THE EFFECT OF BISULFITE ON CERTAIN OXIDIZING ENZYMES

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This is to certify that the

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ABSTRACT

THE EFFECT OF BISULFITE ON CERTAIN
OXIDIZING ENZYMES

By

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Investigations were carried out on the effects of bisulfite on the following oxidizing enzymes: peroxidase, catalase, lipoxygenase, phenolase, and ascorbic acid oxidase.

It was found that 0.05-0.1M bisulfite retards the inactivation of horseradish peroxidase solutions (0.5-10 purpurogallin units per ml) by weak acids. Spectral analysis indicates that it accomplishes this by stabilizing the linkage between the iron-containing prosthetic group and the protein. Cyanide, azide, and fluoride, which form reversible complexes with peroxidase iron, exert a similar effect; thus, it is inferred that bisulfite also forms a complex with peroxidase iron. A kinetic method was used to calculate a dissociation constant of 0.02M for the bisulfite-enzyme complex.

A solution of bovine liver catalase (2640 units per ml) was nearly inactivated by twelve days of contact

with 0.1M bisulfite. The apparent cause is denaturation of the catalase protein by bisulfite.

The oxidation of linoleic acid by lipoxygenase in dried pea extract is slightly accelerated in the first 20-25 minutes of the reaction by 1.6×10^{-4}M bisulfite, and later inhibited. The enhancement may be due to the bisulfite destruction of free radicals and hydroperoxides which may inhibit the enzyme. The bisulfite inhibition, which occurs later in the reaction, is probably caused by an attack on the enzyme by the bisulfite itself.

The oxidation of monophenols by phenolase is stimulated by low levels (10^{-5}M) of catechol and other reducing agents and inhibited by low levels (10^{-5}M) of bisulfite. This inhibition does not appear to be due to any enzyme-bisulfite interaction because the extent of the inhibition is nearly independent of enzyme concentration for low cresolase preparations. The bisulfite inhibition is overcome by reducing agents, and conversely, the stimulation of monophenol oxidation by reducing agents is diminished by bisulfite. This indicates that monophenol oxidation by phenolase involves a reaction not directly controlled by the enzyme, but in which a reducing agent participates and bisulfite interferes. A mechanism for this reaction is proposed.

A crude preparation of ascorbic acid oxidase (0.0026 units per ml) was readily inhibited by 10^{-4}M

bisulfite, apparently because of denaturation of the enzyme protein by the bisulfite.

ADDENDUM: The effect of 50-480 ppm SO_2 solutions on the growth of Saccharomyces cerevisiae was also investigated. Evidence is presented to show that the sulfurous acid molecule, and not bisulfite or sulfite, is the antiseptic agent. Its antiseptic potency depends on its rate of uptake by the yeast cells and not on the total amount taken up.

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I. INTRODUCTION

Sulfur dioxide, along with its salts, is probably the most versatile of all the chemical agents used in the food industry. It is used widely as a preservative to reduce or prevent spoilage by microorganisms and as a selective inhibitor of undesirable organisms in the fermentation industries. It is also used to inhibit enzymatic and non-enzymatic oxidative discoloration of many food products, and to protect ascorbic acid, carotene, and other biologically active components. The pronounced bleaching action of SO_2 has been employed in the preparation of specialty products such as Maraschino cherries, and for decolorizing sugar-cane and sugar-beet juices in sugar manufacture. Sulfiting of grapes reduces molding and may repel insects. Treatment of flour with sulfur dioxide modifies its baking characteristics by breaking disulfide bonds in the gluten molecules.

The main disadvantages of sulfur dioxide are its unpleasant flavor and its destructive action in thiamine (vitamin B_1).

In spite of its wide commercial use, the chemical mechanisms involved in the various effects of SO_2 are not

well understood. The purpose of this study was to clarify some of the mechanisms relative to the effect of SO_2 on certain oxidizing enzymes important in the food field.

Since there is no one common name for sulfur dioxide and its salts, the term "bisulfite" will be used in describing the SO_2 solutions referred to in this thesis, because bisulfite is the dominant form among the various molecular species of SO_2 in the mildly acidic pH range of most interest in the food field.

II. REVIEW OF LITERATURE

Peroxidase

Peroxidase (Doner: H_2O_2 oxidoreductase 1.11.1.7) is a hemiprotein which catalyzes the reaction:

$$ROOH + H_2x \text{ (or } 2x^m) \longrightarrow ROH + H_2O + x \text{ (or } 2x^{m+1})$$

where H_2x is an oxidizable substrate such as ascorbic acid, a phenol, a short chain alcohol, etc. and x^m is a metallic ion (Mahler and Cordes, 1966). Therefore, its presence in fruit and vegetables can result in discoloration and loss of vitamin C.

The effect of sulfurous acid and its salts on peroxidase has not been extensively studied. Klebanoff (1961) reported that sulfite activated the nonperoxidative oxidation of NADH and NADPH as catalyzed by peroxidase. Chmielnicka (1963, 1964) and Monikowski and Chmielnicka (1964) found that SO_2 inhibited peroxidase activity both in pure systems and in raw vegetables and their products.

Maehly (1952) noted that acids in general split the iron-containing prosthetic group of horseradish peroxidase from its protein, thereby inactivating the enzyme. The half-life of the active enzyme in acidic solution varies

inversely with the pH of the solution and with the ionic strength.

The purpose of our study was to observe and explain some of the effects of bisulfite on the enzymatic activity of horseradish peroxidase.

Catalase

Catalase (H_2O_2:H_2O_2 oxidoreductase 1.11.1.6) is an iron-containing protein which catalyzes the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase is quite similar to peroxidase in that it contains a protohematin prosthetic group and catalyzes a similar type of oxidation-reduction reaction. Also, the linkage between the iron atom of the prosthetic group and the protein is susceptible to rupture by acid (Lewis, 1954).

The question then arises as to whether bisulfite can exert a protective effect on catalase in acid media as it does with peroxidase. To clarify this issue, a study was made on the effect of bisulfite on the enzymatic activity of catalase.

Lipoxygenase

Lipoxygenase, or lipoxidase, (1.99.2.1) catalyzes the oxidation of linoleic, linolenic, and arachidonic

acids. The reaction proceeds as follows:

$$\underset{\text{cis}}{-CH=CH-CH_2-}\underset{\text{cis}}{CH=CH-} + O_2 \longrightarrow \underset{\text{cis}}{-CH=CH-}\underset{\text{trans}}{CH=CH-}\underset{\text{cis}}{CH(OOH)-}$$

A complex series of free radicals and hydroperoxides are formed in this reaction, and some of these may inhibit the enzyme even as it produces them. Evidence for this may be seen in the fact that hydrogen peroxide readily inhibits lipoxygenase (Mitsuda, et al., 1967).

Lipoxygenase occurs in green peas and may be responsible for chlorophyll and flavor deterioration in this product (Eriksson, 1967). Therefore, a method for inhibiting lipoxygenase may have some practical value. In this work, bisulfite was investigated as a possible inhibitor of this enzyme.

Phenolase

Phenolase (o-diphenol: O_2 oxidoreductase 1.10.3.1) is a copper-containing enzyme which catalyzes the reaction:

$$2 \text{ o-diphenol} + O_2 \longrightarrow 2 \text{ o-quinone} + 2 H_2O$$

However, it also appears to catalyze the o-hydroxylation of monophenols:

$$\text{monophenol} + 2e^- + O_2 \longrightarrow \text{o-diphenol} + O^=$$

although there is some evidence that the monophenol is converted directly to the o-quinone (Dressler and Dawson, 1960):

$$\text{monophenol} + O_2 \longrightarrow \text{o-quinone} + H_2O$$

The copper moiety of the enzyme is absolutely essential for enzymic activity and no other element can substitute for it (Kubowitz, 1938).

The hydroxylating function of phenolase is referred to as its "cresolase" activity while the oxidative function is its "catecholase" activity. The relationship between these activities is unknown and is a matter of some dispute. The following mechanisms have been proposed:

1. The monophenol is hydroxylated nonenzymatically by the highly reactive o-quinones produced by enzymic oxidation of an o-diphenol (Kertesz and Zito, 1962). However, this could not involve an oxidation-reduction reaction between the monophenol and quinone as in the equation:

$$\text{monophenol} + \text{o-quinone} + H_2O \longrightarrow 2\ \text{o-diphenol}$$

because Mason et al. (1955) have shown that the hydroxyl group added to the monophenol molecule is derived from molecular oxygen and not from water.

2. The enzyme possesses one copper-containing active site. The copper catalyzes the oxidation of an o-diphenol while being itself reduced from the cupric to the cuprous state. The cuprous copper then catalyzes the hydroxylation of a monophenol (Mason, 1956).

3. The enzyme possesses two separate, copper-containing active sites, one for oxidizing diphenols to

o-quinones and the other for the hydroxylation of monophenols (Dressler and Dawson, 1960).

4. Phenolase is a mixture of two different enzymes, one for the oxidation of o-diphenols and the other for the hydroxylation of monophenols (Macrae and Duggleby, 1968).

None of these theories satisfactorily accounts for all the enzymatic properties of phenolase. Theory 1 fails to explain why the enzyme produces only o-diphenols (or o-quinones) when nonenzymatic hydroxylations are always randomly directed. Theory 2 would have difficulty showing, if there were only one active center, why bisulfite has a much more adverse effect on the cresolase activity than on the catecholase activity (Markakis and Embs, 1966; Muneta, 1966). Theories 3 and 4 do not account for the fact that minute amounts of an o-diphenol added to a phenolase-monophenol mixture greatly enhance the rate of monophenol hydroxylation.

In the present work we investigated the effect of bisulfite on the phenolase-monophenol reaction and attempted to construct a hypothesis which would account for this effect and also elucidate some aspects of the phenolase-monophenol reaction.

Ascorbic Acid Oxidase

Ascorbic acid oxidase (l-ascorbate:O_2 oxidoreductase 1.10.3.3) is a copper-containing protein that catalyzes the

aerobic oxidation of L-ascorbic acid. It also catalyzes the oxidation of a limited number of phenolic compounds, such as 2,6-dichloroindophenol (Dawson, 1965).

Means of preventing vitamin C loss by this enzyme may have practical significance for fruit and vegetable processors. Therefore, bisulfite was investigated as a possible inhibitor of this enzyme.

III. METHODS AND MATERIALS

The peroxidase used in this work was "Type II" horseradish peroxidase from the Sigma Chemical Company. Its activity was 110 purpurogallin units per mg.

Enzymatic activity was determined by adding 0.1 ml. of the peroxidase solution, properly diluted (usually 1:40), to a mixture of 1 ml of 0.5M phosphate buffer, pH 6.5, 1 ml of 20 mM guaiacol, 0.1 ml of 10mM H_2O_2, and 0.8 ml H_2O. The reaction mixture was in a 1-cm-path Beckman cuvette and the enzyme solution was added with a square teflon plunger provided with a groove and three orifices and connected to a stainless steel handle. Color formation in the solution was measured at 470 nm with a Beckman DU spectrophotometer connected to a Ledland log-converter and a Sargent SR recorder. The rate of reaction was measured by the tangent at the origin of the curve obtained with the recorder.

Spectra were obtained with a Bausch & Lomb Spectronic 505 recording spectrophotometer.

Twice crystallized bovine liver catalase from the Sigma Chemical Company was used.

Catalase activity was determined spectrophotometrically by incubating 1 ml of 0.059M H_2O_2 with 2 ml of catalase

solution, properly diluted, in a 1-cm-path Beckman cuvette. The reaction was followed at 240 nm. The rate of reaction was taken as the tangent at the origin of the curve obtained with the recorder, in the same manner as in the peroxidase work.

Pea lipoxygenase extracts were made by grinding dried, split peas in a Wiley mill with a 40-mesh sieve, washing the powder with petroleum ether to remove lipids, and then shaking a five gram quantity of the powder with 50 ml of 0.05M phosphate buffer, pH 7.0, for thirty minutes. The extract was filtered with No. 1 filter paper before use.

Linoleic acid solution was made up as follows: 0.5 ml of linoleic acid and 0.5 ml of Tween 80 were shaken vigorously with 10 ml of 0.2M borate buffer, pH 9.0. Then 1.3 ml of 1M NaOH was added and the solution shaken until clear. Finally, 90 ml of 0.05M phosphate buffer, pH 7.0 were added and the solution diluted to 200 ml with demineralized water. This method of solubilizing linoleic acid was taken from Surrey (1964).

Lipoxygenase activity was determined manometrically by adding 2.0 ml of the linoleic acid solution and 0.5 ml of water (or bisulfite solution) to the main compartment of a Warburg flask and 0.5 ml of the pea extract to the side arm. A Warburg apparatus thermostated at 30° C was used in the subsequent measurements of oxygen uptake.

The "low cresolase" enzyme was mushroom tyrosinase from the Worthington Biochemical Corporation. The "high cresolase" enzyme was mushroom tyrosinase from the Sigma Chemical Company. Its activity was listed as 1050 units per mg. (1 unit = 0.001 absorbancy increase per min. at 280 nm, in 0.16M phosphate buffer, pH 6.5, 25° C, containing 3 x 10^{-4}M L-tyrosine).

Browning rates were determined at 470 nm with a Beckman DU Spectrophotometer, log converter, and recorder in the same manner as in the peroxidase and catalase work. The browning rate was taken as the tangent at the origin of the curve obtained with the recorder, and was expressed in arbitrary units.

In paper chromatography, No. 1 Whatman paper was employed, with n-butanol-acetic acid-water (25:6:25) as solvent, in descending irrigation at 25° C.

The juice squeezed from fresh cucumber peel was used as the source of ascorbic acid oxidase.

Ascorbic acid oxidase activity was determined manometrically by adding 1.5 ml of buffer (0.2M phosphate-0.1M citrate, pH 5.7), 0.5 ml of gelatin solution (750 mg gelatin in 150 ml water), 0.5 ml of ascorbic acid solution (250 mg of ascorbic acid and 50 mg of metaphosphoric acid in 50 ml of water), and 0.9 ml of water (or bisulfite solution) in the main compartment of a Warburg flask and 0.1 ml of the peel juice in the side arm. Subsequent measurements

of oxygen uptake were carried out with a Warburg apparatus thermostated at 30° C.

IV. RESULTS AND DISCUSSION

Peroxidase

When horseradish (HRPR) solutions were held for thirty hours at room temperature at the pH range 4.0 - 7.0, there was a loss of enzymatic activity at the acidic end of the range (Table 1), presumably because of the loss of the prosthetic group as described by Maehly (1952). With sodium bisulfite present in the solutions, however, there was a considerable retention of activity at the lower pH levels. The opposite effect would be expected as the increase in ionic strength, caused by the addition of bisulfite, should accelerate the detachment of the prosthetic group by acid. This is illustrated in Table 2, where it can be seen that the addition of sodium sulfate hastens the loss of enzymatic activity at pH 4.0, in contrast to the addition of bisulfite.

The protective effect of bisulfite was also observed at pH levels below 4.0 (3.5 and 3.0), where the inactivation of peroxidase is much more rapid, taking place in only a few hours. The protective effect also exists in the absence of oxygen (Table 1). This indicates that the effect is not due to the antimicrobial action of

Table 1.--The effect of bisulfite on the activity of horseradish peroxidase solutions.

pH	Without Bisulfite	Containing 0.067M Bisulfite
4	37	87
5	80	92
6	100	96
7	100	98

Each solution contained 0.5 mg HRPR and 0.66M citrate-phosphate buffer in a total volume of 3 ml. The figures are the percent enzymic activity remaining after 30 hours incubation under anaerobic conditions.

Table 2.--The effect of sulfate and bisulfite on the activity of HRPR solutions at pH 4.0.

Time (hr)	No SO_4 or HSO_3	With 0.05M SO_4	With 0.05M HSO_3
0	100	100	100
1.5	91	69	94
3.0	80	45	86
4.5	72	29	82
6.5	59	17	78
8.5	43	8	72
10.5	34	0	71

Each solution contained 0.03 mg HRPR in 6 ml 0.05M citrate buffer, pH 4.0. The figures are the percent activity remaining after varying periods of incubation.

bisulfite, in that the bisulfite preserves the enzyme by discouraging the growth of microorganisms (e.g., mold) in the solutions.

A similar protective effect on the Soret absorption band (360-450 nm) was observed when bisulfite was present in the HRPR solutions at pH 4.0. Line 1 of Figure 1A represents the normal absorbance spectrum of HRPR in the Soret region, with a peak at 403 nm. Line 2 represents the spectrum of the same material six hours later: the absorbance had fallen off because a portion of the enzyme had lost its protohematin prosthetic group. Line 3 represents the spectrum after twelve hours and line 4 after twenty-four hours of incubation. By that time the enzyme had become nearly colorless and inactive.

Figure 1B illustrates the spectral curve of a similar solution of peroxidase at pH 4.0 with bisulfite added. It can be seen that the curve did not fall with time and after twenty-four hours the only change was a shift of the peak from 403 nm to about 395 nm.

Both solutions were dialyzed for twenty-four hours at 5°C against demineralized water and their Soret spectra then compared. (Figure 1C). The peroxidase which had been treated with bisulfite had an almost normal spectrum, with its peak shifted back to 402 nm, whereas the untreated peroxidase had a flattened spectrum.

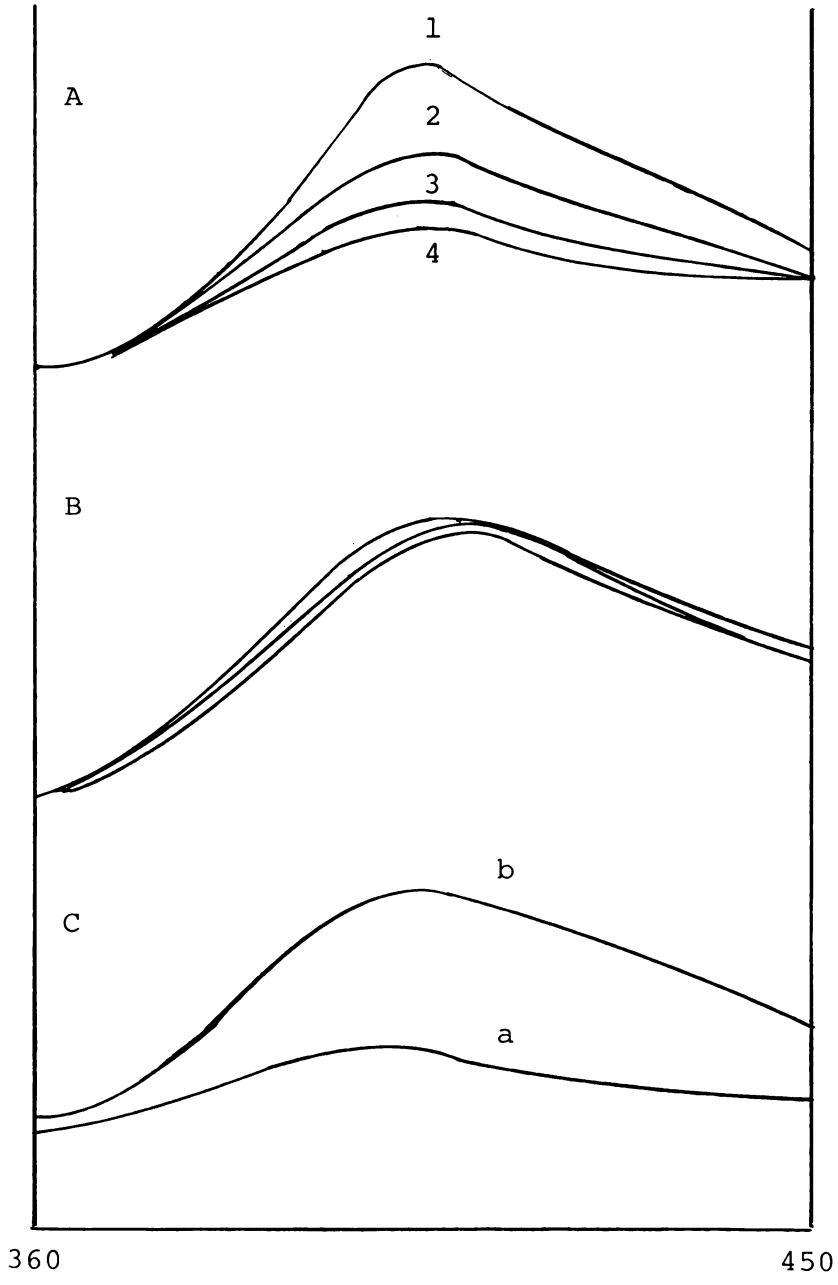


Figure 1.--Effect of bisulfite on the spectrum of HRPR. (A) Spectra of a solution containing 1 mg HRPR in 5 ml of 0.04M citrate buffer, pH 4.0. Lines 1, 2, 3, and 4 correspond to the spectra at 0, 6, 12 and 24 hours of incubation, respectively. (B) Spectra of same solution containing 0.1M bisulfite. (C) Line a is the spectrum of solution A, and b of B, both after 24-hour incubation followed by 24-hour dialysis.

The enzymatic activities of these solutions at the times their spectra were determined are given in Table 3.

These findings indicate that bisulfite retards the loss of the prosthetic group by acid. The reversible shift in the spectral peak caused by bisulfite suggests a reaction between the bisulfite and the protohematin group, which stabilizes the linkage between the iron atom and the protein. This reaction could be a reversible complex formation between bisulfite and the enzymic prosthetic group.

To test this hypothesis, four peroxidase solutions with the same HRPR and buffer compositions as those in Table 1 were held for twenty-four hours at pH 4.0. Solutions 1, 2, and 3 also contained 0.033 m KCN, 0.033M NaN_3, and 0.033M NH_4F, respectively; the fourth solution was a control. Cyanide, azide, and fluoride all form spectroscopically distinctive complexes with peroxidase (Keilin and Hartree, 1951).

After twenty-four hours incubation the four solutions were dialyzed for thirty-six hours against demineralized water at 5°C. With the removal of the complexing agents the normal Soret spectrum of each peroxidase solution was restored, but the spectrum of the control solution was very flat in comparison. When the enzymatic anitivities of the dialyzed solutions were determined, it was found that solution 1 retained 81% of its original activity; solution

Table 3.--Loss of enzymatic activity of the solutions A and B of Figure 1 with time.

Incubation Time	Solution A	Solution B
0	100	100
6	45	96
12	25	80
24	15	75

Figures are percent activity remaining after incubation.

2, 72%; and solution 3, 87%; the control retained only 14% of its original activity.

It appears, therefore, that complexing agents in general stablilize the enzyme against attack by weak acids, and it may be inferred that bisulfite, since it exerts a similar stabilizing effect, is also a complexing agent for peroxidase. The fact that the bisulfite-peroxidase complex is active, whereas the other complexes are not, may be due to the rapid oxidation of the bisulfite upon addition of H_2O_2 for the assay.

Assuming the bisulfite-peroxidase complex is exactly as active as free peroxidase, its dissociation constant could be determined by the kinetic method of Mildvan and Leigh (1964). This method is applicable in cases where an enzyme is gradually inactivated by some inhibitory agent and a cofactor or other substance combines with the enzyme and retards the inactivation. The key equation is as follows:

$$\frac{1}{k_{app}} = \frac{1}{k} + \frac{[lig]}{k\ K_d}$$

where K_d is the dissociation constant of the ligand-enzyme complex and [lig] is the molar concentration of the complexing agent. k and k_{app} are, respectively, the actual and apparent rate constants for the inactivation of the enzyme. k_{app} is defined by the equation:

$$\log \frac{[Eo]}{[E]} = \frac{k_{app} t\ [I]}{2.3}$$

where [Eo] is the total enzyme concentration and [E] is the active enzyme concentration after time t of incubation with an inhibitor present in concentration [I].

K_d is determined by plotting $1/k_{app}$ against [lig] and the intercept on the x axis has the value of $-K_d$.

In this study, four HRPR solutions containing varying amounts of bisulfite were deoxygenated by passing nitrogen through them and incubated for twenty-four hours at pH 4.0 at room temperature. The activities of the solutions were determined both at zero time and after incubation. The solutions were then held for an additional twenty-four hours and their activities again determined. These data were used to determine a simplified k_{app}, to be designated as k'_{app}, in the following manner: the value of Eo/E was taken as the ratio of the activity at zero time to that after a given incubation time and log Eo/E was considered equal to k'_{app}; this simplification is based on the fact that incubation time, t, and acid concentration, [I], do not enter into the final value of K_d.

Figure 2 gives the plot of $1/k'_{app}$ vs $[HSO_3]$ for both the twenty-four hour and forty-eight hour periods. The intercept on the X axis indicates a value of about 0.02M as the dissociation constant of the bisulfite-HRPR complex.

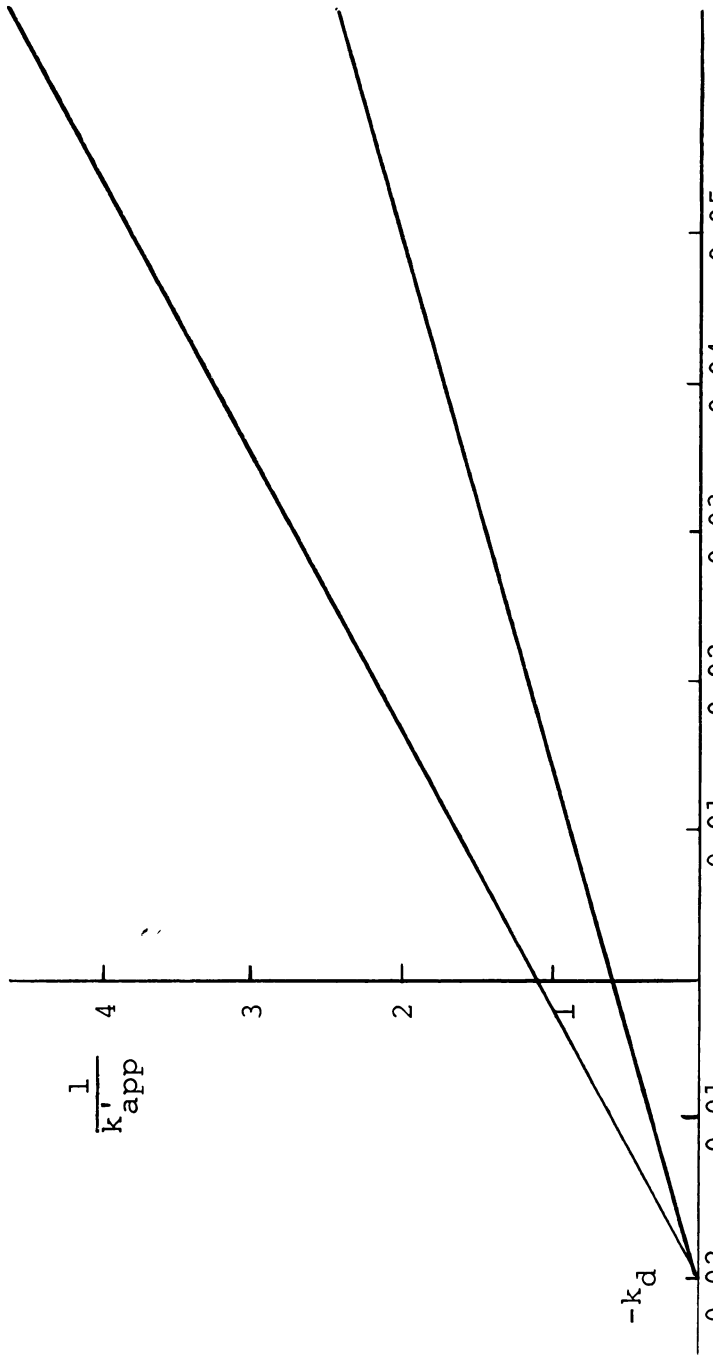


Figure 2.--Mildvan-Leigh Plot of $1/k'_{app}$ vs $[HS\bar{O}_3]$. Each reaction mixture contained 0.5 mg of HRPR, 0.05M citrate buffer, pH 4.0 and varying quantities of bisulfite, in a total volume of 4 ml. The enzymatic activity was measured at 24 and 48 hours of incubation.

This determination was based on the assumption that the bisulfite ion did not inhibit the enzyme directly, e.g., by breaking disulfide bridges in the enzymic protein. However, there is evidence of such an inhibition, since Chmielnicka (1963) reported that 0.48 mg of SO_2 in solution at pH 4.5 will inhibit the activity of one microgram of horseradish peroxidase after preincubation for 10 hours. But for the purpose of our work, this loss was considered negligible.

Catalase

One milliliter of commercial beef liver catalase preparation was added to each of three 250-ml volumetric flasks. The first flask was made to volume with demineralized water, the second with 0.1M Na_2SO_4, and the third with 0.1M $NaHSO_3$. No buffer was added, but the pH of each solution was about 5.0 because of dissolved carbon dioxide.

The mixtures were allowed to stand at room temperature for twelve days. The water suspension retained a constant degree of turbidity during that time, as judged by visual observation, while the sodium sulfate solution remained clear; apparently the catalase was "salted in." The $NaHSO_3$ solution, however, developed an increasingly heavy turbidity and ultimately a white precipitate appeared on the bottom of the flask.

On the twelfth day of preincubation, the enzymatic activities of the catalase solutions were determined. The

catalase treated with sulfate had 85.5% of the activity of the catalase in water suspension. This loss could be due to a splitting of the protohematin prosthetic group from the catalase protein by the acid (H_2CO_3) in the solution; this process would be accelerated by the presence of sodium sulfate. The catalase treated with bisulfite had only 9.5% of the water-treated enzyme. The bisulfite obviously caused extensive denaturation of the catalase protein, and therefore no "protective effect" of bisulfite on catalase could be observed.

Lipoxygenase

The aerobic oxidation of linoleic acid by pea lipoxygenase was studied manometrically in the presence of zero and 1.6×10^{-4}M bisulfite. The results are depicted in Figure 3. Other bisulfite concentrations studied were: 1.6×10^{-5}M, 3.3×10^{-5}M, 3.3×10^{-4}M, and 1.6×10^{-3}M. For purposes of clarity, however, reaction curves corresponding to these concentrations were not included in Figure 3 because they would crowd the space around the two curves already illustrated.

Two conclusions can be drawn from these results: (a) bisulfite slightly enhances the lipoxygenase reaction during the first 20-25 minutes of the oxidation; and (b) it inhibits the reaction after that point. It may be argued that the enhancement is only apparent, that the bisulfite

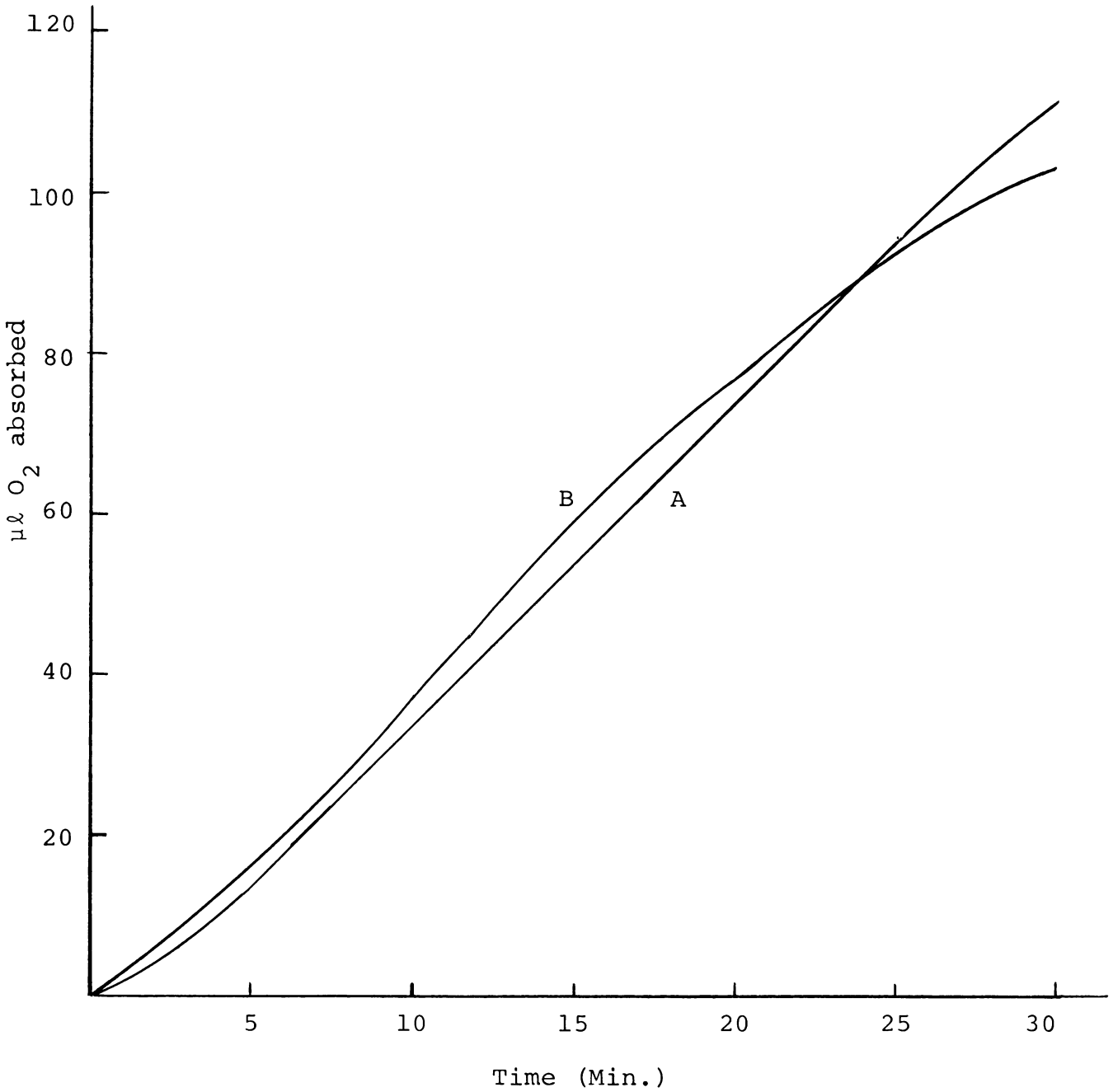


Figure 3.--Effect of bisulfite on the oxidation of linoleic acid by lipoxygenase.
Line A: No bisulfite in reaction mixture.
Line B: Reaction mixture contained 1.6×10^{-4}M $NaHSO_3$. Each reaction mixture contained 0.1% linoleic acid at pH 7.0, and 3% crude pea extract.

autooxidizes and contributes to the oxygen uptake of the system. However, control experiments show that very little of the bisulfite autooxidizes in thirty minutes under the conditions of the reaction.

An alternative explanation is that the bisulfite acts as a scavenger, destroying free radicals and hydroperoxides and relieving the enzyme of some reaction inhibition. Ultimately, however, the bisulfite attacks the enzyme itself, presumably by breaking disulfide bonds in the enzymatic protein, and in the long run inhibits the oxidation reaction.

Phenolase

When our "low cresolase" enzyme was incubated with a quantity of tyrosine in a solution buffered at pH 6.5, there was a long period of time (exceeding an hour) during which the mixture remained colorless and after which a brown color slowly appeared. However, when a small quantity of catechol was included in the reaction mixture, the colorless lag period was reduced to only a few seconds and the browning reaction (melanin formation) proceeded at a greatly accelerated rate. This catalytic effect of o-diphenols (and other reducing agents, such as ascorbic acid) has been observed by several investigators, and is explained by Bright (1963) as a triggering effect of the reducing agent on the enzyme, in which the reducing

agent furnishes the electrons necessary to initiate the conversion of monophenol to diphenol. If this were true, then adding catechol should shorten the lag period and enhance the monophenolase reaction until enough catechol was added to reduce the enzyme fully, after which no further increase in reaction rate would occur.

The shortening of the lag period by o-diphenols has been noted by Mason (1956), and the increase in browning rate caused by catechol is illustrated in Table 4, where it can be seen that the browning rate of a phenolase-tyrosine mixture is strictly dependent on the amount of catechol initially present. It should be emphasized that the concentrations of catechol used in these reactions are at catalytic levels, and the brown products derived from the catechol are negligible in amount compared with those from tyrosine (as measured by absorbance at 470 nm).

A difficulty with Bright's explanation arises from a simple mathematical calculation: the reaction mixtures in Table 4 each contained 0.05 mg tyrosinase, and assuming that the enzyme contained 0.2% copper (Kertesz and Zito, 1965), each mixture would contain 1.6×10^{-9} equivalents of copper. The lowest concentration of catechol used would then be about thirty times the minimum amount necessary to reduce all the enzymic copper, and consequently the reaction should proceed at its maximum rate. However, Table 4 shows that additions of larger amounts of catechol increase the rate of reaction.

Table 4.--Effect of catechol on the browning rate of phenolase-tyrosine reaction mixtures.

Concentration of Catechol	Browning Rate
1.66×10^{-5}M	6
3.32×10^{-5}M	12
6.64×10^{-5}M	22
9.96×10^{-5}M	31

Each reaction mixture contained 3.3×10^{-3}M tyrosine, 0.05 mg of "low cresolase" enzyme, 0.16M phosphate buffer, ph 6.5, and varying quantities of catechol, in a total volume of 3 ml. Browning rates are expressed in arbitrary units.

A further difficulty arises with respect to bisulfite. Since this compound is capable of reducing cupric ions to the cuprous state, it would also be expected to catalyze the cresolase reaction. However, previous work in this laboratory (Embs and Markakis, 1965; Markakis and Embs, 1966) has shown that while bisulfite in small quantities has little or no effect on the catecholase activity of phenolase, it profoundly inhibits cresolase activity as measured by O_2 uptake. This is also true for the browning reaction as shown in Table 5.

It is interesting to note that bisulfite influences the rate of browning even though it disappears before the brown products appear; i.e. it combines with the enzymatically produced quinones to form colorless addition products, and when the supply of bisulfite is exhausted, brown products appear (Embs and Markakis, 1965). Of course it may be suggested that the bisulfite addition products themselves inhibit the cresolase reaction, though to a lesser extent than does bisulfite. There is no evidence for this, in theory at least, since the addition products are probably cyclic sulfates and sulfonic acids whose molecular structures resemble those of the smaller melanin molecules.

Another possibility is that bisulfite forms a reversible complex with the enzymic copper, as is the case with cyanide or cysteine, and somehow inhibits the cresolase activity without disturbing the catecholase. This

Table 5.--Effect of bisulfite on the browning rate of phenolase-tyrosine-catechol reaction mixtures.

Concentration of Bisulfite	Browning Rate
0	10
1.66×10^{-5}M	6
2.49×10^{-5}M	4
3.32×10^{-5}M	0

Each reaction mixture had the same composition as those in Table 4, except that each contained 3.32×10^{-5}M catechol and varying amounts of bisulfite. Browning rate is expressed in arbitrary units.

hypothesis is suggested by the fact that the bisulfite inhibition is a temporary one, that the cresolase reaction proceeds very slowly until the bisulfite is consumed by the quinones and is no longer an obstacle to the reaction. Some of the bisulfite is also probably oxidized by the disulfide bridges of the enzyme.

This hypothesis was tested by determining the reaction rates of a phenolase-catechol-tyrosine-bisulfite mixture in the presence of varying amounts of cuprous ions. The results are shown in Table 6. If bisulfite forms a complex with copper, excess cuprous ions should relieve the enzyme of some of the inhibition. However, it can be seen that there was no significant increase in reaction rate resulting from the added copper. When excess phenolase was added instead of excess copper, the reaction increased somewhat, but fell short of what would be expected if bisulfite were simply a complexing agent.

As a comparison, the same experiments were repeated with cysteine, a known copper binding agent. The results in Table 7 show that the addition of cuprous ions readily overcomes the cysteine inhibition.

Table 8 shows that the addition of increasing amounts of catechol to a phenolase-tyrosine-bisulfite reaction mixture abolishes the inhibition by bisulfite. Therefore, it would seem, from all the foregoing evidence, that bisulfite does not react with the enzyme itself but

Table 6.--Effect of cuprous ions on the bisulfite inhibition of the browning rate of a phenolase-tyrosine-catechol reaction mixture.

Concentration of Cu	Browning Rate
0	9
1.66×10^{-5}M	12
3.32×10^{-5}M	12
4.98×10^{-5}M	13

Each reaction mixture had the same enzyme, tyrosine, buffer, and catechol concentration as those in Table 5. Also, each mixture contained 3.3×10^{-5}M $NaHSO_3$. The browning rate without bisulfite was 22.

Table 7.--Effect of cuprous ions on the cysteine inhibition of the browning rate of a phenolase-tyrosine-catechol reaction mixture.

Concentration of Cu	Browning Rate
0	11
1.66×10^{-5}M	31

Each reaction mixture was the same as those in Table 6 except that each contained 6.7×10^{-5}M cysteine instead of bisulfite. Apparently once the copper relieved the cysteine inhibition, the cysteine enhanced the browning rate in its capacity as a reducing agent.

Table 8.--Effect of catechol on the bisulfite inhibition of browning of a phenolase-tyrosine reaction mixture.

Concentration of Catechol	Browning Rate
3.33×10^{-5}M	0
6.7×10^{-5}M	13
1.0×10^{-4}M	22
1.3×10^{-4}M	31

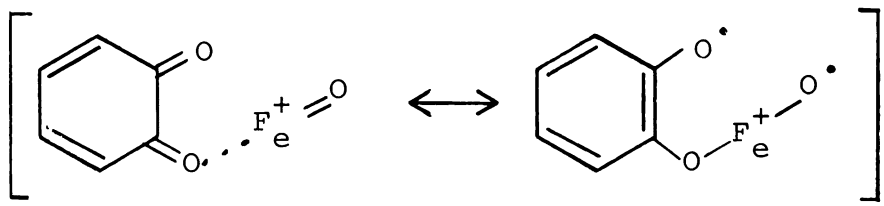
Each reaction mixture had the same composition as those of Table 4 except that each also contained 3.3×10^{-5}M bisulfite and varying amounts of catechol.

interferes with some intermediate reaction crucial for the hydroxylation process, and that an o-diphenol (or some other reducing agent) is involved in this reaction. Also, this intermediate reaction is not a reduction of the copper by the reducing agent.

The only known reaction to occur among the reactants: phenolase, catechol, and bisulfite, is an oxidation of the catechol to o-quinone followed by a reaction with bisulfite to form the colorless addition product or products. Thus a very simple explanation of the bisulfite effect can be proposed if the nonenzymatic hypothesis for the cresolase action is accepted (see Review of the Literature): The 0-quinone is responsible for the hydroxylation, and the bisulfite interferes by converting it to the inactive addition product.

This would explain why bisulfite and o-diphenol act in opposite directions in the cresolase reaction. However, a mechanism must be proposed to account for the possibility of hydroxylation caused by quinone, in which molecular oxygen is inserted into the monophenol molecule.

One such mechanism could be suggested by the work of Hamilton et al. (1966), who found that a ferric ion-catechol mixture will catalyze the hydroxylation of aromatic compounds by peroxide. The active agent is thought to be:



in which the oxygen atom on the right hand side of the iron ion is derived from peroxide and is the hydroxylating agent. One could imagine a similar species composed of o-quinone, copper ions, and molecular oxygen performing the same function with monophenols in a cresolase reaction. The quinone, forming on the enzyme molecule, would draw copper ions from the enzyme, complex with molecular oxygen, and attack a monophenol molecule which would be held in place by the enzyme. The result could be a diphenol or quinone. Put in schematic form:

1. $E\text{-}Cu + O_2 \longrightarrow E\text{-}Cu - O_2$
2. $E\text{-}Cu - O_2 + 2D \longrightarrow E\text{-}Cu\text{-}2Q + 2H_2O$
3. $E\text{-}Cu - 2Q + O_2 \longrightarrow E + Q\text{-}Cu\text{-}O_2 + Q$
4. $Q\text{-}Cu - O_2 + M \dashrightarrow Q\text{-}Cu + Q + H_2O$
5. $Q\text{-}Cu + Q \longrightarrow$ melanin

where E = enzyme, Q = quinone, D = diphenol, and M = monophenol.

The following observations lend indirect support to this hypothesis:

1. Reaction of phenolase with catechol causes partial inactivation of the enzyme, and this inactivation is believed to be a reaction between quinone and enzyme (Brooks and Dawson, 1965).

2. Phenolase loses copper during reaction with o-diphenols (Dressler and Dawson, 1960).

3. The oxidation of catechol in the presence of cupric ions results in a dark purple color rather than the usual yellow color of the melanins. The purple color does not form if the cupric ions are added after the oxidation has taken place. This indicates that copper forms a complex with some early product of the oxidation, probably a quinone.

This hypothesis suggests that the hydroxylation could be carried out in the absence of enzyme. Accordingly, a solution containing 2×10^{-4}M catechol, 5×10^{-3}M p-coumarate, 4×10^{-4} bisulfite, and 2×10^{-4}M cuprous ions was allowed to stand at room temperature for 3-4 hours at pH 6.5. A control solution, containing the same ingredients but also a small amount of phenolase, was allowed to stand for the same length of time. Afterwards the two solutions were streaked on chromatographic paper which was then irrigated overnight and dried. Under ultraviolet light the area of the paper corresponding to the control solution exhibited a series of fluorescent bisulfite addition products of characteristic sizes, colors, and

distribution. The pattern of fluorescent products closely resembled that derived from a solution of caffeate and bisulfite under oxidative conditions, since caffeate is the o-dihydroxy analog of the monophenol p-coumarate (Embs and Markakis, 1965).

The test solution (without phenolase) also yielded addition products, but of a different pattern than those of the control. These products were fewer in number, rather uniformly blue in color, and considerably fainter in intensity.

A repetition of the above experiment using a test solution without cuprous ions yielded the same pattern, but only after four days of standing. However, solutions containing any combination of ingredients other than those mentioned above (e.g. cuprous-p-coumarate-bisulfite, cuprous-catechol-p-coumarate, etc.) did not yield detectable fluorescent products of any kind.

The atypical products from the nonenzymatic reaction could have been derived from nonspecific hydroxylation of the p-coumarate, resulting in a mixture of di, tri, or even tetrahydroxy compounds, some of which may not be fluorescent. If this is true, it would seem that the enzyme is necessary to insure that the cresolase reaction produces only 3,4 dihydroxy compounds from their 4-hydroxy parents. The enzyme could hold the monophenol molecule in position and labilize the proton ortho to the hydroxyl group for attack by the quinone-copper-oxygen complex.

The fact that addition products were also obtained from the reaction without either copper or enzyme indicates a possible contamination by traces of copper ions, or even that the quinones, derived from autooxidation of catechol, alone are able to cause a hydroxylation reaction, as Kertesz asserts.

Of course it may be argued that the atypical bands are actually catechol addition products which have absorbed some p-coumarate and became fluorescent. Since the composition of the products is still unknown, this argument cannot be entirely discounted. However, it may be mentioned that when o-coumarate, a brilliantly fluorescent monophenol, is allowed to react with catechol and bisulfite under oxidative conditions, no fluorescent products can be separated from the solution by paper chromatography, with either enzyme or copper present in the reaction mixture. Therefore, if the catechol addition products do not derive fluorescent from o-coumarate, they probably would not from p-coumarate.

Two problems remain to be discussed: the fact that nonphenolic reducing agents, such as ascorbic acid, also stimulate the cresolase reaction and overcome inhibition by bisulfite (Markakis and Embs, 1966); and also that certain mushroom phenolase preparations are able to carry out the cresolase reaction very efficiently in the absence of any apparent reducing agent.

Kertesz and Zito (1962) suggested that a phenolase-ascorbate combination could carry out the cresolase reaction with the mechanism proposed by Udenfriend et al. (1954), in which an ascorbate - H_2O_2 reaction product hydroxylates aromatic compounds, with the help of ferric ions. This would be analogous to the quinone-copper-oxygen mechanism mentioned above, except that phenolase does not oxidize ascorbic acid. It may be argued that H_2O_2 forms spontaneously from the autooxidation of ascorbic acid, and that it rids the system of bisulfite and promotes hydroxylation of monophenols. However, our previous work (1966) has shown that fluorescent addition products form in a phenolase-p-coumarate-bisulfite-ascorbate reaction mixture, indicating that the bisulfite was not oxidized in the reaction by peroxide. Obviously the effect of ascorbic acid on the cresolase reaction is complex and needs further study.

Highly purified mushroom phenolase preparations will catalyze the rapid oxidation of tyrosine and other monophenols without the addition of reducing agent. These "high cresolase" preparations are sensitive to inhibition by bisulfite, but unlike the low cresolase type, addition of larger concentrations of enzyme readily overcomes the inhibition (Table 9). Therefore, it appears that the high cresolase enzyme is equivalent to a mixture of low cresolase enzyme and a reducing agent, and it may be suggested

Table 9.--Effect of increasing enzyme concentration on the bisulfite inhibition of browning by phenolase-tyrosine-reducing agent reaction mixtures.

A. Low cresolase enzyme

Amount of Enzyme in Mixture	Browning Rate
0.05 mg	0
0.10	1.7
0.15	0
0.20	2
0.25	3

Each reaction mixture had the same composition as those in Table 4 except that each had 3.3×10^{-5}M bisulfite, 3.3×10^{-5}M catechol, and varying amounts of enzyme.

B. High cresolase enzyme

Amount of Enzyme in Mixture	Browning Rate
0.05 mg	2.5
0.10	6
0.15	9
0.20	14
0.25	18

Each reaction mixture contained: 1.67×10^{-3}M tyrosine, 0.16M phosphate buffer, pH 6.5, 3.3×10^{-6}M dihydroxyphenylalanine, 3.3×10^{-5}M bisulfite, and varying amounts of phenolase in a total volume of 3 ml.

that the mushrooms phenolase molecule contains a residue which acts as a reducing agent, possibly dihydroxyphenylalanine (dopa) or even dopa-quinone, if quinones are necessary for hydroxylation.

In terms of the cresolase mechanism mentioned above it may be suggested that high cresolase enzymes contain quinone residues which hold copper ions and oxygen molecules, and carry out the cresolase reaction. If this is true, the enzyme should not lose its copper as it does during the catecholase reaction, and this indeed was found to be the case by Dressler and Dawson (1960) with their high cresolase preparations. The fact that a high cresolase preparation can be converted to a low cresolase state by partial denaturation (e.g. by heating or prolonged contact with bisulfite) may be explained as a disruption of the spatial relationship between the quinone-copper-oxygen complex, and the binding site of the monophenol molecule on the enzyme. In such a case, a trace of added o-diphenol, oxidized to o-quinone, would substitute for the enzyme-bound quinone.

The foregoing discussion is admittedly largely speculative. Further lines of research into this problem may be suggested: (1) a better elucidation of the molecular structure of phenolase to reveal the existence of a quinone residue; (2) an investigation of the possibility of a quinone-copper complex as a hydroxylating agent;

(3) an investigation of the effect of bisulfite on the iron-ascorbate hydroxylating system; and (4) a repetition of the work of Dressler and Dawson using low cresolase enzyme, a monophenol, and a trace of o-diphenol, to see if the enzyme loses its copper. If the above mechanism is correct, it would; and if Dressler and Dawson's two-site theory is correct, it would not.

Ascorbic Acid Oxidase

When 0.1 ml amounts of cucumber peel juice, which contains ascorbic acid oxidase, were incubated with solutions containing 4.8×10^{-3}M ascorbic acid and varying amounts of bisulfite at pH 5.7 (volume of each solution was 3.0 ml), a loss of enzymatic activity in direct proportion to the amount of bisulfite present was observed (Figure 4). However, in contrast to its effect on the cresolase function of phenolase, the bisulfite did not cause a lag period followed by increased activity, but merely a general reduction in the rate of ascorbic acid oxidation. This indicates a direct inhibition of the enzyme by the bisulfite. To substantiate this conclusion, 0.1 ml of cucumber juice was preincubated with 0.1 ml of 10^{-2}M $NaHSO_3$ solution for 30 minutes and then its ascorbic acid oxidase activity was determined in the same manner as in Figure 4. The results, shown in Figure 5, indicate that the bisulfite caused an extensive loss of enzymatic activity.

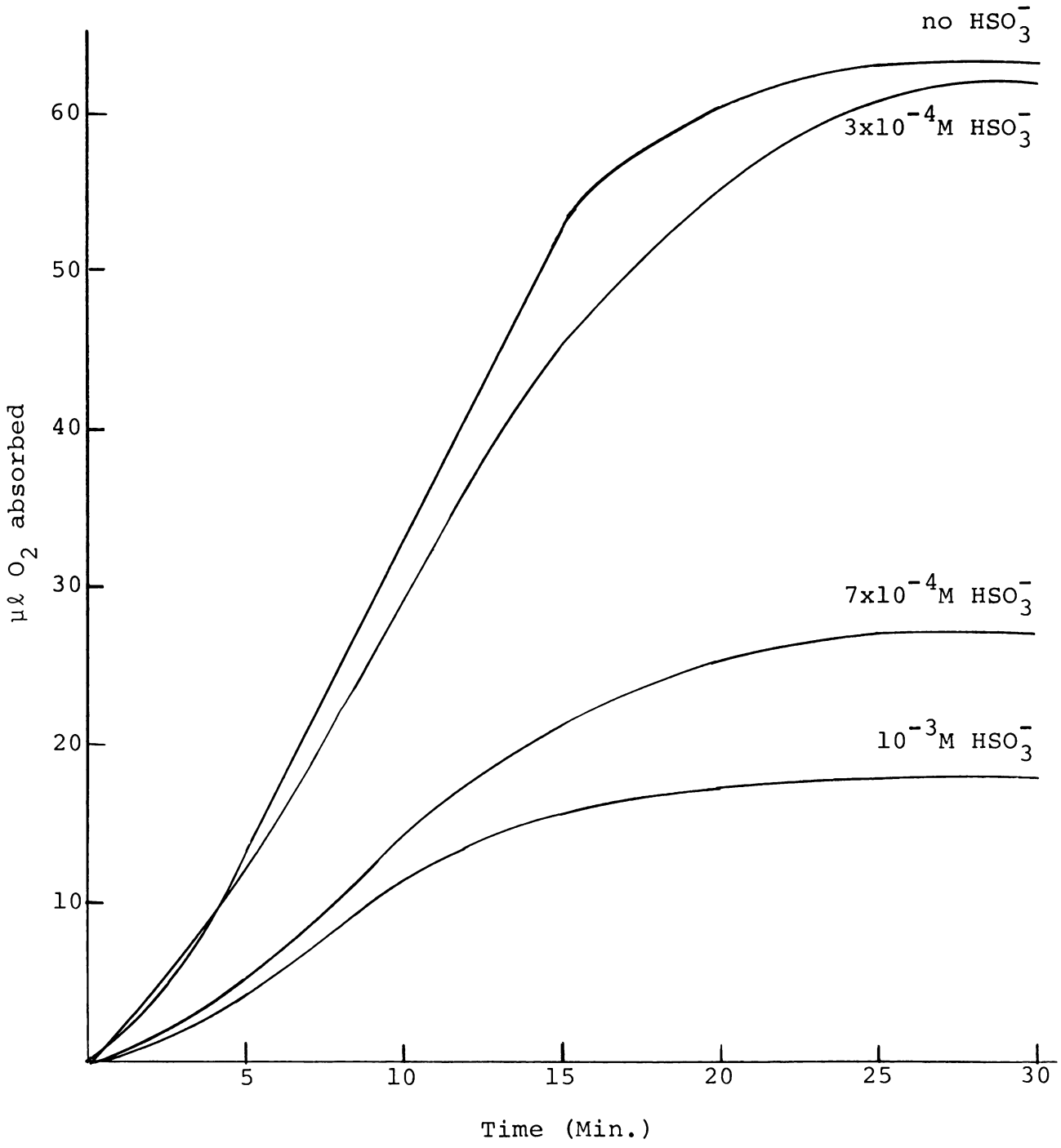


Figure 4.--Effect of varying concentrations of bisulfite on the rate of ascorbate oxidation by ascorbate oxidase in 0.1 ml cucumber peel juice. Each reaction mixture contained 14.4 micromoles ascorbate at pH 5.7.

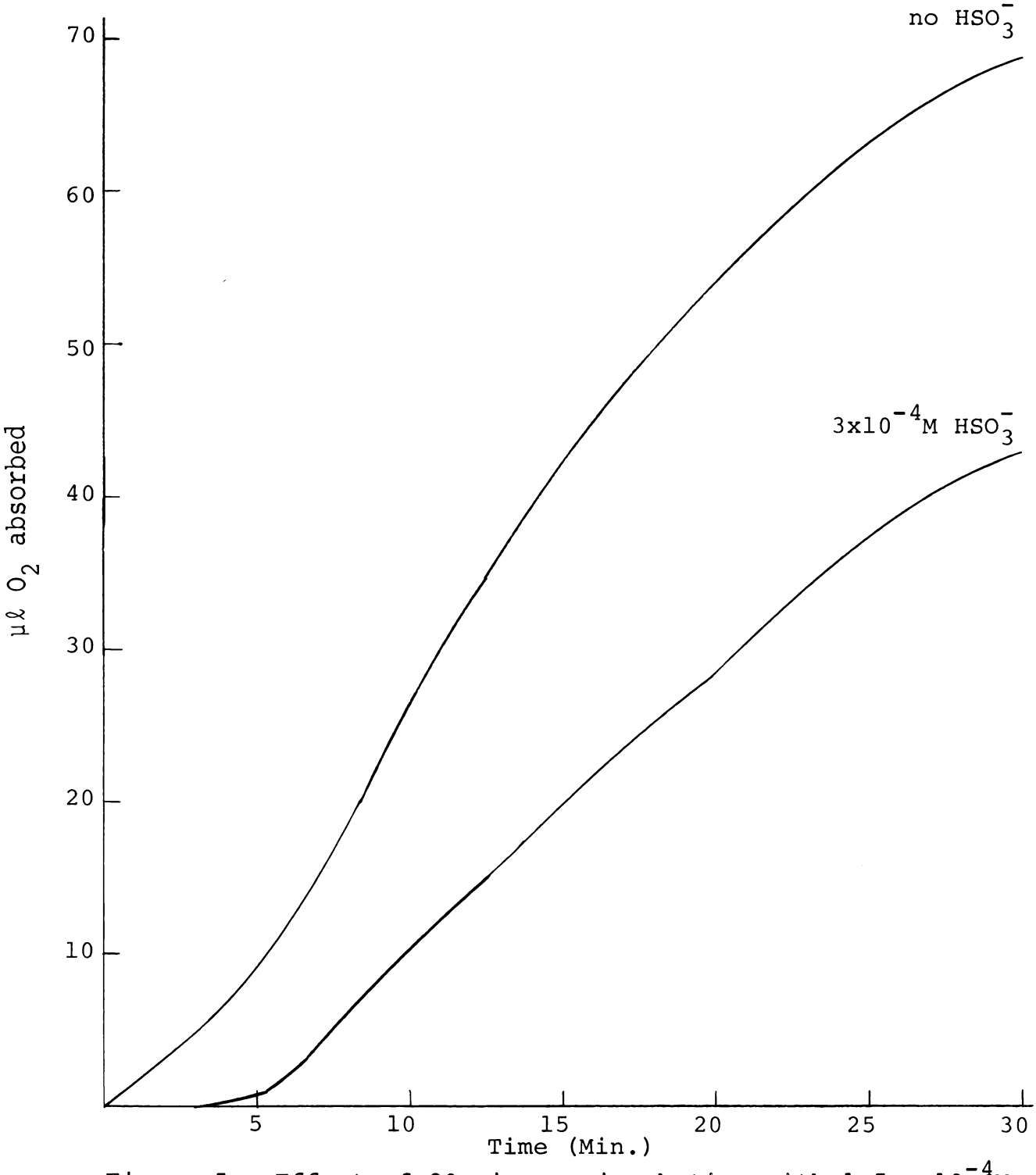


Figure 5.--Effect of 30 min. preincubation with 1.5 x 10^{-4}M bisulfite on the ascorbate oxidase activity of 0.1 ml cucumber peel juice.
For composition of reaction mixtures, see Figure 4.

This loss of activity is quite similar to the gradual inhibition of phenolase by bisulfite (Embs and Markakis, 1965). In both cases the cause is apparently a denaturation of the enzymic protein by breakage of disulfide linkages.

ADDENDUM: The Effect of Bilsulfite on the Growth of *Saccharomyces cerevisiae*

Review of Literature

Sulfur dioxide solutions are widely used in the food industry to control the growth of yeasts, molds, and bacteria. Their antiseptic potency is strongly pH dependent, in that they are powerfully inhibitive to microbial growth under acidic conditions and ineffective at neutrality. This could possibly be explained by the assumption that the nonionic form of the compound (H_2SO_3), which exists at lower pH levels, more rapidly penetrates the cell wall of the microorganism than the ionic forms (HSO_3^-, $SO_3^=$) do, and consequently greater amounts can enter the cell and interfer with its life processes (Bosund, 1962).

Rehm and Wittmann (1963) studied the effect of SO_2 solutions on yeast growth at various pH levels, and concluded that the bisulfite ion, as well as the undissociated H_2SO_3, had an appreciable antiseptic effect on yeast. However, their conclusions are dubious because they used an obsolete pK_2 value for sulfurous acid in calculating the variation of bisulfite concentration with pH.

In the present work attempts were made to recheck the work of Rehm and Wittmann using the proper pK_2, and

also to determine the accuracy with which Bosund's theory describes the mechanism of inhibition of yeast growth by SO_2.

Methods and Materials

All yeast used in these experiments was Saccharomyces cerevisiae taken from yeast cake, Red Star brand. No attempt was made to use an individual variety of this yeast. The yeast was allowed to reach an actively growing stage in growth media before use.

All growth media used was distilled water containing 0.67% yeast nitrogen base, 2% sucrose, and 0.05M citrate-phosphate buffer. The pH was adjusted with 5N NaOH using a pH meter. Fifty ml portions of the media were used in 125 ml Erlenmeyer flasks plugged with cotton. Sterilizations were done with a steam autoclave, and after inoculation each flask was allowed to rock gently on a mechanical shaker overnight.

Growth assays were determined either by plating with a yeast nitrogen base agar mixture or by determining the turbidities of the growth media 24 hours after inoculation, using a Beckman DU Spectrophotometer. Turbidities were expressed as per cent transmittance at 600 nm.

Radioactive SO_2 was prepared by boiling $H_2{}^{35}SO_4$, with copper, trapping the radioactive SO_2 in 0.1N NaOH,

then distilling it after acidification with H_2SO_4, and receiving it in cold 0.1N NaOH.

SO_2 uptake studies were performed by adding 0.35 -0.4g of actively growing yeast cells to 30 ml of 0.01M citrate-phosphate buffer containing radioactive SO_2. At measured time intervals, 4 ml aliquots of the well mixed liquid were withdrawn and added to 25 ml polyethylene scintillation bottles. The bottles were immediately placed in a Servall high speed centrifuge and the yeast precipitated by centrifugation. The supernatant liquid was poured off and the yeast washed three times with distilled water, centrifuging between washings. The yeast cells were then suspended with Cab-o-sil in a toluene based scintillation medium (6 g PPO and 0.2g POPOP in one liter toluene) and their radioactivity counted with a Packard Tri-Carb Liquid Scintillation Spectrometer.

In the growth studies, aseptic techniques were employed throughout. At measured time intervals 0.1 ml aliquots were withdrawn from the buffered $^{35}SO_2$ solutions and added to media for overnight growth.

In the experiment involving 600 PPM $^{35}SO_2$, each scintillation bottle received 5 ml of 0.03% H_2O_2 before the radioactive aliquots were added. The purpose of the H_2O_2 was to oxidize the SO_2 to $SO_4^{=}$, which would be less readily absorbed by the yeast.

Results and Discussion

Figure 6 shows the distribution of the three species, H_2SO_3, HSO_3^-, and $SO_3^=$, over the pH range 0-8. It can be seen that the nonionic form, H_2SO_3, predominates at the lower end of the pH scale, where SO_2 solutions have their greatest antiseptic potency. According to the theory of Bosund the H_2SO_3 form is antiseptic because, being non-charged, it is the only one of the three species which can penetrate the lipo-protein barrier in the cell wall. However, Rehm and Wittmann (1963) reported that bisulfite (HSO_3^-), though charged, also had antiseptic properties. Therefore, the first step in this study was to check the validity of this report by investigating the effect of SO_2 solutions at various pH levels on the growth of yeast cells.

Equal numbers of yeast cells were innoculated into flasks of growth media which had been adjusted to pH 3, 4, 5, 6, and 7, and containing 50 to 400 ppm SO_2. Saccharomyces cerevisiae was used because it grows throughout the pH range 3-7, though not equally well at every pH (Figure 7). The following observations were made: at pH 3 and 4, no yeast growth was evident even if the flasks were allowed to stand for several days with 50 ppm SO_2; at pH 6 and 7 the cells grew as well as the controls when observed 18 hours after innoculation at 400 ppm SO_2; at pH 5 the cells grew after undergoing a lag period the length of which

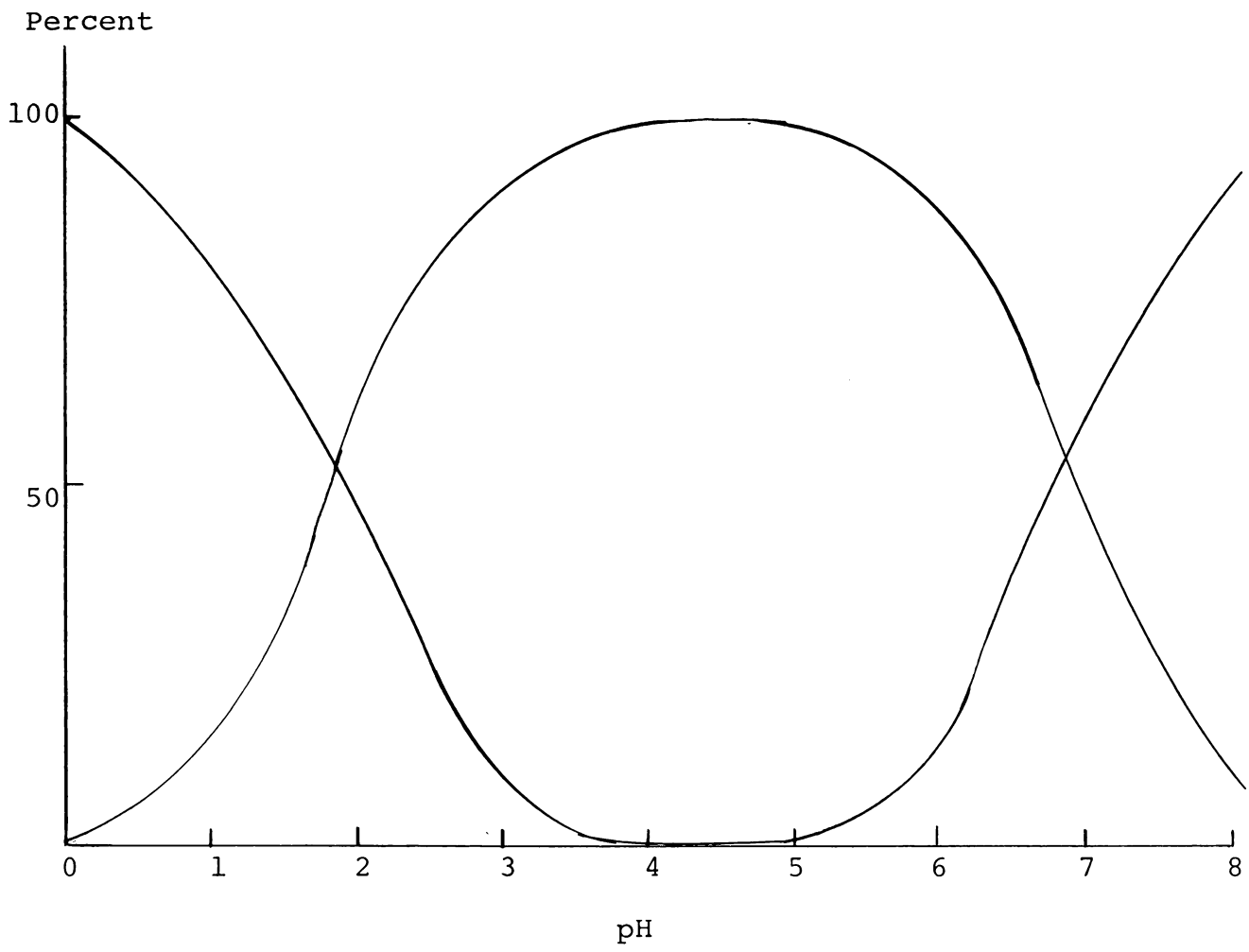


Figure 6.--Variation of the ionic species, H_2SO_3, HSO_3^-, and $SO_3^=$, with pH.

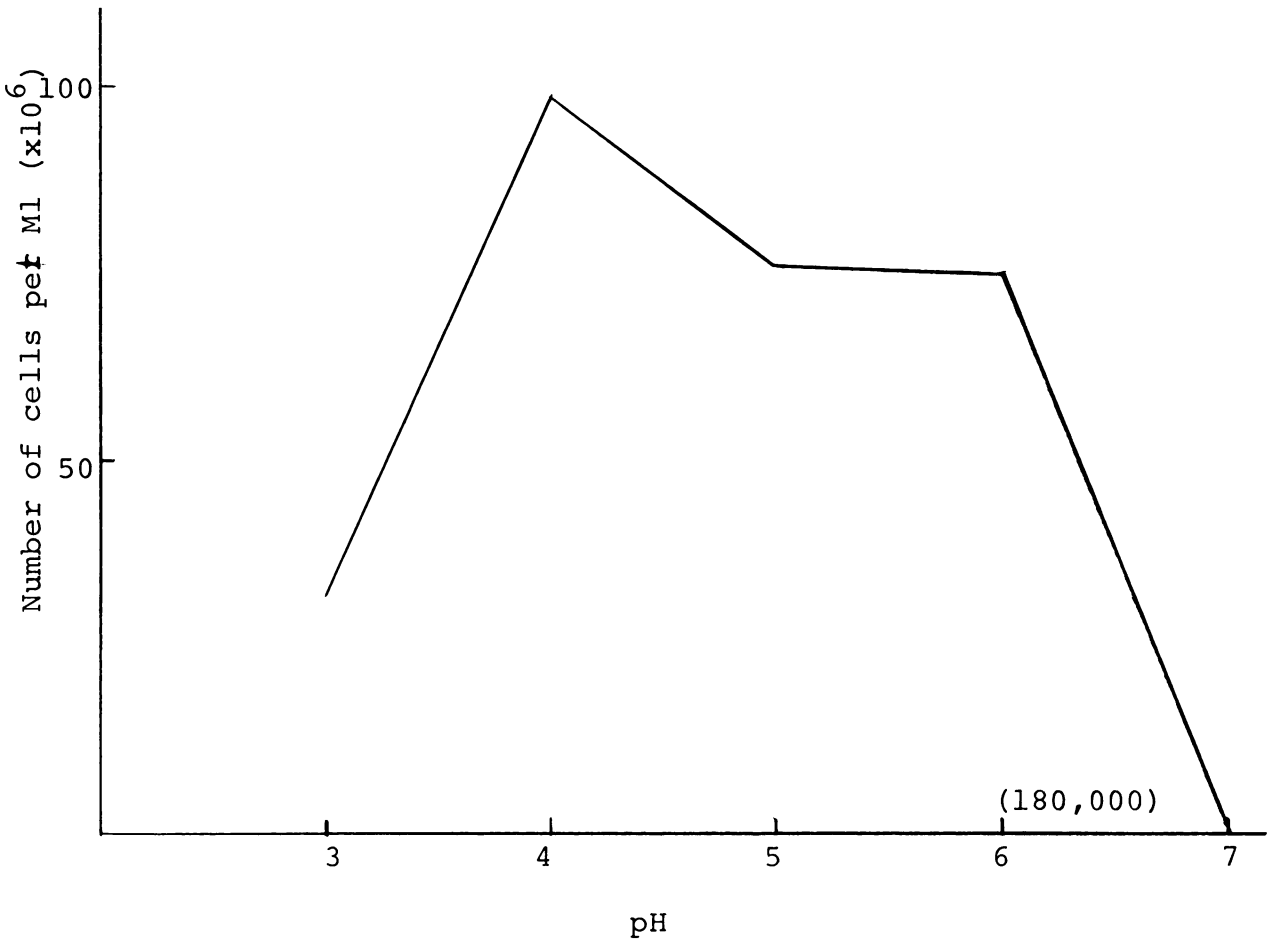


Figure 7.--Growth of yeast in the pH range 3-7. (Media buffered at pH's 3, 4, 5, 6, and 7 were inoculated with S. Cerevisiae. After overnight growth, cell count was determined by plating.)

was proportional to the SO_2 concentration. At 50 ppm SO_2 the lag was a few hours in length; at 100 ppm, it was about 40 hours long; at 200 ppm, 60-70 hours; at 300 ppm about 4 days; and at 400 ppm about 5 days. Once the yeast recovered from the lag period, its growth was as full as that of the controls.

On the basis of these results, the following conclusions can be drawn:

1. The bisulfite ion has very little antiseptic potency. At pH 6, 88% of the total SO_2 is in the bisulfite form, and since even 400 ppm SO_2 will not prevent yeast growth at that pH, it means that yeast can grow without difficulty in a solution containing 0.044% HSO_3^-.

2. The sulfurous acid molecule is an extremely potent antiseptic. At pH 4 the yeast cells would not grow at 50 ppm SO_2, which at that pH constitutes a solution of 0.0062% HSO_3^- and 0.000044% H_2SO_3. Since at 50 ppm SO_2 the solution does not contain enough bisulfite to affect yeast growth, all its antiseptic potency must be derived from the sulfurous acid molecule.

3. A comparison of the figures, 0.000044% H_2SO_3 and 0.044% HSO_3^- indicates that H_2SO_3 is well over one thousand times more potent than HSO_3^-. The latter may even have no antiseptic potency at all. Therefore, the report of Rehm and Wittmann is in error, probably because of their use of an obsolete pK_2 value in calculating the bisulfite content of their systems.

If the sulfurous acid molecule is the only form that actually enters the cell, then a direct relationship should exist between the amount of sulfur absorbed by the cell and the antiseptic potency of the SO_2 solution at a given pH. This possibility was investigated by exposing yeast cells to labeled sulfur dioxide solutions ($^{35}SO_2$) at various pH's, times, and SO_2 concentrations in the following manner: yeast cells were grown in a suitable medium overnight, then collected and washed asceptically and added to buffered sterile solutions of $^{35}SO_2$. At periodic time intervals aliquots were taken and added to fresh media for growth and simultaneously other aliquots were taken for radioactive assay. This was done over the pH range 3-7; at time intervals of less than 5 minutes, 2, 4, and 8 hours; and at concentrations of 200 and 480 ppm SO_2.

The results are shown in Table 10 and 11, and in Figures 8 and 9. It can be seen that uptake of labeled sulfur occurred at every pH, but that the uptake was more extensive at lower pH's. Tables 10 and 11 showed that the yeast cells died rapidly when exposed to SO_2 at pH 3; at pH 4 and above they withstood the full 8 hours exposure with apparent ill effect. In spite of this, less SO_2 was absorbed at pH 3 than at pH 4 or 5. This indicates that the antiseptic power of an SO_2 solution does not depend on the total amount of SO_2 absorbed by the microorganism but perhaps on the rate of absorbance. It may be suggested

Table 10.--Growth of yeast cells after exposure to 200 ppm SO_2 at pH's 3, 4, 5, 6, and 7.

	pH of Exposure				
Time of Exposure (Hr.)	3	4	5	6	7
0	3	3	2	2	2
2	22	3	2	2	2
4	86	5	3	2	2
8	85	3	2	2	2

The figures represent the transmittance values of the growth media at 600 nm. Figures below 10 represent substantial growth and figures above 80 represent no growth in 24 hours after inoculation.

Table 11.--Growth of yeast cells after exposure to 480 ppm SO_2 at pH's 3, 4, 5, 6, and 7.

	pH of Exposure				
Time of Exposure (Hr.)	3	4	5	6	7
0	2	2	1	1	1
2	77	2	1	1	1
4	74	2	1	1	1
8	78	3	1	1	1

These figures represent transmittance values of growth media, at in Table 4.

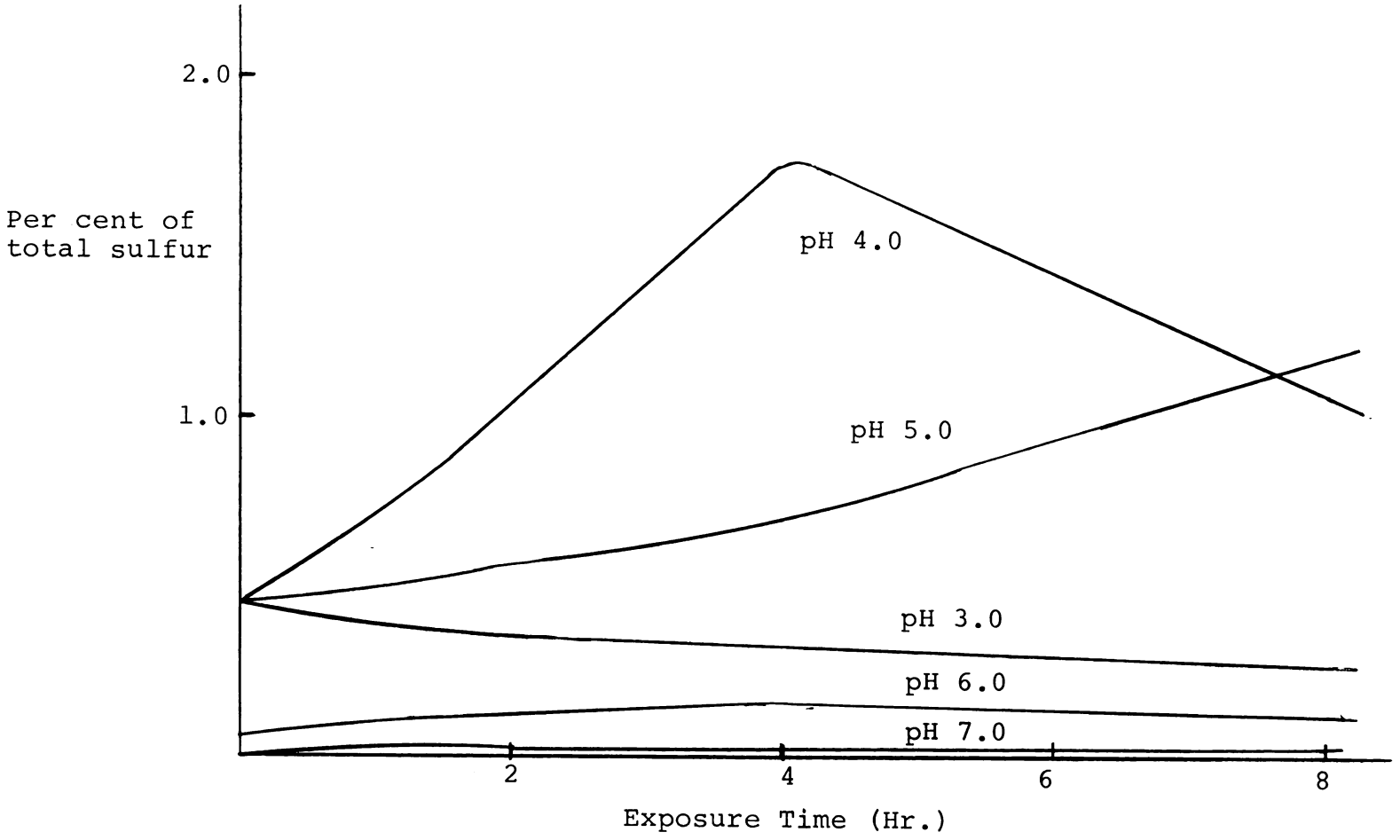


Figure 8.--Uptake of sulfur by yeast cells exposed to 200 PPM SO_2 at pH's 3, 4, 5, 6, and 7.

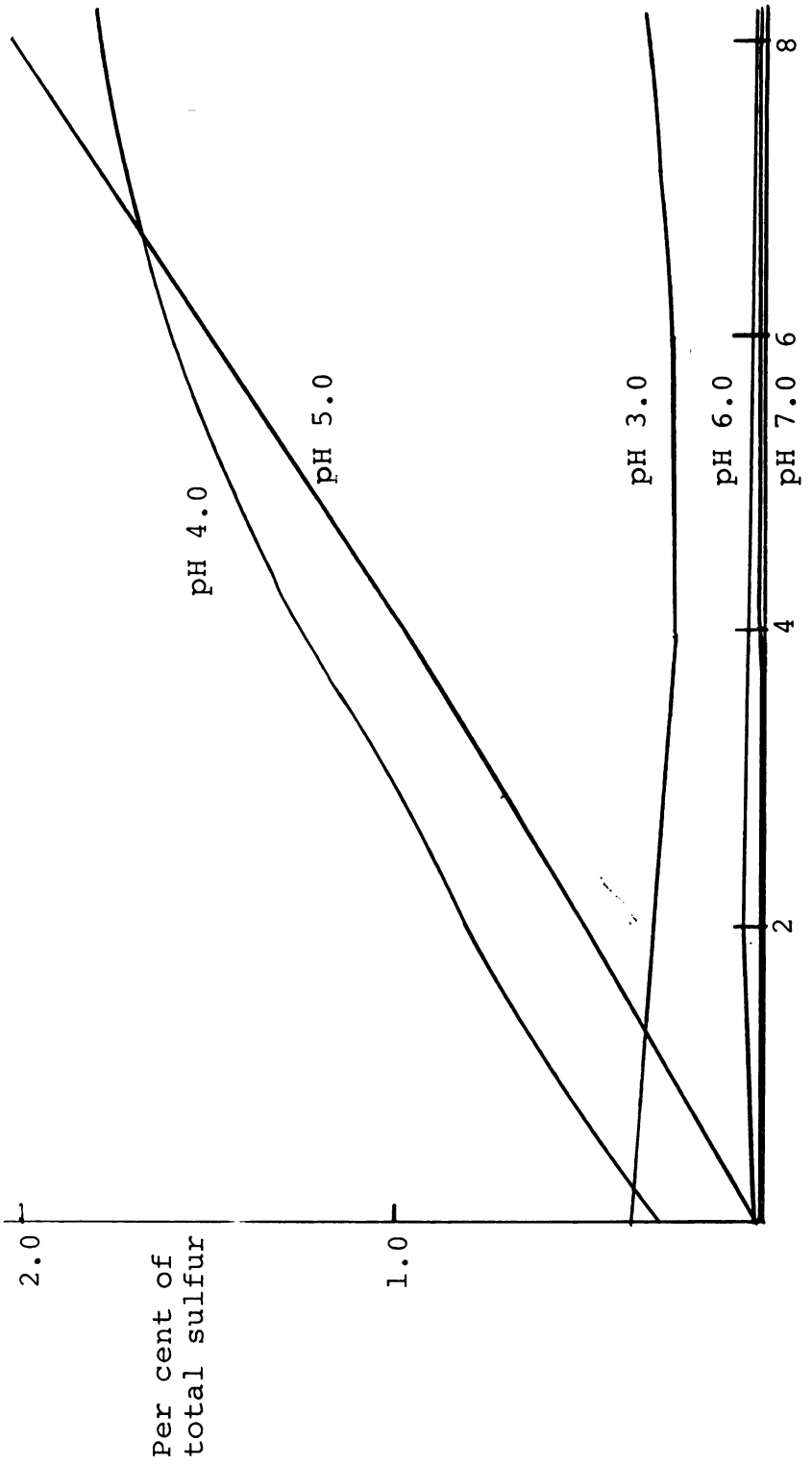


Figure 9.--Uptake of sulfur by yeast cells exposed to 480 PPM SO_2 at pH's 3, 4, 5, 6, and 7.

that pH 3 the SO_2 floods the cell so rapidly that its vital functions are paralyzed in the first few minutes of exposure, after which it absorbs no more SO_2. At pH 4 and 5 the yeast apparently can absorb and store large quantities of SO_2, and even expel some back into solution after holding it for a period of time.

In an attempt to substantiate these points, the experiments described above were repeated with the exception that aliquots were taken only for radioactive assay, and none for growth studies. This meant that aseptic procedures were not needed and consequently aliquots could be taken much more rapidly than before, thus making possible a greatly reduced time scale. The results were given in Figure 10. Here it can be easily seen that at pH 3 the yeast almost instantly reaches its maximum load of SO_2 while at pH's 4 and 5 the build-up is gradual.

To summarize: the antiseptic potency of an SO_2 solution toward yeast depends on the rate of uptake of SO_2 by the cell and not on the total amount absorbed. A more gradual rate of uptake means a longer survival time for the cell. Lower pH's cause higher uptake rates probably because of the greater proportions of H_2SO_3 molecules, which can easily penetrate the cell wall.

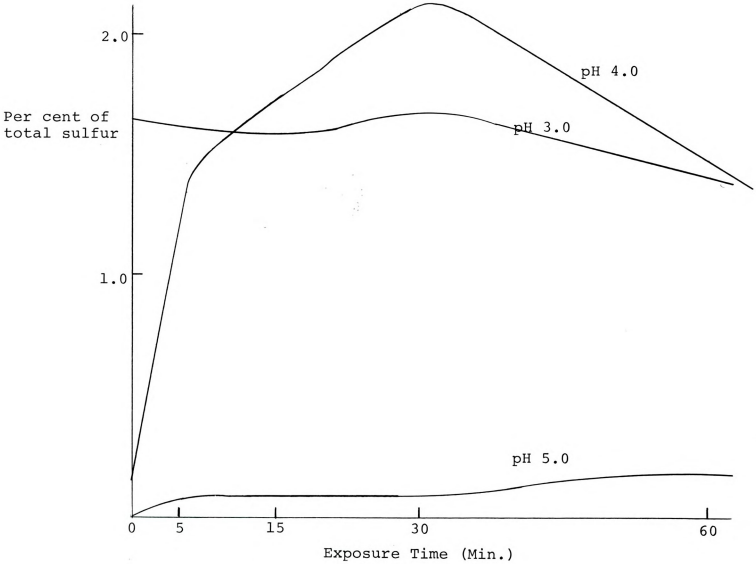


Figure 10.--Uptake of sulfur by yeast cells exposed to 600 PPM SO_2 at pH's 3, 4, and 5.

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