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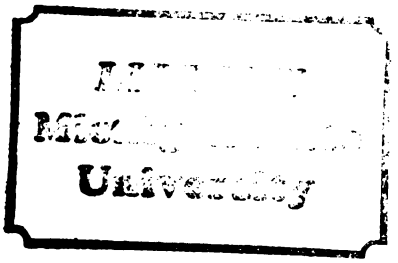


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thesis entitled

**Bisulfite oxidation in homogenates of young and old cucumber leaves and its potential role in $SO_2$ injury**

presented by

**Joel Ernest Ream**

has been accepted towards fulfillment
of the requirements for

M.S. degree in Botany

Lloyd G Wilson
Major professor

Date April 27, 1982

# BISULFITE OXIDATION IN HOMOGENATES OF YOUNG AND OLD CUCUMBER LEAVES AND ITS POTENTIAL ROLE IN $SO_2$ INJURY

By

Joel Ernest Ream

A THESIS

Submitted to
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ABSTRACT

BISULFITE OXIDATION IN HOMOGENATES OF YOUNG AND OLD CUCUMBER LEAVES AND ITS POTENTIAL ROLE IN $SO_2$ INJURY

By

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Bisulfite oxidation is a major mechanism by which the excess sulfur absorbed by a leaf as $SO_2$ is metabolized. The bisulfite oxidation activity of homogenized cucumber leaves, as measured with an oxygen electrode, was found to occur by a dark and a light-dependent process. Part of the dark activity was resolved as a single heat-sensitive peak on a Sephadex G-200 column. The light-dependent activity was linked to the formation of superoxide anions by photosynthetic electron transport because of its inhibition by DCMU and superoxide dismutase. Thirteen percent of the total bisulfite oxidation from young leaves (resistant to $SO_2$) and 42% from old leaves (sensitive to $SO_2$) occurred by this light-dependent process. The greater sensitivity to $SO_2$ in older cucumber leaves may be due to injury from free radicals produced by their increased light-dependent activity.

to Stephanie

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | bovine serum albumin |
| CHL | chlorophyll |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| DCPIP | 2,6-dichlorophenol-indophenol |
| EDTA | ethylenediaminetetraacetate |
| MV | methyl viologen |
| PS I | photosystem I |
| SOD | superoxide dismutase |

# INTRODUCTION

Sulfur dioxide is a major constituent of air pollutants in industrial countries around the world. It is produced mainly from the combustion of fossil fuels (26) and reaches especially high concentrations near industrial areas. Plants are particularly susceptible to injury by airborne sulfur dioxide (39), exhibiting the classical injury symptoms of interveinal chlorosis when exposed to toxic concentrations. The concentration of sulfur dioxide and length of exposure required to cause injury varies widely in the plant kingdom. Susceptible plants (e.g. spinach, cucumber, and oats) are damaged by exposure to 0.05 to 0.5 parts per million (ppm) sulfur dioxide for eight hours while resistant plants (e.g.maize, celery, and citrus) require concentrations in excess of 2 ppm for eight hours for visible injury to occur (26). Besides this diversity in susceptibility among different species, leaves within a single plant vary in their susceptibility to sulfur dioxide and to ozone injury (4, 16, 39, 40). The younger, unexpanded leaves are generally more resistant to injury.

Despite a long awareness of the deleterious effects of sulfur dioxide on plants (39), the primary event leading to cell damage remains unknown. The manner in which a plant

can avoid this unknown lethal event can be divided into two categories: stomatal mechanisms and biochemical mechanisms. Since most gas exchange by a leaf occurs through the stomata this is the first point where a plant can react to minimize the damage caused by sulfur dioxide. Much of the variability in the sensitivity of different plants to sulfur dioxide can be accounted for by differential absorption. Bressan et al. (5) exposed two cultivars of Cucumis sativus L. and two cultivars of Cucurbita pepo L. to sulfur dioxide and noted a wide range of sensitivities to identical exposures. When the amount of sulfur dioxide taken up was measured, it was found to vary in the same manner; that is, the more resistant cultivars absorbed less sulfur dioxide than the more sensitive ones. Factors which affect stomatal opening (e.g. light, humidity, and $CO_2$ concentration) affect the amount of sulfur dioxide absorbed by a leaf.

Not all mechanisms of resistance can be explained by stomatal factors, however. Bressan et al. (5), in the same study, also found a difference in sensitivity among different leaves of the same plant, the younger leaves being more resistant than the older leaves. In contrast to the differences between cultivars, this difference in susceptibility could not be accounted for by differences in sulfur dioxide absorption. This suggests there are biochemical factors involved in leaf sensitivity to sulfur dioxide, an idea supported in work by Guderian (16). Winner and Mooney (41)

evaluated the role of stomatal and non-stomatal factors in predicting sulfur dioxide sensitivities and concluded that non-stomatal components do play a role. From their study they predicted the plant with the higher intrinsic photosynthetic capacity will be more sensitive.

Once sulfur dioxide has passed through the stomates it readily dissolves in the apoplastic space of the substomatal cavity, dissociating into the bisulfite ($HSO_3^-$) and sulfite ($SO_3^{2-}$) ions. The distribution of these two ionic species depends on pH (30). They are quite reactive and it is thought that it is in these forms that sulfur dioxide acts to cause injury.

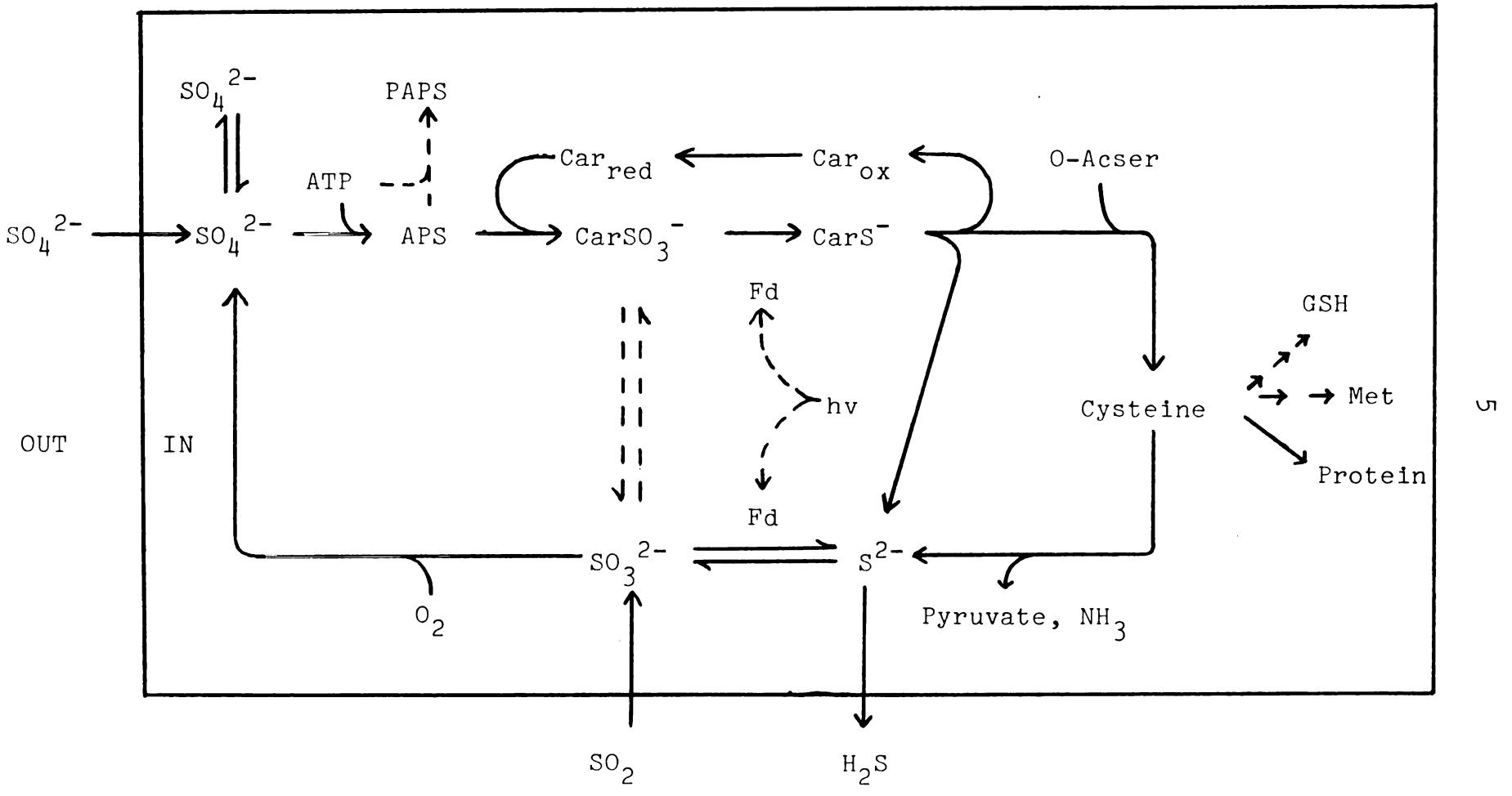
There are many known reactions of the bisulfite and sulfite ions that could account for their toxicity. Sulfite reacts with aldehydes and ketones to form hydroxysulfonates which have been found to be enzyme inhibitors (26). Sulfite reacts with olefinic compounds by a free radical mechanism to produce sulfonic acids (26). Sulfite reacts with disulfides, such as those found in cystine, producing a thiol and S-sulfonate: $RSSR + SO_3^{2-} \rightleftharpoons RS^- + RSSO_3^-$ (26). Sulfite can also disrupt the genetic machinery of the cell, converting uracil and cytosine to 5,6-dihydrouracil-6-sulfonate derivatives (18, 32). The bisulfite ion has been shown to inhibit ribulose bisphosphate carboxylase and phosphoenolpyruvate carboxylase activity by acting as a competitive inhibitor with the bicarbonate ($HCO_3^-$) ion (27, 43, 44). Finally, the presence of bisulfite has been

linked to phosphorylase inhibition (21), phosphodiester bond cleavage (19), and IAA oxidation (42). Which, if any, of these potentially deleterious reactions is responsible for the primary lethal event in sulfur dioxide injury is not known. Some of these reactions require much higher bisulfite concentrations than would be expected to result from exposure to sulfur dioxide.

Because of the many potentially injurious reactions of the bisulfite ion, the metabolic manner in which a plant rids itself of this toxic species has been proposed as a possible explanation for biochemical mechanisms of resistance. Bisulfite can interact with the normal sulfur metabolism of the leaf (Figure 1) (52). Sulfur is absorbed as sulfate ($SO_4^{2-}$) by the roots, transported to the leaf, reduced to the level of a sulfide using the reducing power of ferredoxin, and used to synthesize cysteine from which other sulfur-containing compounds are made. Sulfite from sulfur dioxide exposure can be oxidized to sulfate. In this manner, it can serve as a sulfur source in a sulfur deficient plant (10, 24). Sulfite can also be reduced by the action of sulfite reductase (35) and there is evidence for sulfite reacting directly with the reduced carrier to form a thiosulfonate (17). Cucumber leaves have been shown to emit hydrogen sulfide ($H_2S$) in response to $SO_2$ fumigation (31).

Determining the fate of sulfur introduced to a plant is accomplished by fumigating with $^{35}S$-$SO_2$ and noting

Figure 1. Interaction of sulfur dioxide with leaf cell sulfur metabolism[1].




1. modified from Schmidt (52)

the $^{35}S$-labelled products formed. When this type of study is done, much of the absorbed $^{35}S$ can be accounted for as $^{35}S\text{-}SO_4^{2-}$. De Cormis (9) fumigated tomato plants with sulfur dioxide and could account for 98% of the introduced sulfur as sulfate. Ziegler (45) recovered 43% of $^{35}S$ as $^{35}S$-sulfate 7 hours after fumigating spinach leaves with $^{35}S\text{-}SO_2$. Kondo et al. (22) showed, using ion chromatography, that almost all sulfite was transformed into sulfate in castor bean extracts. With cucumber, Sekiya et al. (31) accounted for 60% of sulfur introduced as $^{35}S\text{-}SO_2$ as $^{35}S$-sulfate 3 hours after fumigation.

The in vivo labelling data strongly supports the principle that the major metabolic fate of excess sulfur introduced to a plant as sulfur dioxide is oxidation to sulfate. Since sulfite has been estimated to be thirty times more toxic to a plant than sulfate (37), it is tempting to speculate that this conversion of toxic sulfite to relatively non-toxic sulfate, i.e. sulfite oxidation, is a major mechanism of biochemical defense against the injurious effects of sulfur dioxide. This concept was first proposed by Thomas et al. (38) in 1950. Subsequent researchers have correlated the degree of sulfite-oxidizing activity in leaves to their resistance to $SO_2$ injury (22, 25).

Despite the obvious magnitude of the bisulfite to sulfate conversion after $SO_2$ exposure, little is known as to how this process takes place in plant leaves. In general, bisulfite has been proposed to be oxidized by a

a free radical chain mechanism (Table 1) (29). The process is initiated by formation of superoxide anion ($O_2^{-\cdot}$) and bisulfite ($HSO_3\cdot$) radicals. The reaction proceeds through a series of free radical-generating steps leading to the production of $SO_3$ which can then be hydrated to form sulfate. Many of the proposed ways in which a plant can oxidize bisulfite (Table 2) are thought to be indirect due to an initiation of this non-enzymatic mechanism through the production of the initiating free radicals (17) (Table 2, part B). Metal ions have been shown to be excellent catalysts for sulfite oxidation. Iron ($Fe^{3+}$) was shown to be a true catalyst at $10^{-6}$M (6). Ultraviolet radiation and illuminated dyes (12) also will initiate sulfite oxidation. Some enzymes oxidize sulfite through their production of superoxide anion radicals. Xanthine oxidase (11) and ferredoxin-NADP reductase (28) have been shown to initiate sulfite oxidation in this manner. Peroxidase, cytochrome oxidase, aldehyde oxidase, lipoxygenase, and amine oxidase have also been linked to the stimulation of sulfite oxidation (1). Asada and Kiso (1) demonstrated that illuminated chloroplasts will also initiate the aerobic oxidation of sulfite. This activity is inhibited by DCMU and superoxide dismutase (EC 1.15.1.1), an enzyme catalyzing the disproportionation of superoxide anion radicals ($O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$). It was concluded that the activity is due to the univalent reduction of oxygen by chloroplast electron transport to form the sulfite

Table 1. Proposed mechanism for bisulfite oxidation (29).

| | | |
|---|---|---|
| (1) | $Mn^{2+} + O_2 \longrightarrow Mn^{3+} + O_2^{\cdot-}$ | INITIATION |
| (2) | $Mn^{3+} + HSO_3^- \longrightarrow Mn^{2+} + HSO_3\cdot$ | |
| (3) | $O_2^{\cdot-} + HSO_3^- + 2H^+ \longrightarrow HSO_3\cdot + 2OH\cdot$ | CHAIN PROPAGATION |
| (4) | $HSO_3^- + OH\cdot + H^+ \longrightarrow HSO_3\cdot + H_2O$ | |
| (5) | $HSO_3\cdot + O_2 \longrightarrow SO_3 + H^+ + O_2^{\cdot-}$ | SULFATE FORMATION |
| (6) | $HSO_3\cdot + OH\cdot \longrightarrow SO_3 + H_2O$ | |
| (7) | $2HSO_3\cdot \longrightarrow SO_3 + HSO_3^- + H^+$ | |
| (8) | $SO_3 + H_2O \longrightarrow SO_4^{2-} + 2H^+$ | |

Table 2. Possible ways to oxidize sulfite in plant leaves (17).

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A. Direct oxidation

--sulfite oxidase (E.C.1.8.3.1)

$$SO_3^{2-} + O_2 + H_2O \longrightarrow SO_4^{2-} + H_2O_2$$

B. Indirect oxidation: initiating radical formation

--non-enzymatic initiation

e.g. metal ions

$$Mn^{2+} + O_2 \longrightarrow Mn^{3+} + O_2^{\cdot-}$$

--enzymatic initiation

e.g. xanthine oxidase (E.C.1.2.3.2)

$$\text{xanthine} + H_2O + O_2 \longrightarrow \text{uric acid} + O_2^{\cdot-}$$

--illuminated chloroplasts

$$\text{chloroplast} + h\nu \longrightarrow O_2^{\cdot-}$$

oxidation-initiating superoxide anion radical. All of these systems have in common the feature of indirectly stimulating the non-enzymatic free radical chain reaction by producing the initiating radicals.

Sulfite can also be oxidized directly by sulfite oxidase (EC 1.8.3.1) (Table 2, part A). This enzyme has been purified and characterized from bovine liver by Cohen and Fridovich (7). They showed that the oxidase activity involved a two-electron reduction of oxygen to hydrogen peroxide without the formation of any detectable radical intermediates. The activity of this enzyme has been linked to sulfur dioxide resistance in rats. Cohen et al. (8) fumigated rats deficient in sulfite oxidase with sulfur dioxide and found them to be more susceptible than rats with normal enzyme activity. They concluded that sulfite oxidase is instrumental in counteracting the toxic systemic effects of bisulfite.

There is evidence for the participation of enzymatic sulfite oxidation in plants. A sulfite-oxidizing enzyme was found in oat mitochondria by Tager and Rautenen (34) in 1955. It required the addition of $Mg^{2+}$ and cytochrome c for activity. Fromageot et al. (15) reported the presence of an enzymatic system for oxidizing sulfite in wheat roots that was inhibited by metal chelating agents. The extent to which these systems may operate in a plant leaf undergoing sulfur dioxide uptake is not known. Recently, a sulfite-oxidizing enzyme was isolated from castor bean

leaves by Kondo et al. (22). This enzyme differed from those previously described in that no cofactors were required. Furthermore, the sulfite-depleting activity of leaf extracts, partly attributable to this enzyme, was correlated with resistance to injury by sulfur dioxide.

The different mechanisms available to a plant leaf to oxidize bisulfite suggest a paradox in evaluating the protective value of bisulfite oxidation in $SO_2$-exposed plants. If the bulk of the oxidation proceeds by the non-enzymatic free radical chain mechanism, being initiated by the systems described that produce the superoxide anion radical (Table 2, part B), numerous free radicals could be produced. Bisulfite ($HSO_3\cdot$), hydroxyl ($OH\cdot$), and superoxide ($O_2^{-}\cdot$) radicals are produced in the chain propagation steps of the proposed mechanism (Table 1). These radical intermediates are highly reactive (14) and have the potential to cause more injury than the unoxidized bisulfite ion. Tanaka (36) states that $10^{-3}$ to $10^{-2}$ M sulfite causes toxicity while only $10^{-8}$ to $10^{-7}$ M $O_2^{-}\cdot$ causes plant damage. This suggests bisulfite oxidation could actually be an injury-causing reaction in plants. In support of this concept, Peiser and Yang (29) linked the oxidation of sulfite to chlorophyll destruction. They discuss evidence for two chlorophyll-destroying systems; one requires light and $O_2$ with chlorophyll acting as the photosensitizer and in the other, chlorophyll is destroyed in the dark when in presence of $Mn^{2+}$, glycine, and $O_2$. They suggest that in both systems

chlorophyll is destroyed by free radicals, including superoxide anions, produced during the aerobic oxidation of bisulfite. This conclusion came from results obtained using free radical scavengers to inhibit the reaction. Lizada and Yang (23) proposed that sulfite oxidation led to the peroxidation of lipids with subsequent ethane and ethylene emission by spinach chloroplasts. Again, this activity was inhibited by free radical scavengers. Shimazaki et al. (33) found free radical-linked chlorophyll destruction and lipid peroxidation in response to spinach leaf fumigation with sulfur dioxide. They speculate that the increased production of superoxide anions could be responsible, in part, for the phytotoxic effects of $SO_2$.

Plants possess natural mechanisms to scavenge free radicals produced during normal metabolism. Superoxide dismutase has been suggested as providing a natural defense against the deleterious effects of superoxide anion radicals (2, 13, 14). It is present in chloroplasts and its activity has been proposed as playing an important role in protecting leaf cells from free radical injury due to sulfur dioxide exposure (3, 36). Tanaka and Sugahara (36) compared superoxide dismutase activities in young (resistant to $SO_2$ injury) and old (susceptible to $SO_2$ injury) poplar leaves. They found the young leaves contained five times more superoxide dismutase activity. Furthermore, when the leaves were sprayed with diethyldithiocarbamate, an inhibitor of superoxide dismutase activity (20), their resistance to $SO_2$

injury decreased. Their findings suggest that the toxic effects of sulfur dioxide are due in part to the superoxide radical and that the presence of superoxide dismutase is a means of protecting the leaf against its toxicity.

It is apparent that bisulfite oxidation in plant leaves can, in principle, proceed by at least two different mechanisms. The manner in which it actually occurs may eminently determine whether bisulfite oxidation will be a protective or a lethal process. If toxic bisulfite is oxidized to relatively non-toxic sulfate by a direct enzymatic process whereby no free radical intermediates are released (e.g. sulfite oxidase), then bisulfite oxidation could serve as a protective reaction. However, if oxidation occurs mainly through the initiation of the non-enzymatic free radical chain mechanism, then it is potentially injurious. If this were the case, the ability of the affected plant cell to effectively scavenge the free radicals produced may be paramount in determining whether injury will occur.

Cucumber (Cucumis sativa L.) leaves vary in susceptibility to sulfur dioxide with age, the younger leaves being more resistant (5). From $SO_2$ absorption studies, this difference is attributable to biochemical factors, not stomatal ones. A comparative study of the biochemical means of coping with excess sulfur from sulfur dioxide in young and old cucumber leaves may reveal mechanisms by which plants resist $SO_2$ injury.

Bisulfite oxidation is the major mechanism by which sulfur absorbed by leaves as sulfur dioxide is metabolized. It is not clear how plants execute this reaction and the significance to $SO_2$ injury attributed to it is contradictory. In light of this, the major goals of this project were 1) to elucidate the manner in which a cucumber leaf oxidizes bisulfite, and 2) to compare this process in young and old cucumber leaves. This comparison should help in determining what role bisulfite oxidation plays in resistance and/or susceptibility to $SO_2$.

# MATERIALS AND METHODS

## Plant Material

Cucumber (*Cucumis sativus* L.) inbred line SC 25 seeds were sown in 300 ml plastic pots, 3-4 seeds per pot, in a mixture of equal parts sterilized soil, peat, and Turface (granular calcined absorbent clay). Plants were watered twice daily, once with deionized water and once with one-half strength Hoagland nutrient solution. Plants, thinned to one per pot after two weeks, were grown with vertical support in a growth chamber at 33°C for 16 hours with full light (light from fluorescent and incandescent lamps totalling 7.5 mw $cm^{-2}$) and at 15°C for 8 hours with 2 hours light from incandescent lamps (2.4-4.7 mw $cm^{-2}$) at the beginning and end of the cool period.

Plants were used when they were 4 to 7 weeks old, having 6 to 10 distinct leaves. When necessary, leaves were designated as "young" and "old". The expanding leaves, usually the first and second from the apex, were considered "young". The healthy, fully expanded leaves farthest from the apex, usually the fifth and sixth, were considered "old".

## Chemicals

Type I bovine blood superoxide dismutase, bovine serum albumin, Triton X-100, xanthine, and Grade IV milk xanthine oxidase were obtained from Sigma Chemical Company (St. Louis, Missouri). All other chemicals used were of reagent grade.

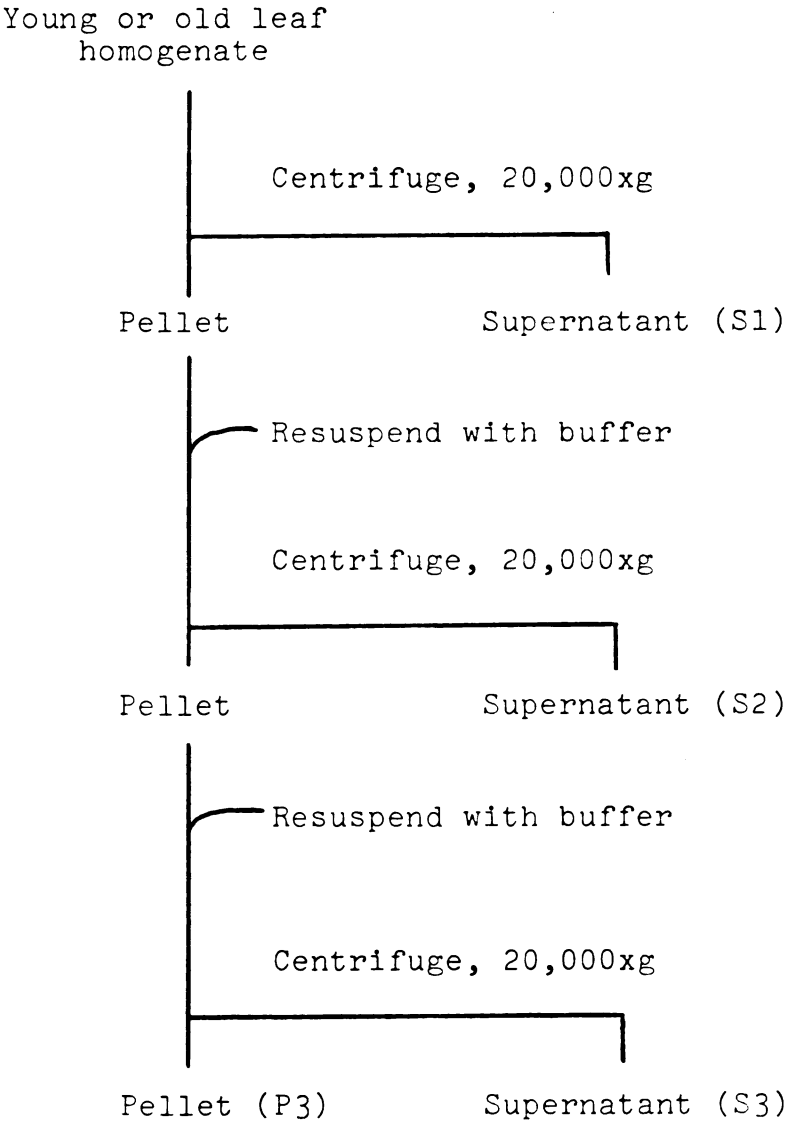
## Preparation of Leaf Homogenates

Young and old cucumber leaves were cut from the plant, rinsed with distilled water, and the midrib removed with a sharp razor blade. Leaf sections were sliced into thin strips (approximately 1 mm x 20 mm), weighed, and homogenized 1-2 minutes in 50 mM potassium phosphate (pH 7.0) + 1 mM EDTA (5:1, vol buffer:g fresh wt) at $4^{\circ}C$ with a Waring blender at top speed. This phosphate-EDTA buffer was used in all experiments except where indicated. Homogenized material was squeezed through a single layer of premoistened Miracloth. The filtered homogenate was stored on ice until ready for analysis or fractionation.

## Fractionation of Leaf Homogenate

Young and old leaf homogenates were separated into soluble (S1, S2, S3) and washed pellet (P3) fractions as diagrammed in Figure 2. Centrifugations were carried out at 20,000 g for 30 minutes. Pellet fractions were resuspended in cold phosphate-EDTA buffer, in a volume equivalent to

Figure 2. Fractionation of leaf homogenate.



that of the supernatant, by repeatedly drawing and expelling the solution through a Pasteur-type pipette. On some occasions clumps of particulate matter in the P3 fraction were dissipated with a ground glass homogenizer before analysis with the oxygen probe assembly. All steps were done at 4°C and fractions were kept on ice until assayed for activity.

Assay for Bisulfite Depletion

Bisulfite-depleting activity was determined by incubating samples at 30°C in the presence of 1 mM or 0.1 mM $KHSO_3$. When 1 mM $KHSO_3$ was used, the reaction was stopped by diluting 0.1 ml samples to 5 ml with 1 mM EDTA. When 0.1 mM $KHSO_3$ was used, 0.5 ml samples were diluted to 5 ml with 1 mM EDTA. Bisulfite ion concentration before and after the incubation was determined using the basic fuchsin method (48). The absorbance was measured at 585 nm.

Assay for Bisulfite Oxidation

Oxygen consumption of samples was measured with a Clark polarographic-type oxygen probe (Yellow Springs Instrument, Model 5331) connected to a suitably amplified chart recorder. Samples were placed in reaction vessels immersed in a water-jacketed stirrer assembly. The temperature was usually kept at 30°C ($\pm$ 0.02°C). The rate of oxygen consumption of 4 ml samples was measured before and after injecting samples with $KHSO_3$. "Bisulfite

oxidation" was determined as the difference between these two rates. "Light treatment" consisted of illuminating samples (at 54 mw $cm^{-2}$) with a 100 w incandescent bulb. For assaying gel chromatography fractions the oxygen monitoring apparatus was commercially modified to use 1 ml samples.

Column Chromatography

Procedures used to separate soluble components containing bisulfite-depleting activity with Sephadex G-25 and G-200 were modified from those described by Kondo et al. (22). The soluble component of the cucumber leaf homogenate (S1) was prepared as previously described, except that leaves were homogenized (4:1, vol buffer:g fresh wt) in 100 mM potassium phosphate (pH 7.0) + 1 mM EDTA. The S1 fraction was concentrated by lyophilizing overnight, stored at -10°C until ready for use, and then dissolved in a small volume of buffer. Five percent (w/v) sucrose was added to the sample just before chromatography. Concentrated sample was applied to a 2.6 x 26 cm column packed with Sephadex G-25 (medium) (Pharmacia Fine Chemicals) equilibrated with 50 mM potassium phosphate (pH 7.0) + 1 mM EDTA. The sample was eluted at 4°C with the same buffer and 4.5 ml fractions were collected.

For fractionation with Sephadex G-200, the S1 sample was concentrated as before and applied to a G-25 (coarse) column. The activity corresponding to the void volume was

pooled and concentrated by lyophilization. The sample was applied to a 1.6 x 50 cm column packed with Sephadex G-200 equilibrated as before. It was eluted in the same buffer at 4°C and 4.5 ml fractions were collected.

Superoxide Dismutase Activity

Preparation of the sample for the determination of superoxide dismutase activity was slightly modified from that described by Tanaka and Sugahara (36). The soluble leaf homogenate fraction (S1), prepared as previously described, was dialyzed 22 hours against 2 liters of 20 mM potassium phosphate (pH 7.8) with two changes of the dialysis buffer. Material remaining in the dialysis bag was centrifuged at 10,000g for 30 minutes. All procedures were done at 4°C. The superoxide dismutase activity of the supernatant was measured at 25°C as described by McCord and Fridovich (50) and modified slightly by Tanaka and Sugahara (36). Total volume of the assay was 1.05 ml. It contained 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.01 mM cytochrome c, 0.1 mM xanthine, 30 µg xanthine oxidase (suspended in 2M ammonium sulfate, 1 mM EDTA, pH 8.0), and enzyme sample. The reaction was initiated by the addition of xanthine oxidase. Cytochrome c reduction was monitored as the increase in absorbance at 550 nm with a Gilford 2000 recording spectrophotometer. One unit of superoxide dismutase activity was defined as the amount of enzyme required to inhibit the reduction rate of cytochrome c

by 50% under the described conditions. Units of activity were determined as V/v - 1, where V and v are the reduction rates in the absence and presence of enzyme, respectively (47).

Protein Determination

One volume of sample was combined with one volume 20% TCA to precipitate the protein. The sample was heated at 100°C for five minutes, brought to 5 ml with 10% TCA, and centrifuged. The pellet was washed with 95% ethanol, collected by centrifugation, dried under nitrogen, and dissolved in 1 N NaOH. Protein content of this sample was determined by the method of Lowry *et al.* (49).

Chlorophyll Determination

Chlorophyll was extracted from the sample into 80% (v/v) acetone. The extraction was facilitated by drawing the solution into a Pasteur-type pipette several times. The sample was centrifuged 5 minutes at top speed in a clinical centrifuge and the absorbance of the supernatant solution measured at 645 and 663 nm. Chlorophyll concentration was determined by the equation of Arnon (46).

# RESULTS

## Activity of Leaf Homogenates

A significant problem encountered in studying bisulfite oxidation in plant material is the tendency for bisulfite to readily become oxidized non-specifically. As shown in Table 3, tap water by itself has a very high bisulfite-oxidizing activity. Distilled water has very little. It is particularly important to buffer the sample being assayed due to the change in bisulfite and sulfite ion distribution with pH (30). However, 50 mM potassium phosphate itself has significant oxidizing activity. This activity is most likely due to the presence of small amounts of metal ions, strong catalysts of bisulfite oxidation, in the potassium phosphate. As shown in Table 3, this non-specific activity can be completely eliminated by the addition of 1 mM EDTA to the buffer.

The general response of cucumber leaf homogenate analyzed with the oxygen probe is illustrated in Figure 3, part A. The homogenate, with no additions, has a small intrinsic oxygen consumption rate that is unaffected by light. When bisulfite as $KHSO_3$ is introduced into the reaction vessel in the dark, the slope of the trace changes sharply, reflecting the increased rate of oxygen consumption

Table 3. Oxidation of bisulfite by tap water and potassium phosphate buffer.

| Content of sample | Bisulfite oxidized ($\mu l\ O_2\ min^{-1}\ ml^{-1}$) |
|---|---|
| Tap water | 18.70 |
| Distilled water | 0.01 |
| 50 mM potassium phosphate | 1.29 |
| 50 mM potassium phosphate + 1 mM EDTA | 0.00 |

Four ml samples at 30°C were injected with 10 µl 0.4 M $KHSO_3$.

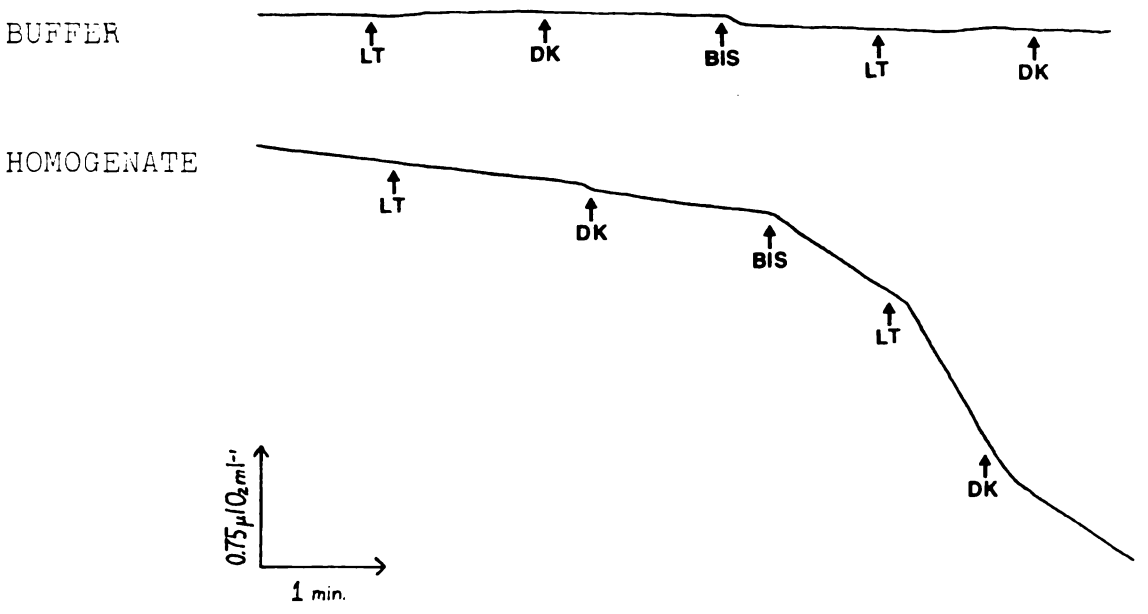
Figure 3. Oxygen probe traces of leaf homogenate bisulfite oxidation.

Oxygen consumption versus time was monitored for 4 ml samples in the dark (DK) and light (LT) in the presence and absence of 1 mM $KHSO_3$ (BIS).

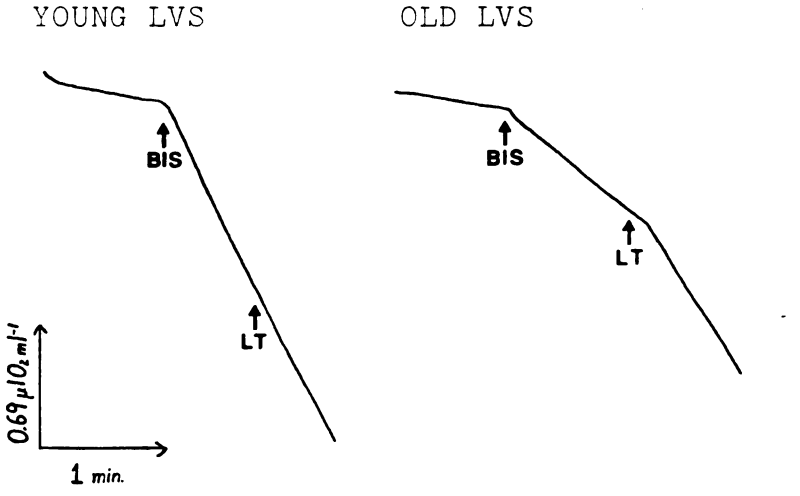
A. Samples of buffer and leaf homogenate, prepared from randomized leaves, at 25°C were injected with 40 µl 0.1 M $KHSO_3$ to give a final concentration of 1 mM.

B. Samples of young and old leaf homogenate at 30°C were injected with 10 µl 0.4 M $KHSO_3$ to give a final concentration of 1 mM.

A.


BUFFER
LT
DK
BIS
LT
DK
HOMOGENATE
LT
DK
BIS
LT
DK
0.75 µl O2 ml-1
1 min.

B.


YOUNG LVS
OLD LVS
BIS
LT
BIS
LT
0.69 µl O2 ml-1
1 min.

due to the oxidation of bisulfite. When the sample is illuminated, this rate of oxidation increases sharply. When the light is turned off, the oxygen consumption rate returns to approximately the original rate observed in the dark. The buffer, containing 1 mM EDTA, shows no oxygen consumption under the same conditions.

A striking difference is noted in oxygen consumption when that of young leaf homogenates is compared with that of old leaf homogenates (Figure 3, part B). The rate of bisulfite oxidation does not increase upon illumination in young leaf homogenates; whereas, there is a distinct increase due to light in old leaf homogenates. The results from many determinations of this type are summarized in Table 4. In all cases, and under varying conditions, the dominant difference in bisulfite oxidation activities in young and old leaf homogenates is the considerably greater degree of light-dependent activity in the older leaves. The difference in the degree of dark activity is small, but on the average, younger leaf homogenates appear to have more.

When a gradient of leaf ages from a plant is analyzed (Figure 4), there is a clear trend toward increasing light-dependent activity with increasing leaf age. Dark activity decreases with leaf age. It is interesting to note that, with the exception of the very youngest leaves, the total rate of oxidation in the light does not change with leaf age, yet the distribution of the dark and light-dependent components to this total rate changes significantly.

Table 4. Bisulfite oxidation in homogenates from young and old leaves.

Samples, maintained either in the dark or in the light, were injected with 10 µl 0.4 M $KHSO_3$. Light-dependent activity was determined by subtracting the dark activity from that in the light. The buffer contained 1 mM EDTA in all experiments except Experiment VII which contained 0.1 mM EDTA. The assay temperature was 30°C in all experiments except I and II where it was 25°C. Protein content of young and old leaves (as determined in Experiments VI and VII) averaved 5.8 and 4.4 mg g fresh $wt^{-1}$, respectively. The chlorophyll content from the same tissues averaged 419 and 515 µg g fresh $wt^{-1}$, respectively. Data given is based on the average of two replicates in Experiments III and IV with an average standard error of ±3.7.

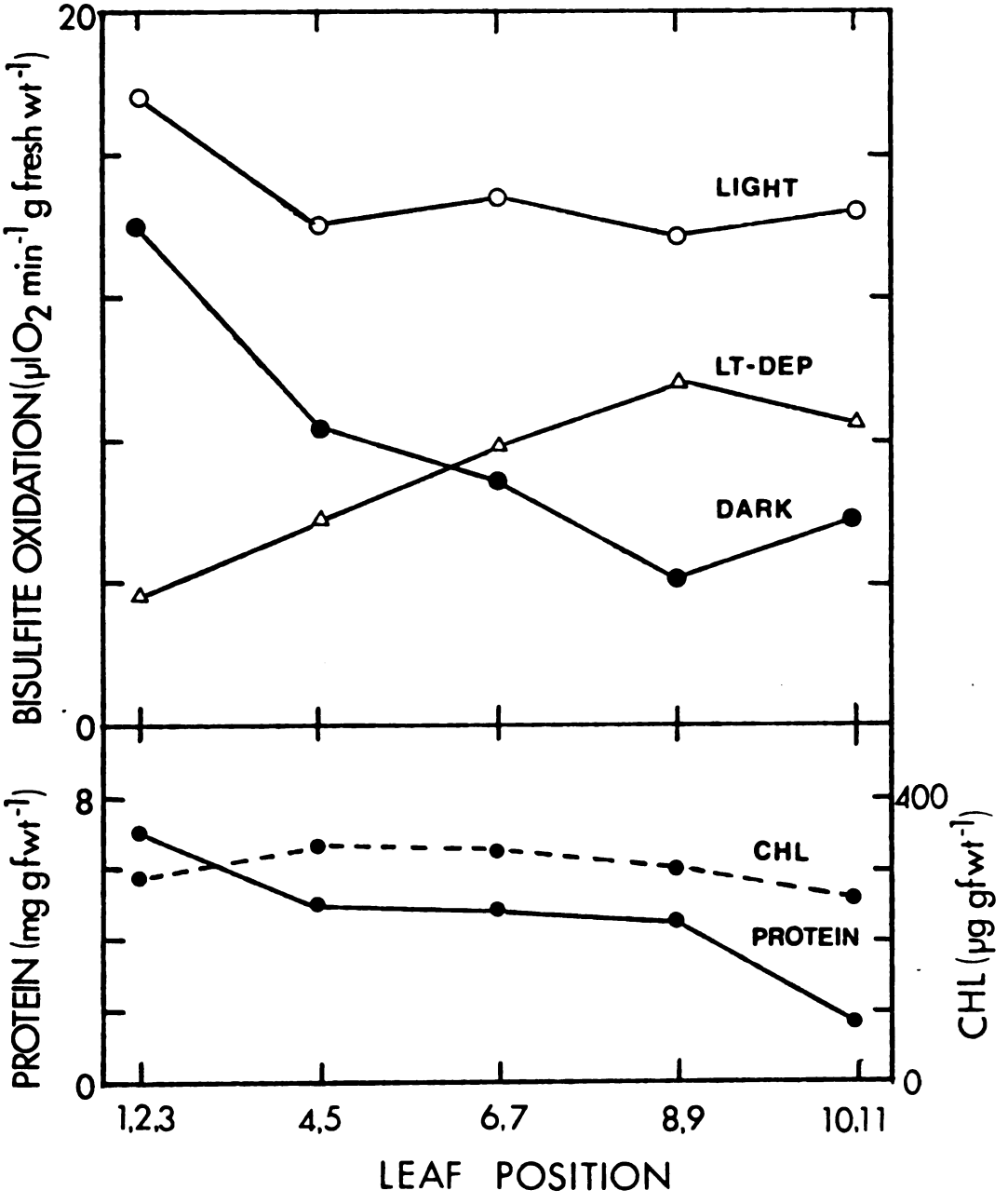
Table 4.

| Experiment | Leaf age | Bisulfite oxidation ($\mu l\ O_2\ min^{-1}\ g\ fresh\ wt^{-1}$) | |
|---|---|---|---|
| | | Dark | Light-dependent |
| I | Young | 17.4 | 0.5 |
| | Old | 23.2 | 9.7 |
| II | Young | 26.1 | 2.4 |
| | Old | 28.5 | 19.5 |
| III | Young | 22.1 | 2.3 |
| | Old | 10.1 | 11.0 |
| IV | Young | 14.6 | 6.6 |
| | Old | 17.9 | 10.4 |
| V | Young | 24.1 | 4.6 |
| | Old | 21.3 | 13.0 |
| VI | Young | 20.4 | 2.9 |
| | Old | 15.7 | 18.5 |
| VII | Young | 63.1 | 8.3 |
| | Old | 35.2 | 23.3 |

Figure 4. Bisulfite oxidation by leaf homogenates versus leaf position.

Leaves whose positions were numbered sequentially, beginning at the apex, were taken from two 8-week-old cucumber plants. They were homogenized in buffer and 4 ml samples were injected with 10 µl 0.4 M $KHSO_3$ (to give a final concentration of 1 mM) at 30°C. Light-dependent activity (LT-DEP) was calculated by subtracting the activity in the dark from that in the light. For bisulfite oxidation measurments, each point represents the mean of two replicates with an average standard error of ±0.8. Protein and chlorophyll (CHL) concentrations were determined from aliquots of the leaf homogenates.

Figure 4.


20
0
BISULFITE OXIDATION(μlO2 min-1 g fresh wt-1)
LIGHT
LT-DEP
DARK
8
0
PROTEIN (mg g fwt-1)
400
0
CHL (μg g fwt-1)
CHL
PROTEIN
1,2,3
4,5
6,7
8,9
10,11
LEAF POSITION

The chlorophyll content of these leaves did not change to a great degree. The protein content of leaf homogenates decreased with increasing leaf age.

Fractionation of Leaf Homogenates

Young and old cucumber leaf homogenates were fractionated by centrifugation into several components and the bisulfite oxidation activities of these fractions were determined (Table 5). The majority of the dark bisulfite oxidation activity is recovered in the first supernatant solution (S1) and in the washed pellet (P3). The total amount of dark activity recovered in all of the fractions is approximately equal to that measured in the homogenate before fractionation under the described conditions. There is no light-dependent activity in any of the soluble fractions (S1, S2, and S3) and a small amount in the pellet (P3) from both young and old leaves. In the young leaves the amount of this light-dependent activity recovered in the fractions is about the same as that measured in the homogenate; whereas, for the older leaves there is a significant loss in light-dependent activity. If the S1 and P3 fractions are combined, a significant amount of the original light-dependent activity is recovered (Table 6). This suggests the light-dependent activity requires both a particulate and a soluble component.

It is important to characterize these different components of bisulfite oxidation in leaf homogenates

Table 5. Bisulfite oxidation in fractions of homogenates from young and old leaves.

| Sample | | Bisulfite oxidation ($\mu l\ O_2\ min^{-1}\ g\ fresh\ wt^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Expt I | | Expt II | | Expt III | |
| | | Dark | Lt-dep | Dark | Lt-dep | Dark | Lt-dep |
| Unfract'd Homogenate | Young | 22.1 | 2.3 | 24.1 | 4.6 | 20.4 | 2.9 |
| | Old | 10.1 | 11.0 | 21.3 | 13.0 | 15.7 | 18.5 |
| 1st Supern. (S1) | Young | 7.8 | -- | 14.4 | -- | 15.2 | -- |
| | Old | 4.1 | -- | 16.7 | -- | 10.5 | -- |
| 2nd Supern. (S2) | Young | 3.2 | -- | 3.2 | -- | 1.9 | -- |
| | Old | 1.8 | -- | 0.9 | -- | 1.9 | -- |
| 3rd Supern. (S3) | Young | 0.9 | -- | -- | -- | 0.5 | -- |
| | Old | 0.5 | -- | -- | -- | 1.0 | -- |
| Washed Pellet (P3) | Young | 3.7 | 3.2 | 7.9 | 4.2 | 6.2 | 1.9 |
| | Old | 4.1 | 1.8 | 4.6 | 3.2 | 4.8 | 0.0 |
| Sum of Fractions | Young | 15.6 | 3.2 | 25.5 | 4.2 | 23.8 | 1.9 |
| | Old | 10.5 | 1.8 | 22.2 | 3.2 | 18.2 | 0.0 |

Four ml samples at 30°C were injected with 10 µl 0.4 M $KHSO_3$ in the dark and the light. Data reported for Experiments I and II represents the average of two replicate assays.

Table 6. Reconstitution of the light-dependent activity of a homogenate from old leaves.

| Sample | Lt-dep bisulfite oxidation ($\mu l\ O_2\ min^{-1}\ g\ fresh\ wt^{-1}$) |
|---|---|
| Unfract'd homogenate | 18.5 |
| S1 | 0.0 |
| S2 | 0.0 |
| S3 | 0.0 |
| P3 | 0.0 |
| P3 + S1 | 8.1 |

Samples of 0.4 ml were diluted to 4 ml with buffer before being assayed. For reconstitution, 0.4 ml S1 was added to 0.4 ml P3 and this mixture diluted to 4 ml.

because different activities can have different effects on a plant in regards to injury by $SO_2$. Toward this goal, the unfractionated homogenate and component activities were compared for their sensitivities to heat treatment using the bisulfite depletion and bisulfite oxidation assays (Table 7). When activity was determined by measuring bisulfite depletion, 34% of the homogenate activity was heat stable and nearly all of this could be accounted for in the S1 fraction. In sharp contrast, all of the activity measured as bisulfite oxidation was heat labile. The bisulfite depletion assay measures the disappearance of bisulfite ions from the treatment solution with no indication as to where they are going, while the bisulfite oxidation assay measures the actual oxidation of bisulfite to sulfate. It is evident that plant leaf homogenates contain soluble bisulfite-depleting activity that cannot be attributed to oxidation. At least part of this activity is stable to heat treatment.

In order to further characterize the S1 fraction activity it was fractionated on a G-25 column. Two distinct peaks of bisulfite depletion activity were resolved (Figure 5). The predominant peak is the high molecular weight one associated with the void volume, Peak A. There is also at least one lower molecular weight activity peak, B. The presence of a third peak is uncertain due to its lack of activity when assayed a second time. This G-25 activity profile is similar to that obtained by Kondo *et al.* (22)

Table 7. Stability to heat of bisulfite depletion and bisulfite oxidation activities in the homogenate and in its fractions.

| | | Percent activity heat stable | |
|---|---|---|---|
| | | Assay | |
| Sample | | $HSO_3^-$ depletion | $HSO_3^-$ oxidation |
| Homogenate | Dark | 34 | 0 |
| | Lt-dep | -- | 0 |
| S1 | Dark | 33 | 0 |
| P3 | Dark | 0 | 0 |
| P3 + S1 | Dark | -- | 0 |
| | Lt-dep | -- | 0 |

Activities of samples placed in 100°C water bath for 5 minutes were compared to those of samples kept on ice. Bisulfite depletion was determined by incubating 1 ml heated and unheated samples in 1 mM $KHSO_3$ at 30°C for 45 minutes. Bisulfite oxidation was determined by injecting 4 ml heated and unheated samples at 25°C with 40 µl 0.1 M $KHSO_3$ to give a final concentration of 1 mM.

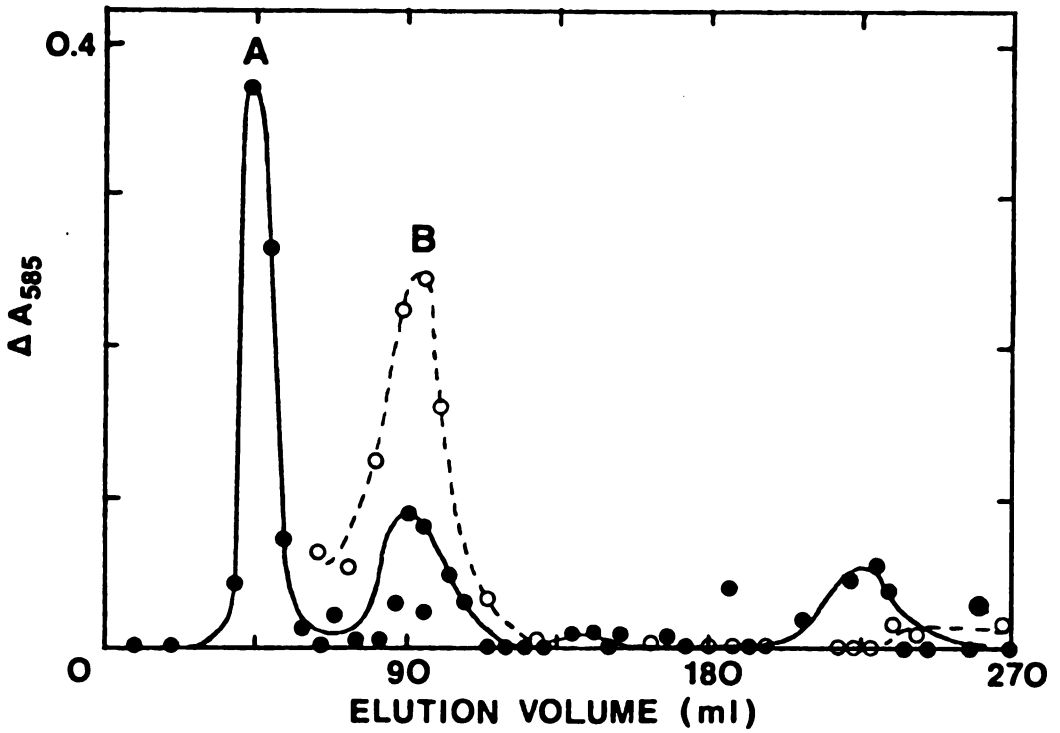



Figure 5. Chromatography of leaf extract on Sephadex G-25.

Concentrated S1 fraction from leaf homogenate was applied to a 2.6 x 26 cm column of Sephadex G-25. The sample was eluted at 50 ml $hr^{-1}$ at 4°C. Fractions of 4.5 ml were collected and assayed for bisulfite depletion activity. Replicate 0.5 ml fraction aliquots were incubated at 30°C for 45 minutes in the presence of 2 mM $KHSO_3$ (●—●). Selected fractions were assayed the next day in 1 mM $KHSO_3$ with a 90 minute incubation (o--o). Absorbance was determined at 585 nm.

using castor bean extract.

The two activity peaks, A and B, have entirely different characteristics (Table 8). Bisulfite-depleting activity of Peak A is almost entirely heat labile while that of Peak B is almost entirely heat stable. When assayed for bisulfite oxidation, Peak A clearly has activity whereas none is measurable in Peak B. These results suggest that the S1 fraction contains at least two bisulfite-depleting activities: a heat stable, lower molecular weight substance that depletes bisulfite without concurrent oxidation and a higher molecular weight, heat-sensitive substance that contains significant bisulfite oxidation activity. The presence of these two different activities supports the observations made on S1 activity from Table 7.

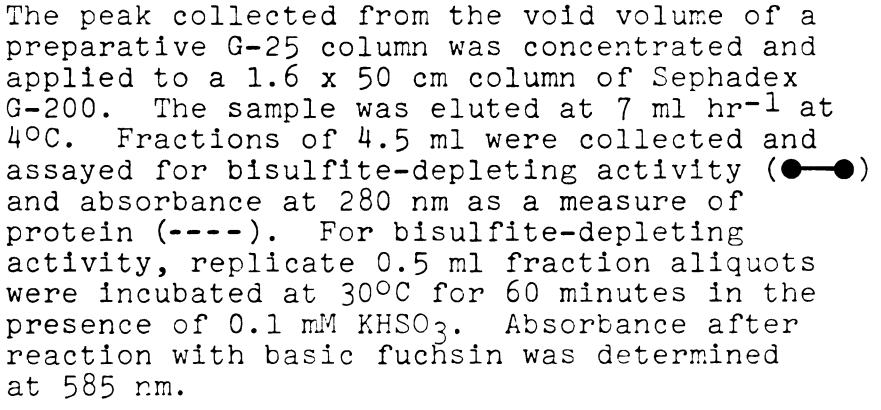
When the higher molecular weight peak, A, is fractionated on a Sephadex G-200 column, it resolves into one major bisulfite depletion activity peak separate from the two major protein peaks (Figure 6). There are two minor peaks of activity corresponding to the protein peaks. These two minor peaks may involve bisulfite depletion by reaction with disulfide groups of proteins. This elution profile is also similar to that obtained by Kondo *et al.* (22) using castor bean extract. In the present study, the pooled major-activity peak contained measurable bisulfite oxidation activity. There is then a heat-sensitive, high molecular weight compound, resolvable into a single G-200 column peak, that oxidizes bisulfite without the requirement of additional

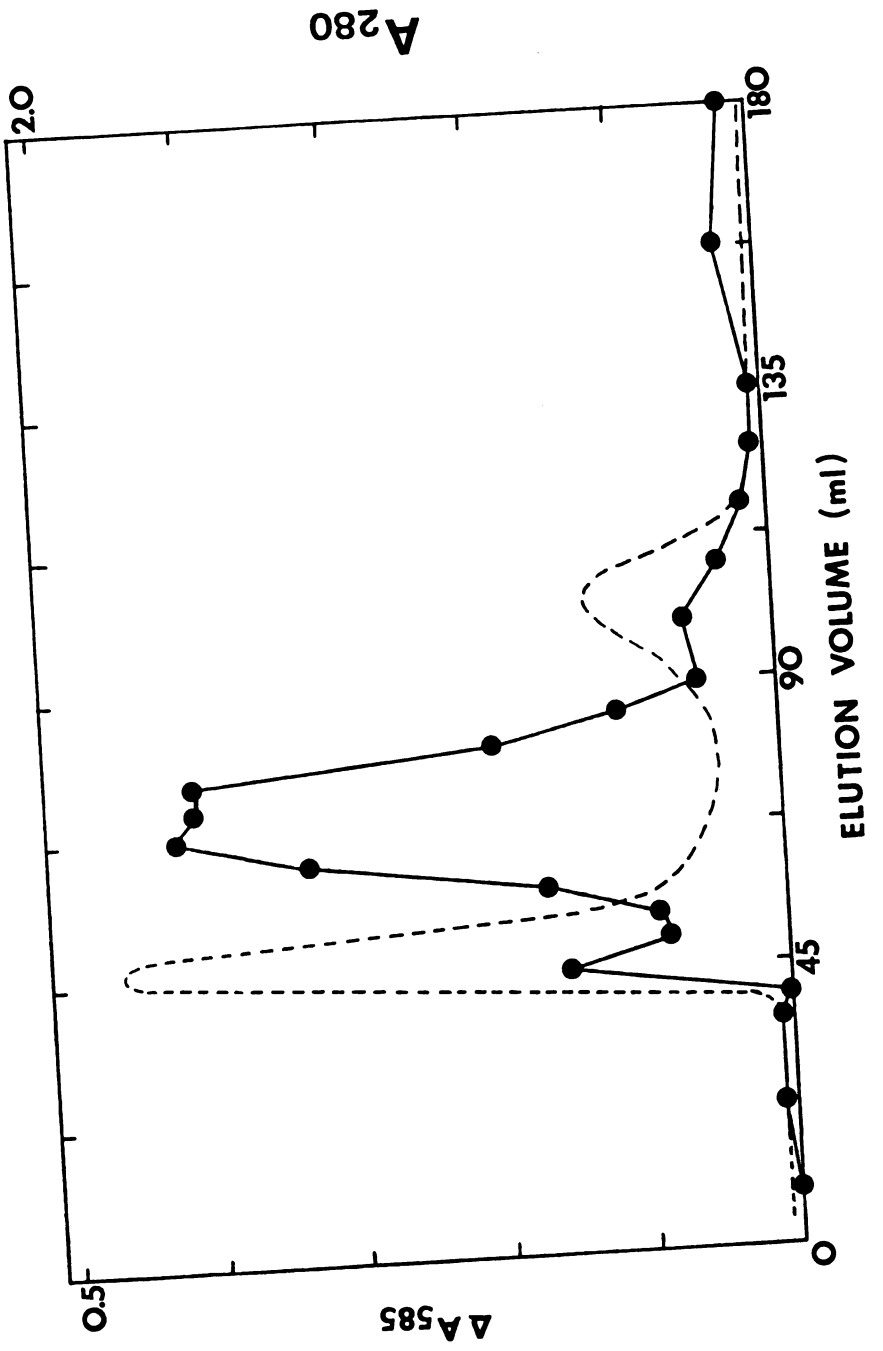
Table 8. Characterization of Sephadex G-25 peaks: heat sensitivity and bisulfite oxidation activity.

| | Bisulfite depletion (nmole $HSO_3^-$ $min^{-1}$ $ml^{-1}$) | | Bisulfite oxidation (µl $O_2$ $min^{-1}$ $ml^{-1}$) |
|---|---|---|---|
| Sample | Untreated | Boiled | |
| Peak A | 992 | 56 | 0.44 |
| Peak B | 163 | 154 | 0.00 |

Pooled peaks of activity, A and B, from a G-25 column were assayed for bisulfite depletion activity by incubating 0.5 ml samples in 1 mM $KHSO_3$ at 30°C for 90 minutes. "Boiled" samples were placed in 100°C water bath for 5 minutes before being assayed. Bisulfite oxidation was determined by injecting 1 ml samples at 30°C with 10 µl 0.1 M $KHSO_3$ to make a final concentration of 1 mM.

Figure 6. Chromatography of leaf extract on Sephadex G-200.




A280
2.0
180
135
90
45
0
ELUTION VOLUME (ml)
0.5
ΔA585

substances for activity.

The particulate (P3) dark activity is shown in Table 7 to be completely heat sensitive. In an attempt to further characterize this fraction, it was subjected to a mild detergent treatment with Triton X-100 (Table 9). A decrease in pellet-associated activity with detergent treatment and concurrent increase in soluble activity would suggest one can extract a membrane-associated protein from the pellet fraction. When the washed and resuspended pellet (P3) was treated for 24 hours with and without Triton X-100 and separated into soluble and particulate components, there was a large decrease in the activity associated with the pellet in both the young and old leaf samples. However, the unusually large degree of activity in the supernatant solution was apparently due to Triton X-100 itself strongly stimulating non-enzymatic bisulfite oxidation; therefore it is not known whether active bisulfite-oxidizing substances from the pellet were solubilized by this treatment. The small amount of activity found in the pellet fraction after treatment could, in fact, all be due to trace contamination with Triton X-100 as it was not rinsed prior to the activity assay.

## Inhibitor Studies

DCMU, an inhibitor of photosynthetic electron transport, potently inhibits the light-dependent bisulfite oxidation activity of leaf homogenates (Figure 7). It apparently has

Table 9. Effect of Triton X-100 on bisulfite oxidation activity of particulate and soluble components of the P3 fraction.

| Sample | | Bisulfite oxidation ($\mu l\ O_2\ min^{-1}\ g\ fresh\ wt^{-1}$) | |
|---|---|---|---|
| | | Buffer only | + Triton X-100 |
| P3, young | | | |
| | pellet | 33.6 | 11.4 |
| | supern. soln. | 23.8 | 120 |
| P3, old | | | |
| | pellet | 16.6 | 7.2 |
| | supern. soln. | 17.6 | 133 |
| Buffer | | 0.0 | 340 |

P3 fractions prepared from homogenates of young and old cucumber leaves were isolated and resuspended in 50 mM potassium phosphate containing 0.1 mM EDTA. Triton X-100 (1%, v/v) was added to half the P3 samples and all were stored at 4°C with occasional shaking. After 24 hours, samples were separated into pellet and supernatant solution fractions by centrifugation. Bisulfite oxidation activity was measured in the dark at 30°C. Activities for young and old P3 fractions before incubation were 45.0 and 22.0 $\mu l\ O_2\ min^{-1}\ g\ fresh\ wt^{-1}$, respectively.

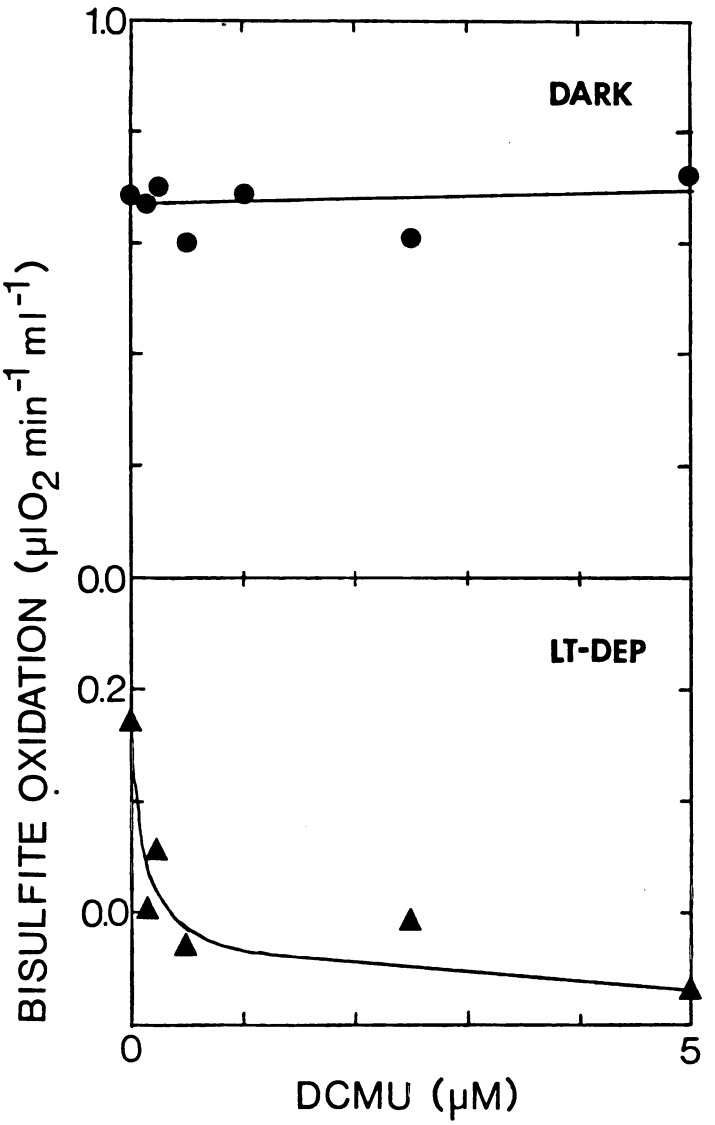



Figure 7. Effect of DCMU concentration on bisulfite oxidation by leaf homogenate.

Varying concentrations of DCMU were injected into 4 ml samples of leaf homogenate prepared from old leaves at 30°C during dark and light bisulfite oxidation.

no effect on the dark activity at concentrations up to 5 μM. The specific inhibition of the light-dependent activity by 1 μM DCMU is clearly shown in Table 10. DCMU injected during bisulfite oxidation in the light (Table 10) reduces the rate to approximately that of the original dark oxidation. When injected during dark oxidation (Table 10), DCMU causes no change in the original rate.

Superoxide dismutase at 1 mg $ml^{-1}$ almost completely inhibits light-dependent activity in old leaf homogenates (Table 11). Bovine serum albumin, added as a protein control, inhibits light-dependent activity to a degree, but not to the extent of inhibition by superoxide dismutase. The small increase in light-dependent activity in young leaf homogenate is probably not significant as the oxygen trace used for this particular calculation was not linear. There was a stimulation of dark bisulfite oxidation in the old leaf homogenates in the presence of superoxide dismutase, whereas BSA significantly inhibited dark oxidation in both young and old leaf homogenates.

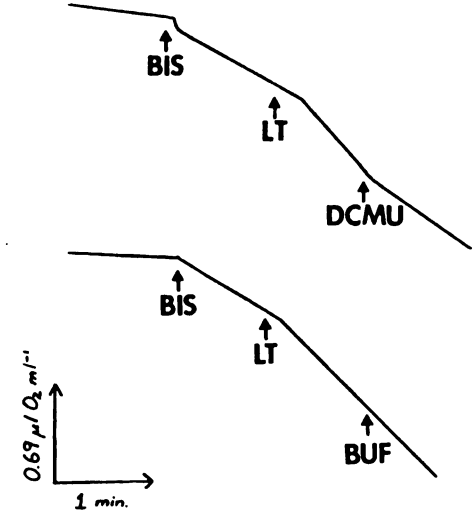
Methyl viologen is thought to exert its herbicidal activity by accepting electrons from PS I and forming a stable radical which can then be oxidized by oxygen (51). When added to leaf homogenate, it inhibits the dark bisulfite oxidation activity with no apparent effect on the leaf-dependent activity (Figure 8). As shown in Table 12, 1 mM methyl viologen inhibits S1 and P3 dark oxidation activities similarly in both young and old leaves.

Table 10. Effect of DCMU on leaf homogenate activity.

A leaf homogenate from old cucumber leaves was prepared and assayed in buffer containing 0.1 mM EDTA. Samples at 30°C were first injected with 10 µl 0.4 M $KHSO_3$ (BIS) (to make a final concentration of 1 mM) and then with either 40 µl 100 µM DCMU (to make a final concentration of 1 µM) or with 40 µl buffer (BUF). Tabular data given corresponds to adjacent $O_2$ trace.

A. Effect on light-dependent activity

| Treatment | $O_2$ consumption ($\mu l\ O_2\ min^{-1}\ ml^{-1}$) | Percent of "Dark + $KHSO_3$" rate |
|---|---|---|
| Dark | 0.09 | -- |
| Dark + $KHSO_3$ | 0.41 | 100 |
| Light + $KHSO_3$ | 0.74 | 180 |
| Light + $KHSO_3$ + DCMU | 0.50 | 122 |
| | | |
| Dark | 0.05 | -- |
| Dark + $KHSO_3$ | 0.43 | 100 |
| Light + $KHSO_3$ | 0.74 | 172 |
| Light + $KHSO_3$ + Buffer | 0.77 | 179 |




B. Effect on dark activity

| | | |
|---|---|---|
| Dark | 0.03 | -- |
| Dark + $KHSO_3$ | 0.46 | 100 |
| Dark + $KHSO_3$ + DCMU | 0.41 | 89 |
| Light + $KHSO_3$ + DCMU | 0.44 | 96 |
| | | |
| Dark | 0.07 | -- |
| Dark + $KHSO_3$ | 0.43 | 100 |
| Dark + $KHSO_3$ + Buffer | 0.36 | 84 |
| Light + $KHSO_3$ + Buffer | 0.70 | 163 |

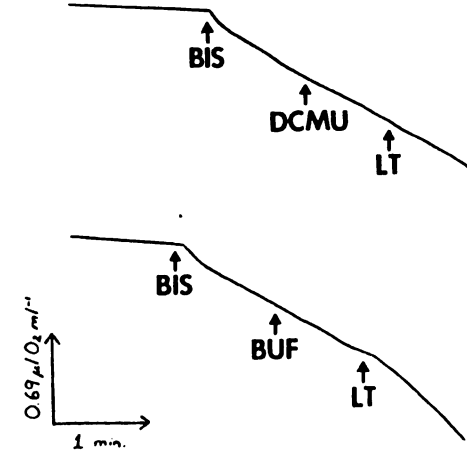
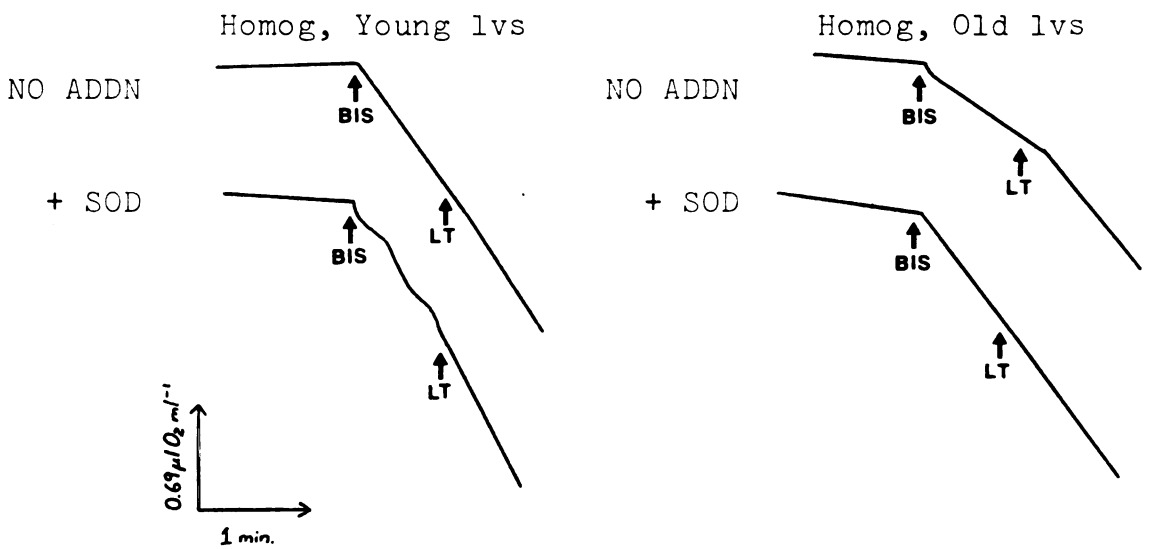


Table 11. Effect of superoxide dismutase on bisulfite oxidation activity of leaf homogenates from young and old leaves.

| Sample | | Bisulfite oxidation ($\mu l\ O_2\ min^{-1}\ ml^{-1}$) | | |
|---|---|---|---|---|
| | | No addn. | +BSA | +SOD |
| Homog., Young lv. | | | | |
| | Dark | 1.06 | 0.35 | 1.03 |
| | Lt-dep | 0.09 | 0.06 | 0.20 |
| Homog., Old lv. | | | | |
| | Dark | 0.43 | 0.14 | 0.77 |
| | Lt-dep | 0.34 | 0.17 | 0.03 |




Leaf homogenates from young and old leaves were prepared and assayed in buffer containing 0.1 mM EDTA. Samples (4 ml) were equilibrated to 30°C. Powdered superoxide dismutase (SOD) or bovine serum albumin (BSA) was added to a concentration of 1 mg $ml^{-1}$. Oxygen consumption was measured in the continuous presence of the BSA or SOD. Samples were injected with 10 µl 0.4 M $KHSO_3$ to make a final concentration of 1 mM.

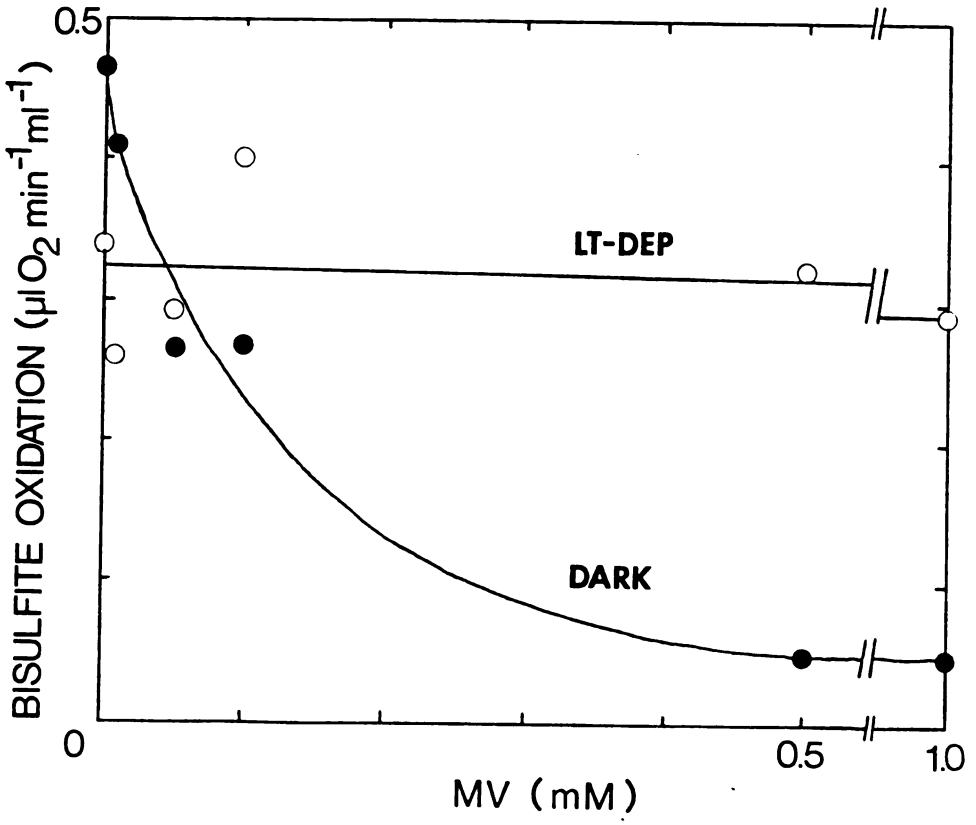


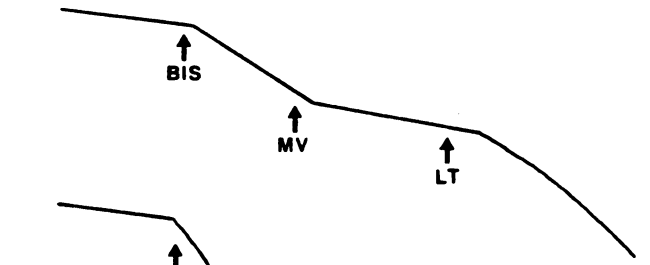

Figure 8. Effect of methyl viologen (paraquat) on bisulfite oxidation by leaf homogenates.

Varying concentrations of methyl viologen (MV) were injected into 4 ml samples of homogenate prepared from old leaves at 30°C during dark and light bisulfite oxidation. Light-dependent (LT-DEP) activity was determined by subtracting activity in the dark from that in the light.

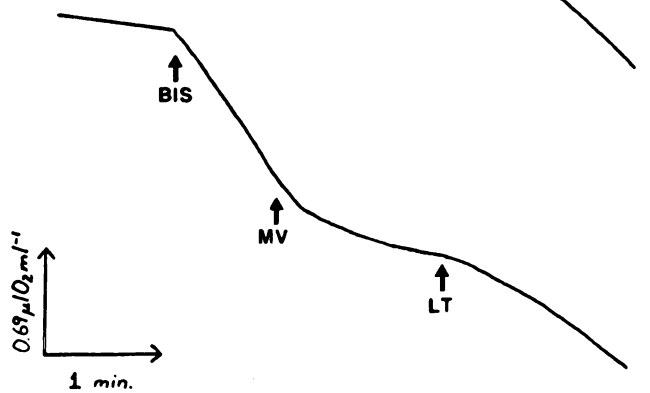
Table 12. Effect of 1 mM methyl viologen (paraquat) on bisulfite oxidation by leaf homogenate and its fractions.

| Sample | | Bisulfite oxidation (µl $O_2$ $min^{-1}$ $ml^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Dark | | | Lt-dep | |
| | | -MV | +MV | % Inhbn | -MV | +MV |
| Homogenate | Young | 1.04 | 0.21 | 80 | 0.07 | 0.22 |
| | Old | 0.39 | 0.14 | 64 | 0.46 | 0.40 |
| S1 | Young | 0.58 | 0.09 | 84 | | |
| | Old | 0.62 | 0.10 | 84 | | |
| P3 | Young | 1.03 | 0.19 | 82 | | |
| | Old | 0.53 | 0.12 | 77 | | |

Homog, Old lvs




Homog, Young lvs




Homogenates and fractions were prepared from young and old leaves and assayed in buffer containing 0.1 mM EDTA. Samples (4 ml) at 30°C were injected with 10 µl 0.4 M $KHSO_3$ (BIS) (to make a final concentration of 1 mM) followed by an injection of 10 µl 400 mM methyl viologen (MV) (to make a final concentration of 1 mM) during the dark and light.

Other Factors

The endogenous activity of superoxide dismutase in young and old leaf extracts (S1) was determined (Table 13). There is slightly more activity in the older leaves.

A preliminary determination of the intrinsic rates of photosynthetic electron transport in young and old leaf homogenates was done by measuring rates of DCPIP, an artificial electron acceptor, reduction. DCPIP reduction was followed by measuring the rate of absorbance change at 600 nm. The results (data not shown) suggest older leaf homogenates have significantly higher rates of photosynthetic electron transport.

Table 13. Superoxide dismutase activity of young and old cucumber leaves.

| Sample | Superoxide dismutase activity | | |
|---|---|---|---|
| | units/ml | units/g fresh wt | units/mg protein |
| Young lvs | 11 | 55 | 11 |
| Old lvs | 11 | 59 | 16 |

Soluble fractions (S1) were prepared from young and old cucumber leaves. Samples were assayed for superoxide dismutase activity. Protein concentration of samples from young and old leaves were 1.0 and 0.7 mg $ml^{-1}$, respectively.

# DISCUSSION

Other studies of bisulfite oxidation in plants have dealt with either the dark activity alone (22, 34) or with the light-dependent activity (1). This study represents the first time both activities have been studied together in one system. The major advantage to this approach is that one can assess, to a degree, the relative magnitudes of these different activities. Futhermore, other studies have investigated the role of bisulfite oxidation in protecting a plant from $SO_2$ injury (22, 25) or in its possible role in causing injury (23, 29). This is, to my knowledge, the first study undertaken with the perspective that both protective and injurious reactions may be taking place simultaneously and it may be the balance between these two types of activities that determines whether bisulfite oxidation overall will be protective or injurious.

The major goals of this research project were 1) to elucidate the manner in which a cucumber leaf oxidizes bisulfite, and 2) to compare this process in young and old cucumber leaves in hopes of obtaining insight into the role that bisulfite oxidation plays regarding $SO_2$ injury. In cucumber leaf homogenates, bisulfite oxidation was found to occur by a dark and a light-dependent process. The dark

activity was separated into a soluble (S1) and a particulate (P3) component by centrifugation (Table 5). The soluble (S1) component was resolved to a single, heat-sensitive activity peak on a G-200 column (Figure 6). This activity is different from enzymatic systems previously described (15, 34) in that there does not appear to be a cofactor requirement. The particulate (P3) dark activity was also found to be heat-sensitive and had no apparent cofactor requirement. Most of this activity could be stripped from the pellet by treatment with the mild detergent, Triton X-100 (Table 9).

The light-dependent activity of leaf homogenates requires both a soluble and particulate component (Table 6) and is strongly inhibited by DCMU (Figure 7 and Table 10) and superoxide dismutase (Table 11). This suggests the activity is through photosynthetic electron transport production of superoxide anion radicals; these then initiate non-enzymatic bisulfite oxidation. This appears to be the same activity as that described by Asada and Kiso (1) using isolated chloroplasts.

When bisulfite-oxidizing activities of homogenates from young leaves were compared to those from old leaves, the outstanding difference was found to be that there was considerably more light-dependent activity in the leaf homogenates from the old leaves. This was supported by the results from seven different comparisons (Table 4) and from an experiment comparing a gradient of leaf ages

(Figure 4). The younger leaves appeared to contain more of the dark bisulfite oxidation activity.

Since superoxide dismutase inhibits the light-dependent activity (Table 11), one explanation for the absence of this activity in young leaf homogenates could be that they contain more superoxide dismutase than old leaf homogenates. However, when soluble superoxide dismutase activities of young and old homogenates were determined, they were found to be nearly equal (Table 13). Another explanation could be that the old leaf homogenates produce more of the initiating superoxide anions. This may be the case, as the old leaf homogenates were preliminarily found to have a higher rate of photosynthetic electron transport as measured by DCPIP reduction.

The different means of oxidizing bisulfite in young and old leaves may relate to their relative susceptibilities to $SO_2$ injury. The light-dependent activity described here appears to be the same as that linked to chlorophyll destruction (29) and lipid peroxidation (23), causing injury by producing free radicals during non-enzymatic bisulfite oxidation (Table 1). The old leaves, which are more susceptible to $SO_2$ injury, contain significantly more of this activity as a homogenate than the less susceptible young leaves. The degree of light-dependent activity could therefore provide an explanation for the differences observed in $SO_2$ susceptibility between leaves of different ages. Winner and Mooney (41) predicted that a plant with a

higher photosynthetic capacity will be more sensitive to $SO_2$. This is interesting in light of the greater degree of photosynthetic electron transport observed in homogenates from old cucumber leaves. In ultrastructural studies, the very first manifestation of injury in $SO_2$-fumigated leaves is a swelling of the thylakoids in the choroplast (45). It is interesting that this is where potentially injurious free radicals produced during photosynthetic electron transport-initiated bisulfite oxidation would be expected to be in greatest abundance. It may be that the light-dependent bisulfite oxidation activity described here is the primary manner by which $SO_2$ exerts its toxicity in plants.

The dark bisulfite oxidation activity described here can be attributed, in part, to an enzyme. An enzymatic system could stimulate bisulfite oxidation through either direct or indirect means (Table 2). The indirect manner possible is through the enzymatic production of the initiating superoxide anion (e.g. xanthine oxidase). If this were occurring, it too could cause injury through free radical production from the non-enzymatic chain mechanism. There is no evidence indicating that this is what is happening in cucumber homogenates. If it were, additional substrates or cofactors would be expected to be required for activity. The enzyme studied in this system does not have this requirement. Furthermore, superoxide dismutase would be expected to inhibit the dark activity if it were occuring by this process. As shown in Table 11, superoxide dismutase did not inhibit

the dark activity at a concentration that completely inhibits the light-dependent activity. This dark oxidation, then, appears to be due to direct oxidation of bisulfite by an enzyme. In the case of the sulfite oxidase characterized from bovine liver, the oxidase activity was shown to involve a two-electron reduction of oxygen to hydrogen peroxide without the formation of any detectable free radicals (7). If this is the case in the cucumber system, then this represents a way to get rid of the potentially toxic bisulfite ion without causing free radical injury. It could then be a biochemical means of resisting $SO_2$ injury. It may be significant that young leaves (resistant to $SO_2$) contain higher amounts of this potentially protective dark activity than the susceptible older leaves.

There appears to be two major bisulfite oxidation activities in cucumber leaves, one that is potentially injurious and another that is potentially protective. The older leaves contain more of the injurious (light-dependent) activity and the young leaves contain more of the protective (dark) activity. As shown in Figure 4, the distribution of these two activities changes with leaf age even though the total amount of oxidation in the light remains about the same. Therefore, the critical factor in assessing the role of bisulfite oxidation in plants may not be the magnitude of the oxidation activity, but how the bisulfite is oxidized.

This light-dependent oxidation of bisulfite would not be injurious if the plant leaf had the means to scavenge all

the extra free radicals produced. Since superoxide dismutase is an important means of scavenging superoxide anions (3), its activity may be crucial to determining if injury will occur. The amount of activity of this enzyme has been correlated with resistance to $SO_2$ injury (3, 36). This does not explain the difference in sensitivities of young and old cucumber leaves, however, as they contain similar levels of superoxide dismutase activity (Table 13).

This study on bisulfite oxidation and its possible role in $SO_2$ injury was done on one plant variety using young and old homogenates. To extend this study, several different plants should be tested for bisulfite oxidation activities. Also, this study should be extended to an *in vivo* system. The use of isolated chloroplasts would be an obvious next system due to their ease in assaying with an oxygen electrode and because the light-dependent activity should be expressed. Finally, further characterization of the soluble and particulate dark activity is necessary.

This research suggests three features of a plant leaf that could act to minimize the toxic effects of $SO_2$: 1) enzymatic oxidation of the toxic bisulfite ion to sulfate; 2) a low endogenous rate of superoxide anion production to decrease the possibility of initiating the injurious non-enzymatic bisulfite oxidation; and 3) possession of sufficient free radical scavenging ability (*e.g.* superoxide dismutase) to protect the cell from free radical injury. How these factors relate to $SO_2$ toxicity may vary for

different plants; in the young and old cucumber leaves, the first and second seem to play the important role.

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