Effects of the Air Pollutant SO$_2$ on Leaves[1]

Inhibition of Sulfite Oxidation in the Apoplast by Ascorbate and of Apoplastic Peroxidase by Sulfite

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ABSTRACT

After SO$_2$ has entered leaves of spinach (*Spinacia oleracea*) through open stomata and been hydrated in the aqueous phase of cell walls, the sulfite formed can be oxidized to sulfate by an apoplastic peroxidase that is normally involved in phenol oxidation. The oxidation of sulfite is competitive with the oxidation of phenolics. During sulfite oxidation, the peroxidase is inhibited. In the absence of ascorbate, which is a normal constituent of the aqueous phase of the apoplast, peroxidative sulfite oxidation facilitates fast additional sulfite oxidation by a radical chain reaction. By scavenging radicals, ascorbate inhibits chain initiation and sulfite oxidation. Even after exposure of leaves to high concentrations of SO$_2$, which inhibited photosynthesis, the redox state of ascorbate remained almost unaltered in the apoplastic space of the leaves. It is concluded that the oxidative detoxification of SO$_2$ in the apoplast outside the cells is slow. Its rate depends on the rate of apoplastic hydrogen peroxide generation and on the steady-state apoplastic concentrations of phenolics and sulfite. The affinity of the peroxidase for phenolics is higher than that for sulfite.

The widespread use of coal and other fossil fuels for the generation of electric power and other energy sources in industrialized countries has led and still leads to the emission of large quantities of sulfur dioxide into the atmosphere. In dry air, this potentially acidic gas enters the leaves of plants through the stomata. It dissolves inside the leaves in the aqueous phase of cell walls (apoplastic space) and may then diffuse across the biomembrane barrier of the plasmalemma into the cytosol of leaf cells. It is hydrated to form divalent sulfurous acid (pK values about 1.8 and 7), which, depending on the pH of the apoplast or the cytosol, is neutralized, yielding the anions bisulfite and sulfite.[4] These anions are cytotoxic, owing to their reactivity. Detoxification can be achieved by oxidation to sulfate or by reduction to the sulfide level and incorporation of the reduced sulfur into organic compounds such as cysteine. It is not known to what extent oxidative detoxification can take place already outside of the cells in the apoplast before cytotoxic reactions in the cytoplasm that compete with detoxifying reactions produce cellular damage. However, it is known that apoplastic peroxidases, which normally use phenolics as oxidizable substrates, can oxidize sulfite to sulfate as long as hydrogen peroxide is available as a reducible substrate (11).

Peroxidases occur in several cellular compartments. In chloroplasts, the function of a peroxidase is to scavenge the hydrogen peroxide that is formed in the light during oxygen reduction in the Mehler reaction (2). This peroxidase uses AA[5] as an electron donor. Its rapid inactivation in the absence of AA can be used to distinguish it from other peroxidases (2) that are localized in the large central vacuole of plant cells (3, 9, 15, 16) and outside the cells in the apoplast (4, 5, 10, 13, 18). The apoplastic peroxidases are involved in the formation of cross-linkings within cell wall constituents. Some of them are bound to the cell wall, whereas others are soluble (7). These enzymes may also scavenge hydrogen peroxide in a way similar to that employed by vacuolar peroxidases, which first oxidize phenolics (14). The oxidation products are then reduced by AA, which acts as secondary electron donor. If this view is correct, cross-linking reactions can proceed only in the presence of very low ascorbate levels in the apoplast.

In the present communication, we analyze the role that peroxidases play in the apoplastic detoxification of sulfite and bisulfite. We show that these reactive anions compete with phenolics as substrates of soluble apoplastic peroxidase. Formation of sulfite radicals during oxidation accounts for additional inhibition of phenolic peroxidase. Rapid sulfite oxidation in a radical chain reaction, which would give rise to uncontrolled oxidative reactions in apoplast and plasmalemma, occurs only after apoplastic AA has been fully oxidized. Not much oxidation was observed in fumigation experiments with leaves, although SO$_2$ concentrations sufficient to inhibit photosynthesis were employed. We conclude that AA prevents uncontrolled radical chain reactions in the apoplastic space.

MATERIALS AND METHODS

Spinach (*Spinacea oleracea*) was grown in a greenhouse under 10-h day and 14-h night cycles. Intercellular washing

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[4] When the term sulfite is used in the context of sulfite additions to reaction mixtures, it should be noted that it describes the sum of both sulfite and bisulfite because, depending on pH, more or less bisulfite is formed in the solutions by protonation of sulfite.
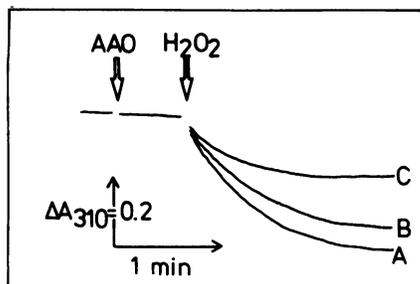
[5] Abbreviations: AA, ascorbate; AAO, ascorbate oxidase; IWF, intercellular washing fluid.

Figure 1. Effects of sulfite on the time course of hydrogen peroxide-dependent oxidation of ferulic acid. The reaction mixture (1.0 mL) contained 0.1 mL of IWF, 40 μM ferulic acid, and 90 mM Na-phosphate (pH 6.8). AAO (1 unit) was added to remove AA before the addition of 0.5 mM hydrogen peroxide. Trace A, No sulfite added; trace B, 0.5 mM sulfite present; trace C, 1 mM sulfite present.

fluid (IWF) was prepared by infiltrating leaves with 100 mM KCl and centrifuging them at 160g for 5 min at 4°C; 0.3 to 0.4 mL of IWF were usually obtained from 1 g of leaves. The pH of IWF was close to 6.5. IWF was kept on ice until used.

Hydrogen peroxide-dependent oxidation of ferulic acid was measured spectrophotometrically at 310 nm in a reaction mixture (total volume 1 mL) that contained ferulic acid, as indicated in the legends to the figures, 0.5 mM hydrogen peroxide, and 90 mM sodium phosphate, pH 6.8. Hydrogen peroxide-dependent oxidation of AA (0.04 mM) by IWF (0.1 mL) in the presence or absence of added phenolics was followed at 265 nm in the same reaction medium. Sulfite was added, as shown in the legends to the figures.

Hydrogen peroxide-dependent changes of pH were recorded by a pH electrode. The reaction mixture (1 mL) contained undiluted IWF or IWF diluted with 100 mM KCl and additions, as indicated in the legends of the figures. When necessary, oxidation of AA in IWF was measured simultaneously in the same medium.

Fumigation of spinach leaves with SO_2 was performed in a sandwich-type cuvette. The gas flow rate was 30 L h^{-1}. SO_2 concentrations in air were adjusted by Tylan flow controllers (Tylan, Eching, Germany). CO_2 and water exchange was measured by infrared gas analysis using a Binos analyzer (Haereus, Hanau, Germany). Calculated transpiration rates

permitted calculation of SO_2 uptake under the assumption that the intercellular concentration of SO_2 is close to zero. Illumination was provided by glass fiber optics. IWF was obtained and levels of reduced and oxidized AA were determined in IWF spectrophotometrically in the 265-nm region using AAO and DTT, respectively, as reported previously (17).

RESULTS

Effects of Sulfite on Oxidation of Ferulic Acid in IWF by Hydrogen Peroxide

Figure 1 shows time courses of the oxidation of ferulic acid by the peroxidase of IWF in the absence and presence of sulfite. To eliminate AA from the reaction mixture, AAO (1 unit) was added before the addition of hydrogen peroxide. AA rapidly rereduces phenolics that had been oxidized by peroxidases (14), obscuring the oxidation reaction. The K_m value for ferulic acid in the presence of 0.5 mM hydrogen peroxide and in the absence of sulfite was about 45 μM, and that for hydrogen peroxide in the presence of 60 μM ferulic acid was about 100 μM. Lower affinities have been reported for apoplastic peroxidase from needles of Norway spruce (12). As shown in Figure 1, sulfite inhibited both the initial rate and the final extent of oxidation of ferulic acid. Inhibition by sulfite was competitive with respect to ferulic acid and noncompetitive with respect to hydrogen peroxide (Fig. 2).

During the measurements, the rate of oxidation of ferulic acid decreased in the absence and presence of sulfite. The slowing down of the rate in the absence of sulfite was due to depletion of the electron donor because the concentration of the substrate used was lower than the K_m value. The reaction recovered when more ferulic acid was added (data not shown). However, in the presence of sulfite, no recovery was observed, suggesting that inhibition of the peroxidase reaction by sulfite is not due only to the competitive action of sulfite, but also has another cause.

In the presence of both ferulic acid and AA in the reaction mixture, no oxidation of ferulic acid was observed. Instead, AA was oxidized. The rate of its oxidation was nearly constant during incubation (Fig. 3). Sulfite inhibited the oxidation of AA. The extent of inhibition increased with incubation time. Initially, it was smaller in the presence of both AA and

Figure 2. Inhibition of IWF-dependent ferulic acid oxidation by sulfite. Left panel: Lineweaver-Burk plot of ferulic acid (FA) oxidation as a function of concentration of ferulic acid. The reaction mixture (1 mL) contained 0.1 mL of IWF, 0.5 mM hydrogen peroxide, 1 unit of AAO, ferulic acid, and 90 mM Na-phosphate (pH 6.8). Right panel: Lineweaver-Burk plot of ferulic acid oxidation as a function of hydrogen peroxide concentration. The reaction mixture contained 0.1 mL of IWF, 60 μM ferulic acid, 1 unit of AAO, hydrogen peroxide, and 90 mM Na-phosphate (pH 6.8). O, In the absence of sodium sulfite; ●, in the presence of 0.5 mM sodium sulfite.
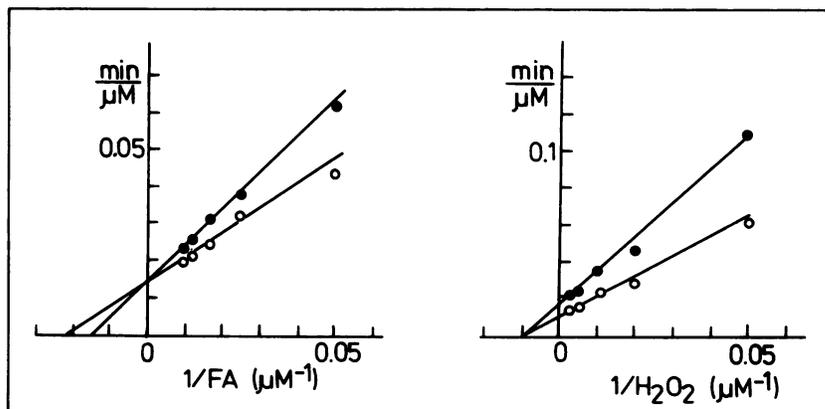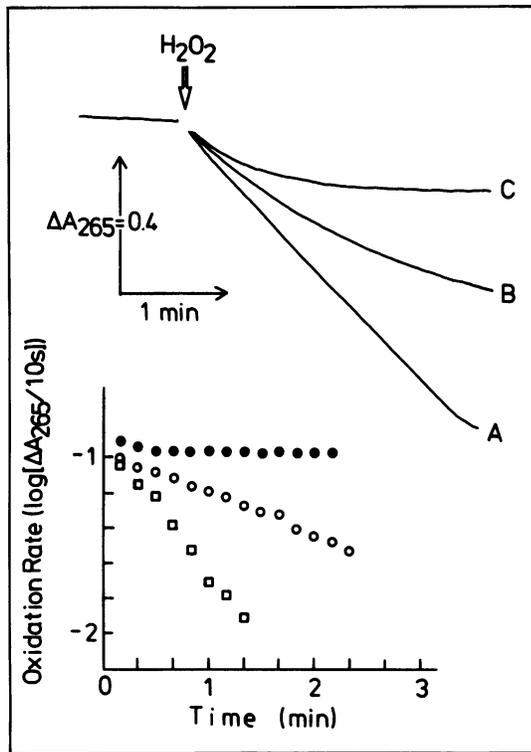
Figure 3. Effect of sulfite on hydrogen peroxide-dependent oxidation of AA. The reaction mixture (1 mL) contained 0.1 mL of IWF, 20 μM ferulic acid, 40 μM AA, and 90 mM Na-phosphate (pH 6.8). Reactions were started by adding 0.5 mM hydrogen peroxide. Upper panel: trace A, No sulfite added; trace B, plus 0.5 mM sodium sulfite; trace C, plus 1.0 mM sodium sulfite. Lower panel: Semilogarithmic plots of oxidation rates as a function of reaction period. ●, No addition; ○, 0.5 mM sodium sulfite; □, 1.0 mM sodium sulfite.

ferulic acid than in the presence of ferulic acid only. The K_i value for sulfite was about 1 mM in the presence of ferulic acid only. It was increased in the presence of both AA and ferulic acid.

By plotting the reaction rate semilogarithmically, it can be seen that inhibition by sulfite follows first-order kinetics. After sulfite had inhibited the AA oxidation by IWF in the absence of ferulic acid, AA in the reaction medium was removed by AAO. Ferulic acid now added was no longer oxidized. Apparently, the peroxidase contained in the IWF had become inactive. There are two possibilities to explain inactivation: (a) sulfite might act by itself on the enzyme; or (b) competitive inhibition of ferulic acid oxidation by sulfite suggests that sulfite can act as a substrate of the enzyme donating electrons to hydrogen peroxide. A product of electron donation is the reactive sulfite radical. The radical or radicals generated from it may react with the enzyme causing inactivation. The first possibility is ruled out by the observation that incubation of IWF with 1 mM sulfite for 5 min in the absence of hydrogen peroxide did not cause any change in peroxidase activity. This leaves the second possibility. We conclude that sulfite not only inhibits the oxidation of phenolics by acting as a competitive substrate for the peroxidase, but also by direct inactivation of the enzyme as a consequence

of the reaction between enzyme and radicals formed accompanying sulfite oxidation.

Relation of AA Oxidation to Sulfite Oxidation

Figure 4 shows time courses of absorbance at 265 nm by IWF and simultaneous pH changes. The addition of 0.3 mM Na₂SO₃ to IWF did not cause any absorption changes at 265 nm. In fact, the absorption spectrum of IWF was not affected by sulfite (data not shown). The addition of 20 μM hydrogen peroxide decreased absorption at 265 nm due to oxidation of the AA that is contained in IWF (12, 17). A further addition of hydrogen peroxide caused no further absorption decrease because all AA had been oxidized.

When sodium sulfite was added to IWF, the pH of the only slightly buffered reaction mixture increased as a result of bisulfite formation according to $SO_3^{2-} + H_2O \rightarrow HSO_3^- + OH^-$. The addition of hydrogen peroxide caused a slow and slight further increase of pH, which is attributed to the oxidation of AA. After most of the AA was oxidized, a decrease of pH was observed. The H⁺-producing reaction proceeded in a sigmoidal fashion. A further addition of hydrogen peroxide resulted in a further decrease of pH, but the reaction was now slower. It reestablished the original pH. The complete reversibility of the pH change suggests that sulfite is completely oxidized to sulfate according to the two reactions $SO_3^{2-} + \frac{1}{2} O_2 \rightarrow SO_4^{2-}$ (no proton production) and $HSO_3^- + \frac{1}{2} O_2 \rightarrow SO_4^{2-} + H^+$ (proton production).

It is important to note that no sulfite oxidation was indicated by H⁺ production as long as endogenous ascorbate was present in the IWF. No phenolics had been added to the IWF, although a low level of endogenous phenolics is likely to have been present. Only after available AA had been largely oxidized by the low concentration of added hydrogen peroxide was sulfite first rapidly and then more slowly oxidized to sulfate.
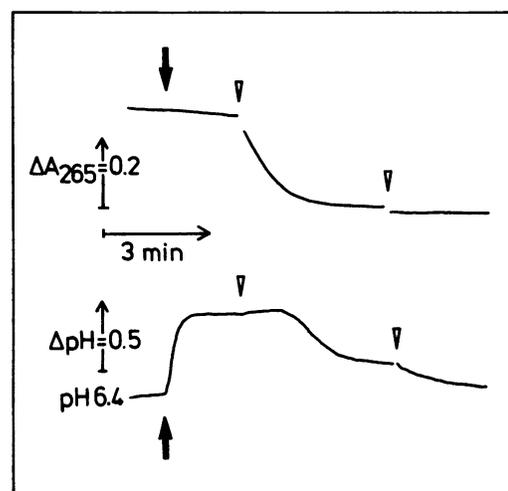

Figure 4. Hydrogen peroxide-dependent oxidation of AA and pH changes of IWF. The reaction mixture (1.0 mL) contained 0.5 mL of IWF in 100 mM KCl. Upper trace, absorption changes at 265 nm; lower trace, pH changes of IWF. First, 0.3 mM sodium sulfite (black arrows) and then two additions of 20 μM hydrogen peroxide (white downward arrows) were added.

Figure 5. The relationship between oxidation of AA and oxidation of sulfite as indicated by acidification. The reaction mixture (1.0 mL) contained 0.5 mL of IWF in 100 mM KCl. Downward arrows indicate the addition of 5 μM hydrogen peroxide. Upward arrows indicate the addition of 0.3 mM sodium sulfite (black upward arrows) or 20 μM sodium ascorbate (white upward arrows). Upper trace: 265-nm absorption change, no sulfite addition; middle trace, same, but sulfite added twice; lower trace, pH, sulfite added twice.
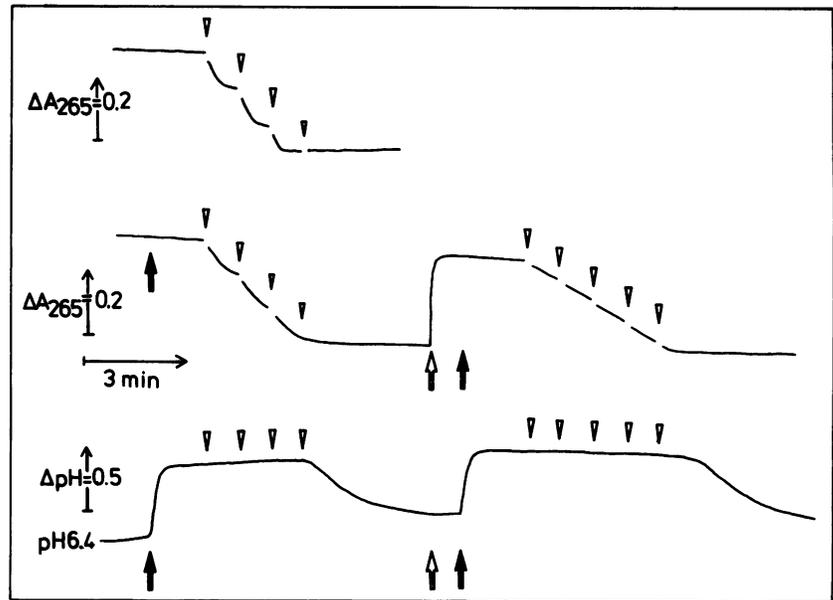

Figure 5 analyzes in more detail the relationship between ascorbate oxidation, as shown by the decrease in 265-nm absorption, and sulfite oxidation, as shown by acidification. The addition of small amounts of hydrogen peroxide (5 μM) caused only small absorption changes at 265 nm and very slight alkalization. In the presence of sulfite, initial rates of the absorption decrease slowed down as a function of the number of titrations. No such decrease in rate was seen in the absence of sulfite. Oxidation of sulfite was indicated by proton production only after AA had been oxidized. The addition of sodium ascorbate (20 μM) after its oxidation to reestablish a level of AA similar to the initial level did not cause a pH change in the reaction mixture. The addition of sodium sulfite after the addition of AA did not change absorbance, but increased pH. The second titration by hydrogen peroxide also caused absorption decreases at 265 nm, but the rates of AA oxidation were slower than those observed during the first titration sequence. The slow rates of AA oxidation did not decrease further during the second titration sequence, as they had done during the first sequence. Obviously, endogenous phenolics had largely become oxidized in the course of the experiment in a form that does not permit fast reduction by AA. In both titration sequences, acidification of the medium was observed only after AA had been oxidized, but the rate of acidification after the second titration was slower than that after the first titration. This may be due to both the inactivation of peroxidase and the oxidation of phenolics. Phenolics can stimulate peroxidase-dependent oxidation of sulfite (11).

After IWF had been dialyzed against 100 mM KCl to remove or dilute low molecular weight solutes such as AA or phenolics, hydrogen peroxide added after sodium sulfite caused immediate acidification (Fig. 6, upper trace). AA added before sulfite to the dialysate inhibited the acidification induced by hydrogen peroxide until all available AA was oxidized (Fig. 6, lower trace). Ferulic acid (14 μM) failed to inhibit the acidification (data not shown).

When ascorbate present in undialyzed IWF was oxidized by an excess of hydrogen peroxide before the addition of sodium sulfite, some alkalization was observed (Fig. 7, lower trace). Sodium sulfite increased the pH further. In this case, however, the pH did not stabilize as it did in the control experiment (Fig. 7, upper trace), but decreased again, indicating immediate oxidation of sulfite to sulfate. In the control, the pH decreased only after available AA had been oxidized.

In another experiment, in which the endogenous AA of IWF had been destroyed by AAO, hydrogen peroxide added
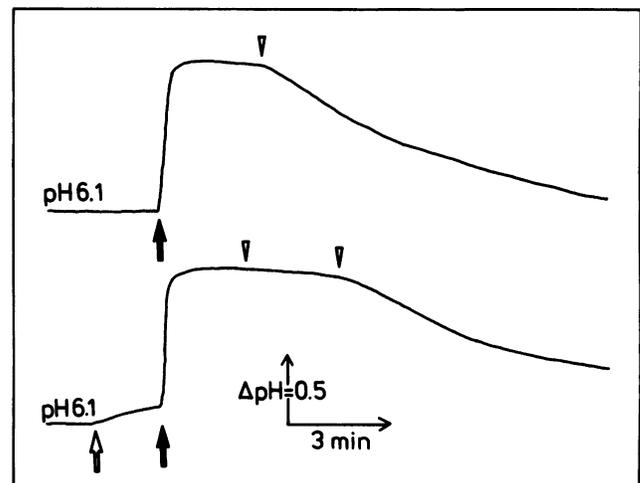

Figure 6. Effect of dialysis of IWF on the time course of acidification by hydrogen peroxide. IWF (2.5 mL) was dialyzed against 200 mL of 100 mM KCl overnight at 4°C. Upper trace: sodium sulfite (0.43 mM) and hydrogen peroxide were added successively. Lower trace: same as the upper trace, but sodium ascorbate (14 μM) was added before the addition of sodium sulfite and hydrogen peroxide. Two additions of hydrogen peroxide (14 μM) were required in this case to initiate acidification.
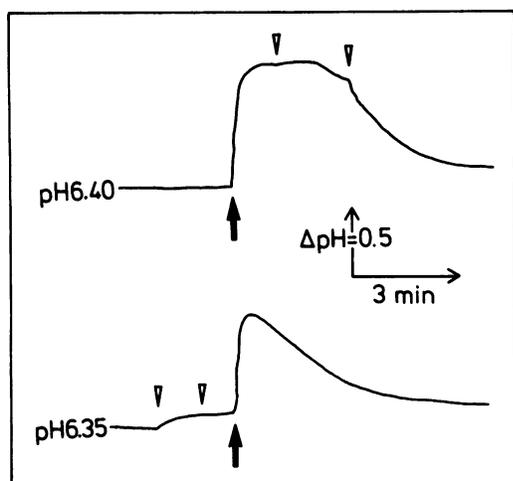
Figure 7. Effects of sequence of addition of hydrogen peroxide and sodium sulfite on kinetics of the oxidation of sulfite, as indicated by acidification. IWF was used without dilution. Upper trace: sodium sulfite was added before two additions of hydrogen peroxide. Lower trace: sodium sulfite (1 mM) was added after two additions of hydrogen peroxide.

after sodium sulfite immediately caused acidification. The observations were similar to those shown in Figure 7 (lower trace).

Level of AA in the Apoplastic Space of Leaves after Fumigation with SO₂

When illuminated leaves of spinach were fumigated with a high concentration of SO₂ (10 μL SO₂ L^{-1} air) for periods as short as 6 or 15 min, photosynthesis became partially inhibited, indicating that SO₂ and its hydration products entered the mesophyll cells and acted on the photosynthetic apparatus (Table I). The first effects of SO₂ on photosynthetic

reactions were observed after less than 1 min of fumigation (data not shown). The extent of photosynthesis inhibition increased with SO₂ concentration and time of exposure. As long as damage was not extensive, inhibition was slowly reversed after the fumigation was terminated, revealing capabilities for detoxification and repair. For this reason, fumigation experiments with SO₂ were limited to 6 or 15 min, and IWF was rapidly prepared from the leaves. It contained levels of AA comparable to levels in the IWF of control leaves. Also, ratios of reduced to total ascorbate were similar in IWF from control leaves and from leaves fumigated for 6 min. After 15 min of fumigation, when photosynthesis was inhibited by more than 60%, oxidation of AA appeared only somewhat increased.

DISCUSSION

The oxidation of phenolics by apoplastic peroxidase is inhibited by sulfite both as a result of competition between ferulic acid and sulfite for donation of electrons to the peroxidase (Fig. 2) and by the direct action of sulfite radicals on the peroxidase (Figs. 3 and 5). When ferulic acid is an electron donor, ferulic acid radicals may be formed. This is indicated by the increase in the level of monodehydroascorbate when ferulic acid is present together with ascorbate in a peroxidase/hydrogen peroxide system (17). The ferulic acid radical can also oxidize sulfite to the sulfite radical. Another source of sulfite radicals is the peroxidase-mediated electron abstraction from sulfite during H₂O₂ reduction. The inability of AA to prevent inactivation of the peroxidase suggests that the inactivating reaction occurs very close to the site where the radicals are generated. Phenolics seem to have a function in the inactivation reaction, because peroxidase activity in leaf extracts of spinach was not inactivated by sulfite plus hydrogen peroxide when ascorbate was present but phenolics were absent (data not shown). Radical-dependent inactivation of peroxidase has been reported for the AA peroxidase of chloroplasts by Cheng and Asada (6). Other possibilities to ex-

Table I. *Effect of SO₂ on Photosynthesis and on the Redox State of Ascorbate in the Apoplastic Space of Spinach Leaves*

The leaves were freshly cut and CO₂ assimilation was measured before and during fumigation with 10 μL L^{-1} SO₂. The photon flux density was 1000 μmol m^{-2} s^{-1}.

	CO₂ Assimilation before Fumigation	CO₂ Assimilation after Fumigation	SO₂ Absorbed	Inhibition of Assimilation by SO₂	Total Ascorbate Reduced
	nmol cm^{-2} s^{-1}		*nmol cm^{-2}*		%
Controls					
Experiment 1	0.99				76.7
Experiment 2	0.96				80.3
Experiment 3	1.54				77.4
6-min SO₂					
Experiment 1	1.67	0.91	30.3	45.5	82.4
Experiment 2	1.68	0.92	26.1	45.2	87.5
Experiment 3	1.34	0.82	23.6	38.8	91.6
Experiment 4	1.20	0.72	23.6	40	86.7
15-min SO₂					
Experiment 1		0.39	48.3		71
Experiment 2		0.51	45.3		68

plain inhibition have been considered by Acosta et al. (1) for horseradish peroxidase.

The data in Figures 4 to 7 show that as long as AA is present in IWF, acidification, which indicates the oxidation of sulfite to sulfate, does not occur. Rather, a slight alkalization is observed in the presence of AA. Oxidation of AA liberates hydroxyl ions. However, the failure to observe acidification in the presence of AA cannot be interpreted to indicate the absence of any sulfate formation from sulfite. The observed pH changes obviously depend on the ratio of the oxidation of AA to the oxidation of sulfite. At pH values close to 7, the oxidation of sulfite liberates only about 0.5 H^+ per transferred electron, whereas the ratio for ascorbate is not far from 1 OH^- per electron.

The acidification of IWF seen after the full oxidation of AA was triggered by only small amounts of hydrogen peroxide (Figs. 4–7). For example, 14 μmol of hydrogen peroxide caused the oxidation of 430 μmol of sulfite. This means that oxidation of sulfite proceeds in the absence of ascorbate by a radical chain reaction. AA inhibits chain initiation by scavenging phenoxy radicals, sulfite radicals, and superoxide radicals, which can be formed by the reaction of sulfite radicals with oxygen. AA is well known to be an effective radical scavenger. Thus, AA has an important function in controlling the rate of the oxidation of sulfite to sulfate in the apoplast and in preventing uncontrolled radical reactions.

In spinach leaves fumigated with SO_2, no appreciable changes in the apoplastic redox state of AA were observed. Levels of AA did not appear to be reduced even after exposure of leaves to concentrations of SO_2 that caused photosynthesis inhibition. This shows that fast oxidation of SO_2 to sulfate by a radical chain reaction cannot occur in the apoplastic space of leaves. Rather, sulfite oxidation is slow. Its rate will be limited by the apoplastic production of hydrogen peroxide, which then oxidizes either phenolics or sulfite. Which substrate is preferred depends on the relative affinities of the enzyme for phenolics and sulfite and the availability of both substrates in relation to one another. In this respect, it is important to note that K_m values for phenolics such as ferulic acid are in the micromolar range, whereas K_i values for sulfite are in the millimolar range. Appreciable sulfite oxidation by apoplastic peroxidase should be expected to lead to inactivation of this enzyme. Indeed, it has been observed that lignification of cell walls in the vicinity of guard cells, which are exposed to much higher SO_2 concentrations than mesophyll cells, has been inhibited when plants were exposed to air polluted with SO_2 (8). Peroxidase is involved in lignin formation.

ACKNOWLEDGMENT

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