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Chloroplast Response to Low Leaf Water Potentials

III. DIFFERING INHIBITION OF ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION[1]

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ABSTRACT

Cyclic and noncyclic photophosphorylation and electron transport by photosystem 1, photosystem 2, and from water to methyl viologen ("whole chain") were studied in chloroplasts isolated from sunflower (*Helianthus annus* L. var Russian Mammoth) leaves that had been desiccated to varying degrees. Electron transport showed considerable inhibition at leaf water potentials of −9 bars when the chloroplasts were exposed to an uncoupler *in vitro*, and it continued to decline in activity as leaf water potentials decreased. Electron transport by photosystem 2 and coupled electron transport by photosystem 1 and the whole chain were unaffected at leaf water potentials of −10 to −11 bars but became progressively inhibited between leaf water potentials of −11 and −17 bars. A low, stable activity remained at leaf water potentials below −17 bars. In contrast, both types of photophosphorylation were unaffected by leaf water potentials of −10 to −11 bars, but then ultimately became zero at leaf water potentials of −17 bars. Although the chloroplasts isolated from the desiccated leaves were coupled at leaf water potentials of −11 to −12 bars, they became progressively uncoupled as leaf water potentials decreased to −17 bars. Abscisic acid and ribonuclease had no effect on chloroplast photophosphorylation. The results are generally consistent with the idea that chloroplast activity begins to decrease at the same leaf water potentials that cause stomatal closure in sunflower leaves and that chloroplast electron transport begins to limit photosynthesis at leaf water potentials below about −11 bars. However, it suggests that, during severe desiccation, the limitation may shift from electron transport to photophosphorylation.

Although stomatal closure generally occurs when higher plants are desiccated (28), chloroplast activity also changes (4, 5, 7, 12, 22) and, in some instances (4), may be more limiting to photosynthesis than is the reduced supply of CO_2 due to stomatal closure. In several studies, chloroplasts isolated from leaves that were moderately desiccated had a reduced capacity for electron transport measured as Hill activity (12) or oxygen evolution (7, 25) in the presence of DCIP.[3] During severe desiccation, cyclic photophosphorylation was also inhibited (22), but it was not clear whether this effect occurred only during severe desiccation or during moderate desiccation as well. This study was therefore undertaken to compare the inhibition of photophosphorylation and electron transport at the same water potentials that bring about the early effects of desiccation on photosynthesis in intact leaves.

MATERIALS AND METHODS

Sunflower (*Helianthus annuus* L. var. Russian Mammoth) plants were grown from seed in soil under conditions previously described (25). Except where it is indicated otherwise, all the experiments were done with 3- to 3.5-week-old plants. Recently mature leaves, usually at the third and fourth nodes from the bottom of the plant, were washed while intact and permitted to dry before the experiments. One-half of each leaf was then excised and desiccated to varying degrees, usually for less than 1 hr, under growth conditions. The other leaf-half served as the control and remained attached. Control activity was unaffected by attachment or detachment of the leaf, providing the leaf remained well hydrated. During the treatment, leaf temperatures were within 2 C of the ambient air regardless of the severity of desiccation.

Leaf Water Potential. A thermocouple psychrometer was used to measure ψ_w by isopiestic technique (8), as previously described (4). Samples for ψ_w were obtained from all the tissue to be used for a particular chloroplast isolation.

Chloroplast Isolation. After sampling for ψ_w, the remainder of the leaf tissue (1–2 g) was cut into a micro Waring Blendor containing 20 ml of isolation medium at 0 C made up of 0.33 M sorbitol; 0.1 M KCl; 5 mM $MgCl_2$; 20 mM Tricine, pH 8.0; and 5 mM sodium ascorbate (18) and was ground for 15 sec. The brei was strained through nylon net (10 μM openings), the filtrate was centrifuged at 500g for 4 min, and the pellet was resuspended in ascorbate-free isolation medium. The Chl content of this suspension was determined according to Arnon (1).

Chloroplast Assays. The activity of chloroplasts for electron transport was determined by three basic assays. In the first, the rate of photoreduction of DCIP was used as a measure of the activity of photosystem 2. Assay conditions were: 2 ml of assay medium containing 0.1 M KCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 45 μM DCIP, and 10 μg of Chl. The side of the sample was irradiated with actinic light from a slide projector fitted with an infrared filter and red Corning filter No. 2403 to give an intensity of 0.74 cal cm^{-2} min^{-1} which was saturating. The initial rate of DCIP reduction at 19 C was determined spectrophotometrically at 600 nm (photomultiplier was fitted with a blue blocking filter Corning No. 5433). In some experiments, interference filters provided red (Baird Atomic 645 nm, 10 nm half-band width) and far red (Baird Atomic 715 nm, 10 nm

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[3] Abbreviations: DCIP: sodium 2,6-dichloroindophenol; MV: methyl viologen; PMS: phenazine methosulfate; ψ_w: leaf water potential; PS: photosystem.

half-band width) actinic light and were used in place of Corning filter No. 2403 (31).

In the second type of assay, electron transport through photosystem 1 was measured by using the electron donor reduced DCIP in the presence of MV. Assay conditions were: 2.9 ml of assay medium containing 1 mM ADP, 1 mM K_2HPO_4, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM DCMU, 80 μM DCIP, 1 mM sodium ascorbate, 0.5 mM MV, 0.5 mM sodium azide (prepared daily), and 10 μg/ml of Chl (17). The assay mixture was irradiated with saturating (1.0 cal cm^{-2} min^{-1}) incandescent light that had been filtered through 20 cm of water. The early steady rate of oxygen uptake was measured with a Clark-type oxygen electrode (7, 17, 21) at 19 C.

In the third assay, electron transport from water to MV was measured and will be termed "whole chain" transport for convenience. Assay conditions were identical to those for PS 1, except that DCMU, DCIP, and sodium ascorbate were deleted from the medium.

In addition to the assays for electron transport, two assays were used to determine the photophosphorylating activity of the chloroplasts. In the first, noncyclic photophosphorylation was determined in an assay medium (4.5 ml) of the same composition as that used for whole chain electron transport. The assay mixture was irradiated with the same light source used for assays of PS 1 and whole chain electron transport. The rate of ATP production was measured with a pH meter as the initial, stable rate of hydrogen ion production in the assay medium at 19 C (23).

In the second photophosphorylation assay, cyclic photophosphorylation was measured in the same way as noncyclic photophosphorylation, except that 5 μM PMS was substituted for MV and sodium azide.

The optimum pH for conducting these assays was near 8.0, and consequently all assays were carried out at pH 8.0 (electron transport) or at an initial pH of 8.0 (photophosphorylation).

RESULTS

Figure 1 shows that PS 2 activity was reduced relative to that of the well watered controls ($\psi_w = -3$ bars) when chloroplasts were isolated from leaf-halves having ψ_w below -11 bars. By comparison with tissue from 3- to 3.5-week-old plants, the inhibition was more severe when leaf tissue was obtained from the same plants at a later time (3.5–4 weeks), even though the leaves were comparably developed (Fig. 1). In the tissue from the older plants, there appeared to be a low residual activity which remained during severe desiccation and was independent of ψ_w.

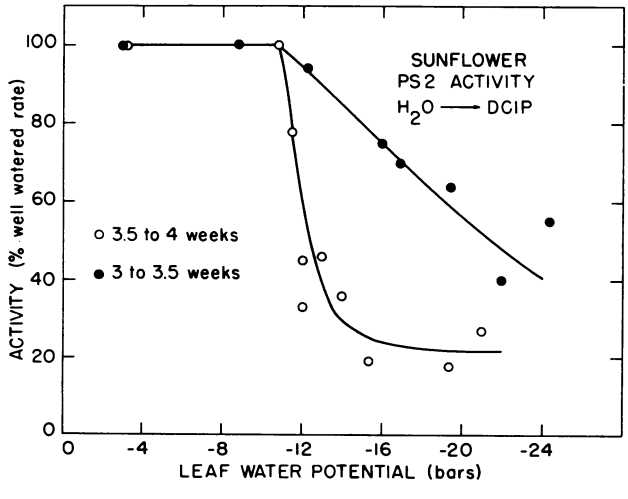

FIG. 1. Photoreduction of DCIP by PS 2 in sunflower chloroplasts isolated from leaf-halves that had been desiccated to varying degrees. Measurements at 3 to 3.5 weeks and 3.5 to 4 weeks were made with the same plants on comparably developed leaves. Control rates were 200 to 280 μmoles e^-/hr·mg Chl.

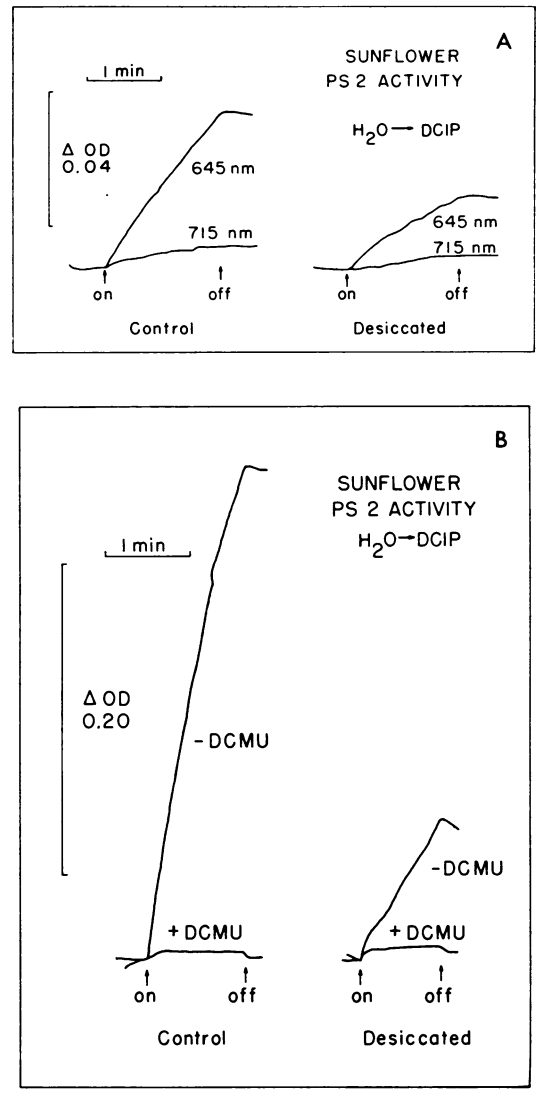

FIG. 2. Photoreduction of DCIP by sunflower chloroplasts isolated from leaf-halves having (A) a water potential of -3 bars (control) and -15.5 bars (desiccated) in light at 645 nm (5.2×10^{-2} cal cm^{-2} min^{-1}) and 715 nm (5.2×10^{-2} cal cm^{-2} min^{-1}) and (B) a water potential of -3 bars (control) and -21.0 bars (desiccated) in the presence and absence of 0.1 mM DCMU in red light (Corning filter No. 2403, which included both 645 and 715 nm light) at a saturating intensity of 0.74 cal cm^{-2} min^{-1}.

Although DCIP accepts electrons primarily from PS 2 (19, 26), there also may be a contribution from PS 1 under certain conditions (15, 19). To test whether PS 1 became more involved in the assay when sunflower chloroplasts were isolated from desiccated leaves, two experiments were done. In the first, the photoreduction of DCIP by the chloroplasts was determined in actinic light at 715 nm, which stimulated PS 1, and at 645 nm, which stimulated PS 2 and, to a lesser extent, PS 1 (20). Regardless of ψ_w, however, the photoreduction occurred seven times more rapidly at 645 nm than at 715 nm (Fig. 2A). In the second experiment, DCMU, which blocks

electrons flowing from PS 2 to PS 1, was added to the reaction medium. Electron transport was completely inhibited by this addition, whether or not the tissue had been desiccated (Fig. 2B). These experiments show that electrons moved through PS 2, and that PS 1 alone could not photoreduce DCIP appreciably. Previous work (25) demonstrated that the stoichiometry for reduced DCIP/O_2 is 2 at all ψ_w, so that there was no change in electron donation by water as a result of leaf desiccation. Consequently, the over-all route of electrons in this assay was unaltered by desiccation, and the photoreduction of DCIP remained primarily due to PS 2 (19, 26). Apparently, our previous assays of O_2 evolution and DCIP reduction can be interpreted as PS 2 assays as well (7, 25).

Electron transport by PS 1 and by the whole chain were inhibited in chloroplasts isolated from leaves with ψ_w below −11 bars (Fig. 3, A and B). As with PS 2 activity, there was a stable residual activity which remained at ψ_w below −17 bars. The residual activity for the whole chain appeared to be less than that of either PS 2 or PS 1 alone. To investigate this possibility more thoroughly, the residual activity for electron transport by the whole chain, PS 2, and PS 1 were assayed with the same suspension of chloroplasts from 3-week-old sunflower. Three replicate determinations showed whole chain, PS 2, and PS 1 activities of 15, 40, and 70%, respectively. Thus, a portion of whole chain transport between PS 2 and PS 1 appeared to be more inhibited than either of the photosystems themselves. Plastocyanin, a particularly labile component of the electron transport chain between PS 2 and PS 1 (2), failed to relieve these effects on the residual activity when it was added in concentrations that returned electron transport of plastocyanin-depleted chloroplasts to the maximum rate.

Both cyclic and noncyclic photophosphorylation were inhibited when chloroplasts were isolated from leaf tissue with a ψ_w below −11 bars (Fig. 3, C and D). The decline began at virtually the same ψ_w as that for coupled electron transport (Fig. 3, A, B, C, and D). In contrast to the findings with electron transport, photophosphorylation became zero and there was no stable, residual photophosphorylation at ψ_w below −17 bars. The sharp decline in photophosphorylation was highly reproducible and similar for both cyclic and noncyclic forms.

The stability of electron transport compared with photophosphorylation in chloroplasts from leaves desiccated below −17 bars suggests that desiccation ultimately uncoupled the chloroplasts. This possibility was explored by measuring coupled and uncoupled electron transport in the same chloroplast preparation. Table I shows that in well watered tissue,

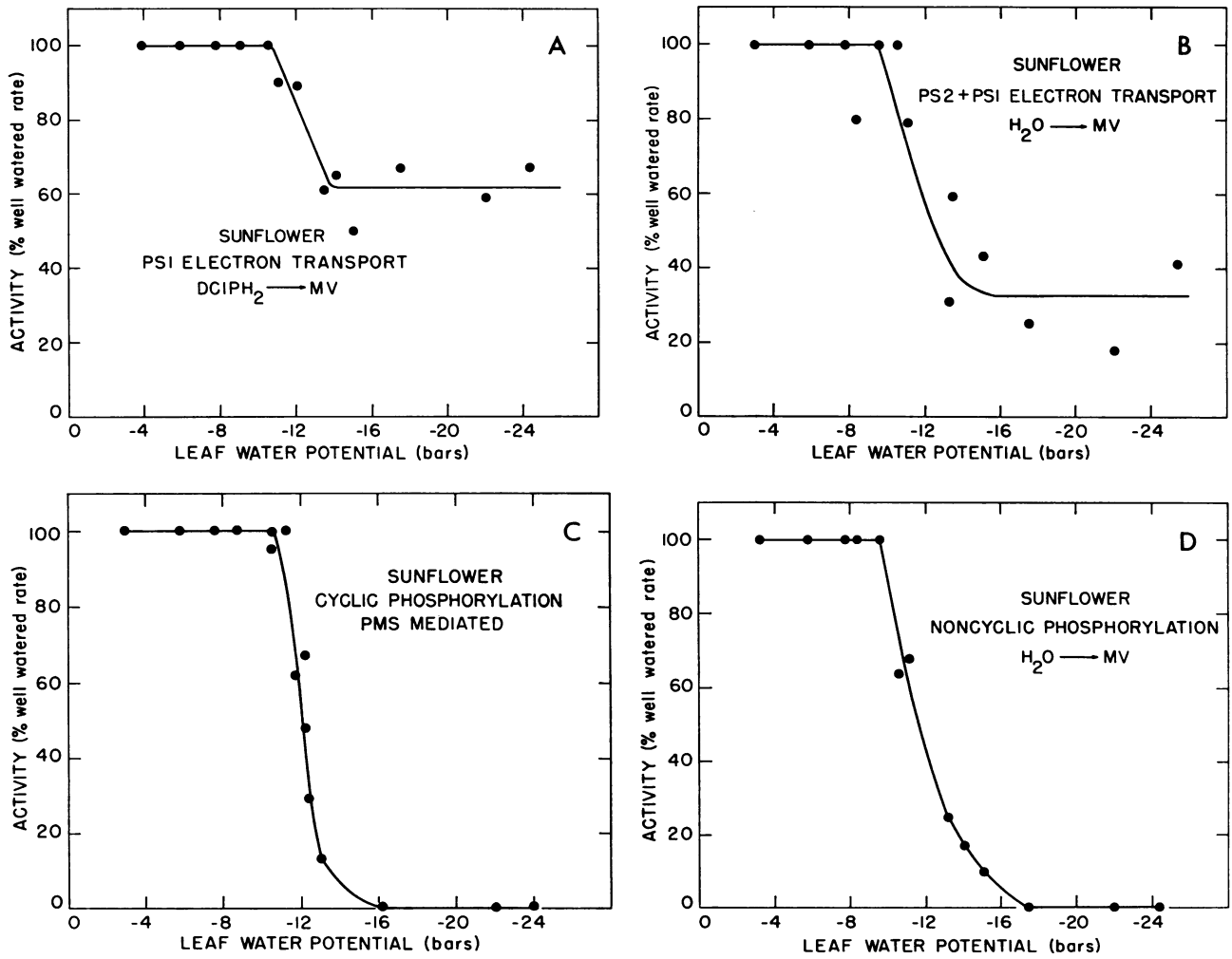

FIG. 3. Activity of sunflower chloroplasts isolated from leaf-halves that had been desiccated to varying degrees. A: photosystem 1 electron transport (control rates = 745 to 2890 μmoles e^-/hr·mg Chl); B: electron transport from water to methyl viologen (control rates = 306 to 1360 μmoles e^-/hr·mg Chl); C: cyclic photophosphorylation (control rates = 785 to 1930 μmoles e^-/hr·mg Chl); D: noncyclic photophosphorylation (control rates = 560 to 1100 μmoles e^-/hr·mg Chl).

Table I. *Coupled and Uncoupled Electron Transport in Chloroplasts Isolated from Well Watered and Desiccated Sunflower Leaf-halves*

Assay conditions are as described under "Materials and Methods," except in uncoupled chloroplast assays which contained, in addition, 0.5 mM NH_4Cl. Well watered leaf-halves were from the same leaf as the desiccated leaf-halves for each comparison. Well watered tissue had a leaf water potential of −3 to −4 bars.

Leaf Water Potential	Coupled Activity		Uncoupled Activity		Uncoupled/ Coupled Rates of e⁻ Flow
	μmoles e⁻ / mg Chl·hr	% of well watered rate	μmoles e⁻ / mg Chl·hr	% of well watered rate	
					ratio
PS 1 electron transport ($DCIPH_2$ ——→MV					
Well watered	780	100	1670	100	2.14
−11.7 bars	785	100	1420	85	1.81
Well watered	705	100	1560	100	2.21
−14.3 bars	522	74	890	57	1.71
Well watered	855	100	1490	100	1.74
−16.8 bars	410	48	780	52	1.90
Well watered	820	100	1820	100	2.22
−20.9 bars	335	41	446	24	1.33
Whole chain electron transport (H_2O—→ MV)					
Well watered	615	100	1210	100	1.97
−9.0 bars	650	106	930	77	1.43
Well watered	354	100	780	100	2.20
−14.3 bars	260	74	465	60	1.78
Well watered	223	100	408	100	1.83
−17.5 bars	149	67	149	37	1.00
Well watered	316	100	558	100	1.76
−25.4 bars	130	41	149	27	1.14

uncoupling the chloroplasts with ammonium ion approximately doubled the rate of electron flow. In the desiccated tissue, however, uncoupling had less effect as ψ_w decreased. Apparently, at these ψ_w, both whole chain and PS 1 electron transport had already been uncoupled by desiccation and subsequent addition of an uncoupler did not alter the rate of electron flow.

Apart from the effect of desiccation on chloroplast coupling, however, Table I also shows that the response of electron transport to desiccation was different for coupled and uncoupled chloroplasts. For both whole chain and PS 1 electron transport, coupled activities were unaffected at ψ_w of −9 and −11.7 bars, but uncoupled electron transport was inhibited by 23 and 15%, respectively. Thus, uncoupled electron transport was affected before any of the other assays.

Since photophosphorylation ultimately was highly sensitive to leaf desiccation, it was used to study some possible means by which desiccation might cause changes in chloroplast activity. It has been shown that the effects of desiccation on oxygen evolution and DCIP reduction are unlikely to result from changes in the free energy of water (9, 25). An alternative would be that desiccation brought about a change in the cytoplasmic content of some substance(s) which then interacted with the chloroplasts. Two such substances known to undergo changes in concentration during desiccation are ABA and RNase. Growth regulators can influence the photosynthetic activity of whole plants (27, 29) and RNase is known to uncouple mitochondria (13, 14). However, ABA failed to have an effect (6) on cyclic or noncyclic photophosphorylation when sunflower leaves were floated on ABA solutions (0.1 mM, plus sodium phosphate buffer, 10 mM; pH 6.5) for 1 to 3 hr or when chloroplasts were assayed directly in the presence of ABA (1 μM). ABA had entered the leaf tissue, since measurements with a porometer indicated stomatal closure had occurred (tissue floating on buffer without ABA was used as the control). RNase (bovine pancreatic RNase, concentrations as high as 100 μg/ml in the assay medium) also did not have any influence on cyclic photophosphorylation.

DISCUSSION

In attached sunflower leaves, the rate of photosynthesis decreases at ψ_w below −8 bars and becomes negligible at ψ_w of −20 to −25 bars (3, 4, 7). The present data show that, in chloroplasts from leaves having these ψ_w, both photophosphorylation and electron transport are inhibited. Uncoupled electron transport (PS 1 and whole chain) was appreciably inhibited at ψ_w of −9 bars. Coupled electron transport and photophosphorylation then began to decrease at ψ_w of about −11 bars. Except for PS 2 in young plants, the final activity in all the assays was reached at ψ_w of about −17 bars. Between ψ_w of −11 and −17 bars, the activity measured for whole chain electron transport and both types of photophosphorylation decreased at least as much on a percentage basis as did photosynthesis in the intact leaves (3, 4, 7). At ψ_w below −17 bars, however, electron transport was generally independent of leaf desiccation despite the ultimate decline in photosynthesis to zero in attached leaves. This residual electron transport was so stable that we have detected PS 2 activity in chloroplasts from nonliving, air-dried leaf tissue.

Unlike the situation with electron transport, no photophosphorylation could be detected at ψ_w below −17 bars. Since photosynthesis continued at a low and declining rate in intact leaves at these ψ_w, photosynthesis *in vivo* may have been dependent on some source other than photophosphorylation for ATP. In sunflower, dark respiration continues at appreciable rates at ψ_w lower than −17 bars (3) and possibly could represent a source of ATP for photosynthesis, although some form of high energy phosphate would have to cross the outer membrane of the chloroplasts for this mechanism to operate. Alternatively, the chloroplasts could have lost a factor essential for photophosphorylation during isolation and the decline of photophosphorylation to zero might not have occurred *in vivo*. Nevertheless, photophosphorylation represents the only partial reaction which we have found thus far that can account for the eventual decline of *in vivo* photosynthesis to zero (3, 5).

Although the chloroplast assays may have been subject to artifacts such as the loss of essential factors during isolation, we do not think this problem constituted a major source of error for the following reasons. First, the inhibition of electron transport can be detected as a decrease in the photochemical activity of the intact leaves (4, 7, 9). The decrease was of the same magnitude for both PS 2 and photochemical activity when we used similar leaf material for the *in vitro* and *in vivo* experiments (7). Second, both photochemical activity (5) and electron transport (25) recover *in vivo* when the tissue is rehydrated. Consequently, the changes in electron transport which were measured *in vitro* also appeared to occur *in vivo*.

Because the inhibition of PS 2 showed a large dependence on the age of the plant material, it is not possible presently to

determine which partial reaction of electron transport limited *in vivo* photosynthesis the most. However, PS 2 and whole chain electron transport clearly appeared to be more affected than PS 1.

The effect of age is also important in another sense, because it suggests that the inhibitory effects of desiccation on chloroplasts may not be a biological constant dependent only on the species, but may be influenced by the degree of development of the plant or leaves or possibly the specific conditions under which the plants were grown. The age effect serves to illustrate the point that quantitative extrapolations from chloroplasts to the whole leaf level must be approached with caution unless measurements at the two levels have been carried out with tissue that is comparable in every way. To obtain high control activities, the present work was done with sunflower that was younger than that used in our earlier studies (3–5, 7) and consequently the quantitative inhibition of chloroplast activity differs slightly from that in the earlier work. Nevertheless, the water potentials at which the chloroplasts are affected and the general response of the partial reactions are consistent with the behavior of photosynthesis noted earlier.

Although stomatal closure occurs in sunflower as photosynthesis decreases during desiccation, neither the diffusive resistance of the stomata nor other diffusive resistances limit photosynthesis at ψ_w below -11 bars (4). We have presented evidence that the inhibition at these ψ_w may instead be due to some aspect of electron transport (4, 7). The present data generally confirm this idea, since virtually every aspect of electron transport which was assayed showed an inhibition due to desiccation at these ψ_w. However, since there was residual activity for electron transport at severe desiccation levels (ψ_w of -20 to -25 bars) but photosynthesis *in vivo* showed complete inhibition, electron transport cannot account for the total loss of photosynthetic activity that eventually occurs at ψ_w of -20 to -25 bars. In view of the insensitivity of leaf photosynthesis to temperature at ψ_w between -11 and -17 bars (4), it seems most likely that electron transport limits photosynthesis initially but, as desiccation becomes severe (ψ_w of -20 to -25 bars), photosynthesis probably is affected more by some other factor, most likely the loss in phosphorylating activity of the chloroplasts.

At least a part of the shift from limitation due to electron transport to limitation due to phosphorylation can probably be attributed to the uncoupling of the chloroplasts during desiccation. In the isolated chloroplasts, electron transport increased in rate in the presence of an uncoupler (Table I). Since there was no evidence of an increase in electron flow when chloroplasts from moderately desiccated tissue were compared with those from control tissue, no uncoupling *in vivo* must have taken place during early desiccation (ψ_w of -11 to -12 bars). At ψ_w below -17 bars, however, the chloroplasts were almost totally uncoupled (Table I and Fig. 3, A, B, C, and D).

Plaut (24) has suggested that *in vivo* changes in the activity of photosynthetic enzymes, particularly phosphoribulokinase, may be responsible for the decline in photosynthesis due to desiccation. The assay which he used depended on rapid rates of photophosphorylation to supply ATP for the reaction. Thus, the decline in photophosphorylation which we have found may also explain the apparent decline in the activity of phosphoribulokinase in intact chloroplasts. Plaut (24) was unable to detect differences in the activity of the extracted enzyme. Huffaker *et al.* (16) also found no appreciable differences in the extractable activity of several enzymes of the carboxylative phase of photosynthesis.

The effects of desiccation on sunflower chloroplasts were detectable within 5 to 10 min after the initiation of desiccation. Such a rapid response indicates that the chloroplast changes were close to the early, primary metabolic events that take place as ψ_w decreases. Apparently, these events do not involve the direct interaction of the free energy of water with the chloroplasts (9, 25), although an indirect effect of the free energy of water acting through some other metabolic system remains an open possibility. The rapidity of the response makes it unlikely that senescence was involved either, since symptoms of senescence usually require several days to appear in desiccated sunflower. It is perhaps not surprising, therefore, that RNase did not reproduce the uncoupling of chloroplasts by desiccation, even though it is associated with senescence (10) and can be an uncoupler in other systems (11, 13, 14). Although ABA increases in leaves within 1 to 2 hr during desiccation (30), it also did not affect photophosphorylation within this time span.

The present assays were conducted in media which were identical for all desiccation levels and the chloroplasts should have had identical activities if activity were a simple function of the reversible effects of hydration. Although small, reversible effects of hydration have been described for these chloroplasts, the effects of *in vivo* desiccation are persistent and much larger (25). Since both chloroplast activity and photosynthesis in the intact plant recover when rehydration occurs *in vivo*, however, the *in vivo* inhibition is not due to an irreversible disruption of chloroplasts but rather to a phenomenon which, at least so far, requires the intact cell to reverse. It is noteworthy that the inhibition occurs whether or not chloroplasts have been isolated by methods that preserve the limiting membrane (7, 25). Thus, the effects of desiccation appear to occur at the thylakoid membranes.

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