Inhibition of Oxygen Evolution in Chloroplasts Isolated from Leaves with Low Water Potentials

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ABSTRACT
Chloroplasts were isolated from pea and sunflower leaves having various water potentials. Oxygen evolution by the chloroplasts was measured under identical conditions for all treatments with saturating light and with dichlorodiphenyl oxide as oxidant. Evolution was inhibited when leaf water potentials were below -12 bars in pea and -8 bars in sunflower and the inhibition was proportional to leaf water potential below these limits. Inhibition was more severe in sunflower than in pea chloroplasts. In sunflower, it could be detected after 5 minutes of leaf desiccation, and, up to 1 hour, the effect was independent of the duration of low leaf water potential.

In high light, the reduction in activity of sunflower chloroplasts paralleled the reduction in CO$_2$ fixation by intact sunflower plants having low leaf water potentials. Stomatal apertures and transpiration rates were also reduced under these conditions and were probably limiting. In low light, intact sunflowers required more light per unit of CO$_2$ fixed when leaf water potentials were low than when they were high. This increased light requirement in the intact system was of a magnitude which could be predicted from the reduced oxygen evolution by the isolated chloroplasts. It was concluded that moderately low leaf water potential affects photosynthesis in at least two ways: first, through an inhibition of oxygen evolution by chloroplasts and, second, by closure of stomata in intact leaves.

In higher plants, photosynthesis is inhibited when leaves have low water potentials. The effect is usually attributed to stomatal closure (7, 9, 18) or, occasionally, to increased resistance to CO$_2$ diffusion in the photosynthesizing cells (17). In spite of numerous observations of this inhibition, there is little known about chloroplast changes which may accompany it. In the only in vivo study available, inhibition of NADP and P-glycerate reduction was observed when tissue water contents were below 60% of saturation (15). In vitro, chloroplasts lost all Hill and photophosphorylating activity when dried to 1% of their original water content and rehydrated (16). Inhibition of photochemical activity has also been noted at tissue water contents of 72 and 35% (13). Water contents below 70% are rarely met in nature, however, and it is difficult to assess whether significant changes in chloroplast behavior would be observed under more moderate conditions. The following work was therefore undertaken to determine whether oxygen evolution by chloroplasts is affected when leaves are exposed to moderate desiccation. The general plan of the experiments consisted of rapid desiccation of leaves to varying degrees, measurement of their water potential, and immediate assay of oxygen evolution by chloroplasts isolated from the tissue under conditions which were identical for all treatments.

MATERIALS AND METHODS
Chloroplasts were isolated from leaves of 14-day-old pea (Pisum sativum L. var. Alaska) and 3-week-old sunflower (Helianthus annuus L.) plants which were grown in soil in a controlled environment chamber (peas: temperature = 20 ± 1°C day and night; light intensity = 200 ft-c, fluorescent light; photoperiod = 12 hr; sunflower: temperature = 25 ± 1°C day, 21 ± 1°C night; relative humidity = 50–70%; light intensity = 3000 ft-c (fluorescent) for first 10 days, 1000 ft-c (fluorescent) for remaining growth period; photoperiod = 14 hr). The longer growth period for sunflower was necessary to obtain high chloroplast activity.

For an experiment, approximately 2 g of whole pea leaves were divided into two subsamples, one serving as the control and the other as the desiccation treatment. Much the same procedure was followed for sunflower except that each leaf was cut longitudinally, one-half being used for the control and the other for the treatment. The tissue to be desiccated was excised and placed in the growth chamber for varying lengths of time. Immediately prior to chloroplast isolation, the tissue was sampled for an isopiestic measurement of leaf water potential with a thermocouple psychrometer (4, 6). The sample consisted of several leaves (pea) or a leaf disc (sunflower) placed in a psychrometer chamber that had been coated with melted and re-solidified petrolatum to reduce the adsorption of water vapor on the walls (5).

Immediately after sampling for water potential measurements, chloroplasts were isolated by cutting the remaining tissue into a micro Waring blender containing 20 ml of a solution at 0°C made up of sorbitol, 0.33 M; MgCl$_2$, 5 mM; sodium ascorbate, 2 mM; and Na$_2$P$_2$O$_7$·10H$_2$O, 10 mM, adjusted to pH 6.5 at 0°C with HCl (8). For sunflower, 20 mM NaCl was also present in the isolation medium to promote dispersion of the chloroplasts. The tissue was ground for 7 sec (peas) or 15 sec (sunflower), and the material was poured into a bag made of two layers of cotton cloth. The cloth was of a sufficiently close weave to retain large particles and whole cells but permit chloroplasts to pass through (14). The resulting filtrate was centrifuged for 2 min at 500g at 2°C, the supernatant fluid was discarded, and the pellet was resuspended by placing the tube on a vortex mixer. The suspension was then stored in an ice bath during measurement of chlorophyll concentration. The entire isolation was carried out at low light.

Measurement of Oxygen Evolution by Chloroplasts. Oxygen
evolution was assayed in a medium containing chloroplasts equivalent to 100 μg of chlorophyll. The 2.9-ml assay medium consisted of sorbitol, 0.33 M; NaEDTA, 2.0 mM; MgCl₂, 1.0 mM; MnCl₂, 1.0 mM; and HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 50 mM, adjusted to pH 7.6 at 20 C with NaOH (8). For sunflower, 20 mM NaCl was also present in the assay medium.

Sodium 2,6-dichloroindophenol, 0.88 mM, served as oxidant in all assays and was added to the medium immediately prior to injection of the chloroplasts. Evolution of oxygen was measured with a Clark-type (12) electrode separated from the magnetically stirred assay medium by a Teflon membrane. The difference between the output of the electrode in water in equilibrium with air and water in equilibrium with pure nitrogen was considered to represent 0.28 μmole of O₂·ml⁻¹ in the assay medium. There was negligible difference in electrode output in water equilibrated with air and in assay medium equilibrated with air.

After injection of dichloroindophenol and chloroplast suspension into the assay medium, the medium was illuminated from opposite sides with two 300-W incandescent spotlights filtered through 25 cm of water. Light intensity was measured with a Moll thermopile at the surface of the assay vessel without water present. This intensity was then corrected for light absorption by 25 cm of water measured with the same detector and light source. With water present in the assay system, the lights provided 0.9 cal cm⁻² min⁻¹ on both sides of the assay vessel. This intensity was saturating under the conditions of the experiment. Water temperature was controlled so that the assay medium was maintained at 20 ± 0.05 C. The rate of oxygen evolution was determined from the initial slope of electrode output as a function of time.

Measurement of CO₂ Fixation by Intact Plants. Carbon dioxide fixation by intact sunflower plants was measured for comparison with oxygen evolution from sunflower chloroplasts. Fixation was followed with an infrared gas analyzer which sampled air in an assimilation chamber having the following conditions: air temperature = 25 ± 0.25 C; relative humidity = 77 ± 2%; CO₂ concentration = 250 ± 7 μl/liter except during a measurement of photosynthetic rate; wind speed = 1.7 m sec⁻¹. Air was circulated between the chamber and the analyzer in a semiclosed circuit. The shoot of the plant to be tested was sealed in the assimilation chamber so that CO₂ exchange by the roots, which were outside, did not affect the measurement. Leaf temperature was within 0.6 C of chamber temperature. Seven 300-W incandescent spotlights provided a light intensity of 1.6 cal cm⁻² min⁻¹ (measured with a Moll thermopile) at leaf height and was saturating under these conditions. Net photosynthesis was determined at approximately 10 min intervals by measuring the time required for the shoot to decrease the CO₂ concentration in the system from 270 to 230 μl/liter. Each determination of net photosynthesis required a few seconds to as much as 2 min. The short times required for CO₂ determinations were similar to the times required for O₂ evolution in the isolated chloroplasts.

Transpiration was measured at the same time by collecting the water condensed from the chamber for humidity control. After steady rates of photosynthesis and transpiration were obtained, the chamber was opened and a leaf disc was rapidly removed from a lower leaf and placed in a thermocouple psychrometer chamber for an isopiestic measurement of leaf water potential (4, 6).

Subsequent to sampling for the psychrometer, leaf porosity was followed with a modified viscous flow psychrometer (1) which permitted rapid determinations and used a small vacuum. The time required for a standard change in porometer vacuum is related to the diffusive resistance of the leaf stomata by (2, 10):

\[ r = k \sqrt{R_s} = \frac{K}{\ln(P_i/P)} \sqrt{t} \]

where \( r \) is the diffusive resistance, \( k \) and \( K \) are proportionality constants, \( R_s \) is the resistance to viscous flow of air through the stomata, \( P_i \) and \( P \) are the initial and final pressures, \( t \) is the time in seconds, and \( n \) is a constant having a value between 2 and 3 depending on the leaf (10). In the present work, the diffusive resistance of the stomata was estimated in relative terms from the cube root of the time required for sufficient movement of air through the leaf to cause a change in vacuum from 3 to 2 cm of water.

RESULTS

Figure 1 shows that oxygen evolution was reduced at low leaf water potentials in both sunflower and pea chloroplasts. Inhibition occurred at potentials below -12 bars in peas and -8 bars in sunflower and was roughly proportional to leaf water potential below these limits. At -18 bars, evolution was inhibited 20 and 80% in the two species, respectively. Control rates were 23 to 36 μmoles of O₂·hr⁻¹·mg⁻¹ of chlorophyll in peas and 30 to 53 μmoles of O₂·hr⁻¹·mg⁻¹ of chlorophyll in sunflower. Desiccation periods were as long as 16 hr in peas but only 45 min in sunflower, which dried much faster. Inhibition could be detected after 5 min of desiccation in sunflower.

The moderate potentials and short times required for inhibition suggested that the effect was not due to secondary metabolic
Fig. 3. Comparison of oxygen evolution in sunflower chloroplasts at high light with CO₂ fixation in intact sunflower plants at high light over a range of leaf water potentials. Rates expressed as a percentage of well watered controls (control rates of CO₂ fixation: 31–34 mg of CO₂·hr⁻¹·100 cm⁻² of leaf area; control rates of oxygen evolution: 30–55 μmoles of O₂·hr⁻¹·mg⁻¹ of chlorophyll).

Fig. 4. CO₂ fixation, transpiration, and relative diffusive resistance of the stomata (shown as the cube root of time for viscous flow) at various leaf water potentials. Rates are expressed as a percentage of well watered controls in intact sunflower plants.

changes resulting from excision of the tissue. However, to test this idea, leaf tissue was removed from sunflower plants, oxygen evolution was measured in the controls, and the other leaf half was desiccated and stored in a dark chamber at 100% relative humidity for various times before assay. There was no time effect up to 1 hr (Fig. 2) in sunflower leaf tissue at either −5 or −11 bars. Thus, inhibition of oxygen evolution appeared to result from desiccation rather than the length of time the tissue was excised.

Comparison of oxygen evolution by chloroplasts with CO₂ fixation by whole sunflower plants under high light showed that the two were similarly affected (Fig. 3). Transpiration was reduced at low leaf water potentials (Fig. 4), probably as a result of reduced stomatal apertures (shown as an increase in relative diffusive resistance, Fig. 4). It is therefore likely that the reduction in photosynthesis of intact plants resulted largely from the smaller stomatal apertures rather than reduced chloroplast activity, since the measurements were made at high light where leaf diffusive resistance may be limiting (9).

However, at low light intensities, the effect of reduced chloroplast activity should be apparent. Figure 5 shows that in low light, the rate of total photosynthesis was less when the plant was desiccated than when the same plant was hydrated. Considerable care was taken to assure that the arrangement and exposure of the leaves when wilted duplicated those of the turgid leaves. Photosynthesis becomes increasingly independent of the leaf diffusion resistance as light levels drop below saturation, and the response of the process to light at very low levels provides a measure of the photochemical activity of the system (11). The reciprocal of the slope of the low light data in Figure 5 shows the quantity of light required for a unit change in rate of photosynthesis.

Under these conditions, the data indicate that the light requirement of desiccated leaves (~19.5 bars) was 3.7 times that of the well watered leaves (~5 bars). The chloroplast data of Figure 1 predict that the light requirement of the desiccated leaves should have been 4.3 times the light requirement of the same leaves when well watered. The chloroplast data therefore predict the increased light requirement in sunflower and suggest that chloroplast inhibition occurs in vivo.

**DISCUSSION**

The results show that oxygen evolution by chloroplasts was reduced in tissue which had been moderately desiccated. The reduction was evident even though chloroplasts isolated from tissue having various water potentials were studied in media having the same potential for all treatments (osmotic potential of isolation and assay media = −10 to −11 bars). If chloroplast activity were solely a function of the potential of the isolation media, the assays should have shown no differences in chloroplast activity. Since differences did appear, the changes must have occurred while chloroplasts were still in the leaf tissue and must have been irreversible or only slowly reversible.

Although the mechanism by which such changes might occur is unclear, reduction seemed to be more dependent on the degree of desiccation than on the duration of desiccation. In sunflower, inhibition occurred rapidly and could be detected in as little as 5 min. Moreover, the rates of oxygen evolution at a given leaf water potential remained constant even though the tissue had been stored for up to 1 hr at that potential.

The magnitude of the inhibition depended on the species— inhibition was considerably less in pea than in sunflower at the same leaf water potential. This difference may have been associated with differences in the growth conditions for the two species or to the presence of NaCl in the chloroplast medium for sunflower.

The data suggest that low leaf water potential affects photosynthesis in at least two ways: first, through an inhibition of oxygen evolution by chloroplasts and, second, by closure of stomata in intact leaves. The stomatal effects have been well documented (7, 9, 17, 18), but the inhibition of oxygen evolu-
tion has not been described previously for moderate desiccation levels. The leaf water potentials at which both stomatal closure and chloroplast inhibition occur are high enough so that both should be of frequent occurrence in nature.

The similarity between inhibition of chloroplast activity and reduction of CO$_2$ fixation by the intact plant in high light was unexpected and could have been fortuitous. The parallelism may reflect a chance similarity in sensitivity of stomata and chloroplasts to low leaf water potential in sunflower. On the other hand, since stomatal behavior is linked to the photosynthetic activity of the guard cells (19), it is also possible that there is some relationship between stomatal aperture and chloroplast inhibition that would account for the similarity in the two sets of data.

In saturating light, the reduction in stomatal aperture probably explains much of the reduction in intact plant photosynthesis as leaf water potentials drop. Below saturation, photosynthetic rates should have been increasingly independent of resistances to CO$_2$ diffusion. Consequently, possible increases in the so-called cytoplasmic resistance to CO$_2$ diffusion (9) should have had little effect on the measurements of photosynthesis in low light. Under these conditions, reductions in photosynthesis are probably associated with chloroplast changes. Reductions in photosynthesis that could not be attributed to stomatal closure have been reported previously (3, 17), but it has not been clear whether these instances were due to increased cytoplasmic resistance (17) or to some change in the chloroplasts (3). Although the results presented here do not indicate whether the resistance to CO$_2$ diffusion in the cytoplasm is subject to change during desiccation, the data obtained with isolated chloroplasts show that the physiological activity of the chloroplasts changes. Since the effect was apparent in the intact system when incident light was as high as 0.8 cal cm$^{-2}$ min$^{-1}$ (shown as inhibition of photosynthesis at below-saturation light levels, Fig. 5), nonstomatal reduction of photosynthesis would occur commonly in nature even on relatively bright days. The question of whether chloroplast activity or stomatal aperture is most limiting during desiccation would appear to depend primarily on light intensity.

The magnitude of the light requirement in the intact system when desiccated was similar to that predicted from the isolated chloroplast system. Both the reduction in oxygen evolution by chloroplasts and the concomitant increase in light requirement are consistent with the idea that some part of photosynthetic electron transport is affected by exposure of chloroplasts to low water potentials within the leaf. This conclusion is supported by the low levels of NADPH which occur in desiccated tissue (15).

**LITERATURE CITED**