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Discoloration Pattern of Lettuce Leaf Disks as Influenced by Sulfur Dioxide

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亞黃酸에 의한 상치 잎組織의 變色패턴

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ABSTRACT

Development of a model system for mode of action studies of SO_2 was attempted with a plant tissue. Leaf disks, 1.0 cm diameter, cut from the lamina of lettuce leaves, were floated on the testing medium and placed in light or dark condition to investigate the discoloration pattern with various sources of SO_2.

Discoloration of leaf disks tended to be more serious with higher concentrations of SO_2 and on exposure to the light. Leaf disks were more severely discolored at lower pH with constant SO_2 concentration. These discoloration patterns were highly reproducible and similar in all sources of SO_2. Spectrophotometric evidence suggested that light-mediated discoloration of leaf disks in the presence of SO_2 might occur mainly through chlorophyll *a* degradation.

INTRODUCTION

Sulfur dioxide (SO_2) is known to be one of the most common phytotoxic air pollutants and to cause extensive injury to vegetation (Heggestad and Heck, 1971). Even though the relation of SO_2 to plant injury has been investigated by various workers (Adams, 1956; Brandt and Heck, 1967; Daines, 1968; Darley and Middleton, 1966; Han, 1973; Katz, 1949), the mode of action of this contaminant is not fully understood.

Sulfur dioxide is readily absorbed by plant leaves and dissolves on the moist surfaces of mesophyll cells, as it is freely soluble in water. Phytotoxicity of SO_2 appears to result from a plasmolytic effect due to the local build-up of sulfurous acid on the mesophyll cell or to reducing properties of sulfite in the course of oxidation (Thomas *et al.*, 1950). It is well known that a toxic dose of SO_2 bleaches interveinal tissues to light ivory or tan color (Adams, 1956; Brandt and Heck, 1967; Daines, 1968; Darley and Middleton, 1966; Heggestad and Heck; 1971).

Attempts were made to develop a reproducible model system simulating exposure of moist plant tissues to SO_2 for mode of action studies of this contaminant. A series of experiments were conducted with leaf disks floated on media containing sulfurous acid or its salts. It was the main purpose of this investigation to examine visible symptoms of SO_2 injury systematically.

METHODS and RESULTS

The first experiment was conducted to find out the effect of various concentrations of SO_2 on visible changes in leaf disks. Five different concentrations of SO_2, i.e., 200, 400, 600, 800 and 1,000 $\mu g/ml$ and a negative control were chosen as treatments, and sources of SO_2 included sulfurous acid, sodium bisulfite, sodium metabisulfite, and sodium sulfite.

Prior to medium preparation, sulfurous acid was always assayed for SO_2 adopting West and Gaeke's colorimetric method (West and Gaeke, 1956) and then was diluted to desired concentrations since this reagent appeared to be severely vaporized. In other sources, all SO_2 concentrations were based on stoichiometric values. Doubly deionized water was used for the preparation of media throughout the experiments.

The testing media consisted of designated concentrations of SO_2 containing solutions prepared from different sources. The pH of all media involved in this experiment was adjusted to 7 with sodium hydroxide or sulfuric acid.

Ten leaf disks were floated on 25 m*l* water or testing medium in petri dishes. The leaf disks were 1.0 cm in diameter and were cut from the lamina of lettuce leaves (*Lactuca sativa* L. var. Grand Rapids). A set of covered petri dishes was wrapped with two sheets of paper to interrupt light, and another set of petri dishes was unwrapped. The former will be referred to as the dark condition and the latter, as the light condition. A duplicate set of petri dishes containing 50 m*l* of medium without leaf disks was used to follow pH change of medium. All prepared petri dishes were placed under 500 ft-c fluorescent light at 20−25°C. This experiment was replicated twice.

After 20 hours, the only visible symptom on leaf disks was discoloration, even though injurious effect such as blistering was occasionally shown on severely discolored leaf disks. Therefore evaluation of visible symptoms of SO_2 injury was limited to discoloration. Extent of discoloration of leaf disks was rated with a scale of 1—5, where 1 is almost whitish discoloration and 5, no discoloration.

Table 1 shows the discoloration pattern of lettuce leaf disks floated on various SO_2 concentrations of medium. It is apparent that discoloration of leaf disks tends to be more serious with increasing SO_2 concentration and on exposure to the light. The discoloration pattern is very similar in all sources of SO_2.

In the control, a greenish tint of leaf disks remained almost intact both in light and dark conditions. However, in the SO_2-containing medium, the discoloration pattern was entirely different depending on the light condition. On exposure to the light, the margin of leaf disks was discolored forming a narrow, whitish ring at the lowest concentration of SO_2. The affected margin was gradually widened with increasing SO_2 concentration, and only a small portion of the center of leaf disks remained green at the higher ranges of SO_2 concentration. In most cases, the whole surface turned whitish at the highest concentration of SO_2.

In the dark condition, no distinguished discoloration was detectable except at the highest concentration of SO_2. Even at the higest concentration of SO_2, only a narrow margin of the leaf disks turned slightly light green. At the low ranges of SO_2 concentration, the margin of the leaf disks tended to be locally invaded showing a water soaked appearance.

The pH of media without leaf disks was measured concomittantly with rating of the discoloration pattern. As shown in Fig. 1, final pH of all concentrations of SO_2-containing media fell to approximately equal values around 3. The pH change was very similar in all sources of SO_2, in all SO_2 concentrations, and in light or dark condition.

The second experiment was done to examine an effect of initial pH of the medium on the discoloration pattern of leaf disks. All materials and procedures were the same as described in the previous experiment, except that initial pH of medium was varied and the SO_2 concentration of all media was kept constant. Initial pHs of media used were 3, 5, 7, 9 and 11 with an SO_2 concentration of 600 $\mu g/ml$. A 50 m*l* portion of testing solution was added to all petri dishes to measure final pH of media with or without leaf disks.

According to Table 2, leaf disks were more severely discolored in the low range of initial pH of medium and on exposure to light. In light, leaf disks turned completely whithish at pH 3, and discoloration was slightly reduced as initial pH of medium reached neutrality. At pH 9 and 11, a water soaked appearance was infrequently detected and no real discoloration was shown on the majority of leaf disks. In the dark, discoloration of leaf disks was less severe compared with those exposed to light at equivalent pH in the acidic to neutral range. It was noted that leaf disks tended to show slightly more water soaked appearance in the dark than in the light.

It was interesting to know whether leaf disks influence the pH change of the media. Consequently, the final pH of the medium was measured both with and without leaf disks. Fig. 2 depicts initial and final pH of media, with or without leaf disks, prepared from all sources of SO_2. With no leaf disks, initially acidic to neutral media reached approximately equal final pHs around 3, while alkaline media showed a decrease of 2—3 pH units. With leaf disks, the tendency of pH change was similar to that without leaf disks; however, pH values of media with leaf disks tended to be somewhat higher than without leaf disks. When

Table 1. Discoloration pattern of lettuce leaf disks floated on various concentrations of media prepared from several sources of SO_2 in light or dark condition

Stoichiometric SO_2 concn (μg/m*l*)	Extent of discoloration[1]							
	Sulfurous acid		Sodium bisulfite		Sodium metabisulfite		Sodium sulfite	
	In light	In dark	In light	In dark	In light	In dark	In light	In dark
0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
200	4.3	4.9	4.8	4.8	4.5	4.9	4.0	4.9
400	3.8	4.4	3.3	4.7	3.9	4.4	3.2	4.5
600	3.0	4.3	2.7	4.8	3.0	3.8	2.0	4.3
800	2.0	4.0	1.8	4.2	3.0	3.5	1.3	3.9
1,000	2.0	3.8	1.0	3.5	2.3	3.4	1.5	3.9

[1] Rated 20 hrs after floating of leaf disks with a scale of 1—5, where 1=almost whitish discoloration; 5=no discoloration. Based on 10 leaf disks in each of 2 replications. Initial pH of medium was adjusted to 7.

Table 2. Discoloration pattern of leaf disks floated on media with various pHs prepared from several sources of SO_2 in light or drak condition

Initial pH of medium	Extent of discoloration[1]							
	Sulfurous acid		Sodium bisulfite		Sodium metabisulfite		Sodium sulfite	
	In light	In dark	In light	In dark	In light	In dark	In light	In dark
3	1.0	3.0	1.0	3.0	1.0	2.5	1.0	2.0
5	2.5	3.5	1.8	3.0	1.3	2.8	1.5	2.8
7	2.9	4.0	1.8	3.5	2.3	3.3	2.3	3.3
9	5.0	4.9	5.0	4.7	4.8	4.7	4.9	4.7
11	4.9	4.7	4.9	4.6	4.9	4.3	4.9	4.7

[1] Rated 20 hrs after floating of leaf disks with a scale of 1—5, where 1=almost whitish discoloration; 5=no discoloration. Based on 10 leaf disks in each of 2 replications. Stoichiometric SO_2 concentration of all media was 600 μg/m*l*.

leaf disks were floated, final pH of the medium was generally higher in dark than in light.

In preceding experiments, the discoloration pattern of leaf disks due to SO_2 concentration or due to initial pH of the medium was similar in all sources of SO_2. However, it was not possible to make a direct comparison of the discoloration pattern among sources based on earlier experiments which were done at different times.

Accordingly the third experiment was conducted to make a comparison of discoloratin patterns among different sources of SO_2 at constant concentration, when initial pH was adjusted or not adjusted. In this experiment, all leaf disks were exposed to light. As shown in Table 3, there were no great differences in discoloration among various sources of SO_2, when initial pH of medium was adujsted to 7. Final pH of the medium fell to approximately 3 without leaf disks and to somewhat higher values with leaf disks.

When initial pH of the medium was not adjusted, it was noticeable that the discoloration pattern of leaf disks was entirely dependent upon pH of each source material. Leaf disks were severely discolored in acidic media prepared from sulfurous acid, sodium bisulfite, or sodium metabisulfite, even though extent of discoloration varied somewhat. On the other hand, leaf disks maintained greenish color in an alkaline medium prepared from sodium sulfite. Final pH of sulfurous acid, sodium bisulfite and sodium metabisulfite-containing media was around 3 and that of sodium sulfite-containing medium was neutral when no leaf disks were floated. In case of sodium bisulfite and sodium metabisulfite, final pH of the medium was slightly higher with leaf disks than without leaf disks (Table 3).

Under acidic conditions, the magnesium atom is removed from the center of tetrapyrrole structure of chlorophyll, forming pheophytin (Eskin *et al.*, 1971; Rao and LeBlanc, 1965). The question arose of whether higher pH values in media with leaf disks than in media without leaf disks were attributable to magnesium leaching from leaf disks due to chlorophyll degradation.

Table 4 shows final pH and magnesium content of media with and without leaf disks in light or dark condition. This time one hundred leaf disks were floated on 50 m*l* of medium. Sodium bisulfite was used as the source of SO_2, and the concentration of SO_2 used was 600μg/m*l*. As chlorophyll is known to be susceptible to acid action, initial pH of the medium was adjusted to 5. Magnesium content of the testing medium was determined by the titan yellow method(Löhnis, 1960).

Final pH was evidently higher in the media with leaf disks than the one without leaf disks, and somewhat higher in dark than in light. No magnesium was detected in the media without leaf disks, while concentrations of 15.9 μg/m*l* in light and of 12.4μg/m*l* in dark were found in the media with leaf disks (Table 4).

In all of preceding experiments, two sheet-covered petri dishes were referred to as the dark condition. Since complete darkness was not obtained with two sheet-covering, further information on discoloration pattern under varied light condition was desirable. Accordingly, the final experiment was designed to illustrate an effect of various light conditions on discoloration of leaf disks floated on SO_2-containing medium. Sodium bisulfite was utilized as the source of SO_2, and the concentration was 600μg/m*l*. Initial pH of the medium was adjusted to 5. Fifty leaf disks were floated on 25 m*l* of medium. Leaf disks floated on doubly deionized water in open light were used as a control.

In this experiment, uncovered (open light), one or two sheet-covered (relative), and aluminum foil-covered (complete dark) petri dishes were placed under 500 ft-c fluorescent light. As a result of measuring light intensity by a Toshiba photocell illuminometer (Type SPI—1), it was noted that one sheet-cover decreased light intensity approximately to one-tenth, and two sheet—cover to less than one-hundredth. Aluminum foil-cover resulted in complete darkness.

Objective measurement for the extent of discoloration of leaf disks was based on the spectropho

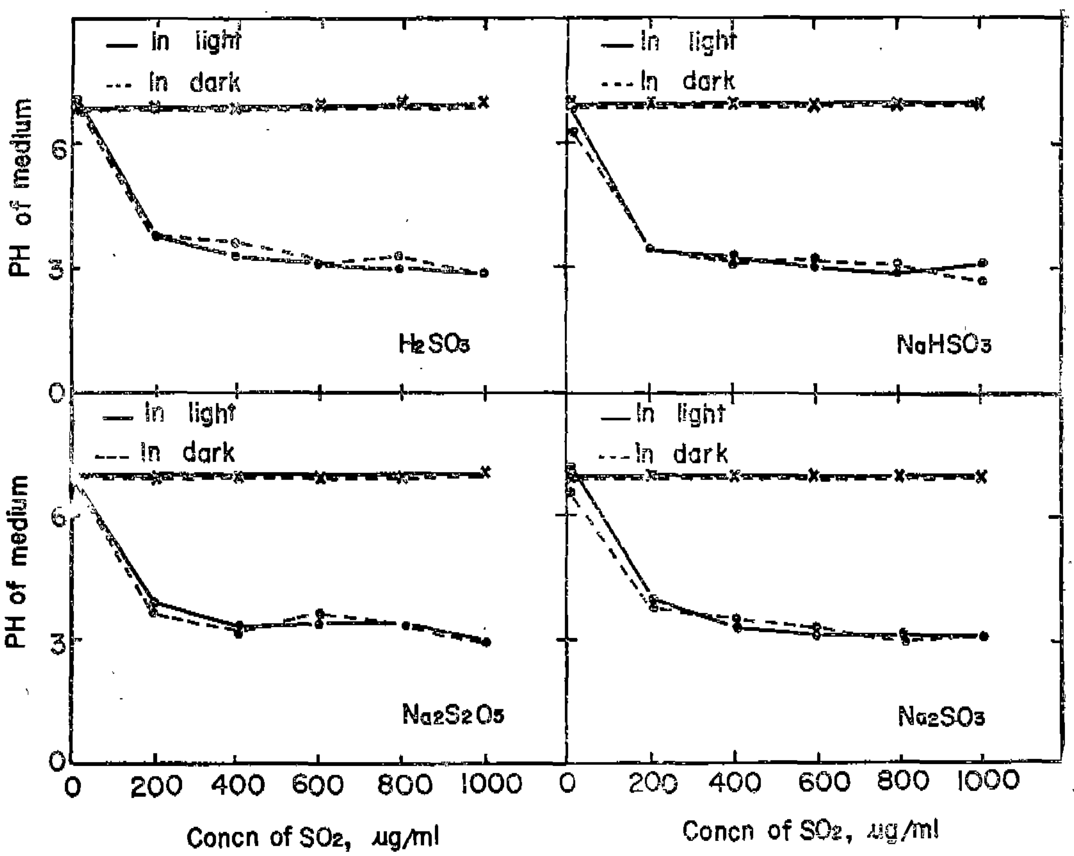

Fig. 1. Initial and final pH in various concentrations of SO_2 without leaf disks, prepared from several sources of SO_2 in light or dark condition. Initial pH of medium (×—×, ×……×) was adjusted to 7. Final pH of medium (●—●, ●……●) was measured after 20 hrs.

tometric procedure described by Vernon (1960). Aqueous acetone extracts of plant tissue were treated as a four-component system composed of chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and pheophytin *b*. Leaf tissue was extracted into 80% acetone, and centrifuged for 10—15 minutes at 15,000×*g*. Original samples and samples totally converted to pheophytin were required for spectrophotometric measurements of each extraction. The samples were prepared by diluting 3.0 m*l* of 80% acetone or saturated oxalic acid in 80% acetone to 100 m*l* with the prepared extract. Both samples were kept in the dark at room temperature for 3 hours. Absorbance was read in Beckman DU—2 spectrophotometer. The content of chlorophyll was calculated directly assuming no conversion of chlorophylls. Pheophytin content was estimated due to the difference between total chlorophyll and the chlorophyll actually present in the extract.

Fig. 3 shows absorption spectra of 80% acetone extracts of leaf disks floated on SO_2-containing media under varied light conditions. In all light conditions, absorption maxima of both original and converted samples were observed at 665 nm. It is

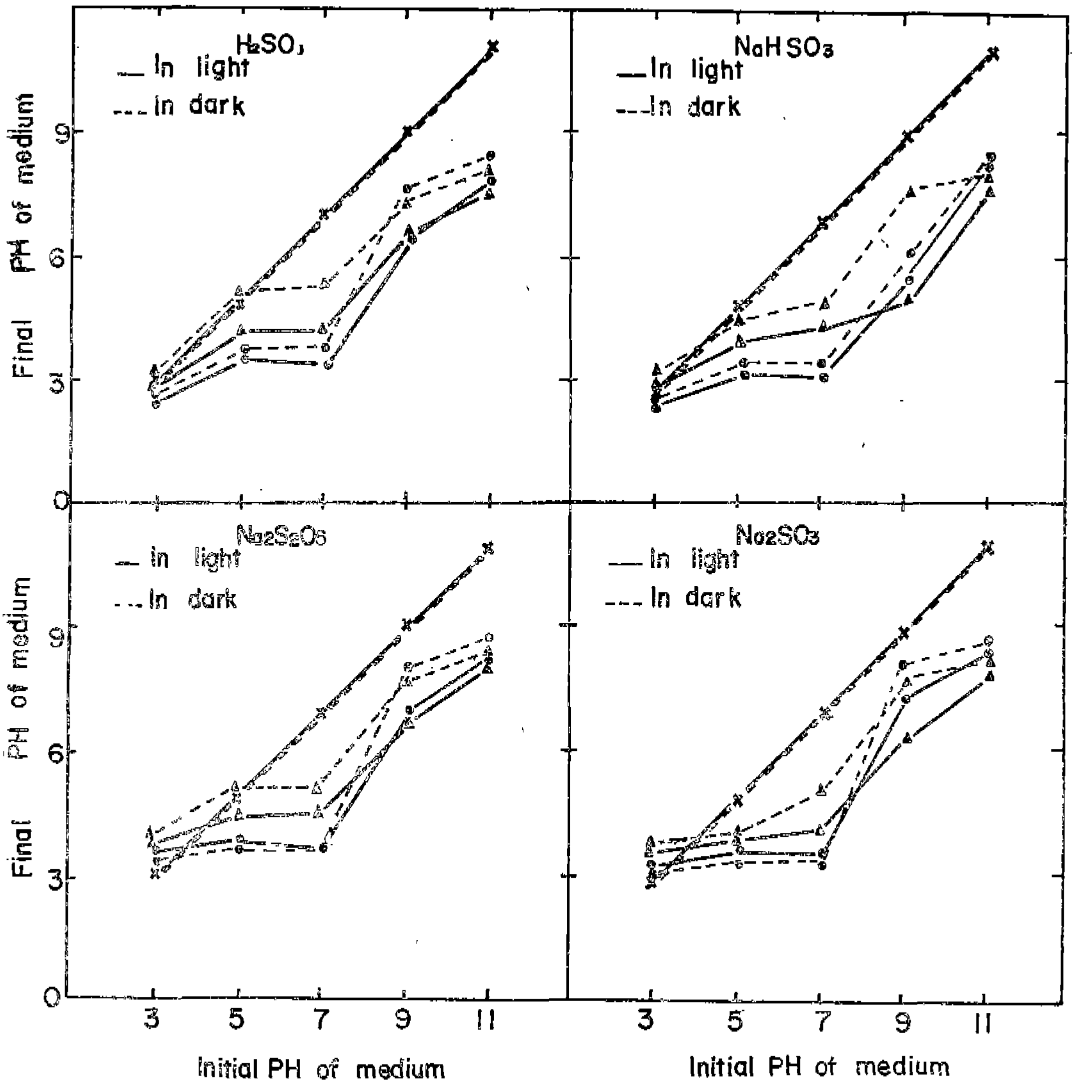

Fig. 2. Initial and final pH of medium, with or without leaf disks, prepared from several sources of SO_2 in light or dark condition. Initial pH of medium (×——×, ×……×) was adjusted to 3, 5, 7, 9 and 11 respectively. Final pH of medium with (▲——▲, ▲……▲) or without (◎——◎, ◎……◎) leaf disks was measured after 20 hrs.

noted that the peaks in relative and complete dark treatments are distinctly lower compared with the ones in the control, and the peaks in open light are further reduced.

Estimated contents of chlorophyll and pheophytin of leaf disks floated on SO_2-containing media under varied light conditions are seen in Table 5. Chlorophyll *a* content was appreciably decreased in relative and complete dark conditions compared with the control, and further reduced in the open light condition showing severe discoloration of leaf disks. Chlorophyll *b* tended to be reduced only in open light. A greater amount of pheophytin *a* than pheophytin *b* was detected from leaf disks under varied light conditions in the presence of SO_2.

DISCUSSION and CONCLUSIONS

In aqueous solution, SO_2 is partly combined with water and forms sulfurous acid, even though the forward reaction is incomplete, and sulfurous acid cannot be isolated in pure form. Sulfurous acid solutions are characterized by their reducing properties. Sulfurous acid captures oxygen from the water molecule and is oxidized to sulfuric acid, producing nascent hydrogen. The bleaching action of sulfurous acid has been ascribed to the reduction of colorless compounds by nascent hydrogen (Partington, 1950). Since salts derived from sulfurous acid, such as soluble sulfite and hydrogen sulfite (bisulfite), are dissociated into sodium hydroxide and sulfurous acid in aqueous solution, the same explanation might be applicable to these salts.

As summarized by Thomas *et al.*(1950), actual injury of SO_2 is assumed to occur mainly through its absorption and oxidation. It is essential for maximal absorption of SO_2 that external surfaces of leaves are wet. Presumably the oxidation rate of absorbed SO_2 is influenced by a number of internal and external factors. Based on this knowledge, floating of leaf disks on aqueous solution of SO_2 under controlled conditions might be a convenient system to study plant response as influenced by sulfur dioxide. Concentrations of

Table 3. Discoloration pattern of lettuce leaf disks and pH changes in the media prepared from various sources of SO_2 in light condition

Sources of SO_2	pH was adjusted			pH was not adjusted		
	Initial pH of medium	Extent of discoloration[1]	Final pH of medium[2]	Initial pH of medium	Extent of discoloration[1]	Final pH of medium[2]
Sulfurous acid	7.0	2.5	3.4(4.4)	2.4	1.0	2.7(2.8)
Sodium bisulfite	7.0	2.5	2.9(4.1)	4.5	1.4	3.3(4.1)
Sodium metabisulfite	7.0	1.9	3.1(4.2)	4.3	2.3	3.6(4.2)
Sodium sulfite	7.0	2.3	3.1(4.3)	9.0	5.0	7.2(6.8)

[1] Rated 20 hrs after floating of leaf disks with a scale of 1—5, where 1=almost whitish discoloration; 5=no discoloration. Based on 10 leaf disks in each of 2 replications. Stoichiometric SO_2 concentration of the medium was 600 μg/m*l*.

[2] Final pH of medium with leaf disks is shown in parenthesis.

Table 4. Final pH and magnesium content of medium with and without leaf disks in light or dark condition[1]

	Final pH of medium		Mg content of medium(μg/m*l*)	
	In light	In dark	In light	In dark
No leaf disks floated	2.9	3.2	—	—
Leaf disks floated	4.2	5.6	15.9	12.4

[1] Final pH and Mg content of medium were determined 20 hrs after floating of leaf disks. SO_2 concentration and initial pH of medium were 600 μg/m*l* and 5 respectively. One hundred leaf disks were floated on 50 m*l* of medium.

SO_2, pH of floating medium and light conditions were the only variables studied in the present experiments.

Table 1 shows that discoloration of leaf disks tends to be more serious with increasing concentrations of SO_2 and on exposure to light. Since final pH of the medium revealed that oxidation of aqueous solutions of SO_2 decreased pH values around 3 regardless of SO_2 concentrations (Fig.1), the extent of discoloration of leaf disks might be positively correlated with nascent hydrogen production. Light is generally considered to act as a catalyst in oxidation reactions yielding free radicals. Accordingly, more serious discoloration of leaf disks in light than in dark might be attributed to the greater amount of nascent hydrogen produced. However, it is difficult to explain why the final pH of the medium without leaf disks in light is not greatly different from that in dark (Fig. 1). One reason may be that it is impossible to detect the conversion of sulfurous acid into sulfuric acid by pH determination.

It has been generally recognized that the open stomata are the sole or principal avenue for SO_2 absorption and that SO_2 results in more injury to plants during daylight when stomata remain open. In the present experiments, discoloration started in the outward portion of leaf disks punched from the lamina, suggesting that SO_2 was primarily absorbed through the cut injury. Under this experimental condition, it seemed likely that the main effect of light with regard to SO_2 injury was independent of stomata openings. The same conclusion had been reached earlier (Daines, 1958).

Table 2 reveals that discoloration of leaf disks tends to be more serious at lower initial pH of medium. The result appears reasonable since more acidic conditions could hasten oxidation of aqueous solutions of SO_2. Without leaf disks, oxidation yielded a pH of 3 in initially acidic to neutral media, but the pH declined far more slowly in alkaline medium (Fig. 2).

According to Gilbert (1968), the degree of ionization in an aqueous solution of SO_2 and the rate of oxidation to sulfate are greatly influenced by pH, and phytotoxicity of SO_2 might be dependent upon the predominant form present in aqueous solution. He suggested that bisulfite ions were more toxic than sulfite or sulfate ions and that unionized sulfurous acid was the most toxic.

Vass and Ingram (1949) illustrated distribution of various constituents of sulfurous acid at various pH levels. Considering discoloration patterns of leaf disks from the viewpoint of nascent hydrogen production, the predominant species in aqueous solution might also be of significance. It is known that nascent hydrogen occurs when unionized sulfurous acid is oxidized to sulfuric acid, by combining with water, and not by atmospheric oxygen (Partington, 1950). Nascent hydrogen production might be proportional to the number of unionized sulfurous acid molecules in aqueous solution. Accordingly, it is probable that greater amount of nascent hydrogen would be produced as pH becomes lower. Recently Puckett *et al.* (1973) have reported that phytotoxic effect of dissolved SO_2 was due to the oxidation property of the pollutant at lower pH.

The results in Table 3 show that initialy acidic

Table 5. Rough estimation of chlorophyll and pheophytin of lettuce leaf disks floated on SO_2-containing medium under varied light condition[1]

Light condition	Estimated content of chlorophyll and pheophytin			
	Chlorophyll *a* (mg/*l*)	Chlorophyll *b* (mg/*l*)	Pheophytin *a* (mg/*l*)	Pheophytin *b* (mg/*l*)
Control[2]	15.3	3.1	—	2.5
In open light Uncovered	3.6	1.1	2.4	1.0
In relative dark One sheet covered	7.5	3.2	4.5	1.7
Two sheets covered	7.0	2.4	8.8	2.4
In complete dark Aluminum foil covered	7.2	2.8	7.1	0.9

1) The content of chlorophyll and pheophytin was determined 20 hrs after floating of leaf disks. Sodium bisulfite was used as the SO_2 source. SO_2 concentration and intitial pH of medium was 600 μg/m*l* and 5 respectively.

2) Leaf disks floated on distilled water in open light were regarded as control for comparison.

media such as sulfurous acid, sodium bisulfite and sodium metabisulfite solutions resulted in severe discoloration of leaf disks, while initially alkaline media such as sodium sulfite solution resulted in almost no discoloration. It is conceivable that the varied extent of discoloration due to sources of SO_2 depends entirely upon initial pH of the medium.

According to Table 4, magnesium was detected only in the medium with leaf disks, and this indicates that chlorophyll degradation of leaf disks occurs in the presence of SO_2. The final pH of media with leaf disks tends to be somewhat higher than that without leaf disks (Fig. 2; Table 4). Assuming that sulfurous acid is rapidly neutralized by organic bases in the leaf and that cations leached from leaf disks, particularly magnesium removed by chlorophyll breakdown, raise the final pH of the medium, slightly higher final pH of medium in dark than in light seems contradictory to the fact that light discolors leaf disks more severely compared with dark. The progress of oxidation in the medium and amount of magnesium leached could be implicated, since the composition of the medium is complex.

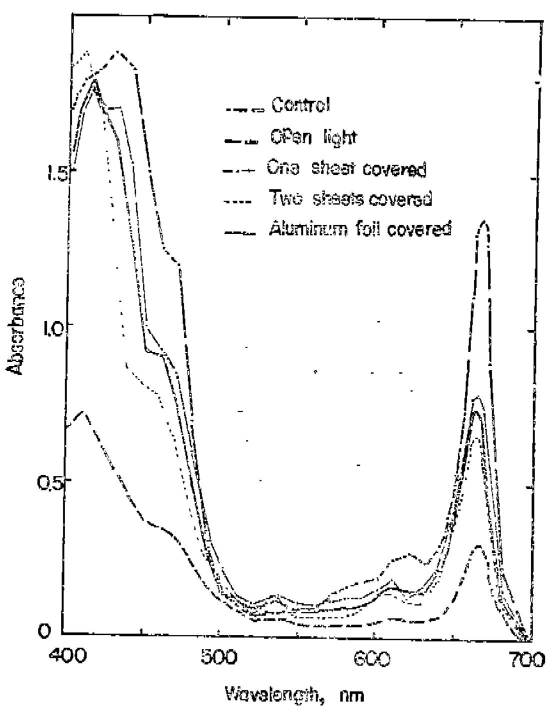

Fig. 3. Absorption spectra of 80% acetone extract of leaf disks floated on sodium bisulfite-containing medium under varied light condition. Fifty leaf disks were floated for 20 hrs on 25 m*l* of medium in which SO_2 concentration and initial pH of medium were 600 μg/m*l* and 5 respectively. Leaf disks floated on distilled water in open light were regarded as control for comparison.

The final experiment investigated whether various light conditions influenced the content of chlorophyll and pheophytin of leaf disks in the presence of SO_2. In the original sample, the absorption maximum appeared somewhat shifted toward longer wavelengths, and was located at 665 nm the same as in the converted sample (Vernon, 1960). It is probable that the absorption peak of the original sample is mainly due to chlorophyll *a*, but the location of the absorption maximum might indicate the existence of the corresponding pheophytin in the original preparation.

As expected, absorption spectra of 80% acetone extract of leaf disks showed reduced peak heights in relative and complete dark and further reduced peak heights in open light compared with the control. These spectra illustrate that the presence of SO_2 in the floating medium slightly discolors leaf disks in relative and complete dark but discolors severely in open light.

A rough estimation of chlorophyll and pheopytin content of leaf disks floated on SO_2-containing medium under varied light conditions is given in Table 5. Since fluctuations are quite large in the data, these results are not satisfactorily reliable. Although pheophytin could be produced during the grinding and extraction processes (Vernon, 1960), the results of Table 5 and the location of absorption maximum shown in Fig. 3 suggest that light mediated discoloration of leaf disks in the presence of SO_2 occurs mainly through chlorophyll *a* degradation. A low amount of pheophytin in open light suggests that chlorophyll degradation either is more advanced than pheophytin or might occur by some pathway other than that in dark. Previous workers reported that SO_2 was responsible for converting chlorophyll into pheophytin in the acutely injured leaves (Adams, 1956).

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摘 要

식물체에 대한 亞黃酸의 被害機作 연구를 위하여 model system의 확립을 시도하였다. 직경 1.0 cm의 상치 잎조직을 여러가지 급원의 亞黃酸 용액에 띄워 光 혹은 暗黑條件에 방치한 후 그 패턴을 조사하었다.

잎조직의 變色은 亞黃酸의 농도가 증가함에 따라 그리고 光에 노출시킨 경우 더욱 심하였으며 동일한 농도의 용액에서는 pH가 낮을수록 잎의 變色이 더욱 심하였다. 이들 變色패턴은 고도로 再現性이 있었으며 亞黃酸의 급원에 상관없이 비슷하였다. 光條件下에서 亞黃酸에 의한 잎조직의 變色은 주로 엽록소 *a*의 분해를 통하여 일어나는듯 보였다.

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