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Direct Observation of Reversible and Irreversible Stomatal Responses of Attached Sunflower Leaves to SO_2

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ABSTRACT

The effects of SO_2 on stomatal aperture of attached sunflower leaves were observed with a remote-control light microscope system that permitted continuous observation of stomatal responses over periods of several hours. The relationship between actual stomatal aperture and stomatal conductance, measured with a porometer, also was examined on leaves before and after exposure to SO_2.

A distinction between uninjured and injured regions was clearly visible on leaves after exposure to 1.5 microliters per liter SO_2 for less than an hour. During the exposure, the mean value of apertures for many stomata, which indicates stomatal conductance and transpiration rate, tended to decrease simultaneously in the uninjured and injured regions. However, the rate of decrease in the injured region was slower than that in the uninjured region because of a transient opening induced by water-soaking in the injured region. The transient opening was less common in stomata near veins and veinlets.

There was a good correlation between pore width and stomatal conductance measured with a porometer before exposure to SO_2. This correlation continued in leaves exposed to SO_2 until visible, irreversible injury occurred, but then it disappeared.

The results of these experiments indicate the necessity of continuous observation of individual stomata under the microscope to understand the effects of air pollutants such as SO_2 on stomatal behavior.

Stomata play an important role in regulating the uptake of air pollutants such as SO_2 (5, 10, 19, 20, 22, 30), and stomatal aperture therefore is a major factor controlling the amount of leaf injury. Stomatal behavior also is affected by SO_2 and stomatal response to SO_2 usually has been evaluated by measurement of stomatal conductance or transpiration rate. However, such measurements represent the average behavior of many stomata, and they may be modified by such SO_2-induced leaf injury as disorganization of cells and excessive water loss through the epidermis, independently of stomatal aperture. As a result, measurements based on stomatal conductance or transpiration rate provide no information concerning the response of individual stomata to SO_2. They also may not even represent the average response of stomata after the appearance of leaf injury. Omasa and Onoe (27) using a remote-control light microscope, recently observed a transient opening of sunflower stomata exposed to 2 $\mu l \cdot l^{-1}$ of SO_2, varying greatly in extent even in neighboring stomata, and occurring before water-soaking was visible in the subsidiary cells. This phenomenon could not have been found without direct, continuous observation of stomatal behavior.

There are many reviews of the interrelationships between stomatal response to SO_2 and leaf injury (4, 8, 9, 14, 17, 22, 31), and several mechanisms have been proposed to explain stomatal responses. However, these explanations are inconsistent with one another and inadequate because they do not fully explain the effects, or differentiate between reversible and irreversible effects. We therefore examined the reversible and irreversible effects of SO_2 on stomata of attached sunflower leaves by means of a remote-control light microscope system and attempted to organize the data so as to explain stomatal behavior. We also examined the relationship between stomatal aperture and stomatal conductance during exposure to SO_2 to learn the effect of leaf injury on stomatal conductance.

MATERIALS AND METHODS

Plant Materials. Sunflower (*Helianthus annuus* L. cv Russian Mammoth) plants were grown in pots (10 cm in diameter and 20 cm in height) in a controlled-environment greenhouse (1) at 25/20°C day/night temperature and 70% RH under natural

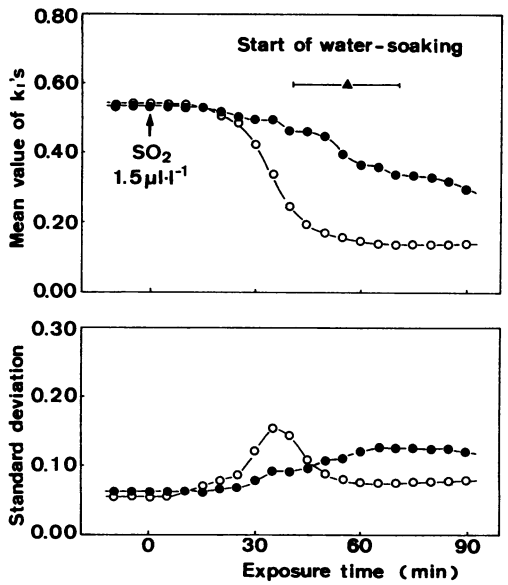

FIG. 1. Changes in the mean value of k_l's for about 30 stomata in uninjured and injured regions of attached leaves exposed to 1.5 $\mu l \cdot l^{-1}$ SO_2. (○), Uninjured region; (●), injured region. Symbol ▲ represents the mean value of the starting time of water-soaking in the injured region and the horizontal bar indicates ±SD. Environmental conditions: air temperature, 25.0°C; RH, 60%; light intensity, 600 $\mu E \cdot m^{-2} \cdot s^{-1}$.

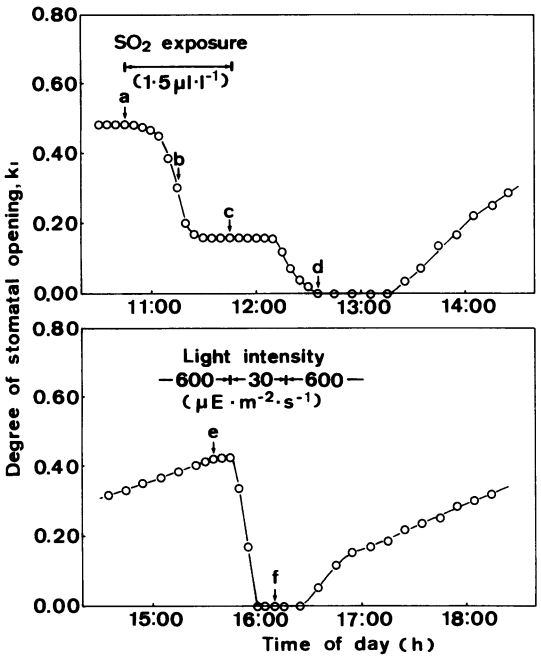

FIG. 2. Typical response to SO_2 of a single stoma in the uninjured region. Environmental conditions were the same as those in Figure 1. Letters refer to Figure 3.

lighting for 4 to 6 weeks. The pots were filled with a 4:2:4:1 (v/v) mixture of vermiculite, perlite, peat moss, and fine gravel. The plants were watered daily and nutrient solution was supplied every other day. After the test plants were moved to a controlled-environment chamber designed for studies of air pollution effects on plants (1) and allowed to acclimate to the new environmental conditions for about 5 h, attached mature leaves of the intact plants were used in the experiments.

Observation of Stomatal Responses to SO_2. Stomatal responses of attached leaves of growing plants to SO_2 were observed continuously, using a remote-control light microscope system (25). A light microscope with a long working distance (about 13 mm) at high magnification (a × 50 objective, × 1.5 and × 2 amplifiers and a TV adapter lens) and a TV (SIT[1]) camera head with high sensitivity were set in the chamber. An attached leaf of a well-watered plant was fastened to a movable microscope stage and illuminated until its stomatal apertures attained a steady state, after which the leaf was treated with SO_2. During the experiments, the microscope image was projected onto a TV monitor outside the chamber, giving × 800 to × 1,600 magnification with high resolution and little distortion. The images were recorded photographically on black-and-white film for later study. Camera sensitivity, microscope focus, and movement of the microscope stage were adjusted by a remote control system outside of the chamber.

Air temperature and RH in the chamber and around the leaf were maintained at 25.0°C and 60%. Leaf temperature was about 24°C before the SO_2 exposure and increased with stomatal closure after the exposure. The SO_2 concentration was measured with a pulsed fluorescent SO_2 analyzer (Thermo Electron, further developed version of Model 43) and adjusted with a program controller. It was maintained at 1.0, 1.5, or 2.0 $\mu l \cdot l^{-1}$ during 60 to 90 min after rising about 10 min. Deviations in both steady state and transient conditions were within about ±5% of the

[1] Abbreviations: k_1, degree of stomatal opening expressed by l_a/l_{bmax}; l_a, width of a stomatal pore; l_{bmax}, maximum length of a fully opened stomatal pore; SIT, silicon intensifier target.

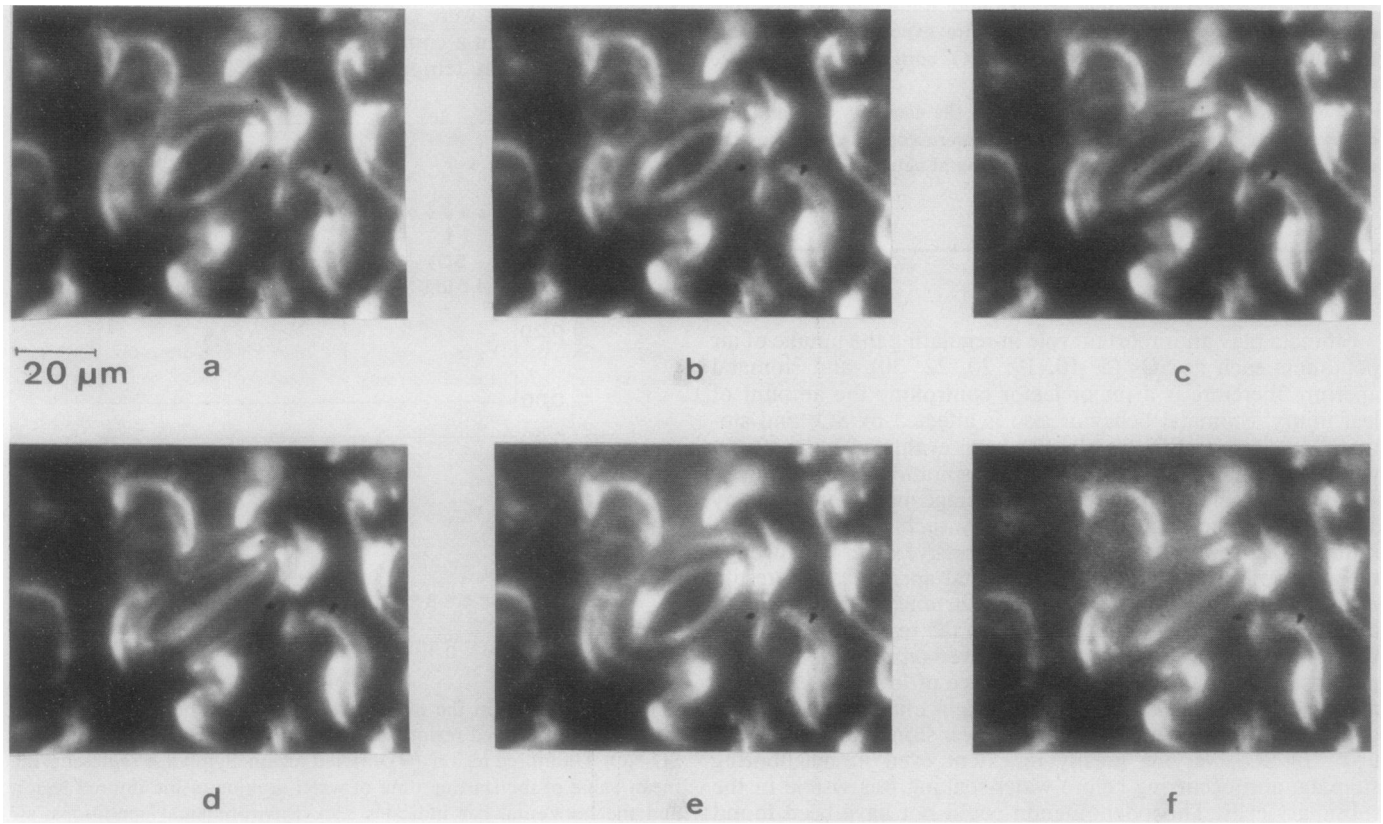

FIG. 3. Photomicrographs of the stoma shown in Figure 2. (a) to (f) correspond to time points (a–f) in Figure 2.

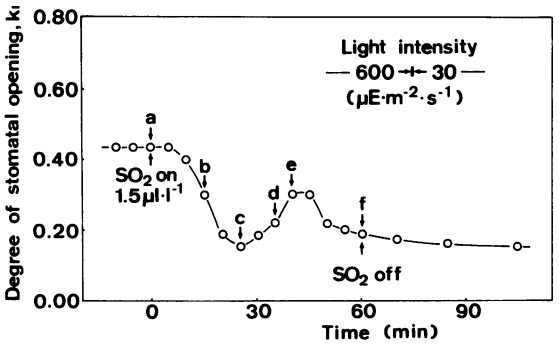

FIG. 4. Typical responses of a single stoma in the injured region. Environmental conditions were the same as those in Figure 1. Letters refer to Figure 5.

final desired value, although these varied according to concentration. The leaf was illuminated through heat-absorbing glass filters by tungsten lamps equipped with autotransformers in addition to metal halide vapor lamps installed within the chamber. The light intensity was measured with a quantum sensor (LI-COR, Model LI-185).

The stomatal response was evaluated by the degree of stomatal opening k_1, which is the ratio l_a/l_{bmax}, where l_a is the width of a stomatal pore and l_{bmax} is the length l_b of the fully opened pore, that is, the maximum value of l_b (27).

Measurement of Stomatal Conductance. An attached leaf of the test plant was held on the microscope stage under a constant light intensity. After the stomatal aperture attained steady state, about 30 stomata in an area about 15 mm in diameter of the leaf were randomly photographed, and then the stomatal conductance of the area was quickly measured with a porometer (LI-COR, Model LI-1600). This procedure was repeated for the same area at different light intensities from 0 to 600 $\mu E \cdot m^{-2} \cdot s^{-1}$. Thereafter, the light intensity was maintained at about 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ and after the stomatal aperture attained steady state, the SO_2 exposure was carried out. During the exposure, the above procedure was repeated for the same area again.

RESULTS

Figure 1 shows changes in the mean value of k_1's for many stomata in uninjured and injured regions of attached leaves exposed to 1.5 $\mu l \cdot l^{-1}$ SO_2. The leaves were healthy before the exposure but became clearly divided into uninjured and injured regions after the exposure. In the injured region, water-soaking appeared in about an hour after the start of the exposure and then the injury progressed irreversibly with chlorosis and necrosis and sometimes slight water-soaking without cell collapse. The mean values of k_1's tended to decrease steadily, but the rate of decrease in the injured region was slower than in the uninjured region. Both standard deviations were large in transient conditions.

Figures 2 to 5 show behavior of individual stomata instead of the average of many stomata. Figures 2 and 3 show typical responses of a single stoma in the uninjured region. After the start of exposure (a), the stoma began steady closure within 10 min and reached constant aperture (c) after 45 min. This aperture continued until 25 min after the end of the exposure. The stoma closed completely after 50 min (d), but then began to reopen after about 90 min and recovered to the same aperture as before the exposure. The reopened stoma responded normally to change in light intensity, as shown in the lower part of Figure 2. During the experiment, the guard and epidermal cells maintained normal turgor (Fig. 3). These phenomena were observed in repeated

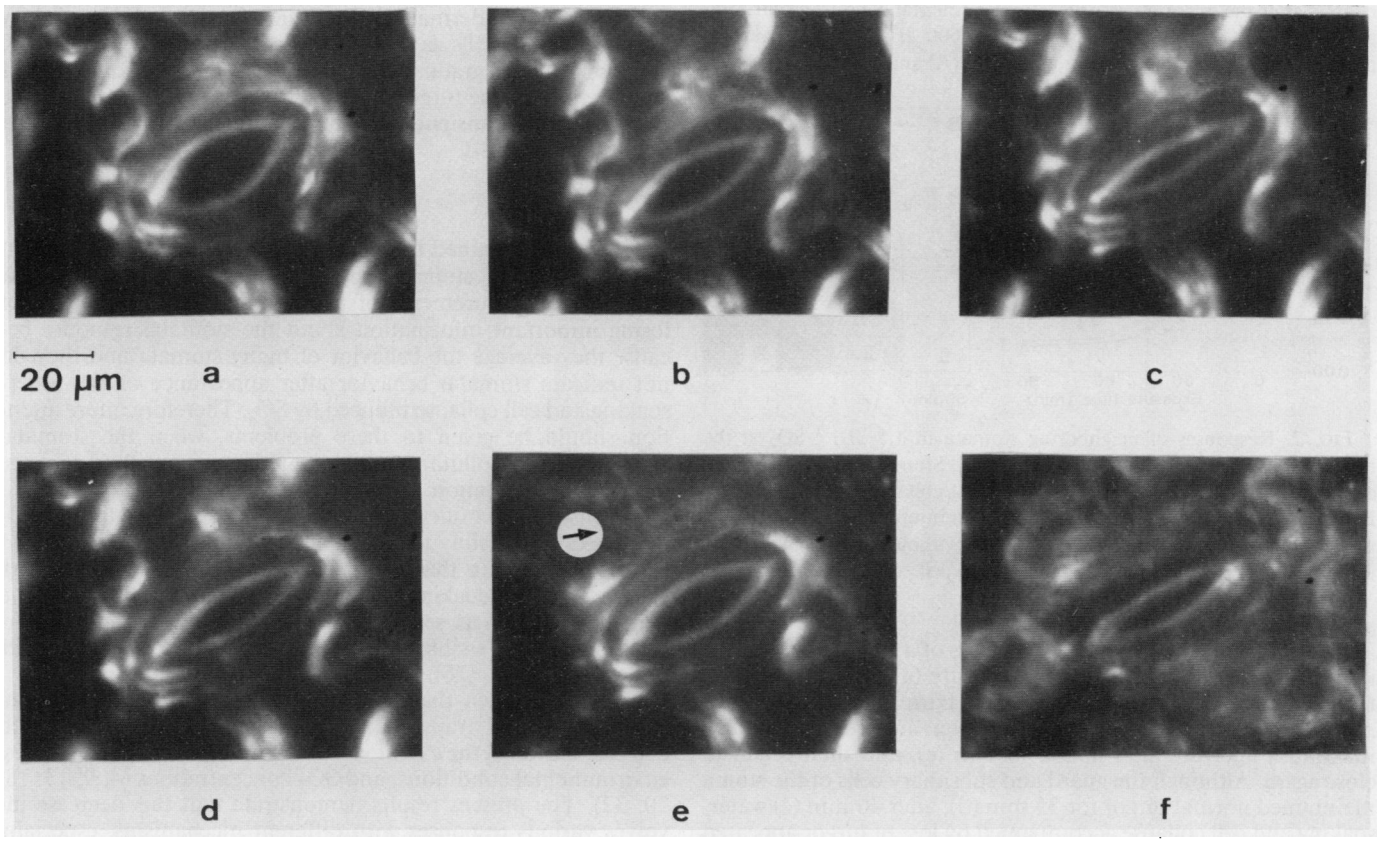

FIG. 5. Photomicrographs of the stoma shown in Figure 4. (a) to (f) correspond to time points (a–f) in Figure 4. The arrow (↗) in (e) indicates water-soaking and cell collapse.

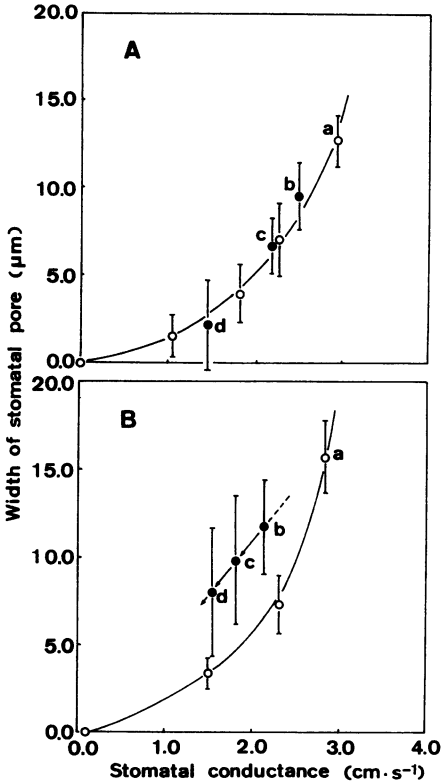

FIG. 6. Relationships between widths of the stomatal pores in uninjured and injured regions and their stomatal conductances. A, Uninjured region; B, injured region. (O), Data measured at different light intensities before SO_2 exposure; (●), data after the exposure. (a) to (d) show time courses after the exposure in order. Environmental conditions: air temperature, 25.0°C; RH, 60%, light intensity, 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ during exposure to SO_2; SO_2 concentration, 1.0 $\mu l \cdot l^{-1}$ (A) and 2.0 $\mu l \cdot l^{-1}$. (B).

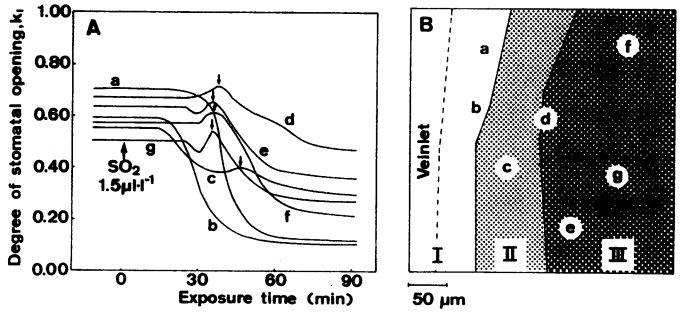

FIG. 7. Responses of neighboring stomata to 1.5 $\mu l \cdot l^{-1}$ SO_2 at the border of an injured region near a veinlet. A, Stomatal responses; small arrows (↓) show when water-soaking and cell collapse began to appear. B, Development of injury at the region; I, uninjured region; II, injured region where only cell collapse appeared, without water-soaking; III, injured region where both water-soaking and cell collapse appeared.

experiments.

Figures 4 and 5 show typical responses of a single stoma in the injured region. After the start of exposure (a), the stoma began to close within 10 min and reached a maximum closure after 25 min (c). Thereafter, it began to reopen and reached a new maximum aperture after about 40 min (e), and then began to close again. Although the guard and subsidiary cells of the stoma maintained normal turgor for 35 min (d), after 40 min (e) water-soaking and cell collapse accompanied by loss of turgor appeared in the subsidiary cells, and then expanded rapidly from the surrounding epidermal cells to the guard cells (f) (Fig. 5). At the end of the exposure, the stoma did not completely respond to change in light intensity because both the guard and epidermal cells were disorganized. Each of the injured stomata, used to calculate the mean value in Figure 1, also had a transient opening before the occurrence of water-soaking, although the extent of the opening varied among the stomata. In Figure 1, however, this phenomenon was lost by the averaging and appeared as a slow continuous closure.

Figure 6 shows relationship between the widths of the stomatal pores in the uninjured (A) and injured regions (B) and their stomatal conductances. The data measured at different light intensities before exposure to SO_2 indicated a good correlation between pore width and stomatal conductance (O, Fig. 6, A and B). This correlation was maintained during exposure to SO_2 in those regions of the leaves where the guard and epidermal cells maintained normal swelling during the exposure (Fig. 6A). As shown in Figure 6B, the correlation existing before exposure did not continue after water-soaking and cell collapse appeared in the epidermal cells at time point 'b' and expanded into all cells at time points of 'c' and 'd.' This means that the porometer measurements did not indicate the stomatal behavior of leaves injured by SO_2 as reliably as the direct observation method because of collapse of stomatal cavity and cell and their dehydrations.

Figure 7 shows varying responses of the stomata to 1.5 $\mu l \cdot l^{-1}$ SO_2 at the border of the injured region near a veinlet. These stomata continued to maintain constant apertures until about 15 min after the start of the exposure, and then, a wide variety of stomatal responses began. Stomata in area I contiguous to the veinlet (Fig. 7B) showed rapid and continuous closure and the guard and epidermal cells maintained normal turgor as in Figures 2 and 3. Stomata in area III at a distance from the veinlet had a transient opening after either closing or keeping a constant aperture because of the appearence of water-soaking and cell collapse in the epidermal cells, and then they closed as the injury expanded to all cells as seen in Figures 4 and 5. In area II cell collapse occurred gradually without water-soaking from the cells near area III by the turgor loss of cells in area III. Therefore, the appearance of transient stomatal opening in area II was later than that in area III.

DISCUSSION

The results obtained from the present study demonstrate that measurements of stomatal responses based on porometry or gravimetric measurements of transpiration involve the risk of losing important information about the stomatal response because they average the behavior of many stomata and they do not indicate stomatal behavior after appearance of the water-soaking and cell collapse induced by SO_2. Therefore, more attention should be given to these problems, when the stomatal responses to air pollutants are measured by porometry or change in rate of transpiration.

The visible leaf injury induced by a high concentration of SO_2 progresses irreversibly through water-soaking, cell collapse, and loss of pigments in that order, culminating in necrosis (2, 26). The severe water-soaking expands irreversible cell collapse to the neighboring cells as seen in Figure 7. However, slight water-soaking is a reversible effect which returns to normal after removing the SO_2 (26).

It is well known that at high SO_2 concentrations, stomatal conductance and transpiration rate both show a tendency to decrease although the extent varies according to the plant species, environmental conditions, and SO_2 concentrations (4, 9, 13, 18, 20, 32). The present results demonstrate that this decrease involves various responses with different mechanisms, especially in relation to the reversible and irreversible injuries. Although various mechanisms have been proposed to explain the stomatal

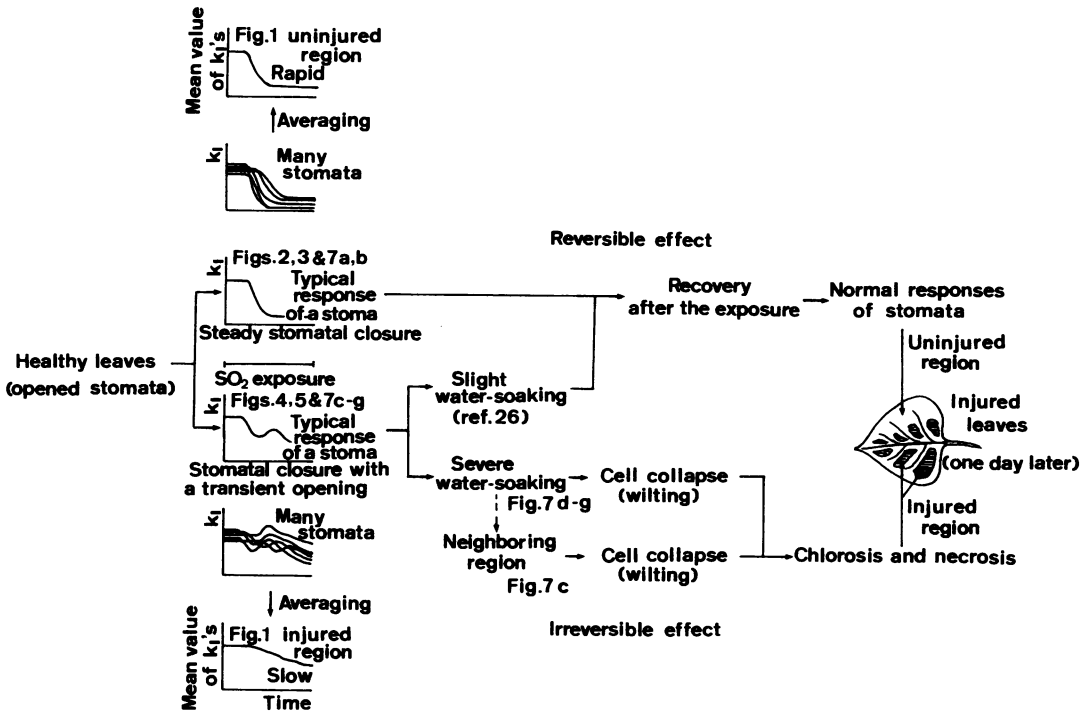

FIG. 8. Model showing relationship of reversible and irreversible effects of SO_2 on stomata.

responses to SO_2 (9, 12, 14, 18, 28, 31), these were inconsistent with one another and inadequate because the phenomena could not be classified adequately. Therefore, we organized the phenomena graphically in Figure 8 to show the progression from reversible to irreversible effects.

Rao *et al.* (28) claimed that SO_2-induced stomatal closure resulted from a combination of the immediate effects on the activity of P-enolpyruvate carboxylase and NAD- and NADP-malate dehydrogenase in the epidermis, which are key enzymes controlling the malate content and the osmotic pressure of the guard cells, and delayed effects arising from the inhibition of photosynthesis in the mesophyll. Decreased photosynthesis in the mesophyll may lead to CO_2 accumulation in the intercellular spaces which should induce stomatal closure (29, 33). Therefore, the initial and reversible closure of stomata (a to c in Figs. 2–5) are probably induced by inhibition of key enzymes and CO_2 accumulation. ABA content and the pH of the cells also are related to stomatal closure by some scientists (11, 12, 18, 29, 33).

Stomatal opening arises either from an increase in guard cell turgor or from a loss of turgor in the epidermal cells, or both (7, 11, 15, 16, 29, 33). The SO_2-induced reversible effects such as slight increase in permeability of epidermal cell membranes and alteration of the osmotic pressure, modulate a balance in turgor between the guard and subsidiary cells and may cause stomatal opening although the extent varies with atmospheric humidity and soil water content (9, 14, 31). A constant aperture before and after time point c in Figures 2 and 3 probably is a new balance resulting from a combination of change in factors affecting closing and opening. After removing SO_2, the SO_2-induced activity causing opening began to decrease within 20 to 30 min and the stomata closed completely by 50 min (c to d in Figs. 2 and 3). The SO_2-induced activity causing closing also began to decrease about 40 min after the complete closure and the stomata reopened to the same aperture as before the exposure by about 3 h (d–e in Figs. 2 and 3).

On the other hand, a large increase in permeability of the epidermal cell membranes and alteration of the osmotic pressure may promote greater opening activity. This activity probably opened stomata in opposition to the activity causing closing (c–e in Figs. 4 and 5), but after the appearance of the irreversible injury such as water-soaking and cell collapse the stomata closed again (e–f in Figs. 4 and 5). This closure probably was caused by a fall in the guard cell turgor resulting from severe injury irrespective of the turgor of the epidermal cells (3, 7, 15, 31).

Stomatal closure before the occurrence of irreversible injury commonly decreases the SO_2 uptake and reduces the leaf injury (6, 13, 18, 20, 23). However, the transient opening increases the SO_2 uptake and probably accelerates the increase in irreversible injury. In fact, the average stomatal closure in the injured leaf region was slower than that in the uninjured region because of the transient opening (Fig. 1). Omasa *et al.* (21, 24) evaluated the spatial distributions in stomatal resistance (1/conductance) and SO_2 uptake on a leaf from the leaf temperature image measured with a thermal image instrumentation system and found that a high concentration of SO_2 caused irreversible injury such as necrosis and chlorosis in interveinal leaf regions with slow stomatal closure and an aperture greater than the threshold for the SO_2 uptake. This evidence suggests that the transient opening is a major factor inducing the interveinal leaf injury which is clearly distinguishable from uninjured regions.

SO_2-induced injury usually is very slight in the regions near veins (2, 27). The SO_2 uptake of the region near the vein was less than that of the interveinal region because of the small stomatal conductance of this region (24). This phenomenon may relate not only to the density and size of the stomata in the region, but also to their responses to SO_2. Since regions near the veins and veinlets have ample water, the turgor in the epidermal cells may maintain a moderate value irrespective of SO_2 uptake. The sulfite usually absorbed in the tissues also may be diluted with water and diffused into the veins and veinlets near the tissues. As a result, the stomata close promptly, preventing irreversible injury (Fig. 7).

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