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Sulfur Dioxide Inhibition of Photosynthesis in Isolated Spinach Chloroplasts

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ABSTRACT

Photosynthetic oxygen evolution by isolated spinach (*Spinacia oleracea* L.) chloroplasts approached complete inhibition in the presence of a 5 mM concentration of sulfur dioxide. A similar inhibition was observed in the presence of equimolar concentrations of bisulfite ions, suggesting a parallel mode of action. In contrast, an equimolar concentration of sulfite ions was markedly less inhibitory and sulfate ions caused negligible inhibition of apparent photosynthesis. The mode of action of sulfur dioxide and related sulfur anions in inhibiting photosynthesis was found to be essentially independent of direct hydrogen-ion effects. Supplements of inorganic pyrophosphate lessened the inhibition of oxygen evolution caused by sulfur dioxide and the sulfur anions.

Sulfur dioxide and the sulfur anions were almost equally effective in inhibiting cyclic and noncyclic photophosphorylation in chloroplast suspensions. However, the extent of the inhibition of these photosynthetic reactions does not appear sufficient to account for the inhibition of photosynthetic oxygen evolution by sulfur dioxide.

Sulfur dioxide is a major atmospheric contaminant resulting primarily from the combustion of sulfur-containing fossil fuels. The phytotoxic behavior of SO_2 has been described in numerous studies during the past few decades (10, 13, 27). While factors influencing the degree of "visible" injury to plants have received much attention, it has become apparent that ambient levels of SO_2 in many areas may affect physiological processes without causing visible injury. For example, reduction in rates of apparent photosynthesis (7, 24) by ambient levels of SO_2 may cause slight reductions in crop yield which may go unnoticed. In recent years, studies have been conducted to determine the effects of SO_2 on physiological processes (10, 19, 29).

Much of the SO_2 absorbed by leaves enters through stomata and dissolves in the moist surfaces of meosphyll cells (27). The resulting sulfurous acid dissociates into H^+, HSO_3^-, and SO_3^{2-}. Sulfate ions result from a free radical chain reaction in the cytoplasm involving HSO_3^- and SO_3^{2-} (21). Therefore, plant cells having absorbed SO_2 will experience an accumulation of HSO_3^-, SO_3^{2-}, SO_4^{2-}. The three sulfur anions are known to uncouple photophosphorylation associated with light-driven electron transport (3, 11, 20). Sij (24) suggested that SO_2 inhibition of apparent photosynthesis may result from this uncoupling effect of the sulfur anions.

The present investigations were conducted to measure the comparative inhibition of photosynthetic O_2 evolution in isolated chloroplasts by SO_2 and the anion solution products HSO_3^-, SO_3^{2-}, and SO_4^{2-}. Similar comparative studies were conducted using chloroplast suspensions active in cyclic and noncyclic photophosphorylation.

MATERIALS AND METHODS

Preparation of Chloroplast Suspensions. Fresh spinach leaves (30–40 g) purchased locally were preconditioned by placing them in a cool water bath in light as described by Cockburn *et al.* (8). Intact chloroplasts suitable for measurement of photosynthetic O_2 evolution were isolated from the preconditioned leaves by methods adapted from earlier workers (9, 15). The isolation medium contained 0.35 M mannitol, 25 mM HEPES buffer, 2 mM EDTA, and 2 mM sodium isoascorbate; the latter was added following adjustment of pH to 7.6 with NaOH. Chlorophyll concentrations were determined according to Arnon (1). Preparation of chloroplasts for measurement of photophosphorylation followed the same procedure, using an isolation medium adapted from Jagendorf *et al.* (14).

Measurement of Photosynthetic O_2 Evolution by Chloroplasts. A Gilson Medical Electronics Oxygraph, Model KM, equipped with a Clark type electrode fitted into a water-jacketed cell, was used as described by Cockburn *et al.* (9). An incident light intensity of 1100 ft-c was provided to the reaction cell by a 150 w reflector flood lamp.

The reaction medium was adapted from Kalberer *et al.* (15), and contained 0.35 M mannitol, 50 mM HEPES buffer, 4 mM sodium pyrophosphate, 10 mM $NaHCO_3$, and 17 μM sodium isoascorbate. The pH was adjusted to 7.6 with NaOH. Following introduction of this medium into the water-jacketed cell, the reaction was initiated by addition of an aliquot of chloroplast suspension representing 300 μg of Chl.

Supplements of HSO_3^-, SO_3^{2-}, SO_4^{2-}, and SO_2 were added within 30 sec following additions of chloroplast suspensions to the reaction media contained in each cell. A 50-μl Hamilton syringe was used to introduce the appropriate volumes of 0.5 M or 1 M solutions of $NaHSO_3$, Na_2SO_3, or Na_2SO_4. Since these additions did not increase the reaction volume by more than 3%, effects of the increased volume were assumed to be negligible.

A 3-ml gas-tight syringe was used to introduce appropriate volumes of a SO_2-air mixture (Union Carbide Corp., Pittsburgh, Pa.) into the chloroplast suspension from a Saran bag (Anspec Co., Ann Arbor, Mich.) equipped with serum cap. Samples of 0.4 ml or less were bubbled slowly through the magnetically stirred suspension within 30 sec following addition of the chloroplasts. A gas sample of like volume was bubbled through a H_2O_2

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solution for use in titrimetric analysis of SO_2 (16). Considering the aqueous solubility of SO_2, it was assumed that most of the SO_2 was dissolved via the interfaces of the small bubbles produced from the syringe needle and the remainder through subsequent stirring action in the reaction chamber.

The slope of the recorded response curve during the 4- to 7-min elapsed time period was used in calculating rates of O_2 evolution. Rates measured in response to HSO_3^-, SO_3^{2-}, SO_4^{2-}, and SO_2 were expressed as a percentage of subsequent control rates.

Measurement of Cyclic Photophosphorylation. Cyclic photophosphorylation by chloroplast suspensions was measured in a water-jacketed cell equipped with a magnetically driven stirring bar. The reaction mixture was adapted from Jagendorf and Avron (14), and contained the following components added separately to the reaction cell in the order listed: (*a*) reaction medium (1 ml), containing (in μmoles) tris-HCl (pH 7.8), 30; NaCl, 88; $MgCl_2$, 10; and phenazine methosulfate, 0.1; (*b*) chloroplast suspension (0.1 ml), containing 50 μg Chl; (*c*) substrates of cyclic photophosphorylation (0.2 ml), containing 8 μmoles of ADP and 2 μmoles of KH_2PO_4. Immediately following addition No. 2 (chloroplast suspension), aliquots of HSO_3^-, SO_3^{2-}, or SO_4^{2-}, or SO_2 were added by methods already described. Then, 1 min was allowed to elapse before addition No. 3 (substrates of photophosphorylation). The chloroplasts were thus exposed to the sulfur anions or SO_2 for 60 sec prior to the start of a 3-min measurement period. The 1.3-ml reaction mixture was maintained at 20 C, and a light intensity of 4000 ft-c was provided by a 150-w General Electric No. 150-PAR/SP spotlight.

Rates of cyclic photophosphorylation were determined by the method of Hill and Walker (12). Rates measured in the presence of SO_2 or sulfur anions were then expressed as a percentage of control rates.

Measurement of Noncyclic Photophosphorylation. The methods and conditions used here are identical to those used in the study of cyclic photophosphorylation with the exception of the composition of the reaction mixture. The reaction mixture was formulated to meet the needs of the present study and contained the following: (*a*) reaction medium (1.0 ml) containing (in μmoles) tris-HCl (pH 7.8), 25; sucrose, 190; $MgCl_2$, 10; potassium ferricyanide, 2; and $NaHCO_3$, 1.04; (*b*) chloroplast suspension (0.1 ml) containing 50 μg of Chl; (*c*) substrates of noncyclic photophosphorylation (0.2 ml), containing 3 μmoles of ADP and 2 μmoles of KH_2PO_4.

RESULTS

Inhibition of Photosynthetic Oxygen Evolution. In control samples, the rates of photosynthetic O_2 evolution by isolated spinach chloroplasts averaged from 200 to 300 μl O_2 mg Chl^{-1} hr^{-1}. In the presence of 5 mM concentrations of either SO_2 or HSO_3^-, photosynthetic O_2 evolution was almost entirely inhibited (Fig. 1). The inhibition by SO_3^{2-} and SO_4^{2-} was significantly less, being unable to fully account for SO_2 inhibition of photosynthesis.

To distinguish possible H^+ effects from the effects of the sulfur anions themselves, changes in pH caused by addition of the inhibitors were measured. Of the four inhibitor forms shown in Figure 1, only SO_2 caused a change in pH of the reaction mixture. The original pH of 7.6 was lowered to pH 7.3 by the highest concentration of SO_2. To determine whether or not this pH alteration by SO_2 was in part responsible for the inhibition of photosynthesis by SO_2, control rates of O_2 evolution at pH 7.6 were compared to rates in response to addition of HCl, lowering the pH to 7.3. No change in rates was detected.

Inorganic Pyrophosphate Effect. Figure 2 shows the effect of SO_2, SO_3^{2-}, and SO_4^{2-} on rates of O_2 evolution by chloroplasts

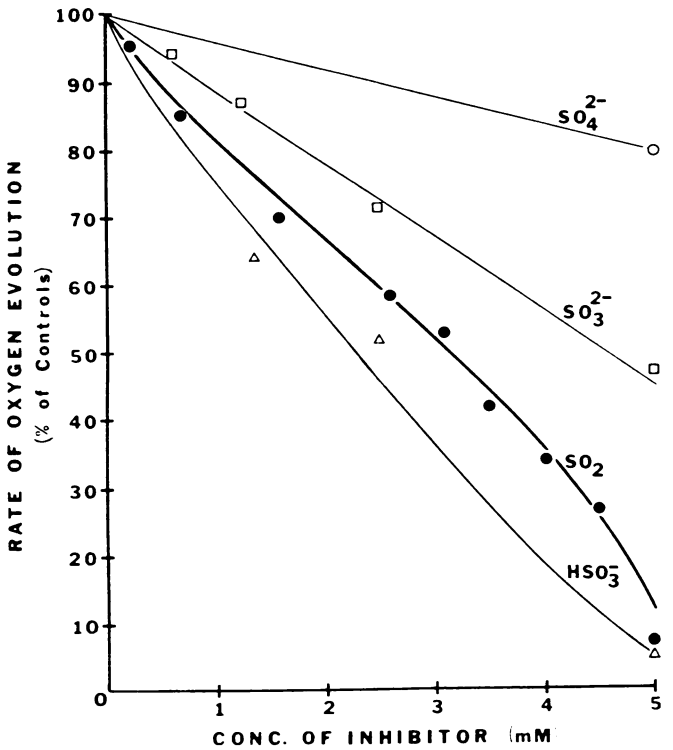

FIG. 1. Oxygen evolution by isolated spinach chloroplasts as a function of HSO_3^-, SO_3^{2-}, SO_4^{2-}, and SO_2 concentration. Control rates ranged from 200 to 300 μl O_2 mg Chl^{-1} hr^{-1}. The illuminated reaction mixtures contained 40 μmoles of PPi, and other components listed under "Materials and Methods." Fifty percent inhibition of O_2 evolution was observed in the presence of 12 mM SO_4; complete inhibition at 30 mM.

in a reaction mixture containing 4 μmoles of PPi. The effect of the inhibitors in the presence of 40 μmoles of PPi is shown in Figure 1.

Effects of SO_2 on Photophosphorylation. Rates of cyclic photophosphorylation in broken chloroplasts averaged 400 to 600 μmoles ATP formed mg Chl^{-1} hr^{-1} in controls; rates of noncyclic photophosphorylation averaged 100 to 200 μmoles ATP mg Chl^{-1} hr^{-1}. The response of cyclic and noncyclic photophosphorylation to a range of SO_2 concentrations from 0 to 10 mM is shown in Figures 3 and 4. The inhibition (Figs. 3 and 4) curve for SO_2 serves as a reference to compare with the inhibition by equimolar concentrations of sulfur anions potentially formed when the gas is absorbed. The curve labeled HCl indicates the response of photophosphorylation to additions of HCl as a source of H^+.

DISCUSSION

In this study the highest concentration of SO_2 or sulfur anions introduced into the chloroplast suspensions was 5 mM (Fig. 1). A question that must be considered is whether chloroplasts in the normal cytoplasmic environment of the leaf may experience such a concentration of the sulfur anions as a result of exposure of the plant to ambient levels of SO_2 in industrially polluted air. A precise answer cannot be given, but an approximation is possible.

According to the calculations as cited by Puckett *et al.* (19), 0.04 μl/l SO_2 at 25 C yields, in equilibrium with water, a solution of 35 mg/l SO_2. Therefore, the ambient atmospheric concentration of 1 μl/l SO_2 recorded in certain industrial areas (19, 25) would yield, at equilibrium, an aqueous solution with 875 mg/l SO_2, or

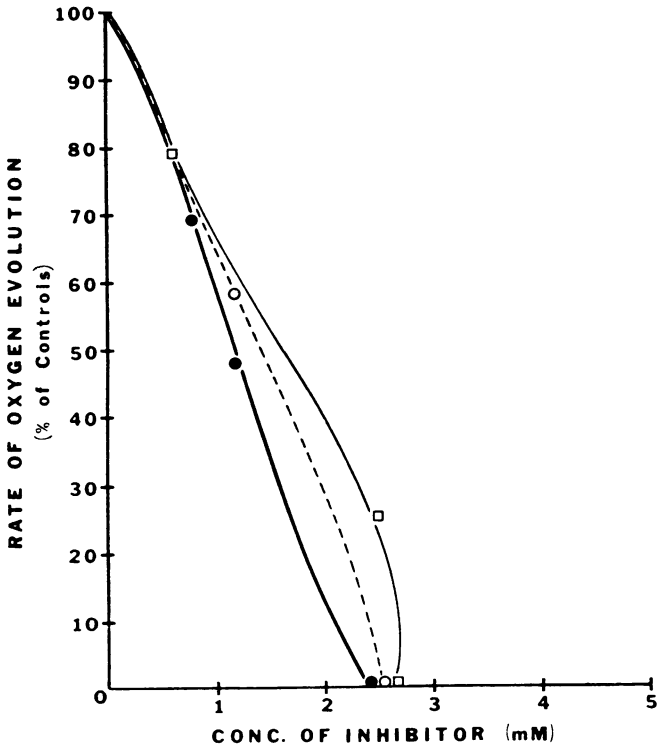

FIG. 2. Oxygen evolution by isolated spinach chloroplasts as a function of SO_3^{2-}, SO_4^{2-}, and SO_2 concentration. Control rates ranged from 200 to 300 μl O_2 mg Chl^{-1} hr^{-1}. The illuminated reaction mixtures contained 4 μmoles of PPi, and other components listed under "Materials and Methods." SO_3^{2-} (□——□); SO_4^{2-} (○---○); SO_2 (●——●).

13.4 mM. While the leaf-diffusive capacity is less than that for the surface of an aqueous solution, the high solubility of SO_2 coupled to high foliar absorption rates (8) allows rapid accumulation of sulfur anions in mesophyll cells. In support of this proposal is the discovery that pine needles, normally containing less than 500 mg/l of sulfur, contained up to 3700 mg/l in samples collected near a coal-fired power station (25). It seems reasonable to suggest that SO_2 and sulfur anion concentrations to which chloroplasts were exposed in the present study may indeed be attained within mesophyll cells of leaves exposed to certain reported ambient levels of SO_2.

Dissolved SO_2 at physiological pH produces both HSO_3^- and SO_3^{2-} in less than a 1:5 ratio (19). The relative effects of the gas and these two anions shown in Figure 1 suggest that the inhibition may indeed be due to HSO_3^- alone, or in combination with SO_3^{2-}. Studies using HCl as a source of H^+ suggest that SO_2 concentrations of 5 mM or less must inhibit photosynthetic O_2 evolution from chloroplast suspensions apart from apparent H^+ effects. The observation that SO_3^{2-} was more inhibitory than SO_4^{2-} is consistent with the report by Thomas and Hendricks (26). They noted that plant injury caused by SO_2 was more severe when SO_3^{2-} accumulated in the leaves. This accumulation occurred when SO_2 absorption by leaves exceeded the rates of conversion of SO_3^{2-} to the less toxic SO_4^{2-}.

It is apparent that higher concentrations of PPi exert a protective effect against the inhibition of photosynthetic O_2 evolution by SO_2 and the sulfur anions (Figs. 1 and 2). Baldry *et al.* (6) have studied the effect of added Pi and PPi on the degree of SO_4^{2-} inhibition of O_2 evolution by chloroplast suspensions. They suggested that PPi, being an effective complexing agent, might cause inactivation or prevention of penetration of sulfur anions to the site of inhibition. The studies by Lüttge *et al.* (17) to be discussed below are interesting in this connection. In addition, while PPi apparently cannot enter the intact chloroplast (23), an active pyrophosphatase released from ruptured chloroplasts may release Pi from PPi. The Pi may then act to reverse sulfur anion inhibition as observed by Baldry *et al.* (6).

In chloroplast suspensions used in photophosphorylation studies, SO_2 appears to have altered the pH to an extent that an apparent H^+ effect becomes significant. However, only SO_2 concentrations greater than 5 mM caused pH changes great enough to inhibit significantly photophosphorylation as shown by the HCl curve. This suggests two distinct kinds of inhibition. At concentrations of SO_2 greater than 5 mM, the H^+ effect becomes predominant and inhibition parallels increasing acidity. At lower concentrations, inhibition of photophosphorylation corresponds closely to that of the anion solution products.

Of the three sulfur anions, only HSO_3^- altered the pH. Rates of cyclic and noncyclic photophosphorylation, resulting from the same pH change duplicated by adding HCl, were 88% and 100% of controls, respectively. This suggests that the apparent H^+ effect accounts for little or none of the inhibition caused by HSO_3^-. It appears that SO_2 and the sulfur anions in solution caused measurable reduction in rates of photophosphorylation apart from H^+ effects. The same was true for the more inclusive process of photosynthetic O_2 evolution (Fig. 1).

Sulfite and bisulfite ions each inhibited cyclic and noncyclic photophosphorylation to a similar extent. Measurements of photosynthesis in the presence of SO_4^{2-} and HSO_3^- (Fig. 1) show the latter to be much more inhibitory. Since photosynthesis depends on the function of many component reaction systems in addition to photophosphorylation, this differing extent of inhibition would be expected if additional component reactions are being affected. Further support for this hypothesis arises

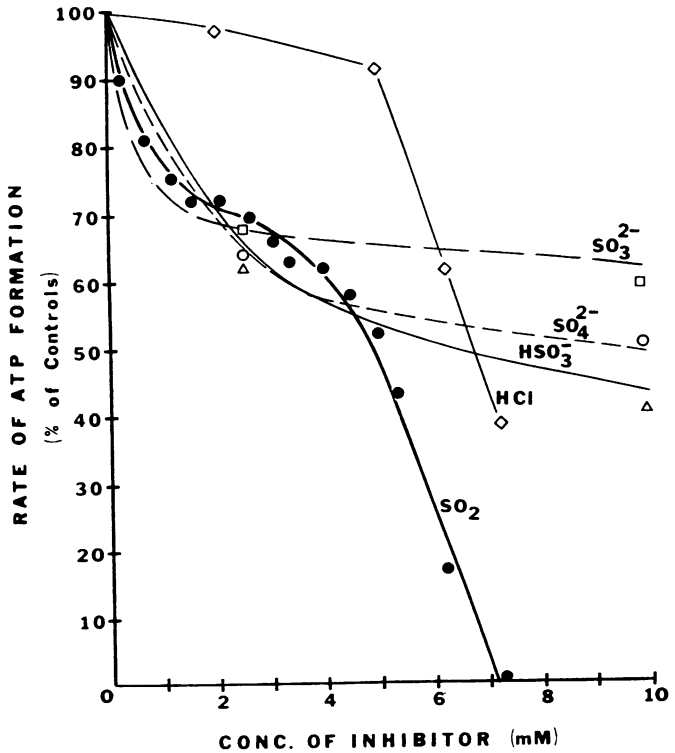

FIG. 3. Phenazine methosulfate-mediated cyclic photophosphorylation in broken spinach chloroplasts as a function of concentration of HSO_3^-, SO_3^{2-}, SO_4^{2-}, and SO_2. HCl was added in separate experimental runs to duplicate the effect of pH alteration produced in the presence of 2.1, 5, 6.2, and 7.3 mM concentrations of SO_2. Control rates averaged 400 to 600 μmoles ATP mg Chl^{-1} hr^{-1}.

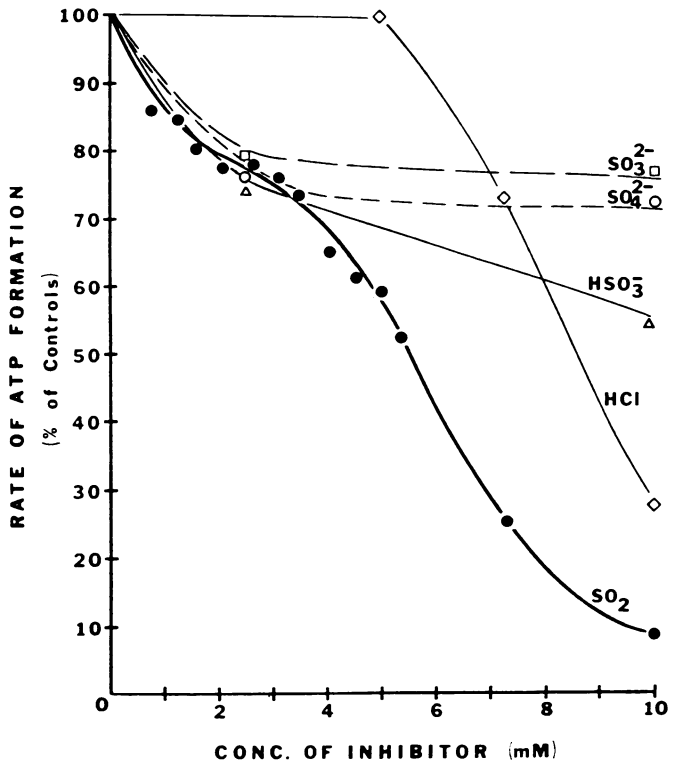

FIG. 4. Ferricyanide-mediated noncyclic photophosphorylation in broken spinach chloroplasts as a function of concentration of HSO_3^-, SO_3^{2-}, SO_4^{2-}, and SO_2. Hydrochloric acid was added in separate experimental runs to duplicate the effect of pH alteration produced in the presence of 5, 7.3, and 10 mM concentrations of SO_2. Control rates averaged 100 to 200 μmoles ATP mg Chl^{-1} hr^{-1}.

from a comparative study of SO_2 inhibition of O_2 evolution *versus* photophosphorylation. Neither phosphorylation reaction was inhibited as drastically as over-all photosynthetic O_2 evolution (Figs. 1, 3 and 4). Experiments have shown that there is a high correlation between the effective concentrations of a given inhibitor of photosynthesis in intact chloroplasts and its direct effect on photophosphorylation in broken chloroplast preparations (2, 22). It is doubtful that the nearly complete inhibition of photosynthesis in the presence of 5 mM SO_2 or HSO_3^- (Fig. 1) can be explained entirely by their effect on photophosphorylation, the rates of which were reduced by less than 50% at the same concentration (Figs. 3 and 4).

Several other possible targets of SO_2 inhibition of photosynthesis are suggested by work in various laboratories. Asada *et al.* (4) measured the effect of SO_3^{2-} and glyoxalbisulfite, an α-hydroxy-sulfonate compound, on photophosphorylation by broken chloroplasts. They report that 10 mM concentrations of both compounds reduce ATP synthesis by 50%. The same 10 mM concentration of the two compounds completely inhibited $^{14}CO_2$ fixation in spinach chloroplasts. To account for the greater inhibition of CO_2 fixation, they suggest that additional photosynthetic reactions are being affected (*e.g.*, carbon fixation pathway). Murray and Bradbeer (18) have demonstrated that inhibitory effects of α-hydroxy-suflonates may be quite general and widespread. Ziegler (29) has shown that SO_3^{2-} is a competitive inhibitor of bicarbonate attachment to ribulose-1,5-diP-carboxylase. This may represent a key mechanism by which solution products of SO_2 directly compete in CO_2 fixation.

Lüttge, *et al.* (17) and Murray and Bradbeer (18) have proposed separately that chloroplast membranes may be attacked by bisulfite and sulfonate compounds "prior to any measurable effect on enzymatic reactions." The uncoupling of photophosphorylation caused by these inhibitors may be just one example of nonspecific alteration of membrane integrity (17), since this process is a highly complex membrane phenomenon (5). Disruption of chloroplast membrane ultrastructure by SO_2 was recently reported by Wellburn *et al.* (28), who studied electron micrographs prepared from SO_2-fumigated leaves.

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