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CONFORMATIONAL CHANGES OF CHLOROPLASTS INDUCED BY ILLUMINATION OF LEAVES *IN VIVO*

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SUMMARY

Upon illumination leaves showed both fast absorbance changes and a slow increase in their apparent absorbance (maximum at about 530 mμ) which appeared to be caused by a light-dependent shrinkage reaction of chloroplasts. The extent of shrinkage was strongly influenced by the quality and intensity of exciting light and by the presence or absence of electron acceptors. The following observations pertain to the control of electron flow within the electron transport chain of photosynthesis.

1. In N_2, shrinkage was promoted by either far-red light or low intensity red light which caused a mediated cyclic electron flow to occur in Photosystem I. Shrinkage was inhibited by illumination with high intensity red light.

2. Addition of red to a beam of far-red light illuminating a leaf under N_2 stimulated shrinkage if the intensities of both beams were sufficiently low and led to inhibition of shrinkage if the red beam was intense. A dark period was required to relieve inhibition.

3. In CO_2-free air, O_2 was an electron acceptor and caused shrinkage at higher intensities of red but not at low intensity far-red light. Shrinkage promoted in N_2 by far-red or low intensity red light was strongly suppressed by O_2 which appeared to interrupt cyclic electron transfer by oxidizing an electron acceptor in the pathway between Photosystem I and $NADP^+$. This view was supported by measurements of cytochrome *f* changes at 420 mμ. Action spectra indicated also that shrinkage in N_2 was a Photosystem I reaction, while in the presence of O_2 Photosystems I and II cooperated to produce shrinkage.

4. While shrinkage was greatly stimulated by low concentrations of O_2, when both photosystems were sufficiently excited, increasing O_2 concentrations suppressed shrinkage increasingly in the investigated plant species possibly by inhibiting electron flow.

5. The affinity of the shrinkage reaction for O_2 was high. Half maximal stimulation or inhibition of shrinkage was obtained at an O_2 concn. of about 1.4 μM in the tissue or 1200 ppm in the gas phase. Half maximal fluorescence quenching by O_2 occurred at a similar concentration. The kinetics of shrinkage and of fluorescence during a change in the gas atmosphere from N_2 to CO_2-free air or *vice versa* were

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluorophenylhydrazone.

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similar indicating that the effects of O_2 on the redox state of the quencher and on the shrinkage were indirect and were both mediated by the same reaction of O_2 with a component of the electron transport chain beyond Photosystem I.

6. Shrinkage caused by electron flow to O_2 in air or by the cyclic electron flow in N_2 was effectively suppressed by CO_2. However, CO_2 relieved the inhibition of shrinkage caused by high intensity red light under N_2 probably by stimulating electron flow. The affinity of the system for CO_2 as judged by the ability of CO_2 to act as a fluorescence quencher in N_2 in blue light or as an inhibitor of shrinkage in far-red light was higher than that for O_2. Half maximal response was obtained in leaves capable of high rates of photosynthesis at a CO_2 concn. in the gas phase of 10–20 ppm.

7. The results indicate that CO_2 and O_2 *in vivo* both act as electron acceptors of photosynthesis; O_2 reduction probably supplies ATP in a pseudocyclic type of photophosphorylation. Cyclic electron transfer in the presence of O_2 is unlikely to occur except under conditions where the reaction reducing O_2 is saturated. The observations support the series model of photosynthesis and do not seem to fit into a model with separate and independent photoreactions.

INTRODUCTION

Isolated chloroplasts exhibit light-dependent, reversible shrinkage and swelling phenomena caused by ion and water movements across the membranes of the grana compartments (survey of literature[1,2]). Light-dependent shrinkage of chloroplasts in the living cell has been demonstrated by electron microscopy, by flashlight photography and by spectrophotometry[3–6] indicating that the observations with isolated organelles reflect, at least in part, physiological processes. In the present study properties of light-dependent conformational changes of chloroplasts in intact leaves as revealed by absorbance measurements have been investigated. The results suggest a relationship between the conformational changes to phosphorylation and a control of cyclic by noncyclic electron flow and by the availability of electron acceptors *in vivo*. A brief account of some aspects of this work has been given elsewhere[7].

MATERIALS AND METHODS

Transmittance changes were generally observed using leaves of *Spinacia oleracea, Mimulus cardinalis, Mimulus verbenaceus, Oenothera hookeri, Chenopodium gigantospermum, Plantago lanceolata* and *Pisum sativum*. Normally the leaves were freshly removed from the plant; however, spinach leaves were stored at 5° before use. Pale green mutants of *Pisum sativum* (1206 A and 130 A) lacking chlorophyll *b* but capable of normal photosynthesis were grown in the green house. Transmittance changes were recorded on an oscillograph as the change in the photocurrent produced by a measuring beam (usually at 530 mμ, band width 1 mμ, intensity 9.4 ergs·cm^{-1}·sec^{-1}) which passed through the leaf. Conformational changes resulting in transmittance changes were induced by illuminating the leaf with a broad band of red (RG 2 filter, Schott, Jena) or far-red light (RG 8 filter). Additional filters were 27 mm water and 1 mm Calflex (Balzer). The filters of the red beam transmitted light from

about 620 mμ to 820 mμ (half band width from 633 to 762 mμ), those of the far-red beam from about 690 to 820 mμ (half band width from 702 to 762 mμ). The phototube (Emi 9558 B) was protected from exciting light by two No. 9782 Corning filters and by BG 18 (Schott, Jena). For the action spectra, narrow bands in the red region were isolated by B-type interference filters from Balzer, Liechtenstein (half band width 15 mμ) which were added to a RG 2 cutoff filter. The intensity was adjusted to give equal incident quanta for the different spectral regions.

For the measurements, a leaf was placed on a Lucite light pipe in a closed Lucite vessel directly above the phototube and was gassed with a stream of 99.997% pure moist N_2, with moist CO_2-free air or with a mixture of both (approx. 0.8–1.2 l/min). For experiments where high rates of photosynthesis were desired, leaves were left attached to the plant, and only a leaf area of about 6 cm^2 was enclosed in a chamber and was gassed from both sides. The O_2 concentration was monitored with a Beckman O_2 electrode. Different concentrations of CO_2 in N_2 or in air were obtained by adding a stream of CO_2-free air or N_2 to another stream of approx. 500 ppm CO_2 in either air or N_2. Temperatures were measured with a thermocouple inserted in the leaf.

Absorption changes induced by light were distinguished from scattering changes by comparing transmission of light by a leaf (leaf between phototube and measuring beam) with its absorption in an Ulbricht sphere (phototube measuring the total light coming from the sample). Light intensities were measured with a silicon photocell calibrated with a thermopile.

3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea (DCMU) and uncouplers of phosphorylation were fed to sligthly wilted leaves by floating them for up to 14 h on solutions containing different concentrations of DCMU or uncoupler. Inhibiton of electron flow by DCMU was indicated when absorbance changes induced by strong red or far-red light at 420 mμ either were not or only slowly reversed in the dark or in weak light (*i.e.* when no photoreduction of cytochrome *f* could be observed).

RESULTS

1. *Slow changes in the apparent absorbance of leaves*

Leaves exhibit slow and relatively large reversible changes in the amount of 530 mμ light transmitted to a photomultiplier, *i.e.* in their apparent absorbance, when they are illuminated and darkened or when air in the gas phase is replaced by N_2 or *vice versa* under continuous illumination with red or far-red light (Fig. 1). The changes show several phases and, depending on the geometry of the sample arrangement, may correspond up to 0.04 absorbance unit in spinach leaves. Fig. 2 shows a difference spectrum for the apparent increase in the absorbance of a leaf of *Chenopodium gigantospermum* observed on replacing N_2 by air under continuous illumination with a broad band of red light. There is a broad maximum at about 525 mμ which in spinach and Pisum leaves is shifted to 530 mμ or 535 mμ and a second small peak around 455 mμ. Minima are apparent at 425 mμ and 470 mμ. Light–dark difference spectra of the slow absorbance changes caused by high intensity red light in CO_2-free air or by low intensity red light in N_2 are similar to the difference spectrum displayed in Fig. 2.

Practically the same difference spectrum is seen when a suspension of isolated

chloroplasts in 0.025 M Tricine buffer containing 0.1 M sodium acetate and $7 \cdot 10^{-5}$ M phenazine methosulfate (pH 7.6) is illuminated. The large light-induced increase in the absorbance of such chloroplast suspensions is caused by light scattering and reflects the light-induced chloroplast shrinkage[2]. From these data, from results of uncoupler experiments and from the large reduction in the extent of the absorbance changes in the Ulbricht sphere, it is concluded that the large slow absorbance changes of leaves around 530 mμ are caused by light scattering and reflect light-induced changes in the chloroplast volume.

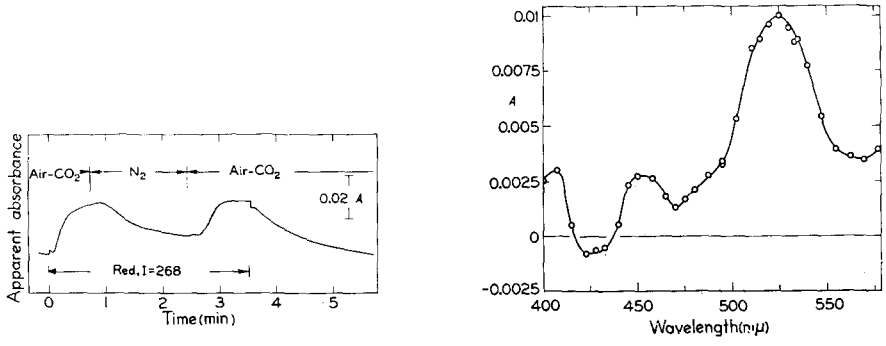

Fig. 1. Changes in the apparent absorbance at 530 mμ of a leaf of *Mimulus cardinalis* caused by illumination with high intensity red light in CO_2-free air or by gassing with N_2 under continuous illumination. Illumination causes a large increase in apparent absorbance under air which is reversed under N_2. Definition of figure labels (valid also for other figures except when indicated): red, red light transmitted by 3 mm of RG 2 cutoff filter combined with 1 mm of Calflex C and 27 mm water; far-red, far-red light transmitted by 3 mm of RG 8 cutoff filter combined with 1 mm of Calflex C and 27 mm water; *I*, light intensity in kergs $\cdot$ cm^{-2} $\cdot$ sec^{-1}. An increase in apparent absorbance denoting shrinkage is shown as an upward deflection of the trace, decrease in apparent absorbance denoting swelling as a downward deflection.

Fig. 2. Difference spectrum of the increase in apparent absorbance of a leaf of *Chenopodium gigantospermum* caused by a change in the gas phase from N_2 to CO_2-free air. Measurements of the differences 40 sec after admission of CO_2-free air. Continuous illumination with red. $I = 112$.

A light-induced reduction in the volume of chloroplasts has been observed in both intact leaves[3,4,6] and isolated chloroplasts[8]; and because a reduction in the volume of light scattering particles results in an increase in apparent absorbance[9], a corresponding slow increase in the apparent absorbance of leaves will tentatively be identified as a shrinkage reaction of chloroplasts, thus a decrease as a swelling reaction. There is also the possibility that changes in the apparent absorbance are caused, in part, by light-dependent intracellular movements of whole plastids. However, as these changes are strongly suppressed, among others, by very low concentrations of CO_2 in air and as their kinetics appear to be too fast and too coordinated for chloroplast movements, it is assumed that the main factor responsible for them is a change in the conformation of chloroplasts. In agreement with this, leaves of *Abutilon striatum* for which the absence of chloroplast movements has been reported[10] show the same changes in their apparent absorbance as other leaves. Mitochondria are not involved since no scattering changes are observed at 530 mμ when N_2 is replaced by air or *vice versa* in the dark with only the measuring beam on.

2. Kinetics

The scattering changes seen at 530 mμ are normally superimposed on fairly large absorbance changes, which probably are caused in part by chlorophyll *b* (maximum at about 513 mμ (ref. 11)). A typical trace is shown for spinach in Fig. 3A. To avoid most changes due to absorption, measurements have been made with a mutant of *Pisum sativum* lacking chlorophyll *b* (Fig. 3B). On illumination with high

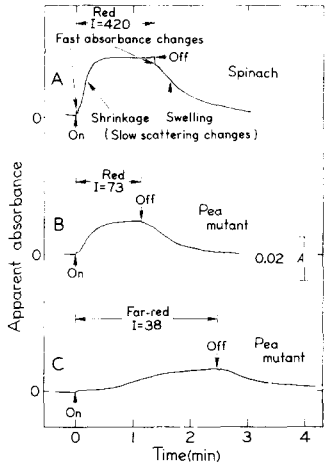

Fig. 3. Changes in the apparent absorbance induced in leaves of spinach and a chlorophyll *b*-free pea mutant by red and far-red light. A slow increase is due to photoshrinkage, a slow decrease to swelling presumably of the grana compartments of chloroplasts. Fast (vertical) changes are caused by the alteration of pigments. Measuring beam at 530 mμ.

intensity red light in CO_2-free air, the shrinkage reaction becomes apparent only after a lag phase of a few sec. This lag is longer at lower light intensities and is especially pronounced in N_2 when the leaf is given far-red light (Fig. 3C). Maximum shrinkage is reached in high intensity red light and air after 20–60 sec. After darkening there is another lag phase before the reaction is reversed; this becomes shorter when the light intensity is reduced. Lowering the temperature results in an extension of both lag phases; shrinkage and its reversal proceed slower. At 0° in CO_2-free air or in N_2, some swelling instead of shrinkage may be observed in the light. A swelling reaction preceding shrinkage can be seen on exposure to low intensity far-red in N_2 at room temperature suggesting that the observed gross changes are composed of a shrinkage and a swelling reaction occurring simultaneously and thus complicate the kinetics of the changes.

3. Intensity dependence

Fig. 4A shows the intensity dependence of the shrinkage reaction of a leaf of *Mimulus verbenaceus*. In red light with CO_2-free air in the gas phase, shrinkage increases with intensity up to high light intensities. In N_2 at lower intensities, light is more effective in producing the shrunken condition than it is in air. After a maximum is reached, however, shrinkage is progressively reversed until the change can no longer be seen. On the other hand, far-red light does not inhibit shrinkage even at high intensities. In CO_2-free air, far-red light fails to produce the shrunken condition

at low intensities; only at high intensities which excite both Photosystems I and II (ref. 12) does shrinkage occur. Intensity dependence similar to that shown for Mimulus has been observed with *Oenothera hookeri, Pisum sativum* and spinach. Spinach required more light than the other plants to produce the effects (Fig. 4B). Correction for the different absorption of leaves in red and far-red light reduces the large differences seen in Fig. 4 between the extent of shrinkage under red and far-red illumination.

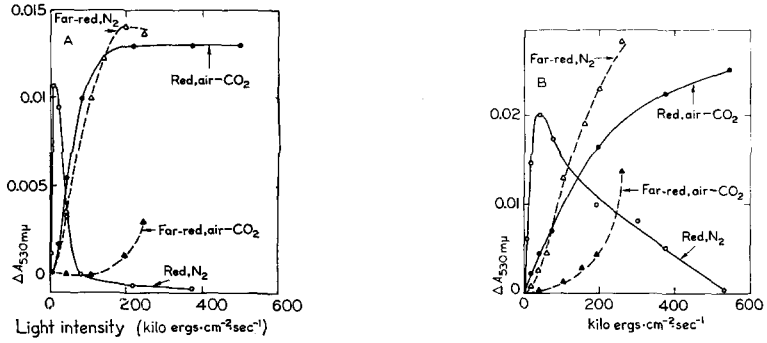

Fig. 4. Extent of photoinduced shrinkage in CO_2-free air or N_2 as a function of the intensity of exciting red or far-red light. In air red promotes shrinkage at high, in N_2 only at low intensities. Far-red produces shrinkage readily in N_2, but in air only at higher intensities. It should be noted that especially the far-red beam includes a considerable proportion of light which is not absorbed by the pigment system of photosynthesis and that the red beam contains also far-red light of a spectral composition similar to that of the far-red beam. (A) Leaf of *Mimulus verbenaceus*. (B) Leaf of *Spinacia oleracea*.

During a change in the gas phase from N_2 to CO_2-free air under continuous illumination, shrinkage is reversed and swelling occurs at low intensity red light; shrinkage is stimulated at high intensity red light. Opposite changes are seen on replacing N_2 by CO_2-free air. Under far-red illumination, shrinkage is always reversed on admission of O_2 to the anaerobic system. Thus it is possible to predict from Fig. 4 the direction and extent of the change in shrinkage which will occur when the gas phase is changed from N_2 to CO_2-free air or *vice versa* under red or far-red illumination at any light intensity.

4. Affinity for O_2

Fig. 5 shows the extent of the scattering change seen at 530 mμ under continuous red or far-red illumination when N_2 is replaced by different concentrations of O_2 in N_2. As has been mentioned in the previous section, the direction of the change (shrinkage or swelling) is determined by the intensity and wavelength of the exciting light. At high intensity red light, low concentrations of O_2 cause a large shrinkage reaction; increasing the O_2 concentration has little effect probably because the system is maximally shrunken. At lower intensities of red light the system responds to low O_2 concentrations first with shrinkage and then to increasing O_2 concentration gradually with swelling (*i.e.* with the reversal of shrinkage). Low intensity red light produces a large swelling reaction at a low O_2 concentration which is increased gradually at higher concentrations. Illumination with far-red light caused related effects although now the inhibiting action of higher O_2 concentrations on shrinkage is more pro-

nounced. Only at the lowest intensity of far-red light this gradual inhibition is not apparent because shrinkage already is completely reversed to the dark state by the lowest concentration of O_2; no further suppression is possible. Monochromatic light of 678 mμ or 709 mμ as a source of excitation yields results similar to those observed when broad bands of red or far-red light are used.

If, for low O_2 concentrations, O_2 concentration over the extent of the scattering change (values taken from Fig. 5) is plotted against O_2 concentration, results depicted in Fig. 6 are obtained. Connection of the experimental points results in lines which intercept with the abscissa at about the same point. Half saturation of the initial response caused by O_2 (contraction and swelling alike) is accordingly at about 0.1% O_2 in N_2 or 1.4 μM O_2. Although extent and direction of the response are strongly influenced by intensity and wavelength of the exciting beam, the affinity appears to be the same under all conditions.

O_2 is known to cause a large fluorescence depression in leaves which were previously kept in N_2 (ref. 13). In order to see whether this O_2 effect is related to its influence on scattering, simultaneous measurements of fluorescence and scattering were performed during changes in the gas atmosphere. At exciting intensities sufficient to suppress scattering in N_2 at least partially fluorescence quenching by air is ac-

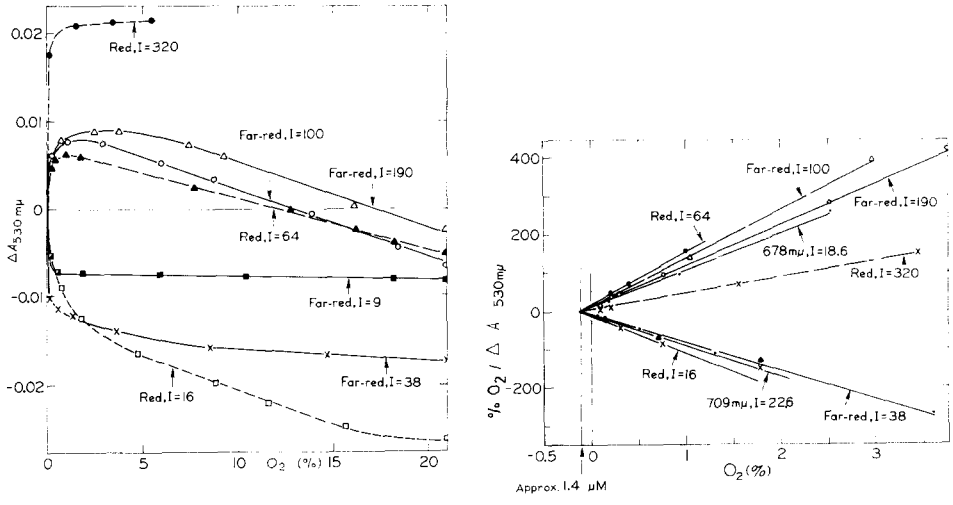

Fig. 5. Extent and direction of the scattering change induced in a spinach leaf under continuous illumination as a function of the concentration of O_2 and of light intensity. Positive changes in the absorbance denote shrinkage, negative change swelling. Reference (ΔA = 0) is the shrinkage condition produced in N_2. Fig. 4 should be consulted for the state of shrinkage of the reference at different light intensities. Two effects of O_2 can be distinguished: a large response, either shrinkage or, at low intensity red or far-red, swelling, to low concentrations of O_2, and a gradual suppression of shrinkage by higher concentrations of O_2.

Fig. 6. Michaelis–Menten plot ($[S]/v$ against $[S]$)* of data shown in Fig. 5 and of other scattering data. The intersection with the abscissa reflects the high affinity of the system for O_2 and gives the concentration of O_2 that produces the half maximal scattering response. Note that the affinity is the same regardless of whether O_2 produces shrinkage or swelling (*cf.* Fig. 5) or whether red or far-red is the source of excitation.

* The extent of the scattering change at low O_2 concentrations has been taken as a measure of the interaction of O_2 with the system.

companied by a shrinkage reaction; the kinetics is almost identical with but antiparallel to that of the fluorescence depression (Fig. 7A). If the light intensity is low and insufficient to excite Photosystem II significantly, the opposite effect is observed: A small fluorescence depression in air is accompanied by inhibition of shrinkage (swelling) and the traces show a parallel course (Fig. 7B). Dissimilarities in the kinetics of fluorescence and scattering are observed at intermediate intensities of exciting light.

5. Influence of CO_2

Changes in shrinkage of a spinach leaf on addition of 0.03% CO_2 are shown in Fig. 8 as a function of light intensity. In the presence of O_2, CO_2 completely reverses shrinkage to the dark state at low intensities of red light and partially at high intensities. In N_2, shrinkage is suppressed at low and stimulated at high light intensities.

The dependence of shrinkage on the CO_2 concentration is analyzed in Fig. 9

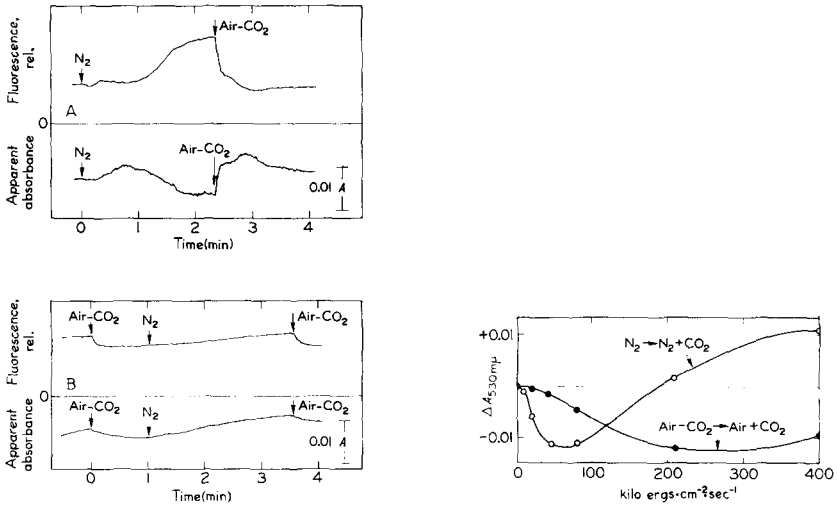

Fig. 7. Simultaneous measurements of the kinetics of fluorescence and scattering by an illuminated leaf of *Pisum sativum* during changes of the gas atmosphere. A downward deflection of the upper trace shows a decrease in fluorescence, an upward deflection of the lower trace an increase in scattering, *i.e.* photoshrinkage. Note the similarities in the kinetics of fluorescence and scattering. (A) Excitation with blue light (3.5 mm Corning filter 5113, peak transmission at 405 mμ, half band width 60 mμ), $I = 12$. (B) Excitation with blue light (Corning filters 5562, 3.5 mm and 3850, 3.5 mm, peak transmission at 410 mμ, half band width 70 mμ, $I = 2.5$). Fluorescence was recorded by a photomultiplier at an angle of about 45° to the exciting beam. The multiplier was protected by the following filters: 2030 (Corning), RG 2 and RG 5 (Schott u. Gen.), Calfex C (Balzer) and an interference filter (Balzer) with a maximum transmission at 684 mμ (half band width 15 mμ). Scattering was recorded in the usual arrangement, but the multiplier was protected from exciting light by two interference filters (Balzer, maximum transmission 534 mμ and 537 mμ, respectively, half band width 15 mμ) and Corning filters No. 3384, 3484 and 3486.

Fig. 8. Effects of CO_2 on the scattering condition produced in a spinach leaf by different intensities of red. Gas phase N_2 or air. CO_2 concentration in N_2 320 ppm, in air normal CO_2 content. Negative changes in absorbance indicate suppression of shrinkage, positive changes stimulation. In air CO_2 suppresses shrinkage up to high intensities, but only at low intensities in N_2. Higher intensities promote shrinkage in N_2.

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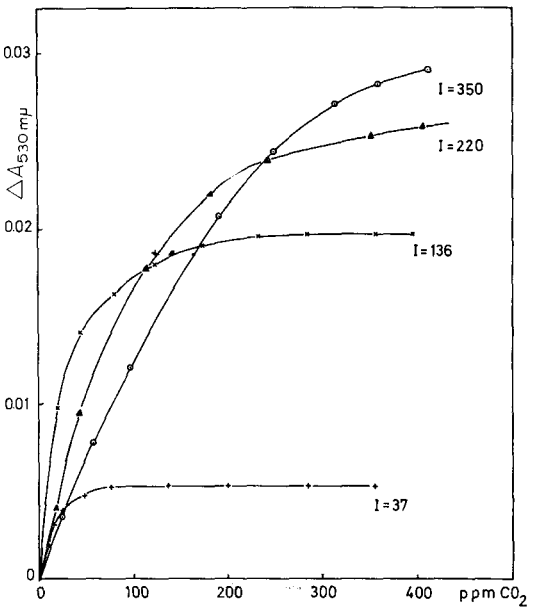

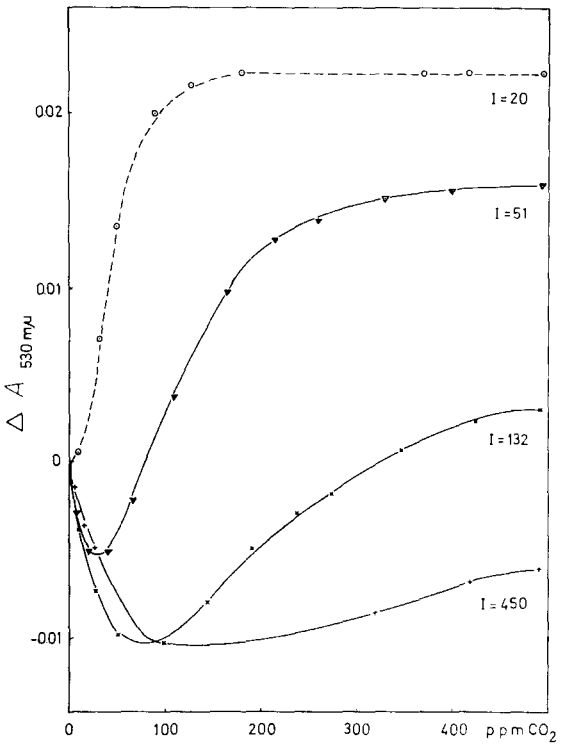

Fig. 9. Inhibition of photo-induced shrinkage in a leaf of *Mimulus cardinalis* by CO_2 as a function of CO_2 concentration and of the intensity of illumination with red. Gas phase: 21 % O_2 in N_2. Measurements were performed with attached leaves. Reference (ΔA = 0) is the shrinkage condition produced in CO_2-free air. Fig. 4 should be consulted for information on the state of shrinkage of the reference at different light intensities. Shrinkage is almost completely reversed to the dark state at the point of saturation of the curves. Ordinate: reversal of photo-induced shrinkage (= swelling) measured as a change in the apparent absorbance of a leaf.

Fig. 10. Inhibition or stimulation of shrinkage in leaves of *Mimulus cardinalis* by CO_2 as a function of CO_2 concentration and of the intensity of illumination with red. Gas phase: N_2. Measurements were performed with attached leaves. Reference (ΔA = 0) is the shrinkage condition produced in N_2. Fig. 4 should be consulted for information on the state of shrinkage of the reference at different light intensities. Negative changes in the apparent absorbance denote stimulation of shrinkage by CO_2, positive changes inhibition of shrinkage (= swelling).

for a leaf of *Mimulus cardinalis* kept under aerobic conditions. Under low intensity illumination, low CO_2 levels are sufficient for a complete reversal of shrinkage to the dark state, while increasingly higher concentrations are required at higher intensities to suppress shrinkage effectively. At extremely high light intensities (not shown), CO_2 no longer causes swelling. In N_2 (Fig. 10) at very low red light intensities shrinkage is suppressed as it is in air; at higher intensities shrinkage is stimulated by low CO_2 concentrations and increasingly suppressed by higher concentrations. At very high intensities, a stimulation of shrinkage persists even at high CO_2 concen-

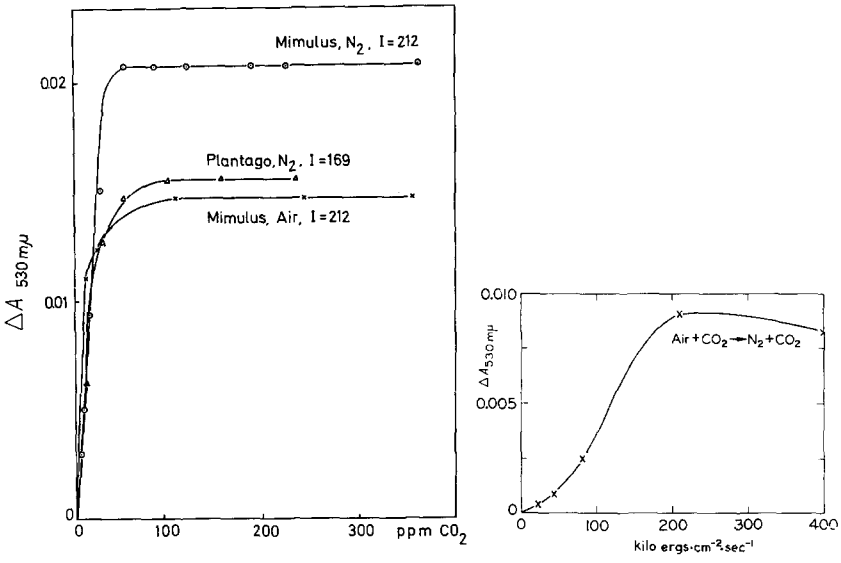

Fig. 11. Inhibition of photoinduced shrinkage under far-red by CO_2 in air or N_2 as a function of CO_2 concentration. Measurements were performed with attached leaves of *Mimulus cardinalis* and *Plantago lanceolata*. Reference is the shrinkage condition produced in N_2 or CO_2-free air. Shrinkage is completely suppressed at the point of saturation in the curve.

Fig. 12. Shrinkage produced in a spinach leaf by replacing normal air by N_2 containing 320 ppm CO_2. Shrinkage is higher in N_2 than in air up to high light intensities. Illumination with different intensities of red.

TABLE I

EFFECTS ON PHOTO-INDUCED SHRINKAGE OF CHLOROPLASTS IN LEAVES CAUSED BY A TRANSITION FROM ONE GAS PHASE TO ANOTHER*

Light	*Intensity*	*CO_2-free air $\rightarrow N_2$*	*CO_2-free air $\rightarrow$ air + CO_2*	*$N_2 \rightarrow N_2 + CO_2$*	*Air + $CO_2 \rightarrow N_2 + CO_2$*
Red	Low	Stimulation	Suppression	Suppression	Little effect
	High	Suppression	Suppression or no effect	Stimulation	Stimulation
Far-red	Low	Stimulation	No effect	Suppression	—
	High	Stimulation	Suppression	Suppression	—

* Changing the gas atmosphere in a direction opposite to that given in Table I results in effects opposite to those shown. The changes are thus reversible.

trations. Under far-red illumination, shrinkage is suppressed both in the presence of O_2 and in N_2 already by very low concentrations of CO_2 (Fig. 11).

On changing the gas atmosphere from air *plus* CO_2 to N_2 *plus* CO_2, shrinkage is increased indicating that the high O_2 content of air is inhibitory (Fig. 12, also RESULTS 4).

Table I summarizes the effects of changes in the gas atmosphere on shrinkage as discussed in RESULTS (3 and 5).

6. Action spectra

In Fig. 13 action spectra of the shrinkage reaction in the absence of CO_2 are shown. In air a distinct maximum is observed around 678 mμ which corresponds to the absorption maximum of chlorophyll *a in vivo*. A shoulder at about 650 mμ is indicative of chlorophyll *b* absorption. The relatively slow decline towards longer wavelengths may be false as the interference filters used transmit some light of wavelengths shorter than indicated by the experimental points. Therefore, the decline beyond 678 mμ is probably somewhat steeper than shown in Fig. 13. Excitation at 700 mμ no longer causes any shrinkage.

Measurements performed in N_2 with the same leaf show a very pronounced peak at 695–700 mμ. However, the action spectrum for the shrinkage reaction in N_2 is distorted because of the inhibiting effect of red light on the change as has been demonstrated in RESULTS 3 and because shrinkage in far-red light is much slower than in red light. The shape of the action spectrum measured in N_2 does not, therefore, represent relative rates of a reaction underlying shrinkage. In spite of these shortcomings, it is apparent that shrinkage is excited in air by red light or light of shorter wavelengths in N_2 and predominantly by far-red light. In addition, light energy is utilized more effectively in producing the shrinkage reaction in N_2 than in air.

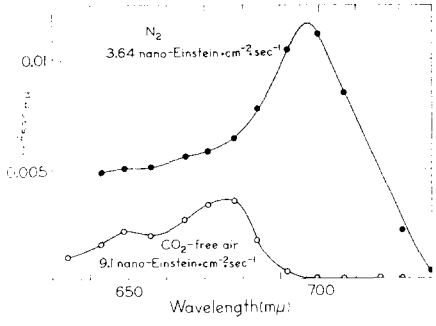

Fig. 13. Action spectra for the shrinkage reaction produced in a spinach leaf under N_2 or CO_2-free air by very low intensities of monochromatic light. In comparing this figure with Fig. 4 it should be noted that the results shown in Fig. 4 were obtained with broad bands of far-red light and of red light containing also far-red.

7. Inhibition of scattering

The scattering changes produced by illumination are completely inhibited by 10^{-4} M DCMU. Mutants of Oenothera having a nonfunctional Photosystem II (ref. 14), but an apparently intact Photosystem I as judged by their ability to photooxidize

cytochrome *f* do not show the scattering effect. A mutant with an impaired Photosystem I has a drastically diminished scattering response. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-trifluorophenylhydrazone (FCCP), uncouplers of phosphorylation which are known to suppress scattering in isolated chloroplasts[15,16] inhibit scattering of leaves completely at 10^{-4} M (CCCP) or saturation (FCCP) (concentrations of solutions, on which leaves were kept floating for approx. 12 h). Atebrin, an uncoupler of phosphorylation, which enhances shrinkage in isolated chloroplasts[17], does not inhibit shrinkage of leaves at low concentrations and only slightly at high concentrations ($5 \cdot 10^{-3}$ mM). To ensure uptake, the compound was fed to leaves through the stalk. Pale green mutant leaves serving as uptake controls became yellow during feeding.

8. Antagonistic and synergistic effects of red and far-red light

Depending on the intensity of the exciting light and on the order of addition, pronounced synergistic and antagonistic effects may be observed on adding red to far-red light or *vice versa*. Strong inhibition of shrinkage is seen on addition of red to far-red light in N_2, if the former is of sufficiently high intensity. The system remains inhibited even after the red beam is turned off. A short dark period is required to restore far-red induced shrinkage. If far-red light of the same intensity is given to red light, shrinkage does not occur. Stimulation of shrinkage is seen in N_2 on adding

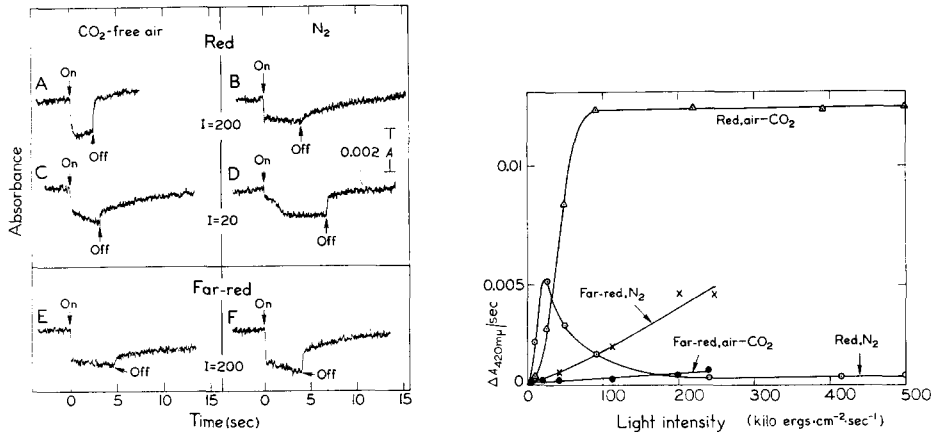

Fig. 14. Kinetics of photooxidation and dark reduction of cytochrome *f* in a leaf of *Mimulus verbenaceus* under anaerobic and aerobic conditions. Measuring beam at 420 mμ. Downward deflection of the trace shows oxidation of cytochrome, upward deflection reduction. A, illumination with red (I = 200) in CO_2-free air. Fast photooxidation and fast dark reduction. B, illumination with red (I = 200) in N_2. Less extensive oxidation and very slow dark reduction. C, illumination with red (I = 20) in CO_2-free air. Biphasic dark reduction with a small fast and a large slow phase. D, illumination with red (I = 20) in N_2. Biphasic oxidation and dark reduction, the latter distinctly faster than in air. E, illumination with far-red (I = 200) in CO_2-free air. Fast oxidation and very slow reduction. F, illumination with far-red (I = 200) in N_2. Biphasic dark reduction with a large fast and a small slow phase.

Fig. 15. Dark reduction of photooxidized cytochrome *f* in a leaf of *Mimulus verbenaceus* as a function of previous illumination with red or far-red light. The rate of dark reduction was calculated from the slope of the initial decay of the signal seen after darkening. Note the similarity to the scattering response as a function of light intensity depicted in Fig. 4A.

low intensity red to low intensity far-red light. In air, synergistic effects of red and far-red light are observed within a proper range of light intensities.

9. Intensity dependence of cytochrome f oxidation

As will be discussed in a later section, the scattering data suggest the occurrence of Photosystem I-dependent cyclic electron flow in N_2 and its inhibition by O_2 or excitation of Photosystem II. If this is correct, it should be reflected by the behavior of a typical Photosystem I reaction. Cytochrome *f*, a component of the reaction center of Photosystem I (ref. 18) exhibits a shift to a more oxidized state upon illumination which is followed by measuring absorbance changes in the Soret region of its spectrum. Traces of absorbance changes at 420 mμ caused by oxidation of cytochrome *f* as indicated by the occurrence of a sharp peak at 420 mμ in the difference spectrum are shown in Fig. 14. In air, oxidation in the light and reduction in the dark proceed fast at high intensities of the exciting beam (Fig. 14A) and somewhat slower at lower intensities (Fig. 14C). In N_2, the downward deflection of the trace (oxidation) is smaller as compared with the reaction in air, and a significant upward deflection of the trace indicating fast initial reduction is seen only at low (Fig. 14D), not at high (Fig. 14B), exciting intensities. Under illumination with far-red light, dark reduction of photooxidized cytochrome *f* is much faster in N_2 (Fig. 14F) than in air (Fig. 14E), which agrees with the concept of cyclic electron flow under N_2 and its inhibition by O_2.

The dependence of the dark reduction on the intensity of previous illumination is shown in Fig. 15. Reduction in air follows a saturation curve, while reduction in N_2 reaches a maximum at low and falls off at higher intensities. This behavior is similar to the intensity dependence of shrinkage in N_2 and air (Fig. 4A).

DISCUSSION

1. Control of cyclic electron flow by Photosystem II and electron acceptors

An interpretation of the scattering phenomena described in the preceding sections for intact leaves relies on related work with isolated chloroplasts. Similar scattering changes have been observed and explained as a consequence of the transfer of protons into the interior of thylakoids during electron transport. During the energy-dependent inflow of protons into grana compartments, electroneutrality partially is maintained by an outflow of cations and of undissociated weak acids which were present as anions[2,19,20]. This decreases the osmotic potential inside the thylakoids and causes an outflow of water resulting in the observed volume decreases and scattering increases. Under phosphorylation conditions, *i.e.* in the presence of ADP and phosphate, volumetric decreases and scattering increases were significantly smaller presumably because the proton flow into the grana compartments partially was compensated by a loss of protons due to phosphorylation[15].

Assuming that the theoretical framework explaining the observations with isolated chloroplasts reflects the situation correctly, the following hypothetical explanation for the effects seen with intact leaves is offered.

Because electron flow is a prerequisite of photo-induced shrinkage in isolated chloroplasts, the scattering changes seen in intact leaves in CO_2-free air or in N_2, signify electron transport in chloroplasts driving ion and water movements. In CO_2-

free air O_2 serves as an electron acceptor, probably in a Mehler-type reaction. Evidence for this has been presented[21] and more will be discussed later on. The action spectrum for shrinkage in air (Fig. 13) is consistent with this view.

In N_2 no external electron acceptor is available and electron transport occurs cyclically. Indeed far-red light, *i.e.* excitation of Photosystem I alone, is sufficient to cause shrinkage. The action spectrum for the changes in N_2 is markedly different from that obtained in air and reflects a distorted Photosystem I spectrum. In isolated chloroplasts phenazine methosulfate-supported cyclic electron transport is known to be a Photosystem I reaction.

In red light and in N_2, a complicated intensity dependence of shrinkage has been observed in leaves (Fig. 4); shrinkage is seen only at low and is progressively inhibited at higher intensities. This effect appears to be caused by the different excitation characteristics of the two photosystems. Even at low intensities excitation of Photosystem I induces cyclic electron flow resulting in shrinkage. Photosystem II is not yet significantly excited to inhibit Photosystem I and thereby shrinkage; only at higher light intensities does it inhibit the cyclic electron flow. The mechanism of this inhibition is unclear; one possibility is "over-reduction". Cyclic electron flow requires oxidized electron carriers on the oxidizing side of the reaction centers, and at higher intensities Photosystem II activity shifts the redox state of these carriers towards reduction. As they become more reduced, they are no longer available as electron acceptors for Photosystem I generated electron flow and cyclic electron transport stops, although some photooxidation of cytochrome *f* is still seen on illumination (Fig. 14B). This view assumes cyclic electron transport to be a quasi-reversible system with controlled states of electron transfer *in vivo*.

If this explanation of cyclic electron flow inhibition by Photosystem II is correct, less turnover of cytochrome *f* which is the electron donor to the reaction centers of Photosystem I should occur under high than under low intensity red light in N_2, while the opposite should be true in air. This expectation is verified in the experiment shown in Fig. 15.

Cyclic electron flow is controlled by the availability of electron acceptors. Under far-red and under low intensity red illumination both O_2 and CO_2 inhibit cyclic electron transfer effectively as can be seen from their effect on shrinkage. Consistent with this is the slow dark reduction of far-red oxidized cytochrome *f* in air and the faster reduction in N_2 (Figs. 14E and 14F). The results indicate that in leaves cyclic electron transport occurs at low light intensities only under N_2, while under aerobic conditions O_2 is preferably reduced. Under high light intensities the rate of reduction of electron acceptors and the redox state of electron carriers between the photosystems will determine whether cyclic electron flow can occur. From the high affinity of the electron transport chain for O_2, it may be concluded that cyclic electron transfer is only possible when the reaction with O_2 is saturated. It should be noted that noncyclic electron flow to O_2 is coupled to phosphorylation[22] and that light-dependent O_2 consumption has been shown to occur *in vivo*[23].

The antagonistic and synergistic effects of red and far-red light on shrinkage of leaves (described in RESULTS 8) are a direct consequence of the intensity dependence of shrinkage in N_2 and in air and from the results shown in Fig. 4 can be explained in terms of inhibited or stimulated electron transfer.

It should be noted that the intensity dependence of shrinkage under N_2 and

CO_2-free air and the effects observed on adding a red beam to a far-red beam can scarcely be accommodated in a model of photosynthesis which involves two photoreactions with independent electron transport pathways such as proposed by ARNON *et al.*[21]. Rather the observations add to the already abundant evidence in favor of a scheme with two photoreactions acting in series.

However, although most results fit easily into such a scheme, some facts remain unexplained. Photosystem II deficient mutants of Oenothera[14] and DCMU poisoned normal leaves do not show the shrinkage reaction in either N_2 or air although their Photosystem I appears to be unimpaired as judged by their ability to photooxidize cytochrome *f*. Chloroplasts isolated from the mutants can form ATP in a cyclic type of photophosphorylation with phenazine methosulfate as a cofactor, again indicating functioning of Photosystem I (ref. 25), and DCMU poisoned leaves appear to be capable of cyclic phosphorylation[26]. Why then do the mutants and the DCMU poisoned leaves not exhibit shrinkage under N_2? Why is there considerable cytochrome *f* oxidation in high intensity red light under N_2 (Fig. 14B), although cyclic electron transport is inhibited under these conditions, presumably by "over-reduction" of electron carriers between Photosystems I and II?

While most of the pieces of the puzzle fit into place, some might need readjustment.

2. Interaction of O_2 with the electron transport chain

There are two effects of O_2 on the leaf system. As shown in Fig. 5, a large and sensitive initial response is followed by an almost linear suppression of shrinkage with an increasing O_2 concentration. A linear inhibition of photosynthesis by O_2 has repeatedly been noticed[27,28]. Whether there is a direct relation to the gradual reversal of shrinkage by O_2 requires further experimentation.

The sensitive initial response may, depending on the quality and intensity of the exciting light, be either shrinkage or swelling. In both cases half saturation of the effect is at about 1.4 μM O_2 suggesting that the same reaction of O_2 with the electron transport chain causes shrinkage when both photosystems are involved and swelling, when only Photosystem I is involved. An interpreation for this has been given in DISCUSSION I.

Where does O_2 interact with the electron transport chain? As far-red light is sufficient to cause shrinkage in N_2 and O_2 suppresses it, obviously a component of Photosystem I is oxidized. CO_2 causes the same suppression of far-red induced shrinkage as does O_2 (*cf.* Figs. 5 and 11), and it is likely that O_2 interacts with one of the same electron donors that ultimately reduce CO_2. In agreement with this isolated chloroplasts exhibit O_2 uptake in a Mehler-type reaction even after addition of DCMU, if an electron donor system is present[21,29].

In intact leaves O_2 causes a large fluorescence depression[13]. While for different batches of spinach leaves obtained from a local market somewhat lower affinities were calculated from fluorescence measurement (apparent Michaelis constants for fluorescence quenching, *i.e.* the O_2 concentration that produces half maximal quenching, were 5–7 μM (ref. 21)), the same high affinity for O_2 (apparent K_m 1–2 μM) as was seen for the scattering change in freshly picked spinach leaves was also measured for the fluorescence depression by O_2. This suggests that the same reaction with O_2 causes both the fluorescence depression and the scattering response. This view is

supported by simultaneous measurements of fluorescence and scattering during changes of the gas atmosphere, which at higher intensities of exciting light show almost identical although antiparallel kinetics (Fig. 7A) and at lower intensities parallel kinetics (Fig. 7B). In the former case shrinkage on admission of O_2 marks the onset of noncyclic electron transfer to O_2; in the latter suppression of shrinkage by O_2 indicates the inhibition of cyclic electron transfer. The fluorescence quenching can be considered as an indirect effect of O_2 which acts primarily on a component of Photosystem I and only secondarily *via* a shift in the redox state of electron carriers on the quencher causing a drop in fluorescence.

CO_2, in accordance with its role as the main electron acceptor in photosynthesis, quenches fluorescence even more effectively than does O_2. Measurements performed with attached leaves in N_2 at very low CO_2 concentrations show that half maximal fluorescence quenching occurs at approx. 20 ppm CO_2. This compares with an O_2 concn. of approx. 1000–2000 ppm that gives half maximal fluorescence quenching.

From the affinities of the electron transport chain for CO_2 and O_2, it is suggested that the relative rates of electron flow to these acceptors are regulated by the redox state of $NADP^+$ and the demand for ATP. Reduction of 1 mole CO_2 to the sugar level requires 2 moles of NADPH and at least 3 moles of ATP. $NADP^+$ reduction by isolated chloroplasts under phosphorylating conditions results in ATP formation in a 1:1 ratio. From this there would appear to be a shortage of ATP *in vivo*, if electron transport to $NADP^+$ were the sole source of ATP formation. As phosphorylation is coupled to electron transport, a shortage of ATP would increase the NADPH to $NADP^+$ ratio. This would direct electrons to O_2 with concomitant ATP formation. Only when the reaction with O_2 is saturated would the cyclic pathway be open for ATP formation. ATP supply would then, through CO_2 reduction, lower the NADPH to $NADP^+$ ratio and $NADP^+$ would again become available as an electron acceptor. Under steady state conditions, a balanced flow of electrons would result to CO_2 and O_2 and under high intensity illumination perhaps also within the cyclic pathway.

3. Effects of CO_2 on shrinkage

Shrinkage appears to be closely related to phosphorylation in that the chemical potential generated in the light across the chloroplast membranes, if not used for and partially quenched by phosphorylation, gives rise to the movements of osmotically active material which result in shrinkage[2]. The relation to phosphorylation is also demonstrated by uncoupler work[1,2] with isolated chloroplasts. Uncouplers of phosphorylation strongly affect shrinkage[16]. Although experiments involving feeding of uncouplers to intact tissue should be interpreted with reservation, results with CCCP, FCCP and atebrin fed to leaves are in line with reported work on isolated chloroplasts in that the former compounds completely eliminated shrinkage without abolishing absorbance changes; the latter left shrinkage largely unaffected.

The effects of CO_2 on shrinkage can be explained if shrinkage is understood as an energy requiring reaction taking place only in the absence of active phosphorylation. More specifically, if high ATP and low ADP levels do not permit significant rates of phosphorylation, the proton gradient established by illumination will lead to shrinkage. The latter cannot be expected to reach considerable proportions if the proton gradient is used for efficient phosphorylation. This will be the case in the presence of CO_2 whose reduction requires in addition to electrons phosphate energy

and provides ADP and phosphate. Consequently in air under low intensity illumination shrinkage is effectively suppressed by CO_2. At higher intensities shrinkage will persist at low CO_2 levels which do not permit efficient photosynthesis; at higher levels it will be suppressed due to fast phosphorylation. These relations may explain the CO_2 dependence of shrinkage in air (Fig. 9).

In N_2 the situation is more complicated. Low intensities of red light in N_2 result in cyclic electron transport and shrinkage. CO_2 acts inhibitory as it induces photosynthesis stimulating phosphorylation and thereby lowering shrinkage. At higher intensities of red light, however, cyclic electron flow and shrinkage are inhibited in N_2 by Photosystem II activity. Addition of low levels of CO_2 relieve this inhibition stimulating electron transport. Cyclic electron transport may become possible in addition to the noncyclic electron flow to CO_2 and lead to shrinkage. The latter becomes progressively inhibited by higher concentrations of CO_2 simply because the proton gradient is used for phosphorylation rather than for the shrinkage reaction (Fig. 10).

Under far-red illumination, CO_2 probably inhibits shrinkage by intercepting electrons and by interrupting Photosystem I-mediated cyclic electron flow. The affinity of the system for CO_2 is surprisingly high. Under far-red illumination (Fig. 11), half maximal inhibition of shrinkage occurs at a CO_2 concn. of about 10–20 ppm in the gas phase. This is the same concentration which results in the half maximal fluorescence quenching of attached leaves in N_2 again indicating that the fluorescence quenching is an indirect effect caused primarily by the oxidation of a Photosystem I component (*cf.* DISCUSSION 2).

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