AN ABSTRACT OF THE THESIS OF

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Title: A Study of Sulfite Mutants of *Saccharomyces cerevisiae*

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Alan T. Bakalinsky

Sulfite mutants representing five complementation groups, previously derived from an ethyl methanesulfonate-treated haploid strain of *Saccharomyces cerevisiae* were studied. Although the wildtype *S. cerevisiae* strain used (isogenic to X2180-1A) had a basal tolerance for sulfite (7 μM free H_2SO_3), the sensitive and resistant mutants were found to tolerate less than 3 to 5.5, or greater than 19 μM free H_2SO_3, respectively. No apparent correlation was found between the response to sulfite and generation time in rich (YEPD) or minimal media. Resistant mutant 11-1 had an extended lag phase relative to wildtype. Mutant and wildtype proteins were labeled with ^{35}S-methionine to determine differences in banding patterns due to sulfite-specific induction or disappearance of polypeptides. No obvious differences following SDS-PAGE and autoradiography were observed upon induction with 0.213 μM free H_2SO_3. No consistent correlations were found between the sulfite phenotypes and responses to other reducing agents. Sensitive mutant 35-2 appeared to be three to ten times more sensitive to dithiothreitol than wildtype and sensitive mutant 47-9 was three to four times more sensitive to sodium nitrite and three to seven times more sensitive to sodium thiosulfate than wildtype. Log phase cells of sensitive mutant 33-2 were found to have significantly less glutathione than wildtype. Wildtype contained 62.6

nmol min^{-1} mg $protein^{-1}$ (62.6 mU mg $protein^{-1}$) glutathione reductase (GR) and 2.78 nmol min^{-1} mg $protein^{-1}$ (2.78 mU mg $protein^{-1}$) glutathione S-transferase (GST). Log phase cells of one resistant mutant showed a significantly higher level of GR than wildtype, 135%. The resistant mutants as well as some of the sensitive mutants had reduced GST levels. Survival rates of the mutants in buffer in the presence of sulfite did not correlate with their sensitive or resistant phenotypes, suggesting that survival and growth in the presence of sulfite are not necessarily related functions. Relative to wildtype, survival upon prolonged storage at 4° C was markedly reduced for two of the four sensitive mutants, one of which was 33-2, and was enhanced for one resistant and another sensitive mutant.

A Study of Sulfite Mutants of *Saccharomyces cerevisiae*

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LITERATURE REVIEW

IMPORTANCE OF SULFUR DIOXIDE

Humans are exposed to sulfur dioxide (sulfite) through three main sources: combustion of sulfur-containing fossil fuels, as an additive in foods, beverages and pharmaceuticals and endogenous production from catabolism of sulfur-containing amino acids. In the U.S., sulfite is regulated by the EPA (environmental sources- emissions to the atmosphere), FDA (foods and pharmaceuticals) and BATF (wine and beer). During the latter 1970's and early 1980's, SO_2 and sulfites emerged as a major issue due to its threat to human health.

Long-term epidemiological studies have shown a consistent association of human illness and death with exposure to enhanced levels of inhaled SO_2 (Shapiro, 1977; 1983). From 1976 to 1985, 13 deaths occurred and 500 known cases of adverse reactions, allegedly to sulfites, were reported (Walker, 1988). When used as a preservative in foods and pharmaceuticals sulfite is capable of causing flushing, throat swelling, itching of the mouth and skin, and asthma (Bush *et al.*, 1986b; Stevenson and Simon, 1981a). Other symptoms include: urticaria, pruritus, swelling of the tongue, difficulty in swallowing, tightness in the chest, eczema and hypotension (Stevenson and Simon, 1981b; Taylor *et al.*, 1986).

The first reported cases of SO_2-induced asthma (see Kochen, 1976; Prenner and Stevens, 1976; Freedman, 1977; cited in Taylor *et al.*, 1986) generated very little attention. It wasn't until later reports by Allen and Collett in 1981 (cited in Taylor *et al.*, 1986) and

Stevenson and Simon (1981a), linking SO_2 ingestion in food and drugs with asthmatic episodes in several patients, that this issue generated more interest. Sulfite sensitivity is generally restricted to asthmatics. In 1985 the Federation of American Societies for Experimental Biology reported that there was no evidence that sulfiting agents represent any hazard at current levels of use for the majority of the population (Bush *et al.*, 1986a). Reports vary as to the percentage of sulfite-sensitive individuals though; from 5-10 % of all asthmatics - about 450,000 in the U.S. (Simon *et al.*, 1982; Stevenson and Simon, 1981a), to 5-10% of severe or steroid-dependent asthmatics - about 90,000 in the US (Bush *et al.*, 1986a), to less than 3.9% of all asthmatics (Bush *et al.*, 1986b). The 3.9% was subdivided into non-steroid-dependent asthmatics (0.8%) and steroid-dependent asthmatics (8.4%).

Taylor and Cumming (1985) described sulfite-induced asthma as a food idiosyncrasy: "an adverse reaction to a food or food component that occurs through unknown mechanisms which even include psychosomatic illnesses". The most common circumstances in which sulfites produce adverse reactions are from ingestion of restaurant-prepared foods to which a sulfiting agent has been added. In 1986, the FDA declared that sulfites can no longer be added to raw or fresh fruits and vegetables served to the public, in salad bars for example.

Studies have been performed to determine sulfite-sensitivity with two main vehicles of ingestion: by acidic solutions (see Freedman, 1977; cited in Taylor *et al.*, 1986) and Sprenger *et al.* (1989); by encapsulation (Stevenson and Simon, 1981a,b; see Allen and Collett, 1981; cited in Taylor *et al.*, 1986) ; or by both (Delohery *et al.*, 1984; Towns and Mellis, 1984; Bush *et al.* 1986). Inorganic sulfites, such as potassium metabisulfite, are usually used in the challenges. In 1982, Werth reported on the existence of two groups of sulfite-sensitive asthmatics, those who respond to inhaled but not ingested SO_2 and those who respond to both. Delohery *et al.* (1984) determined that more asthmatics are sensitive

to acidic solutions of sulfite than to encapsulated sulfites. This correlates with Goldfarb and Simon's 1984 report (cited in Taylor *et al.* 1986) that the minimal provoking dose for a beverage challenge was approximately one half that of the capsule challenge. Town and Mellis (1984) reported that sulfites appear to provoke symptoms only when given in solution or swilled around the mouth. Allen and Delohery (1985) reported that the reaction appeared to be prevented if the test subject held his breath until the solution was swallowed. This correlates with reports by Nadel *et al.* in 1965 and Sheppard *et al.* in 1980 (cited in Taylor *et al.*, 1986) that sulfites in solution can release sulfur dioxide. By implication, it is the inhaling of SO_2 fumes that triggers asthma in such individuals, as it does in asthmatics who are exposed to ordinary fog. Objective studies need to be done that require more complex assessment methods such as the measurement of inflammatory mediators in blood or urine after cumulative challenge doses (Lessof *et al.*, 1988). These methods can be used both for studying the effects in healthy individuals and to provide objective confirmation of the diagnosis in individuals with supposed food additive-induced reactions. However, information gained by this means cannot be assumed to be relevant to the levels of food additives that can be taken safely by the population at large.

Studies to understand the mechanisms for adverse sulfite reactions lead to three theories. First, some asthmatic patients may not be able to metabolize sulfiting agents properly, due to a deficiency of sulfite oxidase. Jacobsen *et al.* (1984) assayed the sulfite oxidase levels of sulfite-sensitive asthmatics in skin fibroblasts, and in many of the subjects low levels were found relative to normal or non-sulfite-sensitive asthmatics. Secondly, Delohery *et al.* (1984) speculated that bronchospastic response may be mediated through the stimulation of airway irritant receptors by inhaled SO_2, since asthmatic symptoms were provoked in five asthmatics challenged by sulfite mouthwash but not by gastric challenge. Lastly, the adverse reaction may be immunologically based; ie. specific IgE antibodies may be involved (Simon, 1986). There have been three separate case reports and one series of

five patients in which IgE sensitivity to sulfites was documented by positive immediate skin tests, Prausnitz-Kustner (PK) transfer and/or *in vitro* basophile histamine release (see Prenner and Stevens, 1986; Twarog and Leung, 1982; Simon and Wasserman, 1986; cited in Simon, 1986). Data by Bush *et al.* (1986) suggested the existence of an IgE - mediated reaction, but a specific antibody was not identified. In opposition to this theory, Sprenger *et al.* (1989) found that sulfites do not stimulate *in vitro* release of histamine from basophiles suggesting that sulfite reactions are not mediated by an immune mechanism.

At present, there are no animal models for studying mechanisms of sulfite sensitivity (Jacobsen, 1991). Extrapolation of toxicological results from rats to humans is difficult because the normal pathway of sulfite metabolism is by sulfite oxidase and rat liver has about a 20-fold greater activity than human liver (Johnson and Rajagopalan, 1976a,b). Gunnison (1981) suggested use of sulfite oxidase-deficient rats in sulfite toxicity studies since sulfite oxidase contains molybdenum in its active site and rat tissues can be depleted of sulfite oxidase activity by maintaining animals on a diet high in tungsten and low in molybdenum (Gunnison *et al.*, 1981). Hui *et al.* (1989) found that sulfite oxidase-deficient rats were more susceptible than normal rats to the toxic effects of free sulfite and acetaldehyde hydroxysulphonate. Mammalian tissues and cells with very low sulfite oxidase activity, such as lung and macrophages, were also found to demonstrate a pH-dependent sulfite sensitivity, indicated by a significant decrease in ATP at pH 6.0 (Beck-Speier *et al.*, 1985).

Food is not the only source of exogenous sulfites as coal burning in industrial areas can also be a source of SO_2. Urban SO_2 exposure of humans has been correlated with increased mortality (Shapiro, 1977), bronchitis, asthma and other respiratory diseases. The Federal - Provincial Advisory Committee on Air Quality (FPACAQ) in Canada (1987), Lippmann (1980) and the U.S. Environmental Protection Agency (EPA) (1982), have done extensive reviews of epidemiological studies on the effects of particulate matter and sulfur

oxides on human health. The results of these studies must be interpretated cautiously though, since SO_2 from air pollution is usually accompanied by increased quantities of particulate matter and other common pollutants such as nitrogen dioxide, nitric acid and ozone.

The FPACAQ (1987) reported that no consistent effects have been observed in healthy subjects exposed for brief periods to concentrations of SO_2 less than 2600 $\mu g/m^3$ (0.4 ppm). At levels greater than this, increased respiratory rate and airway and nasal airflow has been observed along with decreases in air intake volume, forced expiratory flow and nasal mucociliary flow rate. Increased tracheobronchial clearance was noted when SO_2 levels exceeded 14,000 $\mu g/m^3$ (5.38 ppm). Variance in results on exact levels causing adverse effects are due to differences in the sensitivity of the study population, experimental conditions, patterns of exposure and exercise, temperature and relative humidity, routes of exposure (mouthpiece versus natural breathing) and time course of manifestation of the effect. The FPACAQ also reported that natural breathing of SO_2 can cause some asthmatics to experience symptoms and increase airway resistance at concentrations of 1000 $\mu g/m^3$ (0.4 ppm) with sufficiently heavy exercise. Increases in airway resistance have also been observed in exercising asthmatics exposed to low levels administered through a mouthpiece; but, these results were not considered relevant since they were obtained by unnatural means of exposure. In most subjects, the adverse effects disappear in less than one hour with rest, even if SO_2 exposure continues. There is also evidence that tolerance develops following repeated exposure of asthmatics to SO_2 (FPACAQ, 1987).

Lippmann (1980) has found that 1) high concentrations of SO_2 can slow bronchial clearance and 2) sulfur oxide pollutants may be a factor in benzo(a)pyrene (BaP) carcinogenesis. Leung *et al.* (1989) found that sulfite reacted with glutathione to form

glutathione S-sulfonate, a known inhibitor of glutathione S-transferases, which mediates the conjugation of glutathione and BaP epoxides. GSH conjugation represents the major pathway of elimination of BaP epoxides. Of less immediate concern are the effects of sulfur oxides on other lung diseases: eg. SO_2 affects the clearance of insoluble particles from the alveolar region; SO_2 acts as a cofactor in the development of bronchial carcinoma and combined exposures to H_2SO_4 and O_3 can increase mortality in mice infected with streptococci (Lippmann, 1980). He also stated that SO_2 exposure required to produce the afore mentioned effects were many orders of magnitude higher than ambient levels.

The EPA (1982) reviewed many epidemiological studies and reported that some of the studies provided meaningful quantitative information on health effects associated with ambient air exposures to particulate matter and SO_2, while many others contained ambiguous interpretations of the investigators' findings. The EPA reported strong evidence for induction of severe health effects, such as mortality and respiratory disease, by marked elevations of atmospheric levels of particulate matter and SO_2 in certain populations at special risk (ie. elderly and adults with chronic pre-existing cardiac or respiratory disease such as bronchitis).

USES OF SULFITES IN FOODS AND BEVERAGES

Many forms of sulfiting agents are used in the food industry: sulfur dioxide (SO_2), potassium bisulfite ($KHSO_3$), potassium metabisulfite ($K_2S_2O_5$), sodium bisulfite ($NaHSO_3$), sodium metabisulfite ($Na_2S_2O_5$) and sodium sulfite (Na_2SO_3). All are considered as GRAS (Generally Recognized as Safe) in the United States Code of Federal Regulations. The most frequently used agents are sodium bisulfite and potassium bisulfite,

due to their stability toward autoxidation in the solid phase (Wedzicha, 1984). Alternatives to sulfiting agents in foods generally provide a narrower range of benefits, are less effective and usually more expensive. Sulfiting agents perform several functions in food processing: they act as inhibitors of non-enzymatic browning and enzyme-catalyzed reactions, as inhibitors and/or controllers of microorganisms, as antioxidants, as reducing agents and as bleaching agents. Sulfites may also have antinutritional effects.

Non-enzymatic or Maillard browning occurs when certain foods are heated due to the formation of carbonyl intermediates and brown polymeric pigments from the reaction of amino acids and reducing sugars without involving oxidizing enzymes. Sulfur dioxide inhibits this formation by reacting with the carbonyl intermediates (Joslyn and Braverman, 1954; Taylor *et al.*, 1986).

Sulfite also helps prevent undesirable enzymatic reactions catalyzed by polyphenol oxidase, ascorbate oxidase, lipoxygenase, peroxidase and thiamine-dependent enzymes (Haisman, 1974; Taylor *et al.*, 1986). These enzymes are involved in enzymatic browning, degradation of ascorbic acid in plant tissues, formation of off-flavors during storage of vegetables. Under anaerobic conditions, the terminal respiratory pathway of vegetable tissues is blocked causing production of alcohol; addition of sulfite prevents this occurrence

Sulfur dioxide has been shown to be an antimicrobial agent and its effect is dependent on pH, concentration, species, cell number and SO_2 binding capacity (King *et al.*, 1981). Undissociated sulfurous acid (H_2SO_3) is the only effective molecular species, since it is the only form that passes into cells. In winemaking, sulfur dioxide is used to control undesirable organisms such as acetic acid and lactic acid bacteria. Strains of *Saccharomyces cerevisiae* that are used as starter cultures in winemaking, have been selected over time for their high tolerance for sulfur dioxide. Sulfite has also been used to prevent mold damage in fruits prior to jam production and in the prevention of postharvest

deterioration of fruits used in juice production, although the former is not common in the U.S. (Taylor *et al.*, 1986).

As an antioxidant, sulfur dioxide prevents oxidation of essential oils and carotenoids which otherwise can generate off-flavors (Baloch *et al.*, 1977; Roberts and McWeeny, 1972). Sulfites are also used as dough conditioners in the baking industry since they break the disulfide bonds in the gluten fraction of the dough (Wedzicha, 1984).

Sulfur dioxide is often used as a bleaching agent. It can reduce many colored compounds to colorless species, especially anthocyanins, as well as prevent discoloration or color formation in some food and beverages. When cherries are bleached during maraschino cherry and glacé fruit production, sulfites and anthocyanins react to form colorless sulfonates (Joslyn and Braverman, 1954; Taylor *et al.*, 1986). Sulfur dioxide is also used in pectin production for its bleaching ability (Roberts and McWeeny, 1972) and as an acidulant (Wedzicha, 1984). Sulfites also prevent color formation during sugar manufacture from beets and cane and discoloration of shrimp during iced storage (Wedzicha, 1984).

Sulfite destroys thiamine and folic acid (Pizzoferrato *et al.*, 1988). Therefore, in the United States sulfites are not permitted as an additive in foods considered prime thiamine sources, such as meats (Taylor *et al.*, 1986). Other water soluble vitamins react negatively with sulfites such as pyridoxal, nicotinamide and riboflavin (Wedzicha, 1984). The reaction of pyridoxal and sulfite results in the reduction of its carbonyl group reactivity. NAD^+ reacts with sulfites to form $NADSO_3^-$ which competes with NAD^+ for the coenzyme binding site on NAD-dependent enzymes. ß-carotene contained in plants can also be destroyed by sulfites and *in vivo* the hepatic vitamin A reserve decreases after prolonged exposure (Pizzoterrato *et al.*, 1988).

Although sulfite is usually added during food processing, it can also occur naturally in some fermented foods and beverages. This occurrence has been most thoroughly

studied in alcoholic beverages such as beer and wine. The ability of yeast to produce sulfite has been known since the nineteenth century and arises from the reductive assimilation of sulfate. During winemaking, certain strains of *Saccharomyces cerevisiae* can generate 10 - 30 ppm sulfur dioxide with some strains producing greater than 100 ppm (Eschenbruch, 1974; Taylor *et al.*, 1986).

METABOLISM OF SULFUR DIOXIDE

In Humans

The major pathway for endogenous and exogenous sulfite metabolism in humans is by sulfite oxidase (Gunnison, 1981), more precisely known as sulfite:cytochrome-c oxidoreductase or sulfite:O_2 oxidoreductase. It is a molybdohemoprotein found in the intermembrane space of mitochondria in most mammalian tissues, with highest activity found in liver (MacLeod *et al.*, 1961; Johnson *et al.*, 1977). The enzyme catalyzes electron transfer from sulfite to the molybdenum (Mo 6+) site in the enzyme, to the heme moiety to cytochrome c, which is a constituent of the mitochondrial respiratory chain, in the final step in the catabolism of sulfur containing amino acids (cysteine and methionine). The ultimate products are sulfate (which can be rapidly excreted in the urine) and H_2O - from the reduction of 1/2 O_2, (Taylor *et al.*, 1986). See Figure 1. Endogenous production of SO_2 via catabolism of cysteine and methionine is the greatest source of human exposure. It is estimated that humans excrete approximately 25 mmol (2400 mg) sulfate in their urine daily and up to 24 mmol is generated from endogenous sulfite (IFT Expert Panel on Food Safety and Nutrition, 1975).

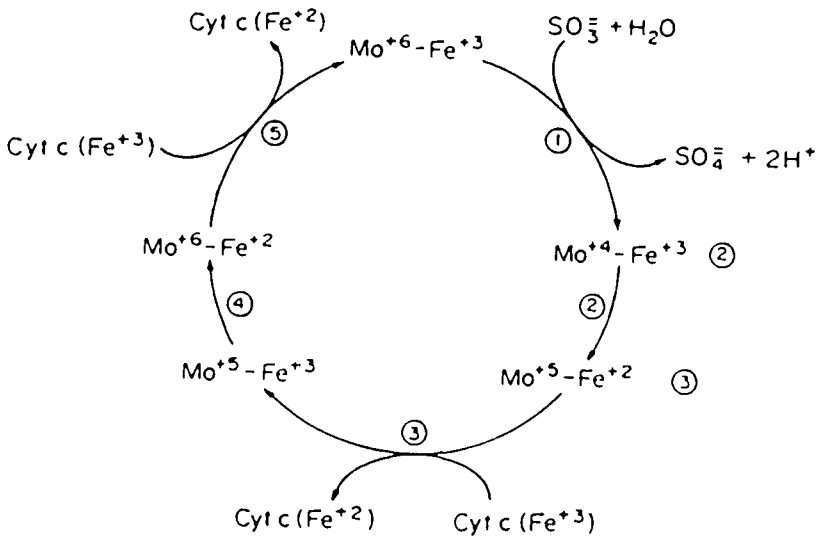

Figure 1. Suggested reaction mechanism of sulfite oxidase. The steps are (1) oxidation of sulfite by Mo^{6+} and formation of Mo^{4+}; (2) 1 e transfer from Mo^{4+} to the heme; (3) reoxidation of reduced heme by cytochrome c; (4) electron transfer from Mo^{5+} to the heme; (5) formation of oxidized enzyme by 1 e transfer to cytochrome c. (Rajagopalan, 1984).

Several researchers have proposed that defects in sulfite metabolism among certain segments of the human population may put them at greater risk to the possible toxic effects of sulfur ingestion (Jacobsen *et al.*, 1984; Taylor *et al.*, 1986). Although sulfite oxidase is the most common pathway for sulfite metabolism, profound sulfite oxidase deficiency has been reported on several occasions in humans (Taylor *et al.*, 1986). Deficiency symptoms have included dislocated ocular lenses, severe neurological abnormalities resulting in mental and physical retardation (Irreverre *et al.*, 1967) and at least one case of infantile death has been reported (IFT Expert Panel of Food Safety and Nutrition, 1975). There have been no reported cases of sulfite deficiency in adults. Sulfite oxidase deficiency may also be characterized by increased urinary excretion of sulfite, thiosulfate and cysteine S-sulfonate and decreased excretion of sulfate. The molecular basis for the pathology of the disease is not known but two possibilities include toxicity from the higher levels of sulfite in some critical organs and the absence of sulfate required for the formation of sulfated lipids, proteins and small molecules (Rajagopalan, 1984).

Minor pathways for sulfite metabolism exist in humans also. One involves the reaction of sulfite with 3-mercaptopyruvate (3-MP) catalyzed by 3-mercaptopyruvate sulfurtransferase to form S-sulfonate compounds as by-products (Westley, 1980), Figure 2. Another involves the non-enzymatic formation of S-sulfonate compounds by the reaction of sulfite with the disulfide bonds of proteins, cysteine and glutathione, (sulfitolysis) $RSSR + SO_3^{-} \rightleftharpoons RS^{-} + RSSO_3^{-}$, (Cecil, 1963). Gunnison *et al.* (1981), Johnson *et al.* (1980) and Shih *et al.* (1977) reported the detection of urinary cysteine S-sulfonate in humans and rats with sulfite oxidase deficiency, even though it cannot be detected in normal subjects of both species. Since S-sulfonates can be formed in extracellular compartments, the latter pathway may have some significance in the removal of exogenous sulfite, even if high levels of sulfite oxidase exist. Low molecular weight S-sulfonates can be excreted in the urine but the mechanism for clearance of protein S-

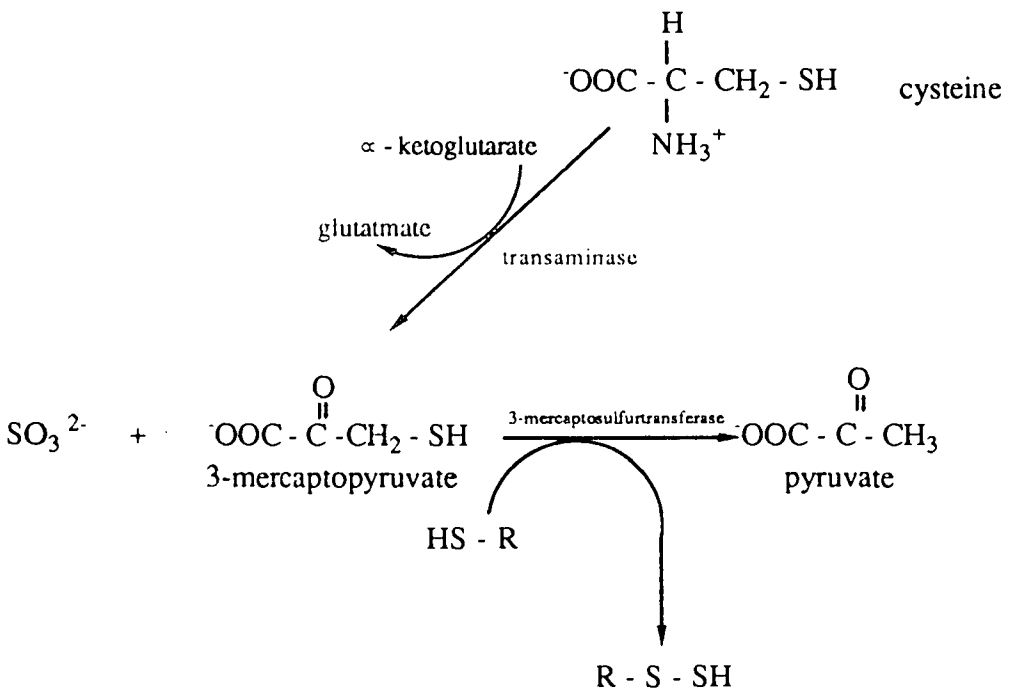

Figure 2. Conversion of inorganic sulfite to S-sulfonates. (Westley, 1980).

sulfonates from the body is not known (Taylor *et al.*, 1986). A third pathway is the conversion of inorganic sulfite to S-sulfocysteine by reaction with glutathione (Kågedal *et al.*, 1986), Figure 3. Significant concentrations of S-sulfocysteine have been found in body fluids (Gunnison *et al.*, 1981; Irreverre *et al.*, 1967; Kågedal *et al.*, 1986). S-sulfoglutathione (formed as an intermediate product) is a potent competitive inhibitor of glutathione S-transferase and thus may impair the detoxification of carcinogenic compounds. A mechanism for its removal may be of physiological importance (Kågedal *et al*, 1986).

In Plants

Plants are exposed to sulfur through soil and air pollution. Normally, sulfur is taken up by plants from soil in the form of sulfate and reductively assimilated into various compounds such as cysteine, methionine, proteins, glutathione, biotin, thiamine and coenzyme A (FPACAQ, 1987). Sulfur dioxide absorbed from air, through stomata, has been shown to rapidly undergo oxidation to sulfates inside plant tissues. Plant chloroplasts are able to both oxidize and reduce SO_2 in light-dependent reactions (Ghisi *et al.*, 1990). Oxidation increases acidification in the plant since sulfuric acid (product of oxidation) is a stronger acid than sulfurous acid (product of hydration). The cytoplasm becomes burdened with sulfate anions which possibly inhibit photosynthesis at higher levels if not removed. Reduction results in H_2S and formation of sulfur-containing amino acids, which are used in protein synthesis. This is a detoxifying step since it consumes sulfite and protons (Ghisi *et al.*, 1990). In plants, sulfates are metabolized as seen in Figure 4 with the transport of sulfate into the plant vacuoles requiring ATP.

In excessive amounts, sulfur can have a deleterious effect. Current information demonstrates that vegetation is generally more sensitive than humans to sulfites (FPACAQ, 1987). Sulfur dioxide is toxic to metabolic processes taking place in plant mesophyll cells.

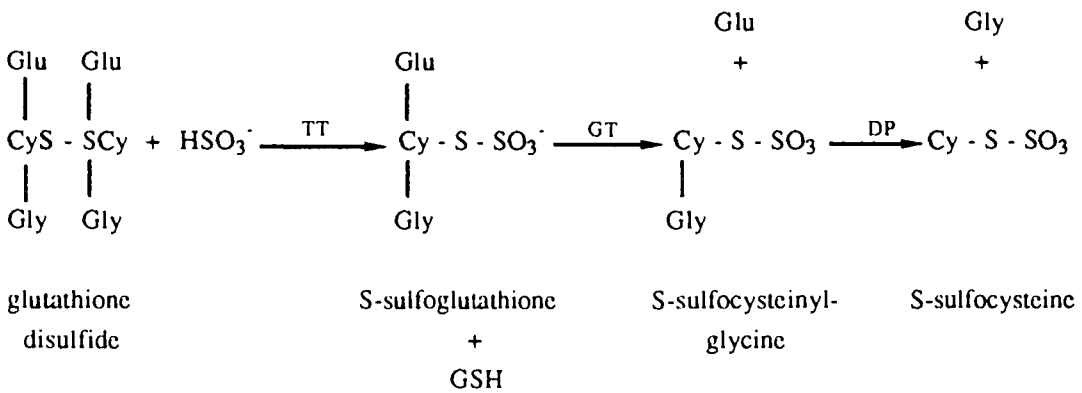

Figure 3. Conversion of inorganic sulfite to S-sulfocysteine. TT = thiol transferase. GT = γ-glutamyltranspeptidase. DP = dipeptidase. (a) = glutathione disulfide. (b) = S-sulfoglutathione. (c) = S-sulfocysteinylglycine. (d) = S-sulfocysteine. (Kågedal *et al.*, 1986).

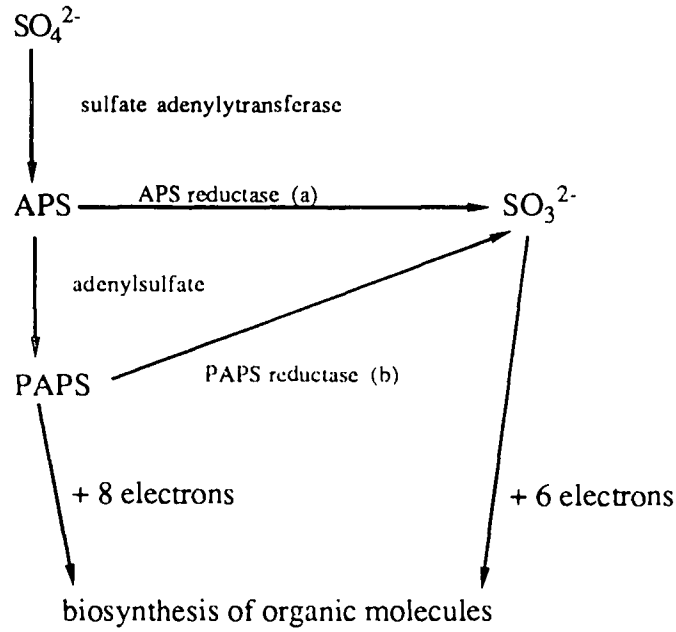

Figure 4. Metabolism of sulfate in plants. Pathway (a) is for photosynthetic organisms and (b) for non-photosynthetic organisms. APS = adenosine-5'-phosphosulfate. PAPS = 3'-phosphoadenosine-5'-phosphosulfate. (Goodwin and Mercer, 1983).

Acute injury can be caused by rapid accumulation of bisulfite and sulfite, and chronic injury may occur when sulfate accumulates beyond a threshold value (FPACAQ, 1987). Thomas (1951) reported that sulfate is about 30 times less toxic than sulfite in plants.

In Yeast

Yeasts are exposed to sulfite through three main routes. They can make it by reductive assimilation of sulfate, by catabolism of sulfur-contatining amino acids, or they can import sulfur dioxide from the environment. The first step in sulfate metabolism in yeast is transport into the cells. Stratford and Rose (1986) proposed that SO_2 was transported into the cell by simple diffusion. In the range of pH 3.0 - 5.0, they reported four indicators signifying a lack of protein involvement in transport 1) lack of saturability, 2) near vertical Woolfe-Hofstee plots at pH 3.0 and 4.0 (initial velocity of SO_2 accumulation versus initial velocity/ $[SO_2]$), 3) the inability of inhibitors such as iodoacetamide and *p*-chloromercuribenzoate to affect the initial velocity and 4) the absence of an affect of pH on the process other than that predicted by changes in substrate concentration (see Figure 5). Stratford and Rose (1986) found that excluding sugar from growth medium had no effect on initial velocities of SO_2 accumulation. They observed that total SO_2 accumulation increased in the presence of glucose, but explained this as being due to the maintenance of a relatively high internal pH value as a result of glycolysis.

After sulfite (H_2SO_3) enters the cell, it encounters an environment around pH 6.5, resulting in a large proportion being converted into HSO_3^- (Schimz, 1980; Pilkington and Rose, 1988). This explains the ability of yeasts to concentrate sulfite intracellularly. The charged species of sulfite may be responsible for intracellular reactions with metabolites, or with DNA by acting as a mutagen primarily causing C-G to T-A transitions (Schimz, 1980).

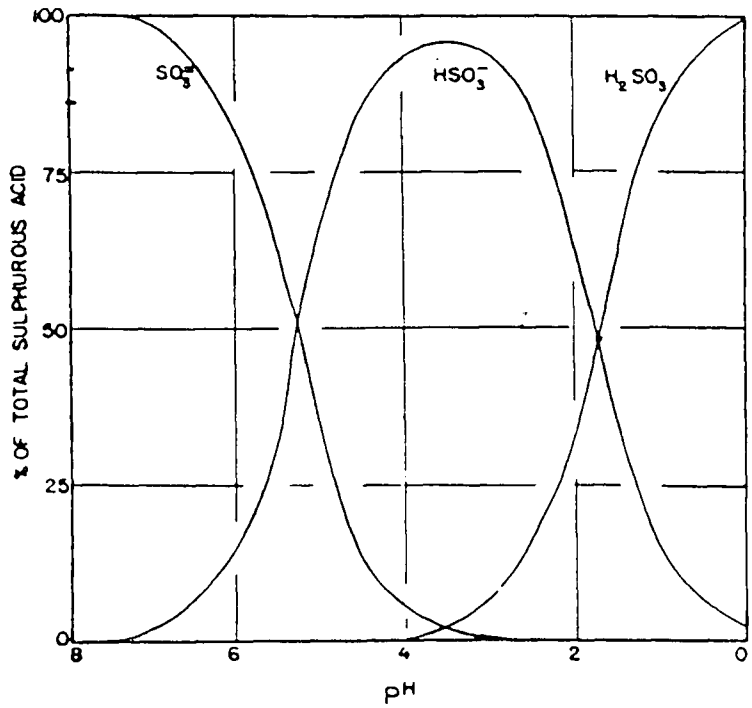

Figure 5. Distribution of various species of sulfurous acid at various pH values. (Joslyn and Braverman, 1954).

As in plants and mammals, yeast needs sulfur for amino acid biosynthesis. The reductive assimilation pathway of sulfate is shown in Figure 6.

EFFECT OF SULFUR DIOXIDE ON YEAST

A short period of tolerance to sulfite before irreversible damage occurs has been reported in yeast (Schimz and Holzer, 1979; Schimz, 1980; Maier *et al.*, 1986). The length of tolerance and rate of damaging effect depends on sulfite concentration, pH, temperature, physiological state of cell and incubation time (Schimz, 1980). The period of insensitivity toward sulfite is shortened by increasing the sulfite concentration or temperature, decreasing the pH or decreasing the storage time of the pre-culture used for incubation (Schimz and Holzer, 1979).

Various reasons have been proposed for differences in sulfite sensitivities in yeasts. Pilkington and Rose (1988) found that the decrease in internal pH following accumulation of sulfite was not of the same magnitude in all yeast strains, suggesting that the internal buffering capacity of the organism might be related to sulfite sensitivity. The differences in the lipid plasma membrane composition and in the rate and amount of aldehyde production have also been proposed to explain sensitivity differences (see Stratford *et al.*, 1987; cited in Casalone *et al.*, 1989).

Two different mechanisms for sulfite actions on energy metabolism have been postulated, in glycolysis and in respiratory chain phosphorylation (Maier *et al.*, 1986). Schimz and Holzer (1979) and Schimz (1980) both reported that millimolar concentrations of sulfite caused a rapid depletion of the ATP content of yeast cells at low pH values. Glycolysis is effectively impaired by low sulfite concentrations (Maier *et al.*, 1986). A variety of glycolytic enzyme activities were assayed in yeast cell extracts incubated with sulfite and glyceraldehyde-3-phosphate-dehydrogenase (GPD) was found to be the most

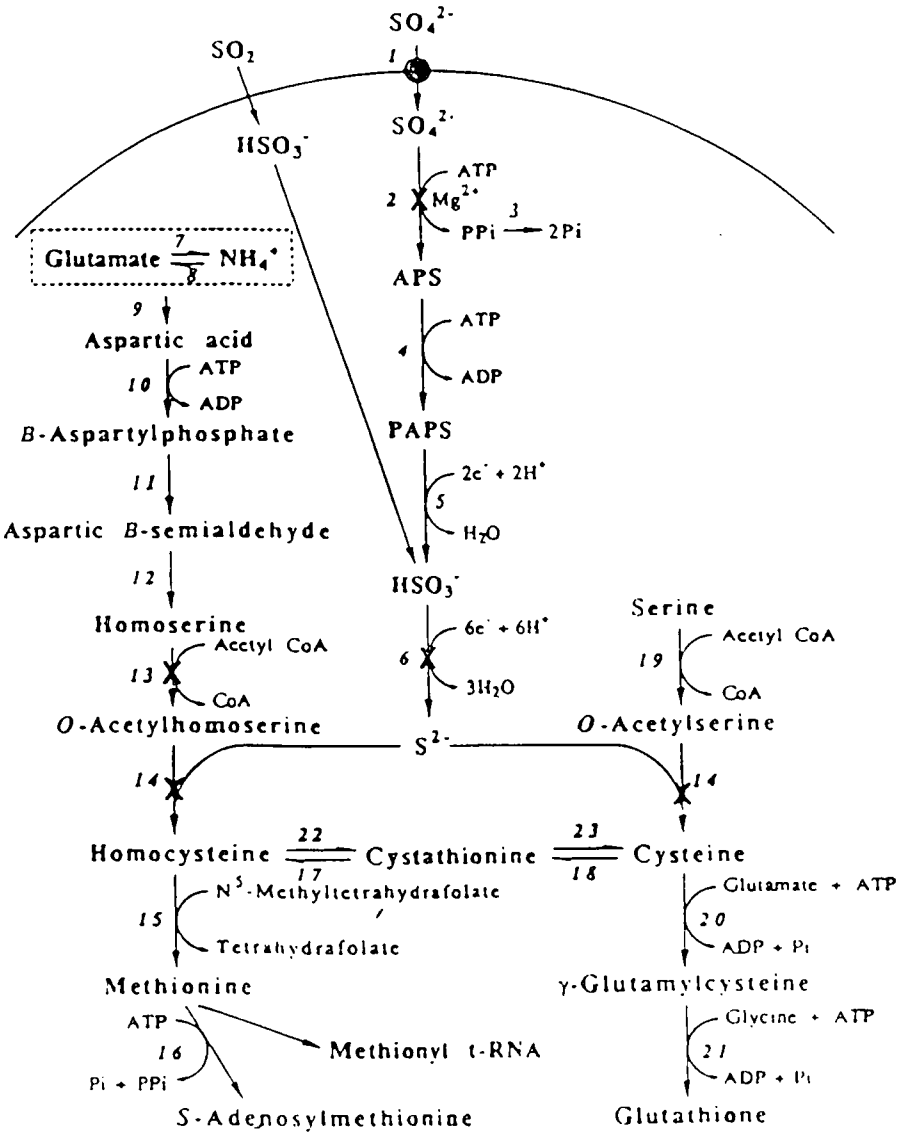

Figure 6. Pathway for sulfur metabolism in *Saccharomyces cerevisiae*. Sites subject to repression by methionine metabolites are indicated with an X. Numbers correspond to the following enzymes: 1 - sulfate permease I and II; 2 - ATP-sulfurylase; 3 - pyrophosphatase; 4 - APS kinase; 5 - PAPS reductase; 6 - sulfite reductase; 7 - NAD-dependent glutamate dehydrogenase; 8 - NADP-dependent glutamate dehydrogenase; 9 - aspartate transaminase; 10 - aspartate kinase; 11 - ß-aspartylphosphate dehydrogenase; 12 - homoserine dehydrogenase; 13 - homoserine *O*-acetyltransferase; 14 - OAS-OAH sulfhydrylase; 15 - homocysteine methyltransferase; 16 - methionine adenosyltransferase; 17 - ß-cystathionase; 18 - cystathionine γ-synthase; 19 - serine *O*-acetyltransferase; 20 - γ-glutamylcysteine synthetase; 21 - glutathione synthetase; 22 - cystathionine ß-synthase; 23 - γ-cystathionase (Henschke and Jiranek, 1991).

sensitive (Hinze and Holzer 1985a,b, 1986; Maier *et al.*, 1986). GPD catalyzes the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. When GPD is inhibited, ATP formation is also inhibited and therefore,the ATP supply is depleted (Hinze and Holzer, 1985b). The reaction of GPD with sulfite also caused the intracellular steady-state concentration of glyceraldehyde-3-phosphate to increase 10 to 100-fold over the normal concentration (Hinze and Holzer, 1985b).

Maier *et al.* (1986) found alcohol dehydrogenase to be inhibited by sulfite. It catalyzes the reduction of acetaldehyde to ethanol by NADH. In this step, NAD^+ is regenerated and subsequently acts as an electron acceptor in the reaction catalyzed by GPD. Therefore, if alcohol dehydrogenase is inhibited, NAD^+ is not produced and the oxidation of glyceraldehyde-3-phosphate is inhibited, causing a depletion of ATP. The most obvious result of alcohol dehydrogenase inhibition is the blocking of ethanol formation.

Respiratory chain phosphorylation is also inhibited by sulfites. Oxygen consumption at pH 3.6 of glucose-starved yeast cells, in the presence of different sulfite concentrations was measured by Maier *et al.* (1986). Respiration was found to be significantly reduced between 0.05 and 0.8 mM sulfite and the sulfite-dependent decrease in ATP correlated well with the reduction in oxygen consumption. This indicates a close relationship between respiration and ATP generation in glucose-starved yeast. Maier *et al.* (1986) suggested that the impairment of respiration by sulfite might be caused by the inhibition of flavoproteins, such as cytochrome b_2.

The proton-motive force (PMF) in yeast may also be disrupted by sulfite entering the cells (Pilkington and Rose, 1988). The PMF consists of a proton concentration gradient and a membrane potential gradient. Normally the inner mitochondrial membrane is impermeable to protons; however, the action of the respiratory electron-transport chain complex generates a proton gradient across the inner mitochondrial membrane. During

ATP synthesis, ATP-synthetases use the PMF to drive phosphorylation of ADP. This is the immediate source of energy for ATP synthesis (Darnell *et al.*, 1986). As sulfite enters the cells, the intracellular pH declines causing the transmembrane pH gradient to decrease (Pilkington and Rose, 1988). This causes dissipation of the PMF across the plasma membrane, resulting in retardation or inactivation of processes that require energy from the PMF, such as active transport of solutes (Pilkington and Rose, 1988).

Maier *et al.* (1986) investigated the effects of sulfite on ATP-hydrolyzing systems. They found that the intracellular proton concentration of glucose-starved yeast cells increased 100-fold with the addition of 1 mM sulfite, at pH 3.6. This intracellular acidification may stimulate an ATP-driven proton pump, thus contributing to ATP depletion (Maier *et al.*, 1986).

GLUTATHIONE

Glutathione (GSH) is a tripeptide thiol (L-γ-glutamyl-L-cysteinyl-glycine) found in virtually all cells. It is an important intracellular metabolite involved in various processes in both prokaryotes and eukaryotes. GSH functions in the reduction of disulfide linkages in proteins and other molecules, in synthesis of deoxyribonucleotide precursors of DNA, and in the protection of cells against the effects of free radicals and of reactive oxygen intermediates, such as peroxides, that are formed naturally during aerobic metabolism. It also serves as a storage and transport form of cysteine (for reviews see Meister, 1983, 1984, 1988; Meister and Anderson, 1983). Kågedal *et al.* (1986) reported the possible involvement of glutathione in the detoxification of sulfite as described previously (Sulfite Metabolism In Humans section).

The synthesis of glutathione in cells is unique in two ways; it is mRNA-independent and the glutamic acid residue is attached to the cysteine residue by an unusual linkage of the γ-C-atom (Kistler *et al.*, 1990). Due to its structure, GSH is protected from proteolytic cleavage. The γ-glu moiety prevents action of intracellular dipeptidases on the cysteine-glycine bond and the C-terminal glycine residue protects GSH from cleavage by γ-glutamylcyclotransferase (Meister, 1988). GSH is synthesized in two enzymatic, ATP-consuming steps from the non-essential amino acid precursors glutamic acid, cysteine and glycine (Figure 7). The two enzymes are γ-glutamyl-cysteine synthetase, which catalyzes the formation of the dipeptide γ-glutamyl-cysteine, and GSH synthetase, which catalyzes the coupling of glycine with the dipeptide.

Although glutathione is involved in many cellular processes, it is not essential in prokaryotes and there is doubt as to whether it has an essential function in eukaryotes. *Entamoeba histolytica* is a eukaryote which grows normally without producing or using GSH (Fahey *et al.*, 1984). This lower eukaryote lacks mitochondria and the usual aerobic respiratory pathways. This might suggest that eukaryotes acquired GSH metabolism at the same time they acquired mitochondria (Fahey *et al.*, 1984). Therefore, GSH may be essential to mitochondrial functions in eukaryotes. Another difference between *E. histolytica* and higher eukaryotes is that it uses a more primitive form of cell division and appears to lack microtubules.

Most of the research on the biological role of GSH has been conducted in animal cells using selective inhibitors of the enzymes involved in its metabolism. Yeast offers the unique chance to isolate GSH-deficient mutants (Glaeser *et al.*, 1991). Little is known about the function of glutathione and the regulation of glutathione biosynthesis in *S. cerevisiae* (Ohtake *et al.*, 1990).

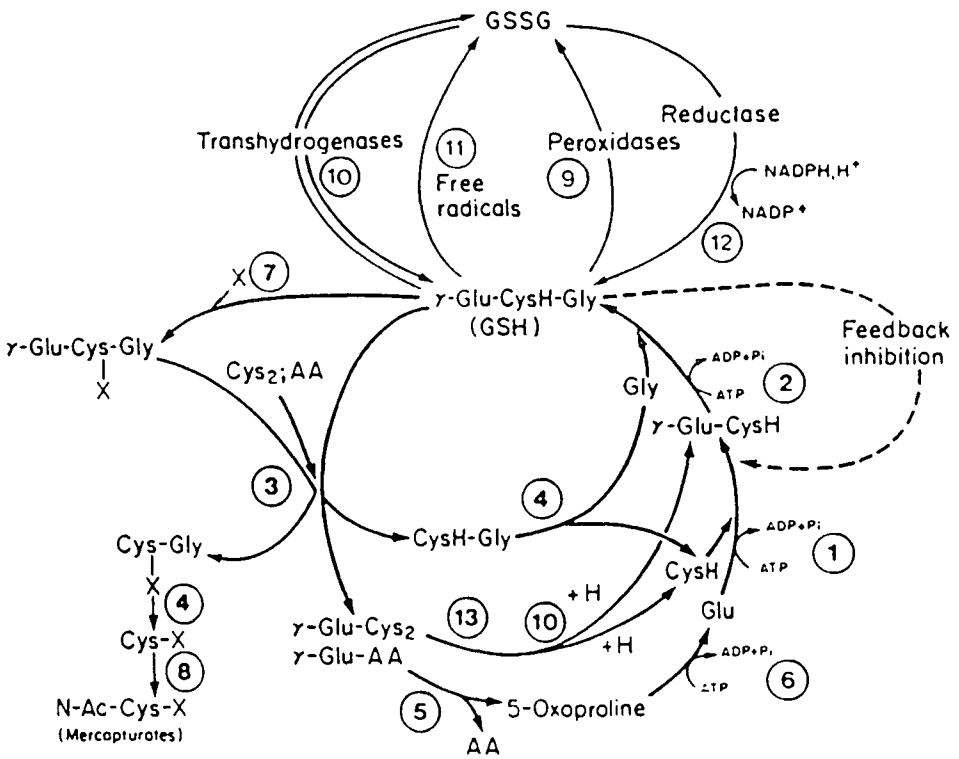

Figure 7. Outline of the biochemistry of GSH. AA - amino acids; X - compounds that react with GSH to form conjugates. 1 - γ-glutamylcysteine synthetase; 2 - GSH synthetase; 3 - γ-glutamyltranspeptidase; 4 - dipeptidases; 5 - γ-glutamylcycltransferase; 6 - 5-oxoprolinase; 7 - GSH S-transferases; 8 - N-acetyltransferases; 9 - GSH peroxidases; 10 - GSH thiol transferases; 11 - reaction of free radicals with GSH; 12 - glutathione disulfide (GSSG) reductase; 13 - transport of γ-Glu-$(Cys)_2$ (Meister, 1988).

Two methods have been used to isolate glutathione-deficient mutants of yeast: by their resistance towards the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and by their sensitivity to methylglyoxal. Glaeser *et al.* (1991) employed filter disks soaked in an MNNG-solution that were placed on actively-growing *Schizosaccharomyces pombe* lawns on glucose complete agar. After four hours of incubation, a zone of inhibition became apparent and after seven days papillae were growing in this zone. These papillae were picked and purified and shown to contain cells deficient in GSH. Kistler *et al.* (1986) also used MNNG to produce a zone of inhibition on YEPD plates containing lawns of actively- growing *S. cerevisiae*, which had been previously UV-irradiated. Ohtake *et al.* (1990) used sensitivity to methylglyoxal as a means for isolating GSH biosynthesis-deficient mutants of *S. cerevisiae*. Methylglyoxal is normally toxic to wildtype cells and is degraded by the glyoxalase system and/or by methylglyoxal reductase. The glyoxalase system consists of two enzymes (glyoxalase I and II) which both require glutathione as a coenzyme. *S. cerevisiae* is inhibited by methylglyoxal at the mM level. Lack of glutathione will impair the glyoxalase system and increase the sensitivity of yeast to methylglyoxal (Ohtake *et al.*, 1990).

The phenotypes that accompany glutathione deficiency have been examined by many investigators. Glaeser *et al.* (1991) found that GSH-deficient *S. pombe* mutants were also sensitive to cadmium (Cd), since the heavy metal-detoxifying phytochelatins are synthesized from glutathione. Kistler *et al.* (1986) found that GSH deficiency in *S. cerevisiae* led to an extension of the lag phase, a decrease in the growth rate and reduced biomass in cells grown in liquid YEPD or synthetic media. They also found that a residual GSH level of 1%, relative to wildtype, lead to serious impairment of the plating efficiency of the cells and their respiration ability, accompanied by a loss of mitochondrial DNA. Ohtake *et al.* (1990) and Glaeser *et al.* (1991) reported an increase in generation time of GSH-deficient mutants. Ohtake *et al.* also found that mutants were more sensitive than

wildtype to chemicals such as thiol-reactive agents (iodoacteamide) and metal-chelating agents (8-hydroxyquinoline). Many of the glutathione-deficient mutants described by these researchers share some of the same phenotypes as exhibited by the sulfite mutants in the present study.

MATERIALS AND METHODS

YEAST STRAINS

The sulfite mutants examined in this study (Table 1) were previously derived from an ethyl methanesulfonate-treated haploid strain of *Saccharomyces cerevisiae*, 2407-1a (Xu and Bakalinsky, 1990).

MEDIA

YEPD is 2% Bacto peptone, 1% Bacto yeast extract and 2% glucose. Minimal medium (M) is 0.67% Bacto Yeast Nitrogen Base Without Amino Acids supplemented with 2% glucose. Media were solidified by addition of 1.7% agar (Moorhead). YEPD + TA is YEPD containing 75 mM tartaric acid, pH 3.5, prepared by autoclaving the agar separately from the YEPD and tartaric acid. Components were mixed 5-10 minutes after their removal from the autoclave and the plates poured after 30-40 minutes. M + TA is M containing 75 mM tartaric acid, pH 3.5, prepared as above. YEPD + TA plates containing different concentrations of SO_2 were prepared by spreading an appropriate amount of freshly-prepared Na_2SO_3 stock solution onto a solid plate and allowing it to dry and diffuse overnight.

Table 1. Yeast Strains

Strain	Genotype	Sulfite Tolerance[a]	SO_2 Phenotype[b]
2407-1a[c]	a *gal2 mal mel CUP1*	6.8	W
16-1	a *sur1-15*	19.6	R
18-8	a *sur1-18*	19.6	R
28-1	a *sus1-1*	5.5	S
33-2	a *sus2-6*	2.9	S
35-2	a *sus3-7*	4.2	S
47-9	a *sus4-11*	2.9	S
8-2	a *sur1-17*	n.d.[d]	R
11-1	a *sur1-2*	n.d.	R
20-5	a *sur1-13*	n.d.	S
29-3	a *sus1-2*	n.d.	S
34-1	a *sus1-4*	n.d.	S
46-5	a *sus1-10*	n.d.	S
3005-3a	a *sus1-9*	n.d.	S

[a] Maximum level of sulfur dioxide tested that permitted growth in M +TA, expressed as μM free H_2SO_3 and calculated by relating free sulfite (H_2SO_3 + HSO_3-) at pH 3.5 with pKa_1 of sulfurous acid, 1.77 (King *et al.*, 1981).
[b] Sulfite tolerance on YEPD + TA plates: W=wildtype, <2 mM; R=resistant, >2.5 mM; S=sensitive, <1 mM SO_2.
[c] 2407-1a is isogenic with X2180-1A.
[d] n.d.- not determined.

GROWTH RATES

Cells taken from YEPD slants were grown overnight in 10 ml liquid YEPD or M at 30° C and 200 rpm (LAB-LINE Orbit Environ-shaker, Melrose Park, IL). The overnight cultures were diluted in 50 ml of the same media in 300 ml culture side-arm flasks to give initial A_{600} readings of 0.10. Absorbance (A_{600}) was measured every 30 minutes until the cultures reached approximately 0.35. All measurements were made using a Spectronic 20, either Milton Roy (Seattle, WA) or Bausch & Lomb (Rochester, NY). Doubling times were determined by plotting $\log_{10}$ absorbance versus time.

Lag times were measured for 2407-1a and 11-1 grown in YEPD and M. Cultures were grown to stationary phase (2 x 10^8 cells/ml), washed twice in 100 mM potassium phosphate buffer, pH 6.9, resuspended in 4 ml of phosphate buffer and stored at 4° C. Lag times were determined for cells held for 0, 24, 48 and 96 hours of storage at 4° C.

PROTEIN LABELING, EXTRACTION, SDS-PAGE AND AUTORADIOGRAPHY

Cells were grown in 80 ml M in 250 ml flasks at 30° C and 250 rpm and divided into two equal volumes at A_{600}= 0.3 - 0.4. Sodium sulfite was added to one culture to a final concentration of 0.2 mM. The cultures were kept at 30°C and 200 rpm and after 30, 60 and 180 minutes, 8 ml samples were taken from each flask, transferred to 13 x 100 glass test tubes and centrifuged in a table top clinical centrifuge (International Equipment Company, Boston,MA) at 335 x g for 5 min. The cells were resuspended in 1 ml M, transferred to 1.5 ml microfuge tubes to which 5 µl ^{35}S-methionine (10 mCi/ml, ^{35}S-trans labeled, New England Nuclear Research Products, EI DuPont de Nemours and Company, Boston, MA) was added and the cultures were incubated for another 20 minutes at 30° C and 250 rpm. The tubes were centrifuged at 11,000 x g for 5 min in a Microspin Sorvall

centrifuge (Dupont Company, Wilmington, DE), the supernatants discarded and the cells cooled on ice. To each tube were added: three volumes of 50 mM Tris-HCl, pH 7.5, 2 µl - 100X protease inhibitor mix (Ausubel *et al.*, 1989, 13.13.7) modified by addition of 1 mg phenylmethylsulfonyl fluoride (PMSF) per 0.5 ml inhibitor mix and four volumes of 0.45 mm acid-washed glass beads. To break open cells, the tubes were vortexed at highest speed for 3 x 30 seconds with 30 second intervals on ice and centrifuged at 11,000 x g for 5 min. The supernatants were transferred to clean microfuge tubes, 0.5 ml Tris-HCl buffer was added and the tubes were centrifuged as before. The supernatants were transferred to another microfuge tube to which one-tenth volume of 100% trichloracetic acid (v/v) was added. The mixture was cooled on ice for 10 minutes, recentrifuged and the supernatants were discarded. The protein pellets were washed twice with 0.5 ml ice-cold acetone with centrifugation after each wash. The tubes were warmed briefly on a heating element to evaporate the acetone, 100 µl of 2X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Sambrook *et al.*, 1989) was added and the samples were frozen in liquid nitrogen and stored at -70° C. SDS-PAGE was carried out using 0.75 mm, 12% polyacrylamide gels as described by Sambrook *et al.* (1989) and O'Farrell (1975). The stacking and resolving gels were run at 15 and 25 mAmp, respectively. The gels were fixed for at least 15 minutes in 50% methanol, 7% HOAc and then enhanced in 1 M sodium salicylate pH 5.5 for 10-12 minutes. They were dried onto filter paper and an autoradiograph was taken for 4-8 hours at -70° C using Kodak Diagnostic Film X-OMAT™ AR (Eastman Kodak Company, Rochester, NY).

SENSITIVITY TO OTHER REDUCING AGENTS

The sensitivity of the mutants to reducing agents other than sulfur dioxide was determined. Ascorbic acid, L-cysteine, sodium nitrite, sodium thiosulfate (pentahydrate), reduced glutathione and sodium selenite were from Sigma (St. Louis, MO) and 1,4-dithiothreitol (DTT) from Boehringer Mannheim (Indianapolis, IN).

Cells taken from YEPD slants were grown to stationary phase in 10 ml liquid YEPD, centrifuged at 850 x g for 5 min in a Beckman TJ-6R table top centrifuge (Palo Alto, CA), washed twice with 100 mM potassium phosphate buffer pH 7.0 and stored in 5 ml of buffer at 4° C. The highest concentration of each reducing agent in which each strain could grow was determined in phosphate-buffered (100 mM, pH 7.0) M containing an apppropriate concentration of reducing agent and a starting inoculum of 0.4 - 1.4 x 10^7 cells in a final volume of 2 ml. Cells at this initial concentraion caused no apparent turbidity. The tubes were incubated at 30° C and 200 rpm and growth was monitored visually over 4 days. The reducing agents were prepared immediately before use and sterile-filtered (Millipore 0.45 µm membrane filters, Bedford, MA). Tubes containing ascorbic acid were incubated in the dark due to its light sensitivity. Both the glutathione and the cysteine were made to volume at pH 7.0 with 5 N sodium hydroxide using a Beckman Ø™ 44 (Palo Alto, CA) with an Orion ROSS semimicro combination pH electrode (Cambridge, MA) before filtration.

GLUTATHIONE AND GLUTATHIONE-METABOLIZING ENZYMES

Cells were grown in liquid YEPD and harvested by centrifugation during mid-log (A_{600}= 0.93-2.2) and early stationary (A_{600}= 5.0-7.6) phases. Protein extracts were prepared by cell disruption using glass beads, frozen in 300 µl aliquots in liquid nitrogen and stored at -70° C (Ausubel *et al.*, 1989). A sample of the medium, with cells removed

by centrifugation, was also saved and stored at -70° C. Protein assays were performed as described by Bradford (1976) with a Varian DMS 80 spectrophotometer equipped with a Varian model 9176 recorder (Varian Techtron Pty. Limited, Mulgrave, Australia). Glutathione was measured by the method of Akerboom and Sies (1981) on both the Varian spectrophotometer and on a Beckman DU-40 spectrophotometer using a Beckman DU series 60 Spectrophotometer Kinetic Soft-Pac™ module P/N 598273 (Beckman Instruments, Inc., Fullerton, CA). The values obtained are the sums of the reduced and oxidized forms of glutathione (GSH and 2 GSSG). Glutathione reductase was measured spectrophotometrically by following the decrease in absorbance of NADPH at 340 nm (Racker, 1955; Mavis and Stellwagen, 1968). Glutathione S-transferase was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and the formation of conjugated derivatives was followed spectrophotometrically at 340 nm (Habig *et al.*, 1974). Glutathione reductase and glutathione S-transferase were measured using the Varian spectrophotometer. Glutathione was also measured by HPLC (Reed *et al.*, 1980) using a Spectra-physics model 770 spectrophotometric detector with a SP 8800 ternary HPLC pump and a SP 4200 computing integrator equipped with a 3-amino propyl-spherisorb column. This method measured both the reduced and oxidized forms.

The sample preparation for the HPLC method was as follows. A fully-grown culture (25-50 µl) containing 8-15 nmole of protein was transferred to a microfuge tube and the volume brought to 200 µl with buffer (121 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl), pH 7.45. To each tube, 50µl of 70% perchloric acid was added and the tubes centrifuged at 11,000 x g for 2 min. A small amount (approx. 1 mg) of sodium bicarbonate was placed in the bottom of 16 x 125 mm screw-top test tubes. A 200 µl aliquot of the supernatant was transferred to the test tubes and the bicarbonate was allowed to bubble. When the bubbling ceased, a small amount of bicarbonate was left in the test tube or more was added if necessary. A 50 µl aliquot of iodoacetic acid (IAA, 15

mg/ml H_2O) was added and the tubes were allowed to incubate at room temperature for one hour. At the end of this period, the tubes were again checked for the presence of bicarbonate which was added if necessary. To each tube, 200 µl of Sanger's reagent (0.75 ml 2,4-dinitrofluorobenzene in 49.25 ml 100% ethanol, stored in a brown bottle at 4° C) was added. The tubes were capped and stored in the dark for at least 24 hours at 4° C before analysis. Samples can be kept for two weeks under these conditions without a change in results. The GSH and GSSG standards were prepared as above except 500 µl of sample were transferred to the screw-cap tubes and 50 µl of IAA and 500 µl of Sanger's reagent were used.

The glutathione analysis was based on two different reactions. The enzymatic determination involved the coupled reaction:

$$2\,GSH + DTNB \xrightarrow{\text{nonenzymatic}} GSSG + 2\,TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^+$$

$$DTNB + NADPH + H^+ \longrightarrow 2\,TNB + NADP^+$$

in which the rate of formation of 5-thio-2-nitrobenzoate (TNB) was followed spectrophotometrically at 412 nm. The HPLC method was based on the reaction of iodoacetic acid with thiols to form S-carboxymethyl derivatives followed by chromophore deriviatization of amino groups with Sanger's reagent (1-fluoro-2,4-dinitrobenzene). The derivatives were then rapidly separated by HPLC and detected at 365 nm.

SULFUR DIOXIDE TOLERANCE

Cells were grown to stationary phase in 10 ml YEPD (30° C at 200 rpm), centrifuged (850 x g for 5 min), washed twice in 75 mM tartaric acid buffer, pH 3.5, and stored in 5 ml of the buffer at 4° C. Cell concentrations were determined by making appropriate serial dilutions, plating on YEPD and counting colonies after 3 - 4 days at 30° C. The maximum amount of sulfur dioxide that permitted growth was determined in M containing 75 mM tartaric acid pH 3.5, with a starting inoculum of 0.5 - 1.6 x 10^7 cells in a total volume of 2 ml (in a one dram screw-top vial) as described in the "Sensitivity to Other Reducing Agents" section. Growth (turbidity) was monitored for 3 consecutive days.

Free sulfite was measured by a modification of the Ripper iodometric method (Amerine and Ough, 1974). To a 2.5 ml sample, 1 ml 1:3 H_2SO_4:H_2O and 1 ml 1% potato starch were added and the solution titrated with 0.002 N iodine. The endpoint was the first darkening of the solution to a bluish color which persisted for 1 minute. The amount of free H_2SO_3 at the maximum concentration permitting growth was calculated from the measured free sulfite, the Henderson - Hasselbalch equation, pKa_1 = 1.77 of sulfurous acid (King *et al.*, 1981) and the equation: free sulfite = H_2SO_3 + HSO_3^-.

EFFECT OF SULFUR DIOXIDE ON CELL SURVIVAL

Cells were incubated in 75 mM tartaric acid buffer pH 3.5 and 0.15 mM Na_2SO_3 at 30° C and 250 rpm. Samples (50 µl) were withdrawn immediately, 2, 4, 6 and 8 hours after SO_2 addition, diluted and plated in duplicate on YEPD to yield 25 - 250 colonies / plate. An addition of water replaced SO_2 for the control. The plates were incubated at 30° C and colonies counted after 3 - 5 days.

CELL STORAGE STUDY

Survival curves for cells stored in 75 mM tartaric acid buffer pH 3.5 at 4° C were determined over a period of 30 days. Cells were grown, harvested and stored as described in the "Sulfur Dioxide Tolerance" section. Cells were sampled periodically, serially diluted and plated on YEPD. Colonies were counted after 2 - 4 days at 30° C.

TESTING FOR REVERSION

Approximately every 6 months the cultures were tested to confirm that their sensitivity or resistance to SO_2 had not changed. Cells were spotted onto a single YEPD master plate and incubated at 30° C. After 2 - 3 days, the master plate was replica-plated to YEPD + TA plates containing 0, 1.5, 2.5, 3.0 and 3.5 mM SO_2. The plates were incubated at 30° C and scored for growth after 1 and 2 days.

STATISTICAL ANALYSIS

Data were analyzed with the Student's *t*-test to determine significant difference at the 95% confidence interval, $p \leq 0.05$ (Zar, 1984).

RESULTS AND DISCUSSION

GROWTH RATES

Doubling times were determined in rich (YEPD) and minimal (M) media, Table 2. No apparent correlation was found between the sulfite phenotype and generation time. Resistant mutants 16-1 and 18-8 grew at significantly slower rates than wildtype in YEPD and 16-1 was significantly slower in M. Resistant mutant 8-2 had a doubling time 175% of wildtype but it was not statistically significant due to a large standard error (30%). All but two of the sensitive mutants tested grew significantly slower than wildtype in M, YEPD or both media. Sensitive mutant 47-9 had the slowest doubling time in YEPD and M, which may have been due to the fact that its sulfite sensitivity co-segregated with a petite phenotype.

During the course of determining the doubling times, it was noted that mutant 11-1 had a long lag time relative to the other strains. Therefore, the lag time of 11-1 was compared to that of wildtype (Table 3). Strain 11-1 showed a dramatic increase in lag time in M after being stored for 24, 48 and 96 hours (discussed subsequently in the Glutathione section).

Table 2. Doubling times in two media at 30° C and 200 rpm, expressed as % wildtype[a].

SO_2 PHENOTYPE[b]	STRAIN	YEPD	M
R	8-2	175	129
R	11-1	115	97
R	16-1	130*	140*
R	18-8	135*	97
R	20-5	129	94
S	28-1	129	121
S	29-3	130*	125
S	33-2	154*	215*
S	34-1	183*	167*
S	35-2	129	162*
S	46-5	129*	100
S	47-9	194*	400*[c]
S	3005-3a	111	128

[a] Values are means of two to four replicates. Doubling times for wildtype in YEPD and M were 88 min and 144 min, respectively.

[b] W = wildtype, R = resistant, S = sensitive.

[c] One replicate only.

* Significantly different from wildtype at 95% confidence interval.

Table 3. Lag times (in minutes) for 2407-1a and 11-1 in two media at 30° C and 200 rpm[a].

	2407-1a		11-1	
STORAGE[b]	YEPD	M	YEPD	M
0	144	141	60	96
24	183	180	129	411
48	201	162	120	399
96	156	150	114	363

[a] Means are based on one or two replicates.

[b] Held at 4° C in 100 mM potassium phosphate buffer, pH 6.9, for the indicated number of hours prior to inoculation.

PROTEIN LABELING, EXTRACTION, SDS-PAGE AND AUTORADIOGRAPHY

The purpose of this experiment was to determine if there was a difference in protein banding patterns due to sulfite-specific induction or disappearance of polypeptides. Cells were labeled with ^{35}S-methionine since most proteins contain methionine to some extent. Proteins lacking methionine residues would not be expected to appear as bands on the autoradiograph. Sulfite was added to the cultures while the cells were actively growing, early-to-mid-log growth phase.

Two trials were performed: 1) to determine the effect of incubation period on the response to sulfite and 2) to determine if there was a concentration effect. Wildtype (2407-1a), resistant (18-8) and sensitive (28-1) strains were chosen based on their similar growth rates in M. To determine the effect of incubation period, sulfite was added at 0.2 mM and cells were harvested after 30, 60 and 180 minutes. An autoradiograph of proteins separated by SDS-PAGE (Figure 8), revealed no obvious differences in banding patterns. To examine the effect of concentration, two sub-lethal doses, 0.2 and 1.0 mM, were added and cells were harvested after 60 minutes. Again, no obvious differences in banding patterns appeared.

Because the medium in this experiment was not buffered, the pH changed upon addition of sulfite: M = pH 4.16, M + 0.2 mM SO_3 = pH 4.32 and M + 1.0 mM SO_3 = pH 5.42. The amount of free H_2SO_3 was determined as described in the "Sulfur Dioxide Tolerance" section of the Materials and Methods. King *et al.* (1981) reported that the lethal effect of sulfur dioxide was directly proportional to the concentration of undissociated H_2SO_3. The only toxic form of sulfur dioxide is the free H_2SO_3 form, bound SO_2 is not toxic. The amount of free H_2SO_3 was calculated to be 0.213 µM in the 0.2 mM system and 0.122 µM in the 1.0 mM system, almost a two-fold decrease. Therefore, the effect (if

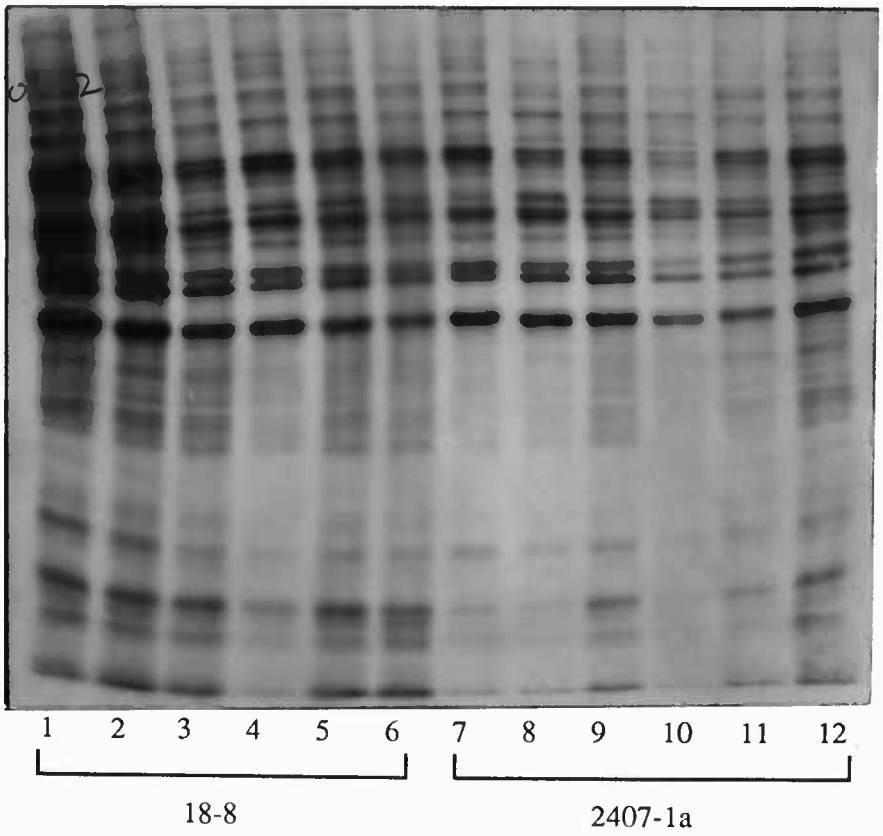

Figure 8. Autoradiograph of ^{35}S-methionine labeled protein extracts separated by SDS-PAGE. Cells were harvested after the specified exposure to 0.2 mM Na_2SO_3. Lane 1: 30 min control (no sulfite). Lane 2: 30 min. Lane 3: 60 min control (no sulfite). Lane 4: 60 min. Lane 5: 180 min control (no sulfite). Lane 6: 180 min. Lane 7: 30 min control (no sulfite). Lane 8: 30 min. Lane 9: 60 min control (no sulfite). Lane 10: 60 min. Lane 11: 180 min control (no sulfite). Lane 12: 180 min.

any) should have been more noticeable in the 0.2 mM system. Both of these levels were well below the sulfite tolerances reported in Table 1.

SENSITIVITY TO OTHER REDUCING AGENTS

Since sulfite is a reducing agent, other reducing agents were tested to determine what affect they might have on the mutants (Table 4). All the strains exhibited the same high tolerance for cysteine, reduced glutathione (GSH) and ascorbic acid. No obvious correlations were found between the SO_2 phenotypes and the responses to the other reducing agents. The resistant strains did not appear to be more resistant to the reducing agents than wildtype and the sensitive strains did not appear to be more sensitive than wildtype. Sulfite- sensitive strain 35-2 was far more sensitive to dithiothreitol (DTT) than the other strains and approximately three to ten times more sensitive than wildtype. Sulfite-sensitive strain 47-9 was also more sensitive to sodium nitrite ($NaNO_2$) and sodium thiosulfate ($Na_2S_2O_3$) than the other strains. It was three to four times more sensitive to $NaNO_2$ than wildtype and three to seven times more sensitive to $Na_2S_2O_3$. This mutant has a petite phenotype which co-segregates with the sulfite sensitivity and causes slow growth relative to the other mutants and to wildtype.

A precipitate formed in the tubes in which cells were incubated with cysteine. Presumably, cysteine was oxidized during incubation to cystine which precipitated. This precipitate sedimented to the bottom of the test tube so when the tube was vortexed to determine growth (turbidity), a definite difference could be seen between the precipitate and cells.

Table 4. Growth of yeast strains in phosphate-buffered (100 mM, pH 7.0) M in the presence of various reducing agents.

		REDUCING AGENT (mM) [a]							
SO_2[b]	STRAIN	DTT		$NaNO_2$		$Na_2S_2O_3$		Na_2SeO_3	
		+	-[c]	+	-	+	-	+	-
W	2407-1a	10	15[d]	200	250	750	n.d.[e]	20	25
R	16-1	20	25	175	200	300	500	20	25
R	18-8	5	10	250	300	750	n.d.	25	50
S	28-1	10	15	75	100	500	750	10	15
S	33-2	5	10	100	150	400	500	20	25
S	35-2	1	5	75	100	400	500	20	25
S	47-9	15	20	50	75	100	200	10	15

[a] All strains grew at the same concentrations of cysteine, 250 mM; GSH, 600 mM; and ascorbic acid, growth at 1.0 M and no growth at 1.4 M.

[b] SO_2 phenotype: W = wildtype, R = resistant, S = sensitive.

[c] (+) indicates growth and (-) no growth at the next highest concentration tested.

[d] Means are based on two to five replicates.

[e] n.d. - not determined.

In the presence of sodium selenite, all strains turned red 24 hours after noticeable turbidity. Whanger (1991) suggested that the red color was due to elemental selenium or phosphoselenide, which can form from the reduction of Se. Izuka *et al.* (1988) also observed a red pigment when yeast was grown in the presence of selenite, but in parallel with increasing extracellular glutathione. They suggested that the color was due to the accumulation of elemental Selenium (Se°) via the following reaction:

$$H_2SeO_3 + 4\,GSH \rightarrow GSSeSG \rightarrow GSSeH \rightarrow Se^{\circ} + GSH$$

where H_2SeO_3 is selenious acid, GSSeSG is glutathione selenotrisulfide and GSSeH is glutathione selenopersulfide. They also found glutathione reductase activity (known to be involved in the reduction of GSSeSG to GSSeH) increased in the presence of selenite.

GLUTATHIONE

Glutathione is widely-distributed in animals, plants and microorganisms (Meister, 1988). It is the most prevalent cellular-thiol and the most abundant low molecular weight peptide. Two methods are commonly used to measure glutathione, spectrophotometrically (Tietze, 1969; Akerboom and Sies, 1981) and by HPLC (Reed *et al.*, 1980; Fahey *et al.*, 1984; Ritchie and Lang, 1987). The spectrophotometric methods are based on the same reaction (see the Material and Methods section) but the samples are prepared differently. Akerboom and Sies (1981) employed perchloric acid to precipitate proteins followed by its removal by precipitation at neutral pH as the potassium salt. Tietze's (1969) method is based on protein precipitation with trichloroacetic acid and removal of the precipitate by ether extraction. The method of Akerboom and Sies (1981) was chosen because it was simpler. The HPLC methods differ by the derivatives formed and detected. Reed *et al.* (1980) reacted iodoacetic acid with thiols to form S-carboxymethyl derivatives followed by

chromophore derivatization of amino groups with Sanger's reagent (1-fluoro-2,4-dinitrobenzene). Fahey *et al.* (1984) analyzed thiol components by fluorescent labeling of the thiol with monobromobimane and separation of the resulting derivatives. Ritchie and Lang (1987) combined HPLC with dual electrochemical detection (HPLC-DEC). Samples were deproteinized with metaphosphoric acid, diluted with the assay solution after centrifugation and injected. Thiols were detected by monitoring the current at the down-stream Au/Hg electrode of the DEC, which is set at a thiol-specific potential. The method of Reed et *al.* (1980) was chosen due to the availability of the instrument and column.

In the spectrophotometric analysis, the two spectrophotometers used gave comparable results, therefore the data were pooled. According to this method, all of the sensitive mutants and the stationary phase cells of 16-1 had significantly less total glutathione than wildtype. According to the HPLC method, only the log phase cells of 33-2 had significantly less glutathione, Table 5. Casalone *et al.* (1989) found GSH concentrations in their UV-induced and spontaneous sulfite-resistant mutants to be higher than in the parental strain.

To explain the differences in glutathione values measured by the two methods, possible explanations were considered: freshness of samples, presence of mixed disulfides, sample preparation time and standard deviations. Freshness of samples is a major factor in accurate glutathione determination. All replicates per instument/method, were assayed on the same day and within 36 days overall. Several investigators have reported data suggesting the occurrence of mixed disulfides between GSH and protein or other thiols (Meister and Anderson, 1983). In mammalian tissues, Higashi *et al.* (1985) found that less than one percent of the total glutathione was bound to proteins by a disulfide bond. To prevent mixed disulfides from forming, the HPLC method utilized iodoacetic acid to form S-carboxymethyl derivatives of thiols eliminating thiol disulfide interchange within a few

Table 5. Glutathione (GSH + GSSG) in yeast cell extracts measured by two methods[a].

		SPECTROPHOTOMETRIC[b]		HPLC[c]	
SO_2[d]	STRAIN	LOG PHASE	STATIONARY	LOG PHASE	STATIONARY
W	2407-1a	100 (69.2±5.4)	100 (110.7±17.7)	100 (43.8±5.6)	100 (38.8±7.2)
R	16-1	102 (70.8±7.0)	68* (75.7±6.3)	91 (39.6±4.4)	115 (44.5±0.4)
R	18-8	132 (91.4±12.2)	88 (97.8±13.8)	113 (49.5±1.5)	135 (52.5±4.7)
S	28-1	44* (30.4±3.2)	37* (41.4±5.0)	78 (34.0±0.6)	68 (26.5±1.1)
S	33-2	24* (16.5±2.5)	28* (31.0±4.9)	52* (22.6±0.7)	67 (26.1±1.3)
S	35-2	50* (34.8±2.9)	32* (35.2±3.0)	82 (36.0±0.2)	n.d.[e]
S	47-9	70* (48.4±4.7)	40* (44.1±3.6)	84 (36.9±7.4)	70 (27.3±0.6)

[a] Expressed as percent of wildtype and (nmol mg protein^{-1} ± std error).
[b] Akerboom and Sies, 1981. Means are based on 2 samples with 4-8 replicates each.
[c] Reed *et al.*, 1980. Means are based on 2-3 replicates.
[d] SO_2 phenotype: W = wildtype, R = resistant, S = sensitive.
[e] n.d.- not determined.
* Significantly different from wildtype at 95% confidence interval.

minutes after cell disruption (Reed *et al.*, 1980). With the spectrophotometric method, dithiothreitol was added to the cell lysing buffer to prevent disulfides from forming. The sample preparation time for the spectrophotometric and HPLC methods varied considerably, with the HPLC method requiring the least amount of time. The covariance for the spectrophotometric method ranged from 24 to 62%, while for the HPLC method it was much lower, ranging from 0.6 to 26%. In considering all this, the author believes the HPLC method most accurately measured the amount of glutathione present in the cell extracts.

Decreased glutathione levels in 33-2 coupled with its slower growth rate is consistent with the findings of Kistler *et al.* (1986) and Ohtake *et al.* (1990), who worked with *S. cerevisiae*, and Glaeser *et al.* (1991), who worked with *Schizosaccharomyces pombe*. All the studies reported that glutathione deficient mutants grew more slowly than wildtype.

The long lag time of mutant 11-1 is apparently not related to a decrease in glutathione. Although the glutathione content of this particular mutant was not measured, levels in resistant mutants 16-1 and 18-8, both of which are allelic to 11-1, were no different than wildtype. Kistler *et al.* (1986) found that an extension of the lag phase of *S. cerevisiae*, along with a decrease in the growth rate may be due to lowered GSH levels. Glaeser *et al.* (1991) found a long lag phase for cells grown in glucose complete media and a reduced growth rate in minimal media with GSH-deficient *Schizosaccharomyces pombe* mutants as well. The data of Murata and Kimura (1986) also confirms the findings of Kistler *et al.* (1986), although their conclusions did not agree with their data. The generation times of most of the sulfite-sensitive mutants in the present study were significantly greater than wildtype.

The growth media, with cells removed by centrifugation, were also analyzed for glutathione by the spectrophotometric method; but none was found. No reports are known

to this author about the excretion of glutathione by yeast except for leakage of the tripeptide from polyacrylamide gel-entrapped *S. cerevisiae* cells and secretion by yeast cells grown in the presence of selenite (Izuka *et al.*, 1988).

GLUTATHIONE REDUCTASE AND GLUTATHIONE S-TRANSFERASE

Glutathione reductase (GR) catalyzes the reduction of GSSG to GSH with NADPH as a cofactor. GR values are reported as percent wildtype and nmol mg protein^{-1} min^{-1} in Table 6. Only log phase cells of sulfite-resistant mutant 18-8 showed a significantly higher GR level than wildtype. Casalone *et al.* (1989) also found that sulfite resistant mutants of yeast had a higher GR than wildtype. The quantity of GR in wildtype was 62.6 nmol min^{-1} mg^{-1} which compares well with the 57.5 nmol min^{-1} mg^{-1} measured by Casalone *et al.* (1988) who used late-log phase *S. cerevisiae* cells disrupted with a cap vibrator apparatus. Their method proved to be more efficient than the one this author used in extracting protein, 13.3 mg/ml versus 6.36 mg/ml.

Glutathione S-transferases (GST) are a group of enzymes involved in the detoxification of xenobiotics. The resistant mutants as well as some of the sensitive mutants had reduced GST levels, except for 35-2 and 47-9, Table 6. In wildtype, the amount of GST measured, 2.78 nmol min^{-1} mg^{-1}, was similar to that found by Casalone *et al.* (1988), 2.4 nmol min^{-1} mg^{-1}. Glaeser *et al.* (1991) found that most of their glutathione-deficient mutants of *Schizosaccharomyces pombe* had reduced levels of GST activity. But, Casalone *et al.* (1989) found sulfite resistant mutants of *Saccharomyces cerevisiae* had GST activity comparable to wildtype. Glaeser *et al.* (1991) speculated that reduced levels of GR and GST activities were due to a pleiotropic effect caused by glutathione depletion.

Table 6. Glutathione reductase and glutathione S-transferase in yeast cell extracts[a].

		GLUTATHIONE REDUCTASE		GLUTATHIONE S-TRANSFERASE	
SO_2[b]	STRAIN	LOG PHASE	STATIONARY	LOG PHASE	STATIONARY
W	2407-1a	100 (62.6±5.5)	100 (77.2±13.0)	100 (2.78±0.2)	100 (3.29±0.6)
R	16-1	159 (100.3±20.2)	105 (81.4±10.1)	59* (1.63±0.2)	40* (1.32±0.2)
R	18-8	135* (84.9±5.4)	105 (81.0±6.5)	29* (0.80±0.1)	31* (1.02±0.1)
S	28-1	89 (55.7±7.1)	84 (64.8±4.1)	39* (1.07±0.3)	27* (0.89±0.1)
S	33-2	117 (74.0±2.4)	92 (70.6±34.5)	55* (1.54±0.2)	43* (1.41±0.2)
S	35-2	n.d.[c]	n.d.	113 (3.14±1.3)	44 (1.46±0.4)
S	47-9	n.d.	n.d.	94 (2.61±0.5)	45 (1.49±0.4)

[a] Expressed as percent of wildtype and (nmol substrate mg protein^{-1} min^{-1} ± std error). Means are based on 2 samples with 2-3 replicates each.

[b] SO_2 phenotype: W = wildtype, R = resistant, S = sensitive.

[c] n.d.- not determined.

* Significantly different from wildtype at 95% confidence interval.

SULFUR DIOXIDE TOLERANCE

The sulfur dioxide tolerance of the mutants is reported in Table 1. Prior to this experiment, a puzzling observation was made. Wildtype and sensitive mutants 33-2 and 4709 were incubated in M + TA + 1.25 or 1.0 mM total sulfite, respectively. Samples plated after one and two days did not contain viable cells but samples plated after 3 days incubation were viable. This pattern of growth was observed whether cells were plated on M + TA, YEPD + TA, or YEPD. A possible explanation for growth after an extended period may be that sulfite is acted as a mutagen (Shapiro, 1977) and after a few days, sulfite-resistant mutants or revertants were selected that were able to survive and to grow on the solid media containing no sulfite. This possibility was not confirmed by genetic analysis.

Two procedures were examined for use in determining free sulfur dioxide: 1) the spectrophotometric method of Owades (1963) involving p-rosaniline HCl as the coloring agent and 2) a modification of the Ripper method (Amerine and Ough, 1974) involving acidification of the sample with H_2SO_4 followed by titration with iodine using a starch indicator. Due to the small number of samples and for simplicity, the modified Ripper method was used. A standard curve relating added sulfite to "free sulfite" was made based on samples taken immediately, 2 and 24 hours after sulfite addition. The amount of free sulfur dioxide was highest immediately after the sulfite addition and then decreased, presumably as it began to bind to components in the media. The curves based on sampling 2 and 24 hours after addition were approximately the same indicating that most of the sulfite was bound in the first 2 hours. The standard curve based on sampling immediately after addition was chosen for use since this was the initial amount of undissociated H_2SO_3 that cells were exposed to (Figure 9).

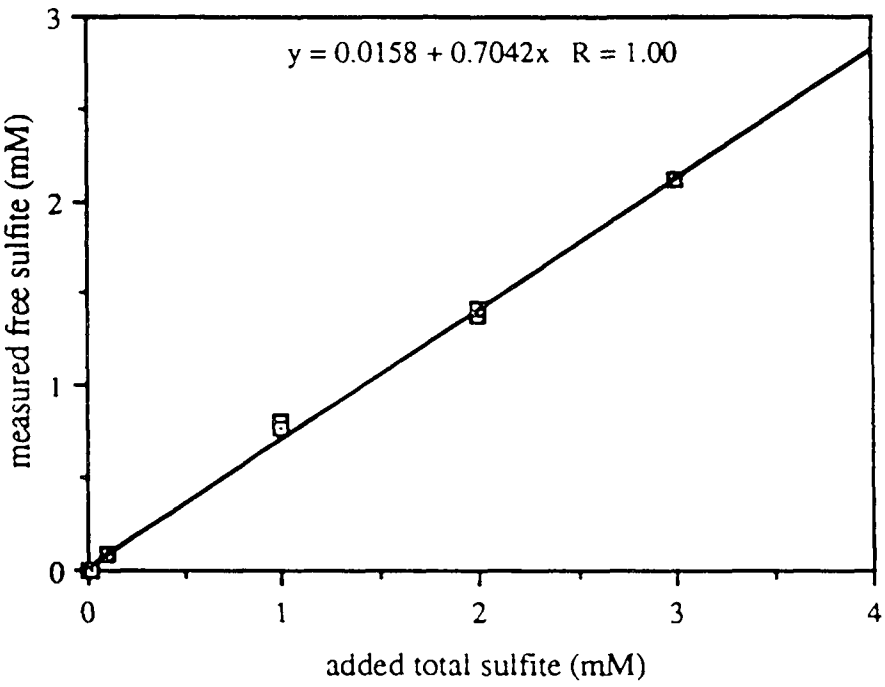

Figure 9. Standard curve for free SO_2 measured by a modified Ripper method. Samples were assayed immediately after addition of Na_2SO_3 to buffered M (75 mM tartrate, pH 3.5).

EFFECT OF SULFUR DIOXIDE ON CELL SURVIVAL

Initially, various sulfite concentrations (0.75, 0.5, 0.2 and 0.05 mM) and incubation times (0, 2, 4, 6, 8, 24, 48 and 72 hours) were tested to determine the proper conditions for this experiment. Eventually, all strains were incubated in the presence of 0.15 mM Na_2SO_3 and samples were plated after 0, 2, 4, 6 and 8 hours (Figure 10). The expectation was that at the same sulfite concentration, the resistant mutants would die at a slower rate than wildtype and the sensitive mutants would die more rapidly. This was not true and may be related to the fact that the mutants were initially isolated based on their ability or inability to grow rather than survive at various sulfite concentrations. The survival curves of most of the strains (except 35-2 and 47-9) appeared to be biphasic with little or no kill during the first 2 hours followed by an increased exponential rate.

A log transformation of the data was performed and the slopes, based on samples taken from 2 to 8 hours, were compared using the Student's T-test (Zar, 1984). In the case of 35-2 and 47-9, the slopes were based on all data points. There was a significant difference between the experimental and control slopes of each strain, indicating sulfite did have a toxic effect.

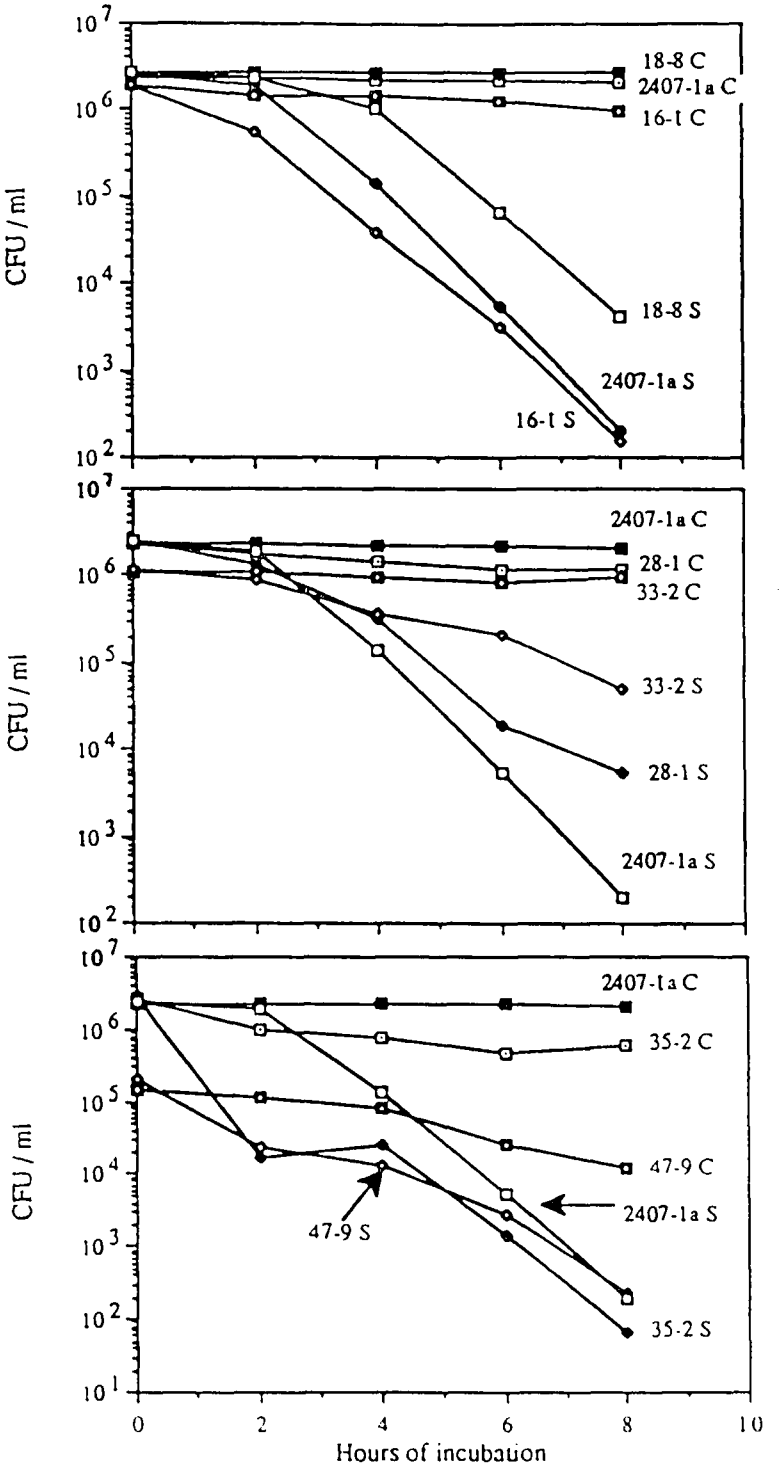

Figure 10. Effect of sulfur dioxide on cell survival. Cells were incubated in 75 mM tartaric acid, pH 3.5 at 30° C and 250 rpm. 'C' = control with water. 'S' = 0.15 mM Na_2SO_3.

CELL STORAGE STUDY

Because some of the mutants appeared to be dying during storage, the survival of the cells during prolonged storage in buffer at 4° C was determined (Figure 11). Three classes of survival rates were found. Strains 18-8 and 35-2 appeared to survive well during storage with little loss of viability. Strains 28-1, 2407-1a (wildtype) and 16-1 appeared to die at an exponential rate, and strains 33-2 and 47-9 appeared to die at two different exponential rates: quickly at the beginning and slowly after about 15 days. The time for 90% loss of viability was calculated for the mutants using either the exponential rate of cell death or the initial rapid decrease in cell viability (if the cell death rate was biphasic). The approximate times were: 2407-1a = 4.5 days; 16-1 = 8 days; 28-1 = 1 day and 47-9 = less than one day. There was no obvious correlation between nitrite sensitivity and the rate of viability loss in the mutants. Kolter (1992), studied starved *E. coli* cultures, and found a biphasic death curve, with viable counts dropping by one or two log units in the first 4 or 5 days of incubation in a rich medium. Similar results were observed for *Serratia marcescens* and *Sarcina lutea* (Steinhaus and Birkeland, 1939).

After 3 days of growth on YEPD plates, it was noted that strains 16-1 and 33-2 produced colonies of two sizes. Cells from representative colonies from both strains were examined under the microscope. Cells from the two types of colonies of 16-1 had a normal appearance, while both sizes of 33-2 included normal and abnormally elongated cells. Fresh single colony isolates were restreaked onto YEPD and after 24 hours examined under the microscope. Cells from the new colonies appeared to be the same as before.

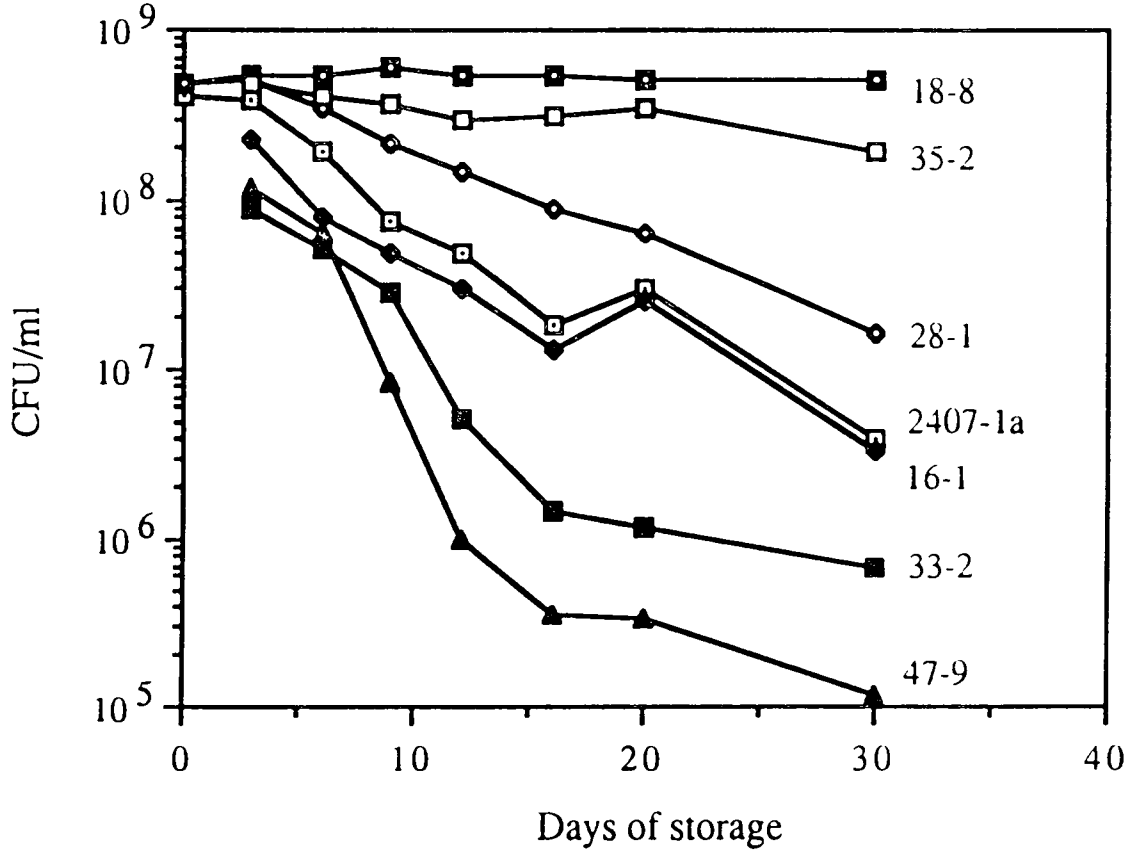

Figure 11. Cell survival during storage. Cells were grown in YEPD, harvested by centrifugation , washed twice with 75 mM tartaric acid, pH 3.5 and stored in the buffer at 4° C. Data points are means of duplicate counts.

CONCLUSIONS

Because no cure for sulfite sensitivity in humans is known, the only recourse available to sensitive individuals is avoidance of foods and pharmaceuticals to which sulfite has been added. While recent government actions concerning labeling of foods have made this approach practical, rational treatment and cure require an understanding of the mechanistic basis for the sensitivity. The sulfite mutants of yeast examined in this study offer a promising system in which to model the condition, the basis of which remains obscure. As a microbial eukaryote, *Saccharomyces cerevisiae* shares essential characteristics of higher organisms while offering a far more tractable genetics. While yeast lacks sulfite oxidase, this is probably an experimental advantage, since it is very likely that inadequate sulfite oxidase activity is a prerequisite to sulfite sensitivity in humans. A more serious weakness of the yeast model is the lack of an immune system. However, at present, evidence in favor of an immune-related mechanism in humans is lacking.

The responses of the yeast mutants to a variety of reducing agents suggest that the sulfite-sensitivity or -resistance is indeed a sulfite-specific response and not a general reaction to a change in redox potential. The finding that one sensitive mutant (33-2) had approximately 50% of the wildtype level of glutathione during active growth suggests that glutathione may mediate sulfite detoxification *in vivo*. Published correlations between sulfite sensitivity in humans and reductions in glutathione are unknown to this author.

Future work will be directed towards the cloning of genes implicated in the aberrant responses to sulfite and determination of the functions of the products they encode. Because of the potential relevance of glutathione deficiency to sulfite sensitivity in humans, initial efforts will be made to determine the molecular basis for the deficiency in mutant 33-2.

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