TRANSPORT AND TOXICITY OF SULFUR DIOXIDE IN THE YEAST SACCHAROMYCES CEREVISIAE VAR. ELLIPSOIDEUS

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A B S T R A C T

TRANSPORT AND TOXICITY OF SULFUR DIOXIDE IN THE YEAST *SACCHAROMYCES CEREVISIAE VAR. ELLIPSOIDEUS*

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Investigations were carried out on the transport and toxicity of sulfur dioxide in the yeast *S. cerevisiae var. ellipsoideus*.

It was found that SO_2 was transported inside the cell by a mediated transport system.

The transported SO_2 was not all retained inside the cell and a certain portion of it could be removed by washing. The amount of sulfur dioxide taken up by the cells as well as that which could not be washed out was increased as the pH value of the incubation mixture was decreased.

The SO_2 transport system operated very rapidly. The SO_2 reached its maximum level in about two minutes. There were two equilibria involved in this transport. The first was a chemical equilibrium which regulated the availability of substrate (molecular SO_2) to this transport. The second was a transport equilibrium which regulated the inward and outward fluxes of SO_2 across the cell membrane.

Kinetic data showed that SO_2 transport was saturable and conforms to the Michaelis-Menten type kinetics. Also the transport system was apparently temperature dependent and it was irreversibly heat inactivated.

Molecular SO_2 is the only transported form. The HSO_3^- and $SO_3^=$ ions are not taken up by this yeast.

In regard to SO_2 toxicity, it was found that the suppression of cell growth was dependent upon the amount of molecular SO_2 and the time during which this form remained in contact with the cell. The curve which relates cell viability and time to SO_2 exposure is linear on a semilog plot.

A preliminary study on the localization of ^{35}S in cells exposed to $^{35}SO_2$ showed that sulfur dioxide was not actively metabolized. Practically all SO_2 which reacted inside the cell, was found in the small molecule fraction. Most likely, SO_2 reacts with compounds containing carbonyl groups as well as with other molecules inhibiting various steps which causes termination of cell growth.

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CHAPTER I

INTRODUCTION

The problem of finding satisfactory methods for preserving foods has been a challenge to mankind for centuries and our evolution to an industrial society has not lessened this problem.

Sulfur dioxide, bisulfite and sulfite salts are used extensively in a wide variety of foods and food products for the purpose of preservation from microbial deterioration. Literally hundreds of millions of people have consumed sulfur dioxide in the low concentrations permitted in foods without apparent harm. It is one of the most common beverage preservatives (Jacobs, 1944; Braverman, 1963). In the fermentation industries SO_2 is used as a selective inhibitor of undesirable organisms (kean and Mash, 1956). A rather unique application of sulfur dioxide involves the bleaching of specialty products such as Maraschino cherries (Bullis and Wiegand, 1931) and the decolorizing of sugar-cane and sugar-beet juices in the sugar industry. Treatment of grapes with sulfur dioxide reduces molding and repels insects (Joslyn, 1952). In the case of flour it modifies the baking characteristics of flour

by breaking disulfide bonds in the gluten molecule. Sulfur dioxide, bisulfite and sulfite salts are also used to prevent enzymatic and non-enzymatic browning and preserve the color of various food products (Vas and Ingram, 1949; Joslyn and Braverman, 1954; Kyzline et al., 1961). Various pharmaceutical formulations use SO_2 as an antioxidant (Jenkin et al., 1951; Remington, 1961).

Contrary to its wide commercial uses sulfur dioxide is one of the most harmful air pollutants (West, 1970); it affects both living organisms and inanimate structures. Flue gases from smelters or power stations may contain sufficient sulfur dioxide to cause serious damage to vegetation. It is also toxic to the human and animal respiratory system (Fairhall, 1957) and it has been implicated in eye irritation (Whittenberger and Frank, 1963). In the food field the main disadvantage of sulfur dioxide are its unpleasant flavor and its destructive action on thiamine (Thomas and Berryman, 1949).

Despite the importance of SO_2 as a food preservative the mechanism of transport of SO_2 inside the cell, as well as its metabolism in the living cell are not well understood. The purpose of this study is to clarify some of the mechanisms relative to transport, toxicity and metabolism of sulfur dioxide in yeast cells.

CHAPTER II

LITERATURE REVIEW

For many centuries SO_2 has been used for protection of foods from microbial deterioration caused by yeasts, molds and bacteria. According to Joslyn and Braverman (1954), fumes of burning sulfur were used as a sanitizing agent in wine-making by the ancient Egyptians and Romans. However, the literature on the mechanism of its antimicrobial action is very limited. Although much knowledge has been obtained on the inhibition of various metabolic processes in microbial cells by certain food preservatives, the factor which determines the antimicrobial effect is not clear (Wyss, 1948; Bosund, 1962). Lipid solubility and surface activity were correlated to antimicrobial actions of various series of compounds (Meyer, 1937; Cavill and Vincent, 1949; Cavill et al., 1949; Danielli, 1950; Hirai, 1957).

Wyss (1948), pointed out that the preserving effect of SO_2 solutions is derived from their inhibiting influence on enzymes of the living cells. Enzymes containing S-S bonds may be inactivated by reducing substances, e.g. by sulfur dioxide which breaks S-S bonds and reduces them to SH-groups. Furthermore, the formation of sulphonates of sulfur dioxide with carbonyls, e.g. acetaldehyde, is well known.

Oka (1960 a-e; Oka, 1962 a-f) studied the antimicrobial effect of various food preservatives and classified them into two groups, according to the manner by which this effect is determined. In the first group belong the acid preservatives, salicylic, benzoic, sorbic and dehydroacetic acids, their esters and phenols; the second group includes quinones and nitrofurane. Sulfur dioxide aqueous solutions belong to the first group. The acid antiseptics affect all stages of microbial growth. The lag phase is extended, the specific growth rate during logarithmic phase decreases and the total yield also decreases (Huntington, 1945; Nomoto et al., 1955). The antimicrobial effect of acid antiseptics is strongly pH-dependent in that they are powerful inhibitors of microbial growth under acidic conditions and ineffective at neutrality. This appears to be dependent on the amount of unionized molecular fraction which increases as the pH value decreases. At any pH, equal concentrations of unionized molecules of these compounds show similar antimicrobial effects (Rahn and Conn, 1944). In the case of SO_2 solutions Bosund (1962) explained this by assuming that the unionized form penetrates the cell wall of the microorganisms more rapidly than do the ionic forms (HSO_3^-, $SO_3^=$). Consequently greated amounts can enter the cell and interfere with its life processes.

Studies on the effect of SO_2 solutions on the growth of different microorganisms at various pH levels showed

that not only the unionized but also the ionized forms (HSO_3^-, $SO_3^{=}$) had an appreciable antiseptic effect on those microorganisms. This work indicated that the unionized form was greated than 1,000-fold more active against Escherichia coli than the HSO_3^- ions. In the case of Saccharomyces cerevisiae the unionized form was only 100 to 500-fold greated than the HSO_3^- ions; the effect of the unionized form against Aspergillus niger is only 100-fold greater than that of the ionized forms (Rehm and Wittmann, 1962). However, the conclusions of this work are dubious because an inaccurate pK_2 value was used in calculating the distribution of the three molecular forms of SO_2 in solution as a function of pH.

Yeast cells suspended in solutions of acid antiseptics caused a decrease in the concentration of these compounds. The rate of this decrease was very high and the concentration attained an equilibrium state in a very short time. The transported quantity of these acid antiseptics was considered to be in quilibrium with the concentration of unionized molecules of these compounds in the medium (Bosund, 1960). Studies on the distribution of the acid preservatives between yeast cells and the surrounding medium have shown that except for dehydroacetic acid, these preservatives were absorbed on the solid phase in equivalent quantities under equilibrium conditions. In this case the quantities

dissolved in the water and in the lipid phase of the cells varied greatly according to the kind of preservatives (Oka, 1960 b-e). Essentially the same results have been obtained with E. coli and Staphylococcus aureus (Oka, 1960 a). Oka (1964) concluded that the antimicrobial effect of the acid preservatives depended upon the adsorption of the compound on the solid phase of microbial cells. Since the food products to which the acid preservatives are applied consist also of water, lipid and solid phases, the adsorption on the solid phase of microbial cells is in equilibrium with the preservative dissolved in the water phase of the food and not the average concentration in the food.

Rehm (1964) investigated the antimicrobial effect of SO_2 and found that it decreased due to the formation of hydroxy-sulphonates with different carbonyl compounds. However, the magnitude of this decrease did not correspond, in all cases, to the extent of the binding of SO_2 with the carbonyl. This difference was due to the antimicrobial action of hydroxy-sulphonates which were formed by reaction of SO_2 with acetaldehyde, pyrurate, a-keto-glutarate and acetone and which had significant inhibiting effects on the respiration of S. cerevisiae.

Sulfur dioxide affects both respiration and fermentation. As mentioned in the beginning, SO_2 is a potent inhibitor of SH-group bearing enzymes. The high sensitivity of different

SH-enzymes to sulfites is caused by a primary inhibiting action against nicotinamide adenine dinucleotide (NAD), according to Pfleiderer et al. (1956). Sulfur dioxide forms an addition product with NAD as demonstrated by spectrophotometric methods (Meyerhof et al., 1938). SO_2 can also destroy thiamine and split different disulfides, e.g. cystine to cysteine (Tehn, 1964).

Rehm (1964) investigated the effect of SO_2 on fermenting cells of S. cerevisiae and E. coli. He first examined the following NAD dependent steps in glycolysis:

1. The step from 3-phospho-glyceraldehyde to 1,3 diphosphoglycerate.
2. The reaction from pyrurate to lactate.
3. The reaction from acetaldehyde to ethanol.

In the case of reaction (1), simultaneous addition of 3-phosphoglycerate and ATP to SO_2-inhibited cultures of S. cerevisiae restored fermentation.

Step (2) is strongly inhibited by SO_2 in E. coli. This inhibition was due to the formation of an addition product between SO_2 and NAD. The back reaction, that is from lactate to pyrurate, was also inhibited by sulfur dioxide.

Yeast ethanol dehydrogenase which may catalyze Step (3) is slightly inhibited in vitro (Pfleiderer et al., 1956). Rehm has found that, in equilibrium, the fermentation was inhibited almost to the same extent as in the case when

free SO_2 bound acetaldehyde. Most likely, yeast ethanol dehydrogenase is not inhibited directly, since fermentation of ethanol in this step is not possible as free SO_2 is bound by acetaldehyde.

Rehm (1964) also investigated the effect of SO_2 on respiration. Respiring cells of E. coli are strongly inhibited by sulfur dioxide in vitro. The step from malate to oxaloacetate was strongly inhibited by SO_2 in E. coli. In the Krebs cycle and from oxalosuccinate to α-ketoglutarate there is a pyridoxal-dependent decarboxylating reaction which is inhibited by SO_2. Another step of respiration which is inhibited by SO_2 is that from α-ketoglytarate to succinate through succinyl-CoA. Between succinyl-CoA and succinate there is a thiamine-dependent decarboxylation which is always strongly inhibited by SO_2. Addition of succinate activated the respiration of cells of E. coli. Besides these significant inhibitions of SO_2 on fermentation and respiration, an inhibition by formation of addition products must be considered. The formation of these addition products begins at the level of glucose and may also include 3-phospho-glyceraldehyde, dihydroxy-acetonphosphate, pyrurate, acetaldehyde, oxalacetate and α-ketoglutarate.

Shapiro (1970) in a preliminary in vitro experiment showed that SO_2 may cause point mutations in nucleic acids by converting cytosine to uracil under mild conditions.

The same investigator, in an unpublished work, had found that HSO_3^- can change up to 90 percent of the cytosine residues of yeast RNA into uracil residues. In another preliminary experiment, also unpublished, Makai (1970), has succeeded in producing bacterial mutations with $HaHSO_3^-$.

CHAPTER III

METHODS AND MATERIALS

Organism

The microorganism used in this study was the yeast Saccharomyces cerevisiae var. ellipsoideus ATCC 14824.

Synthetic growth media

a. Solid medium. The synthetic medium used for the stock culture was the same used for the survival experiments. This is the medium which Etchells et al. (1953) called Synthetic Agar B and prepared (Wickerham, 1951) as follows: Difco yeast nitrogen base (DYNB) solution, to which glucose was added to reach a concentration of 4%, and a 3% agar solution were separately heat-sterilized (121°C for 15 min) and mixed at equal volumes just prior to plating. The ready-to-use medium had a concentration of 2% glucose, 1.5% agar and a pH of 5.

b. Liquid medium. This medium was similar to that used by Schultz and McManus (1950). One liter of this medium contained:

I. Sugar and Salts

Sucrose	12.52 g
KH_2PO_4	0.57 g
KCl	0.44 g
$MgCl_2$	0.13 g
$CaCl_2.2H_2O$	0.13 g
NH_4Cl	1.57 g
$FeCl_3$	0.01 g
$NaSO_4$	0.04 g

II. Buffer (McIlvaine's Citrate buffer).

Citric acid	12.823 g
$NaHPO_4.7H_2O$	22.196 g

III. Growth factors

Inositol	16.30 mg
Calcium Pantothenate	10.50 mg
Biotin	0.05 mg
Thiamine	0.05 mg
Pyridoxin	0.05 mg
Nicotinic acid	0.05 mg

The liquid medium was prepared as follows: The sugar, the salts and the buffer walte were dissolved in 900 ml distilled water. The growth factors, dissolved in 25 ml water were added to the above solution and the volume was made up to 1 liter with distilled water. The pH of this medium was 4.2. The medium was then filter sterilized.

Preparation of radioactive SO_2

Sulfur dioxide is a readily condensable, colorless gas. It was prepared by the reduction of hot concentrated sulfuric acid by means of copper.

$$Cu + 2H_2{}^{35}SO_4 \longrightarrow Cu^{35}SO_4 + 2H_2O + {}^{35}SO_2$$

A flask provided with a safety tube and exit tube was filled about one third with copper turnings which were covered with labelled sulfuric acid, specific activity 1 μ Ci per ml.

Sulfur dioxide was evolved when the flask was heated and it was trapped in 0.1 N NaOH.

$$^{35}SO_2 + NaOH \longrightarrow NaH^{35}SO_3$$

The $NaH^{35}SO_3$ was redistilled by reacting with moderately concentrated sulfuric acid in order to further purify the labelled SO_2.

$$NaH^{35}SO_3 + H_2SO_4 \longrightarrow NaHSO_4 + H_2O + {}^{35}SO_2$$

Again the evolved $^{35}SO_2$ was trapped in cold 0.1 N NaOH and kept as sodium disulfite ($NaH^{35}SO_3$) under atmosphere of nitrogen in order to avoid oxidation during storage in the freezer.

Molecular species of SO_2 in aqueous solutions

Sulfur dioxide is soluble in water providing sulfurous acid.

$$SO_2 + H_2O \rightleftharpoons H_2SO_3$$

The solubility of SO_2 in H_2O depends upon temperature. At room temperature (20°C) and atmospheric pressure it is 11.3% (w/w) (Handbook of Chemistry and Physics, 1959).

Ley and Koning (1938) have questioned the existence of the compound sulfurous acid in aqueous solutions. Ultraviolet absorption spectra of aqueous solution of sulfur dioxide suggested the following equilibrium.

$$SO_2 + H_2O \rightleftharpoons HSO_3^- + H^+$$

Infrared spectral studies (Falk and Giguere, 1958;

Jones and McLaren, 1958) have demonstrated that molecular sulfurous acid (H_2SO_3) is not present in aqueous solutions of sulfur dioxide. Falk and Giguere (1958) inferred that sulfur dioxide is dissolved in the molecular state since no stable H_2SO_3 molecules were found in aqueous solution. Eigen et al. (1961) studied the rate of hydrolysis of sulfur dioxide by sound absoprtion technique and concluded that sulfurous acid is not an intermediate product. The hydration of sulfur dioxide appears to be one of the most rapid hydrolytic reactions known (DeMeyer and Kustin, 1963).

Raman spectra (Simon and Waldmann, 1956) of aqueous solutions of sulfur dioxide of concentrations greater than 1M contain lines attributable to pyrosulfite (S_2O_5). Dilute solutions contain lines assigned to SO_2 and HSO_3^- (Simon and Kriegsmann, 1956). Raman spectra of dilute aqueous solutions of alkali bisulfites ($NaHSO_3$) show the presence of HSO_3^- while more concentrated solutions contain increased concentration of pyrosulfite (Simon and Waldmann, 1956).

Ionization constants of sulfurous acids have been reported by Tartat and Barretson (1941). The values they found appear to be reliable in spite of the fact that modern evidence indicates that aqueous solutions of sulfur dioxide contain no detectable sulfurous acid molecules. The unionized sulfur dioxide species in aqueous solutions of sulfur dioxide is almost entirely free SO_2 molecules. The first dissociation of sulfur dioxide in aqueous solutions at 25^oC is:

$$SO_2 + H_2O \rightleftharpoons H^+ + HSO_3^-$$

$$K_1 = \frac{a_{H^+}\, a_{HSO_3^-}}{a_{SO_2}} = \frac{[H^+]\,[HSO_3^-]\,\gamma H^+ \gamma\, HSO_3^-}{[SO_2]\qquad \gamma SO_2} = 1.54 \times 10^{-2}$$

and the second dissociation at 25°C

$$HSO_3^- \rightleftharpoons H^+ + SO_3^=$$

$$K_2 = \frac{[H^+]\,[SO_3^=]\,\gamma\ H^+\ \gamma\ SO_3^=}{[HSO_3^-]\ \gamma\ HSO_3^-} = 1.02 \times 10^{-7}$$

Applying the Henderson-Hassebalch equation to SO_2 dissociations the following equations are obtained:

$$pH = pK_1 + \log \frac{[HSO_3^-]}{[SO_2]}$$

$$pH = pK_2 + \log \frac{[SO_3^=]}{[HSO_3^-]}$$

The sum of all three forms adds to 100% and since $pK_1 = 1.81$ and $pK_2 = 6.91$ (Handbook of Chemistry and Physics, 1970-1971)

$$pH = 1.81 + \log \frac{[HSO_3^-]}{[SO_2]} \qquad (a)$$

$$pH = 6.91 + \log \frac{[SO_3^=]}{[HSO_3^-]} \qquad (b)$$

$$[SO_2] + [HSO_3^-] + [SO_3^=] = 100 \qquad (c)$$

This system has 3 equations and 4 unknowns, namely pH, $[SO_2]$, $[HSO_3^-]$ and $[SO_3^=]$.

Solving (a) and (b) for $[HSO_3^-]$ and $[SO_3^=]$ as a function of SO_2 and substituting those values to (c) the following equation is obtained:

$$1 + 10^{(pH-1.81)} + 10^{(2pH-8.72)} = \frac{100}{[SO_2]} \qquad (I)$$

In the same way:

$$1 - 10^{(8.72-2pH)} - 10^{(pH-6.91)} = \frac{100}{HSO_3^-} \qquad (II)$$

$$1 - 10^{(8.72-2pH0} - 10^{(6.91-pH)} = \frac{100}{SO_3^=} \qquad (III)$$

Equations (I), (II) and (III) provide a direct relationship between pH and each form of SO_2. On the other hand these three equations can not be solved when any one of these three SO_2 forms gets 0 or 100% value. The latter is proved mathematically as follows:

If $[SO_2] = 0$, then substitution to (I) gives:

$$[SO_2]\ [10^{(pH-1.81)} + 10^{(2pH-8.72)} + 1] = 100$$

$$\text{or } [0]\ [10^{(pH-1.81)} + 10^{(2pH-8.72)} + 1] = 100$$

or $0 = 100$. This is impossible.

In the same way $[HSO_3^-]$ and $[SO_3^=]$ cannot have a zero value. Therefore, all three SO_2 forms are present in all pH values and none of them disappears completely. This also means that none of those forms can have a 100% value. The latter is mathematically proved as follows:

Substitution to (I) for $[SO_2]$ = 100 gives:

$$1 + 10^{(pH-1.81)} + 10^{(2pH-8.72)} = \frac{100}{100} = 1$$

$$\text{or } 10^{pH}\left(\frac{1}{10^{1.81}} + \frac{10^{pH}}{10^{8.72}}\right) = 0 \qquad (d)$$

Equation (d) has the following solutions:

$$10^{pH} = 0$$

$$\text{or} \quad \frac{1}{10^{1.81}} + \frac{10^{pH}}{10^{8.72}} = 0 \qquad (e)$$

$$\text{or} \quad 10^{pH} = -\frac{10^{8.72}}{10^{1.81}} \qquad (f)$$

Both (e) and (f) equations are mathematically impossible and therefore SO_2 never assumes the value of 100%.

In the same way HSO_3^- and $SO_3^=$ do not become 100%

Therefore none of the three SO_2 forms gets a value of 100% and none of them disappears at any pH value. The amount each of those forms takes at pH values between 0 and 14 appears in Table 1, and Figure 1 shows the distribution of these forms at the same pH values. Molecular SO_2 predominates at low pH values, it has its maximum value at pH = 0 and approaches assymptotically to zero as pH gets closer and closer to pH = 14. Bisulfite (HSO_3^-) form has its maximum value at about pH 4.4 and decreases as pH value goes above or below its maximum, approaching zero assymptotically as pH approaches zero or 14. Finally

sulfite $SO_3^=$ gets its maximum value at pH = 14 and approaches assymptotically zero as pH value approaches zero.

Analytical Determination of SO_2

The importance of SO_2 in many fields has been responsible for the development of a number of analytical methods. The following methods were used in this study:

a. Iodometric method. This determination is based upon oxidation of sulfurous to sulfuric acid by iodine (Treadwell and Hall, 1946):

$$SO_2 + 2H_2O + I_2 \longrightarrow 4H^+ + 2I^- + SO_4^=$$

The iodine solution was previously standardized by $Na_2S_2O_3$. The latter compound was in turn standardized against KIO_3, which served as a primary standard.

$$1 \text{ liter of } 0.1 \text{ N iodine} = \frac{SO_2}{20} = 3.203 \text{ g } SO_2$$

b. Spectrophotometric method. This method was described by Scoggins (1970). The sulfur dioxide solution, containing not more than 0.08 mole SO_2, is transferred into a 50-ml volumetric flask and diluted to 40 ml with water. Then 5 ml of a 5M sulfuric acid solution is added to the flask, the volume is made up to 50 ml with H_2O and the absorbance of the solution is measured at 276 nm vs a reagent blank. The SO_2 concentration is calculated from a standard curve.

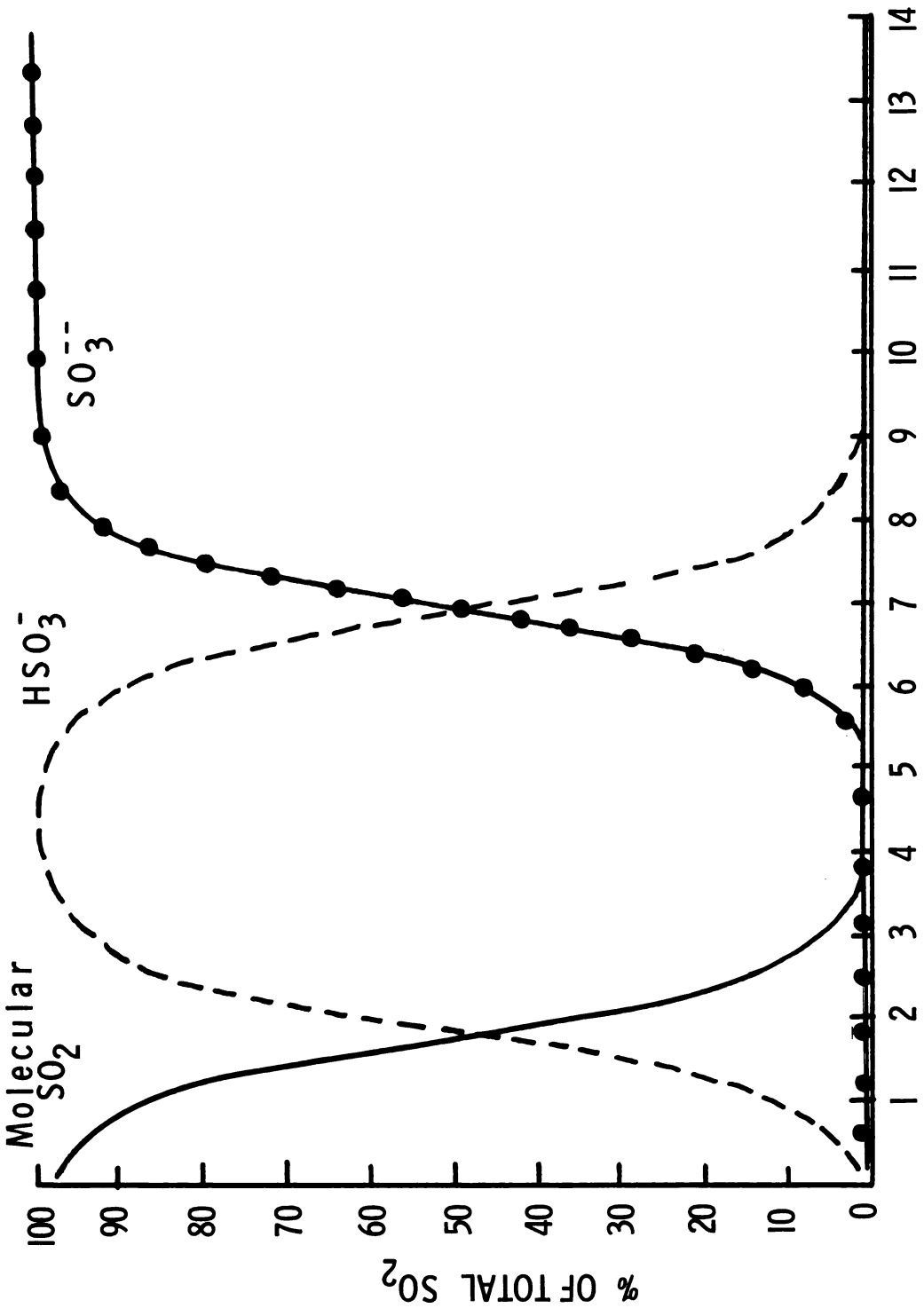

Figure 1. Percentage distributions of molecular SO_2, bisulfite (HSO_3^-) and sulfite ($SO_3^{=}$) forms as a function of pH in aqueous solutions of SO_2.

Table 1. Percent distribution of the three SO_2 molecular species in aqueous solution as a function of pH.

pH	Molecular SO_2	Bisulfite (HSO_3^-)	Sulfite ($SO_3^=$)
0.2	97.60	2.39	0.00
0.4	96.26	3.73	0.00
0.6	94.19	5.80	0.00
0.8	91.10	8.89	0.00
1.0	86.59	13.40	0.00
1.2	80.29	19.70	0.00
1.4	71.99	28.00	0.00
1.6	61.86	38.13	0.00
1.8	50.58	49.41	0.00
2.0	39.23	60.76	0.00
2.2	28.95	71.04	0.00
2.4	20.45	79.54	0.00
2.6	13.95	86.04	0.00
2.8	9.28	90.71	0.00
3.0	6.06	93.92	0.01
3.2	3.91	96.07	0.01
3.4	2.50	97.46	0.03
3.6	1.59	98.36	0.04
3.8	1.01	98.91	0.07
4.0	0.64	99.24	0.11
4.2	0.40	99.40	0.19
4.4	0.25	99.44	0.30
4.6	0.16	99.35	0.48

pH	Molecular SO_2	Bisulfite (HSO_3^-)	Sulfite ($SO_3^=$)
4.8	0.10	99.13	0.76
5.0	0.06	98.72	1.21
5.2	0.04	98.05	1.90
5.4	0.02	96.98	2.99
5.6	0.01	95.32	4.66
5.8	0.00	89.04	10.95
6.0	0.00	83.68	16.31
6.2	0.00	76.39	23.60
6.4	0.00	67.12	32.87
6.6	0.00	56.30	43.69
6.8	0.00	44.83	55.16
7.0	0.00	33.89	66.10
7.2	0.00	24.45	75.54
7.4	0.00	16.96	83.03
7.6	0.00	11.40	88.59
7.8	0.00	7.51	92.48
8.0	0.00	4.87	91.12
8.2	0.00	3.13	96.86
8.4	0.00	2.00	97.99
8.6	0.00	1.27	98.72
8.8	0.00	0.80	99.19
9.0	0.00	0.51	99.48
9.2	0.00	0.32	99.67

pH	Molecular SO_2	Bisulfite (HSO_3^-)	Sulfite ($SO_3^=$)
9.4	0.00	0.20	99.79
9.6	0.00	0.12	99.87
9.8	0.00	0.08	99.91
10.0	0.00	0.05	99.94
10.2	0.00	0.03	99.96
10.4	0.00	0.02	99.97
10.6	0.00	0.02	99.97
10.8	0.00	0.01	99.98
11.0	0.00	0.00	99.99
11.2	0.00	0.00	99.99
11.4	0.00	0.00	99.99
11.6	0.00	0.00	99.99
11.8	0.00	0.00	99.99
12.0	0.00	0.00	99.99
12.2	0.00	0.00	99.99
12.4	0.00	0.00	99.99
12.6	0.00	0.00	99.99
12.8	0.00	0.00	99.99
13.0	0.00	0.00	99.99
13.2	0.00	0.00	99.99
13.4	0.00	0.00	99.99
13.6	0.00	0.00	99.99
13.8	0.00	0.00	99.99

Preparation of the Samples

The culture received from the American Type Culture Collection was transferred to DYNB-glucose-agar slants and served as a stock culture.

Cells from the stock culture were used to inoculate the 500 ml liquid medium contained in 1 liter Erlenmeyer flasks. The cells were grown aerobically by shaking on a gyratory shaker (300 rpm) for about 36 hours at constant temperature (25°C). The cells were then centrifuged (10,000 xg for 3 min) and washed 3 times with demineralized water.

A simple experimental procedure for studying SO_2 transport in a microbial system would be to incubate the cells with labelled SO_2 for a certain period of time, interrupt the incubation either by filtration or centrifugation, wash the cells and measure the radioactivity of those cells. This procedure was tried but gave inconsistent results. The reason for the inconsistency was radioactivity losses during washing of the cells after incubation. The following two methods proved to be more satisfactory for the study of the SO_2 transport in this yeast:

a. Indirect method. 0.4 ml of a dense cell suspension containing about 4.8×10^8 cells of S. cerevisiae var. ellipsoideus were incubated with 0.4 ml of a solution containing the desired concentration of labelled SO_2 and 0.2 ml McIlvain's buffer (0.25 M citric acid and 0.5 M disodium

phosphate). The incubation was terminated by filtering the cell suspension through 0.6 μ Millipore filter, 1.4 cm in diameter. With this technique approximately 0.3 ml of clear filtrate could be obtained in 2 to 4 sec.

The radioactivity present in the filtrate was measured by placing 0.2 ml of the filtrate in a polyethylene vial, containing 15 ml of a dioxane base scintillation liquid. The scintillation liquid was prepared by diluting in 2.7 liters 1,4-dioxane, 300 ml of absolute ethanol, 21 g of 2,5-diphenyloxazol, 750 mg 1,4 bis-2-(5 phenyl-oxazolyl) benzene and 375 g of naphthalene. The vials were counted in a Packard Tri - Carb liquid scintillation counting system, model 3310.

In the indirect method it was necessary to determine the volume occupied by the cells and the intercellular space. In determining cell volume and intercellular space a procedure similar to that followed by Black and Gerhardt (1961) was employed. Water-suspended yeast cells were distributed into four tared 35-ml round-bottom glass centrifuge tubes and packed at 15,000 xg for 30 minutes in a clinical centrifuge at 5°C. The results of packing are illustrated in Figure 2. Detailed values are presented in Appendix, Table 1.

This curve determines the plateau region between time and centrifuge force. This plateau region was used for all cell packing experiments carried out in this study. Four

ml of water-suspended yeast cells, representing an average dry weight of 200 mg, were packed at 15,000 xg for 30 min at 5°C. Four replicates were used. After centrifugation the supernatant water was decanted and the inside of tubes were wiped free of water with absorbent tissue. Weighed to the nearest 0.0001 gr, each tube contained about 1 gr of packed yeast cells. Following packing, the cells were resuspended in 3 ml of a 3% water solution of dextran (M.W. 500,000). This type of dextran does not penetrate the yeast cell wall, because of its high molecular weight. The cells, after being suspended in the dextran solution, were agitated vigorously with the aid of a LAB-LINE supermixer for 1 minute at 5°C in order to allow equilibration and finally repacked at 15,000 xg for 30 minutes. The supernatant fluids were decanted into centrifuge tubes, clarified by additional centrifugation and assayed for dextran concentration. This assay was carried out by measuring the refeactive index in a Bausch & Lomb refractometer at constant temperature (27°C). The degree of dilution of dextran solution due to intercellular water was calculated by the means of a standard curve of dextran concentration vs refractive index. The results appear in Table 2. Detailed results appear in Appendix, Table 2.

The amount of SO_2 transported was calculated from the equation:

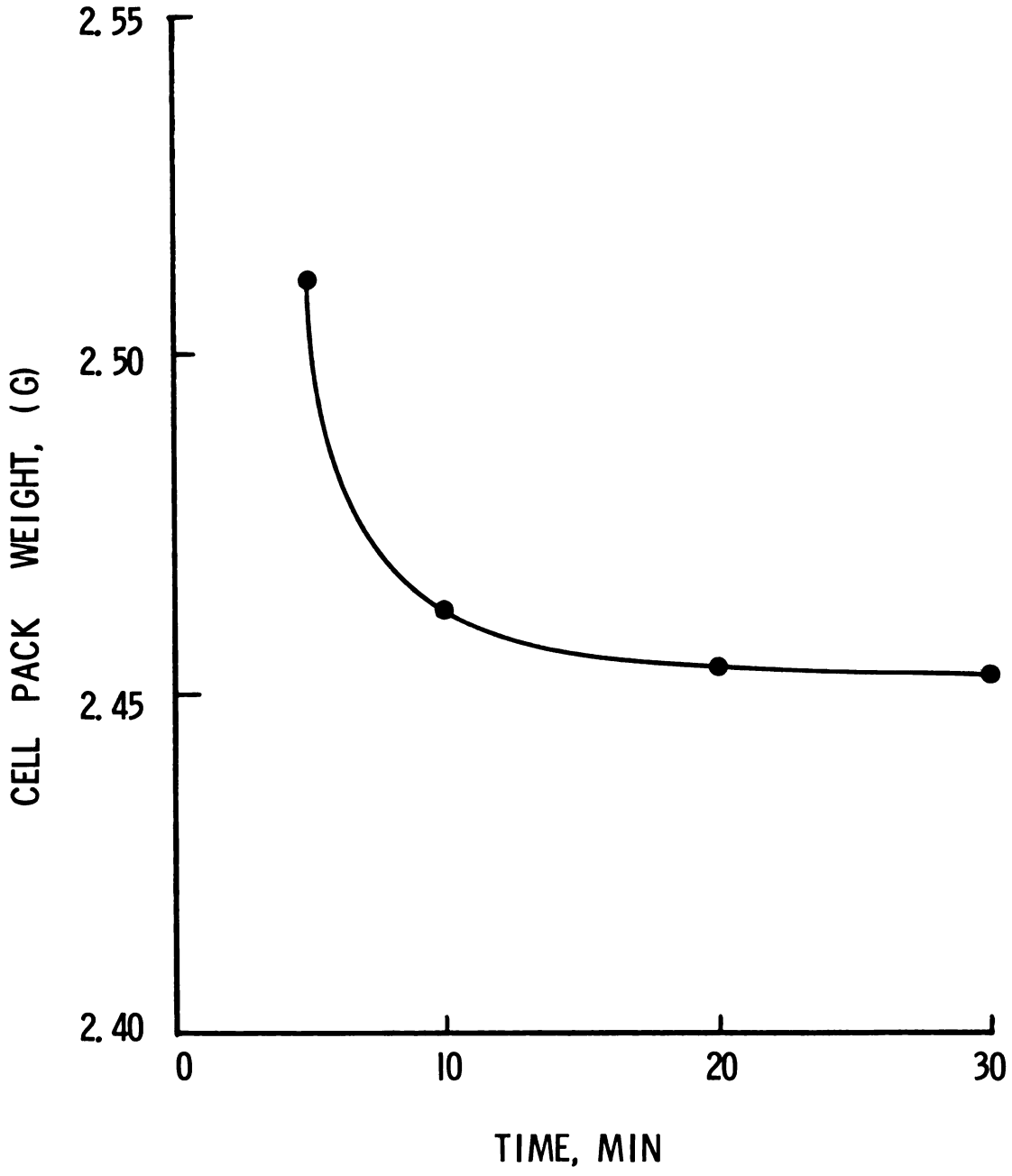

Figure 2. Packing rate of S. cerevisiae var. ellipsoideus cells. Water suspended cells were packed by centrifugation at 15,000xg at different times.

Table 2. Determination of pellet intercellular space and cell volume of S. cerevisiae var. ellipsoideus. Cells suspended in 3% dextran (M.W. 500,000 solution were packed at 15,000xg at 5°C.

Weight of cell pack (g)		Intercellular volume of cell pack (cm^3)	Cells		
			Weight (g)	Volume (cm^3)	Number
Wet	Dry				
1.0107	0.1926	0.3211	0.6896	0.626	4.8×10^9

$$\% = \frac{A - B}{A} \times 100$$

where A represents the amount of radioactivity present in the control and B represents the amount of the radioactivity present in the reaction mixture filtrate.

b. Direct method. In order to prove that cells do take up SO_2 the following direct measurement of the transported SO_2 into the cells was employed. The cells were incubated as in the indirect method with the difference that the cell population was 3 or 4 times smaller than that in the indirect method in order to separate the cells from the incubation mixture in less than 4 seconds. After incubation the radioactivity of the cells plus the filter plus the intercellular liquid was measured by dissolving the mixture in 1 ml of Soluene-100™, a commercial solubilizer and using the scintillation liquid described for the indirect method.

The amount of radioactive SO_2 transported in the direct method was calculated from the equation:

$$T = \underbrace{(C - Y) \times F}_{\text{radioactivity present in the filter}} + \underbrace{(C - Y) \times V}_{\text{radioactivity present in the intercellular space}} + Y$$

where T represents the amount of radioactivity present in the filter plus cells plus intercellular space, C the amount of radioactivity present in the control (incubation medium without cells), V the intercellular volume of the yeast cells after filtration, Y the radioactivity present in

cells and F the precentage of radioactivity retained by the fiber. Solving equation (I) for Y gives:

$$Y = \frac{CF+CV-T}{F+V-1}$$

From this equation the radioactivity present in the cells was calculated.

In order to compare the direct and indirect method, cells were incubated with SO_2 at pH=3.8 for 5 and 10 min with each method. The results appear in Table 3, and show that the difference between the direct and indirect method is not significant. Detailed data appear in Appendix, Table 3. However, the indirect method was used in subsequent experiments for the following reasons:

1. Simplicity. The only parameters to be measured are the volume of cells and the radioactivity of filtrate, whereas in the direct method the cell and filter solubilization step complicates the procedure.

2. Precision and accuracy. The possibility of errors caused by differences in the radioactivity retained by the filter and the intercellular space and the losses due to the adhesion of cells on the walls of the container and filtering apparatus decrease the precision and accuracy of the direct method.

Table 3. Comparison between direct and indirect method in measuring transport in S. cerevisiae var. ellipsoideus. Cells representing a dry weight of 20 mg (4.8×10^8 cells) were incubated in 1 ml of 10^{-3} M SO_2 at pH=3.80 and $20^{\circ}C$.

Method	Incubation time (min)	Counts per minute		Uptake %
		Control	Expt	
Direct	5	3,417	751	21.97
	10	3,417	779	22.79
Indirect	5	4,475	1,041	23.26
	10	4,475	979	21.87

CHAPTER IV

RESULTS AND DISCUSSION

Characterization of SO_2 Uptake by Yeast Cells

Oka (1964) considered that the uptake of acid preservatives was the result of adsorption of those antiseptics on the cell surface. Other investigators, however, have demonstrated that SO_2 inhibits microbial growth at the level of respiration and fermentation. Therefore it should enter the cell (Meyerhof et al., 1938; Pfleiderer et al., 1956; Rehm, 1964). In order to determine whether SO_2 enters the cell or absorbs on the cell surface, yeast cells representing 200 mg dry weight were incubated for 5 minutes with 10^{-5} moles SO_2 at pH=4.11. After incubation the cells were washed with distilled water five times until the recovered radioactivity in the supernatant approached background radiation. The cells, after being washed, were mixed with 20 grams of glass beads (0.5 mm diam.) into a previously chilled metal cylinder and shaken for 60 seconds in a mechanical cell homogenizer. Microscopic examination revealed a small portion of broken cells. The homogenate was centrifuged at 3,000 xg for 3 minutes to separate the

beads. Further centrifugation at 15,000 xg for 5 minutes yielded a supernatant free of membrane which was assayed for radioactivity. The results appear in Table 4. Detailed results are presented in Appendix, Table 3. The recovered radioactivity in the supernatant is a direct proof that SO_2 is transported inside the cells.

Indirect evidence that SO_2 is not adsorbed on the cell surface is that metabolic inhibitors, not reacting chemically with SO_2, inhibit the uptake of SO_2. If SO_2 was taken up by adsorption, the use of metabolic inhibitors should not interfere with the adsorption process.

Measurement of Sulfur Dioxide Pool

In studying a transport system it is important to determine whether the substrate leaves the cell, and to what extent, after its entry into the cell. The phenomenon of counterflow was predicted by Widdas (1952) and verified by Rosenberg and Wilbrandt (1957) in their studies of sugar movement across erythrocyte membranes. Dreyfuss (1963) has also described an outward movememt of sulfate upon washing of Salmonella typhimurium cells, loaded with $SO_4^{=}$.

A certain portion of the radioactivity acquired by cells exposed to $^{35}SO_2$ can be removed by washing (Table 5).

Table 4. Fate of $^{35}SO_2$ upon incubation with S. cerevisiae var. **ellipsoideus**. The reaction mixture contained 200 mg cells (dry weight) suspended in 10 ml of 10^{-5} M solution at pH=4.11. Repeated washing resulted in background radiation in the supernatant. The cells were then partly fractionated in a cell homogenizer and after centrifugation (15,000 xg; 5 min), the radioactivity in the supernatant was assayed.

Operation	Counts per 10 min	%
$^{35}SO_2$ removed in the original incubation	240,544	56.74
^{35}S recovered in:		
First wash	57,973	24.10
Second wash	11,598	4.82
Third wash	2,022	1.36
Fourth wash	83	0.03
Fifth wash	81	0.03
Supernatant of broken cells	2,778	1.53

Table 5. Effect of washing of cells of S. cerevisiae var. ellipsoideus after incubation with $^{35}SO_2$. The reaction mixture contained 250 mg of cells (dry weight) suspended in 10 ml of 10^{-5} M SO_2 solution. Incubation time 10 min. The volume of cells was about 0.6 ml and the volume of H_2O used in each washing was 10 ml.

Type of operation	pH=3.19	pH=3.32	pH=4.11
% $^{35}SO_2$ removed in the original incubation	68.01	51.48	16.24
$^{35}SO_2$ (as % of that removed in the original incubation) recovered in:			
First wash	15.77	15.59	17.65
Second wash	8.97	6.55	0.90
Third wash	6.91	2.11	0.22
Fourth wash	1.72	0.15	0.22
Fifth wash	0.48	0.09	0.29
Sixth wash	0.09	0.08	0.19
Seventh wash	0.06	0.08	0.25
Eighth wash	0.05	0.08	0.21
Ninth wash	0.05	0.09	0.20
Tenth wash	0.06	0.08	0.25
Total $^{35}SO_2$ recovered	34.16	24.82	20.38
Total $^{35}SO_2$ remaining in cells after ten washes (as % of that removed in the original incubation)	44.78	38.71	12.93

Qualitative analysis by the method of Scoggins (1970) revealed the presence of SO_2 in the cell washes. The amount of SO_2 recovered in 10 washes as well as the amount of residual SO_2 remaining in the cell after these washes depended upon the pH of the incubation medium. In Appendix, Table 4, detailed values are presented.

SO_2 Transport as a Function of Time

Yeast cells suspended in solution of acid antiseptics especially benzoic acid caused decreases in the concentrations of these antiseptics in the solution (Oka, 1964). In the case of benzoic acid the rate of decrease was very high and the concentration attained an equilibrium stage in less than 5 min. Figure 3 shows the transport of SO_2 as a function of time. Detailed values are presented in Appendix, Table 5. The amount of SO_2 transported in 5, 10, 20, 40, and 60 minutes was practically the same. The yeast reached their maximum load of SO_2 in less than 5 minutes. The pattern of transport was dimilar to that found by Oka (1964) for benzoic acid.

Cells were incubated with labeled SO_2 for 10, 20, 40, 60, 300 and 600 seconds. The initial rate of uptake was very rapid and seemed to follow a linear course up to about 10 seconds (Fig. 4). Beyond this time the uptake started leveling off and reached maximum value in about 2

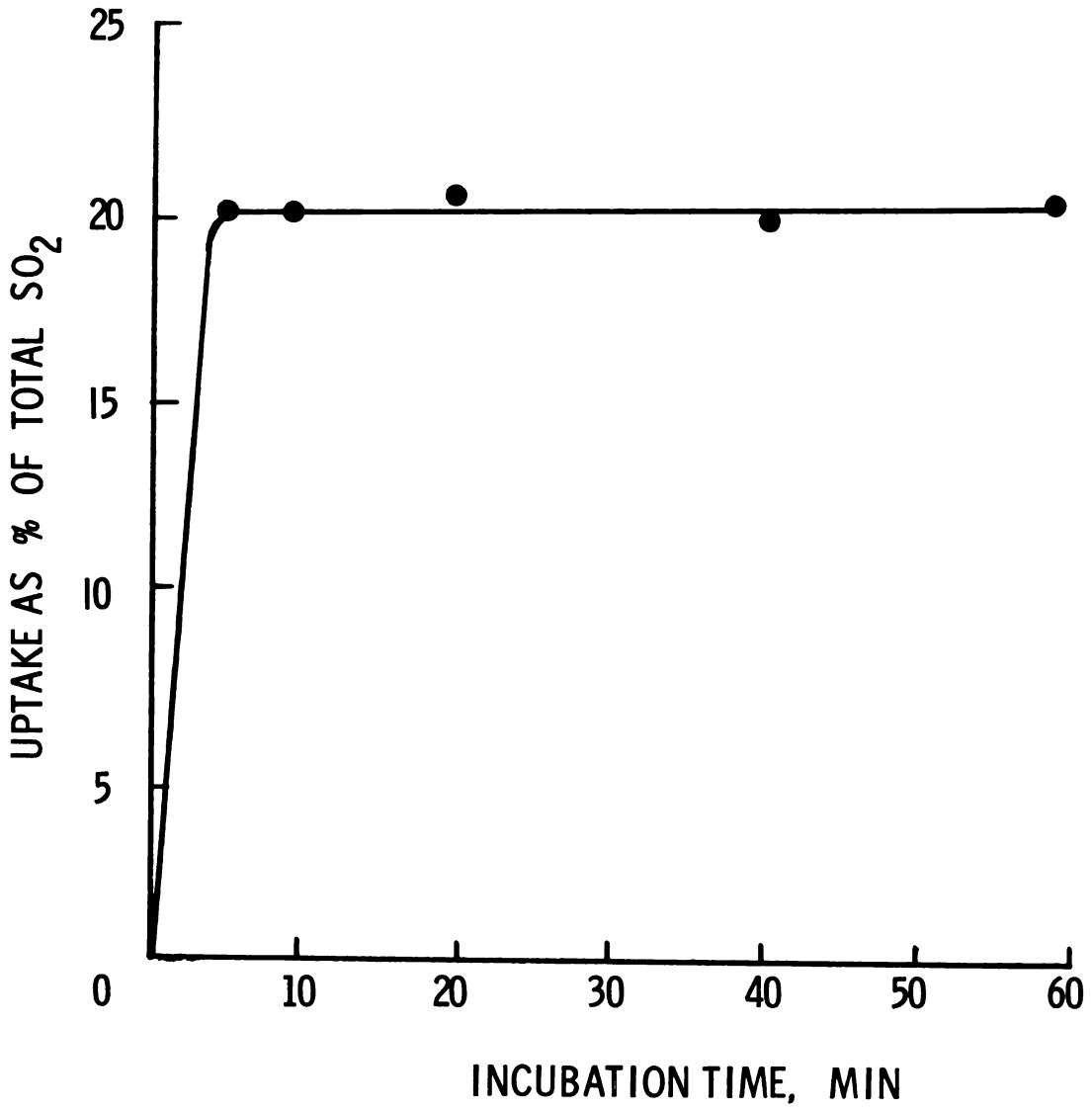

Figure 3. Transport of SO_2 in S. cerevisiae var. ellipsoideus as a function of time. The reaction mixture contained 20 mg of cells (dry weight) or 4.8×10^8 cells suspended in 1 ml of 10^{-3} M SO_2 solution adjusted at pH = 3.80 and 20°C.

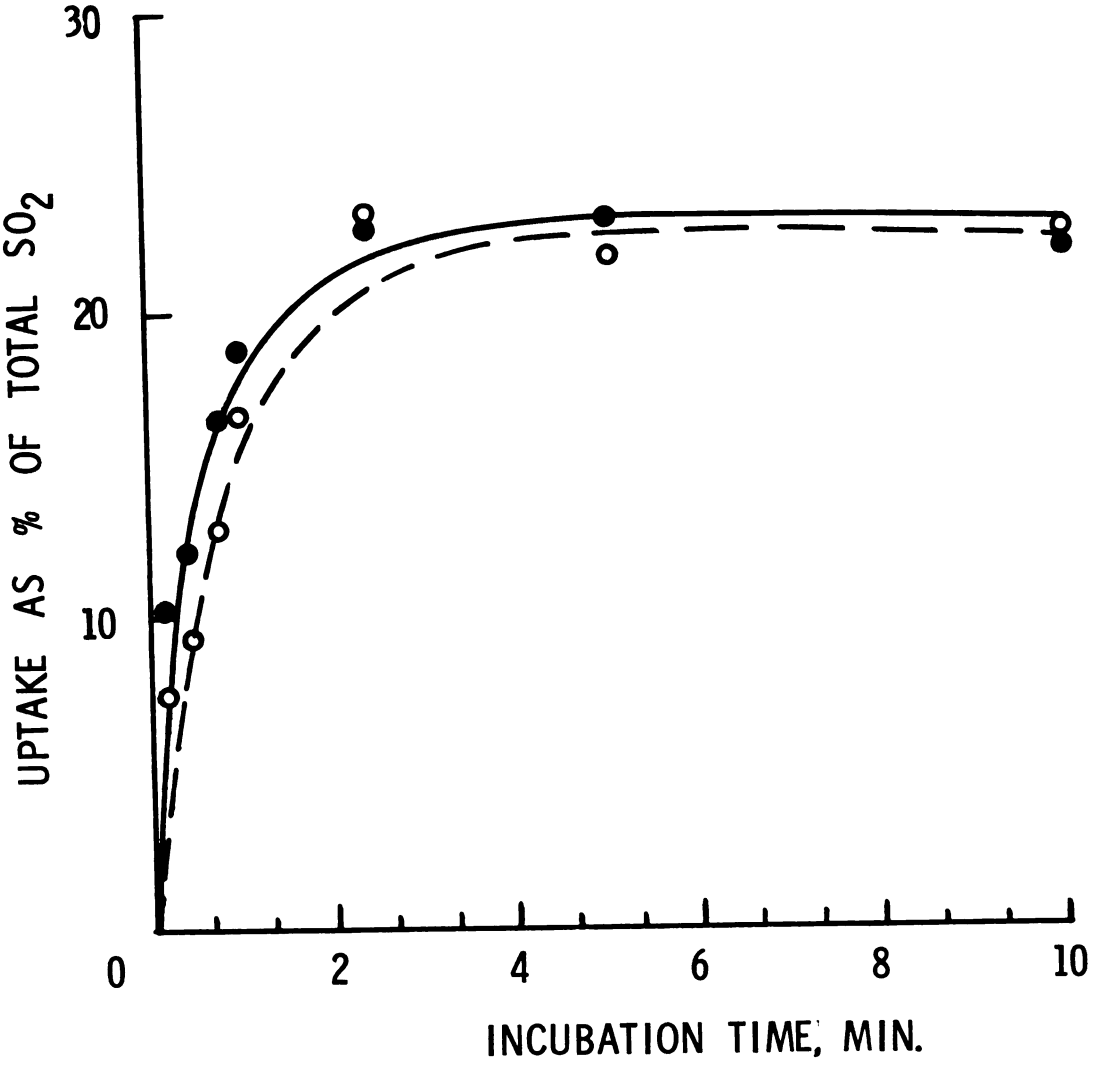

Figure 4. Time course of SO_2 transport in the yeast S. cerevisiae var. ellipsoideus. Cells representing a dry weight of 20 mg (4.8×10^8 cells) were incubated in 1 ml of 10^{-3}M SO_2 solution at pH=3.80 and 20°C. The direct (dotted line) and indirect (solid line) methods were used in measuring uptake.

minutes. After maximum load was attained the SO_2 pool remained at a constant level. In Appendix, Table 6, detailed results are presented.

Identification of SO_2 Transport System

Compounds in solution may undergo a net movement from outside to inside the cell and vice versa by either non-mediated or mediated processes. The non-mediated movement has two characteristics derived from the phenomenon of simple diffusion.

a. The transported compound does not change throughout the transport process.

b. The rate of transport is linearly dependent upon the concentration of the compound being transported.

The mediated transport has a number of identifying characteristics. Those characteristics are often used as experimental criteria and can be studied by measurements of the kinetics of transport through the cell membrane.

In identifying the SO_2 transport in the yeast S. cerevisiae var. ellipsoideus the following criteria were used:

A. Kinetic characteristics:

1. SO_2 transport as a function of SO_2 concentration. While in the case of non-mediated transport the rate of transport is linearly dependent upon the concentration of the compound being transported, mediated transport shows saturation kinetics.

Sulfur dioxide transport in this yeast displayed saturation kinetics, that is the SO_2 transport sites were saturated just as enzymes became saturated with their substrates (Fig. 5). Detailed values are presented in Appendix, Table 7. Plotting of the initial (or close to the initial) rate of SO_2 transport vs SO_2 concentration showed a hyperbolic curve which approached a maximum and was similar to the Michaelis - Menten curve of an enzyme. The rate of transport between 0 and 10^{-2}M SO_2 concentration seemed to be proportional to SO_2 concentration and it was of first order. Beyond this concentration the rate of transport started leveling off up to the point where it became zero order with respect to SO_2 concentration. The pattern of this transport suggested the presence of carriers containing specific sites to which SO_2 bound.

A double reciprocal plot according to the method of Lineweaver - Burk (Fig. 6) gave a straight line with an apparent K_m - 2.65 ± 0.40 x 10^{-2}M and V_{max} = 6.95 ± 0.39 x 10^{-3} M/ 10 sec. The meaning of these two constants in the process of SO_2 transport through the plasma membrane is the following:

The V_{max} expresses the maximum rate of transport that the yeast cell can demonstrate toward SO_2 and depends upon the number and the physiological state of these cells.

The K_m is independent of the number of cells present

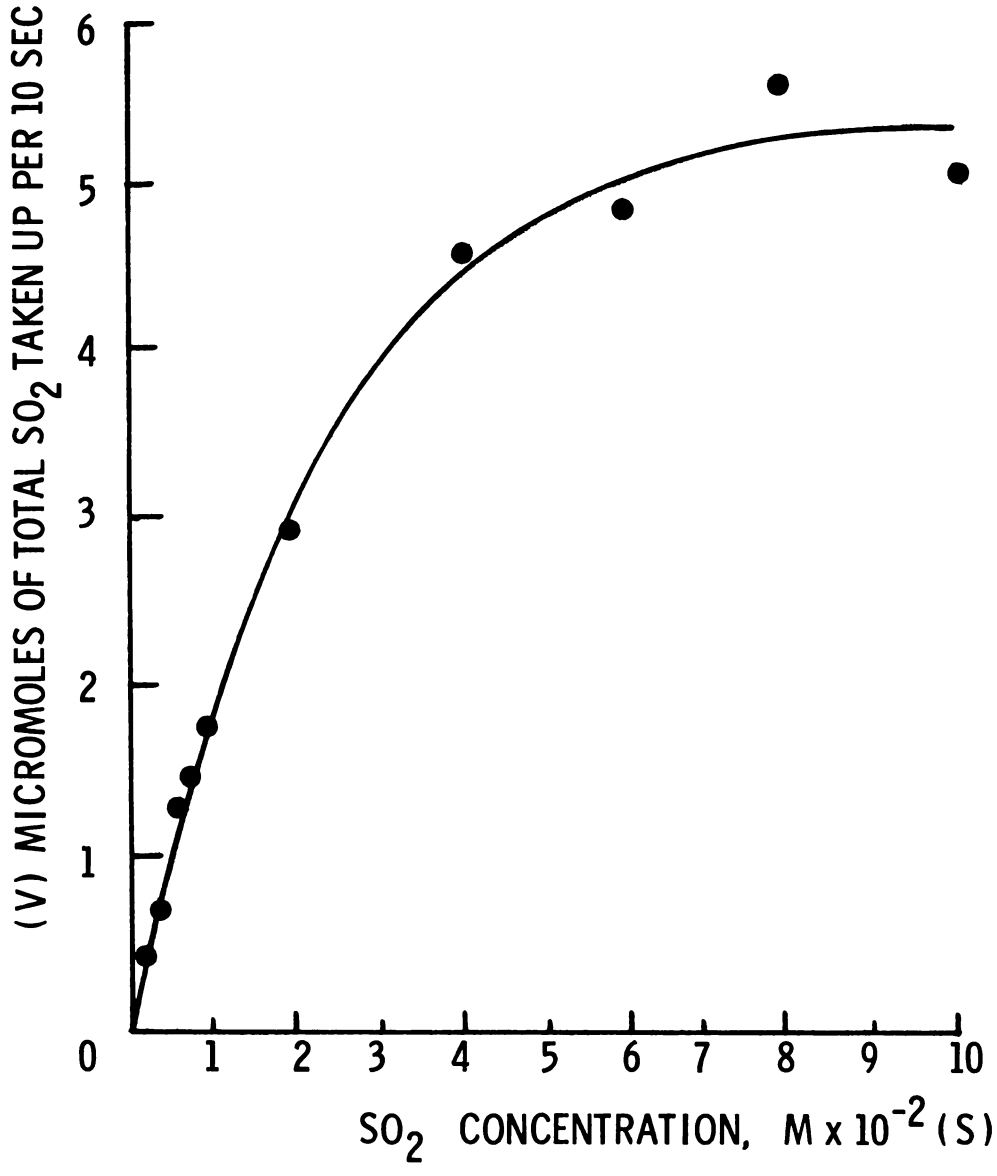

Figure 5. Sulfur dioxide transport in S. cerevisiae var. ellipsoideus as a function of SO_2 concentration. The reaction mixture contained 20 mg of cells (dry weight) or 4.8×10^8 cells suspended in 1 ml SO_2 solution adjusted to pH=3.80. Incubation time: 10^2 sec. at 20°C.

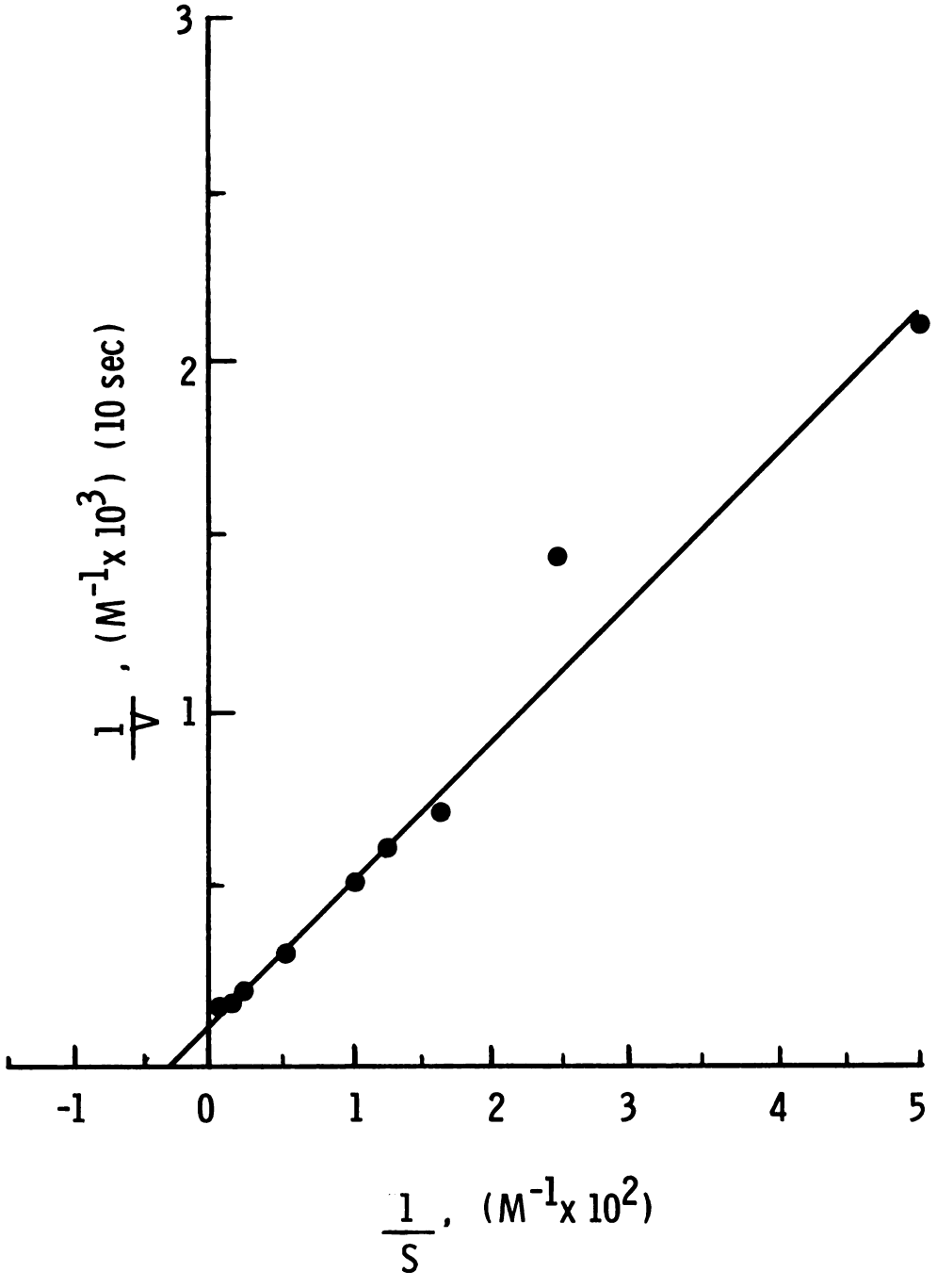

Figure 6. Lineweaver-Burk plot of SO_2 transport in S. cerevisiae var. ellipsoideus.

and expresses the SO_2 concentration at which the rate of transport is one-half of maximum rate of transport (V_{max}).

2. Effect of temperature on SO_2 transport. Another kinetic characteristic of SO_2 transport is the effect of temperature. In the non-mediated transport process the temperature coefficient is usually that of physical diffusion, namely 1.4 per 10°C rise in temperature (Lehniger, 1970).

The pattern of SO_2 transport is affected by temperature as it is shown in Figure 7. Detailed results are presented in Appendix, Table 8. It is clear that the temperature at which the transport system operates has a very pronounced effect on the rate of SO_2 transport in this yeast. Between 0 and 20°C there is no significant difference in the rate of transport. The rate increases sharply with temperature in the range 20-50°C with a maximum temperature coefficient of 2.4. Above the optimum temperature, 50°C, the rate of transport decreases rapidly. At 70°C the transport process is very slow. The effect of temperature on SO_2 transport displays a pattern similar to that observed in enzyme kinetics.

The inactivation of SO_2 transport system is not reversible. Cells were subjected to different temperatures for two minutes and after cooling at 20°C, the SO_2 transport at that temperature was measured. The results appear in Figure 8 and show that the pattern of the SO_2 transport inactivation by temperature, besides being irreversible,

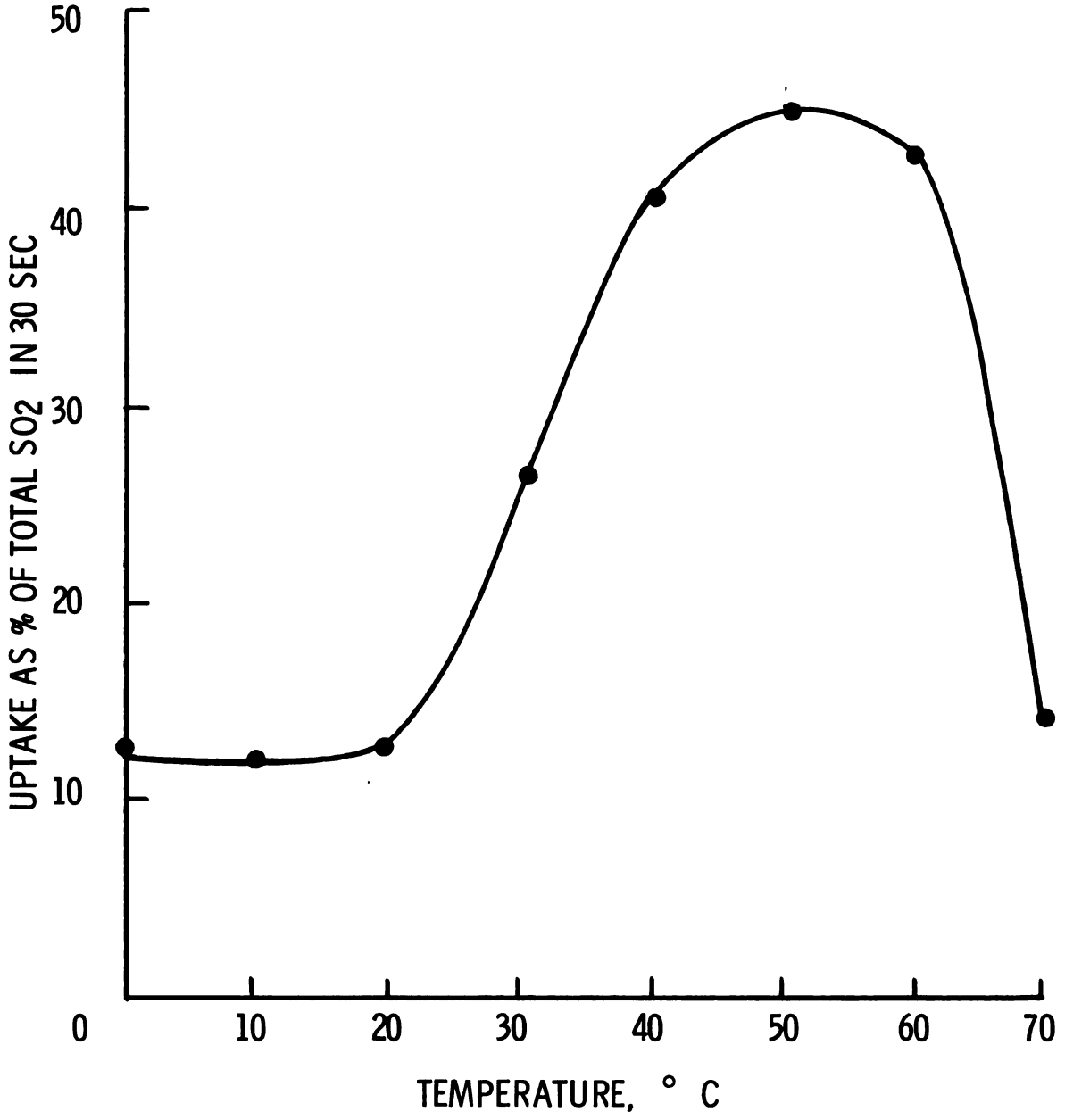

Figure 7. Effect of temperature on SO_2 transport in S. cerevisiae var. ellipsoideus. Cells, 4.8×10^8, 20 mg dry weight were incubated in 1 ml of 10^{-3} M SO_2 at pH=3.80 for 30 sec at different temperatures.

appears to be similar to the pattern of protein denaturation by temperature. In Appendix, Table 9 detailed results are presented.

B. Specificity of transport system.

The specificity of the transport system for the compound transported constitutes the second criterion of mediated transport. The degree of specificity of mediated transport systems starts from structural specificity to stereospecificity. It has been postulated that mediated transport systems reside in membranes and contain binding sites complementary to the compound transported. This process resembles in its specificity the active site of enzyme molecules.

The SO_2 transport system in the yeast S. cerevisiae var. ellipsoideus displays a degree of structural specificity transporting from a mixture of bisulfite (HSO_3^-), sulfite ($SO_3^=$) and the molecular SO_2 only the latter form. The mechanism and results on the transport of molecular SO_2, HSO_3^- and $SO_3^=$ will be discussed later on.

C. Inhibition of transport system.

This is the third criterion of a mediated transport system. In addition to competitive and non-competitive inhibition of transport systems, inhibitors that affect energy metabolism abolish the accumulation of the transported compound inside the cell (Winkler and Wilson, 1966).

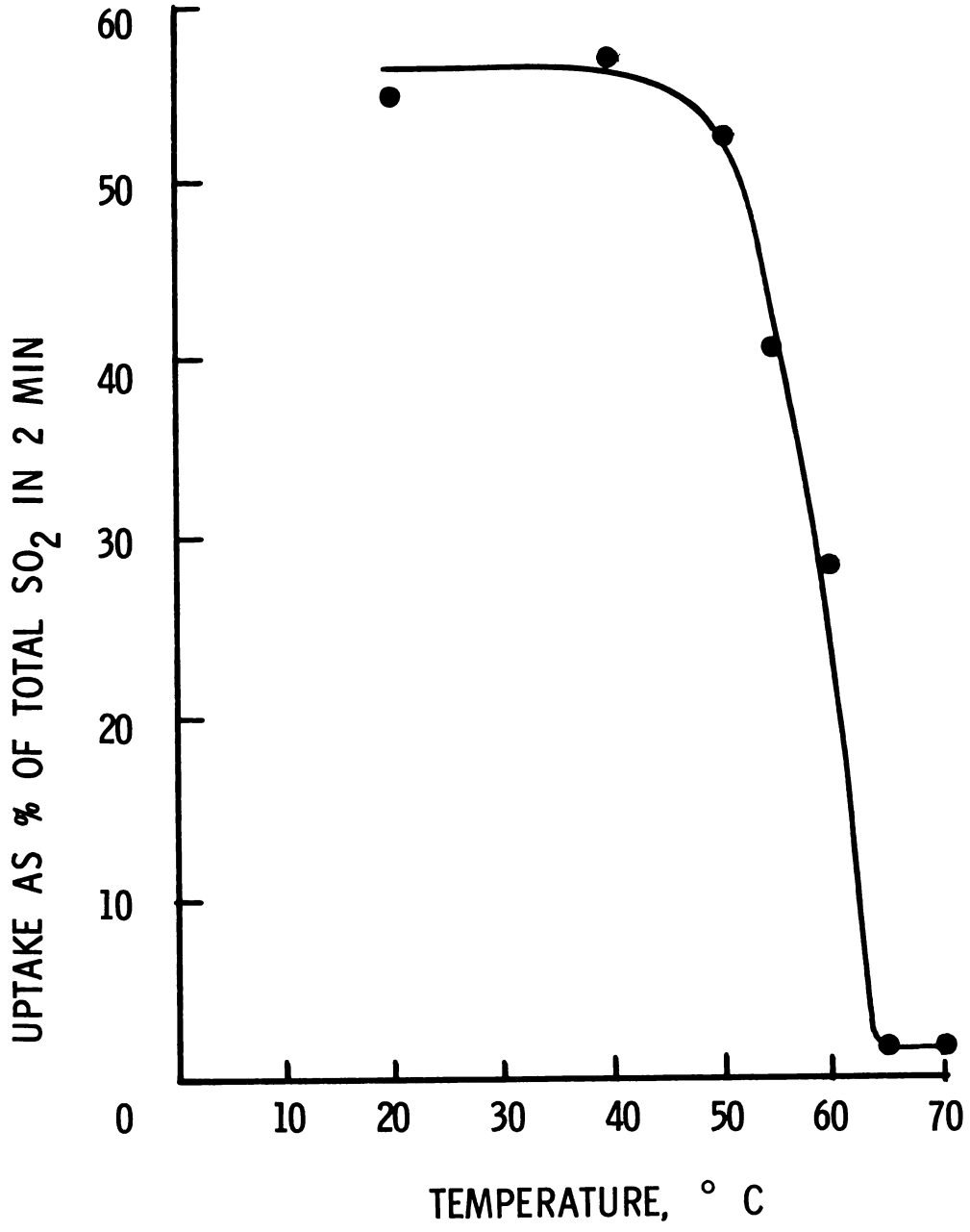

Figure 8. Thermal inactivation of the SO_2 transporting system in S. cerevisiae var. ellipsoideus. Cells representing a dry weight of 20 mg (4.8×10^8 cells) were subjected to different temperatures for 2 minutes and then cooled at room temperature ($20^{\circ}C$). The SO_2 transport was studied by incubating these cells in 1 ml of 10^{-3} M SO_2 at pH=3.19, $20^{\circ}C$, for 2 min.

Inhibitors that block the formation or utilization of high energy phosphate bonds inhibit transport. Details on this inhibition will be discussed later.

All the above criteria characterize SO_2 transport as a mediated transport system. These systems may be divided into the following two types:

Facilitated diffusion, which does not require energy since it merely catalyzes equilibration of solute across the mambrane.

Energy-coupled permeability or active transport, which requires energy since it proceeds against the concentration gradient.

In order to determine whether the SO_2 transport is of the facilitated or energy-coupled type (active) of transport, the following criteria were used:

1. The first criterion refers to whether or not SO_2 transport proceeds against the concentration gradient. This can be easily determined from Figure 4. The transported SO_2 is much higher than that justified by the cell volume. Therefore the transport of SO_2 proceeds against the concentration gradient in this yeast and this is a criterion of active transport. However, this criterion requires not only precise knowledge of the SO_2 concentration inside and outside the cell but also an assurance that the transported substance exists in the same molecular species inside and outside the cell. Since the latter requirement

is very difficult to establish from the analytical measurements alone, the criterion of concentration gradient is not always useful for proving the existence of active transport. In the case of SO_2 transport this is even more difficult because of the complications due to differences in pH inside and outside the cell.

2. The second criterion to characterize whether or not the mediated transport is facilitated diffusion or active transport can be expressed in two ways:

a. The inhibition can produce its effect by acting on the metabolic system involved in the supply of energy for transport or the maintenance of the membrane. Because active transport proceeds against the concentration gradient it must account for thermodynamic work and therefore it should be hooked into some energy producing systems such as the respiration or glycolysis. Inhibitors that block those sources of metabolic energy must also affect active transport.

b. The inhibitor can act on the membrane or transport system directly. Inhibitors reacting with SH groups can inhibit transport by acting on membranes, the protein portion of which contain SH groups, probably important in maintaining the integrity of membranes.

Active transport processes may, for convenience, be considered as consisting of two parts:

I. Exergonic reactions providing the energy required, usually oxidation coupled with ATP production.

II. Endergonic reactions by which the substrate is transported and the energy is utilized (the functional transport system itself).

Inhibitors that affect transport by reducing ATP production (e.g. 2,4-dinitrophenol, sodium azide, etc.) can be considered as exerting a rather nonspecific effect with respect to transport, since any cell function requiring energy will be depressed eventually.

Inhibitors that affect the endergonic system may be more selective, blocking transport and leaving other cell functions intact.

Four metabolic inhibitors, at three different concentrations, were used in the study of SO_2 transport in S. cerevisiae var. ellipsoideus. These inhibitors were found not to react with SO_2 chemically. The results appear in Table 6. In Appendix, Table 10, detailed values are presented.

2,4-Dinitrophenol is one of the best known uncoupling agents of oxidative phosphorylation inhibiting production of ATP from ADP (Lehmniger, 1970). Sodium azide has multiple effects, acting as inhibitor of electron transport, as an uncoupling agent like 2,4-dinitrophenol, and as a phosphorylation blocking agent like oligomycin (Robertson

Table 6. Effect of metabolic inhibitors on SO_2 transport in S. cerevisiae var. ellipsoideus. Cells of 20 mg dry weight were suspended in 1 ml of 10^{-3} M SO_2 containing the quantities of the inhibitor appearing in this Table. The pH was 3.19, 20°C.

Inhibitor	Inhib. concentration (M)	% Inhibition
2,4-Dinitrophenol	5×10^{-4}	18.54
	10^{-3}	30.48
	5×10^{-3}	31.28
$HgCl_2$	10^{-3}	66.86
	5×10^{-3}	103.51
	10^{-2}	104.72
Iodoacetamide	10^{-2}	1.69
	5×10^{-2}	1.41
	10^{-2}	1.66
Sodium azide	10^{-2}	40.04
	5×10^{-2}	64.85
	10^{-1}	72.29

and Boyer, 1955). Both 2,4-dinitrophenol and sodium azide inhibit the production of ATP. Both have a significant effect on SO_2 transport. This effect depends upon the inhibitor concentration.

Iodoacetamide and mercuric chloride are enzyme inhibitors that react with SH groups. Iodoacetamide frequently does not react with SH as readily as mercuric chloride does (Webb, 1966). As far as energy production is concerned, iodoacetamide blocks glycolysis at the level of 3-phosphoglyceraldehyde oxidation and prevents glycolytic generation of ATP.

In essence, iodoacetamide did not inhibit SO_2 transport. Since this inhibitor reacts selectively with SH groups (Webb, 1966) the possible explanation concerning its inability to affect SO_2 transport is that it does not react with SH groups present in the transport system, therefore, leaving the endergonic system intact. On the other hand, since the yeast cells were grown aerobically, the glycolytic generation of ATP was low in comparison to the ATP coming from the respiratory mechanism (Pasteur effect) and therefore one should expect that the presence of iodoacetamide would not have any effect.

Mercuric chloride inhibited SO_2 transport completely. Since it was found that mercuric chloride is a very strong enzyme inhibitor (Webb, 1966) one would assume that mercuric

ions affect the endergonic system of SO_2 transport by reacting with SH groups.

Of these 2 criteria for an active transport system, the movement of SO_2 against a concentration gradient is difficult to defend, since no definite information exists regarding the pH on the cell side of the cell membrane. If the pH inside the cell is high (6 or 7) the cytoplasm could act as a sink for the transported SO_2 by converting it to disulfite and sulfite. The effect of inhibitors does point to an active transport although 2,4-dinitrophenol should have inhibited the SO_2 transport to a greater extent at the concentrations used. At any rate, the possibility of simple diffusion is excluded on the basis of the above findings.

Transport of Molecular SO_2, HSO_3^- and $SO_3^=$

Once it was established that SO_2 was transported inside the yeast cell the question was raised whether all three forms of SO_2 in solution participate in the transport or only one or two.

It has been known that the molecular form of SO_2 is the most toxic of all three forms and predominates at low pH values. Therefore, it was deemed desirable to study it, first from the transport viewpoint. Figure 9 shows the transport of SO_2 at four pH values and clearly demonstrates

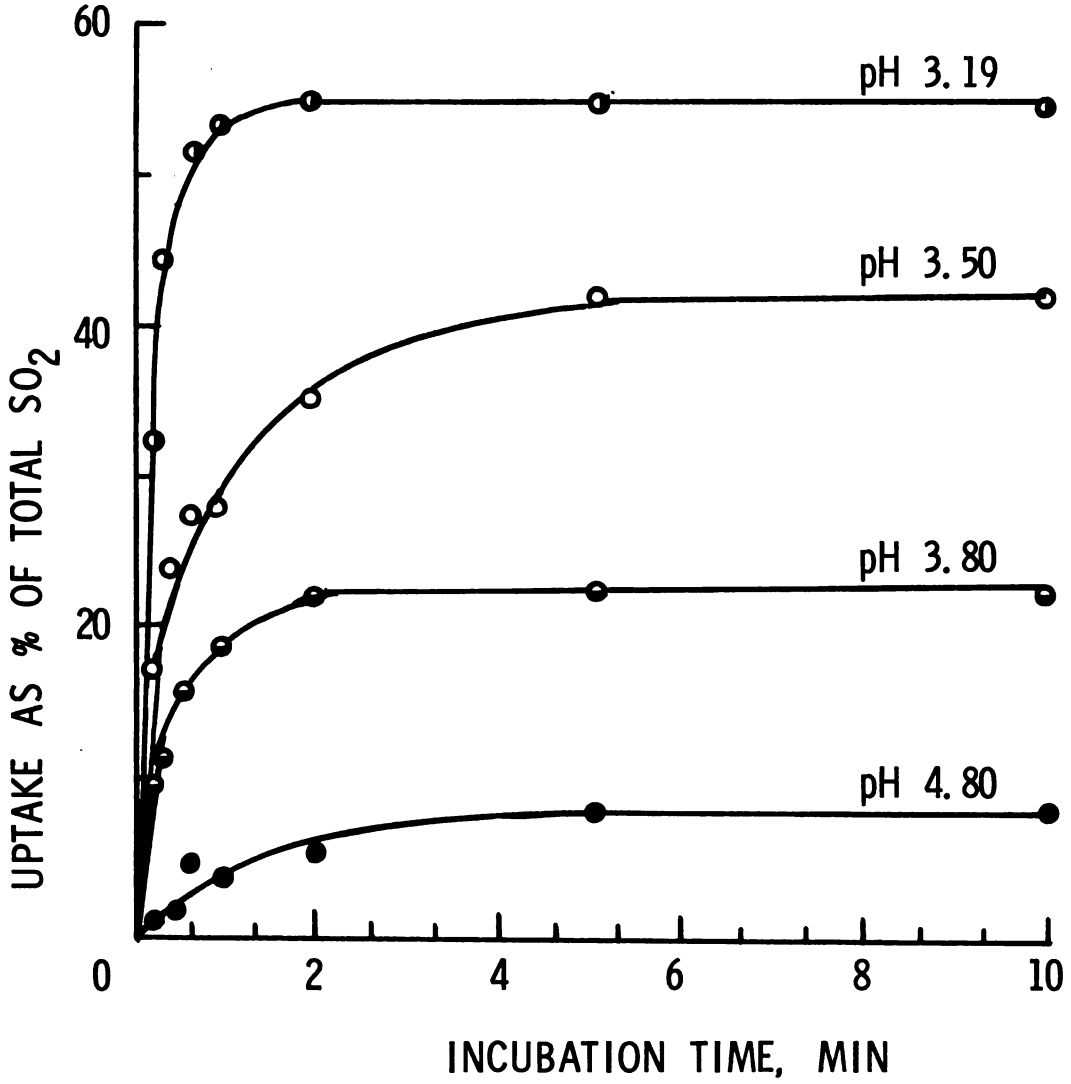

Figure 9. Time course of SO_2 transport in S. cerevisiae var. ellipsoideus as a function of pH. The reaction mixture contained 20 mg cells (dry weight) or 4.8×10^6 cells suspended in 1 ml of 10^{-3} M SO_2 and adjusted at different pH values. Incubation time: 10 sec. at room temperature ($20^{\circ}C$).

that lower pH values increase the SO_2 transport. Detailed values are presented in Appendix, Table 11. The amount of molecular SO_2 present at pH 3.19, 3.50, 3.80 and 4.80 were 4, 2, 1 and 0.1%, respectively.

The effect of pH on transport may be due to the following:

a. The SO_2 transport system is affected by pH directly, thereby influencing the transport itself.

b. The SO_2 transport system is not affected by pH directly; instead, the pH dependent distribution of SO_2 forms in solution is responsible for the change in SO_2 transport.

In order to determine whether the effect of pH is direct or indirect two experiments were carried out as follows:

1. Yeast cells were incubated with labeled SO_2 for 10 sec at five pH values and the rate of transported total SO_2 was plotted against the amount of molecular SO_2 present at those pH values. The results appear in Figure 10. Detailed data appear in Appendix, Table 12.

2. The cells were incubated at the same pH values as above but the total SO_2 concentration was adjusted so that the same concentration of molecular SO_2 at each pH value was obtained. The amount of transported total SO_2 in 10 sec was measured and the results appear in Figure 11. In Appendix, Table 13 detailed values are presented.

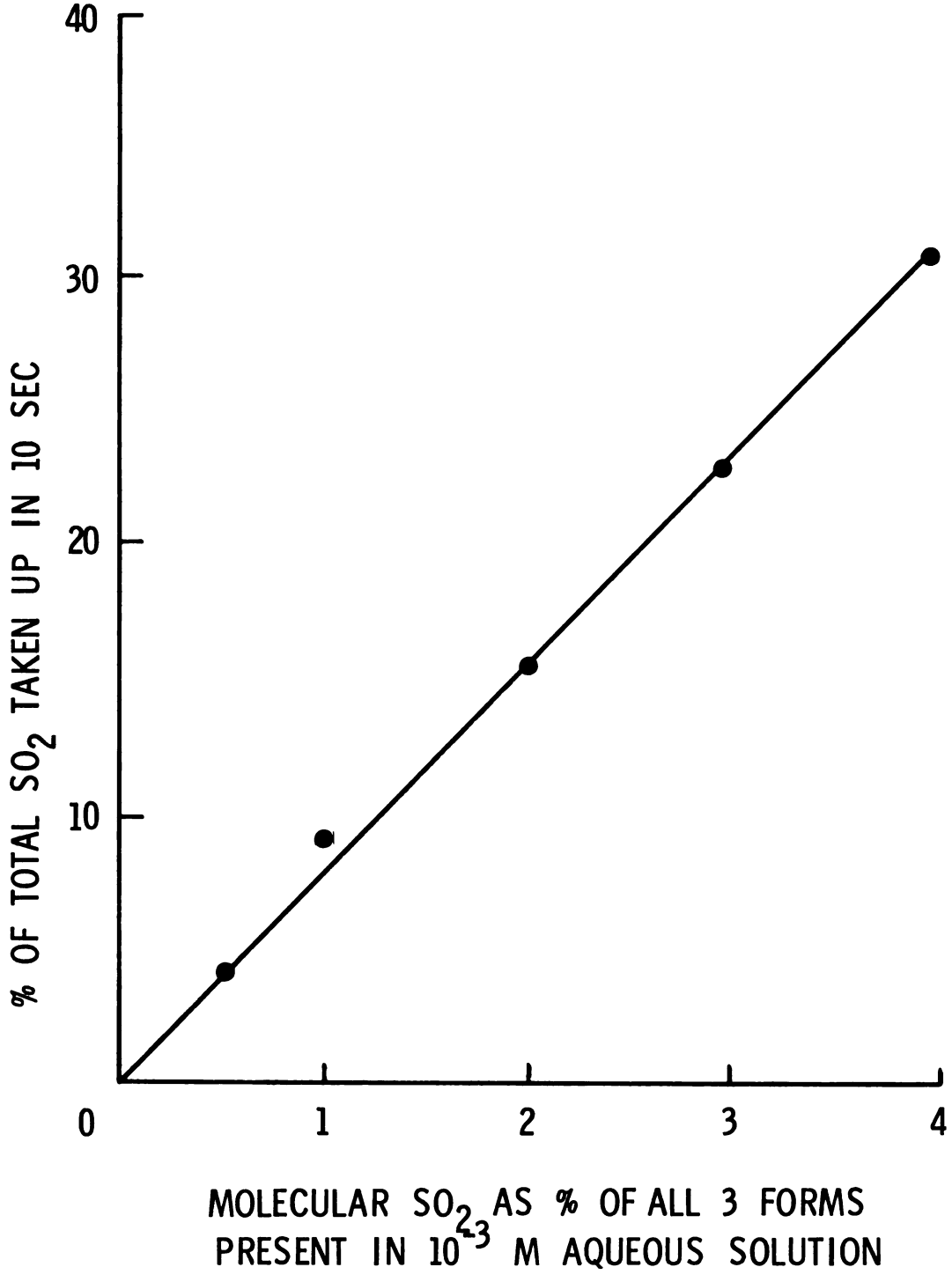

Figure 10. Relationship between rate of SO_2 transport in S. cerevisiae var. ellipsoideus and amount of molecular SO_2. Cells of 20 mg dry weight (4.8×10^8 cells) were incubated in 1 ml of 10^{-3} SO_2, for 10 sec. and at pH 4.11, 3.80, 3.50, 3.32 and 3.19. The amount of molecular SO_2 at those pH values was 0.5, 1.0, 2.0, and 3.0 and 4.0×10^{-5} M, respectively.

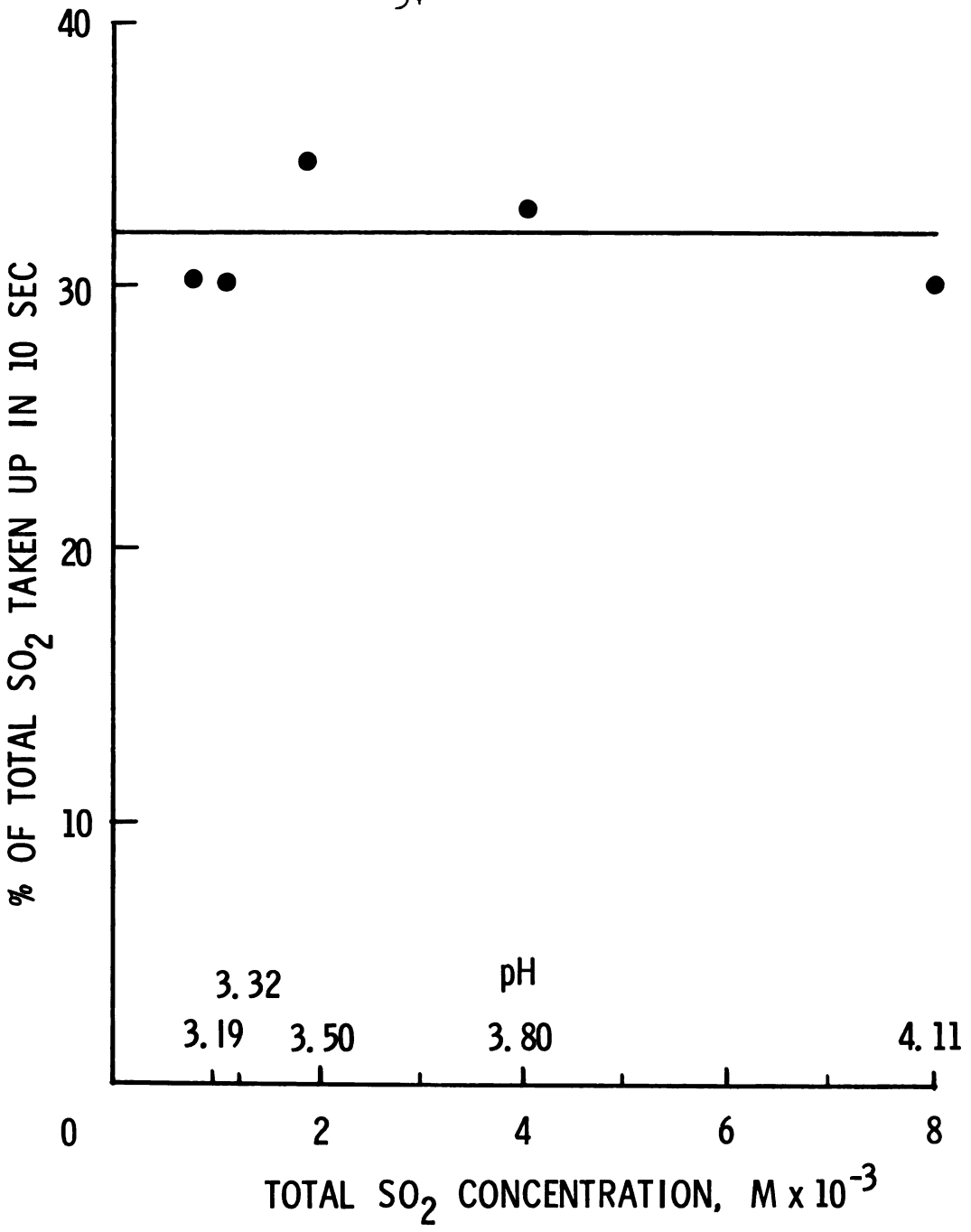

Figure 11. Relationship between rate of SO_2 transport in S. cerevisiae var. ellipsoideus and amount of molecular SO_2. Cells of 20 mg dry weight (4.8×10^8 cells) were incubated in 1 ml of a solution containing 1.0, 1.33, 2.0, 4.0, and $8.0\ 10^{-3}$ M SO_2 at pH 3.19, 3.32, 3.50, 3.80 and 4.11, respectively. The amount of molecular SO_2 at those pH values was the same in all samples 4×10^{-5} M. The incubation time was 10 sec, at 25°C.

The results from both experiments prove that:

A. The effect of pH on the SO_2 transport system is not direct at those pH levels because: (1) As pH decreases the transport rate increases being linearly proportional to molecular SO_2 concentration. (2) When the pH is adjusted so that the molecular SO_2 concentration is the same in all samples the rate of transport remains constant.

B. The molecular SO_2 is the only SO_2 form transported inside the cell. The bisulfite (HSO_3^-) and sulfite ($SO_3^=$) are not transported at all, since only the molecular SO_2 species forms a linear relationship with the amount of total SO_2 transported in 10 sec (Table 7). The HSO_3^- and $SO_3^=$ are not related at all with the observed rate of transport.

The fact that molecular SO_2 is the only transported species aids in further elucidation of transport mechanism. The results appearing in Figure 9, show that the amounts of transported molecular SO_2 at each pH value are much larger than those originally present in the incubation medium. For example at pH 3.8, 1% of the total SO_2 is present as molecular SO_2. After 2 minutes of incubation with the cells at the same pH about 23% of total SO_2 was transported into the cell. Since only molecular SO_2 is transported the amount of this form which disappeared from the solution in 2 minutes should be about 23 times more than the original amount of molecular SO_2. In order for this to happen

and since the pH of the incubation mixture remains constant, sulfite ($SO_3^{=}$) and bisulfite (HSO_3^{-}) must be converted to molecular SO_2 as follows:

$$H^+ + SO_3^{=} \rightleftharpoons HSO_3^-$$

$$H^+ + HSO_3^- \rightleftharpoons H_2O + SO_2$$

As the amount of molecular SO_2 transported inside the cell increases the concentration of the same form outside the cell decreases and the curve describing SO_2 transport as a function of time starts levelling off. This chemical equilibrium aspect is supported by the fact that concentration saturation kinetics (Figure 5) showed that in order to saturate the transport system sites a concentration of $2K_m$=4.70 0.80 x 10^{-2} M total SO_2 was necessary. This value is very high compared to the amount of total SO_2 used, for the same pH (3.80), in the experiment shown in Figure 9. However, molecular SO_2 exhaustion can not itself explain the pattern of SO_2 transport because, if this were the case, the transport curve after reaching a maximum should start gradually going down. Since this was not observed but, instead, a constant transport level was attained, the latter could be understood as a consequence of a transport equilibrium in which equilization of the rate of inward flow to outward flow is obtained. This is supported by the following:

(a) Washing of cells resulted in SO_2 losses (Table 5). The SO_2 that was not washed out reacted inside the cell, whereas the non-reacted SO_2 portion was the one that should be in equilibrium with SO_2 in the incubation mixture. When this equilibrium was attained the rate at which SO_2 entered the cell was equal to the rate at which the same compound left the cell. The mechanism of outward movement of SO_2 is not a diffusion process since the amount of SO_2 released in each cell washing is less than that justified by simple diffusion, as the data of Table 5 show. (b) Cell fractionation data appeared below suggest that SO_2 is not metabolized inside the cell. Therefore SO_2 uptake should not increase continuously with time since the SO_2 transported inside the cell was not used up. (c) Oka (1964) described the same pattern of transport equilibrium for benzoic acid.

SO_2 Transport and Toxicity

Sulfur dioxide is toxic to cells. The inability of yeasts, molds and bacteria to reproduce and form colonies in the presence of this agent has been used as a measure of lethal damage by this chemical.

The toxicity results obtained in this study were expressed as precent survival or percent outgrowth of organism.

$$\% \text{ survival} = \frac{\text{Number of colonies after exposure to } SO_2}{\text{Number of colonies before exposure to } SO_2} \times 100$$

In studying the relationship between transport and toxicity the following factors were examined.

a. The relationship between the amount of SO_2 and toxicity.

b. The dependence of toxicity upon the time during which SO_2 remained in contact with the cells.

Cells representing a dry weight of 4.2 mg (10^7 cells) were incubated with 2.5, 5.0 or 7.5 x 10^{-3} M total SO_2 concentration for 5, 10, 15, 20 and 30 minutes. The effect of SO_2 on the yeast cells was terminated by diluting 0.1 ml of the incubation mixture to 400 ml sterile water. This resulted in that:

a. The SO_2 concentration was diluted 4,000 fold.

b. The level of SO_2 into the cell pool was lowered significantly due to the outward flow of SO_2 upon dilution.

The results appear in Figure 12 and show that the toxicity of SO_2 on the yeast cells depends upon both SO_2 concentration and time during which SO_2 remains in contact with the cells. On the other hand, the relationship between inhibition of growth and time of exposure to SO_2 is exponential. The calculated G values or the time required for 90% growth inhibition under the conditions of this experiment, were G_1=83 min, G_2=25 min and G_3=19 min for total SO_2 concentrations 2.5, 5.0 and 7.5 x 10^{-3} M respectively. Detailed data appear in Appendix, Table 14.

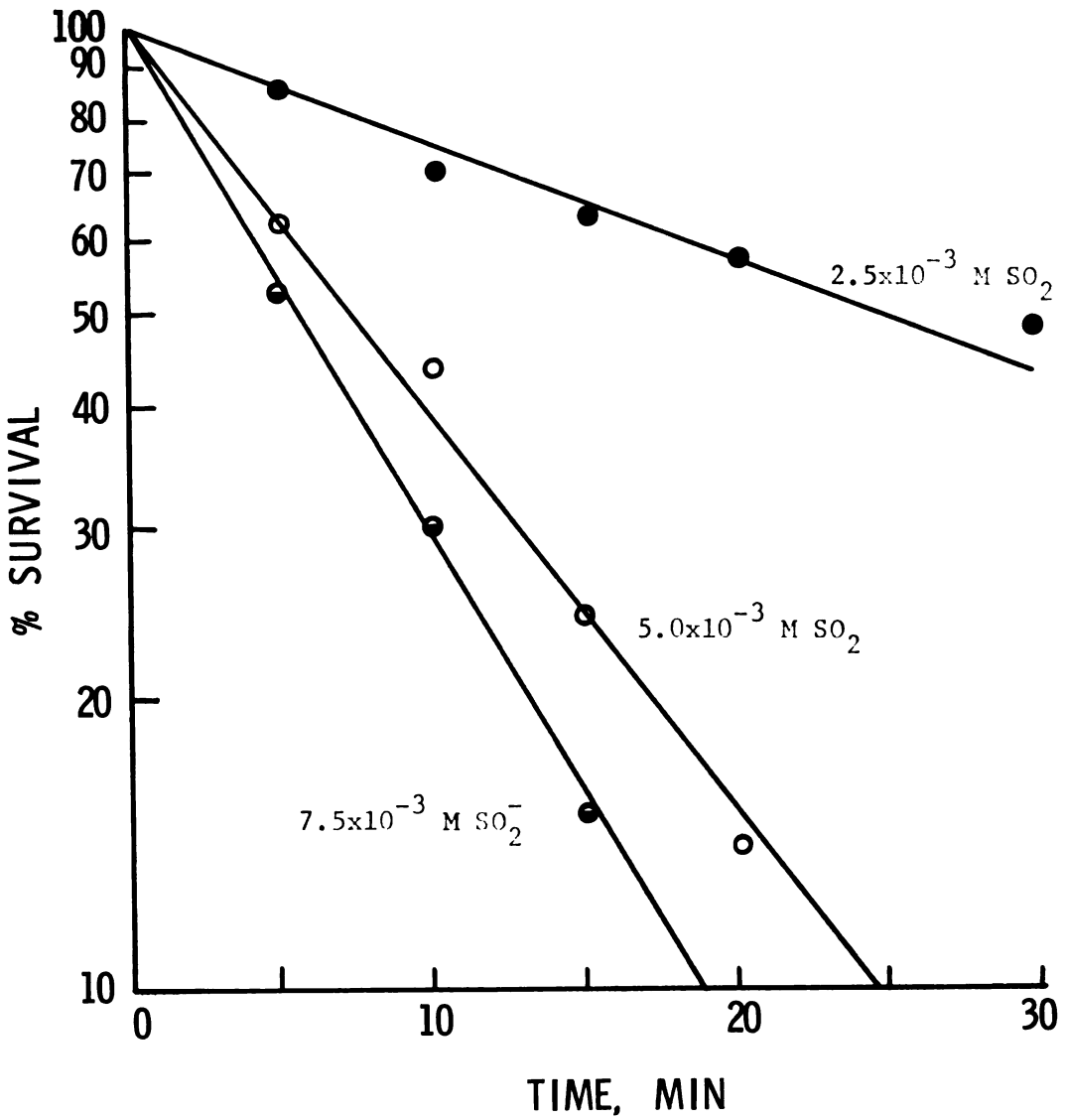

Figure 12. Percent survival of S. cerevisiae var. ellipsoideus incubated with different SO_2 concentrations and times. Cells representing a dry weight 4.2mg (10^7 cells) were incubated in 1 ml SO_2 solution containing 2.5, 5.0 and 7.5 M^{-3} SO_2 at pH 3.19. Incubation was interrupted by diluting 0.1 ml of cell suspension in 400 ml sterile water. Aliquots were plated on glucose-yeast nitrogen base-agar medium.

An experiment was carried out in order to determine the relationship between the three SO_2 forms and toxicity. Since the molecular SO_2 was the only form transported one should expect that this would be the toxic one. Therefore, if molecular SO_2 were the only toxic form, then by changing the pH so that the same amount of molecular form would be present at different total SO_2 concentrations, the same toxic effect would be obtained. The results appearing in Figure 13 show that SO_2 had a very pronounced toxic effect when the concentration of this preservative increased while the pH value was kept constant (pH=3.19). On the other hand, the toxicity remained the same when the pH was adjusted so that the same amount of molecular SO_2 was present although the concentration of total SO_2 was increased 40 fold. Detailed data appear in Appendix, Table 15 and 16. Therefore it is the molecular form only that exerts a significant suppression of growth of S. cerevisiae var. ellipsoideus.

Localization of SO_2 in the Yeast Cell

An attempt was made to determine the fate of SO_2 after it was transported inside the cell. A fractionation was carried out similar to that described by Chaloupka and Babicky (1958). Yeast cells were incubated with labeled SO_2 for 20 minutes at room temperature. The incubation was terminated by centrifugation and the cells were washed

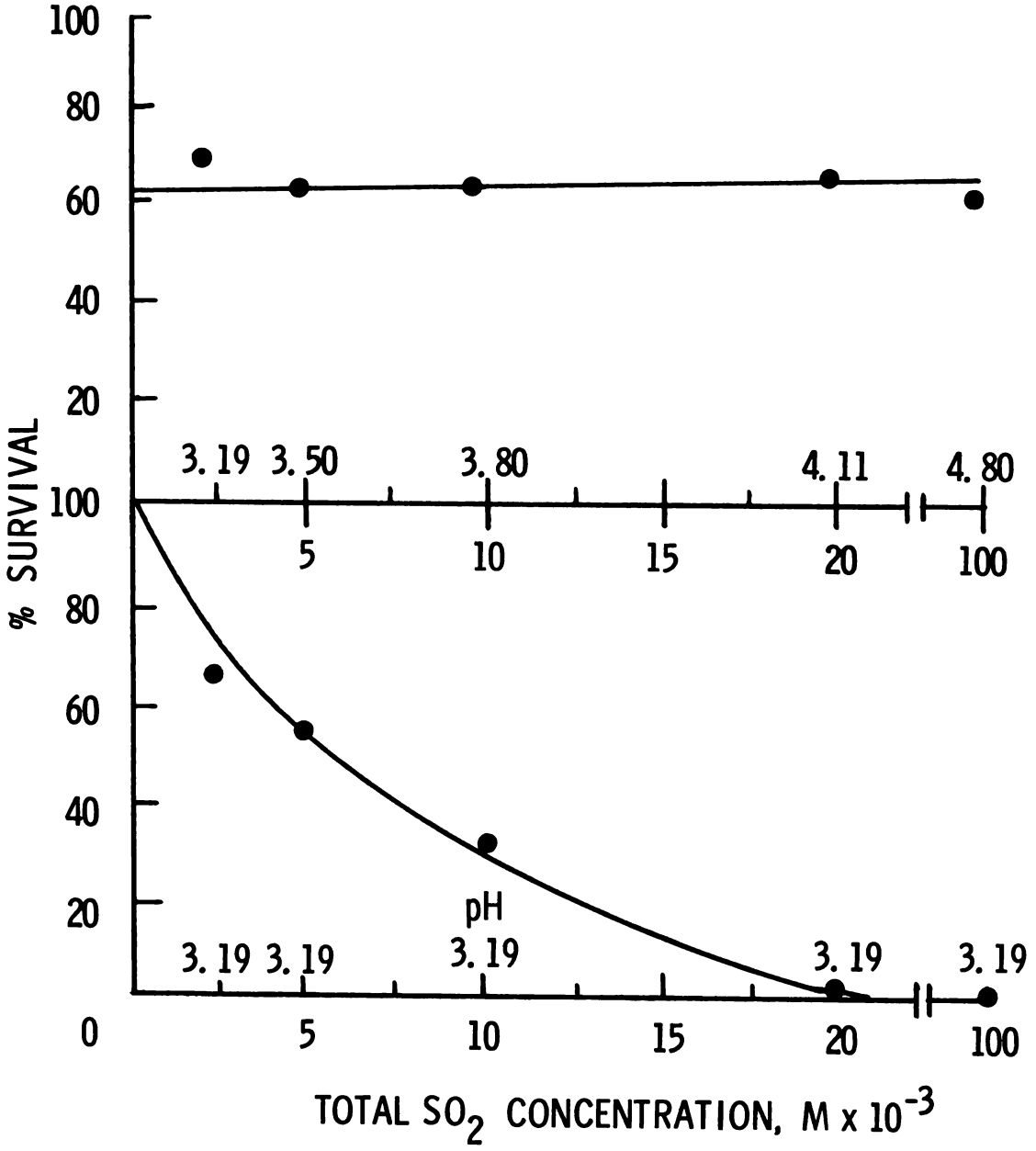

Figure 13. Percent survival of S. cerevisiae var. ellipsoideus incubated at various total SO_2 concentrations and pH levels. Upper curve. Cells representing a dry weight of 4.2 mg (10^7 cells) were incubated in 1 ml containing 2.5, 5.0, 10, 20 and 100×10^{-3} M SO_2 for 5 min at pH 3.19, 3.50, 3.80, 4.11 and 4.80. The incubation period was interrupted by diluting 0.1 ml of cell suspension to 400 ml sterile H_2O and then plating on a glucose-yeast nitrogen-agar medium. Lower curve. Same procedure as above with the difference that the pH of the incubation mixture was 3.19.

six times until practically no radioactivity was recovered in the wash. Trichloroacetic acid (TCA), 10% solution, was added to the washed yeast pellet and the suspension left for 30 minutes at 5°C. It was then centrifuged and the extraction of the yeast pellet was repeated with 5% TCA. The joined extracts contained inorganic ions, nucleotides, free aminoacids and other small molecules. The residue after TCA extraction was suspended twice for 60 minutes in 95% ethanol and once in a mixture of ethanol and ethyl ether (1:1). The extracts were separated from the residue by centrifugation and combined into one solution. This solution contained the lipid sulfur. The residue again was extracted twice for 5 minutes with 1N NaOH to obtain polyphosphate-like compounds and ribonucleic acid and the remaining sediment was finally hydrolyzed in 6N HCl in a glass tube for 15 hours at 110°C. The hydrolyzate was centrifuged and a sample of supernatant was assayed for radioactivity. In addition to the above extracts further fractions were obtained from the TCA extract by precipitation with barium chloride at pH=3.8 (inorganic sulfate and sulfite), at pH=8.2 (nucleotides) and further by cadmium chloride at pH=6.5 (glutatathione).

The results of this fractionation appear in Table 8. Detailed values appear in Appendix, Table 17. In view of the broad spectrum of reactions in which SO_2 can participate inside the cell, as Rehm (1964) and other investigators

Table 7. Distribution of ^{35}S in various fractions of the yeast S. cerevisiae var. ellipsoideus. The reaction mixture contained 1.2 g of cells (dry weight) suspended in 30 ml of 1.2 M SO_2 at pH=3.19 for 20 min. Repeated washing resulted in practically background radiation of the last wash. The radioactivity present in the washed cells was designed as control

Fraction	Total activity (Counts per min/1.2 gr dry weight of cells)	% ^{35}S
Control	15.222	100.00
1. Cold trichloroacetic acid supernatant		
a. Inorganic sulfate and sulfite	6	0.04
b. Nucleotide S.	2	0.01
c. Glutathione	3	0.02
d. Other small molecules	15.143	99.48
2. Lipid S.	21	0.14
3. Alkali-extractable S.	25	0.16
4. 6N-HCl hydrolyzate S.	22	0.15

have demonstrated (Meyerhof et al., 1938; Pfleiderer et al., 1956) the following observations can be derived from this preliminary investigation:

1. ^{35}S reacted with small molecules, the best known of which are carbonyl containing compounds (sugars, pyruvate, acetaldehyde, α-ketoglutarate, etc.), forming hydroxy-sulfonates.

2. Since practically no ^{35}S was incorporated in glutathione or in macromolecules like proteins, one might say that SO_2 is not metabolized in this yeast. On the contrary the sulfur of $^{35}SO_4^=$ fed to the same yeast was found to be present in all cell fractions, obviously because it was actively metabolized (Kotyk, 1959). It is true that $SO_4^=$ is reduced to $SO_3^=$ and then to sulfide ($-SH_2$) prior to incorporation into organic form in yeast. There is some indication that the $SO_3^=$ generated remains bound to the enzyme (Wilson and Bandurski, 1958; Wilson et al., 1969; Asahi et al., 1961; Torrii and Bandurski, 1964) and follows a pathway different from that of SO_2 transported through the cell membrane. The results of this fractionation are in disagreement with those of Shultz and McManus (1950). These investigators showed that SO_2 could be used as sulfur source in the yeast S. cerevisiae var. ellipsoideus but their deductions are dubious, because they incubated the cells with SO_2 overnight without taking into consideration

that SO_2 is oxidated rapidly to sulfate which is one of the best sulfur sources for yeasts.

CHAPTER V

SUMMARY AND CONCLUSIONS

The transport, toxicity and localization of SO_2 in aqueous solution in the yeast S. cerevisiae var. ellipsoideus were investigated in this study. The following conclusions were derived:

A. The SO_2 transport system probably qualifies as an active transport and has the following characteristics. (a) The transport system operates very fast, reaching maximum SO_2 load in about 2 min and then remains at constant level. This can be understood as a two equilibria concept. The first in a chemical equilibrium and regulates the amount of molecular SO_2 being transported. The second one refers to a dynamic state established between the inward and outward flux of SO_2, maintaining the transport at a constant level. (b) The SO_2 transport displays saturation kinetics conforming to the Michaelis-Menten curve of saturation and has a $K_m = 2.65 \pm 0.40 \times 10^{-2}$ M and $V_{max} = 6.95 \pm 0.93 \times 10^{-3}$ M/10 seconds. The pattern of this transport suggests the presence of carriers containing specific sites to which SO_2 binds. (c) The SO_2 transport system is apparently

temperature dependent and displays a pattern similar to that observed in enzyme kinetics. It is also irreversibly inactivated by heat and thus resembles protein denaturation. (d) Metabolic energy is necessary for this transport to proceed. Inhibitors that block the formation and utilization of high energy phosphate bonds abolish the accumulation of SO_2 insdie the cell. (e) Among the SO_2 forms possible in aqueous solution, bisulfite (HSO_3^-), sulfite ($SO_3^=$) and molecular SO_2, the latter is the only form transported. This underlines the high specificity of this transport system.

B. The toxicity of SO_2, expressed as the ability of this chemical to suppress the growth of yeast cells is related to SO_2 transport as follows. (a) The molecular form is the only transported form and the only toxic one. (b) SO_2 toxicity depends upon the amount of the molecular SO_2 as well as the time during which this form remains in contact with the cells. (c) There is an exponential relationship between cell viability and time of exposure to SO_2.

C. The results obtained during fractionation suggested that sulfur dioxide does not seem to be metabolized in the yeast cell. Rather, it reacts with certain small molecules depriving them of their metabolic role; the result is termination of the growth of the cell.

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APPENDIX

Table 1. Packing rate of S. cerevisiae var. ellipsoideus cells.

Centrifugation time (min)	Weight of cell pack (g)				
	Expt I	Expt II	Expt III	Expt IV	Average
5	2.5112	2.4950	2.5180	2.5207	2.5112
10	2.4687	2.4592	2.4610	2.4625	2.4627
20	2.4675	2.4577	2.4377	2.4537	2.4527
30	2.4665	2.4585	2.4372	2.4527	2.4537

Table 2. Determination of intercellular space and cell volume in S. cerevisiae var. ellipsoideus.

Sample	Weight of cell pack (g)		Refractive Index at 27°C						% Dext in supt.	Inter-cellul. space	Cells		
			% Dextran					Supnt. of cell packed in % De.					
No.	Dry	Wet	1.0	1.5	2.0	2.5	3.0			cm^3	Weight g	Vol. cm^3	Number
1	0.1911	1.0054	1.3338	1.3334	1.335	1.3357	1.3364	1.3364	2.69	0.345	0.6604	0.600	
2	0.1920	0.9905	1.3338	1.3334	1.335	1.3356	1.3364	1.3361	2.77	0.249	0.7401	0.673	
3	0.1947	1.2078						1.3360	2.69	0.345	0.6828	0.620	
4	0.1926	1.0194						1.3360	2.69	0.345	0.6744	0.613	
Av.	0.1926	1.0107	1.3338	1.3334	1.335	1.33565	1.3364	1.3361	2.71	0.321	0.6896	0.626	4.8×10^9

Table 3. Fate of $^{35}SO_2$ upon incubation with S. cerevisiae var. ellipsoideus.

Type of Operation	Counts per 10 min						%
	Control			Expt.			
			Aver.	I	II	Aver.	
Original $^{35}SO_2$	437,976	409,892	423,924				100.00
$^{35}SO_2$ removed in the original incubation				239,777	241,165	240,544	56.74
^{35}S recovered in:							
First wash				57,585	58,361	57,937	24.10
Second wash				11,367	11,830	11,598	4.82
Third wash				1,971	2,074	2,022	1.36
Fourth wash				122	44	83	0.03
Fifth Wash				97	65	81	0.03
Supernatant of broken cells				2,832	2,725	2,778	1.52

Table 4. Effect of washing of cells of S. cerevisiae var. ellipsoideus after incubation with $^{35}SO_2$.

pH	Sample	cpm recov. in orig. incub.	Radioactivity recovered in the supernatant — Wash Number										Total SO_2 remaining in cell%
			1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	Total	
4.11	Control	48,180	7,829											
	Expt.													
	1	8,391	1,400	78	18	20	24	17	22	20	16	14		
4.11	2	7,268	1,365	64	18	16	22	13	18	14	16	26		
	Aver.	7,829	1,382	71	18	18	23	15	20	17	16	20		
	%	16.24	17.65	0.90	0.22	0.22	0.29	0.19	0.25	0.21	0.20	0.25	20.38	12.93
	Control	48,180	24,805											
	Expt.													
	1	24,396	4,273	1,267	562	31	23	16	22	17	26	13		
3.32	2	25,214	3,465	1,623	486	45	25	24	18	23	18	29		
	Aver.	24,805	3,869	1,625	524	38	24	20	20	20	22	21		
	%	51.48	15.59	6.55	2.11	0.15	0.09	0.08	0.08	0.08	0.09	0.08	24.82	38.71
	Control	48,180	32,768											
	Expt.													
	1	32,757	5,197	2,986	2,251	585	148	35	28	23	22	20		
3.19	2	32,780	5,141	2,894	2,279	547	172	25	16	25	24	22		
	Aver.	32,768	5,169	2,940	2,265	566	160	30	22	19	23	21		
	%	68.01	15.77	8.97	6.91	1.72	0.48	0.09	0.06	0.05	0.05	0.06	34.16	44.78

Table 5. Transport of SO_2 in S. cerevisiae var. ellipsoideus as a function of time.

Time	Uptake (cpm/20 mg dry weight)				
min	Expt I	Expt II	Expt III	Average	%
0	3,127	2,905	3,027	3,020	---
5	618	620	601	613	20.3
10	621	611	628	620	20.5
20	680	639	619	646	21.4
40	566	561	604	577	19.1
60	713	633	610	652	21.6

Table 6. Time course of SO_2 transport in the yeast.

Time	DIRECT METHOD					INDIRECT METHOD				
	Uptake (cpm/20 mg d.w.)					Uptake (cpm/20 mg d.w.)				
sec.	1	2	3	Av.	%	1	2	3	Av.	%
0	3,430	3,415	3,369	3,417	---	4,439	4,483	4,503	4,475	---
10	260	238	263	254	7.43	412	490	500	467	10.43
20	278	319	347	315	9.21	567	431	641	546	12.20
40	458	433	424	438	12.81	689	856	679	741	16.55
60	558	592	585	578	16.91	820	889	819	843	18.83
120	802	901	651	785	22.97	1,144	982	953	1,026	22.92
300	799	663	790	751	21.97	1,150	993	980	1,041	23.26
600	669	889	781	779	22.79	1,003	1,027	908	979	21.87

Table 7. Sulfur dioxide transport in S. cerevisiae var. ellipsoideus as a function of SO_2 concentration.

Total SO_2 concent. $x10^3M$	Control (cpm)				Uptake cpm/20 mg d.w./10 sec.				$SO_2/10^2$sec.	$\frac{1}{S}$	$\frac{1}{V}$
	I	II	III	Av.	I	II	III	Av.	$x10^3M$		
2	1,932	1,984	1,938	1,951	472	452	---	462	0.47	50.00	2.12
4	4,243	4,208	4,230	4,227	758	683	712	717	0.68	25.00	1.47
6	6,444	6,450	6,418	6,458	1,570	1,322	1,489	1,460	1.35	16.66	0.74
8	7,799	7,621	7,812	7,744	1,303	1,499	1,576	1,459	1.51	12.50	0.66
10	9,687	10,049	9,841	9,859	1,651	1,518	2,232	1,800	1.82	10.00	0.55
20	19,959	19,876	19,782	19,872	3,018	2,869	2,792	2,893	2.91	5.00	0.34
40	39,846	39,539	39,926	39,770	4,509	4,585	4,462	4,516	4.54	2.50	0.22
60	57,242	56,840	56,764	56,948	3,733	4,841	5,152	4,575	4.82	1.66	0.21
80	73,387	73,564	73,309	73,420	4,211	5,215	5,917	5,114	5.57	1.25	0.18
100	90,240	92,687	91,279	91,402	4,764	4,266	4,474	4,592	5.02	1.00	0.20

Table 8. Effect of temperature on SO_2 transport in S. cerevisiae var. ellipsoideus.

Temp.	Counts per minute per 20 mg cells (dry weight)				% of total SO_2 taken up in 30 seconds
	Expt I	Expt II	Expt III	Average	
20 (Control)	11,806	----	11,694	11,750	
0 (Expt)	1,365	1,843	1,372	1,525	12.98
10 (Expt)	1,204	1,295	1,834	1,444	11.28
20 (Expt)	1,386	1,687	1,512	1,528	13.00
30 (Expt)	3,075	3,108	3,122	3,102	26.40
40 (Expt)	4,810	4,693	4,816	4,773	40.62
50 (Expt)	5,487	5,339	5,194	5,340	45.44
60 (Expt)	5,308	5,025	4,863	5,065	43.10
70 (Expt)	1,867	1,872	1,866	1,868	14.98

Table 9. Thermal inactivation of the SO_2 transporting system in *S. cerevisiae var. ellipsoideus*.

Temp. C	Counts per minutes per 20 mg cells (dry weight)				% of total SO_2 taken up in 2 min.
	Expt I	Expt II	Expt III	Average	
20 Contr.	2,982	2,992	3,072	2,985	---
20 Expt.	1,746	1,601	1,651	1,666	55.81
40 Expt.	1,801	1,745	1,808	1,751	58.66
50 Expt.	1,606	1,591	1,623	1,606	53.80
55 Expt.	1,311	1,309	1,112	1,244	41.67
60 Expt.	879	865	815	853	28.57
65 Expt.	43	43	61	49	1.55
70 Expt.	44	44	62	50	1.67

Table 10. Effect of metabolic inhibitors on SO_2 transport in S. cerevisiae var. ellipsoideus.

Inhibitor	Concentration	cpm per 20 mg (d.w.)				% Inhibition
		Expt I	Expt II	Expt III	Aver	
Without	---	3,240	3,343	3,141	3,241	0
Iodoacetamide	10^{-5}M	3,134	3,258	3,163	3,186	1.69
	$5x10^{-5}$M	3,138	3,154	3,244	3,195	1.41
	10^{-4}M	3,216	3,136	3,210	3,187	1.66
Sodium Azide	10^{-5}M	1,949	1,984	1,898	1,943	40.04
	$5x10^{-5}$M	1,196	1,071	1,152	1,139	64.85
	10^{-4}M	921	903	871	898	72.29
2,4-Dinitrophenol	$5x10^{-7}$M	2,660	2,603	2,658	2,640	18.54
	10^{-6}M	2,270	2,279	2,212	2,253	30.48
	$5x10^{-6}$M	2,215	2,193	2,275	2,227	31.28
$HgCl_2$	10^{-6}M	1,299	---	849	1,704	66.86
	$5x10^{-6}$M	-105	-134	-103	-114	103.51
	10^{-5}M	-186	-163	-111	-153	104.72

Table 11. Time course of SO_2 transport in S. cerevisiae var. ellipsoideus as a function of pH.

pH	Sample No.	Control cpm	Uptake (cpm/20 mg cells d.w.)													
			10 sec		20 sec		40 sec		60 sec		120 sec		300 sec		600 sec	
			Expt.	%	Expt.	%	Expt.	%	Expt.	%	Expt.	%	Expt.	%	Expt.	%
	1	4,384	1,402		1,830		2,298		2,326		2,418		2,423		2,434	
3.19	2	4,223	1,471		2,015		2,252		2,365		2,425		2,444		2,359	
	3	4,299	---		2,016		2,303		2,352		2,348		2,401		2,452	
	Average	4,302	1,436	33.37	1,953	45.39	2,284	53.09	2,347	54.55	2,397	55.71	2,422	56.29	2,415	56.13
	1	4,384	709		1,125		1,248		1,262		1,679		1,797		1,773	
3.50	2	4,223	811		1,006		1,175		1,265		1,594		1,891		1,845	
	3	4,299	852		1,122		1,165		1,255		1,405		1,789		1,843	
	Average	4,302	791	18.38	1,084	25.19	1,196	27.80	1,260	29.28	1,559	36.23	1,828	42.49	1,820	42.30
	1	3,430	260		278		458		558		802		799		669	
3.80	2	3,451	238		319		433		592		901		663		889	
	3	3,369	263		347		424		585		651		790		781	
	Average	3,417	254	7.43	315	9.21	438	12.81	578	16.91	785	22.97	751	21.97	779	22.79
	1	4,239	73		127		303		192		205		389		318	
4.80	2	4,235	107		90		258		191		204		352		122	
	3	4,198	---		---		---		---		---		---		---	
	Average	4,224	90	1.82	108	2.55	280	6.62	191	4.52	235	5.56	370	8.75	370	8.75

Table 12. Relationship between rate of SO_2 transport in S. cerevisiae var. ellipsoideus and amount of molecular SO_2.

pH	Total SO_2 x10^3	Molecular SO_2 %	Molecular SO_2 Concentr. x10^4	Uptake (cpm per 20 mg cells, dry weight) Control	Expt.	%	Control	Expt.	%	Control	Expt.	%	Aver. %
4.11	1	0.5	0.5	3,840 3,950 3,889 Av3,893	116 217 184 Av 172	2.9 5.5 4.7 Av 4.4	10,293 10,756 --- Av10,524	510 480 336 Av 442	4.8 4.5 3.1 Av 4.1	- - - -	- - - -	- - - -	4.30
3.80	1	1.0	1.0	 Av3,893	305 270 321 Av 298	7.8 6.9 8.2 Av 7.6	 Av10,524	1,238 972 1,037 Av1,180	11.7 9.2 9.8 Av 10.2	4,439 4,483 4,503 Av4,475	412 490 500 Av 467	9.2 10.9 11.1 Av 10.4	9.45
3.50	1	2.0	2.0	 Av3,893	717 517 522 Av 568	17.1 13.3 13.4 Av 14.6	 Av10,524	1,639 1,848 1,939 Av1,808	15.5 17.5 18.4 Av 17.1	4,384 4,223 4,299 Av4,302	578 682 718 Av 661	13.6 15.8 16.7 Av 15.3	15.72
3.32	1	3.0	3.0	 Av3,893	762 866 737 Av 788	19.5 22.2 18.9 Av 20.0	 AV10,524	2,465 2,885 2,411 Av2,587	23.4 27.4 22.9 Av 24.5	3,708 4,034 3,823 Av3,855	866 839 848 Av 851	22.4 21.7 21.9 Av 22.0	22.96
3.19	1	4.0	4.0	 Av3,893	1,050 1,159 1,140 Av1,116	26.9 29.7 29.2 Av 28.6	 Av10,524	3,017 3,384 3,475 Av3,292	28.6 32.1 33.0 Av 31.2	 Av3,855	1,122 1,226 1,140 Av1,162	29.1 31.8 29.5 Av 30.1	30.03

Table 13. Transport of SO_2 in S. cerevisiae var. ellipsoideus as a function of the molecular SO_2 concentration.

pH	Total SO_3 $x10^3$ M	Molecular SO_2		Control	Uptake (cpm/20mg d.w.)	
		%	Concentr. $x10^{-3}$M		Expt.	%
4.11	8.0	0.5	4	6,075 6,166 6,227 Av. 6,289	1,761 1,960 2,027 Av. 1,916	Av. 30.46
3.80	4.0	1.0	4	Av. 3,144	1,044 1,046 --- Av. 1,045	Av. 33.23
3.50	2.0	2.0	4	Av. 1,572	542 552 549 Av. 558	Av. 34.19
3.32	1.33	3.0	4	Av. 1,025	344 278 316 Av. 313	Av. 30.53
3.19	1.0	4.0	4	Av. 786	254 229 245 Av. 243	Av. 30.91

Table 14. Percent survival of S. cerevisiae var. ellipsoideus incubated with different SO_2 concentrations and times at pH = 3.19, 20°C.

SO_2 Concentration				% Survivors																			
Total		Molecular		5 min.				10 min.				15 min.				20 min.				30 min.			
				Expt.				Expt.				Expt.				Expt.				Expt.			
$x10^3$	ppm	$x10^4$	ppm	I	II	III	Ave.	I	II	III	Ave.	I	II	III	Ave.	I	II	III	Ave.	I	II	III	Ave.
2.5	160	1	6.4	79.4	91.1	88.3	86.3	69.5	72.0	71.0	70.8	70.5	66.4	55.8	64.2	59.3	62.4	54.8	58.8	54.3	51.2	40.1	45.8
5.0	320	2	12.8	64.4	56.8	67.0	62.7	40.6	32.9	48.7	40.7	23.8	21.3	28.4	24.5	11.6	9.1	16.7	12.5	-	-	-	-
7.5	480	3	19.2	53.8	54.9	53.2	54.0	30.2	28.5	31.8	30.2	10.4	10.9	9.8	10.4	-	-	-	-	-	-	-	-

Table 15. Percent survival of S. cerevisiae var. ellipsoideus incubated with various SO_2 concentrations at different pH levels, 20°C.

pH	SO_2 Concentration				% Survivors						
	Total		Molecular		Expt I		Expt II		Expt III		Average
	$x10^{-3}$M	ppm	$x10^{-4}$M	ppm	1	2	1	2	1	2	
3.19	2.5	160	1	6.4	60.29	75.92	62.25	66.17	66.17	71.07	67.15
3.50	5.0	320	1	6.4	65.68	63.23	59.31	62.74	64.70	67.15	63.72
3.80	10.0	640	1	6.4	62.25	66.66	63.23	62.74	64.70	60.78	63.23
4.11	20.0	1,280	1	6.4	69.60	67.15	60.78	57.84	66.17	68.62	65.19
4.80	100	6,400	1	6.4	69.11	50.00	66.17	62.25	72.05	63.72	63.72

Table 16. Percent survival of S. cerevisiae var. ellipsoideus incubated with different SO_2 concentration at pH=3.19 for 5 min.

SO_2 Concentration				% Survivors						
Total		Molecular		Expt I		Expt II		Expt III		Average
$x10^3$	ppm	$x10^4$	ppm	1	2	1	2	1	2	
2.5	160	1	6.4	62.06	66.00	72.41	64.21	65.02	69.45	66.17
5.0	320	2	12.8	62.65	52.70	64.03	62.06	50.73	52.70	57.63
10.0	640	4	25.6	38.91	35.96	25.12	25.61	26.60	27.09	30.04
20	1,240	8	51.2	0	0	0	0	0	0	0
100	6,400	40	256	0	0	0	0	0	0	0

Table 17. Distribution of ^{35}S in various fractions of the yeast S. cerevisiae var. ellipsoideus.

Fraction	Total activity (cpm/1.2 g cells dw)			$\%^{35}S$
	Exp I	Exp II	Aver.	
Control	14,928	15,516	15,222	100.00
1. Cold trichloroacetic acid supernatant				
a. Inorganic sulfate and sulfite	3	9	6	0.04
b. Nucleotide S.	2	2	2	0.01
c. Glutathione	-4	10	3	0.02
d. Other small molecules	14,861	15,427	15,143	99.48
2. Lipid S.	-7	49	21	0.14
3. Alkal-extractable S.	21	29	25	0.16
4. 6N-HCl hydrolyzate	52	-8	22	0.15