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Photosystem II Activity in Agranal Bundle Sheath Chloroplasts from *Zea mays*

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KIRSTEN S. ANDERSEN,[1] JOAN M. BAIN, D. G. BISHOP, AND ROBERT M. SMILLIE
Plant Physiology Unit, Commonwealth Scientific and Industrial Research Organization, Division of Food Research, Ryde, and School of Biological Sciences, Macquarie University, North Ryde, 2113, Sydney, Australia

ABSTRACT

The photochemical activities of chloroplasts isolated from bundle sheath and mesophyll cells of maize (*Zea mays* var. DS606A) have been measured. Bundle sheath chloroplasts are almost devoid of grana, except in very young leaves, while mesophyll chloroplasts contain grana at all stages of leaf development.

Chloroplast fragments isolated from bundle sheath cells showed a light-dependent reduction of potassium ferricyanide, 2,6-dichlorophenolindophenol, mammalian cytochrome *c*, plastocyanin, and *Euglena* cytochrome c_{552}. These activities were inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea at 1.25 micromolar. However, the photoreduction of NADP from water was extremely low or absent, except in chloroplasts from very young leaves, and the capacity for NADP reduction appeared to be related to the degree of grana formation.

Photosystem I activity was present in bundle sheath chloroplast preparations at all stages of leaf growth and senescence examined. However, the activity was lower than in isolated mesophyll chloroplasts. NADPH diaphorase activity was comparable in both types of chloroplast.

Chloroplasts isolated from bundle sheath cells of plants grown under a variety of conditions, including continuous and intermittent light, high and low light intensities, and high temperature, exhibited photosystem II activity.

The chloroplasts of higher plants usually contain numerous appressed lamellae or grana. When such chloroplasts are isolated, either intact or as broken chloroplast preparations containing granal fragments, they possess the capacity to evolve oxygen in the light and to photoreduce NADP. In grana-containing chloroplasts, the grana are joined by unappressed lamellae (stroma lamellae). However, grana are few or even absent in certain plants and algae.

The photosynthetic capacity of unappressed lamellae is not clear, especially with regard to photosystem II activity. It has been reported that the stroma lamellae of spinach chloroplasts lack photosystem II and therefore cannot evolve oxygen (15). After studying a number of mutants of *Nicotiana tabacum*, Homann and Schmid (9) concluded that appressed lamellae are required for photosystem II activity. The appearance of photosystem II activity in developing pea chloroplasts appears to be correlated with the formation of grana, rather than with the production of chlorophyll (5). In leaf sections of plants containing the C4-dicarboxylic acid pathway of photosynthesis, in which both granal and agranal chloroplasts are present, photoreduction of the Hill oxidant, tetranitro blue tetrazolium chloride, was observed only in grana-containing chloroplasts (6, 7). Agranal chloroplasts isolated from the bundle sheath cells of the C4 plants, maize and *Sorghum*, contain photosystem I activity, but do not photoreduce NADP from water, whereas the granal mesophyll chloroplasts carry out this reaction (2, 3, 19).

Similar correlations between photosystem II activity and the presence of grana have been made in algae. The chloroplasts of the green alga *Chlamydobotrys stellata* contain appressed lamellae and carry out normal photosynthesis when grown photoautotrophically. However, when the alga was grown photoheterotrophically on acetate, nearly all the chloroplast lamellae were separated from each other and the chloroplasts lacked photosystem II activity (16, 17). Both photosystem II activity and appression of lamellae were regained when photoautotrophic growth was resumed, again indicating that appressed lamellae are necessary for photosystem II activity (16–18).

In contrast, photosystem II activity is present in a mutant of *C. reinhardi* (*ac-31*) which has chloroplasts that contain only unappressed lamellae (8). Algae belonging to the Rhodophyta and Cyanophyta do not contain appressed lamellae yet evolve oxygen in the light. The disruption of grana by suspension of higher plant chloroplasts in a low salt medium does not result in a loss of photosystem II activity (10). Studies on a mutant of *Chlamydomonas reinhardi* revealed no correlation with Hill activity and grana formation (13).

The occurrence of two morphologically distinct chloroplasts in the leaves of a single plant provided an opportunity to correlate membrane structure with photochemical activity. In this paper, we show that the agranal chloroplasts present in the bundle sheath cells surrounding the vascular tissue in maize possess photosystem II activity. The magnitude of this activity is compared with that of the granal chloroplasts of the mesophyll cells and with the magnitude of photosystem I activity in both types of chloroplasts, using plants grown under a variety of environmental conditions.

MATERIALS AND METHODS

Seeds of *Zea mays* var. DS606A were soaked in tap water overnight without aeration, sown in vermiculite, and grown in a greenhouse or in controlled growth chambers. The seedlings were watered with tap water every second day.

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[1] Visiting scientist from Institute of Genetics, Copenhagen University, Øster Farimagsgade 2A, DK1353, Denmark.

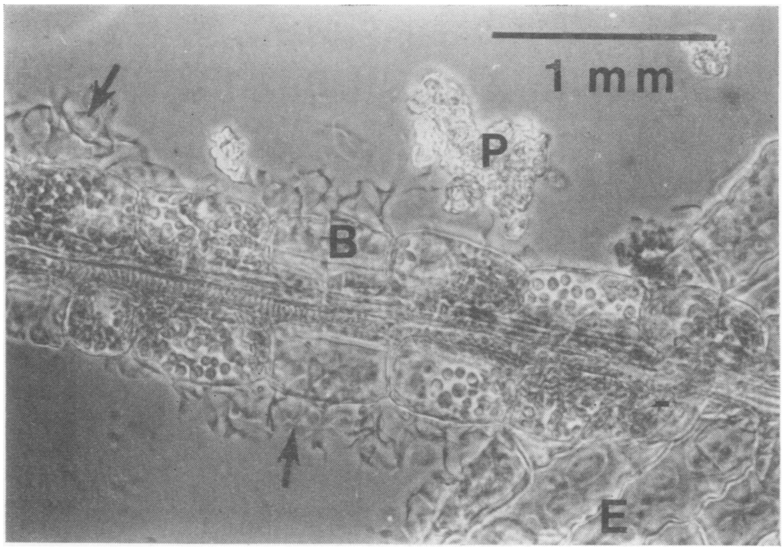

FIG. 1. Micrograph of bundle sheath cell preparation from secondary leaves of 16-day-old greenhouse-grown maize. B: Bundle sheath cells E: epidermal cells; P: Polyclar; arrows: remnants of the mesophyll cell walls along the sheath.

Table I. *Photosystem II Activity in Bundle Sheath and Mesophyll Chloroplasts of Maize*

Chloroplasts were prepared from the secondary leaves of 14-day-old maize. All reactions were sensitive to 1.25 μM DCMU.

Hill oxidant	Rate of Photoreduction in Chloroplast Preparation	
	Bundle sheath	Mesophyll
	μmoles substrate reduced/min·mg chlorophyll	
NADP	0	0.76
Cytochrome *c*	1.93	0.68
DCIP	1.88	2.26
Ferricyanide	2.93	3.56

Mesophyll chloroplasts and bundle sheath chloroplast fragments were prepared according to the procedure of Woo *et al.* (19), except that chloroplast preparations were washed once in suspension medium before assay. Unless stated otherwise, only secondary leaves were used. The purity of bundle sheath cell preparations was checked by light microscopy to insure that they were free of mesophyll contamination before the cells were broken to isolate bundle sheath chloroplast fragments. In Figure 1, a typical portion of a bundle sheath preparation is illustrated. Chlorophyll was measured by Arnon's procedure (1).

Samples for electron microscopy were fixed at 0 C in 6% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight and postfixed in 1% (w/v) osmium tetroxide for 1.5 hr. After embedding in Epon, thin sections were stained with lead citrate and viewed in a Siemens Elmiskop I electron microscope.

Photochemical activities were measured at 23 C with an Aminco-Chance dual wavelength spectrophotometer fitted with a cross illumination attachment which supplied tungsten light filtered with a Corning 2-60 red cutoff filter and two Corning 1-69 infrared filters. Energy incident on the sample was 6.9 × 10^4 ergs cm^{-2} sec^{-1}.

Photoreduction of NADP from water was determined with the measuring beam of the spectrophotometer set at 350 nm and the reference beam at 370 nm. A Wratten 18A filter was inserted between the sample and the photomultiplier tube. The reaction mixture (1.5 ml) contained chloroplasts (equal to 4.6 μg chlorophyll per ml); sorbitol, 300 mM; phosphate buffer, pH 7.4, 10 mM; $MgCl_2$, 1 mM; NADP, 0.67 mM; and ferredoxin (from *Anacystis nidulans*), 3.3 μM. Measurements of the Hill reaction with cytochrome *c*, DCIP,[2] or ferricyanide were carried out in the same reaction mixture, except that ferredoxin and NADP were omitted and horse heart cytochrome *c* (50 μM), DCIP (17 μM), or potassium ferricyanide (330 μM) was added. In these cases the measuring and reference wavelengths were set at 550 and 541 nm, 575 and 525 nm, or 420 and 460 nm, respectively, and a Corning 4-96 filter was inserted between the sample and the photomultiplier tube.

Photosystem I activity was measured by the photoreduction of NADP in a reaction mixture containing chloroplasts (equal to 4.6 μg chlorophyll per ml); sorbitol, 300 mM; phosphate buffer, pH 7.4, 10 mM; $MgCl_2$, 1 mM; NADP, 0.67 mM; ferredoxin, 3.3 μM; DCMU, 1.25 μM; DCIP, 67 μM; and sodium ascorbate, 2.5 mM.

NADPH diaphorase activity was measured by following the oxidation of NADPH in the presence of DCIP. The reaction mixture (1.5 ml) contained chloroplasts (equal to 4.6 μg chlorophyll per ml); sorbitol, 300 mM; phosphate buffer, pH 7.4, 10 mM; $MgCl_2$, 1 mM; DCIP, 17 μM; and NADPH, 0.33 mM.

RESULTS

Photosystem II Activity in Bundle Sheath Chloroplasts. The rates of reduction of four Hill oxidants upon illumination of bundle sheath chloroplast preparations are shown in Table I. Comparisons are also made with the rates obtained from mesophyll chloroplasts. All activities were light dependent. Although the agranal bundle sheath chloroplast preparations failed to reduce NADP, they were capable of reducing potassium ferricyanide, DCIP, and cytochrome *c* (Table I). In other experiments it was demonstrated that oxidized plastocyanin or oxidized *Euglena* cytochrome c_{552} could act as substrate in the Hill reaction of bundle sheath chloroplasts. All of these activities were inhibited by DCMU.

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[2] Abbreviation: DCIP: 2,6-dichlorophenolindophenol.

Thus, the inability of isolated bundle sheath chloroplasts to photoreduce NADP from water cannot be attributed to the absence of photosystem II, but rather, as has been previously suggested (2), to some deficiency in the electron transfer chain between photosystem II and the site of nucleotide reduction. NADP photoreduction by isolated bundle sheath chloroplasts can be demonstrated in the presence of DCMU, DCIP, and ascorbate, showing that photosystem I is present, although the rates are lower than in the case of mesophyll chloroplasts (2, 3).

Treatment of either bundle sheath or mesophyll chloroplasts with 0.2 M tris buffer for 15 min resulted in a loss of the capacity to photoreduce DCIP. The capacity was regained, however, in both types of chloroplast by adding semicarbazide as an electron donor for photosystem II. This reaction also was sensitive to DCMU.

Changes in Photochemical Activities with Leaf Age. The effect of leaf age on the photochemical activities and chlorophyll *a*/*b* ratio of mesophyll and bundle sheath chloroplast preparations is shown in Figure 2. Activities were measured in chloroplasts isolated from secondary leaves harvested between 6 and 21 days after sowing. Between 6 and 16 days after sowing, the leaf fresh weight increased 5-fold (Fig. 2f), but between 16 and 21 days the fresh weight decreased and the leaves began to show signs of senescence, such as yellowing of the leaf tips. Six days after sowing, the chlorophyll *a*/*b* ratios of the mesophyll and bundle sheath chloroplasts were approximately the same, but, while the chlorophyll *a*/*b* ratio of the mesophyll chloroplasts remained fairly constant over the period investigated, that of the bundle sheath chloroplasts increased markedly up to day 12 (Fig. 2e).

Photosystem II activity, measured by the DCMU-sensitive photoreduction of cytochrome *c* (Fig. 2c), showed little variation as a function of leaf age, the rates observed being similar in both meosphyll and bundle sheath chloroplasts. Photosystem I activity was, however, lower in bundle sheath chloroplasts at all stages of growth. Both types of chloroplast had similar NADPH diaphorase activities (Fig. 2d).

In the mesophyll chloroplasts, the activity of NADP photoreduction from water (Fig. 2a) showed a slight increase from day 6 to day 12 and a marked decrease from day 16 to day 21. This decrease corresponded to the yellowing of the leaf tips. Bundle sheath chloroplast fragments from 6-day-old plants exhibited 45% of the rate of NADP photoreduction of mesophyll chloroplasts. This ability of bundle sheath chloroplasts to photoreduce NADP was progressively lost, however, so that from day 6 to day 12 the rate decreased 10-fold, and at day 21 the rate was zero.

The results shown in Figure 2 indicated that the bundle sheath chloroplasts from 6-day-old plants still possessed some capacity to photoreduce NADP from water in contrast to those chloroplasts obtained from older plants. Examination of electron micrographs showed numerous small areas of appressed lamellae in the bundle sheath chloroplasts of 6-day-old plants (Fig. 3a), whereas in chloroplasts from older plants areas of appressed lamellae were rarely seen (Fig. 3c). Extensive grana formation in mesophyll chloroplasts is seen in preparations from both young and old leaves (Fig. 3, b and d). Measurements of the relative lengths of unappressed and appressed lamellae in mesophyll and bundle sheath chloroplasts from plants of different ages are shown in Table II. In the 6-day-old plant the ratio of appressed to unappressed lamellae in the bundle sheath chloroplasts is 35% of that found in the mesophyll chloroplasts. As the leaf ages, the decrease in the ratio of appressed to unappressed lamellae corresponds to the decrease in the capacity of NADP photoreduction from water.

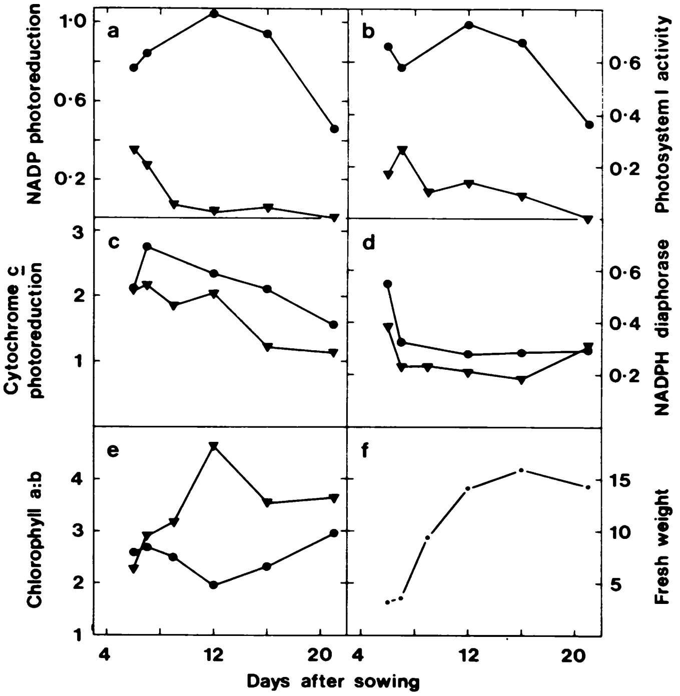

FIG. 2. Photochemical activities of mesophyll and bundle sheath chloroplasts from secondary leaves of greenhouse-grown maize. a: NADP photoreduction from water; b: photosystem I activity measured as NADP photoreduction in the presence of DCMU, DCIP and ascorbate. All activities are expressed in micromoles of substrate reduced or oxidized per min per mg chlorophyll. Fresh weight is given in grams per 100 leaves. ●—●: Mesophyll chloroplasts; ▲—▲: bundle sheath chloroplasts.

Changes in Photochemical Activities with Leaf Senescence. Although photosystem II in bundle sheath chloroplasts was active in growing leaves, it is possible that there could be a preferential degradation of photosystem II relative to photosystem I during leaf senescence. The result of an experiment in which detached secondary leaves of 14-day-old greenhouse-grown maize plants were allowed to senesce under continuous light is shown in Figure 4. Senescence was accompanied by a marked decrease in the capacity of the mesophyll chloroplasts to photoreduce NADP (Fig. 4a) (*cf.* Fig. 2a, 16–21 days), while bundle sheath chloroplasts showed little activity at any stage. Photosystem II activity did not change markedly in either type of chloroplast (Fig. 4c), although a marked decrease in the ratio of chlorophyll *a*/*b* occurred in the bundle sheath chloroplasts as senescence progressed (Fig. 4d).

Effect of Environmental Conditions on Photosystem II in Bundle Sheath Chloroplasts. Since the results recorded above showed relatively small variations in photosystem II activity of bundle sheath chloroplasts in expanding and senescing leaves, we examined the photochemical activities of chloroplasts isolated from plants grown under a variety of environmental conditions in an attempt to produce bundle sheath chloroplasts devoid of photosystem II activity. Plants were grown at temperatures from ambient (16 C) to 38 C and under light intensities ranging from 2 × 10^3 to 4 × 10^5 ergs cm^{-2} sec^{-1}. Both continuous light and intermittent (day/night) light were used in the high and low intensity ranges. In some of the experiments primary leaves as well as secondary leaves were tested, and in one experiment the leaves were taken from a 7-week-old plant grown in the field. Under all growth conditions employed, however, photosystem

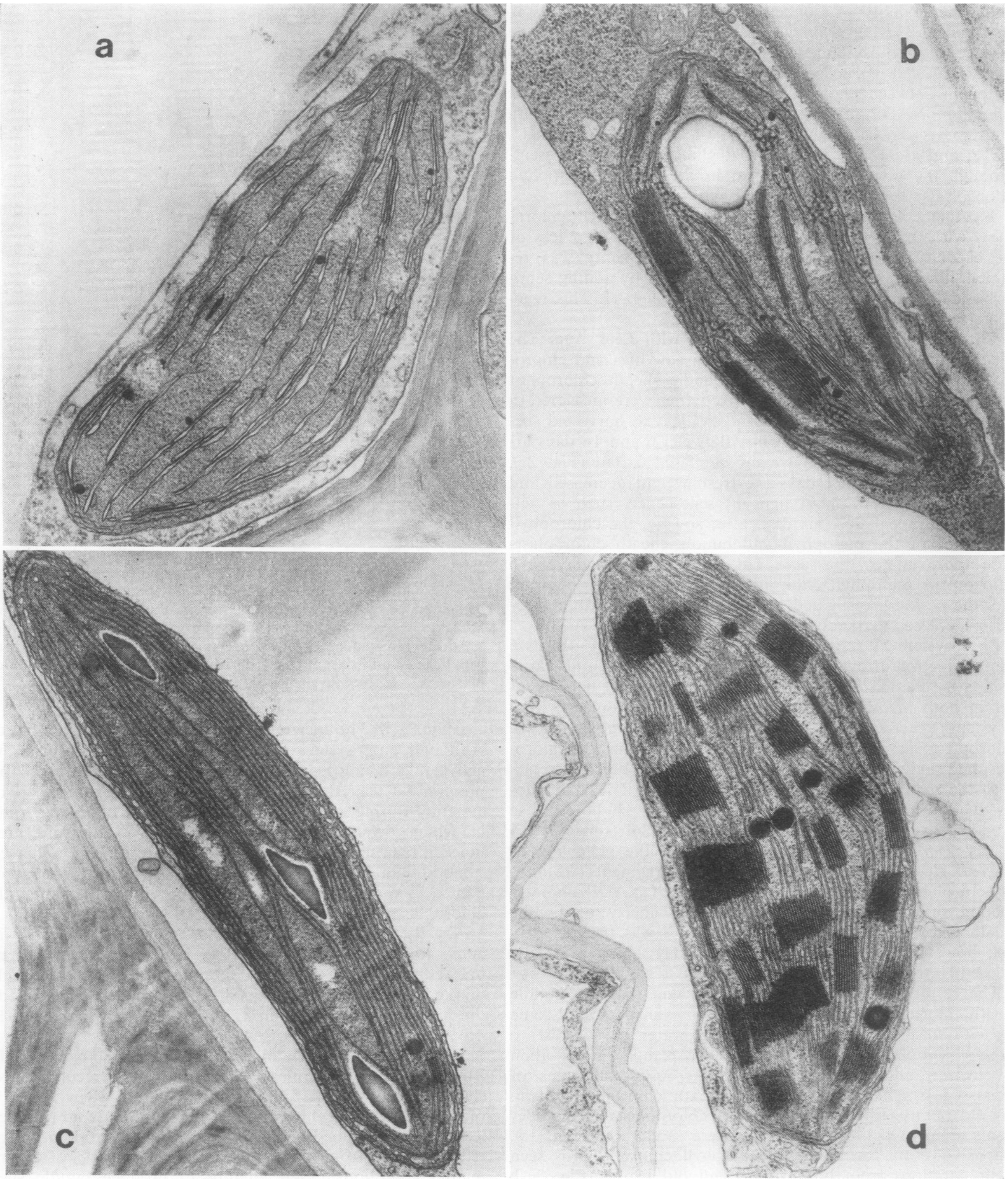

FIG. 3. Electron micrographs of chloroplasts from mesophyll and bundle sheath cells of greenhouse-grown maize. a: Bundle sheath chloroplast from 6-day-old plant (× 20,000); b: mesophyll chloroplast from 6-day-old plant (× 20,000); c: bundle sheath chloroplast from 12-day-old plant (× 20,000); d: mesophyll chloroplast from 12-day-old plant (× 20,000).

II activity in the bundle sheath chloroplasts when measured at pH 7.4 was comparable with that in the mesophyll chloroplasts.

Photo-oxidation of Cytochrome *f*. In granal chloroplasts the oxidation of cytochrome *f* (cytochrome c_{554}) is strongly activated by light which is absorbed by photosystem I only (>700 nm), but not by light which is also absorbed by photosystem II. This dependence upon wavelength is abolished by

DCMU. In contrast, the photo-oxidation of cytochrome *f* in agranal chloroplasts isolated from bundle sheath cells of *Sorghum bicolor* is independent of wavelength (19). Table III shows that this is also the case with isolated bundle sheath chloroplasts of maize with either equal light intensities or intensities which give equal rates of photosystem I at the two wavelengths employed.

DISCUSSION

The results presented in this paper demonstrate that agranal bundle sheath chloroplasts from maize plants contain photosystem II activity under a variety of plant growth conditions and during leaf senescence. However, a number of differences in the photochemical properties of isolated mesophyll and bundle sheath chloroplast preparations are apparent. Isolated bundle sheath chloroplast preparations, except those from very young leaves, have little or no capacity to photoreduce NADP from water and consequently photosystems I and II appear not to be linked for electron flow. These chloroplasts have been reported to lack both cytochrome b_{559} and low temperature fluorescence emission spectral bands characteristic of photosystem II (19). Although both types of chloroplasts show comparable cyclic photophosphorylative activity, noncyclic photophosphorylation with ferricyanide is reported to be low in bundle sheath chloroplast preparations (14). The independence of the photo-oxidation of cytochrome *f* from wavelength shown by bundle sheath chloroplast preparations has been interpreted as evidence of the inactivity of photosystem II (19), but could also be due to reductants produced in photosystem II being unavailable to photosystem I. The question of whether photosystems I and II are linked for electron flow in intact bundle sheath cells is considered in an accompanying paper (4).

In general, the activity of photosystem II in bundle sheath chloroplasts is comparable to that in mesophyll chloroplasts in comparative experiments. At pH 7.4, with either DCIP or ferricyanide as the Hill oxidant, the activity of the mesophyll chloroplasts is somewhat higher (*e.g.*, Table I). In recent experiments, with the use of a slightly modified procedure and reduction of the time taken to prepare the chloroplasts, rates of photoreduction of ferricyanide by mesophyll chloroplasts were increased to about 13 μmoles/min·mg chlorophyll, but at the same time the activity of the bundle sheath chloroplasts was also increased. Since the photosystem II activity in both mesophyll and bundle sheath chloroplasts decays with time after isolation and it takes considerably longer to prepare bundle sheath chloroplasts than mesophyll chloroplasts, direct comparisons of the activities of the two types of chloroplasts are made difficult. However, comparisons based on the decay curves for photosystem II for the isolated chloroplasts indicate that the activity of the bundle sheath

Table II. *Extent of Grana Formation in Mesophyll and Bundle Sheath Chloroplasts of Maize*

The lengths of appressed and unappressed lamellae were measured on electron micrographs of chloroplasts of secondary leaves of greenhouse-grown maize. The value Σ appressed/Σ unappressed represents the ratio of the total lengths of appressed and unappressed lamellae.

Time after Sowing	Chloroplast Type	Grana with >4 Thykaloids	Σ Appressed/ Σ Unappressed
days		%	
6	Bundle sheath	2.1	0.39
	Mesophyll	46	1.10
7	Bundle sheath	3.6	0.19
	Mesophyll	38	1.64
9	Bundle sheath	0	0.07
	Mesophyll	59	1.47
12	Bundle sheath	0.9	0.09

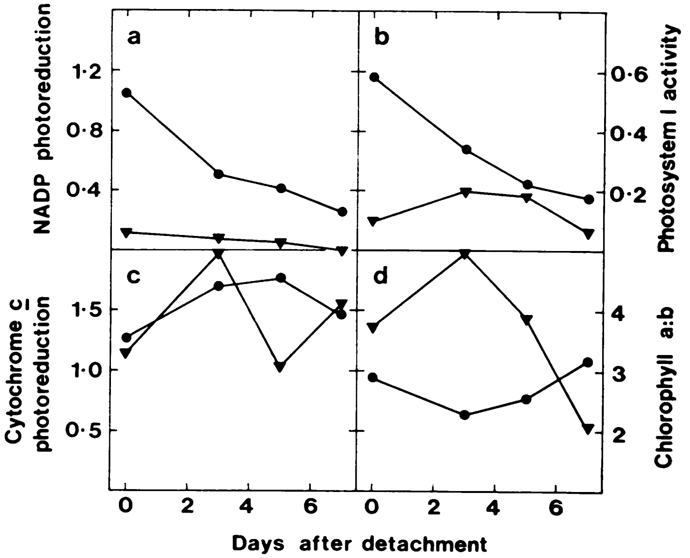

FIG. 4. Effects of senescence on photochemical activities and chlorophyll *a/b* ratios of mesophyll and bundle sheath chloroplasts from secondary leaves of maize. The plants were grown for 14 days in a greenhouse under prevailing light and temperature conditions. On day 0, secondary leaves were detached, and the lower 1 cm was immersed in water. The leaves were allowed to senesce in a growth chamber at 25 C under continuous illumination with white light (1.5×10^4 ergs cm^{-2} sec^{-1}). Activities are expressed in micromoles substrate reduced per min per mg chlorophyll. ●—●: mesophyll chloroplasts; ▲—▲: bundle sheath chloroplasts.

Table III. *Absorbance Changes at 554 nm Induced by Light at 664 and 725 nm*

Mesophyll or bundle sheath chloroplasts were illuminated with monochromatic light at 664 or 725 nm, and the absorbance change due to photo-oxidation of cytochrome f was measured. The reaction mixture contained chloroplasts (28 μg/ml); sorbitol (300 mM); phosphate buffer, pH 7.4 (10 mM); $MgCl_2$ (1 mM); and sodium ascorbate (2.5 mM) in a final volume of 0.75 ml. The measuring wavelength was 554 nm, and the reference wavelength was 541 nm.

Chloroplasts	Actinic Light		Absorbance × 10^4	
	Wavelength	Intensity	No DCMU	2.5 μM DCMU
	nm	*ergs cm^{-2} sec^{-1}*		
Mesophyll	664	8.7×10^3	0.9	2.1
	725	8.7×10^3	1.9	1.9
	664	a[1]	1.2	1.9
	725	a	2.1	2.1
Bundle sheath	664	8.7×10^3	3.9	3.9
	725	8.7×10^3	3.7	3.7
	664	a	3.8	3.7
	725	a	3.9	3.7

[1]a: Intensities used gave equal rates of photosystem I activity (as measured by NADP photoreduction in the presence of DCIP and ascorbate; see "Materials and Methods") at 664 and 725 nm.

chloroplasts at pH 7.5 is 70 to 80% of that of the mesophyll chloroplasts. The activity of the mesophyll chloroplasts was increased to about 21 μmoles/min·mg chlorophyll in the presence of 5 μM methylamine, but this uncoupling agent had little effect on the activity of bundle sheath chloroplasts.

The ability of isolated bundle sheath chloroplasts from young leaves to photoreduce NADP from water and the loss of this activity as the leaf develops correspond with the degree of grana formation in the chloroplast (Fig. 2a and Table I). Bundle sheath chloroplasts of young leaves of maize and sugarcane have been reported to contain grana (11, 12). The present study confirms such observations in maize and shows that between the 6th and 12th day after sowing, when there is a 10-fold decrease in the capacity of isolated bundle sheath chloroplasts to photoreduce NADP from water, there is a similar decrease in grana content. However, photosystem II activity in bundle sheath chloroplasts does not appear to be related to the presence of grana and it cannot be concluded that appressed lamellae are necessary for photosystem II activity in higher plants.

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