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L-ASPARAGINE DEGRADATION IN SACCHAROMYCES CEREVISIAE:
GENETIC CONTROL AND BIOCHEMICAL PROPERTIES OF
YEAST L-ASPARAGINASE

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-iii-

L-Asparagine Degradation in Saccharomyces cerevisiae: Genetic Control and Biochemical Properties of Yeast L-Asparaginase

TABLE OF CONTENTS

ABSTRACT	vi
INTRODUCTION	1
Discovery of asparaginase	1
Asparaginase as an antitumoral agent	2
Properties of asparaginases	4
Mammalian asparaginases	4
Bacterial asparaginases	6
Yeast asparaginase	11
Asparaginase as an adjunct to intermediary metabolism	13
Control of enzyme synthesis and activity in yeast	22
General mechanisms of enzyme control	22
Control of enzymes in aspartate metabolism	24
Scope of the present study	29
MATERIALS AND METHODS	32
Yeast strains	32
Source and life cycle.	32
Nomenclature	33
Media	33
Induction of mutations and isolation of asparaginase mutants.	36
Genetic methods	37
Genetic crosses and tetrad analysis.	37

Complementation analysis	40
Fine-structure mapping	41
Biochemical methods	43
Growth of cells.	43
Preparation of cell extracts	44
Determination of total protein	46
Asparaginase assays.	49
Asparaginase determination in crude extracts.	49
Asparaginase assay in benzene-treated cells	53
Ammonia titration.	57
RESULTS (Genetic).	62
Characteristics of asparaginase mutants	62
Identification	62
Temperature sensitivity.	62
Aspartate synthesis in strains carrying the <u>asp5</u> mutation.	62
Sporulation of diploids homozygous for <u>asp5</u>	64
Petite phenotype of asparaginase mutants	66
Allelism of the asparaginase mutations.	66
Mapping of the asparaginase mutations	70
Independent segregation of the <u>asp1</u> and <u>asp5</u> genes.	74
Fine-structure mapping of the <u>asp1</u> gene	78
Discussion and summary (genetics)	89
RESULTS (Biochemistry)	92
Asparaginase activities in <u>asp1</u> mutants	92

Correlation of <u>aspl</u> mutant phenotype with biochemically determined asparaginase deficiency	92
Lack of inhibitor in extracts from asparaginase mutants . . .	92
Effects of physical factors on asparaginase activity.	94
pH	94
Ionic strength	94
Temperature	98
Asparaginase activity and stability in extract from diploid cells heteroallelic for complementing asparaginase mutations.	102
Effects of chemical factors on asparaginase activity.	103
Substrate concentration	103
Dialysis	107
Amino acids and other metabolites	109
Other potential activators and inhibitors.	114
Human blood.	118
Factors that influence the synthesis of yeast asparaginase. .	118
Conditions of cell growth.	118
Growth medium	120
Asparagine as a carbon or nitrogen source	123
Discussion and summary (biochemistry)	125
ACKNOWLEDGEMENTS	132
APPENDIX I	134
APPENDIX II	136
REFERENCES	137

L-Asparagine Degradation in Saccharomyces cerevisiae: Genetic
Control and Biochemical Properties of Yeast L-Asparaginase

Gary Edward Jones

ABSTRACT

Yeast L-Asparaginase is a multimeric enzyme for which only a single structural gene (aspl) has been found. The gene is located 18 centimorgans from a gene controlling tryptophan synthesis (trp4) on Fragment 2 of the Saccharomyces cerevisiae genetic map. The monomers that comprise the multimeric enzyme have a molecular weight of not less than 8,500 to 10,000.

Fourteen mutants deficient in asparaginase were isolated. Extracts of 12 of these mutants did not contain detectably active asparaginase; extracts from the other two contained activity equal to five to ten per cent of that found in wild-type cells. Wild-type cells contained 5×10^{-2} to 10×10^{-2} International Units of asparaginase activity (μ moles of ammonia evolved per minute per milligram of protein). Lack of asparaginase activity, determined biochemically, was correlated with the presence in cells of an asparaginase mutation.

The pH optimum for yeast asparaginase activity is 8.5, although the enzyme is active over a wide range of pH values. Activity of the enzyme in crude extracts is insensitive to ionic strength over the range $\gamma/2 = 0.05$ to $\gamma/2 = 0.48$ and is not affected by buffer constituents. Q_{10} for the asparaginase reaction is about 2 from 10 C to 30 C but declines slowly above 30 C.

In crude extracts, yeast asparaginase is relatively stable at physiological temperatures; less than 10 per cent of its activity is lost when extract is held at 30 C for six hours. Activity remains constant for months when extract is frozen at -25 C in 0.1 M potassium phosphate buffer at pH 8.0.

The apparent K_m for the asparaginase reaction is about 2.5×10^{-4} M at pH 8.0. Cofactors are not required for asparaginase activity. Activity of the enzyme is not appreciably affected by dialysis of extracts or by the inclusion in reaction mixtures of amino acids, α -keto acids, or potential activators or inhibitors, with one exception. Yeast asparaginase is inhibited by p-chloromercuribenzoate. This inhibition is reversed by reduced glutathione. Neither its substrate nor the products of its activity (aspartate and ammonia) inhibit the enzyme.

The specific activity of asparaginase in yeast cells does not vary by more than a factor of about two regardless of conditions under which cells are grown or of the medium in which they are grown. Even when asparaginase activity provides the sole supply of nitrogen for cell metabolism, the specific activity of the enzyme is not increased by more than a factor of about 1.5.

Active asparaginase is required for the use of asparagine as the sole source of nitrogen in cellular metabolism. Asparagine cannot be used as a sole carbon source.

Asparaginase and glutaminase activities appear to be independent in yeast: glutaminase activity is retained in asparaginase-deficient mutants.

INTRODUCTION

Discovery of asparaginase

The enzyme L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia:

The research summarized in this dissertation concerns the biochemical characteristics of L-asparaginase and the control of its synthesis and activity in the yeast Saccharomyces cerevisiae.

Asparaginases were detected in animal tissues as early as 1904 (52). Clementi (18) studied the distribution of asparaginase in animal tissues and found that the enzyme occurs in many tissues of a variety of herbivorous animals, the blood of guinea pigs being especially rich in the enzyme. Asparaginase is detectable only in the livers of omnivores such as pigs and rats, and not at all in tissues from amphibians, reptiles, carnivorous mammals, monkeys, and man. Other workers have discovered asparagine-hydrolysis activity in bacteria, yeast, molds, and higher plants (30, 49, 124). The first detailed reports on biochemical properties of yeast asparaginase were published in 1928 (30) and 1932 (31).

Asparaginase as an antitumor agent

In 1953, Kidd (47, 48) demonstrated that the growth of certain lymphomas is suppressed and regression of certain established tumors occurs when guinea pig serum is injected into mice and rats carrying the tumors. Interest in asparaginases was stimulated when it was found to be the tumor-suppressing agent in guinea pig serum (2, 6, 7, 8, 10, 14, 63, 113).

Many types of malignant cells have been shown to be deficient in the enzyme asparagine synthetase, which, in mammalian cells, catalyzes the synthesis of asparagine from aspartate and glutamine (10, 12, 40, 86, 87, 92). To grow, the deficient cells require an exogenous supply of asparagine (35, 85, 110). When asparaginase is injected into animals carrying susceptible tumors, it degrades asparagine in the bloodstream and depletes other tissues of the amino acid (11). The asparagine-dependent cells cannot grow and eventually die. Asparaginase causes regression of many tumors in a wide variety of animals (10). Rats, mice, and dogs have been cured of certain tumors by treatment with asparaginase (2, 6, 11, 13, 38, 47, 48, 63, 64, 80, 81, 89, 97, 108, 113, 120). A common feature of cells from these tumors is their requirement for exogenous asparagine.

Exactly why tumor cells die when deprived of asparagine is not known. In mice, regression of tumors is preceded by a marked increase in alkaline ribonuclease activity in the post-mitochondrial cell fraction of the tumors; acid ribonuclease activity increases greatly in the same cell fraction during regression (65). Although the relationship, if any, between increased ribonuclease activity and tumor-cell

death is not understood, Mashburn and Wriston (65) suggest that asparaginase might remove an inhibitor of ribonuclease. Then, because ribonuclease plays a role in the regulation of protein synthesis, the increase in its activity could lead to unbalanced protein synthesis and cell death.

No significant changes occur in ribonuclease activity in normal cells or in tumor cells that are not sensitive to asparaginase. Asparaginase does cause changes in normal cells, but they are usually transitory and not deleterious (1, 10, 11). For example, asparaginase inhibits early waves of mitoses that occur in rat liver after partial hepatectomy, but later mitoses are unaffected (1).

Whether a given asparaginase preparation is effective in reducing tumor growth depends strongly upon the source of the enzyme. Asparaginases that cause tumor regression have been found in guinea pig liver (113) and blood serum (47, 67), and in the sera of some related rodents (81).

The bacterium Escherichia coli synthesizes two different asparaginases, only one of which causes tumor regression (13, 64, 97, 108). Asparaginases from three other bacteria, Serratia Marcescens (104), Erwinia carotovora (117), and Erwinia aroideae (89) also inhibit tumor growth but that from Bacillus coagulans does not (64). Clinical trials with human patients are now being carried out with asparaginases from E. coli (38, 78, 79) and E. carotovora (89).

Broome (9) has reported that partially purified asparaginase from "compressed baker's yeast" is ineffective in the treatment of a mouse lymphoma that is sensitive to guinea pig serum asparaginase. A possible explanation (9) for the ineffectiveness of yeast asparaginase

is discussed below.

Several laboratories are engaged in searching for organisms that might serve as potential sources of asparaginase for large-scale use in therapy, but the screenings have been restricted mainly to bacteria (90, 97). Large quantities of highly purified bacterial preparations are expensive and difficult to manufacture, however, and partially purified extracts are toxic in some circumstances (13, 79, 96, 106). The use of yeast asparaginase in tumor therapy might alleviate these problems, because yeast are easy to grow in large quantities, and partially purified yeast preparations might not elicit the severe antigenic or cytotoxic reactions produced by impure bacterial preparations (22). Knowledge of the biochemical characteristics of yeast asparaginase might indicate ways in which it could be modified to be useful in therapy; knowing how the synthesis and activity of the enzyme are controlled would permit growing cells under conditions leading to optimum production of the enzyme.

Properties of asparaginases

Mammalian asparaginases: Asparagine degradation in rat liver homogenates is stimulated by phosphate and α -keto acids (70). When homogenates are heated to 50 C, loss of activity occurs that is restored upon addition of α -keto acids. The heat-labile, phosphate-activated asparaginase is called asparaginase I. The asparagine-degrading activity that is relatively heat-stable and requires α -keto acids was originally called asparaginase II. However, Meister, et al. (70)

showed that "asparaginase" II activity does not result from the action of a true asparaginase. The degradation is actually due to two sequential enzymatic reactions. The first is transamination between asparagine and an α -keto acid, resulting in the formation of α -ketosuccinamate and a different amino acid (reaction 16 in Figure 1, pg.16). α -Ketosuccinamate is then deamidated by an ω -amidase, producing ammonia and an α -keto acid (reaction 17 in Figure 1).

Guinea pig liver asparaginase has been studied by Suld and Herbut (113). The pH optimum for the enzyme is between 8.6 and 9.6. Fifty-fold purified extracts are stable for at least 20 days when stored at 4 C. The enzyme is not inhibited or activated by 10 mM L-glutamine, 5 mM α -ketoglutarate, 5 mM pyruvate, 10 mM sodium fluoride, 5-10 mM sodium phosphate, or 5 mM sodium sulfate.

The biochemical properties of purified guinea pig serum asparaginase have been extensively investigated (63, 67, 113, 116, 120, 121). The enzyme becomes increasingly unstable as it is purified (63, 113, 116), and the addition of cysteine or glutathione to various purified fractions does not stabilize it (113). The molecular weight of the enzyme is about 138,000 (121), and preparations that were homogeneous by several criteria have been prepared (120, 121). The enzyme is highly specific: only L-asparagine is efficiently hydrolyzed (67, 116). D-Asparagine and a few other amino acids are hydrolyzed by the purified enzyme, but the rates of hydrolysis of these compounds are only 2-10 per cent of the rate of hydrolysis of L-asparagine. L-Glutamine is not hydrolyzed. The pH for optimum activity is about 9.6 (116).

A value of 7.5-8.0 was reported earlier (67), but in the earlier study, activities at higher pH values were probably depressed by the high ionic strength of the buffer that was used. At pH values greater than about 8.0, high ionic strength buffers ($\gamma/2$ greater than 0.4) depress the activity of guinea pig serum asparaginase; the effect is not noticeable below pH 8.0 (116). The apparent Michaelis constant for the enzyme is about 2.2×10^{-3} M (116), and this asparaginase is not inhibited or activated by α -keto acids, phosphate, Mg^{++} , Mn^{++} , Ca^{++} , or most sulfhydryl inhibitors, alkylating agents, or oxidative metabolism inhibitors. p-Chloromercurisulfonate, $HgCl_2$, and Zn^{++} do inhibit the enzyme (116).

Guinea pig serum and liver asparaginases differ in several properties (113). Serum asparaginase is two to three times as effective as liver asparaginase in causing tumor regression. Also, the two enzymes have different pH optima and are eluted from DEAE-cellulose columns in different effluent fractions.

Bacterial asparaginases: Two asparaginases are found in Escherichia coli (10, 13, 15, 16, 97, 108). They differ in several properties, perhaps most markedly in their affinities for asparagine (10, 108). The enzyme with the greater affinity (apparent $K_m = 1.4 \times 10^{-5}$ M (10)), called asparaginase II in E. coli K12 (15), causes regression of tumors, but the asparaginase with the lower affinity (asparaginase I; apparent $K_m = 1.9 \times 10^{-3}$ M (10)) does not. Schwartz, et al. (108), and Cedar and Schwartz (16) have demonstrated that the two activities are distinct. When the enzyme is incubated at 55 C for 10 minutes, 87 per cent of the asparaginase I activity is lost, whereas

only 36 per cent of the asparaginase II activity is lost during the same treatment. The two enzymes are also separable by precipitation with ammonium sulfate.

Enzymes governing the syntheses of the two E. coli asparaginases respond differently to the environment in which the cells are grown (16, 108). Synthesis of asparaginase I is unaffected by conditions of cell growth, but the rate of synthesis of asparaginase II depends greatly upon the conditions under which the cells are grown. When cells are grown anaerobically in media containing high concentrations of a variety of amino acids, 100 to 1000 times more asparaginase II is produced than when cells are grown aerobically. No single amino acid, including asparagine, specifically induces the synthesis of asparaginase II. Even when asparagine is the only nitrogen source in the growth medium, the specific activity of asparaginase II is no higher than if another amino acid and nitrogen source were used. The presence of sugars represses the synthesis of asparaginase II, glucose being the most repressive. Galactose causes much less repression than glucose (16). The greatest amount of asparaginase II is produced in E. coli K12 cells when they are grown anaerobically in medium containing a rich source of amino acids.

E. coli asparaginase II is not inhibited by 10 mM quantities of p-hydroxymercuribenzoate, iodoacetate, iodoacetamide, or N-ethylmaleimide (16, 108). It is stable even when highly purified.

Cells of E. coli strain B produce about three times as much asparaginase as cells of strain K12 (16), but formation of the enzyme is enhanced by anaerobic growth to the same extent in both strains.

E. coli B cells also produce two asparaginases (13, 119), EC-1, which is not effective in tumor therapy, and EC-2, which does cause tumor regression. The two can be separated by salt precipitation or by column chromatography. As in E. coli K12, the relative specific activities of the two enzymes depend greatly upon conditions under which the cells are grown. The two asparaginases differ in their responses to the pH at which activities are measured: EC-1 activity is greatly reduced below pH 8.4, but that of EC-2 shows little change over the pH range 6.0 to 8.4, the optimum pH being about 7.

Whelan and Wriston (119) have purified about 2000-fold a stable asparaginase from E. coli B. Their preparations appeared homogeneous by several physical criteria, including sedimentation in a sucrose density gradient. When the protein concentration in the sedimentation experiments was 0.1 per cent, the molecular weight of the enzyme was about 139,000. When the enzyme concentration was increased to 1 per cent, the apparent molecular weight was 255,000. In dilute protein solution (0.01 per cent), or in 5 M NaCl solution, dissociation occurred that yielded species with an apparent molecular weight of about 64,000. When the enzyme was exposed to 5 M urea, enzymatically inactive material with an apparent molecular weight of 19,000 to 24,000 appeared. These results suggest (119) that the species exhibiting an apparent molecular weight of 255,000 is a multimeric enzyme composed of 12 subunits (monomers) of molecular weight 19,000 to 24,000.

Whelan and Wriston (119) determined the amino acid composition of asparaginase EC-2. The minimum molecular weight of the subunits

was found to be 22,170. Therefore, in vitro, E. coli B asparaginase EC-2 is a multimeric enzyme containing six to 12 monomers, each of molecular weight about 22,000 and consisting of 205 amino acid residues. For the enzyme to be active, its multimeric structure must be intact, because the monomers that make up the enzyme are inactive when separated. The studies of the amino acid composition of the enzyme suggest that all the monomers in the active enzyme are identical. Complementation studies with E. coli asparaginase could be useful for determining, in an independent way, the number of different monomers in the multimer. Such studies have not been possible, because no asparaginase mutants of E. coli have been isolated (16).

L-Asparaginase from E. coli B has been crystallized (39). The crystalline enzyme is homogenous and is active in preventing lymphoma in mice. The specific activity of the crystalline enzyme is 300 ± 15 International Units (One I. U. is the amount of enzyme catalyzing the release of one micromole of ammonia per minute per milligram of protein). At pH 8.5, glutaminase activity of the purified enzyme is only about one per cent of its asparaginase activity.

Roberts and his co-workers (96) have screened several strains of bacteria for asparaginase production. They purified the asparaginase from E. coli strain "HAP" and found it to be effective against a mouse lymphoma. Asparaginase from this strain is synthesized in greatest quantities when cells are grown aerobically in the presence of glutamic acid, methionine, and lactic acid. Glucose represses the synthesis of the enzyme.

Asparaginases in many other species of bacteria have also

been examined (64, 77, 82, 89, 90, 96, 104). Peterson and Ciegler (90) screened 123 species of bacteria and concluded that Erwinia aroideae holds promise as a source of asparaginase for cancer therapy. Only one asparaginase is present in this species, and it exhibits optimum activity at pH 7.5. The apparent K_m for the enzyme is about 3×10^{-3} M. The one asparaginase found in Erwinia carotovora has been crystallized (77). The molecular weight of the enzyme at protein concentrations ranging from 0.05 mg/ml to 3.5 mg/ml was 128, 400 to 145,000. Rowley and Wriston (104) partially purified an asparaginase from Serratia marcescens that causes tumor regression in mice. Cells grown in aerated liquid medium do not produce a satisfactory asparaginase. Partially purified enzyme from this species is not stable to prolonged dialysis or refrigeration.

Asparaginases from two species of mycobacteria have been investigated (82), but their effects on tumors were not determined. The enzymes from M. tuberculosis and M. smegmatis differ in that extracts from M. tuberculosis usually contain more asparaginase than extracts from M. smegmatis. pH optima for the enzymes are 8.5 in both TRIS and histidine buffers. The apparent K_m for M. tuberculosis asparaginase is 1.6×10^{-3} M, whereas that for M. smegmatis enzyme is about 7.4×10^{-4} M. When extracts from the two species are dialyzed against distilled water, some activity is lost that cannot be restored by adding phosphate, pyridoxal phosphate, or α -keto acids to the reaction mixtures. Neither enzyme is inhibited by a wide variety of potential inhibitors (including p-chloromercuribenzoate), with one exception: D-asparagine competitively inhibits the enzyme.

An asparagine-hydrolyzing preparation from a strain of Pseudomonas (species not specified) has been purified several hundred-fold by Ramadan, et al. (93, 94). The preparation exhibits both asparaginase and glutaminase activities that are not separable by a variety of procedures. The molecular weight of the enzyme is about 25,000, and it can be stabilized and kept frozen for long periods of time in a borate-acetamide-glycerol buffer. The pH optimum for glutaminase activity is about 6.6, although high activity obtains over a broad pH range (pH 5 to pH 8). Asparaginase activity is highest at about pH 8.2. Ammonia and aspartate have no effect on the asparaginase activity, but ammonia and glutamate competitively inhibit the glutaminase activity. Both activities are increased by a variety of divalent cations; Fe^{++} and Hg^{++} are inhibitory. Effects of the Pseudomonas enzyme on tumor growth were not investigated.

Yeast asparaginase: Geddes and Hunter (30) described the preparation of extracts of "...bottom yeast, fresh from the brewery..." that were capable of deamidating asparagine. They purified it threefold by precipitation from 50 per cent glycerol with safranin (an azine color base). The amount of asparagine hydrolyzed was approximately proportional to the amount of enzyme in their reaction mixtures, and the pH optimum for the reaction was pH 8. They found that the enzyme is "... exceedingly labile to destruction or inactivation...."

Grassmann and Mayr (31) studied the specificity of brewery yeast asparaginase and measured the effects of some inhibitors on the activity of the enzyme. Of the latter, 10^{-5} M AgCl or HgCl_2 inhibit

activity almost 100 per cent, 10^{-3} M CuCl_2 about 75 per cent, and 0.185 M HCN about 45 per cent. Both Geddes and Hunter and Grassmann and Mayr found their preparations to be very specific for asparagine degradation. A large number of amides, peptides, amino acids, and asparagine analogs were tested as substrates. Only L-asparagine, DL-aspartic acid diamide, and, in the extracts prepared by Geddes and Hunter, glutamine, were found to be degraded. L-Glutamine was not deamidated by the preparations used by Grassmann and Mayr, who prepared extracts by autolyzing yeast in the presence of toluene. Extracts prepared by Geddes and Hunter probably contained glutaminase as a contaminant.

Broome (9) purified yeast asparaginase and tested its effectiveness as an antitumoral agent. Yeast asparaginase becomes increasingly unstable as it is purified, and numerous preparations of "compressed baker's yeast" asparaginase were without effect on tumor growth. The time course of the activity of yeast and guinea pig asparaginases in the bloodstreams of mice were followed. Yeast asparaginase is cleared at a very rapid rate from the blood. Guinea pig serum asparaginase activity can be found for as long as three days after injection, but yeast asparaginase disappears almost completely within an hour of injection.

Broome attempted to measure the molecular weights of the two enzymes by sedimenting them in sucrose density gradients in an ultracentrifuge. Guinea pig serum asparaginase sediments in a rather well defined peak whose position in the gradient indicates that the molecular weight of the enzyme is about 150,000. Yeast asparaginase, on the

contrary, does not sediment in a well defined peak. Higher activities were found at a point in the gradient to which proteins with a molecular weight of at least 800,000 sediment, but some activity sedimented even faster than this. Broome suggested that yeast asparaginase aggregates either with itself or with other proteins during purification and that the aggregates are heterogeneous in size. He attributed the rapid clearance of yeast asparaginase from the blood of mice to the action of the reticuloendothelial system. Aggregates of this size would be rapidly phagocytized and digested by RTE cells and would not have time to deplete asparagine from the bloodstream.

Broome found no loss of yeast asparaginase activity when the enzyme was incubated in vitro in three volumes of mouse blood or serum for one to five hours. This finding again suggests that some component of circulating blood is responsible for the rapid rate of clearance of yeast asparaginase from the bloodstream.

Asparaginase as an adjunct to intermediary metabolism

Asparaginase is interesting for reasons other than its use in cancer therapy. When asparaginase hydrolyzes asparagine, the products are ammonia and aspartate. Thus, asparaginase not only can provide free ammonia in the cell, but it also is one route by which aspartate can be synthesized. Aspartate is a key intermediate in the synthetic pathways of several other amino acids and of the pyrimidines (32, 103), and it functions as a link between amino acid metabolism and the citric acid cycle (33, 105).

An outline of the major pathways of which aspartate is a part

is shown in Figure 1. The relationship of asparagine and asparaginase to these pathways is obscure. Certain tumor cells contain high levels of asparagine synthetase, the enzyme that catalyzes the synthesis of asparagine from aspartate and an ammonia source (reaction 14 in Figure 1). Meister (68) has suggested that, in these tumor cells, asparagine might participate in amide nitrogen transfer reactions of the type known to occur with the amide group of glutamine in normal cells. (In normal cells and in most other organisms, the amide group of glutamine is utilized in the synthesis of a large number of compounds (68).) Why asparagine amide nitrogen should be of particular value in tumor-cell metabolism is not known, but two previously mentioned observations are provocative: some tumor cells synthesize no asparagine, but other tumor cells can synthesize an apparent excess of the amino acid. A third observation emphasizes the lack of understanding of asparagine metabolism: some normal cells are devoid of asparagine synthetase activity but are nonetheless resistant to the action of asparaginase (10). How the asparagine necessary for protein synthesis is maintained in these cells is not known.

Little is known about the control of asparagine metabolism in yeast. Although yeast and other organisms probably metabolize the amino acid in different ways, knowledge of how asparagine degradation is controlled in yeast could indicate ways of attacking questions about the physiological roles of asparagine and asparaginase in other types of cells.

Figure 1. Outline of aspartate metabolism in yeast. Reactions represented by dashed lines are of unknown physiological significance in yeast. Enzymes that catalyze the numbered reactions are listed in Table 1 and Appendix I. Abbreviations are defined in Table 2.

Figure 1.

Table 1. Enzymes involved in aspartate metabolism

Reaction number	Enzyme ^{a, b}
(1)	Aspartate aminotransferase
(2)	Aspartate kinase
(3)	Aspartate semialdehyde dehydrogenase
(4)	Homoserine dehydrogenase
(5)	Homoserine transacetylase
(6)	Homocysteine synthetase
(7)	Homoserine kinase
(8)	Threonine synthetase
(9)	Threonine dehydratase (threonine deaminase)
(10)	Aceto hydroxyacid synthetase
(11)	" " "
(12)	Glutaminase
(13)	Asparaginase
(14)	Asparagine synthetase
(15)	Glutamine-keto acid aminotransferase
(16)	Asparagine-keto acid aminotransferase
(17)	ω -Amidase
(18)	Aspartate ammonia lyase (aspartase)
(19)	Aspartate carbamoyltransferase
(20)	Carbamate kinase

Table 1 (continued).

Reaction number	Enzyme ^{a, b}
(21)	Ornithine carbamoyltransferase
(22)	Argininosuccinate synthetase
(23)	Sulfate adenylyltransferase
(24)	Argininosuccinate lyase
(25)	Dihydro-orotase
(26)	Dihydro-orotate dehydrogenase
(27)	Orotidine-5'-phosphate pyrophosphorylase
(28)	Orotidine-5'-phosphate decarboxylase

a. Trivial names adopted from (125).

b. Systematic names of these enzymes are listed in Appendix I.

Table 2. List of abbreviations.

Abbreviation	Definition
AcHS	<u>o</u> -acethomoserine
α -AHB	α -acethydroxybutyrate
α -AL	α -acetolactate
ALA	alanine
APS	adenosine-5'-phosphosulfate
ARG	arginine
AS	argininosuccinate
ASA	aspartate- β -semialdehyde
ASN	asparagine
ASP	aspartate
ASP-PO ₄	β -aspartyl phosphate
ATP	adenosine-5'-triphosphate
C-PO ₄	carbomyl phosphate
CTL	citrulline
CYS	cysteine
DHO	dihydroorotate
EDTA	ethylenediaminetetraacetate
FUM	fumarate
GLN	glutamine
GLU	glutamate
GLY	glycine

Table 2. List of abbreviations (cont.)

Abbreviation	Definition
HC	homocysteine
HCO_3^-	bicarbonate ion
HIS	histidine
HS or HOMOSER	homoserine
HS-PO_4	<u>o</u> -phosphohomoserine
ISOL	isoleucine
I. U.	International Unit
α -KB	α -ketobutyrate
α -KG	α -ketoglutarate
α -KS- NH_3	α -ketosuccinimide
LEU	leucine
LYS	lysine
MET	methionine
NPD	non-parental ditype
OA	orotate
OAA	oxaloacetate
O. D.	optical density
OMP	orotidine-5'-monophosphate
ORN	ornithine
PAPS	3'-phospho-adenosine-5'-phosphosulfate
PCMB	p-chloromercuribenzoate

Table 2. List of abbreviations (cont)

Abbreviation	Definition
PD	parental ditype
PHA	phenylalanine
PP _i	inorganic phosphate
PRO	proline
PRV	pyruvate
PYR	pyrimidines
R	roentgen
S	survivor
SER	serine
T	tetratype
THR	threonine
TRP	tryptophan
TYR	tyrosine
UMP	uridine-5'-monophosphate
URA	uracil
US	ureidosuccinate
UTP	uridine-5'-triphosphate
VAL	valine

Control of enzyme synthesis in yeast

General mechanisms of enzyme control: Most models explaining how enzymes are controlled have been derived from studies with bacteria. In many bacteria, structural genes governing the synthesis of enzymes in a reaction pathway are often incorporated into an operon (41) in which the genes are adjacent to each other on a chromosome. Synthesis of the enzymes is controlled at the level of transcription by a single operator gene in the operon. When greater quantities of enzymes in the pathway are required, more polycistronic messenger-RNA molecules carrying the information in the operon are synthesized.

This kind of coordinate induction (or repression) of enzymes requires that structural genes associated with the enzymes be adjacent on a chromosome. Many examples of bacterial operons are known in which enzyme synthesis is controlled in this way. Bacteria, however, are more simply organized than yeast cells or the cells of other eucaryotic organisms. Bacteria contain only a single chromosome, and the chromosome is not enclosed in a nuclear membrane. The complexity of eucaryotic cells might allow greater flexibility in enzyme control and, at the same time, might require more sophisticated mechanisms for coordinating cellular metabolism. For example, increased flexibility in the control of enzyme synthesis might result from differential rates of passage of messenger-RNA molecules from the nucleus into the cytoplasm. A more sophisticated coordinating mechanism might be required because structural genes associated with pathways are usually distributed over several chromosomes in these more complex cells. This has been found

to be true of structural genes controlling the synthesis of enzymes in most metabolic pathways of yeast. With few exceptions (23, 29, 98), genes controlling the synthesis of enzymes in yeast are not incorporated into operons and are not controlled by coordinate induction or repression.

An alternate pattern of coordinate enzyme synthesis has been discovered in yeast and in some bacteria. In pathways controlled in this way, the synthesis of some enzymes is induced specifically by products of preceding enzyme reactions in the pathway. This form of control is called sequential induction. Stanier (112) described sequential induction of the enzymes in the mandelic acid synthetic pathway of Pseudomonas fluorescens. Lacroute (50) found a similar type of control mechanism in the synthesis of pyrimidines in yeast (see below). Sequential induction may occur in the mold Neurospora crassa (34) and in mammalian cells (91). In fact, sequential induction may operate in diverse organisms in which genes of synthetic pathways are distributed over several chromosomes.

Although the ways in which the synthesis of enzymes is controlled may differ in detail in yeast and bacteria, the molecular mechanisms by which the control is achieved are probably similar (41): that is, the synthesis of messenger-RNA is controlled by an operator gene responding to a regulator molecule that is activated or inactivated by specific molecules. In induction of enzyme synthesis, the regulator molecule complexes with the inducer and cannot bind to the operator region of the operon or gene. Therefore, the structural gene(s) can participate in messenger-RNA synthesis. In repression of enzyme syn-

thesis, the repressor complexes with the regulator molecule which only then can bind to the operator region. When the complexed regulator molecule binds to the operator region, the structural gene(s) associated with the operator no longer participate in messenger-RNA synthesis.

The synthesis of some enzymes is unregulated (constitutive synthesis), the quantity of enzyme in a cell being nearly constant.

Enzyme activity is probably controlled in much the same way in most living organisms, including yeast; the activity of an enzyme is controlled by changes occurring in its tertiary structure when an inhibitor molecule binds to the enzyme (71). The binding site of the inhibitor molecule is separate from the active site of the enzyme. The separation of the active and inhibitor-binding sites allows molecules not resembling the substrate of the enzyme to act as inhibitors. Such allosteric inhibition (71) controls the activity of many enzymes in diverse living organisms. Many enzymes are inhibited by terminal end-products of pathways of which they are a part, even though the inhibitor molecules bear little structural resemblance to the substrates of the enzymes (feedback inhibition (71)). Some enzymes are also inhibited by the immediate products of their own activity (product inhibition).

Control of enzymes in aspartate metabolism: Because asparaginase activity results in the production of aspartate, control of the activity and synthesis of the enzyme might be accomplished by intermediates or end-products of aspartate metabolism or by aspartate or ammonia, the products of asparaginase activity. A summary of control mechanisms involved in aspartate metabolism will indicate ways in which asparaginase could be affected by intermediates and end-products of these pathways.

(A more detailed review of these and other pathways has been published recently (74)).

The enzyme aspartate kinase catalyzes the first step (reaction 2 in Figure 1) in the sequence leading from aspartate to homoserine and, ultimately, to threonine, methionine, and isoleucine (3, 4, 5, 99, 101). The control of the synthesis and activity of this enzyme have been extensively studied (46, 99, 111, 114). Threonine and homoserine repress the synthesis of aspartate kinase and inhibit its activity. Lysine represses synthesis of the enzyme even though aspartate is not thought to be a precursor of lysine synthesis in yeast (99). The activity of aspartate kinase is usually insensitive to lysine and methionine, but the enzyme synthesized by cells grown in the presence of methionine is partly inhibited by both. Homoserine in the growth medium increases the sensitivity of the enzyme to inhibition by homoserine, but lysine in the growth medium leads to the synthesis of an aspartate kinase that is less sensitive to homoserine inhibition. De Robichon-Szulmajster and Corrivaux (99) suggest that at least three aspartate kinase isoenzymes can be synthesized by Saccharomyces. All are sensitive to threonine, but each has a site of inhibition that accepts a different inhibitor molecule. The relative quantities of the three isoenzymes present in cells depend upon the medium in which the cells are grown.

Homoserine is a branch-point in the pathways leading from aspartate to several end-products. As such, its synthesis and activity are likely control points in the pathways (71). The enzyme homoserine

dehydrogenase, which converts aspartic semialdehyde to homoserine (reaction 4 in Figure 1) has been investigated by Karassevitch and de Robichon-Szulmajster (46). Methionine represses the synthesis of homoserine dehydrogenase, but threonine, another end-product, does not. Both methionine and threonine inhibit the activity of the enzyme. The investigators concluded that homoserine dehydrogenase and aspartate kinase are independently regulated by products of the pathways. The regulation is complex and allows for considerable flexibility in the cells' responsive abilities.

Threonine dehydratase (deaminase) catalyzes the synthesis of α -ketobutyrate from threonine (reaction 9 in Figure 1), α -ketobutyrate being a precursor in the synthesis of isoleucine (100). Complex mechanisms regulate the synthesis and activity of this enzyme. For example, at a concentration of 0.1 mM, isoleucine stimulates the activity of the enzyme, but at higher concentrations, it is inhibitory. The concentration of isoleucine that promotes maximum activity is pH-dependent. Valine reverses the inhibitory effect of isoleucine and also increases the affinity of the enzyme for its substrate, threonine.

The synthesis of valine is carried out by the same set of enzymes as the synthesis of isoleucine. Pyruvate, rather than α -ketobutyrate, is the first precursor in the pathway. Acetohydroxy acid synthetase, the first enzyme common to both pathways, catalyzes the conversion of α -ketobutyrate to α -acetohydroxybutyrate as well as the synthesis of α -acetolactate from pyruvate (45, 56) (reactions 10 and 11 in Figure 1). Valine strongly inhibits a part of the activity of the enzyme, the degree of inhibition depending upon the pH of the incub-

ation mixture. Isoleucine and leucine have no effect on the activity of this enzyme (57).

The isoleucine-valine pathways provide an example of linked metabolic sequences that are controlled not only by their end-products but also by a physical feature (pH) of the environment in which the enzymes function.

Cherest, et al. (17), have described genetic and regulatory aspects of the synthesis of methionine from aspartate and sulfate in S. cerevisiae. Four enzymes in the pathways (reactions 4, 5, 6, and 23 in Figure 1) are repressed by methionine. Two of these enzymes (catalyzing reactions 5 and 6) are simultaneously derepressed in strains carrying a mutant gene (eth2) that conveys resistance to a methionine analog, ethionine. Regulation of the other two enzymes is not affected by the presence of the mutant gene. Structural genes controlling the synthesis of the two enzymes affected by the eth2 gene are unlinked, and neither of the genes is linked to eth2. Therefore, although both enzymes are affected by a mutation in a third gene, they are not incorporated into an operon. The authors propose that the gene eth2, in its wild-type form, "...is responsible for the synthesis of a pleiotropic methionine repressor..." and suggest that at least two different methionine repressors exist in S. cerevisiae.

In Saccharomyces cerevisiae, pyrimidines are synthesized by a sequence of steps beginning with the formation of carbomyl phosphate from bicarbonate ion and glutamine (reaction 20 in Figure 1) (51). This reaction is catalyzed by carbamate kinase. Carbomyl phosphate is linked to aspartate to form ureidosuccinate (reaction 19), which is

ultimately transformed into the various pyrimidines (50). Carbomyl phosphate is also a precursor in the synthesis of arginine (51). Ornithine and carbomyl phosphate are joined to form citrulline (reaction 21), which is then joined to aspartate to form argininosuccinate (reaction 22). Argininosuccinate is split, yielding arginine and fumarate (reaction 24). Lacroute and co-workers (51) have shown that, in yeast, two different enzymes catalyze the synthesis of carbomyl phosphate from glutamine and bicarbonate ion. The two enzymes can be specifically affected by independent mutations, and they differ in their responses to arginine and pyrimidines. One of them is repressed and inhibited by uridine-5'-triphosphate; the other is repressed by arginine. The synthesis of carbomyl phosphate is independently controlled by the two end-products, but once formed, it is freely available to either pathway. Aspartate carbomyltransferase, which catalyzes the linking of carbomyl phosphate and aspartate (reaction 19), is sensitive to feedback inhibition and repression by UTP.

Lacroute (50) found that some enzymes in the yeast pyrimidine pathway are sequentially induced. The first two enzymes (reactions 19 and 20 in Figure 1) are both repressed and inhibited by a pyrimidine and, in fact, seem to form an enzyme complex. Whether the structural genes of the two enzymes form an operon is not known (50): an alternative explanation is that the complex is really a single enzyme with sites of activity for both reactions (50). At least two enzymes that control later steps in the pathway (reactions 25 and 26 in Figure 1) are each induced by their specific substrates and are insensitive to repression by pyrimidines. These enzymes are apparently controlled by

sequential induction.

Aspartate is directly or indirectly involved in all the metabolic pathways discussed above. Because asparagine can be used as a source of aspartate in yeast (see below), the synthesis and/or activity of asparaginase might be controlled by intermediates or end-products of aspartate metabolism. I have tested these possibilities with many of the compounds represented in Figure 1 (the key intermediates and end-products) and with other compounds (mainly amino acids) not directly associated with aspartate metabolism. The effects on asparaginase synthesis and activity of ammonia, the other immediate product of the asparaginase reaction, were also tested.

Scope of the present study

The research summarized in the following chapters had four general objectives:

- (1). to provide a study of genes governing the synthesis of yeast asparaginase;
- (2). to determine how the activity of the enzyme is influenced by the conditions of assay;
- (3). to determine how the synthesis of the enzyme is affected by conditions of cell growth;
- and (4). to suggest possible physiological functions of yeast asparaginase.

Many questions about an enzyme and the genes governing its synthesis can be answered by using mutants that are deficient in the

enzyme. Such mutants are usually isolated by selecting colonies of cells that are unable to utilize a substrate of the enzyme for growth. For example, asparaginase mutants might be isolated by selecting cells that will not grow when exogenously supplied asparagine is the only available source of aspartate for use in cell metabolism.

In yeast, however, asparaginase is not predominantly responsible for synthesizing aspartate under normal growth conditions. Aspartate is normally formed by transamination between glutamate and the α -keto acid oxaloacetate, an intermediate in the citric acid cycle (101, 105) (reaction 1 in Figure 1). If aspartate can be synthesized by the transamination reaction, asparaginase-deficient mutants cannot be identified by their inability to use asparagine as a source of aspartate; sufficient aspartate for growth is supplied by the transamination reaction.

Mutants deficient in aspartate aminotransferase, the enzyme that catalyzes the transamination reaction, have been isolated (73, 101). In strains carrying the mutant form of the gene that codes for aspartate aminotransferase (denoted asp5), growth occurs if aspartate is supplied exogenously. These strains also grow well if asparagine is substituted for aspartate in the growth medium. I attempted to isolate asparaginase-deficient mutants by selecting aminotransferase-deficient cells that are unable to utilize asparagine in place of aspartate.

The asparaginase-deficient mutants were used to answer the following questions:

- (1). how many genes are involved in the synthesis of yeast asparaginase?
 - (2). where are the genes located on the genetic linkage map?
 - (3). is yeast asparaginase made up of more than one polypeptide subunit (that is, is it a multimeric enzyme)?
- and (4). what is the minimum size of monomer(s) that make up the active enzyme?

The effects on wild-type asparaginase of several factors known to affect the activity of enzymes (21) were investigated. Among these factors were substrate concentration, buffer constituents, pH, temperature of assay, exposure to high temperatures, and the presence in reaction mixtures of a variety of metabolites, cofactors, or other molecules that might act as inhibitors or activators.

Factors that could affect the synthesis of the enzyme were investigated as well. Among these were growth medium, oxygenation of cultures during growth, and growth phase of cultures.

An attempt to learn something about the physiological role of asparaginase was made by testing the ability of cells with wild-type or mutant asparaginase to grow when asparagine is the only source of carbon or nitrogen in the growth medium.

Finally, I attempted to demonstrate that active but physically modified asparaginase can be obtained: I tested the heat-lability of hybrid asparaginase produced in diploid cells containing complementing asparaginase mutations.

MATERIALS AND METHODS

Yeast strains

Source and life cycle: All yeast used in this study were heterothallic strains of Saccharomyces cerevisiae obtained from Dr. Robert K. Mortimer, University of California, Berkeley, or derived from strains in his collection. The life cycle of such strains has been described (28, 74). Vegetative growth of haploid strains occurs by budding, mitotic assortment of chromosomes, nuclear division, and separation of the two daughter cells. When haploid cells of opposite mating types (designated a and α) are paired, fusion occurs, and a zygote is formed. After the two nuclei fuse (karyogamy), the resulting diploid cell can be propagated vegetatively for long periods of time. If restrictive culture conditions are imposed upon the diploids, sporulation occurs. During sporulation, crossing over and meiotic assortment of chromosomes take place, and four haploid spores are formed in an ascus. When separated, these four spores can be germinated by culturing them on enriched medium. The resulting haploid strains can be propagated vegetatively almost indefinitely.

All strains were stored on agar slants (YEPAD, see below) at 4 C. (Excess adenine was used in slantagar for reasons not related to the present research.) The slants were transferred not more than once in four months. A permanent stock of a newly isolated strain was started from a single-colony isolate of the strain.

Strains carrying known genetic markers were used to determine the presence in other strains of specific genetic loci. Lack of

complementation between a known mutation and an unknown mutation indicated that the two were allelic.

Nomenclature: Genetic markers and their symbols are listed in Appendix II. The symbols are those proposed at the Osaka Yeast Genetics Conference, Osaka, Japan, 1968 (75). Two numbers are used in the notation: the first number following a symbol designates the particular gene represented by the symbol; the second number designates the particular allele of the gene to which the symbol refers (for example: asp1-15).

Media

YEPD: In one liter of distilled water: Yeast extract (Difco), 10 gm; Bacto-peptone (Difco), 20 gm; dextrose, 20 gm; and agar (Difco), 20 gm. Liquid YEPD contained no agar.

Synthetic Complete (C): In one liter of distilled water: Yeast Nitrogen Base without Amino Acids (Difco), 6.7 gm; dextrose, 20 gm; adenine, 20 mg; L-arginine (free base), 20 mg; L-histidine (free base), 20 mg; L-leucine, 30 mg; L-lysine·HCl, 30 mg; L-methionine, 20 mg; L-threonine (allo free), 150 mg; L-tryptophan, 20 mg; and uracil, 20 mg. L-Threonine was filter-sterilized and added to the other constituents, which were sterilized by autoclaving.

Omission-Addition Media: Synthetic complete medium with one or more constituents omitted and/or with other metabolites included.

C-THR+ASP, for example, is Synthetic complete medium with threonine

omitted but supplemented with L-aspartic acid. L-Aspartic acid and L-asparagine monohydrate were added, when needed, to a final concentration of 100 mg per liter. Concentrated solutions of aspartic acid and asparagine were filter-sterilized and added to the other constituents, which were sterilized by autoclaving.

Petite Medium (PET): In one liter of distilled water: glycerol, 30 ml; dextrose, 0.25 gm; Yeast extract, 10 gm; Bacto-peptone, 20 gm; and agar, 20 gm. Cells exhibiting the petite phenotype (inability to utilize non-fermentable compounds for a carbon source) do not grow or grow only very slowly on this medium.

Pre-Sporulation Medium (GNAP): In one liter of distilled water: dextrose, 50 gm; Yeast extract, 10 gm; Nutrient Broth (Difco), 8 gm; Bacto-peptone, 20 gm; and agar, 20 gm.

Sporulation Media:

Raffinose Acetate Medium (RAO): In one liter of distilled water: potassium acetate, 3.0 gm; raffinose pentahydrate (Difco), 0.22 gm; and agar, 20 gm.

McClary's Medium (66): In one liter of distilled water: potassium acetate, 9.7 gm; dextrose, 1.0 gm; Yeast extract, 2.5 gm; and agar, 20 gm.

Special Sporulation Medium (SP): In one liter of distilled water: potassium acetate, 2.5 gm; Bacto-peptone, 5.0 gm; glycerol, 2.5 ml; Yeast extract, 5.0 gm; L-aspartic acid, 100 mg; and agar, 20 gm. (See RESULTS for discussion.)

Buffers:

Sorensen's buffer: $\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$, 0.067 M, pH 8

Potassium phosphate buffer (K- PO_4): $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$,

molarity and pH adjusted as required by adding the necessary potassium phosphate.

TRIS-HCl buffer (TRIS): Tris-hydroxymethylamino methane (Mann Research Laboratories, New York, N. Y.), molarity, pH, and ionic strength adjusted as necessary with HCl and/or KCl.

Borate-KCl buffer: H_3BO_3 (0.1 M) + KCl (0.1 M), adjusted to the desired pH with NaOH.

Minimal Medium with Vitamins (MV): In one liter of distilled water: Yeast Nitrogen Base without Amino Acids, 6.7 gm; dextrose, 20 gm; and agar, 20 gm.

Slant Agar (YEPAD): In one liter of distilled water: Yeast extract, 10 gm; Bacto-peptone, 20 gm; dextrose, 20 gm; adenine, 40 mg; and agar, 20 gm.

All media were sterilized by autoclaving for about 25 minutes at 15 pounds per square inch pressure, 121 C. Shorter times were used for smaller volumes.

Induction of mutations and isolation of asparaginase mutants

Asparaginase mutations were induced by treating haploid cells of strains X2902-21B (α trp4 asp5) and X2902-67B (a trp4 asp5) with ethyl methanesulfonate (EMS) (54). Cells growing on YEPD agar medium were suspended in 10 ml Sorensen's buffer so that the final titer at the beginning of treatment with the mutagen would be about 10^7 per milliliter. 1.0 ml of 20 per cent dextrose in distilled water (sterile) was added to 8.7 ml of cells in buffer, and the suspension was placed in a 30 C water bath for three to five minutes. When the suspension had warmed, 0.3 ml EMS (Eastman Organic Chemicals, Eastman Kodak Co., Rochester, N. Y.) was added, and the tube was transferred to a shaker in a 30 C incubator. The suspension was incubated at 30 C with gentle shaking for 60 minutes. At the end of the hour, 0.2 ml of the mutagen-treated cells was pipetted into 9.8 ml of six per cent sodium thiosulfate (sterile, in distilled water) that had been prewarmed to 30 C. The thiosulfate suspension was left at 30 C for about ten minutes to inactivate the EMS. 0.25 ml of the suspension was pipetted into 19.75 ml of sterile distilled water, and 0.2 ml of this final dilution was pipetted and spread onto each of 120 to 150 YEPD agar plates. A comparison of viable titer on YEPD plates of treated and untreated cells showed that about 30 per cent of the cells survived the treatment. The cells were incubated at 30 C for three days, and the colonies that developed were replica plated (53) onto MV+TRP+ASN and MV+TRP+ASP media. The replicas were incubated overnight at 30 C.

Colonies that grew on MV+TRP+ASP medium but not on MV+TRP+ASN medium were picked off the original YEPD plates, streaked onto fresh

YEPD plates and tested further. The replicas were left at room temperature (about 24 C) for several days and were again scanned for presumptive asparaginase mutants. Stable clones were selected for lack of growth on MV+TRP+ASN medium by several consecutive single-colony isolations.

Genetic methods

Genetic crosses and tetrad analysis: Crosses were performed by techniques that have been described (73, 74). They were begun by mixing, on a YEPD agar plate, approximately equal portions of 24-hour cultures of parental haploid strains. The mixture was incubated at 30 C for three to five hours, after which a small amount was streaked along one side of a thin YEPD agar slab on a rectangular coverslip. The slab was inverted over a small plastic chamber, and individual zygotes, recognizable by their characteristic "bow-tie" morphology, were separated from the mixture with a glass needle in a micromanipulator and placed on a clear area of the slab. Three or four zygotes were isolated from each cross. After the zygotes were isolated, the slab was removed from the cover slip with a sterile spatula and placed, cells upward, on a YEPD agar plate. The cells were incubated at 30 C until visible colonies of diploid cells derived from the single zygotes had formed. Cells from the colonies were streaked onto agar slants for storage.

Diploid cells were sporulated by growing them overnight on a rich medium (GNAP) and then replica plating cells from the GNAP plate onto sporulation media. In the earlier crosses, diploids were replica plated onto each of the three sporulation media described earlier. Use

of RAC medium was later discontinued, because sporulation was always better on McClary's medium or on SP medium (see RESULTS).

When cells on the sporulation plates had incubated at 30 C for three to four days, portions of the cells were placed onto glass slides and observed under a microscope. If asci were well formed, they were dissected and the haploid segregants isolated as follows (42).

Sporulated cells were suspended in 0.2 ml of Glusulase (Endo Laboratories, Inc., Garden City, New York) that had been diluted 1:40 in sterile distilled water. Glusulase, an extract from the crops of snails, contains glucuronidase, and enzyme that digests ascus walls but not spore capsules (42). The suspension was incubated at 30 C for 30 minutes to an hour and then centrifuged at low speed in a clinical bench centrifuge for about five minutes. The enzyme solution was poured off, and the spores and unsporulated cells were resuspended in the same volume of distilled water. A small amount of the suspension was streaked along one edge of an agar slab on a coverslip, and spore tetrads were dissected under a microscope equipped with a micromanipulator. (Yeast spores adhere to each other, and spores from the same ascus are not separated during digestion of the ascus wall (42).) The spores were separated with a glass needle and aligned on the agar slab so that spores from the same tetrad could be identified. Fifteen to eighteen tetrads were aligned on the same slab in a rectangular array.

When the dissection was completed, the slab was removed from the coverslip and placed, spores upward, on a YEPD agar plate. The spores were incubated at 30 C until they had germinated and haploid colonies had developed. Cells from these colonies were streaked onto

YEPD plates and grown overnight at 30 C. The cells on these master plates were then replica plated onto selective and again incubated overnight at 30 C. Finally, the segregation of nutritional markers was recorded by scoring the growth patterns of the haploid segregants on the selective media.

The methods by which gene-gene linkage and centromere-linkage of genes were analyzed (tetrad analysis) have been described by Mortimer and Hawthorne (73). In a cross involving two genetic characteristics, AB x ab, three classes of asci can be distinguished: Parental Ditype (PD), in which the characteristics are distributed among the four haploid segregants in the arrangement AB, AB, ab, ab; Non-Parental Ditype (NPD), in which the distribution is Ab, Ab, aB, aB; and Tetratype (T), in which it is AB, Ab, aB, ab. If the two genes determining the traits are linked, crossing over between them occurs less frequently than between unlinked genes, and the ratio of PD:NPD asci is significantly greater than unity.

Centromere-linkage of a particular gene is determined by following its segregation relative to known centromere-linked genes (36). After homologous chromosomes pair and form a quadrivalent during meiosis, centromeres segregate at the first meiotic division leading to spore formation. Genes located near centromeres segregate more frequently at the first meiotic division, because the likelihood of crossing over between the gene and its centromere is reduced. Tetratype asci result from segregation of genes at the second meiotic division; therefore, the frequency of tetratype asci is a measure of the centromere linkage of a gene. For randomly assorting gene-pairs, the

ratios of PD:NPD:T asci are expected to be 1:1:4 (73). If one gene is centromer-linked and the frequency of tetratype asci for this gene relative to a second gene is significantly less than two-thirds, the second gene is considered to be centromere-linked as well.

Complementation analysis: Complementation analysis to test for allelism of mutations (25) was performed by mixing haploid stocks of opposite mating types on YEPD medium, allowing the mixtures to incubate overnight at 30 C, and replica plating them onto selective media on which diploid cells would grow only if active enzyme were formed in them. Streaks of cells on the selective media were incubated overnight at 30 C and scored the next day for growth. As a control, the master plates (YEPD) were also replica plated onto non-selective media. To ensure that mating of haploid cells occurred in the original mating mixtures, portions of each mixture were observed under a microscope. Zygotes were found three to four hours after haploid cells were mixed in all mixtures in which mating occurred. Mating failed to take place only very infrequently.

Before the asparaginase mutations could be tested for allelism, each mutation had to be present in cells of both mating types. Each of the asparaginase mutants was crossed to a strain carrying only the asp5 mutation, diploid cells were sporulated, and asci were dissected. A haploid segregant of genotype trp4 asp5 aspl and of mating type opposite to that of the original asparaginase-deficient mutant was selected from each cross. In this way, strains of opposite mating type but with identical (known) genotypes in combination with each of the asparaginase mutations were selected. Therefore, diploid cells carrying

all pairwise combinations of the asparaginase mutations could be used in complementation tests to determine whether the asparaginase mutations were allelic.

Fine-structure mapping: The relative positions of the asparaginase mutations relative to each other in the aspl gene were determined by inducing, with x rays, intragenic, mitotic recombination between the alleles. Manney (60) and Manney and Mortimer (62) showed that the rate at which x rays induce mitotic recombination events between hetero-alleles in diploid cells can be used as a measure of the distance between the mutations in those alleles. Each mutation is mapped relative to two other mutations known to be well separated in the gene. For three mutation, a, b, and c, the distance between a and c (ac) is given either by $\underline{ac} = \underline{ab} + \underline{bc}$ or by $\underline{ac} = \pm (\underline{ab} - \underline{bc})$, depending on whether b is between a and c. The fine-structure map of the asparaginase gene was constructed by assuming additivity of distances between alleles and by normalizing distances between alleles so that intervals between reference alleles were constant.

The unit used to express x-ray map distances is the number of prototrophic cells induced per roentgen per 10^8 surviving cells (60, 62). X-ray doses used in the present experiments were sufficiently low (less than 10 kiloroentgens) that no killing was observed, and the number of prototrophs induced per 10^8 cells was proportional to the exposure dose. The slope of a plot of prototrophs induced per 10^8 survivors versus x-ray dose, then, was the measure of the distance between sites of mutation in two alleles.

Diploid cells carrying two alleles of aspl and homozygous for

asp5 were prepared by isolating zygotes from mixtures of the appropriate strains. In preliminary experiments, alleles were chosen that were suitable for reference allele-pairs. The pairs chosen were aspl-6:aspl-14 and aspl-6:aspl-15. Two pairs were used, because the x-ray mapping technique cannot be used with complementing alleles. When any particular allele was to be mapped, it was crossed to cells carrying reference alleles with which it would not complement.

If diploid cells homozygous for a particular allele are irradiated, prototrophs are induced even though the molecular lesions in the two alleles are presumably at identical sites in the gene. This reversion can contribute to the rate of appearance of prototrophs when heteroallelic diploids are x-irradiated (60, 62). To determine the rate at which reversion events occurred when alleles of the asparaginase gene were irradiated, diploids homoallelic for each of the aspl alleles were grown, plated on selective medium, and irradiated. Because reversion of the asp5 gene would also lead to growth on the selective media (C-THR+ASN or C-THR), diploid cells homozygous for asp5 only were also plated and irradiated.

Heteroallelic diploid cells were grown to stationary phase in liquid YEPD medium, washed twice with distilled water, and diluted in distilled water. About 5×10^5 to 5×10^6 cells were plated onto C-THR+ASN medium. The cells were then exposed to x-ray doses in the range zero to nine kiloroentgens. About 200 cells were plated on YEPD agar plates and irradiated at the same doses to score survival.

The combined rate at which homoallelic reversion of aspl and asp5 occurred was determined by growing the appropriate diploid cells in liquid YEPD medium, washing them and diluting them as described above,

plating 2×10^7 to 4×10^7 cells onto C-THR+ASN medium, and irradiating them with the same doses used in the heteroallelic experiments. The rate of reversion at the asp5 locus alone was measured by plating and irradiating diploid cells on C-THR medium.

X rays were produced by a beryllium-window tube (Machlett OEG 60) operated at 50 kilovolts with a current of 25 milliamperes without added filtration. The dose rate was 250 roentgens per second at the position of the cells (14 cm from the end of the tube) (59).

YEPD plates were incubated for three days at 30 C after which the visible colonies on them were counted. Cells on C-THR+ASN and C-THR media were incubated one day longer before being counted. Survival and recombination data were analyzed with a PDP 8/1 computer (Digital Equipment Corp., Maynard, Mass.). Rates of induction of prototrophs and standard deviations of the rates were calculated directly from plate counts and dilution factors by linear regression analysis. Regression lines were fitted to the data by least-squares statistical techniques (109). In most cases, cells were not killed by the doses of x rays used, and data from cells of each strain plated on YEPD agar medium were pooled to provide a better estimate of the number of cells spread on selective media. The validity of pooling was tested for each strain by regression analysis of the survival data.

Biochemical methods

Growth of cells: Cell growth was followed by reading the turbidity of cultures in 22-mm diameter pyrex test tubes in a Spectronic-20 colorimeter (Bausch and Lomb, Inc., Rochester, N. Y.) used as a turbidimeter. Calibration curves were obtained by diluting and plating cells from cultures of known turbidity to determine the viable

titer and by counting individual cells in a haemocytometer. In nearly all experiments, late exponential phase cultures were used, the cell densities being about 10^8 per milliliter. Occasionally, when a large amount of extract was needed, cultures at densities of 2×10^8 to 3×10^8 cells per milliliter were used. (Stationary phase titers for haploid yeast cells are normally in the range 4×10^8 cells/ml to 6×10^8 cells/ml.)

Preparation of cell extracts: Two methods were used to disrupt cells, extrusion through the orifice of a French pressure cell (American Instrument Co., Silver Springs, Md.), and grinding with powdered alumina (Buehler, Ltd., Evanston, Ill.). When large quantities of extract were needed, the first technique was used. When several separate extracts were to be prepared, grinding with alumina was more convenient.

Pressure-cell disruption: Cells of the appropriate genotype were spread onto YEPD agar plates and grown at 30 C overnight. From these cultures, cells were suspended in distilled water, in buffer, or in the medium in which they were to be grown. Media in Erlenmeyer flasks or Dulong flasks were seeded from the liquid suspensions at cell concentrations of 10^4 to 10^5 cells/ml. Flasks were never filled to more than half their capacity. The cultures were then shaken at 325 revolutions per minute (rpm) in a Psychotherm Incubator (New Brunswick Scientific Co., New Brunswick, N. J.) at 30 ± 0.5 C or on a New Brunswick Rotary Shaker in a warm room (29 ± 1 C). When the cultures had grown to the desired concentrations, they were chilled and transferred to plastic centrifuge tubes or bottles. The containers

were centrifuged for 15 minutes at 5000 rpm (3000 g), 0-4 C, in a Servall refrigerated centrifuge (I. Sorvall, Inc., Norwalk, Conn.). Pellets were resuspended in cold buffer and centrifuged again. A second wash cycle was usually performed, especially if the concentration of substrate was to be varied in an experiment. The final pellet was suspended in 10 to 40 ml of cold buffer, depending on the number of cells being treated, and the suspension was extruded three times at a pressure of 20,000 lbs/sq in through the orifice of the pressure cell. The pressure cell was precooled to 4 C, and all procedures were carried out as rapidly as possible to keep the extract cold. Liquid emerging from the pressure cell was caught in a test tube in a beaker filled with ice.

After the cells were ruptured, the extract was centrifuged at 0-4 C for 30 minutes, 10,000 rpm (12,000 g) in the Servall centrifuge. The supernatant from this final centrifugation, called crude extract in this report, was poured into a cold tube for further processing. When a crude extract was not to be used immediately, it was frozen at -25 to -30 C in polyethylene vials.

Alumina grinding: Cultures were grown and washed as described for pressure-cell extrusion. After the final wash, the pellet of cells from a culture was transferred to a cold mortar containing 2 gm of powdered alumina. Mortars, pestles, and alumina were precooled in a freezer so that the pellet material would freeze when transferred to the mortar. About 0.5 ml of cold buffer was added to the mortar, and the mixture was spread over the bottom of the mortar with the pestle and allowed to freeze. The mortar was left at room temperature; just as the mixture began to thaw, it was ground vigorously for one to two

minutes. The yield of protein was much greater if cells were ground just before and during the thawing process than if thawed or unfrozen cells were ground. After the cells were ground, about three milliliters of cold buffer was pipetted into the mortar, and the mixture was transferred immediately to cold centrifuge tubes. Unbroken cells and alumina were removed by centrifuging the mixtures for 15 minutes at 3000 rpm (1000 g), 0-4 C. Supernatants from these tubes were centrifuged at high speed as discussed above, after which they were treated as crude extracts.

Much less protein was recovered by grinding cells with alumina than by rupturing them in the pressure cell. Protein concentrations in crude extracts prepared by pressure-cell disruption ranged from 5 to 50 mg/ml, depending on the amount of buffer used. This value routinely corresponded to 4 to 5 picograms of protein per cell in the original suspension. Extracts prepared by grinding with alumina had protein concentrations ranging from 0.5 to 5 mg/ml. The lower values were obtained when thawing had progressed too far before the mixtures were ground. Although the absolute protein concentrations were different, specific asparaginase activity (units per milligram of protein) was the same whether cells were disrupted by extrusion from the pressure cell or by grinding with alumina.

Determination of total protein: Enzyme preparations were compared on the basis of units of activity per milligram of protein in the reaction mixtures. The method described by Lowry, et al.(55), was used to measure protein concentrations in crude extracts. Extract was diluted in distilled water to a concentration appropriate for the assay method (usually 30 to 500 μ gm/ml). 0.4 ml of the diluent was

pipetted into an 11-mm diameter test tube containing 2.0 ml of freshly mixed alkaline-copper reagent (2 % Na_2CO_3 in 1 N NaOH + 1 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water + 2 % sodium tartrate in distilled water mixed in the proportions 50 : 0.5 : 0.5 just before use. If alkaline-copper reagent was allowed to stand too long, a precipitate formed that interfered with the measurement.). The protein-alkaline-copper solution was left at room temperature for 15 minutes; then 0.2 ml of Folin-Ciocalteu phenol reagent (Hartman-Leddon Co., Philadelphia, Pa., 2N reagent diluted with an equal volume of distilled water) was added, and the solution was mixed immediately on a vortex mixer. (Mixing must be very rapid-within one second, preferably- because color development decreased rapidly if the protein-alkaline-copper complexes were not accessible to Folin reagent when it was added to the alkaline solution (55)). The final solution was left at room temperature for at least 30 minutes (I routinely allowed an hour), the test tube was inserted into the Spectronic-20 colorimeter, and the optical density at 520 nm of the solution in the tube was read.

A standard protein curve (Figure 2) was obtained by diluting a known concentration of bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) in distilled water and processing as described. Manney (59) showed that using bovine serum albumin for a standard does not introduce error greater than about four per cent over the range of concentrations used in the present work.

Because the calibration curve is not linear and reagents may change slightly with time, protein standards were run with most of the cell extract measurements. All protein determinations were within about 10 per cent of the original calibration curve and showed no con-

DBL 6912 5224

Figure 2. Optical density at 520 nm developed when known concentrations of protein (Bovine serum albumin) were processed by the method of Lowry, *et al.* (99). The protein concentrations given are those of solutions before they were diluted into alkaline-copper reagent.

sistent variation with age of reagents. (The same reagent stock was used throughout the investigation.)

Protein determinations were probably not in error by more than 15 per cent. Reproducibility was somewhat better: simultaneous measurements on a given sample were usually within about five per cent of each other. All experiments that depended upon absolute protein measurements (for example, the kinetic experiments) were done with the same extract stock if possible. Where this was not possible, controls were run to ensure that valid intercomparisons could be made.

Asparaginase assays: L-Asparaginase assays were performed in two ways. Whenever several measurements were to be made with cells grown in the same way, crude extracts were prepared by pressure-disrupting cells or by grinding them with powdered alumina (see above). When cells from several different cultures were to be compared, asparaginase activities were determined using benzene-treated cells (57).

Asparaginase determination in crude extract: Asparaginase activities in crude extracts were routinely measured in reaction mixtures of 2.0 ml total volume. Asparagine monohydrate dissolved in buffer was diluted into buffer in an 11-mm test tube and warmed to the assay temperature in a water bath equipped with a stirrer. An aliquot of extract was added to start the reaction. (Crude extract was not prewarmed. However, the volume of extract added was usually 0.2 ml or less and was rapidly brought to temperature in the 2.0 ml reaction mixture.) After the desired time had elapsed, 0.2 ml of 50 per cent trichloroacetic acid in distilled water (TCA) was added to stop the reaction. The tube was mixed on a vortex mixer and centrifuged in a

clinical bench centrifuge at room temperature for about five minutes to sediment the precipitated material. The concentration of ammonia in the TCA supernatant was determined colorimetrically, as described below.

Reaction mixtures containing buffer without asparagine were incubated simultaneously with those containing asparagine to determine the amount of ammonia in the extract or evolved by reactions not using asparagine as a substrate. This "background" ammonia was accounted for in determining activities in extracts.

The amount of ammonia evolved for a constant reaction time (30 minutes) was directly proportional to the amount of protein added to reaction mixtures for up to at least 5.0 mg in the 2.0 ml reaction mixture volume (Figure 3). When reaction mixtures containing 2.0 mg of protein were incubated for different lengths of time, the amount of ammonia evolved was directly proportional to the time of reaction for at least three hours (Figure 4). Background corrections were proportional to the amount of protein in reaction mixtures but were independent of time of reaction. The temporal independence indicates that ammonia-generating reactions not using asparagine as a substrate did not occur in the mixtures.

Asparaginase specific activities measured in crude extracts are reported in International Units (I. U.) or as optical density developed from ammonia evolved under specified assay conditions. One International Unit of enzyme liberates one micromole of ammonia per milligram of protein in one minute.

2

DBL 6912 5217

Figure 3. Optical density at 420 nm developed from ammonia evolved in reaction mixtures containing different quantities of yeast crude extract. Asparagine concentration: 10 mM; specific activity of extract: 8.5×10^{-2} I. U.

DBL 6912 5218

Figure 4. Optical density at 420 nm developed from ammonia evolved in reaction mixtures incubated at 30 C for various lengths of time. Asparagine concentration: 10 mM; specific activity of extract: 8.5×10^{-2} I. U.

Asparaginase assay in benzene-treated cells: Some experiments required that cells grown simultaneously in many individual cultures be assayed for asparaginase activity. Because of the number of cultures involved, extraction by pressing or grinding was inconvenient. Magee and de Robichon-Szulmajster (57) briefly described a method for measuring activity of acetohydroxy acid synthetase in yeast cells treated with benzene to make the cell membrane permeable to small molecules. This technique worked well for assaying asparaginase activity in yeast.

Cells were grown to the proper phase of growth, and the cultures were cooled to 4 C. The cultures were centrifuged at 4 C and the cells resuspended in cold buffer. They were washed once again in cold buffer and, finally, were suspended in cold buffer at the proper densities. In most experiments, the suspensions contained about 4×10^8 cells per milliliter. Cell densities were made nearly identical in all suspensions to avoid errors that might arise from treating suspensions at different densities with the same volume of benzene.

Aliquots of the final suspensions were diluted and plated onto YEPD agar plates to determine the titer of viable cells. Also, the turbidity of the first dilution tube was measured to provide a further basis for comparing cultures. After the cells on YEPD plates had incubated for three days (30 C), colonies were counted, and all activity measurements were normalized to the same number of cells in the reaction suspensions.

To determine asparaginase activities in cells, suspensions were warmed to room temperature, and an amount of benzene (Allied

Chemical and Dye Corp., New York, N. Y. ; Reagent Grade) equal to one-tenth the volume of the cell suspensions was layered onto the surface of each suspension. The tubes were vigorously mixed at room temperature on a vortex mixer for 30 seconds, set in a water bath at 30 C (without agitation) for 90 seconds, and then kept at 0-4 C until used. Asparaginase activity was measured by pipetting 0.5 ml of a treated suspension into an 11-mm test tube, warming the tubes and their contents to 30 C, and adding 0.5 ml of prewarmed substrate to the tubes. Other tubes containing cell suspension received 0.5 ml of buffer without asparagine. Reaction suspensions were shaken periodically during the incubation period. After the reactions had proceeded as far as was desired, 0.2 ml of 50 per cent TCA was added to each tube to stop the reactions. The tubes were centrifuged for about five minutes in a bench centrifuge, and ammonia concentrations in the TCA supernatants were measured as described in the following section.

When 2×10^8 cells were used in reaction suspensions, the amount of ammonia evolved was proportional to the time of reaction for at least 60 minutes (Figure 5). The evolution of ammonia in a constant time interval (30 minutes) was proportional to the number of cells in a reaction suspension up to about 3×10^8 cells in the 1.0 ml volume (Figure 6). Background corrections were proportional to the number of cells in reaction mixtures but independent of time of reaction.

In all experiments in which this assay method was used, the number of cells in reaction suspensions was 2.5×10^8 or less; reaction times were always 30 minutes (except in the experiment discussed above), and 0.2 ml of TCA supernatant was used to determine ammonia

DBL 6912 5227

Figure 5. Optical density at 420 nm developed from ammonia evolved from benzene-treated cells incubated for various lengths of time at 30 C. Asparagine concentration: 10 mM; Number of cells in 1.0 ml reaction suspension: 2×10^8 .

DBL 6912 5228

Figure 6. Optical density at 420 nm developed from ammonia evolved from reaction suspensions (1.0 ml total volume) containing various numbers of benzene-treated cells. Asparagine concentration: 10 mM; reaction time: 30 min.

concentrations in reaction suspensions. Results of these experiments are expressed as optical density units at 420 nm per 10^8 cells in the reaction mixtures (corrected for "background" ammonia). Because constant reaction times and constant amounts of TCA supernatants were used in the ammonia titration, all the values are directly comparable.

In Table 3 are shown the results of several experiments demonstrating the reproducibility of the benzene-treated cell assay. In Experiment 111, six identical cultures of cells of strain X2902-21B (α trp4 asp5) were grown to late exponential phase, chilled, and assayed. The mean O. D. per 10^8 cells in reaction suspensions was 0.190 with a standard deviation of 0.015 (estimated from the range (109)). Variation among independent experiments was about the same as variation within this experiment. When an asparaginase-deficient mutant (LA21 (α trp4 asp5 aspl-6)) was used for the assay, no activity was observed, and no ammonia was released into reaction suspensions when cells with normal asparaginase activity but not treated with benzene were assayed. Therefore, the release of ammonia in the benzene-treated cell assay requires that active asparaginase be present and that the cell membrane be made permeable to ammonia.

Ammonia titration: The concentration of ammonia in standard solutions or in TCA supernatants of reaction mixtures was measured by titration with Nessler's reagent (direct Nesslerization) (69, 96). Between 0.1 ml and 1.0 ml of solution containing ammonia was pipetted into an 11-mm test tube containing enough distilled water to make a

Table 3. Reproducibility of the benzene-treated cell assay

Exper. no.	Strain, growth conditions, and assay method	Asparaginase activity (O.D./10 ⁸ cells)
100	LA21 (α <u>trp4</u> <u>asp5</u> <u>asp1-6</u>) Aerobic growth in YEPD Benzene-treated cells	0.002 $\pm$ 0.05
100	X2902-21B (α <u>trp4</u> <u>asp5</u>) Aerobic growth in YEPD Benzene-treated cells	0.17 $\pm$ 0.01
100	X2902-21B Aerobic growth in YEPD Cells not benzene-treated	0.005 $\pm$ 0.015
103	X2902-21B Aerobic growth in YEPD Benzene-treated cells	0.200 $\pm$ 0.005
111	X2902-21B Aerobic growth in YEPD Benzene-treated cells	0.190 $\pm$ 0.015 (mean and stand- ard deviation of six cultures)

volume of 3.0 ml. 0.4 ml of Nessler's reagent (prepared from Sigma Ammonia Color Concentrate, Sigma Chemical Co., St. Louis, Mo.) was added, and the solution was mixed on a vortex mixer. Color was allowed to develop at room temperature for 15 minutes, and the optical density at 420 nm was read in the Spectronic-20 colorimeter.

A standard ammonia curve (Figure 7) was prepared by diluting known quantities of ammonium sulfate in distilled water and treating the solutions as described. The optical density developed was proportional to the amount of TCA supernatant in the 3.4 ml (total volume) Nessler titration tubes (Figure 8). The amount of color for a given amount of ammonium ion was the same whether the ion was in distilled water or in TCA supernatant (data not presented). The optical density curves for Nessler color are linear up to an O. D. of at least 0.9.

To account for changes in reagents, standard ammonia determinations were made concurrent with most experimental measurements, and the same stock of Nessler's reagent was used throughout the investigation. Because time of mixing is not so critical with Nessler's reagent, ammonia determinations were considerably more accurate and reproducible than total protein measurements. Overall inaccuracy of ammonia determinations was probably less than 10 per cent, and reproducibility was usually better than 95 per cent. (That is, repeated measurements of ammonia in a given solution were usually within 5 per cent of each other).

In the text that follows, tubes in which ammonia was titrated are referred to as Nesslerization tubes.

DBL 6912 5223

Figure 7. Optical density at 420 nm developed from known concentrations of ammonium ion treated as described in the text. Quantities of ammonia given are the total quantities in Nesslerization tubes containing 3.4 ml total volumes. Dates on which three experiments were performed are given to demonstrate the reproducibility of the assay.

DBL 6912 5225

Figure 8. Optical density at 420 nm developed from ammonia contained in various amounts of TCA supernatant. TCA supernatant was diluted to make 3.4 ml total volumes in the Nesslerization tubes. Asparagine concentration: 10 mM; specific activity of extract: 8.5×10^{-2} I. U.

RESULTS (Genetics)

Characteristics of asparaginase mutants

Identification: Fourteen stable mutants that grew well with aspartate (MV+TRP+ASP) but not with asparagine (MV+TRP+ASN) were selected (44). Nine of these mutants were selected from mutagen-treated cells of strain X2902-21B (trp4 asp5); the other five were derived from cells of strain X2902-67B (trp4 asp5). The fourteen mutants were selected from a total of about 30,000 clones. Approximately 30 per cent of the cells exposed to EMS survived, and of these, nearly 30 per cent exhibited a nutritional deficiency of some kind.

Each mutant strain was assigned a number preceded by the letters "LA." (Each mutant gene was temporarily assigned a number, as well.) After the fourteen mutations were found to be allelic (see below), the alleles were designated asp1-2 through asp1-15. Strain numbers and associated alleles are listed in Table 4.

Temperature sensitivity: The fourteen mutant strains were tested for ability to grow at three temperatures, room temperature (23-25 C), 30 C, and 37 C. No temperature sensitivity was found among the strains at these temperatures.

Aspartate synthesis in strains carrying the asp5 mutation: Segregation of the asp5 gene was followed by testing the ability of haploid cells to grow on C-THR medium. Although this medium can be used to score segregation of this gene, it is not totally restrictive.

Table 4. Strain numbers and genotypic designations of asparaginase mutants and mutations

Strain number	Derived from	<u>aspl</u> allele number
LA6	X2902-21B	2
LA12	"	3
LA18	"	4
LA20	"	5
LA21	"	6
LA25	"	7
LA35	"	8
LA38	"	9
LA46	"	10
LA156	X2902-67B	11
LA158	"	12
LA161	"	13
LA163	"	14
LA165	"	15

The asp5 mutation is suppressible (72), and suppressible mutations usually produce incomplete proteins that are inactive (37, 61). The slow growth of asp5 strains is probably attributable to limited production of aspartate by other synthetic pathways or by the operation in reverse of enzymes involved in aspartate metabolism. Asparaginase mutants were selected by replica plating colonies onto MV+TRP+ASP and MV+TRP+ASN media, which, because they contain fewer metabolites, are more restrictive than C-THR medium. Therefore, the selection of asparaginase mutants was not affected by the slow aspartate production. In the genetic studies, however, omission-addition media (see MATERIALS AND METHODS) based on C-THR were used as the selective media. To avoid interference by the slow production of aspartate in cells plated on these media, growth was scored after the cells had incubated no longer than 1 to 1-1/2 days at 30 C.

Sporulation of diploids homozygous for asp5: Most crosses involving the asparaginase mutations were carried out in cells homozygous for the asp5 mutation so that the asparaginase-deficient phenotype could be easily scored. However, most such diploids failed to sporulate well on the routinely used sporulation media (McClary's medium and RAc medium). When asci did form, spore viability was usually low, often less than 50 per cent. I attempted to remedy this problem by selecting better-sporulating diploids. Two haploid strains carrying the asp5 mutation were crossed to strain S288C, the standard wild-type yeast. By repeatedly crossing asp5 segregants to S288C, I was able to select two haploid strains that formed better-sporulating

diploids when crossed to other asp5 haploid cells. These two strains were XE58-3A (α asp5) and XE59-12B (a asp5).

A more promising approach to obtaining better sporulation of diploid cells homozygous for asp5 was suggested by findings reported by de Robichon-Szulmajster, et al. (102, 115). They observed that S. cerevisiae strains carrying mutations at any one of several genes in the aspartate-to-homoserine pathway, including asp5 (called thr5 in their publication), are deficient in cytochrome oxidase. This deficiency leads to poor sporulation, because active oxidative metabolism, which requires cytochrome oxidase, is necessary for sporulation (102). The deficiency in cytochrome oxidase could be partially overcome by growing cells on medium containing glycerol instead of dextrose for a carbon source or by growing them on medium containing a high concentration of threonine (102). I found that sporulation of diploid cells homozygous for asp5 was not improved by growing them on pre-sporulation medium containing high levels of threonine or by attempting to sporulate them on medium containing threonine. However, if the sporulation medium contained glycerol and aspartate, as many as 70 to 80 per cent of the cells of some strains sporulated, and spore viability was often as high as 100 per cent. This sporulation medium (SP; see MATERIALS AND METHODS) was used routinely throughout the remainder of the investigation to sporulate diploid cells homozygous for the asp5 gene. Although sporulation was greatly improved by this medium, the per centage sporulation still varied among crosses in which asp5 was homozygous. Almost without exception, however, sporulation was better on SP than on the other media.

Petite phenotype of asparaginase mutants: The fourteen mutant strains were tested to determine whether a petite mutation had been induced in any of them. All the strains except LA38 grew well with glycerol as a carbon source (that is, on Petite medium).

To determine whether the petite phenotype of LA38 is chromosomally determined and, if, so, if the petite gene is linked to the asparaginase gene, LA38 was crossed to strain XE59-12B (a asp5), the resulting diploid cells were sporulated, and the segregation of the petite and asparaginase-deficient phenotypes were followed. Segregation of the petite phenotype relative to the mating-type locus, which is loosely centromere-linked (36), was also scored. The petite phenotype of strain LA38 segregates in the 2:2 fashion expected of chromosomal genes, but this petite gene is not linked to the asparaginase gene or to the mating-type locus (Table 5). The petite mutation in this strain did not interfere with experiments in which the strain was used and was not investigated further.

Allelism of the asparaginase mutations

Diploid cells carrying all pairwise combinations of the 14 asparaginase mutations were tested for ability to grow on C-THR+ASN medium (44). The pattern of growth (complementation pattern) is shown in Figure 9. From the pattern, the complementation map in Figure 10 was deduced. The complementation pattern is typical of intragenic (interallelic) complementation: six complementation classes were found, but one of the classes contains alleles that do not complement any of the others. Therefore, only one gene (functional unit) is involved (25).

Table 5. Segregation of the petite gene in strain LA38^{a,b}

Ascus type	Number of asci of given type for petite gene relative to:	
	LA38 asparaginase gene	Mating-type gene
PD	6 (3.5)	1 (2)
NPD	3 (3.5)	2 (2)
T	12 (14)	9 (8)

- a. Cross XE76: LA38 (α trp4 asp5 asp1-9 p) x XE59-12B (a asp5).
b. Numbers in parentheses are those expected if the genes were segregating randomly.

		<u>Allele Number</u>												
		3	4	5	6	7	8	9	10	11	12	13	14	15
<u>Allele Number</u>	2	+	-	+	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	+	+	-	+	-	-	-	-	+
	4	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	+	+	-	+	-	-	-	-	+
	6	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	+	-	+	-	-	-	-	+	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 9. Pattern of growth of diploid cells containing pairwise combinations of mutant asparaginase genes (complementation pattern). "+" indicates that diploid cells carrying a particular pair of alleles grew on C-THR+ASN medium. "-" indicates lack of growth on that medium.

DBL 6912 5226

Figure 10. Complementation map derived from the pattern in Figure 9. Mutations that complement are represented by numbers on lines that do not overlap. Non-complementing mutations are represented by numbers on overlapping lines.

The gene was designated asp1, and the fourteen alleles were called asp1-2 through asp1-15 (see Table 4).

The interpretation of such complementation patterns has been extensively studied (25, 27, 107, 122). Enzymes that participate in interallelic complementation are multimeric proteins consisting of two or more identical polypeptide chains (monomers) (20, 25). In mutant haploid cells and in non-complementing diploid cells, structural changes in the monomers lead to misfolding of the monomers and, subsequently, to inactive protein when the monomers aggregate. In complementing diploid cells, active enzyme is presumably produced because the folding of one type of monomer is partially corrected by the presence in the aggregate of an unaltered, corresponding part of a different mutant monomer (20). Often the hybrid protein is only partially active and exhibits physical properties different from those of the wild-type enzyme (25, 26, 27, 84, 107, 122, 123).

The complementation tests indicate, then, that the fourteen asparaginase mutations were induced in the same gene and that yeast asparaginase is a multimeric enzyme consisting of two or more identical subunits.

Mapping the asparaginase mutations

During the isolation of segregants that contained the asparaginase mutations in cells of opposite mating type to those in which the mutations were originally induced, the trp4 gene seemed to appear in combination with the asparaginase mutations more frequently than would be expected if the two genes were segregating randomly. To test

this suggested linkage, diploid cells from these crosses were sporulated, and the segregation of asp1 relative to trp4 was followed. These data are summarized in Table 6. All the asparaginase mutations are linked to the trp4 gene, which confirms the conclusion derived from the complementation study that only one structural gene for asparaginase synthesis was involved in the tests.

Because the frequency of non-parental ditype asci was low, the distance, x, in centimorgans between the two genes can be calculated from the equation (88):

$$\underline{x} = 50(T + 6 \text{ NPD}) / (PD + \text{NPD} + T) ..$$

PD, NPD, and T refer to the number of PD, NPD, and T asci resulting from the segregation of the two genes. The data for the 14 mutations can be pooled, because the mutations are allelic. The calculation shows that the asparaginase gene, asp1, is 18 centimorgans from the trp4 gene, which has been located on Fragment 2 of the S. cerevisiae genetic map (73, 74). The centromere of Fragment 2 has not been mapped, but several other genes in this linkage group have been identified (73, 74). To map the asp1 gene relative to these other genes, the following cross was performed and analyzed:

XE64: XE61-10A (α ade8 asp5 leu1) x XE47-10C (a asp5 trp4 asp1-2)

ade8, trp4, and asp1-2 are in the Fragment 2 linkage group; leu1 is located near the centromere of chromosome VII (36). Segregation of these four genes was scored (Table 7).

Table 6. Segregation of *aspl* alleles with respect to *trp4*

Allele no.	Number of asci		
	PD	NPD	T
2	35	0	11
3	4	0	4
4	9	1	6
5	8	0	7
6	7	0	2
7	8	0	2
8	9	0	1
9	18	0	8
10	5	0	1
11	4	0	4
12	11	0	5
13	5	0	3
14	8	0	7
15	5	0	7
Totals	136	1	68

Table 7. Segregation of genes in cross XE64^{a,b}

Ascus type	Number of asci of given type			
	<u>asp1-2</u> trp4	<u>asp1-2</u> ade8	<u>asp1-2</u> leu1	<u>ade8</u> trp4
PD	35 (7.7)	10 (6.8)	7 (7.5)	14 (7.3)
NPD	0 (7.7)	5 (6.8)	12 (7.5)	1 (7.3)
T	11 (46.1)	26 (27.3)	26 (30.0)	28 (29.3)

a. Cross XE64: XE61-10A (α ade8 asp5 leu1)
x XE47-10C (a asp5 trp4 asp1-2)

b. Numbers in parentheses are those expected if the genes in a pair were segregating randomly.

asp1 and ade8 segregate randomly (chi-square = 0.36; p greater than 0.5), and the segregation of asp1-2 relative to leu1 demonstrates that asp1 is not significantly centromere-linked (chi-square = 0.81; p greater than 0.5). The sequence of three of the genes on Fragment 2 is therefore ade8-trp4-asp1 (44). Mortimer and Hawthorne (36) measured ascus-type ratios for the segregation of ade8 relative to trp4; the ratios were 32:0:46 (PD:NPD:T). By combining their data with those in Table 7, trp4 can be calculated to be 33 centimorgans from the ade8 gene. Assuming additivity of distances, then, ade8 and asp1 should be about 51 centimorgans apart and would be expected to segregate randomly (36).

A map of chromosomal Fragment 2 is shown in Figure 11. The relative orientations of the bracketed linkage groups has not been established (72). That the two groups are on the same chromosomal fragment was established by scoring mitotic crossing over between them (43, 76).

In accumulating the data in Table 6, asci were included in which either three or four spores produced visible colonies when germinated. Inclusion of incomplete tetrads can lead to erroneous results if the frequency of gene conversion is high. However, of 119 complete tetrads, only two exhibited segregation typical of gene conversion in asp1. Therefore, pooling data from complete tetrads with those from tetrads from which only three spores were viable is justified.

Independent segregation of the asp5 and asp1 genes

The asp1 mutations were induced in strains carrying the asp5

Figure 11. Map of chromosomal Fragment 2 of Saccharomyces cerevisiae . Modified from Mortimer and Hawthorne (74).

gene, the presence of which was necessary for selecting asparaginase mutants by their inability to grow without exogenous aspartate. A necessary, but not sufficient condition for asserting the independence of the two genes is to show that an aspl mutation is conserved when it is carried in a cell that is wild-type for asp5.

When a cell of genotype asp5 aspl is crossed to a cell that is wild-type for both genes, types of asci producing four viable spores can be distinguished by using appropriate diagnostic media. The expected segregation patterns are shown in Table 8. Two of the asparaginase mutants (LA158 and LA165) were crossed to strain S288C (wild-type) (crosses XE101 and XE102, respectively). Diploids from the crosses were sporulated, and the phenotypic segregation patterns were scored. The three patterns predicted in Table 8 were observed. The ratios of PD:NPD:T asci for the segregation of aspl and asp5 were 2:3:4 (both crosses combined). In tetratype asci from these crosses, two haploid segregants cannot be distinguished. They grow on all three media, and their genotypes cannot be deduced. To establish unequivocally that a mutant aspl gene was present in one of the two indistinguishable haploids, those two haploids in a tetratype ascus from cross XE101 (XE101-1A and XE101-1C) and from XE102 (XE102-5B and XE102-5C) were crossed to either X2902-21B or X2902-67B:

XE107: XE101-1A (a trp4 aspl-12?) x X2902-21B (α trp4 asp5)

XE109: XE101-1C (α aspl-12?) x X2902-67B (a trp4 asp5)

Table 8. Expected segregation pattern of asparaginase-deficient trait

Ascus type	Genotype		Growth response		
	asp1	asp5	C-THR +ASP	C-THR +ASN	C-THR
Parental Ditype	-	-	+	-	-
	-	-	+	-	-
	+	+	+	+	+
	+	+	+	+	+
Non-Parental Ditype	-	+	+	+	+
	-	+	+	+	+
	+	-	+	+	-
	+	-	+	+	-
Tetratype	+	+	+	+	+
	+	-	+	+	-
	-	+	+	+	+
	-	-	+	-	-

a. The cross depicted here is: asp1 asp5 x + +

XE108: XE102-5B (a trp4 asp1-15?) x X2902-21B (α trp4 asp5)

XE110: XE102-5C (α asp1-15?) x X2902-67B (a trp4 asp5)

If an indistinguishable haploid cell contains an asp1 allele, the segregation patterns given in Table 9-A would be observed. If an asp1 mutation is not present, the patterns in Table 9-B would be observed. The patterns exhibited by segregants from crosses XE107 through XE110 showed that haploid cells of strain XE101-1A and XE102-5B carried asp1 mutations, but XE101-1C and XE102-5C were wild type for the asp1 gene. Therefore, the asp1 mutations were conserved in the presence of the wild-type asp5 gene.

In crosses XE107 and XE108, the combined segregation ratios (PD:NPD:T) for asp1 relative to asp5 were 6:3:11. The combined ratios from these crosses and crosses XE101 and XE102 are 8:6:15, which are not significantly different from those expected if the two genes were segregating randomly (chi-square = 1.04; p greater than 0.5).

A final demonstration of the independence of asp1 and asp5 involved a biochemical determination of asparaginase activity in haploid strains from tetratype asci from crosses XE101 and XE102 (see below).

Fine-structure mapping of the asp1 gene

The fine-structure x-ray mapping technique developed by Manney and Mortimer (62) was used to determine (1) a minimum size of the polypeptide monomers coded for by the asp1 gene and (2) the relative positions of the asp1 mutations within the gene.

Data for the two alleles found to be at opposite ends of the group (asp1-14 and asp1-15) are summarized in Figures 12 and 13.

Table 9. Expected segregation patterns when a haploid carrying asp5 but wild-type for asp1 is crossed to a haploid that is wild-type for asp5 and either wild-type or mutant for asp1

Ascus type	+ <u>asp1</u> x <u>asp5</u> +		(A)			+ + x <u>asp5</u> +		(B)		
			Growth response					Growth response		
	Segregant <u>asp5</u>	genotype <u>asp1</u>	C-THR +ASP	C-THR +ASN	C-THR	Segregant <u>asp5</u>	genotype <u>asp1</u>	C-THR +ASP	C-THR +ASN	C-THR
PD	+	-	+	+	+	+	+	+	+	+
	+	-	+	+	+	+	+	+	+	+
	-	+	+	+	-	-	+	+	+	-
	-	+	+	+	-	-	+	+	+	-
NPD	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+
	-	-	+	-	-	-	+	+	+	-
	-	-	+	-	-	-	+	+	+	-
T	+	+	+	+	+	+	+	+	+	+
	+	-	+	+	+	+	+	+	+	+
	-	+	+	+	-	-	+	+	+	-
	-	-	+	-	-	-	+	+	+	-

DBL 6912 5230

Figure 12. Dose-frequency curves for x-ray induction of prototrophs in diploid cells heteroallelic or homoallelic for asp1-14 and asp1-15. Error bars represent one standard deviation above and below means. Where no error is shown, one standard deviation is within the point on the figure.

DBL 6912 5229

Figure 13. Dose-frequency curves for x-ray induction of prototrophs in diploid cells homoallelic for asp5, asp1-14, or asp1-15. Cells homoallelic for asp1 alleles were also homoallelic for asp5. Error bars represent one standard deviation above and below mean values.

In Figure 12 are presented dose-frequency data derived from diploid cells heteroallelic for aspl-14 and aspl-15 or homoallelic for one of the two mutations. Data derived from the homoallelic diploids are shown on an expanded scale in Figure 13. (All these diploids were also homozygous for the asp5 gene.) Included in Figure 13 are data derived from diploids homozygous for the asp5 mutation but carrying only the wild-type allele of aspl.

The rate of production of prototrophic cells from diploids heteroallelic for aspl-14 and aspl-15 was 1.65 ± 0.18 prototrophs per 10^8 survivors per roentgen. The rate of induction of prototrophs in cells homoallelic for both aspl-14 and asp5 was 0.14 ± 0.002 prototrophs per 10^8 survivors per roentgen, and the rate was 0.027 ± 0.004 prototrophs per 10^8 survivors per roentgen in cells homoallelic for both aspl-15 and asp5. The rate of induction of prototrophs in cells homozygous for asp5 and wild-type for aspl was 0.013 ± 0.001 prototrophs per 10^8 survivors per roentgen. Therefore, homoallelic reversion contributed less than 2 per cent of the rate of induction of prototrophs in diploid cells heteroallelic for aspl-14 and aspl-15. The data also indicate that homoallelic reversion rates in the aspl gene are similar to that in the asp5 gene. Better additivity of distances was achieved by assuming the distance between aspl-14 and aspl-15 to be 1.8 units. This value is within experimental error of the value determined from the data in Figure 12. Therefore, 1.8 x-ray mapping units represents the distance between the two most widely separated aspl mutations used in this study.

The relative positions of the fourteen mutations in the aspl

gene were deduced from the rates of induction of prototrophs listed in Table 10, and the fine-structure map of the aspl gene is shown in Figure 14. Because homoallelic reversion rates (Table 11) were so low, distances in the map were not corrected for homoallelic reversion. Errors associated with the determination of heteroallelic induction rates were considerably larger than errors incurred by failing to correct for homoallelic reversion.

The relative positions of alleles 3, 4, 6, 7, 11, 14, and 15 are well defined. Alleles 8, 9, and 10 could not be located unambiguously to the "right" or "left" of aspl-6. Diploid cells heteroallelic for aspl-14 and aspl-8, -9, or -10 grew slowly on the selective medium, and rates of induction of prototrophs could not be determined for these allele-pairs. Diploid cells homoallelic for aspl-9 and aspl-10 also grew slowly on the selective medium, preventing the determination of homoallelic reversion rates for these mutations.

I do not know why certain diploid cells carrying aspl-8, -9, or -10 were able to grow on the selective medium (C-THR+ASN). One possible explanation is that weak complementation occurred between aspl-14 and these three alleles. Complementation was routinely scored after only one day of growth of cells on non-permissive medium; weak complementation between alleles might not have been observable after this time. However, complementation is probably not the only reason for anomalous growth of diploid cells heteroallelic for aspl-14 and aspl-9 or aspl-10: cells homoallelic for aspl-9 and aspl-10 also grew slowly on the same medium. Complementation cannot occur between homoallelic mutations.

Table 10. X-ray map intervals for heteroallelic diploids

Diploid no.	Allele pair		Prototrophs ^a 10 ⁸ S · R	Normalized ^b
	α aspl-	a aspl-		
111	6	2	0.50 ± 0.02	0.33
112	14	2	2.01 ± 0.07	1.33
114	6	3	1.05 ± 0.26	1.00
115	14	3	0.09 ± 0.11	0.00
117	6	4	0.32 ± 0.02	0.29
118	14	4	0.80 ± 0.15	0.71
120	6	5	0.61 ± 0.02	0.91
121	14	5	0.05 ± 0.01	0.09
123	14	6	0.85 ± 0.13	1.00
124	15	6	0.73 ± 0.26	0.80
126	6	7	0.08 ± 0.01	0.10
127	15	7	0.71 ± 0.18	0.70
129	6	8	0.12 ^c ± 0.03	-
130	14	8	- -	-
132	6	9	0.13 ^c ± 0.02	-
133	14	9	- -	-
135	6	10	0.08 ^c ± 0.01	-
136	14	10	- -	-

Table 10 (continued).

Diploid no.	Allele pair		Prototrophs ^a 10^8 S . R	Normalized ^b
	α aspl-	a aspl-		
138	6	11	0.42 ± 0.03	0.39
139	14	11	1.50 ± 0.06	1.41
141	6	12	0.01 ± 0.00	0.03
142	14	12	0.46 ± 0.08	0.97
144	6	13	$0.22^c \pm 0.02$	-
145	14	13	5.04 ± 0.11	-
147	6	14	0.88 ± 0.07	1.00
148	15	14	1.65 ± 0.18	1.80
150	6	15	0.95 ± 0.18	0.80
151	14	15	3.34 ± 0.23	-

- a. Regression coefficient $\pm$ standard error of regression coefficient.
- b. Intervals normalized so that the interval between aspl-6 and aspl-14 is 0.8 units and the interval between aspl-6 and aspl-15 is 1.0 units.
- c. Not normalized. See text.

Table 11. Rates of prototroph induction in homoallelic diploids.

Diploid no.	Allele no.	Prototrophs/ 10^8 S. R ^b	
		C-THR+ASN	C-THR
113	asp1-2 ^a	0.018 $\pm$ 0.002	0.016 $\pm$ 0.002
116	-3	0.021 $\pm$ 0.003	0.020 $\pm$ 0.002
119	-4	0.030 $\pm$ 0.005	
122	-5	0.024 $\pm$ 0.002	0.022 $\pm$ 0.001
125	-6	0.010 $\pm$ 0.001	0.013 $\pm$ 0.002
128	-7	0.026 $\pm$ 0.002	0.025 $\pm$ 0.001
131	-8	0.013 $\pm$ 0.010	
134	-9	- $\pm$ -	
137	-10	- $\pm$ -	
140	-11	0.022 $\pm$ 0.004	
143	-12	0.027 $\pm$ 0.011	
146	-13	- $\pm$ -	
149	-14	0.014 $\pm$ 0.002	
152	-15	0.027 $\pm$ 0.004	
153	asp5 only		0.013 $\pm$ 0.001

a. Diploids homoallelic for asp1 alleles were also homozygous for asp5.

b. Regression coefficient $\pm$ standard error of regression coefficient.

DBL 6912 5231

Figure 14. Fine-structure map of the *aspl* gene derived from x-ray induction of mitotic recombination in diploid cells heteroallelic for *aspl* alleles. Intervals are normalized as described in the text. Distances are expressed in units of prototrophs induced per 10^8 survivors per roentgen.

Haploid cells carrying the aspl-8 and aspl-10 alleles were found to synthesize asparaginase that was slightly active when assayed in vitro (see below). Although the strains in which these mutations were originally isolated do not appear to be leaky when grown on C-THR+ASN medium, it is possible that sufficient active asparaginase for slow growth was synthesized in diploid cells carrying the mutations in a different genetic environment. Slight differences in growth between haploid strains of opposite mating type but carrying the same aspl mutation were noticed during the complementation tests. The cells were of identical (known) genotype (except for mating type) in these cases, but other (unknown) genes were probably exerting an effect on the asparaginase-deficient phenotype.

Although the positions of the mutations in aspl-8, -9, and -10 could not be unambiguously assigned, they are probably within 0.1 map unit of aspl-6, as indicated in Figure 14 (see Table 10). The mutation in aspl-2 was only shown to lie in the "left" half of the array. The distance between aspl-6 and aspl-2 was not additive with the distance between aspl-2 and aspl-14. In Figure 14, aspl-2 was placed such that the distances between it and the two reference alleles were proportional to the measured distances. The same technique was used to assign the positions of aspl-5 and aspl-12. Distances measured between these alleles and the reference alleles also were not additive.

The position of the remaining mutation was even more difficult to deduce. The deviation from additivity observed with diploid cells carrying this allele (aspl-13) prevents drawing any stronger conclusion than that aspl-13 is probably nearer to aspl-6 than to aspl-14. On the

map in Figure 14, aspl-13 was arbitrarily placed at the measured distance from aspl-6, distal to aspl-14. The rate of homoallelic reversion of aspl-13 could not be determined. As observed with two other alleles, diploid cells homoallelic for aspl-13 grew slowly on the selective medium.

Discussion and summary (genetics)

The L-asparaginase from Saccharomyces cerevisiae is a multimeric enzyme the monomers of which are coded for by a structural gene (aspl) located on chromosomal Fragment 2 of the genetic map. The gene is 18 centimorgans from the trp4 locus and was estimated to be 51 centimorgans from the ade8 gene. The order of the three genes on the fragment is ade8-trp4-aspl.

Fourteen mutants deficient in asparaginase were isolated. The expression of the aspartate-requiring phenotype of the aspl mutants depends upon the presence in the cells of a second mutant gene, asp5, but the two genes segregate independently. The 14 asparaginase mutations are allelic, which suggests that only a single structural gene is involved in asparaginase synthesis. The pattern of complementation of the 14 mutations is typical of interallelic complementation, which is generally thought to occur when a multimeric enzyme consists of only one kind of monomer. Thus, the complementation pattern of the mutations also suggests the existence of only a single structural gene for asparaginase synthesis.

In mapping the relative positions of the asparaginase mutations in the aspl gene, the most widely separated (aspl-14 and aspl-15)

were found to be about 1.8 x-ray map units apart. One x-ray map unit corresponds to 43 to 50 amino acid residues between sites of mutation in the polypeptide chains coded for by several different genes in S. cerevisiae (60, 62, 83). If this value applies in the present case, yeast asparaginase monomers would contain no fewer than 70 to 80 amino acid residues, and the minimum molecular weight of the monomers would be 8,500 to 10,000.

Estimating monomer size from x-ray mapping data is based on the assumption that most of the gene is included in the interval between the two most distant mutations on the map. To ensure the validity of this assumption, a large number of mutations should be included in the mapping. The mutations should be induced with a variety of mutagens to avoid the possibility of accumulating clusters of mutations at sites that are particularly susceptible to individual mutagens. The number of mutations used in the present study was rather small, and all of them were induced by the same mutagen. It is possible for these reasons alone that the estimate of the size of the asparaginase monomers is low. It seems likely, however, that the asparaginase gene is not as large as might be expected if Broome's (9) estimate of the molecular weight of the enzyme (800,000) were correct. Therefore, the estimate of the size of the aspl gene supports Broome's suggestion that yeast asparaginase clumps to form large aggregates during purification.

The relative positions of seven of the 14 mutations in the aspl gene were well established by the x-ray mapping study, but some rather serious deviations from additivity arose during the mapping of some of the mutations. Although such discrepancies have arisen during the fine-

structure mapping of other genes in S. cerevisiae (24, 72, 83), the causes of the variations have not been satisfactorily explained.

Parker and Sherman (83) found that variation can result from crowding of colonies on selective plates. I found that if fewer cells containing certain pairs of alleles were plated and irradiated, a greater percentage of the cells formed colonies. The increase was usually less than 50 per cent, however, and could not account for the more serious deviations from additivity.

Mortimer (76) has suggested another likely cause of non-additivity of distances derived by the x-ray fine-structure mapping technique. Higher frequencies of prototrophs are obtained if cells are able to divide a few times after they are irradiated (72). Therefore, if certain mutations are slightly leaky, or if allele-pairs are able to weakly complement, more prototrophs would be obtained than for comparable allele-pairs that were non-leaky or non-complementing. Growth on selective medium due to leakiness or weak complementation was observed with several pairs of alleles in the present study. The worst instance of non-additivity occurred with one of these allele-pairs (diploid number XE145, heteroallelic for aspl-14 and aspl-13). The diploid homoallelic for one of these alleles (aspl-13) also grew slowly on the selective medium.

RESULTS (Biochemistry)

Asparaginase activities in aspl mutants

Cells of each of the aspl mutants were grown in vigorously aerated liquid YEPD cultures. The cells were disrupted by grinding with alumina, and the specific activity of asparaginase in each strain was measured. Only the mutants carrying the aspl-8 and aspl-10 alleles exhibited significant activity. The activity in strain LA38 (α trp4 asp5 aspl-8) was $(0.54 \pm 0.05) \times 10^{-2}$ I. U. The activity in strain LA46 (α trp4 asp5 aspl-10) was $(0.45 \pm 0.05) \times 10^{-2}$ I. U. The activities in these strains are about 5 to 10 per cent of activities in wild-type strains, which average about 8×10^{-2} I. U.

Correlation of aspl mutant phenotype with biochemically determined asparaginase deficiency

As a final confirmation that the aspl mutations are responsible for lack of asparaginase activity, activities in cells derived from each of the spores in tetratype asci from crosses XE101 and XE102 (see page 76) were determined by the benzene-treated cell assay. The data in Table 12 show that the lack of asparaginase activity segregates with aspl mutant alleles. Haploids XE101-1A and XE102-5B were shown to be carrying aspl mutations by genetical techniques discussed earlier.

Lack of inhibitor in extracts from asparaginase mutants

An alternative explanation of the correlation between lack of asparaginase activity in the biochemical assay and inability of cells to use asparagine for growth is that extracts from mutant cells contain an inhibitor of asparaginase activity. This possibility was ruled out

Table 12. Segregation of asparaginase activity with wild-type gene

Haploid no.	Growth pattern			Activity ^a (O.D./10 ⁸ cells)	Genotype	
	C-THR +ASP	C-THR +ASN	C-THR		<u>asp1</u>	<u>asp5</u>
XE101-1A	+	+	+	0	-	+
-1B	+	-	-	0	-	-
-1C	+	+	+	0.095	+	+
-1D	+	+	-	0.105	+	-
XE102-5A	+	-	-	0	-	-
-5B	+	+	+	0	-	+
-5C	+	+	+	0.100	+	+
-5D	+	+	-	0.085	+	-

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.

by measuring activity in a mixture of wild-type extract and extract from an asparaginase mutant. If an inhibitor were present in the mutant extract, the activity of the wild-type extract should be reduced. The data in Table 13 show that this is not the case. Activity in wild-type extract is the same (within experimental error) whether mutant extract is present or not.

Effects of physical factors on asparaginase activity

pH: The activity of asparaginase in crude extract was measured at several pH values (Figure 15). The pH optimum is about 8.5, but the breadth of the peak and errors in measurements prevent fixing the value to within better than 0.2 to 0.4 pH unit. The peak is broad, 50 per cent of the maximum activity being obtained at about pH 6.8. The data in Figure 15 also demonstrate that activity is not greatly affected by the type of buffer in which it is measured.

Ionic strength: The concentration of ions in a reaction mixture affects the activities of some enzymes (116). Ion effects are usually presented in terms of ionic strength, $\gamma/2$, defined as follows:

$$\gamma/2 = \frac{1}{2} \sum c_i z_i^2 .$$

c_i is the concentration of the i -th ionic species, and z_i is the charge on the i -th species.

In Figure 16, the activity of asparaginase as a function of ionic strength is shown. In measurements represented by open circles, ionic strength was varied by adding KCl to TRIS-HCl buffer with initial

Table 13. Activity in mixture of wild-type and mutant extract^a

Protein in 2.0 ml reaction mixture (mg)		Net activity (I. U. / 100)
wild-type (X2902-21B)	mutant (LA38)	
0.74	0	8.15
0	0.74	0
0.74	0.74	8.03

a. Activities are means of duplicate measurements. Ranges of duplicate measurements were less than 10 per cent of the means.

DBL 6912 5219

Figure 15. Variation of yeast asparaginase activity at 30 C as a function of pH. Asparagine concentration: 10 mM; specific activity of extract at pH 8.0: 6.0×10^{-2} I. U.

DBL 6912 5222

Figure 16. Variation of yeast asparaginase activity at 30 C, pH 8.0, as a function of ionic strength of reaction mixtures. Asparagine concentration: 10 mM; specific activity of extract at ionic strength 0.2 : 7.1×10^{-2} I. U.

ionic strength 0.05. In measurements represented by closed circles, ionic strength was adjusted by using different quantities of HCl to make the buffer. The line fit to the data by least-squares linear regression analysis has a slope that is not significantly different from zero at the 95 per cent confidence level. The slope is $-3.64 \times 10^{-2} \pm 1.66 \times 10^{-2}$ O.D. units for a change in ionic strength of 1.0. The T statistic for the slope is -2.19 with seven degrees of freedom (p greater than 0.05 but less than 0.10).

Temperature: To determine the stability of yeast asparaginase at various temperatures, samples of crude extract in buffer were held at the temperatures for various lengths of time and then assayed at 30 C. When extract is held at 50 C, activity is rapidly lost, the half-time for the loss being about 24 minutes (Figure 17). The loss of activity is proportional to the activity remaining at a given time (that is, exponential with time of exposure).

At 37 C, the rate of loss of activity is greatly reduced, only 45 per cent being lost in six hours (Figure 18). At 30 C, no loss of activity is detectable after three hours, and a loss of only 10 per cent occurs during exposure to 30 C for six hours. When extract is held at 4 C, no activity is lost, and when frozen at -20 to -30 C in 0.1 M K-PO_4 buffer, pH 8, extract retains its full activity for at least four months.

The activity of yeast asparaginase was measured at several temperatures, too. The results (Figure 19) are presented as an Arrhenius plot (21), in which the logarithm of relative activity is plotted as a

DBL 6912 5215

Figure 17. Stability of asparaginase activity at 50 C. Extract was held at 50 C for the times indicated and then was assayed at 30 C, pH 8.0. Asparagine was not present during the exposure to 50 C. Asparagine concentration during assay: 10 mM; initial specific activity of extract (30 C): 8.5×10^{-2} I. U.

DBL 6912 5221

Figure 18. Stability of asparaginase activity at 37 C. After being held at 37 C for the times indicated, extract was assayed at 30 C. Asparagine was not present during the exposure to 37 C. Asparagine concentration during assay: 10 mM; initial specific activity of extract: 7.2×10^{-2} I, U. For comparison, the dashed line shows the loss of activity when extract was held at 50 C (see Figure 17).

DBL 6912 5214

Figure 19. Arrhenius plot of asparaginase activity at several assay temperatures. Each temperature (C) is shown in parentheses adjacent to the activity obtained at that temperature. Asparagine concentration: 10 mM; specific activity of extract (30 C): 7.4×10^{-2} I. U.

function of reciprocal absolute temperature. From 10 C to about 30 C, the plot is linear, and Q_{10} for the reaction is about 2. (Q_{10} is the ratio of activity at a given temperature to that at a temperature 10 degrees C higher or lower.) From the Q_{10} , the apparent energy of activation of the asparaginase reaction can be obtained (89):

$$E = (RT^2 \ln Q_{10})/10.$$

With $T = 303$ K (30 C), $Q_{10} = 2$, and $R = 1.986$ cal/mole·degree, E (apparent) is about 12,500 cal/mole. This value is not unusual: the hydrolysis of sucrose by yeast "sucrase," for example, has an apparent activation energy of about 11,000 cal/mole; other enzyme reaction apparent activation energies are in the range 5,000 to 20,000 cal/mole (21).

Asparaginase activity and stability in extracts from diploid cells heteroallelic for complementing asparaginase mutations: A goal of this research was to demonstrate that yeast asparaginase with altered physical properties can be obtained. One way in which a physically altered enzyme can be produced is by the formation of active enzyme from monomers that are mutant at different sites (25, 26, 27, 84, 107, 122, 123). Formation of active enzyme in this way is the basis of interallelic complementation (25). To demonstrate that physically altered asparaginase can be synthesized in yeast, two crosses were performed, diploid cells resulting from the crosses were grown, extracts were prepared, and the heat stability of the asparaginases in them were determined. The haploid strains used in one of the crosses were wild-type for the asparaginase gene; the strains used in the second cross carried complementing aspl mutations. The crosses were:

XE153: X2902-21B (α trp4 asp5) x X2902-67B (a trp4 asp5) and

XE154: LA6 (α asp5 trp4 aspl-2) x XE72-10C (a asp5 trp4 aspl-5).

YEPD cultures of each of the diploid strains were grown aerobically to about 5×10^7 cells/ml, and crude extracts were prepared. Aliquots of each extract (in 0.1 M K-PO_4 buffer, pH 8.0) were placed at 47 C for various lengths of time and were then assayed for asparaginase activity at 30 C. The results of the experiment (Figure 20) clearly demonstrate that the hybrid ("complementation") asparaginase is more heat-labile than the wild-type enzyme. The initial specific activity of asparaginase in the wild-type extract was about 5.4×10^{-2} I. U., but that of the hybrid asparaginase was only about 1×10^{-2} I. U., or about 18 per cent of the activity in the wild-type cells. The apparent biphasic nature of the inactivation of hybrid asparaginase is of doubtful significance: the net optical densities obtained were small when mutant enzyme had been held at 47 C for longer times (for example, the net O. D. measured in assaying enzyme held at 47 C for 30 minutes was only 0.010). Because of the small values, the activity measurements at the lower specific activities might be in error by as much as 25 to 50 per cent. If the biphasic nature of the inactivation were real, it could indicate that multimers consisting of different proportions of the mutant monomers are inactivated at different rates.

Effects of chemical factors on asparaginase activity

Substrate concentration: Initial velocities of asparagine degradation were determined at several asparagine concentrations. Results of the experiments are presented in Figure 21-A, in which the init-

DBL 6912 5216

Figure 20. Stability at 47 C of asparaginase activity in extract from diploid cells wild-type for aspl and in extract from diploid cells heteroallelic for the complementing alleles aspl-2 and aspl-5. Activity remaining after exposure to 47 C was measured at 30 C. Asparagine was not present during the exposure to 47 C. Concentration of asparagine during assay: 10 mM; initial specific activity of wild-type extract (30 C): 5.4×10^{-2} I. U. Initial specific activity of "hybrid" extract (30 C): 9.9×10^{-3} I. U. For comparison, the dashed line shows the loss of wild-type asparaginase activity (from haploid cells) at 50 C (see Figure 17).

Figure 21-A. Initial velocity of asparaginase reaction at various initial asparagine concentrations. Error bars represent the ranges of duplicate determinations. Reaction tubes contained 4.0 ml K-PO₄ buffer, pH 8.0; 5.0 ml asparagine in K-PO₄ buffer at twice the final concentration; and 1.0 ml of extract containing 5 mg protein, specific activity 9.1×10^{-2} I. U. At various times after adding extract, 1.0 ml volumes were removed and pipetted into tubes containing 0.2 ml 50 % TCA. Nesslerization tubes contained 0.5 ml TCA supernatant. Initial slopes of reactions were extrapolated to 20 minutes to provide a convenient measure of initial velocity.

Figure 21-B. Lineweaver-Burk plot of data in Figure 21-A. (Reciprocal initial velocity versus reciprocal initial substrate concentration).

DBL 6912 5220

ial reaction velocity, v_o , is plotted against initial substrate concentration, S . The data appear to fit a curve predicted by the Michaelis-Menten kinetic equation (58):

$$v_o = \frac{V_m}{1 + (K_m/S)}$$

v_o is the initial reaction velocity, V_m is the maximum velocity obtained at saturating substrate concentrations, K_m (the apparent Michaelis constant) is the initial velocity when $v_o = V_m/2$, and S is the initial substrate concentration. Because the constants are not conveniently evaluated from the plot in Figure 21-A, the data are presented in Figure 21-B according to the method of Lineweaver and Burk (58). The linear curve fit to the data can be described by the equation:

$$\frac{1}{v_o} = \frac{K_m}{V_m} \cdot \frac{1}{S} + \frac{1}{V_m}$$

The slope of the line is equal to K_m/V_m , the intercept on the abscissa ($1/v_o = 0$) is equal to $-1/K_m$, and the intercept on the ordinate ($1/S = 0$) is equal to $1/V_m$.

The slope of the line fit to the data by least-squares linear regression analysis is 0.763 ± 0.043 (S.D.), and the intercept on the ordinate is 3.20. Therefore:

$$V_m = 1/3.2 \text{ (O.D. units / 20 min)}$$

$$= 0.312 \text{ O.D. units / 20 minutes, corresponding to}$$

a specific activity of 9.4×10^{-2} I. U.

$$\frac{K_m}{V_m} = 0.763 \text{ (mM)} (20 \text{ min} / \text{O.D. unit}),$$

$$\text{and } K_m = 0.238 \sim 0.25 \text{ mM} = 2.5 \times 10^{-4} \text{ M}.$$

Errors in initial velocity measurements were rather large at low substrate concentrations (Figure 21-A); the true value of K_m is probably between 0.2 mM and 0.3 mM. K_m is used only to indicate the reaction velocity when the enzyme is half-saturated. No mechanism of action of the enzyme is implied.

Yeast asparaginase is 95 per cent saturated at an asparagine concentration of about 4 mM. Routine assays were performed with a substrate concentration of 10 mM and for time intervals such that the amount of asparagine in reaction mixtures did not fall below saturating levels during the course of the reactions. In the inhibition studies (see below), lower substrate concentrations were sometimes used, but quantities of enzyme and time intervals were chosen so that rates were constant during the course of the reactions.

The other substrate for asparaginase is water. The concentration of water was always so high that it was not of concern in the analyses.

Dialysis: In Table 14 are presented the results of three separate experiments in which the activities of asparaginase in crude extracts before and after dialysis were measured. In experiment 83, 2 ml of crude extract in buffer (0.1 M K-PO_4 , pH 8.0) was dialyzed for 23 hours at 4 C against 600 ml of the same buffer. In experiment 87, 10 ml of extract was dialyzed at 4 C against 3 liters of buffer (0.1 M

Table 14. Asparaginase activities in dialyzed and undialyzed extracts

Exp. no.	Undialyzed extract		Dialyzed extract	
	Total protein (mg/ml)	Specific activity ^a (I.U./100)	Total protein (mg/ml)	Specific activity ^a (I.U./100)
83	14.3	8.3	12.6	8.4
87	18.5	8.1	17.2	8.5
116	4.9	8.0	3.2	6.9

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.

K-PO₄, pH 8.0) for 19 hours, and in experiment 116, 8 ml of crude extract in Sorensen's buffer was dialyzed for 1.2 hours against 700 ml of distilled water at 4 C and then for 15 hours against 5 liters of distilled water at 4 C. Only in the third experiment was a change in the specific activity of asparaginase observed. The asparaginase activity in this extract declined by only 14 per cent. Total protein concentrations were reduced by dialysis in all three experiments. The reduction was about 10 per cent for the more concentrated extracts and about 35 per cent for the less concentrated one.

In nearly all cases, dialysis of extracts reduced the background ammonia levels in asparaginase activity measurements to less than 20 per cent of the value observed with undialyzed extracts. In experiment 116, however, background was reduced to only 50 per cent of its original value. It is possible that the slight reduction in specific activity observed in this experiment was due to an anomalously high background reading in the determination of asparaginase activity in the dialyzed extract.

Amino acids and other metabolites: Asparaginase activities in dialyzed crude extracts were measured in reaction mixtures containing a variety of amino acids and other metabolites (Table 15). Metabolites involved in aspartate metabolism were tested singly (except arginine, which could not be tested, because it reacts with Nessler's reagent to form a precipitate). Ten other amino acids were tested in two groups of five each. In these reaction mixtures, the asparagine concentration was 2.0 mM, and the ratio of inhibitor concentration to substrate concentration was 12.5. Even weak inhibition (25 to 30 per cent) by a

Table 15. Effect of amino acids and other metabolites on asparaginase activity in crude extracts

Metabolite	Concentration (mM)	Specific activity ^a (I.U./100)	Relative activity (per cent)
L-asn only	2	6.0	100
" + L-asp	25	5.4	90
" + L-glu	25	6.3	105
" + DL-homoser	50	5.1	85
" + L-isol	25	5.7	95
" + L-leu	25	5.5	92
" + L-met	25	5.3	89
" + L-thr	25	5.5	92
" + uracil	25	6.4	107
" + L-val	25	6.1	102
" + L-asn + L-thr 25 each		5.0	83
" + group I ^b	b	8.0	133
" + group II ^c	c	6.2	103
L-asn only ^d	10	7.5	100
" + NH ₄ ⁺	3.5	7.6	101

- a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.
- b. Group I (5 mM each): glycine, L-lys, L-pro, L-ala, and L-cys.
- c. Group II (5 mM each except L-tyr): L-ser, L-pha, L-trp, L-his, and L-tyr (saturated, ~ 0.1 mM).
- d. A different extract was used for the experiment with ammonium ion.

compound would have been detectable.

The effect of 3.5 mM ammonium ion on the asparaginase reaction (asparagine concentration 10 mM) was also tested. A high substrate concentration was necessary to permit accurate measurement of ammonia evolved in the reaction relative to the high "background" of ammonia added to the reaction mixtures.

Yeast asparaginase is not greatly affected by any of these compounds. Because the overall error in the experiments is about 10 per cent, none of the deviations from 100 per cent activity are significant, with the possible exception of the apparent activation that occurred when Group I amino acids were included in reaction mixtures. However, in a series of 13 measurements, the single occurrence of a deviation of this magnitude is not unexpected.

The effects on asparaginase activity of pyridoxal phosphate and of three intermediates in the citric acid cycle were tested (Table 16). Pyridoxal phosphate was included in the reaction mixtures containing α -ketoglutarate and oxaloacetate to determine whether transamination between asparagine and these α -keto acids could affect measurements of asparaginase activity (see Figure 1). None of these compounds significantly affected the enzyme's activity.

L-Glutamine is an analog of asparagine and could have an effect on the asparaginase reaction. When concentrations of asparagine and glutamine were equal, the total rate of evolution of ammonia was simply the sum of the rates observed when the two substrates were tested alone (Table 17). When asparaginase was not saturated (asparagine concentration less than 4 mM), the presence of a higher concentration of

Table 16. Effect of α -keto acids and pyridoxal phosphate on asparaginase activity in crude extracts

Reaction mixture containing:	Specific activity ^a (I.U./100)
asparagine (10 mM)	6.6
asparagine (10 mM) pyridoxal phosphate (10 μ gm/ml)	6.8
asparagine (10 mM) pyridoxal phosphate (10 μ gm/ml) α -ketoglutarate (10 mM)	6.8
asparagine (10 mM) pyridoxal phosphate (10 μ gm/ml) oxalacetate (10 mM)	5.6
asparagine (10 mM) ^b	6.4
asparagine (10 mM) pyruvate (10 mM)	6.1

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.

b. Data for pyruvate were obtained with extract from a different preparation.

Table 17. Asparaginase activity in the presence of L-glutamine

Asn conc. (mM)	Gln conc. (mM)	Specific activity ^a (I.U./100)
10	0	4.8
0	10	5.8
10	10	10.5
2	0	2.4
0	2	1.1
2	2	3.7
2	0	2.6
0	10	5.8
2	10	11.6

a. Means of duplicate measurements. Ranges of duplicates were less than 15 per cent of means.

glutamine (10 mM) resulted in a total rate of ammonia evolution about 37 per cent greater than the sum of the individual rates. The significance of this increase in activity is difficult to assess: reaction rates were not as reproducible at low substrate concentrations as at the higher concentrations. Even if the increased activity were significant, its magnitude is not great.

Glutaminase activity is retained in an asparaginase-deficient strain (LA158) (Table 18). Therefore, separate enzymes are probably responsible for the two activities, but no attempt to physically separate the two activities was made. Although glutamine does not appear to affect the asparaginase reaction greatly, a more detailed investigation of the interactions between asparaginase and glutaminase might be of interest. The apparent three-fold increase in glutaminase activity in the asparaginase mutant is provocative. Such a detailed investigation was beyond the scope of the present investigation.

Other potential activators and inhibitors: The effects on yeast asparaginase activity of five compounds that inhibit or activate a wide variety of enzymes were tested (Tables 19 and 20). Of these compounds, only p-chloromercuribenzoate (PCMB) inhibited asparaginase activity. The inhibition by PCMB did not occur when reduced glutathione was included in reaction mixtures. This result is often interpreted to mean that the enzyme under investigation contains disulfide linkages the integrity of which is necessary for activity. This interpretation is only weakly supported in the present case, however. Such experiments are difficult to interpret when they are performed with enzymes in crude extracts (118). Reactions between the compounds being tested and

Table 18. Asparaginase and glutaminase activities in wild-type and asparaginase-deficient cells

Strain	Genotype	Asparaginase ^a activity (I.U./100)	Glutaminase ^a activity (I.U./100)
X2902-21B	(<u>α</u> <u>trp4</u> <u>asp5</u>)	4.8	5.9
LA158	(<u>a</u> <u>trp4</u> <u>asp5</u> <u>asp1-12</u>)	0	14.7

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.

Table 19. Asparaginase activity in the presence of potential inhibitors or activators

Inhibitor or activator	Specific activity ^a (I.U./100)	Relative activity (Per cent)
None (asn = 2 mM) ^b	6.0	100
Iodoacetamide (10 mM)	5.0	84
Glutathione (10 mM)	5.2	87
Cystathionine (10 mM)	5.8	97
None (asn = 10 mM) ^c	7.9	100
Ethylenediaminetetraacetate (EDTA) (10 mM)	7.7	98

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of the means.

b. Asparagine concentration was 2 mM except for EDTA experiment. Data for iodoacetamide, glutathione, and cystathionine were obtained using dialyzed extract.

c. Data for EDTA obtained using undialyzed extract.

Table 20. Inhibition of asparaginase by p-chloromercuribenzoate^a

PCMB conc. (mM)	Without reduced glutathione		With reduced glutathione	
	Spec. Act. ^b (I.U./100)	Rel. Act. (per cent)	Spec. Act. ^b (I.U./100)	Rel. Act. (per cent)
0	4.4	100	5.4	123
10 ⁻³	4.4	100	5.5	125
5x10 ⁻³	3.8	86	5.6	127
10 ⁻²	2.4	54	5.6	127

a. Dialyzed extract. Asparagine concentration: 4 mM.

b. Means of duplicate measurements. Ranges were less than 10 per cent of means.

constituents of the crude extract other than the enzyme of interest are difficult to control or account for. The experiment does suggest that some knowledge of the structure of yeast asparaginase might be obtained by titrating, with PCMB or a similar compound (118), possible disulfide bonds in the purified enzyme.

Human blood: A possible explanation for the ineffectiveness of yeast asparaginase in tumor therapy (see INTRODUCTION) is that mammalian blood contains inhibitors of the enzyme. This possibility was tested by assaying enzyme activity in reaction mixtures containing whole, human blood. Veinous blood was drawn from the author before breakfast and was used within six to seven hours. (It was refrigerated after being drawn.) To prevent clotting, each five milliliters of blood contained 1 drop of heparin solution. Heparin solution alone had no effect on asparaginase activity. 0.5 ml of heparinized, whole blood was used in 2.0 ml (total volume) reaction mixtures. No significant differences were observed, either at 30 C or 37 C, between activities measured with and without blood in the reaction mixtures (Table 21). This experiment does not exclude the existence in circulating blood of unstable inhibitors that are inactivated when the blood is drawn and stored.

Factors that influence the synthesis of yeast asparaginase

Conditions of cell growth: Asparaginase activities were measured in cells grown both aerobically and anaerobically in liquid YEPD medium. (Cells were grown anaerobically in a sealed flask containing medium that had been purged with nitrogen.) The mean of duplicate

Table 21. Asparaginase activity in the presence of human blood

Temp.	Net activity (I.U./100) ^a	
	Without blood	With blood
30 C	5.2	5.1
37 C	6.5	6.1

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of means.

measurements of activity in anaerobically grown cells was 0.155 ± 0.005 O.D. units per 10^8 cells (benzene-treated cell assay). Means of duplicate measurements of activities in cells grown aerobically in three different cultures ranged from 0.115 O.D. units/ 10^8 cells to 0.190 O.D. units/ 10^8 cells. The mean of the three means was 0.160 O.D. units per 10^8 cells. Therefore, the values for aerobically grown cells are not significantly different from that obtained with anaerobically grown cells.

Asparaginase activities were generally higher in cultures of cells grown to stationary phase than in cultures grown to mid- or late-exponential phase. However, asparaginase activities in the two types of cultures were not different by more than a factor of 1.5 to 2 (Table 22). Specific activities of asparaginase (measured in crude extracts) in exponentially growing cells ranged from about 6.5×10^{-2} I.U. to about 9.3×10^{-2} I.U., but most of the values were between 7.3×10^{-2} I.U. and 8.8×10^{-2} I.U. The range of activities in cultures that were near stationary phase was from about 9×10^{-2} I.U. to 12×10^{-2} I.U. The highest specific activity obtained in any experiment (12.8×10^{-2} I.U.) was in extract from cells grown anaerobically to stationary phase. The lowest, 3.3×10^{-2} I.U., was measured in extract from cells grown aerobically to mid-exponential phase in medium containing glycerol as the primary carbon source (YEPGlycerol; see Table 22).

Growth medium: To test whether yeast asparaginase synthesis is repressed or induced, cells of strain S288C (wild-type) were grown in MV medium supplemented with the metabolites listed in Table 23. All cultures were originally seeded with the same number of cells from

Table 22. Asparaginase activities in crude extracts from cells grown under different conditions.

Exp. no.	Strain ^a	Growth medium	^b	
			Specific activity (I.U./100)	Culture density at end of growth (cells/ml)
24	XE47-10D	YEPD + asn (100 µg/ml)	6.5	3×10^7
38	X2902-21B	YEPD	6.4	1.5×10^8
51	"	YEPGlycerol	3.3	3.5×10^7
51	"	YEPD	8.1	3×10^7
53	"	"	8.8	8×10^7
54	"	"	6.5	2.3×10^8
60	"	"	9.3	1.3×10^8
68	"	"	10.0	3.3×10^8
70	"	"	9.2	Greater than 10^8
70	S288C	"	11.9	" " "
70	X2902-67B	"	10.3	" " "
81	X2902-21B	"	8.3	5×10^7
87	"	"	8.1	1.2×10^8
93	"	YEPD, anaerobic growth to stationary phase	12.8	Stationary phase
104	"	YEPD	7.3	1×10^8

a. S288C is the standard wild-type yeast strain used in these studies. XE47-10D (a asp5) is a segregant from cross:

XE47: X2928-4B (a trp1 ura3 asp5) x LA6 (α trp4 asp5 aspl-2)

X2902-21B (α trp4 asp5) and X2902-67B (a trp4 asp5) are the strains in which the asparaginase mutations were induced.

b. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of the means.

Table 23. Asparaginase activities in cells grown in assorted media

Growth medium	Doubling time (min)	Lag time (hrs)	Net O.D. ^a 10 ⁸ cells	Relative activity (per cent)
MV	126	4.0	0.072	100
MV + ASN ^b	122	3.5	0.116	161
MV + ASP	122	3.5	0.102	142
MV + HS	123	6.0	0.066	92
MV + THR	125	5.5	0.082	114
MV + MET	125	7.5	0.112	155
MV + ARG	120	4.5	0.104	144
MV + URA	123	4.5	0.100	139
MV + GLU	120	3.0	0.114	158
MV + GLN	110	5.0	0.132	183
MV + ISOL	120	7.0	0.138	192
MV + LEU	127	9.5	0.168	233
MV + VAL	128	2.5	0.098	136
MV + (Group I) ^c	113	5.0	0.090	125
MV + (Group II) ^d	113	6.0	0.110	153
Means	121	5.0	0.106	149
Ranges	110-128	2.5-9.5	0.066-0.168	92-233

a. Means of duplicate measurements. Ranges of duplicates were less than 10 per cent of the means.

b. Single amino acids were present at concentrations of 10 mM.

c. Group I (2 mM each): LYS, PRO, ALA, CYS, GLY; (L-amino acids).

d. Group II (2 mM each): SER, PHA, TYR, TRP, HIS; (L-amino acids).

an exponentially growing MV culture. Growth of the cultures was stopped at a density of 10^8 cells per milliliter. In Table 23 are recorded the doubling times and lag times for growth of the cultures as well as asparaginase activities measured in benzene-treated cells from the cultures.

Little difference was observed between asparaginase activities in cells grown in minimal medium and activities in cells grown in the supplemented media. The average activity in cells grown in supplemented media was a factor of about 1.5 higher than in cells grown in minimal medium, but the relative activities in cells grown in supplemented media ranged from less than that in cells grown in MV to about 2.3 times the activity in cells grown in MV. These relative activities are not correlated with either doubling time or lag time of the cultures. Two conclusions may be drawn: the synthesis of yeast asparaginase is not specifically induced or repressed by any of the metabolites tested, but activity of the enzyme may be slightly higher in cells grown in the presence of a variety of amino acids or other nitrogenous compounds.

Asparagine as a nitrogen or carbon source: The effects on the synthesis of yeast asparaginase of different nitrogen sources were tested by growing cells of strain S288C (wild-type) in minimal media containing ammonium sulfate, asparagine, or both as the nitrogen supply for cell metabolism. Asparagine can serve as the only nitrogen source for cell growth (Table 24), but even in this situation, the amount of asparaginase in cells is less than 50 per cent greater than in cells grown in medium containing ammonium sulfate as the nitrogen source. Absolute activities measured in this experiment were greater than those

Table 24. Asparagine as a nitrogen or carbon source for cell growth^a

Growth medium	Doubling time (min). (Exp. 115)	Net O.D. ^b 10 ⁸ cells (Exp. 117)	Relative activity (per cent)
MV-NH ₃	0	-	-
MV-NH ₃ +ASN ^c	122	0.186	143
MV+ASN	104	0.188	145
MV	107	0.130	100
YEPD	83	(~ 0.19; see Table 3)	

a. Strain S288C (wild-type)

b. Means of means of duplicate measurements. Ranges of duplicate measurements were less than 10 per cent of their respective means. Ranges of duplicate means were less than 20 per cent of the overall means.

in cells used in the previous experiment. However, relative activities in cells grown in the presence of asparagine, compared with that in cells grown in medium containing ammonium sulfate, were nearly identical to relative activities observed in cells grown with or without a single amino acid in the previous experiment.

Cells of strain XE101-1A (a trp4 asp1-12) were cultured in medium in which asparagine was the only nitrogen source, but they did not grow. Therefore, asparaginase activity is responsible for the ability of cells to use asparagine as a nitrogen source. Cells of strain S288C (wild-type) could not grow in medium in which asparagine was the only carbon source, demonstrating that asparagine cannot be used as a sole source of carbon in yeast cell metabolism. A similar finding has been reported for bacterial metabolism (16).

Discussion and summary (biochemistry)

Extracts of 12 of the 14 asp1 mutants do not contain detectably active asparaginase. Extracts from the other two mutants, LA35 (α trp4 asp5 asp1-8) and LA46 (α trp4 asp5 asp1-10), contain asparaginase activity equivalent to five to ten per cent of that found in wild-type cells (about 8×10^{-2} I.U.). Activity of wild-type asparaginase is not affected by the presence in reaction mixtures of extract from cells carrying an asp1 mutation; therefore, the lack of asparaginase activity in extracts of cells carrying asp1 mutations is not caused by an inhibitor of the enzyme in those extracts. The lack of activity in crude extracts is correlated with the presence in cells from which the extracts were prepared of asparaginase mutations and is independent of the presence of the asp5 gene.

In the present investigation, the pH optimum for yeast asparaginase activity was found to be 8.5. Geddes and Hunter (30) reported the pH for maximum activity of brewer's yeast to be about 8.0. The difference between my results and theirs can be explained in at least two ways. In my experiments, reactions were allowed to run for only 30 minutes, during which time little loss of activity occurs. Reactions performed by Geddes and Hunter were allowed to proceed for 20 hours (30). Perhaps less loss of activity occurred at the higher pH values in my experiments. It is also possible that asparaginases in the species investigated actually exhibit different pH optima. In my experiments, assays were routinely performed at $\text{pH } 8.0 \pm 0.1$. Because of the breadth of the pH response, errors in activity measurements due to variations in pH of reaction mixtures were small.

Asparaginase activity does not change appreciably over a wide range of ionic strengths of reaction mixtures ($\gamma/2 = 0.05$ to $\gamma/2 = 0.48$). Activity measurements were routinely made in TRIS-HCl buffers of ionic strength 0.05 or 0.054, or in 0.1 M K-PO_4 buffer of ionic strength approximately 0.3. Thus, changes in ionic strength did not affect my experiments significantly. Data obtained during the ionic strength and pH studies also show that the nature of the buffer used did not cause variation in asparaginase activity.

Asparaginase in crude extract is stable at physiological temperatures for hours and is stable for months when frozen. The loss of activity at physiological temperatures could be due either to denaturation of the enzyme or to degradation of the enzyme by proteases in the crude extracts. Broome (9) reported that purified yeast asparag-

inase is stable for five hours when incubated in mouse blood at 37 C. Therefore, the loss of activity observed in the present experiments probably was due to protease activity in the extracts. The exponential loss of activity occurring when crude extracts are held at higher temperatures (47-50 C) suggests that the activity of only a single species of enzyme is measured in the assays. This suggestion is confirmed by the fact that all activity in an extract can be lost as a result of a mutational event in a single gene.

Q_{10} for the asparaginase-catalyzed reaction is about 2, a value that applies to many other enzyme-catalyzed reactions (21). Above a temperature of about 30 C, Q_{10} for the reaction may decrease, possibly because conformational changes begin to occur in the enzyme at higher temperatures.

The affinity of yeast asparaginase for its substrate is rather high compared, for example, with that of guinea pig serum asparaginase. The apparent K_m for the yeast asparaginase reaction is about $2.5 \times 10^{-4} M$ (at pH 8 in 0.1 M K- PO_4 buffer). That of guinea pig serum asparaginase is only about $2.2 \times 10^{-3} M$ (116). Of all the asparaginases discussed in the INTRODUCTION, only the apparent K_m of E. coli asparaginase II is lower than that of S. cerevisiae asparaginase.

Of the several potential inhibitors and activators tested, only p-chloromercuribenzoate inhibits yeast asparaginase activity. None of the amino acids or α -keto acids exert any notable effect on asparaginase activity under the conditions of the assay used in these experiments. Also, activity is not affected when whole, human blood is included in reaction mixtures. Free ammonium ion in the mixtures does not affect

activity. Therefore, yeast asparaginase activity, as determined in the present investigation, is apparently not controlled by the immediate products of its activity or by the major end-products of aspartate metabolism. (The effects of arginine on the reaction could not be tested, however.)

Yeast asparaginase does not require co-factors for its activity. Prolonged dialysis against buffer or distilled water does not appreciably affect specific activities in crude extracts, nor does the presence in reaction mixtures of a chelating agent, ethylenediamine-tetraacetate (EDTA). The addition of phosphate or pyridoxal phosphate to reaction mixtures also does not affect the enzyme's activity.

The specific activity of asparaginase in yeast cells does not vary by more than a factor of about two regardless of culture conditions or medium in which cells are grown. Cells grown in the presence of a variety of amino acids or a pyrimidine possibly contain 1.5 times as much asparaginase as cells grown in an unsupplemented minimal medium. Even when asparaginase activity provides the sole supply of nitrogen for cell growth, the activity of the enzyme in cells is not increased by more than a factor of 1.5. Asparaginase activity may be slightly higher in cells grown to stationary phase than in cells in exponentially growing cultures, but the increase is again less than a factor of two. Increases due to these changes in culture conditions are not additive. These results suggest that yeast asparaginase is a constitutive enzyme the synthesis of which is not greatly affected by culture conditions during cell growth. In these respects, it can best be compared with E. coli asparaginase I, although the two enzymes differ in other characteristics, especially affinity for asparagine.

Asparagine can be used as a sole source of nitrogen for cell metabolism. Cells must be able to synthesize active asparaginase in order to use asparagine in this way. Asparagine cannot be used as the sole carbon source for cell growth. The requirement for active asparaginase for the use of asparagine as a nitrogen source provides another means of selecting asparaginase mutations. Cells carrying asparaginase mutations do not grow on $MV-NH_3+ASN$ medium but grow well on $MV+NH_3+ASN$ medium. Differential growth of cells on these media would be a criterion for selecting asparaginase mutants. This selection method would not require the presence of the asp5 mutation. $MV-NH_3+ASN$ medium could also be used to score the segregation of asparaginase mutations independently of the asp5 gene. This discovery was made too late to be applied in the present investigation.

Asparaginase mutations have not been isolated in bacteria because of the lack of mutants equivalent to the strains of yeast carrying asp5. If bacterial asparaginase functions similarly to yeast asparaginase, bacterial mutants deficient in asparaginase might be able to be selected on the basis of inability to use asparagine for a nitrogen source. Genetic experiments with these bacterial mutants could help clarify the role of the enzyme in bacterial metabolism and in the treatment of tumorous mammalian cells.

Asparaginase and glutaminase activities in yeast appear to be independent. Glutaminase activity is retained in asparaginase-deficient mutants, and glutamine does not appreciably affect asparaginase activity.

Data accumulated during the biochemical studies permit a

comparison of the two assay methods used in the investigation. The average O.D. per 10^8 cells in reaction suspensions containing benzene-treated cells was about 0.19 for cells grown in liquid YEPD medium. Under the conditions of these assays, an O.D. of 0.19 corresponds to the evolution of about 4.8×10^{-10} micromoles of ammonia per cell per minute. When crude extracts were prepared from cells grown under the same conditions, approximately 5×10^{-9} mg of soluble protein was obtained from each cell in the suspension from which the extract was prepared. If one assumes that this value approximately represents the total amount of "soluble" protein in benzene-treated cells, the "specific activity" of asparaginase in benzene-treated cells would be about 9.5×10^{-2} "I.U." This calculated specific activity is well within specific activities measured in crude extracts. The calculation supports the validity of comparing experiments in which the benzene-treated cell assay was used with experiments in which specific activities of crude extracts were determined.

Physiological significance of yeast asparaginase: The significance of asparaginase to yeast mutants that cannot synthesize aspartate by the normal route but that are challenged to grow on medium containing asparagine is obvious: asparaginase is required for cells to use asparagine as a source of aspartate. The same statement applies to cells growing on media containing asparagine as the only nitrogen source. These are rather unusual circumstances, however. The constitutivity of yeast asparaginase and its lack of response to growth conditions suggest that widely varying demands are not usually placed upon the

enzyme in wild-type cells growing under more normal conditions.

At least one possible function of asparaginase in yeast can be considered; it might control the size of the intracellular asparagine pool. If the characteristics of the enzyme in vivo are similar to those determined in vitro, a likely mechanism of this control would be the response of the enzyme to asparagine concentration. As the concentration in the cell approached the range of the apparent Michaelis constant (2.5×10^{-4} M), a greater per centage of the amino acid would be hydrolyzed per unit time, and aspartate and nitrogen would not long be held as the less available amide. An investigation of this possibility would involve studying the fluctuations of the asparagine pool as well as a detailed study of asparagine synthesis in yeast.

Use of yeast asparaginase in cancer therapy: I have examined several characteristics of yeast asparaginase in crude extracts and can only conclude that none of them would prohibit its use in tumor therapy. The enzyme (in crude extracts) is stable and active at the pH (7.4), ionic strength (0.15), and temperature (37 C) of mammalian blood. It is active in the presence of whole, human blood. The affinity of the enzyme for asparagine is greater than those of other asparaginases that are effective in tumor treatment. Therefore, my results support Broome's conclusion (9) that characteristics of the purified enzyme and of circulating blood prevent the use of the enzyme against tumor cells.

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Appendix I. Systematic names^a of enzymes listed in Table 1

Reaction number	E.C. ^b no.	Enzyme ^a
(1)	2.6.1.1	L-Aspartate:2-oxoglutarate aminotransferase
(2)	2.7.2.4	ATP:sulfate adenylyltransferase
(3)	1.2.1.11	L-Aspartate- β -semialdehyde:NADP oxidoreductase (phosphorylating)
(4)	1.1.1.3	L-Homoserine:NAD oxidoreductase
(5)	not listed	
(6)	not listed	
(7)	2.7.1.39	ATP:L-Homoserine <u>O</u> -phosphotransferase
(8)	4.2.99.2	<u>O</u> -Phosphohomoserine phospho-lyase (adding water)
(9)	4.2.1.16	L-Threonine hydro-lyase (deaminating)
(10)	not listed	
(11)	not listed	
(12)	3.5.1.2	L-Glutamine amidohydrolase
(13)	3.5.1.1	L-Asparagine amidohydrolase
(14)	6.3.1.1	L-Aspartate:ammonia ligase (ADP)
(15)	2.6.1.15	L-Glutamine:2-oxoacid aminotransferase
(16)	2.6.1.14	L-Asparagine:2-oxoacid aminotransferase
(17)	3.5.1.3	ω -Amidodicarboxylate amidohydrolase
(18)	4.3.1.1	L-Aspartate ammonia-lyase
(19)	2.1.3.2	Carbomylphosphate:L-Aspartate carbamoyltransferase
(20)	2.7.2.2	ATP:carbamate phosphotransferase

Appendix I (continued)

Reaction number	E. C. no. ^b	Enzyme ^a
(21)	2.1.3.3	Carbamoylphosphate:L-ornithine carbamoyltransferase
(22)	6.3.4.5	L-Citrulline:L-Aspartate ligase (AMP)
(23)	2.7.7.4	ATP:sulfate adenylyltransferase
(24)	4.3.2.1	L-Argininosuccinate arginine-lyase
(25)	3.5.2.3	4,5-L-Dihydro-orotate amidohydrolase
(26)	1.3.3.1	4,5-L-Dihydro-orotate:oxygen oxidoreductase
(27)	2.4.2.10	Orotidine-5'-phosphate:pyrophosphate phosphoribosyltransferase
(28)	4.1.1.23	Orotidine-5'-phosphate carboxy-lyase

a. From reference (125).

b. Enzyme catalog number.

Appendix II. Genes and their associated proteins and phenotypes^a

Gene	Phenotype	Protein
<u>ade8</u>	requires adenine	unpublished
<u>arom1</u>	requires aromatic amino acids	four enzymes involved in the synthesis of aromatic amino acids
<u>asp1</u>	inability to utilize asparagine as source of aspartate or nitrogen	asparaginase
<u>asp5</u>	requires aspartate or threonine and methionine	aspartate aminotransferase
<u>cyt1</u>	cytochrome deficiency	cytochrome-c
<u>eth2</u>	resistance to ethionine	
<u>leu1</u>	requires leucine	unpublished
<u>p</u>	petite (inability to utilize non-fermentable carbon sources)	respiratory enzymes
<u>S</u>	super-suppressor: suppresses nonsense codons	
<u>thr2</u>	requires threonine	aspartate semialdehyde dehydrogenase
<u>trp1</u>	requires tryptophan	N-(5'-phosphoribosyl) anthranilic acid isomerase
<u>trp4</u>	requires tryptophan	phosphoribosyl transferase
<u>ura3</u>	requires uracil	orotidine-5'-phosphate decarboxylase

a. Adapted in part from (74).

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