THE MECHANISM OF THE SULFUR DIOXIDE INHIBITION OF PHENOLASE BROWNING

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AN ABSTRACT

THE MECHANISM OF THE SULFUR DIOXIDE INHIBITION OF PHENOLASE BROWNING

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Sulfur dioxide inhibits the formation of brown pigments (melanins) in fruits and vegetables containing phenolic materials and the tyrosinase enzyme. The purpose of this thesis was to investigate the chemical mechanism of the sulfite induced inhibition of browning.

Model systems containing mushroom tyrosinase, phenolic substrates, varying amounts of sulfite, and on occasion catalytic amounts of ascorbic acid and catechol were used throughout the investigation. They were subjected to manometric, spectrophotometric, electrophoretic, radioactive tracer (with ^{35}S), and chemical analysis.

The investigation resulted in the following conclusions:

1. Sulfite gradually inactivates the monophenolase and polyphenolase functions of mushroom tyrosinase.

2. Sulfite combines irreversibly with the oxidation products (quinones) of the enzymatic reaction to form sulfite addition products and thereby stops the polymerization of the quinones to melanins.

3. Sulfite has an immediate inhibitory effect on the monophenolase function of the enzyme which prevents the enzymatic conversion of monophenols to o-diphenols. However, catalytic amounts of reducing agents greatly reduces this inhibition.

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INTRODUCTION

It is common knowledge that certain fruits and vegetables (apples, peaches, potatoes, etc.) quickly develop a brown discoloration upon bruising, cutting or crushing. This browning is caused by the enzyme-catalyzed oxidation of plant phenols and is generally detrimental to the quality of the product. Therefore, it is of great interest to the food processor to find some means of controlling it.

Since the enzyme (phenolase),* a suitable phenolic substrate, and oxygen must all be present before brown pigment formation can occur, any agent that excludes one of these three factors will prevent browning. Joslyn and Ponting (1951) have listed a number of these. Blanching or acid will inactivate the enzyme. Sugar syrups poured on fruit exclude oxygen from its surface. Chemical inhibitors can inactivate the enzyme (e.g. HCN, urea) or reduce the oxidation products of the reaction and prevent their polymerization to brown pigments (e.g. ascorbic acid, sulfhydryl compounds).

Sulfur dioxide and its salts are extensively used to prevent browning. While one may surmise that it acts by reducing the quinones while being itself oxidized to sulfate, its mechanism of

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*The terms "phenolase" and "tyrosinase" will be used interchangably in this thesis.

action has never been adequately clarified. Hence it is the purpose of this thesis to investigate the means whereby sulfur dioxide prevents phenolase-catalyzed browning.

REVIEW OF THE LITERATURE

The Mechanism of the Phenolase Reaction

The copper-containing enzyme phenolase is capable of catalyzing two apparently dissimilar reactions: the orthohydroxylation of monophenols and the oxidation of o-diphenols to the corresponding o-quinones. This intriguing property and its detrimental consequence in food processing has stimulated an immense amount of research on this enzyme.

Onslow and Robinson in 1928 proposed the theory that phenolase catalyzes the oxidation of catechol to o-benzoquinone and hydrogen peroxide, and that the peroxide is capable of converting monophenols to diphenols. But Dawson and Nelson (1938) found that hydrogen peroxide is not a product of the reaction, and in 1944 Nelson and his co-workers proposed an oxidation reaction with o-quinone and water as its products. This mechanism is generally accepted today.

The mechanism of the enzyme's oxidizing ability has not yet been thoroughly clarified. In 1938 Kubowitz found that the copper moiety of phenolase is necessary for its catalytic action, and that its removal would inactivate the enzyme. LuValle and Goddard (1948) proposed a mechanism involving a complex between the enzyme and

the phenolic substrate. The complex would combine with oxygen and yield a quinone and the oxygenated enzyme; the latter would then oxidize another substrate molecule and lose its oxygen in the process. Mason (1957) proposed a somewhat different theory, involving the following steps: (1) the enzymic copper forms a complex with molecular oxygen; (2) this complex attacks an o-diphenol molecule to yield an o-quinone and a different copper-oxygen complex; (3) this new complex, unlike the first, can either hydroxylate a monophenol or oxidize another diphenol molecule; and (4) after completing one of the reactions in step 3, the copper is free to combine with another molecule of oxygen. In the absence of an o-diphenol the transition from step 1 to step 3 can be effected by any reducing agent, such as ascorbic acid.

Certain phenolases such as bovine adrenal tyrosinase have an absolute requirement for a reducing agent before they can hydroxylate a monophenol (Brenneman and Kaufman, 1964). However, mushroom tyrosinase when highly purified will very rapidly convert a monophenol to an o-diphenol without any apparent need for a reducing agent (Karkhanis and Frieden, 1961). H. J. Bright, in his elaboration of Mason's theory (1963), suggested that mushroom tyrosinase may have a reducing system in its own molecular structure, thus obviating the need of an external reducing agent.

Mason's theory is attractive, but not without difficulties. Coopersmith (1962) and Aerts and Vercauteren (1964) both claim to have found two active sites on phenolase, one for monophenolase and the other for diphenolase activity. Mason's theory suggests that the enzyme has only one active site.

After the o-quinone is produced by the enzymatic oxidation, it rapidly polymerizes in water solution to form melanins. Mason (1959) and Dawson (1963) elaborated theories on the mechanism of this polymerization. Although their theories differ in many important respects, they agree that a number of different quinones arise as intermediate products during the polymerization and that some diphenol substrate is regenerated in the process.

The Mechanism of the Sulfur Dioxide Inhibition of Enzymatic Browning

It has been known since antiquity that sulfur dioxide has the ability to prevent browning in many fruits and vegetables. Ponting and Johnson (1945) reported that fulfur dioxide rapidly disappeared from fruit blends containing phenolase and concluded that the enzyme oxidized the sulfur dioxide in preference to the phenolic substrate. Later, Ponting (1960) decided that the quinones formed during the oxidation were responsible for the oxidation of the sulfur dioxide, thus giving SO_2 the role of antioxidant. Joslyn and Braverman (1954)

suggested that SO_2 may also act by auto-oxidizing and robbing the phenolase system of free oxygen. However, up to the time of the present investigation no well-substantiated theory regarding sulfite inhibition of enzymatic browning has been established.

METHODS AND MATERIALS

Materials and Instruments Used

Phenolase: Mushroom tyrosinase from the Sigma Chemical Co.
Activity: 800 units/mg

Sodium Bisulfite: Merck Reagent

Catechol: Reagent Grade, Eastman Kodak

Ascorbic acid: Reagent Grade, Eastman Kodak

L-Tyrosine: The California Foundation for Biochemical Research

Caffeic acid: Nutritional Biochemicals Corporation

Chlorogenic Acid: Nutritional Biochemicals Corporation

Sulfate--^{35}S: Atomic Energy Commission

Phosphate buffer: 0.5M phosphoric acid solution adjusted to pH 6.5 with NaOH

Preparation of labeled sulfite: A small amount of sulfate--^{35}S was added to 0.5 ml concentrated sulfuric acid in a test tube. The acid was then heated with a small amount of copper wire and the SO_2 evolved was absorbed in 10 ml of cold 2N NaOH. The NaOH was acidified and the SO_2 distilled into cold phosphate buffer to leave behind any sulfate that may have evolved with the SO_2 from the heated sulfuric acid. The SO_2 solution was assayed by titrating with 0.1N iodine solution.

Spectrophotometric measurements were made with a Beckman DU spectrophotometer connected to a Ledland log-converter and a Sargent SR recorder. Except in the preincubation experiments, the enzyme solution was the last component of the reaction mixture to be transferred into the 1 cm light path Beckman cuvette. For the transfer and mixing of the enzyme solution, a square teflon plunger, provided with a groove and three orifices, and connected to a stainless steel handle was used. In the preincubation experiments the substrate solution was added last.

A Warburg apparatus thermostated at 30°C was used for the manometric studies. Paper chromatography was performed with Whatman No. 1 paper with the organic phase of a butanol/acetic acid/water (25:6:25) mixture as the solvent, in descending fashion at 25°C. Paper electrophoresis was performed at 450 volts for two hours with a Spinco Model R cell and accessories. The electrolite was 0.1M formic acid adjusted to pH 2.2 with NaOH. A 4-pi Vanguard 880 radiochromatogram scanner was used in tracing the radioactivity of chromatograms and electropherograms.

Model Systems

I. Manometric determinations

A. Diphenol system

1 ml phosphate buffer, pH 6.5
1 ml 2×10^{-3} M pyrocatechol
0.1 ml mushroom tyrosinase soln. 0.05 mg/ml

X ml 1% $NaHSO_3$
0.8 - X nl H_2O
Total Volume: 3 ml

B. Monophenol system

2 ml 5×10^{-3} M tyrosine made up in phosphate buffer pH 6.5
0.5 ml mushroom tyrosinase soln. 0.5 mg/ml
X ml 5×10^{-3} M Na HSO_3
Y ml 5×10^{-4} M ascorbic acid
Z ml 5×10^{-4} M catechol
0.5 - X - Y - Z ml H_2O
Total Volume: 3 ml

II. Spectrophotometric investigations

1 ml phosphate buffer, pH 6.5
1 ml 4×10^{-3} M pyrocatechol
0.1 ml mushroom tyrosinase, 0.05 mg/ml
X ml 10^{-3} M $NaHSO_3$
0.9 - X ml H_2O
Total Volume: 3 ml

III. Electrophoresis

Solution A:

1 ml phosphate buffer, pH 6.5
1 ml 3×10^{-3} M chlorogenic acid
0.1 ml mushroom tyrosinase soln. 0.05 mg/ml
0.1 ml 3×10^{-3} M sulfite--^{35}S
0.8 ml H_2O
Total Volume: 3 ml

Solution B:

Same as A except that the sulfite was unlabeled and a trace of labeled sulfate was added.

IV. Paper chromatography

Solution A:

1 ml phosphate buffer, pH 6.5
0.1 ml 3×10^{-3} M $NaHSO_3$
0.1 ml mushroom tyrosinase soln. 0.05 mg/ml
1 ml 3×10^{-3} M caffeic acid
0.8 ml H_2O
Total Volume: 3 ml

Solution B:

Same as A except that 1 ml 3×10^{-3} M chlorogenic acid was substituted for the caffeic acid.

RESULTS AND DISCUSSION

The exztmatic oxidation of o-diphenols is represented by the equation:

$$\text{o-diphenol} + 1/2\ O_2 \xrightarrow{\text{phenolase}} \text{o-quinone} \rightarrow \text{intermediates} \rightarrow \text{melanins}$$

On the basis of this mechanism one could postulate three possible means whereby sulfur dioxide delays or prevents the formation of the end products, melanins:

1. sulfur dioxide attacks the enzyme itself and temporarily or permanently inhibits its catalytic action,

2. sulfur dioxide interferes with the exzyme-substrate reaction, as Ponting and Johnson (1945) or Joslyn and Braverman (1954) suggested. There is also the more remote possibility that sulfur dioxide somehow combines with the phenolic substrate itself and renders it unavailable to the enzyme, or

3. sulfur dioxide interferes with the polymerization of the quinones to melanins, either by reducing them back to o-diphenols or by combining with them in some way to form sulfite addition products (Joslyn and Braverman, 1954).

In the case where a monophenol acts as the substrate, one could add a fourth hypothesis, namely that the sulfur dioxide interferes with the enzymatic conversion of the monophenol to the corresponding o-diphenol.

These hypotheses were investigated with model systems. Since all the systems were buffered at pH 6.5, their SO_2 component will be referred to as sulfite, since it exists predominently as $SO_3^=$ under these conditions (Vas and Ingram, 1949).

Preincubation Experiments

To test the first hypothesis, the possible effect of sulfite on the enzyme, phenolase solution was preincubated with sulfite. One tenth ml mushroom tyrosinase solution (40 units/ml) was mixed with 0.1 ml 10^{-3} M $NaHSO_3$ in 1 ml 0.5M phosphate buffer, pH 6.5, and 0.9 ml water, and allowed to stand at room temperature for a measured time interval. Then 1 ml 5 x 10^{-3} M pyrocatechol solution was added and the subsequent browning measured with a recording spectrophotometer at 420 mu. The recorder automatically traced a time-absorbance curve, and the activity of the enzyme was arbitrarily taken as the slope of the curve after the short sulfite-induced lag period. This procedure was repeated for various time intervals and again with 0.2 ml 10^{-3} M $NaHSO_3$ instead of 0.1 ml 10^{-3} M $NaHSO_2$. The activities of the preincubated enzyme solutions were calculated as percentages of the activity of non-preincubated enzyme (i.e., the solution at zero time), and the results plotted against time of preincubation (Figure 1).

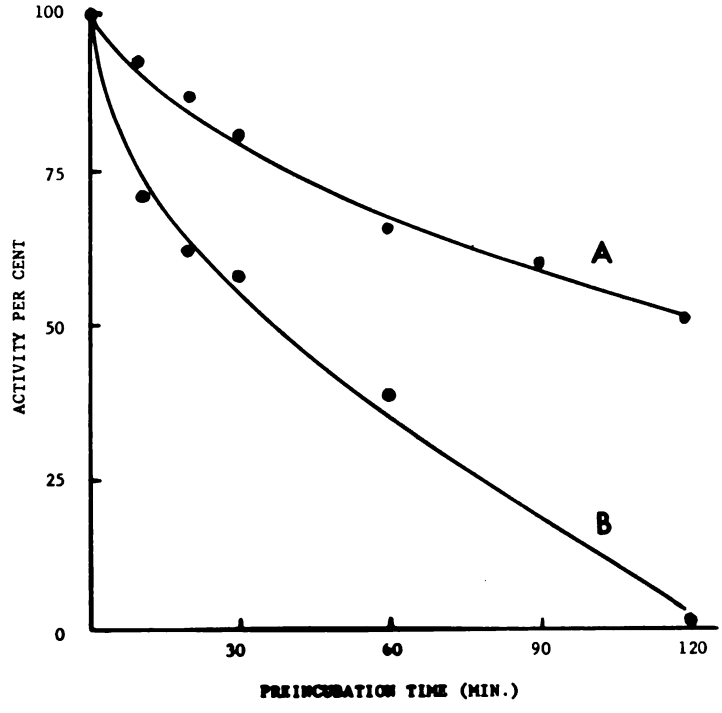

Figure 1.--Effect of preincubation of phenolase with sulfite on the activity of the enzyme.

Solution A: 1 ml phosphate buffer, pH 6.5
0.1 ml enzyme soln. 40 units/ml
0.1 ml 10^{-3} M $NaHSO_3$
0.9 ml H_2O

Solution B: buffer and enzyme same as above
0.2 ml 10^{-3} M $NaHSO_3$
0.8 ml H_2O

Assay mixture: above plus 1 ml 5 x 10^{-3} M pyrocatechol. The activity of the enzyme was measured as increase in absorption at 420 mu.

In a supplementary experiment 1 ml 10^{-3} M tyrosine was added to a solution of preincubated enzyme and the activity measured as before. The result was similar to those of the pyrocatechol experiments: the sulfite had weakened the enzymatic activity. Thus contact with sulfite has a detrimental effect on both the monophenolase and polyphenolase functions of the enzyme.

But the effect is a gradual one, and does not explain the fact that the sulfite inhibition of browning is immediate and complete. Therefore, some chemical mechanism other than a slow poisoning of the enzyme must be involved in the inhibition.

Diphenol Systems -- Manometric and Spectrophotometric Investigations

The oxygen uptake of phenolase-pyrocatechol systems was measured at 30° C with a Warburg manometer in the presence of varying amounts of sulfite. The results are given in Table 1. It will be seen that the three systems absorbed almost equal amounts of oxygen during the first twelve minutes of reaction, although the first system had no sulfite and the third had enough to prevent browning for the whole reaction time.* This is a strong indication that the enzymatic oxidation of the pyrocatechol took place without sulfite interference.

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*No control systems (e.g. without enzyme or with heat-inactivated enzyme) were used since oxygen uptake during twelve minutes of non-enzymatic oxidation of pyrocatechol is negligible.

Table 1.--Oxygen uptake of phenolase-pyrochtechol systems containing varying amounts of sulfite.

General composition of the systems:

1 ml phosphate buffer, pH 6.5
1 ml 2 x 10^{-3} M pyrocatechol
X ml 1% $NaHSO_3$
0.1 ml mushroom tyrosinase solution, 0.05 mg/ml
0.8 - X ml H_2O

The figures below represent microliters of oxygen taken up by the systems (adjusted to S.T.P.)

Time (Min)	X = 0	X = 0.01	X = 0.025
0	0.0	0.0	0.0
2	10.3	11.0	11.0
4	19.2	19.9	18.5
6	24.0	24.7	21.9
8	26.0	27.4	26.0
10	29.5	29.5	29.5
12	30.8	30.1	30.3
	Browning began immediately	Browning after 3-4 min.	No browning occurred

An apparent contradiction may be noted here. It can be seen from Figure 1 that after 10 minutes preincubation with 0.2 ml 10^{-3} M $NaHSO_3$ the enzyme lost roughly ten per cent of its activity. But although the manometric determinations involved greater amounts of sulfite for the same quality of enzyme, the enzymatic activity was apparently undisturbed during the entire twelve minutes of the reaction. This may be explained on the assumption that the sulfite was being consumed during the course of the enzymatic reaction and had

no time to poison the enzyme, as it could during the preincubation experiments.

Phenolase-pyrocatechol systems were investigated with a recording spectrophotometer. The recorder tracings (absorbance-time curves) can be seen in Figure 2. The top row of curves was determined at 420 mu. System A, with no sulfite, showed an immediate rise in absorbance, while system B, with 10^{-4} M sulfite, showed a short lag period. Systems C and D, with two and three times as much sulfite respectively, exhibited greater lag periods. These curves illustrate the sulfite-induced delay in melanin formation.

An examination of these curves reveals a "fanning effect," i.e. as the sulfite concentration increased, the slopes of the curves after the lag period decreased. This same effect was noticed by Goodman and Markakis (1965) in their work on the ability of SO_2 to protect anthocyanin pigments from oxidation in phenolase systems. It may be explained on the assumption that the pyrocatechol was being irreversibly consumed by the enzymatic reaction during the lag period and at the end of the lag the pyrocatechol concentration was depleted, resulting in a lower rate of melanin formation.

When these same pyrocatechol systems were investigated at 290 mu instead of 420 mu, a different picture emerged. Instead of causing a lag in the absorbance increase, the sulfite temporarily

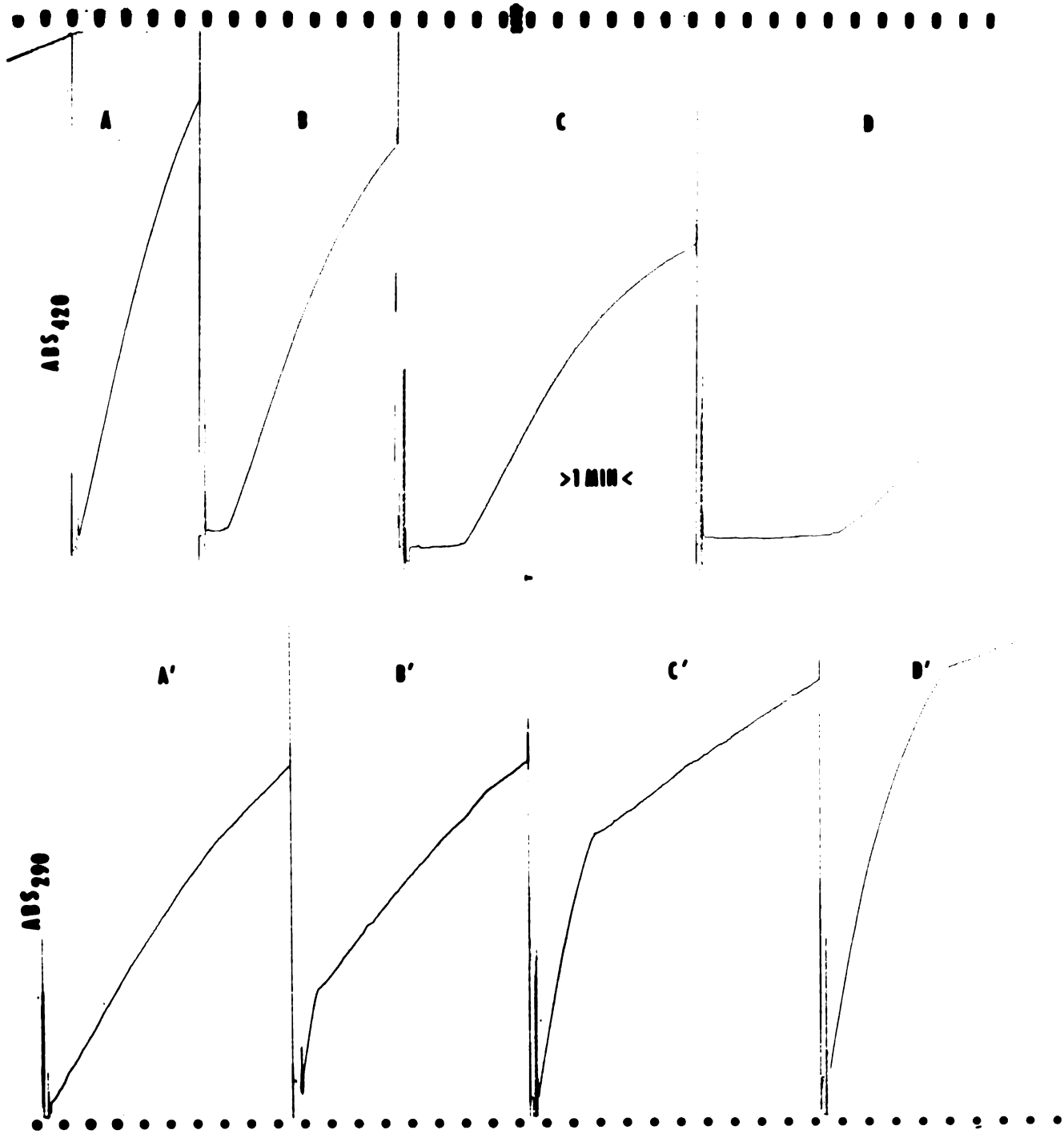

Figure 2.--Automatically recorded absorbance-time curves for the phenolase-pyrocatechol reaction.

Reaction mixture: pyrocatechol 1.3×10^{-3} M; enzyme 1.3 unit/ml sulfite in A, A' none; in B, B' 10^{-4} M; in C, C' 2×10^{-4} M; in D, D' 3×10^{-4} M. Systems buffered at pH 6.5.

accelerated it. (Bottom row, Figure 2) For instance, system B' underwent a rapid rise in absorbance followed by a break and a curve of much lower slope. It is important to note that the duration of this rapid rise was exactly the same as the duration of the lag period at 420 mu for the same system. This suggested that a colorless compound or compounds with high absorbances at 290 mu were being formed in the pyrocatechol-phenolase systems, and that when the compound ceased forming, the melanins appeared.

The possibility of a substrate-SO_2 reaction was tested here. A spectrum was taken with a Spectronic 505 of a dilute catechol solution buffered at pH 6.5. Then a random amount of sulfite was added to the solution and the spectrum taken again. No spectral change was observed, indicating that the sulfite did not affect the chemical structure of the pyrocatechol.

The Quinone-Sulfite Reaction

The results discussed above show that sulfite does not inhibit browning by a rapid attack on the enzyme nor by interfering with the enzyme-substrate reaction. Therefore, it must somehow react with the oxidation products (quinones) of the reaction and prevent their polymerization. It can do this in two ways: (1) by reducing the quinones; and (2) by combining with them.

If sulfite reduces the quinones, then it itself must be oxidized to sulfate. To investigate this possibility, a system containing chlorogenic acid (a fluorescent diphenol), sulfite labeled with ^{35}S, and phenolase was allowed to react until melanins formed (to insure that all the sulfite was consumed) and then a portion of the system was applied to the center of a strip of electrophoretic paper. The paper was dried and subject to electrophoresis in a Durrum type cell. A control system was made with the same composition except that unlabeled sulfite was used and a trace of labeled sulfate was added. This system was also applied to an electrophoretic strip, which was subjected to electrophoresis under the same conditions as the experimental strip. After electrophoresis the strips were scanned with a radiochromatogram scanner to locate their radioactive areas. (See Figure 3) In the control system (B), the labeled sulfate had moved far down the paper under the influence of the electric current. But the experimental system showed no corresponding peak; its radioactivity remained in the fluorescent zone (striated area, A). Thus none of the sulfite had been oxidized to sulfate.

In testing the possibility that sulfite may combine with the quinones to form sulfite addition compounds, it would be highly desirable to isolate these products from a phenolase system. Systems containing fluorescent substrates seemed to be the most appropriate for this purpose. It was noted that while phenolase systems containing

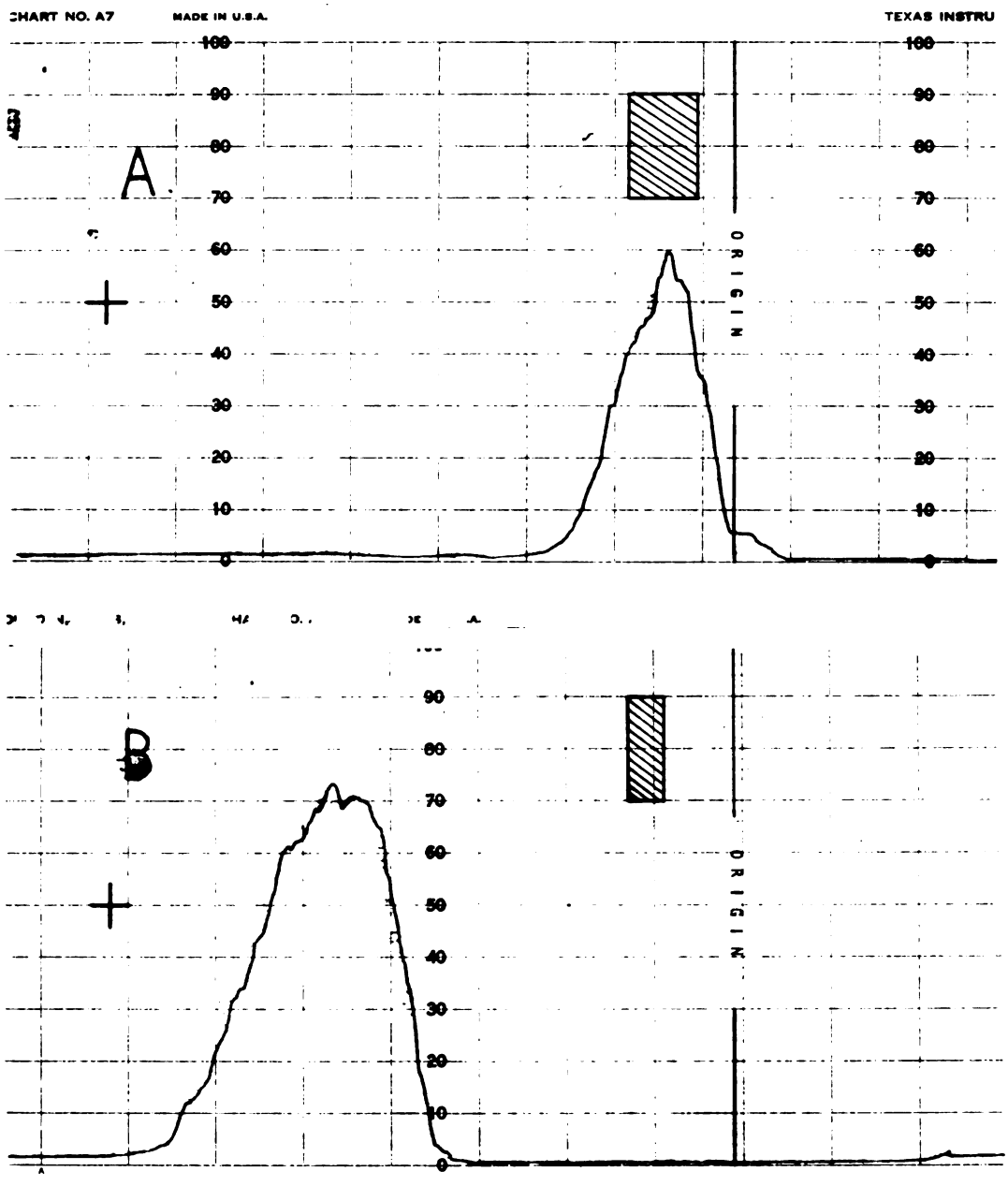

Figure 3.--Radioactivity tracings of electropherograms

System A: 10^{-3} M chlorogenic acid, 1.3 unit/ml enzyme, 10^{-4} M labeled sulfite. Buffered at pH 6.5 About 0.01 ml streaked on standard Spinco paper strip.

System B: same as A, but containing unlabeled sulfite and a trace of labeled sulfate.

Electrophoresis: 450 V. 2 hours, 0.1M formate, pH 2.2
Scanning range: 0 - 300 cpm. Striated areas indicate fluorescent loci.

caffeic or chologenic acid will lose their fluorescence during their reaction, the addition of sulfite would preserve their fluorescence. Therefore, any sulfite addition compounds in the systems would probably be fluorescent.

A system containing caffeic acid and another with chlorogenic acid were allowed to react with phenolase and sulfite and then subjected to paper chromatography (electrophoresis could not separate their fluorescent components; see Figure 3). Both systems yielded a number of fluorescent bands on the paper. Two of the brightest bands from each system were eluted from the papers and subjected to the sodium hydroxide-nickel hydroxide test (Feigl, 1960). The tests were positive. This is a test for sulfonic acids, sulfinic acids, sulfonamides, and sulfones.

Similar systems were allowed to react with phenolase and labeled sulfite until melanins formed (again to insure that no excess sulfite would remain in the systems). The systems were subjected to paper chromatography as before, and strips were cut from the papers and scanned with a radiochromatogram scanner. The tracings are shown in Figure 4. It will be seen that all the radioactivity accumulated in the fluorescent bands on the paper, showing that they were compounds of sulfur.

From these results it must be concluded that sulfite prevents browning in phenolase diphenol systems by combining irreversibly with the o-quinones and interrupting their condensation to

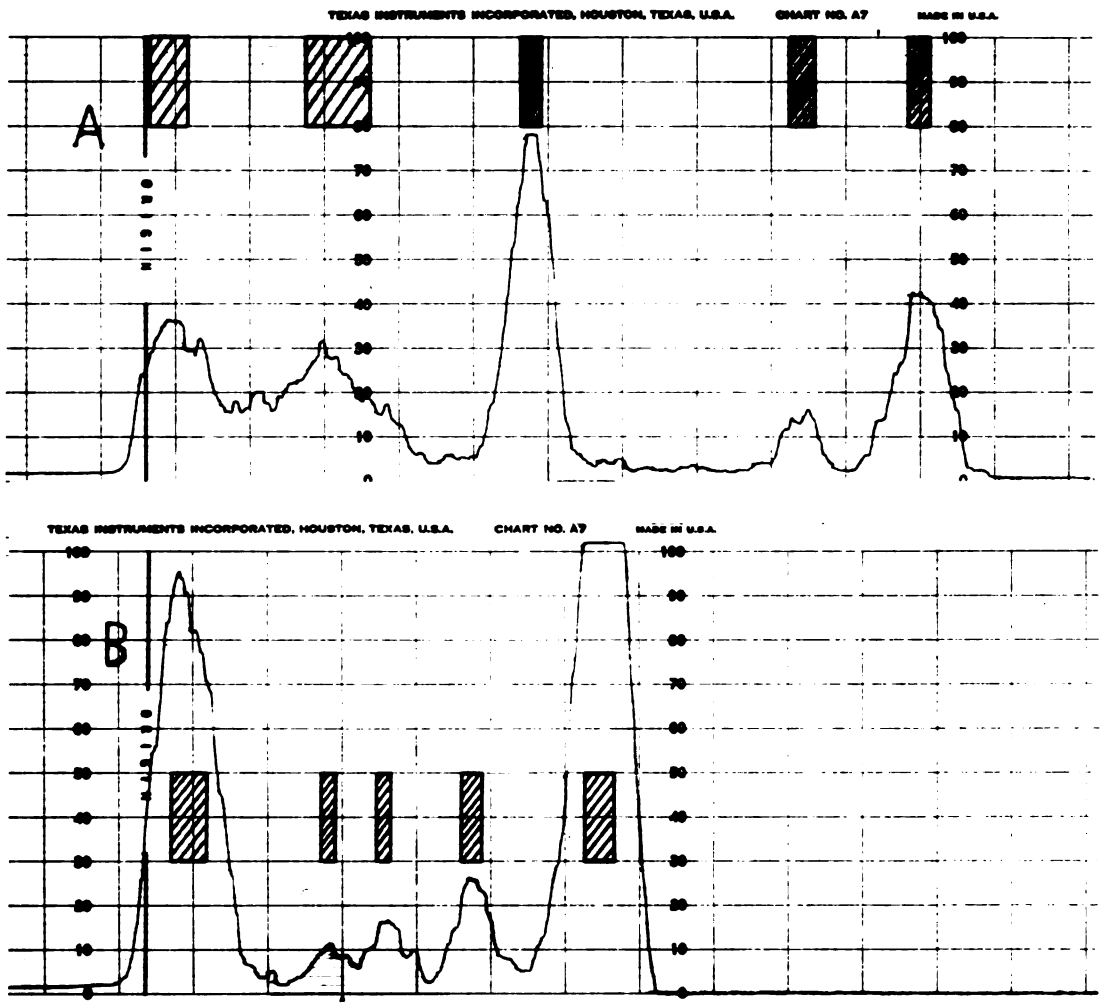

Figure 4.--Radioactivity tracings of paper chromatograms.

System A: 10^{-3} M caffeic acid, 1.3 units/ml enzyme, 10^{-4} M labeled sulfite. Buffered at pH 6.5

System B: same as A except that 10^{-3} M chlorogenic acid replaces caffeic

Paper: Whatman No. 1. Solvent: organic phase of butanol/acetic acid/water (25:6:25). Decending irrigation 24 hrs. Striated areas indicate fluorescent loci.

melanins. The structure of these addition compounds has not been determined. However, LuValle (1952) and Schenck and Schmidt-Thomee (1953) have elucidated the structure of some sulfite-quinone addition products (e.g. some were sulfonic acids, others cyclic sulfates), and LuValle found that some of them absorb strongly in the 290 mu region of the spectrum.

The Monophenol Reaction

Systems containing tyrosine, phenolase and sulfite were studied manometrically and the results are shown in Figure 5a. Unlike the diphenol systems, the oxygen uptake of the tyrosine reaction was delayed by the sulfite, beginning only when melanins formed in the system. Apparently no sulfite addition compounds are formed in monophenol systems. p-Coumaric acid, a fluorescent monophenol, was allowed to react with phenolase and sulfite and then subjected to paper chromatography as had been done with the fluorescent diphenols (see previous section); no series of fluorescent bands were observed on the paper (care was taken not to allow caffeic acid to form in the system, as would be evidenced by a change in its fluorescent color).

The sulfite seems to act by retarding the monophenolase function of the enzyme. If one postulated an attack by the sulfite on the enzymic copper, then one would have to assume, contrary to

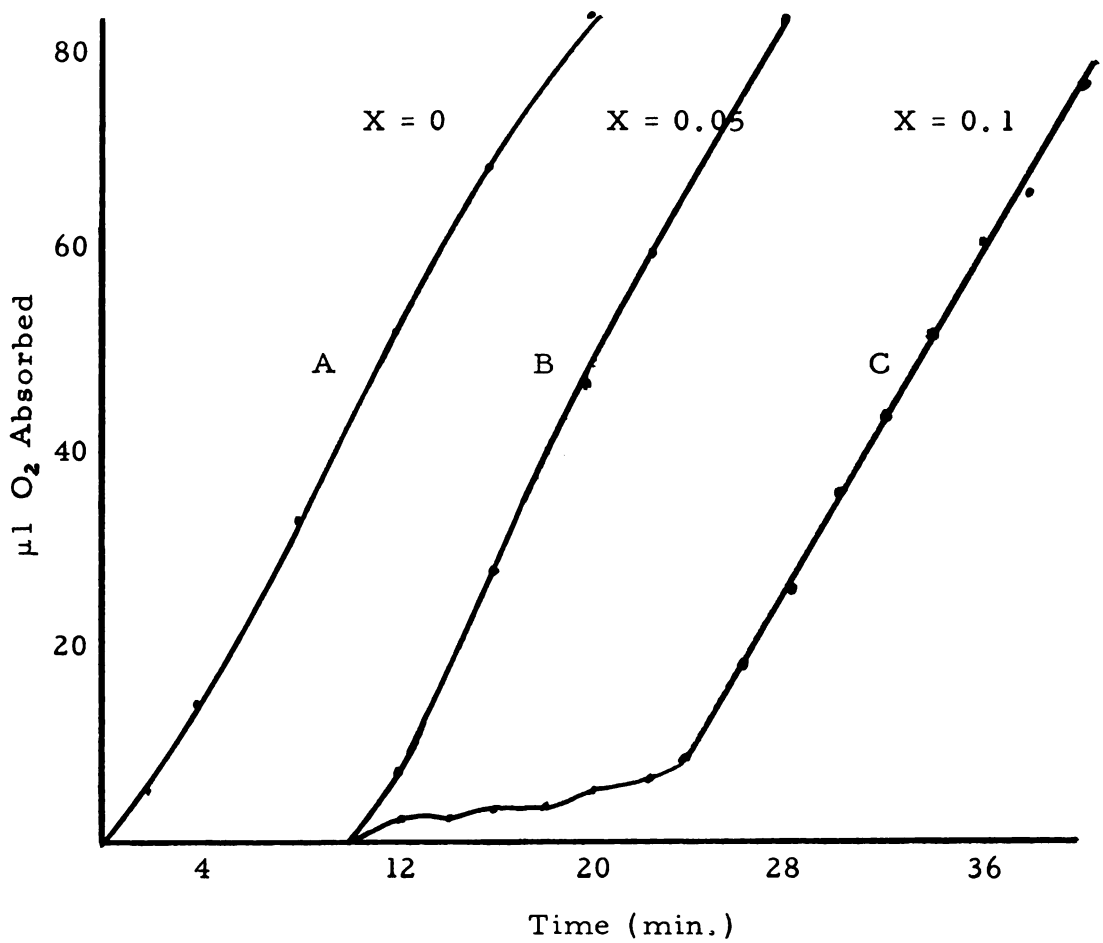

Figure 5a.--Effect of sulfite on oxygen uptake of phenolase-tyrosine solutions.

General composition of systems:
2 ml 5 x 10^{-3} M tyrosine
0.5 ml enzyme 400 units/ml
X ml 5 x 10^{-3} M $NaHSO_3$
0.5 - X ml H_2O
Buffered at pH 6.5

System A: X = 0; browning started immediately
System B: X = 0.05; browning after 10 min.
System C: X = 0.1; browning after 20 min.

Mason's theory, that the enzyme has two active sites and that only the monophenolase site can be inhibited by sulfite.

A clue may be found in the fact that, in the presence of catalytic amounts of ascorbic acid or catechol, the lag period of a tyrosine-phenolase-sulfite system is greatly reduced (Figure 5b). Therefore, an explanation for the action of sulfite may be tentatively proposed. It will be recalled that H. Bright suggested that mushroom tyrosinase had a reducing system in its molecular structure which activates the enzyme for monophenolase activity (see REVIEW OF THE LITERATURE). If one assumes that sulfite acts by attacking this reducing system and inhibiting its action, probably by distorting the enzyme molecule, then a reducing agent such as ascorbic acid or catechol in catalytic amounts can activate the enzyme directly and bypass the blocked reducing system.

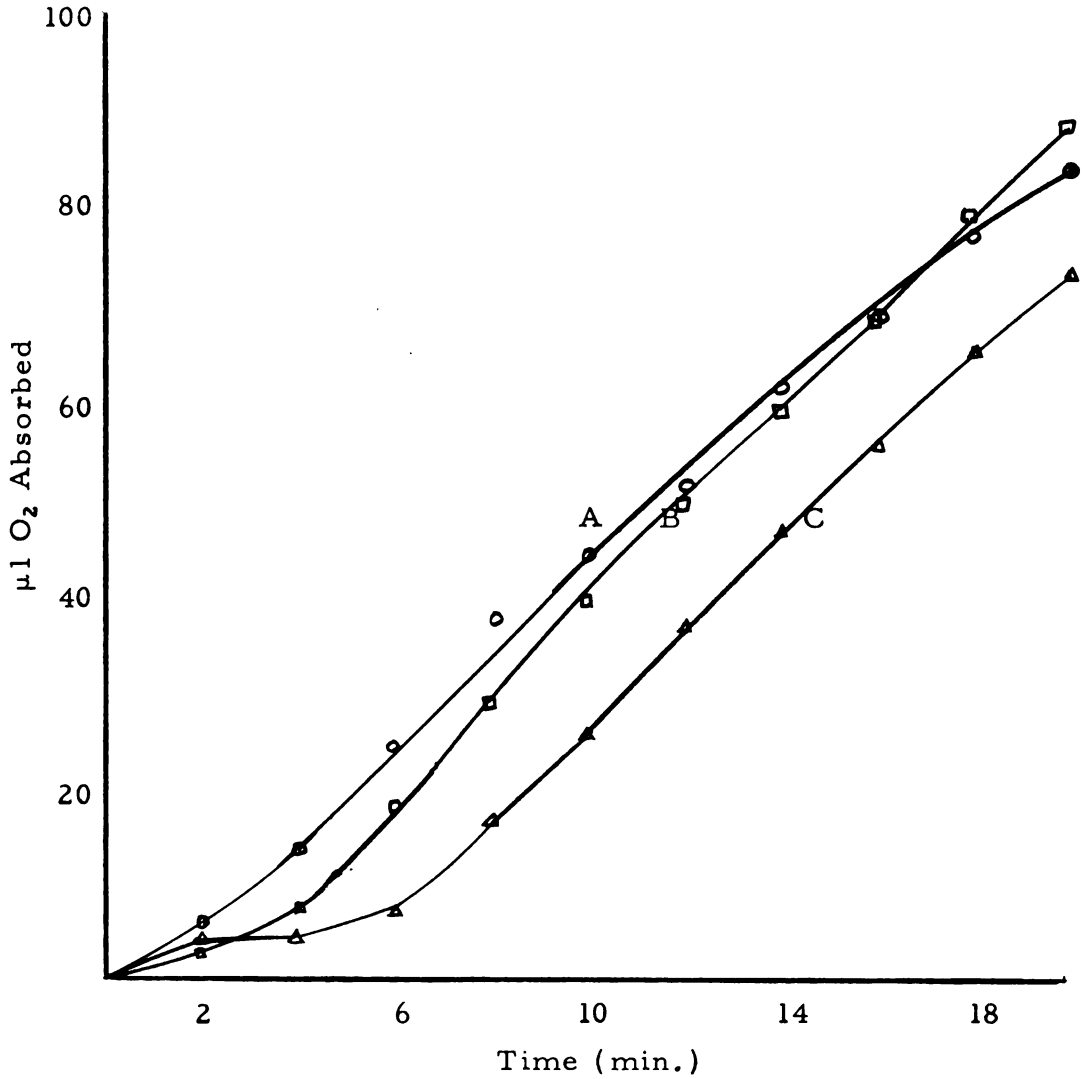

Figure 5b.--Effect of reducing agents on phenolase-tyrosine systems in the presence of sulfite.

General composition of systems:

2 ml 5 x 10^{-3} M tyrosine
0.5 ml enzyme, 400 units/ml
X ml 5 x 10^{-3} M $NaHSO_3$
Y ml 5 x 10^{-4} M ascorbic acid
Z ml 5 x 10^{-4} M catechol
0.5 - X - Y - Z ml H_2O

System A: X, Z = 0; Y = 0.1
System B: X, Y = 0.1; Z = 0
System C: X, Z = 0.1; Y = 0

Browning occurred in all three systems in less than 2 min.

SUMMARY

The enzyme phenolase catalyzes a browning reaction on the surfaces of certain fruits and vegetables. However, if these fruits and vegetables are exposed to sulfur dioxide (or to a solution of sulfurous acid, bisulfite or sulfite) the browning is delayed or completely prevented. The purpose of this theses was to investigate the chemical mechanism responsible for this inhibition of browning by SO_2.

Model systems were used throughout the investigation. The systems contained mushroom tyrosinase, a phenolic substrate (a monophenol or a diphenol), varying amounts of sulfite, and on occasion catalytic amounts of ascorbic acid or catechol. The systmes were always buffered at pH 6.5, the optimum pH for the enzymatic activity.

Preincubation experiments with sulfite and tyrosinase showed that sulfite gradually inactivated the enzyme. However, this did not explain the instantaneous inhibition of browning by SO_2.

Diphenol systems containing sulfite exhibited a "lag period" or delay in browning, the duration of the lag being proportional to the amount of sulfite present. Manometric investigations

of these systems showed that the presence of sulfite did not change their initial rate of oxygen uptake, which indicated that the enzymatic oxidation took place without sulfite interference. Spectrophotometric investigations of the systems at 290 mu showed that the sulfite caused a higher initial rate of absorbancy increase than in systems without sulfite, and that this initial high rate lasted exactly as long as the delay in the browning reaction. This indicated a possible combination of sulfite with the oxidation products (quinones) of the enzymatic reaction to form colorless compounds, probably cyclic sulfates, with high absorbancies at 290 mu. These compounds can be isolated from systems containing fluorescent diphenols (caffeic or chlorogenic acids) and sulfite by paper chromatography, appearing as a series of fluorescent spots on the paper. The use of $^{35}SO_2$ in the reaction resulted in the accumulation of all the radioactivity in the fluorescent spots, indicating that they were compounds of sulfur. It was also shown that no labeled sulfate was formed during the reaction.

Manometric investigations of systems containing a monophenol and sulfite showed that the sulfite caused a lag in their oxygen uptake, the lag being proportional to the amount of sulfite present. Browning was observed in these systems at the point at which they began to absorb oxygen. This indicated that the sulfite attacked the enzyme itself and retarded its monophenolase activity. However, the presence of catalytic amounts of ascorbic acid or catechol in the

systems reduced to a large extent the sulfite-induced lag periods. This may be tentatively explained on the basis of the hypothesis that the enzyme contains a "reducing site" in its mulecular structure which activates the enzyme for monophenolase activity. If sulfite acts by inhibiting this site, the presence of a reducing agent will activate the enzyme directly and bypass the site.

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