Plant Physiol. (1990) 92, 846–849
0032-0889/90/92/0846/04/$01.00/0

Received for publication April 8, 1989
and accepted November 15, 1989

Communication

Oxidation *versus* Reductive Detoxification of SO_2 by Chloroplasts[1]

Rosella Ghisi[2], Andreas P. M. Dittrich, and Ulrich Heber*

Institut für Botanik and Pharmazeutische Biologie der Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg, Federal Republic of Germany

ABSTRACT

Intact chloroplasts isolated from spinach (*Spinacia oleracea* L. cv Yates) both oxidized and reduced added sulfite in the light. Oxidation was fast only when endogenous superoxide dismutase was inhibited by cyanide. It was largely suppressed by scavengers of oxygen radicals. After addition of *O*-acetylserine, chloroplasts reduced sulfite to cysteine and exhibited sulfite-dependent oxygen evolution. Cysteine synthesis from sulfite was faster than from sulfate. The results are discussed in relation to species-specific differences in the phytotoxicity of SO_2.

SO_2 is a gaseous air pollutant that is highly soluble in aqueous phases. It enters leaves through the stomata. After crossing the biomembrane barrier of the plasmalemma it is hydrated. Products of hydration and dissociation are H^+, HSO_3^-, and SO_3^{2-}. The anions are trapped in the cytoplasm of mesophyll cells, which is slightly alkaline, and must be removed to avoid impairment of metabolism. Damage to trees is known to be extensive in regions where air pollution by SO_2 is high. Forest devastation can be seen in wide areas close to the border between Czechoslovakia and the German Democratic Republic. In these regions, grasses such as *Calamagrostis* or *Molinia* may even be luxuriant. Obviously, the capacity of different plant species to cope with SO_2 pollution is different.

Inside the leaves, chloroplasts of the mesophyll are known to be capable of both oxidizing (2) and reducing (15) SO_2 in light-dependent reactions. Oxidation increases acidification, because sulfuric acid, the product of oxidation, is a stronger acid than sulfurous acid, the product of hydration. It also burdens cytoplasm with sulfate anions which, at elevated concentrations, would inhibit photosynthetic reactions (3) if they are not removed. As a matter of fact, sulfate is transported into the vacuoles in an ATP-dependent process (13).

Reduction leads to H_2S and sulfur-containing amino acids, which are finally used for protein synthesis (6). As reduction consumes both sulfite and protons, it may be considered to be a detoxifying reaction. The capacity of chloroplasts for reductive detoxification of SO_2 is ill-defined. In this communication, we briefly consider reduction in relation to oxidation by chloroplasts which were isolated from leaves of spinach (*Spinacia oleracea* L. cv Yates). It will be shown that tolerance of spinach to SO_2 may be explained by the high capacity of this plant for sulfite reduction and its ability to suppress light-dependent sulfite oxidation.

MATERIALS AND METHODS

Plant Material and Chloroplast Isolation

Chloroplasts were isolated from 8 to 12 d old leaves of *Spinacia oleracea* cv Yates that was grown in a greenhouse. Daylight was supplemented by mercury vapor-lamps. The additional intensity was 30 $W \cdot m^{-2}$ and the light period was 13 h/d.

Intact chloroplasts were prepared largely as described by Jensen and Bassham (12). However, the media used were free of phosphate. The percentage of intact chloroplasts in the chloroplast suspensions that had retained their envelopes was measured by the ferricyanide method (9). It ranged between 70 and 90%. After purification in a discontinuous percoll gradient, more than 90% of the chloroplasts had intact envelopes.

Osmotically ruptured chloroplasts were prepared by adding chloroplast suspension to a more than 10-fold volume of 2 mM $MgCl_2$. After 1 min, double-concentrated medium C (minus phosphate, see ref. 12) was added in a ratio of 1:1 (v/v).

Oxygen Exchange

Oxygen exchange was recorded by a Clark-type electrode (Bachofer, Reutlingen, FRG) at 20°C (16). Saturating red light (1000–5000 $\mu E \cdot m^{-2} \cdot s^{-1}$) was used for illumination. The reaction mixture contained 300 mM sorbitol, 1 mM $MgCl_2$, 1 mM $Mncl_2$, 2 mM EDTA, 20 mM $NaHCO_3$, 50 mM Hepes buffer (pH 7.6), and chloroplasts containing 150 or 200 μg Chl in a total volume of 1 mL. Catalase (EC 1.11.1.6) was also added (5200 units $\cdot mL^{-1}$).

[1] This work is part of the research performed within the Sonderforschungsbereich 251 of the University of Würzburg. It has also been supported by the Projektgruppe Bayern zur Erforschung der Wirkung von Umweltschadstoffen.

[2] Present address: Institute of Agricultural and Forest Chemistry, University of Basilicata, Potenza, Italy.

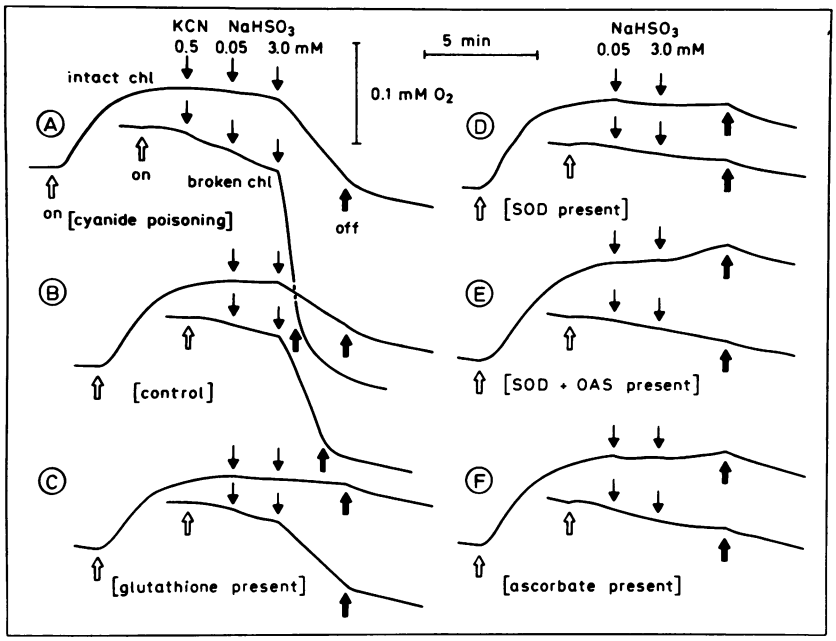

Figure 1. Light-dependent oxygen exchange by osmotically ruptured chloroplasts and by intact chloroplasts. All suspensions contained catalase and 20 mM bicarbonate but no added phosphate. The intact chloroplasts (upper curve in A to F) were preilluminated until photosynthetic oxygen evolution ceased because endogenous phosphate was exhausted. Then 0.05 and 3 mM Na_2SO_3 were added in the light. The broken chloroplasts (lower curve in A–F) were incapable of CO_2 dependent oxygen evolution. A, 0.5 mM KCN added in the light; B, control; C, 2 mM glutathione added in the dark; D, SOD added in the dark (100 units·mL^{-1}suspension); E, SOD and 5 mM OAS added in the dark; F, 10 mM ascorbate added in the dark. Upward directed arrows: light on and off. For further explanations see text.

Cysteine Determination

The reaction mixture was the same as above except for $NaHCO_3$ which was 2 mM. OAS[3] was added to a final concentration of 10 mM and $NaHSO_3$ or Na_2SO_4 as indicated in relevant figures. Usually the reaction was conducted at 20°C for 30 min. The intensity of red light was 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$. Cysteine was determined in the chloroplast suspension with an acid ninhydrin reagent as described by Gaitonde (4), using reagent II. The spectral measurements of reaction products were made against the reagent blank at 560 nm using a spectronic-spectralphotometer (Shimadzu Seisakusho, Kyoto, Japan).

RESULTS AND DISCUSSION

Sulfite-Dependent Oxygen Exchange

Oxidation of sulfite consumes oxygen and can be measured in the oxygen electrode. Figure 1 compares sulfite-dependent oxygen exchange in the light in suspensions of osmotically ruptured and of intact chloroplasts. Although the chloroplasts were isolated in media devoid of phosphate, they still contained some endogenous phosphate. On illumination, the intact chloroplasts reduced CO_2 and evolved oxygen until their supply of endogenous phosphate was exhausted. This caused a decrease in stromal ATP to ADP ratios and an increase of reduced ferredoxin and NADP. After CO_2-dependent oxygen evolution had declined, sulfite was added.

Osmotic rupture of the chloroplast envelope liberated and diluted chloroplast constituents such as enzymes of the Calvin cycle, SOD, ascorbate, and glutathione. Figure 1A shows that the chloroplast thylakoids oxidized sulfite in the light when its concentration was high and when KCN inhibited endogenous superoxide dismutase. Oxidation by intact chloroplasts was slower than that by broken chloroplasts. After prolonged darkening, sulfite-dependent oxygen uptake was insignificant (not shown), although considerable oxidation was observed in the dark immediately after illumination (Fig. 1A). When KCN was absent and catalase present to remove hydrogen peroxide, oxygen uptake in the light was much decreased both in broken and in intact chloroplasts. Once again it was slower in intact chloroplasts (Fig. 1B). It was further reduced when glutathione was added to the system (Fig. 1C). SOD and ascorbate added at high concentrations not only abolished sulfite-dependent oxygen uptake but even caused some net oxygen evolution (Fig. 1D and F). As should be expected, the same was true for combinations of SOD, ascorbate, and glutathione. Interestingly, slow sulfite-dependent oxygen evolution was observed not only with intact chloroplasts but also with thylakoids. When OAS was added to intact chloroplasts in addition to SOD, sulfite-dependent oxygen evolution was faster than in the absence of OAS (Fig. 1E). However, no stimulation of oxygen evolution was observed when OAS was added to a suspension of broken chloroplasts.

Table I shows rates of sulfite-dependent oxygen exchange as calculated from the experiment of Figure 1. Net oxygen evolution of intact chloroplasts was 2 $\mu mol \cdot (mg\ Chl)^{-1} \cdot h^{-1}$ in the presence of OAS and SOD.

The agents effective in suppressing light-dependent sulfite oxidation are scavengers of oxygen radicals which are formed in the light in the Mehler reaction initiating a radical chain reaction in which sulfite is rapidly oxidized to sulfate (1). The ratio of oxygen consumption to sulfite oxidation is 0.5.

The data show that intact chloroplasts are capable of dealing effectively with oxygen radicals, even when ascorbate or glutathione are not added. Addition of SOD which does not enter intact chloroplasts was sufficient to abolish sulfite dependent oxygen uptake. This suggests that much of the oxygen

[3] Abbreviations: OAS, *O*-acetylserine; SOD, superoxide dismutase (EC 1.15.1.1.).

Table I. *Sulfite-Dependent Oxygen Uptake or Evolution by Intact and Broken Chloroplasts in the Light as Affected by Different Additives*

Rates are based on the change in the oxygen trace on addition of 2 mM sodium sulfite in the light. They are given in μmol·(mg Chl)$^{-1}$·h^{-1}. Net oxygen uptake is indicated by (−), net oxygen evolution by (+). Data are from the experiment shown in Figure 1.

Additive	Chloroplasts	
	Broken	Intact
(a) Control	(−) 19.1	(−) 5.6
(b) KCN, 0.5 mM	(−) 83.3	(−) 9.2
(c) Glutathione	(−) 6.5	(−) 0.3
(d) Ascorbate	(+) 1.4	(+) 1.0
(e) SOD	(+) 0.4	(+) 0.8
(f) SOD plus 5 mM OAS	(+) 0	(+) 2.0

uptake observed in these preparations of intact chloroplasts was actually caused by the small percentage of broken chloroplasts contaminating the preparation. Glutathione was much less effective than ascorbate in removing oxygen radicals. *In vivo*, both ascorbate and glutathione are present in chloroplasts together with superoxide dismutase. Under these conditions sulfite oxidation is slow. Its rate will be increased only when reactions competing with oxidation are suppressed so that stromal sulfite concentrations increase.

The slow sulfite-dependent oxygen evolution seen in Figure 1, D, E, and F shows that sulfite reduction takes place concomitantly with oxidation. It becomes visible when oxidation is largely suppressed. Increased oxygen evolution in the presence of OAS by intact, but not by broken, chloroplasts suggests that reduction can lead to the formation of cysteine. OAS provides the carbon backbone for the synthesis of cysteine. In the absence of OAS, sulfite-dependent oxygen evolution is even supported by broken chloroplasts. Sulfite reductase which reduces sulfite to hydrogen sulfide is a thylakoid-bound enzyme (15). Synthesis of cysteine requires a stromal enzyme which is diluted after chloroplasts are ruptured.

Reduction

Figure 2 shows cysteine accumulation in a suspension of intact chloroplasts that was illuminated in the presence of sulfite and OAS. Sulfite reduction was linear for at least 30 min even though intact chloroplasts illuminated in the absence of phosphate are known to suffer extensive photoinhibition (10). Under these conditions the ATP/ADP ratio is low, but ferredoxin is reduced.

Reduction as a function of sulfite concentration is shown in Figure 3. V_{max} of cysteine formation was 3.2 μmol·(mg Chl)$^{-1}$·h^{-1} and an apparent affinity (K_m) of 1.3 mM for sulfite.

Table II shows that cysteine formation from sulfite was enhanced in the presence of oxygen scavengers. It was much faster than cysteine formation from sulfate which, in contrast to sulfite reduction, requires ATP. Sulfate reduction may not involve sulfite reductase (11).

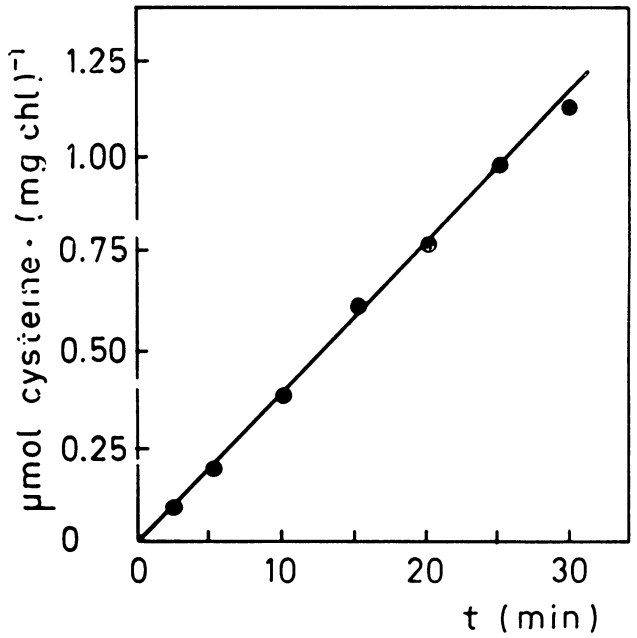

Figure 2. Cysteine accumulation in an illuminated suspension of intact chloroplasts which contained 10 mM *O*-acetylserine and 5 mM Na_2SO_3.

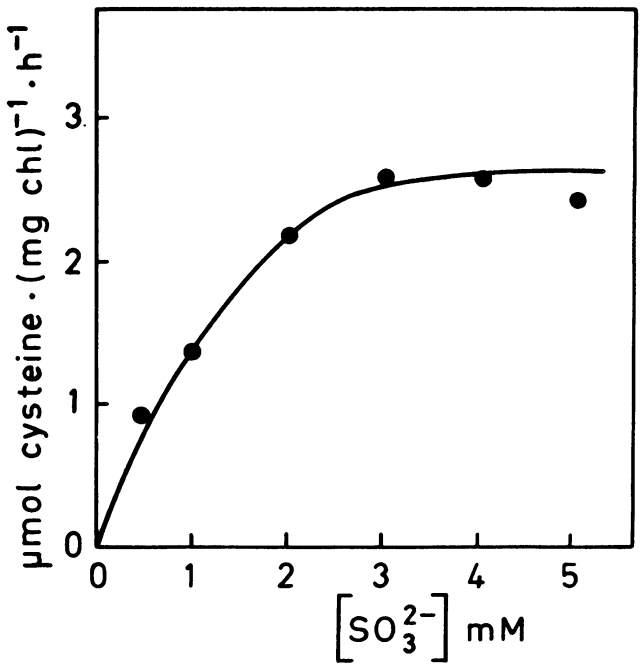

Figure 3. Cysteine formation by a suspension of illuminated chloroplasts as a function of the concentration of sulfite.

CONCLUSIONS

Chloroplasts are the main sites of sulfur metabolism in leaves (15). The purpose of the present work was to investigate whether oxidation or reduction of sulfite formed from SO_2 by hydration predominates under conditions in which light-

Table II. *Cysteine Production from 2 mM Sulfite (a to d) or Sulfate (e) by Illuminated Chloroplasts*

Treatment	Cysteine Production	
	$\mu mol \cdot (mg\ Chl)^{-1} \cdot h^{-1}$	%
(a) Control	1.9	100
(b) SOD	2.4	126
(c) 5 mM Glutathione	2.0	105
(d) 5 mM Ascorbate	2.9	153
(e) Sulfate	0.06	3

dependent formation of oxygen radicals is minimized and availability of the carbon skeleton for cysteine synthesis (OAS) opens the path to the reductive detoxification of sulfite anions. The results of Figure 1 show that light-dependent sulfite oxidation is very slow in intact chloroplasts when ascorbate is available in addition to endogenous SOD. Under these conditions, and if electron pressure is high in the electron transport chain, reduction of sulfite actually outweighs the oxidation to sulfate. The experiments show that chloroplasts of spinach are equipped for the effective synthesis of cysteine from sulfite if OAS is available. However, the affinity of intact chloroplasts for sulfite is not high. Possibly it is determined by the affinity of the phosphate translocator of the inner chloroplast envelope for sulfite, which is known to be capable of slow sulfite transport (7).

The ability of spinach chloroplasts to reduce sulfite to cysteine may help to explain why spinach is not very sensitive to SO_2. However, it must be pointed out that under many environmental conditions, and in many plants, reductive detoxification of SO_2 may be insufficient to prevent damage. In the experiments shown in Figure 1, the chloroplasts were phosphate deficient. In this situation, ferredoxin and NADP were largely reduced. Under normal conditions of photosynthesis, CO_2 competes with sulfite for electrons and ferredoxin and the NADP system is largely oxidized.

Moreover, accumulation of reduced sulfur is not possible *in vivo* as it is in a suspension of isolated chloroplasts (14). Once cysteine is formed *in vivo*, it must be utilized. The main sink for newly formed cysteine is protein synthesis. Plant proteins contain about 1% (w/w) sulfur. Net protein synthesis requires growth. Different plants differ in their growth strategies. Grasses such as *Calamagrostis* or *Molinia* and herbs such as spinach require active protein synthesis for fast growth. Thus, the ratio of nitrogen to carbon in the biomass, and in consequence also of reduced sulfur to carbon, is much higher in spinach than in conifers such as spruce or fir, which specialize in reducing carbon, with a much smaller material and energy investment into protein (8). A reduced requirement for protein synthesis is equivalent to a reduced capacity for the synthesis of sulfur-containing amino acids.

When low temperatures slow down metabolism, the rate of reductive detoxification of SO_2 is also decreased. In spruce, contents of reduced sulfur in needles did not increase with needle age, whereas sulfate levels did (5).

ACKNOWLEDGMENTS

The authors wish to thank Ms. Spidola Neimanis and Ms. Bettina Hilpert for competent assistance and Dr. H. Pfanz for helpful discussions.

LITERATURE CITED

1. **Asada K** (1980) Formation and scavenging of superoxide in chloroplasts, with relation to injury by sulfur dioxide. Res Rep Natl Inst Environ Stud **11:** 165–179
2. **Asada K, Kiso K** (1972) Initiation of aerobic oxidation of sulfite by illuminated spinach chloroplasts. Eur J Biochem **33:** 253–257
3. **Baldry CW, Cockburn W, Walker DA** (1968) Inhibition, by sulphate, of the oxygen evolution associated with photosynthetic carbon assimilation. Biochim Biophys Acta **153:** 476–483
4. **Gaitonde MK** (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J **104:** 627–633
5. **Gasch G, Grünhage L, Jäger H-J, Wentzel K-F** (1988) Das Verhältnis der Schwefelfraktionen in Fichtennadeln als Indikator für Immissionsbelastungen durch Schwefeldioxid. Angew Botanik **62:** 73–84
6. **Giovanelli J, Mudd SH, Datko AH** (1980) Sulfur amino acids in plants. *In* BJ Miflin, ed, The Biochemistry of Plants, 5: Amino Acids and Derivatives. Academic Press, New York, pp 453–505
7. **Hampp R, Ziegler I** (1977) Sulfate and sulfite translocation via the chloroplast translocator of the inner envelope membrane of chloroplasts. Planta **137:** 309–312
8. **Heber U, Laisk A, Pfanz H, Lange OL** (1987) Wann ist SO_2 Nährstoff und wann Schadstoff? Ein Beitrag zum Waldschadensproblem. Allg Forst Zeitschr **42:** 700–705
9. **Heber U, Santarius KA** (1970) Direct and indirect transport of ATP and ADP across the chloroplast envelope. Z Naturforschg **25b:** 718–728
10. **Heber U, Viil J, Niemanis S, Mimura T, Dietz K-J** (1990) Photoinhibitory damage to chloroplasts under phosphate deficiency and alleviation of deficiency and damage by photorespiratory reactions. J Plant Physiol (in press)
11. **Hennies HM** (1975) Die Sulfitreduktase aus *Spinacia oleracea*; ein Ferredoxinabhängiges Enzym. Z Naturforschg **30c:** 359–362
12. **Jensen RG, Bassham JA** (1966) Photosynthesis by isolated chloroplasts. Proc Natl Acad Sci USA **56:** 1095–1101
13. **Kaiser G, Martinoia E, Schröppel-Meier G, Heber U** (1989) Active transport of sulfate into the vacuole of plant cells provides halotolerance and can detoxify SO_2. J Plant Physiol **134:** 756–763
14. **Rennenberg H** (1989) Synthesis and emission of hydrogen sulfide by higher plants. *In* ES Saltzmann, WJ Cooper, eds, Biogenic Sulfur in the Environment, ACS Symposium Ser 393. American Chemical Society, Washington, DC, pp 44–57
15. **Schwenn JD, Depka B, Hennies HH** (1976) Assimilatory sulfate reduction in chloroplasts: evidence for the participation of both stromal and membrane bound enzymes. Plant Cell Physiol **17:** 165–176
16. **Walker D** (1987) The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis. Packard Publishing, Chichester