latter enzyme could afford protection to the mutant. It would seem likely that this organism would be provided some immunity against ethionine, but this is not the case. That this is not unlikely is obvious on close examination of the functional groups of the compounds involved.

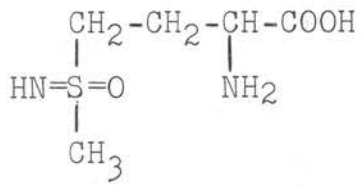
The reacting site for the methionine activating enzyme is at the sulfur atom. In the methionine sulfoximine molecule the configuration around this atom would disallow formation of the hypothetical S-adenosyl-methionine sulfoximine, since no sulfonium configuration can exist. These arguments can best be followed by observing the formulas of the compounds involved:



(methionine)



(ethionine)



(methionine sulfoximine)

A mutation distinguishing between the sulfur of the thioether and that of methionine sulfoximine could involve only very slight alterations of the enzyme's reactive site. The differentiation of the methyl versus the ethyl sustituents on the sulfur atom would demand great enzymatic selectivity. It would be expected that a mutation of the magnitude necessary to confer ethionine resistance would tend to be selective for methionine sulfoximine also. Such was the observation.

Any study of this nature naturally places limitation on the interpretation of the data. All the experiments employed whole cell preparations making it necessary to consider the wide array of physiological variables that are characteristic of such studies. Assumptions and limited speculations were necessary to reasonably explain the phenomena observed in the light of present knowledge on this subject. The data obtained are of great value in the planning of cell-free experiments needed to resolve

the precise mechanism of cellular control reactions in this organism.

# SUMMARY

Preliminary genetic investigations on the methionine mutants isolated in these studies showed no indication of gene-gene linkage between the structural genes responsible for the various enzymes of methionine biosynthesis.

Repression of methionine-synthesizing ability was found to occur during cultivation of the yeast clones in Yeast Complete Medium. Derepression of methionine-synthesizing ability was observed in both the presence and absence of exogenously supplied l-methionine after transfer of the organisms to Wickerham's Minimal Medium. During derepression in the l-methionine supplemented medium, high intracellular concentrations of both l-methionine and S-adenosylmethionine are accumulated indicating that the free forms of these compounds are inactive as repressors of methionine-synthesizing ability. The data suggest that soluble RNA also functions in the repression phenomenon.

The effects of two methionine analogs, ethionine and methionine sulfoximine, on the derepression of methionine-synthesizing ability were also investigated. Mutants were isolated for resistance to these two analogs. Both dominant and recessive ethionine mutants were obtained.

Arguments for the mechanism of repression and analog resistance were presented which were consistent with the

data obtained.

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