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PHOTOCHEMICAL SYSTEMS IN MESOPHYLL AND BUNDLE SHEATH CHLOROPLASTS OF C_4 PLANTS

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SUMMARY

1. The agranal bundle sheath chloroplasts of *Sorghum bicolor* possess very low Photosystem II activity compared with the grana-containing mesophyll chloroplasts.

2. Sorghum mesophyll chloroplasts have a chlorophyll (chl) and carotenoid composition similar to that of spinach chloroplasts. In contrast, the sorghum bundle sheath chloroplasts have a higher chl *a*/chl *b* ratio; they are enriched in β-carotene and contain relatively less xanthophylls as compared to sorghum mesophyll or spinach chloroplasts.

3. Sorghum mesophyll chloroplasts with 1 cytochrome *f*, 2 cytochrome b_6 and 2 cytochrome *b*-559 per 430 chlorophylls have a cytochrome composition similar to spinach chloroplasts. Sorghum bundle sheath chloroplasts contain cytochrome *f* and cytochrome b_6 in the same molar ratios as for the mesophyll chloroplasts, but cytochrome *b*-559 is barely detectable.

4. The chl/P700 ratios of mesophyll chloroplasts of *S. bicolor* and mesophyll and bundle sheath chloroplasts of *Atriplex spongiosa* are similar to that of spinach chloroplasts suggesting that these chloroplasts possess an identical photosynthetic unit size to that of spinach. The agranal bundle sheath chloroplasts of *S. bicolor* possess a photosynthetic unit which contains only about half as many chlorophyll molecules per P700 as found in the grana-containing chloroplasts.

5. The similarity of the composition of the bundle sheath chloroplasts of *S. bicolor* with that of the Photosystem I subchloroplast fragments, together with their smaller photosynthetic unit and low Photosystem II activities suggests that these chloroplasts are highly deficient in the pigment assemblies of Photosystem II.

INTRODUCTION

Studies on the photochemical systems in plants with the C_4-dicarboxylic acid pathway are complicated by the presence of two types of chloroplast-bearing cells; namely, the mesophyll cells, and the bundle sheath cells which surround the vascular bundles[1]. Chloroplasts of the mesophyll cells contain grana, but those of the bundle

Abbreviations: chl, chlorophyll; cyt, cytochrome; DCIP, 2,6-dichlorophenolindophenol; TCIP, 2,3′,6-trichlorophenolindophenol.

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sheath cells exhibit varying degrees of granal development depending on the species. For example, the bundle sheath chloroplasts of *Sorghum bicolor* and *Zea mays* lack grana, but those of *Atriplex spongiosa* show good development of grana.

A differential grinding procedure was developed to separate mesophyll chloroplasts and bundle sheath chloroplast fragments from the leaves of some C_4 plants[2]. The mesophyll chloroplasts of *S. bicolor*, *Z. mays* and *A. spongiosa*, and the bundle sheath chloroplast fragments of *A. spongiosa* gave good rates of oxygen evolution with $NADP^+$ as oxidant. In contrast, the agranal bundle sheath chloroplasts of *S. bicolor* and *Z. mays* were inactive in the Hill reaction with $NADP^+$, but they were able to photoreduce $NADP^+$ if provided with an artificial electron donor to Photosystem I. Furthermore, the agranal bundle sheath chloroplasts of *S. bicolor* contained only traces of cytochrome *b*-559, and they lacked at 77°K the fluorescence bands at 683 nm and 695 nm normally associated with the pigment assemblies of Photosystem II (refs. 2, 3). Woo *et al.*[2] concluded that the agranal bundle sheath chloroplasts are deficient in the pigment assemblies of Photosystem II.

Recently Bishop *et al.*[4] reported that bundle sheath chloroplast fragments from *Z. mays* and *S. bicolor* showed some activity in the Hill reaction either with 2,6-dichlorophenolindophenol (DCIP), ferricyanide or cytochrome *c* as oxidant, but not with $NADP^+$. These authors postulated that Photosystem II was present in the isolated agranal chloroplasts, but not linked to Photosystem I.

Black and Mayne[5] observed that leaf extracts and isolated chloroplasts of a number of C_4 plants had a higher concentration of P700 relative to chlorophyll (chl) and a higher ratio of chl *a*/chl *b* than several species of C_3 plants. They suggested that C_4 plants either have a more active Photosystem I or a smaller photosynthetic unit size.

In the present work, we have determined the pigment composition of mesophyll chloroplasts and bundle sheath chloroplast fragments of *S. bicolor* and *A. spongiosa*. The mesophyll chloroplasts resemble spinach chloroplasts, while the agranal bundle sheath chloroplast fragments of *S. bicolor* are similar to the Photosystem I, subchloroplast fragment obtained from spinach chloroplasts by digitonin fragmentation. Our preparations of bundle sheath chloroplast fragments from *S. bicolor* showed some Hill reaction activity with 2,3′,6-trichlorophenolindophenol (TCIP) or ferricyanide as oxidant, but the rates were very low compared with mesophyll chloroplasts.

MATERIALS AND METHODS

Plant material and chloroplast preparations

Seedlings of *Sorghum bicolor* L. (var. Texas 610), and *Atriplex spongiosa* were grown in a glasshouse for 2–3 weeks. The compositional data reported in this paper was obtained with mesophyll chloroplasts and bundle sheath chloroplast fragments, which were isolated by the differential grind procedure described previously[2]. However, in order to obtain maximal photochemical activities for the mesophyll chloroplasts, it was necessary to modify the isolation procedure.

It was essential to chill the sorghum leaves in ice for 15 min prior to the chloroplast isolation. Dithiothreitol replaced mercaptoethanol in the isolation medium and it was included in the suspension medium. The leaves (10 g) were then cut into strips (2–3 mm wide) in a small dish which contained 100 ml of the isolation medium

(30 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid·HCl–EDTA–NaCl buffer, pH 7.4, 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM dithiothreitol and 0.5 % bovine serum albumin) and quickly transferred to the cup of a Sorvall Omnimixer for a 4 sec blend at 50 % of the line voltage. The brei was passed through 2 layers of Miracloth, the filtrate was centrifuged for 30 sec at 300 × *g*, the pellet discarded and the supernatant centrifuged for 10 min at 1000 × *g*. The chloroplasts were washed by resuspension in 35 ml of suspension medium (10 mM phosphate buffer, pH 7.4, 0.33 M sorbitol, 1 mM $MgCl_2$, 5 mM dithiothreitol and 0.5 % bovine serum albumin) and centrifuged at 1000 × *g* for 10 min. The pellets were suspended in 1 ml of the suspension medium, *minus* the dithiothreitol. The residue from the Miracloth was resuspended in the above isolation medium and the bundle sheath chloroplast fragments were isolated as described previously[2]; they were also washed once by resuspension in the suspension medium. The entire operation was performed in a cold room, maintained at 0–4°.

Spinach chloroplasts were isolated from plants grown in nutrient culture, as described previously[6]. Photosystem I subchloroplast fragments (the fraction sedimented by centrifugation at 144000 × *g* for 60 min) were prepared from spinach chloroplasts by the digitonin procedure[6]. Sorghum mesophyll and bundle sheath chloroplasts (0.35 mg chl/ml) were fragmented by incubation with 0.5 % digitonin for 30 min at 0° as described previously[6].

Photochemical activities

Hill-reaction activities were determined at 20° (ref. 6); ferricyanide reduction was measured at 420 nm and TCIP at 620 nm. White light of 40000 lux was provided by a 250-W photoflood lamp, and passed through a 3 cm layer of water.

Pigment determinations

A chloroplast suspension was extracted into 80 % acetone, the solution clarified by centrifugation and the concentrations of chl *a* and chl *b* determined on a Cary Model 14R recording spectrophotometer using the equations of ARNON[7]. Part of the acetone extract was saponified, and the carotenoids extracted into ether. Total carotenoid concentration was obtained by dividing the absorbance at 442 nm by the factor 0.24 (ref. 8). To obtain the carotenoid compositional data, the pigments in the unsaponified acetone extract were transferred to ether and separated by thin-layer chromatography in the dark at 15° on Kieselgel G (Merck) with benzene–acetone (3:1, v/v) as developing solvent. The individual carotenoids were eluted from the adsorbent, and their concentrations determined from their peak absorbance, using an average specific absorption coefficient of 240 $l \cdot g^{-1} \cdot cm^{-1}$ (ref. 8).

Difference spectra of reduced *minus* oxidized cytochromes were determined on the Cary Model 14R spectrophotometer fitted with a Cary Model 1462 scattered transmission attachment using an EMI 9558 QB photomultiplier. A modified cuvette system of BONNER[9] was used; optical pathlength, 2 mm; volume, 0.28 ml. Chloroplasts were suspended in 0.05 M phosphate buffer, pH 7.2, containing 0.3 M sucrose and 0.015 M KCl. The chlorophyll content of the suspensions was 300 μg/ml.

Total cyt *b* (cyt b_6 + cyt *b*-559) was determined from the dithionite-reduced *minus* ferricyanide-oxidized spectrum after correction for absorption of cyt *f*[8]. Cyt b_6 was estimated from the dithionite-reduced *minus* ascorbate-reduced spectrum. A

difference molar absorption coefficient of $2.0 \cdot 10^4$ was used for cyt b_6 and for cyt b-559.

Cyt f was determined by two methods: (a) from the ascorbate-reduced *minus* ferricyanide-oxidized spectrum of acetone-extracted powders of lypholized chloroplasts[8]; (b) by a more direct method using chloroplasts without prior extraction of chlorophylls, and based on the procedure of PLESNIČAR AND BENDALL[10]. The chloroplast suspension was made to 1 % (v/v) Triton X-100 and the hydroquinone-reduced *minus* ferricyanide-oxidized spectrum determined. A difference molar absorption coefficient of $2.5 \cdot 10^4$ was used for cyt f.

P700 was determined by two methods, using the specific absorption coefficient of chl *a* in 80 % acetone (82 per $1 \cdot g^{-1} \cdot cm^{-1}$) to estimate concentrations of P700: (a) Chloroplasts in 0.05 M phosphate buffer, pH 7.2, and 1 % Triton X-100 were treated with either $2 \cdot 10^{-3}$ M ferricyanide or $2 \cdot 10^{-3}$ M ascorbate for 2 min at room temperature. The final chlorophyll concentration was 50 μg/ml. A ferricyanide-oxidized *minus* ascorbate-reduced difference spectrum was recorded in the Cary Model 14R spectrophotometer (with scattered-transmission attachment) over the wavelength range 680–750 nm. P700 was calculated from the absorbance trough at 698 nm; (b) The light-induced decrease in absorbance at 698 nm was measured in a Chance Aminco dual wavelength spectrophotometer (American Instrument Corp., Maryland, U.S.A.), fitted with a side illumination attachment. The reference wavelength was 739 nm. Actinic light was provided by a 650-W tungsten iodine lamp, and passed through a 3-cm layer of water and a 633-nm interference filter (Balzers; half-band width 10 nm). The intensity of the monochromatic light was $6 \cdot 10^3$ erg $\cdot cm^{-2} \cdot sec^{-1}$. The photomultiplier tube was moved 2 inches away from its normal position to prevent possible fluorescence artifacts. A Corning glass filter 4-77 protected the photomultiplier tube from actinic light. The chloroplasts (25 μg/ml chlorophyll) were suspended in 0.05 M phosphate buffer, pH 7.2, 1 % Triton X-100 and 3 mM sodium ascorbate.

Fluorescence emission spectra at 77 °K were recorded in 60 % glycerol on a fluorescence spectrophotometer incorporating automatic correction for photomultiplier and monochromator responses, and variation in output of light source[11,12].

RESULTS

Photochemical activity

Table I shows Hill-reaction activities of mesophyll and bundle sheath chloroplasts of *S. bicolor*. High rates of photoreduction of TCIP and ferricyanide were obtained with the mesophyll chloroplasts, and these rates were increased about 2-fold in the presence of methylamine, an uncoupler of photophosphorylation. Much lower activities (89–100 μmoles TCIP reduced/mg chl per h and 120–150 μmoles ferricyanide reduced/mg chl per h) were obtained if the chloroplasts were isolated by our original procedure[2], and methylamine–HCl had little or no influence on the rates.

To obtain high activities with mesophyll chloroplasts, it was necessary to take the additional precautions listed in MATERIALS AND METHODS for the isolation of the chloroplasts. These included the prior chilling of the leaves, the cutting of the leaves in the isolation medium, and the inclusion of dithiothreitol in the isolation and suspension media. With this new procedure, the rates of photoreduction of TCIP and ferricyanide by sorghum mesophyll chloroplasts compare favourably with those routinely obtained with spinach chloroplasts in our laboratory.

TABLE I

HILL REACTION ACTIVITIES OF SORGHUM CHLOROPLASTS

For TCIP reduction, the reaction mixture (3 ml) contained chloroplasts (10 μg chlorophyll) and (in μmoles) Tris–HCl buffer, pH 7.8, 40; NaCl, 70; TCIP, 0.06. For ferricyanide reduction, the reaction mixture (3 ml) contained chloroplasts (25 μg chlorophyll) and (in μmoles), Tris–HCl buffer, pH 8.0, 40; NaCl, 70; $MgCl_2$, 10; ferricyanide, 1; methylamine–HCl (MA) if added, 150. Values are the average of 4 experiments.

Sorghum bicolor chloroplasts	*TCIP reduction (μmoles/mg chl per h)*		*Ferricyanide reduction (μmoles/mg chl per h)*	
	−MA	*+MA*	*−MA*	*+MA*
Mesophyll	276	523	361	816
Bundle sheath	37	35	49	51

Previous studies[2] showed that sorghum mesophyll chloroplasts were able to reduce $NADP^+$ in the Hill reaction, but the agranal bundle sheath chloroplasts were inactive. The bundle sheath chloroplasts photoreduced $NADP^+$ with ascorbate–DCIP as electron-donor, indicating an active Photosystem I.

In the present work, the bundle sheath chloroplast fragments possess some Hill-reaction activity with DCIP or ferricyanide as oxidant, but the rates of reduction are low compared with those observed with the mesophyll chloroplasts (Table I). For example, in the presence of methylamine–HCl, the bundle sheath chloroplast fragments reduced TCIP at 6%, and ferricyanide at 6% of the rates observed with mesophyll chloroplast. It is not possible to decide whether the activity observed with the bundle sheath fragments is due to the bundle sheath fragments themselves or to some contaminating mesophyll chloroplasts.

Pigment composition

Mesophyll chloroplasts of *S. bicolor* have a chl *a*/chl *b* ratio of 3.1 which is similar to that of spinach chloroplasts (Table II). In contrast, the bundle sheath chloroplasts of *S. bicolor* have a higher chl *a*/chl *b* ratio, which resembles the ratio of the Photosystem I subchloroplast fragments (D-144) obtained by digitonin fragmentation of spinach. The total chlorophyll/carotenoid ratios are the same for both types of *S. bicolor* chloroplasts and resemble the ratios obtained with both spinach chloroplasts and the Photosystem I subchloroplast fragments.

However, the relative proportions of the individual carotenoids varied for the two types of *S. bicolor* chloroplasts as shown by the xanthophyll/carotene ratios. The bundle sheath chloroplasts are relatively enriched in β-carotene and contain less of the xanthophylls compared with either mesophyll or spinach chloroplasts. Thus, the carotenoid composition of mesophyll chloroplasts is very similar to that of spinach chloroplasts, whereas the agranal bundle sheath chloroplasts resemble rather the Photosystem I subchloroplast fragments (Table II).

Molar ratios of chlorophyll/cytochrome are shown in Table III. Mesophyll chloroplasts of *S. bicolor* have 1 molecule of cyt *f* for every 437 chlorophyll molecules which does not differ significantly from the value of 430 obtained with spinach chloroplasts. The chlorophyll/total cytochrome *b* ratios are also similar for *S. bicolor* mesophyll chloroplasts and spinach chloroplasts. Moreover, sorghum mesophyll chloro-

TABLE II

PIGMENT COMPOSITION OF MESOPHYLL AND BUNDLE SHEATH CHLOROPLASTS OF *S. bicolor*

Chloroplasts were prepared as described in MATERIALS AND METHODS. The carotenoids were separated by thin-layer chromatography. The compositions of spinach chloroplasts and spinach Photosystem I subchloroplast fragments are taken from BOARDMAN AND ANDERSON[8].

	Spinach	*Sorghum bicolor*		*Spinach Photosystem I fragments*
		Mesophyll	*Bundle sheath*	
chl *a*/chl *b*	2.8	3.1	5.7	5.3
$\frac{\text{chl } a + \text{chl } b}{\text{Carotenoid}}$	6.3	6.3	6.3	6.8
$\frac{\text{Xanthophyll}}{\beta\text{-Carotene}}$	2.57	2.85	1.44	1.94
Carotenoids (%)				
β-Carotene	28	26	41	34
Lutein	45	38	26	30
Violaxanthin	17	24	23	23
Neoxanthin	10	12	10	10

TABLE III

MOLAR RATIOS OF CHLOROPHYLL TO CYTOCHROME

The cytochromes were calculated from reduced *minus* oxidized difference spectra as described in MATERIALS AND METHODS. The values are the average from 6 experiments. The molar ratios for spinach chloroplasts and spinach Photosystem I fragments are taken from BOARDMAN AND ANDERSON[8].

Chloroplasts	$\frac{chl}{cyt\ f}$	$\frac{chl}{cyt\ b\ (total)}$	$\frac{cyt\ b}{cyt\ f}$	$\frac{cyt\ b_6}{cyt\ b\text{-}559}$
S. bicolor mesophyll	437	119	3.7	1.1
S. bicolor bundle sheath	320	161	1.9	—
Spinach	430	118	3.6	1.0
Spinach Photosystem I fragments	363	187	1.9	—

plasts contain about equal quantities of cyt b_6 and cyt *b*-559. Thus, mesophyll chloroplasts of *S. bicolor* contain cyt *f*, cyt b_6 and cyt *b*-559 in the approximate molar ratio of 1:2:2, as found previously for spinach chloroplasts[8]. Digitonin fragmentation of spinach chloroplasts showed that cyt *f* and cyt b_6 are localized in Photosystem I and cyt *b*-559 in Photosystem II (ref. 8). Recently, we showed that bundle sheath chloroplasts were highly deficient in cyt *b*-559 as indicated by cytochrome difference spectra at liquid-nitrogen temperature[2]. The bundle sheath chloroplasts have a cyt b_6/cyt *f* ratio of 2 which would be expected for a chloroplast containing only Photosystem I. They contain more cyt *f* and cyt b_6 relative to chlorophyll than the mesophyll chloroplasts and the molar ratio are reasonably similar to those of the Photosystem I subchloroplast fragments prepared by the dilution technique[8].

The chl/P700 ratios, reported in Table IV show good agreement between the two methods. The significant finding is that the bundle sheath chloroplasts of *S. bicolor* contain about twice as much P700 per mole of chlorophyll as compared to the

TABLE IV

CHLOROPHYLL TO P700 RATIOS

P700 was determined by (*a*), oxidized *minus* reduced difference spectrum and (*b*), the light-induced absorbance change as described in MATERIALS AND METHODS. The values shown are the average of at least 6 experiments. Spinach Photosystem I fragments were prepared as indicated in MATERIALS AND METHODS.

Chloroplasts	*chl/P700*	
	(*a*)	(*b*)
S. bicolor mesophyll	496	486
S. bicolor bundle sheath	254	276
A. spongiosa mesophyll	387	389
A. spongiosa bundle sheath	414	456
Spinach chloroplasts	425	460
Spinach Photosystem I fragments	218	240

mesophyll chloroplasts. In contrast, the mesophyll and bundle sheath chloroplasts of *A. spongiosa* have roughly similar P700 contents, but with the mesophyll chloroplasts containing slightly more P700 on a chlorophyll basis. The chl/P700 ratio of mesophyll chloroplasts of *S. bicolor* compare favourably with the ratio of spinach chloroplasts, whereas the bundle sheath chloroplasts resemble the spinach Photosystem I fragments.

Digitonin fragmentation

The above results show that the pigment composition of sorghum mesophyll chloroplasts closely resembles that of spinach chloroplasts. In order to test whether the Photosystems of sorghum mesophyll chloroplasts are similar to those of spinach, we incubated the mesophyll chloroplasts with 0.5 % digitonin under the same conditions as previously used for spinach chloroplasts[5]. Previously a partial fractionation of the Photosystems of spinach chloroplasts was achieved by incubating the chloroplasts with digitonin and separating the subchloroplast fragments by differential centrifugation[6,8].

Table V compares the chlorophyll composition of the subchloroplast particles obtained from digitonin fragmentation of sorghum mesophyll and spinach chloroplasts. It is apparent that the distribution of total chlorophyll among the fractions prepared from sorghum mesophyll chloroplasts was similar to that obtained with spinach chloroplasts. The chl *a*/chl *b* ratios of the sorghum subchloroplast fragments also followed the same pattern as found with the spinach subchloroplast fragments. Thus, the sorghum mesophyll D-10 subchloroplast fragments have a lower chl *a*/chl *b* ratio (2.48) and the D-144 subchloroplast fragments a higher chl *a*/chl *b* ratio (6.40) as compared to that of the mesophyll chloroplasts (3.31). The higher chl *a*/chl *b* ratios of the sorghum mesophyll subchloroplast fragments as compared to the corresponding spinach fragments probably reflects the higher chl *a*/chl *b* ratio of the sorghum mesophyll chloroplasts.

Table V shows the chl/P700 ratios obtained by the two methods; as before, agreement between the methods was satisfactory. For sorghum mesophyll fractions, the D-144 subchloroplast fragment had a chl/P700 of 275 as compared to 491 for the chloroplasts, while the D-10 fraction was considerably higher with 1 P700 for

TABLE V

COMPARISON OF CHLOROPHYLLS, P700 AND FLUORESCENCE EMISSION AT 77° K IN DIGITONIN FRACTIONS OF SORGHUM MESOPHYLL AND SPINACH CHLOROPLASTS

The digitonin subchloroplast fragments were separated by differential centrifugation at 1000 × *g*, 10 min; 10000 × *g*, 30 min; 50000 × *g*, 30 min and 144000 × *g*, 60 min (designated as D-1, D-10, D-50 and D-144, respectively). The values for spinach chloroplasts are given in brackets; chl *a*/chl *b* and % chlorophyll were previously determined[6]. P700 was determined by (*a*) oxidized *minus* reduced difference spectrum and (*b*), the light-induced absorbance change as described in MATERIALS AND METHODS. ϕ_{735}/ϕ_{total} (%) is the percentage of the fluorescence energy emitted at the 735-nm band at 77° K.

Fraction	*Chlorophyll distribution* (%)	*chl a/chl b*	*chl/P700*		$\frac{\phi_{735}}{\phi_{total}}$ (%)
			(*a*)	(*b*)	
Chloroplasts	— (—)	3.31 (2.83)	496 (425)	486 (460)	79 (75)
D-1	12 (19)	2.80 (2.36)	— —	— —	— —
D-10	46 (46)	2.48 (2.27)	790 (814)	820 (876)	65 (60)
D-50	16 (12)	4.41 (4.40)	269 (252)	281 (270)	— —
D-144	14 (12)	6.40 (5.34)	208 (218)	234 (240)	95 (97)
144000 × *g* supernatant	12 (11)	5.54 (3.76)	— —	— —	— —

every 805 chlorophylls. The similarity of the chl/P700 ratios of the sorghum mesophyll subchloroplast particles to those of the spinach fractions gives further evidence for the resemblance of the Photosystems of sorghum mesophyll to those of spinach chloroplasts.

Another parameter used to distinguish between the chlorophylls of the Photosystems comes from fluorescence studies at liquid-nitrogen temperatures. Spinach chloroplasts at 77° K have a characteristic 3-banded fluorescence emission spectrum with peaks at 683, 695 and 735 nm; there is evidence to suggest that the emission bands at 683 and 695 nm arise from Photosystem II chlorophylls and the main band at 735 nm arises primarily from Photosystem I chlorophylls[11]. With spinach chloroplasts some 75 % of the total fluorescence is emitted at 735 nm; the D-10 fraction which is enriched in Photosystem II emits 60 % while the D-144 Photosystem I fragments emit 97 % of their fluorescence at 735 nm. The values obtained with the sorghum mesophyll chloroplast fractions were similar to those of spinach fractions.

To confirm that sorghum bundle sheath chloroplasts contained mainly Photosystem I as indicated by the close resemblance of their composition to that of spinach Photosystem I subchloroplast fragments, we incubated sorghum bundle sheath chloroplasts with digitonin. Some 60 % of the total chlorophyll was located in the D-10 fraction and the remaining 40 % in the lighter fractions. But there were no marked differences in the composition of the fragments: chl *a*/chl *b* (chloroplasts, 6.4; D-10, 6.2; D-144, 8.3), chl/P700 (chloroplasts, 250; D-10 262; D-144, 200) and ϕ_{735}/ϕ_{total} (chloroplasts, 95 %; D-10, 93 %; D-144, 97 %).

The small variations between the D-10 and D-144 fractions suggest a very limited fractionation of the Photosystems, but we are not sure whether sorghum bundle sheath chloroplasts have a small amount of Photosystem II or whether the limited fractionation is due to some contamination of the bundle sheath chloroplasts with mesophyll chloroplasts.

DISCUSSION

Before discussing the results, it is appropriate to comment on the grinding procedure used to separate mesophyll and bundle sheath chloroplasts[2]. The method is dependent on the resistance of the bundle sheath cells to breakage as compared with the more fragile mesophyll cells. A very short initial grind of maize or sorghum leaves releases intact mesophyll chloroplasts relatively uncontaminated by bundle sheath chloroplasts. The residue of the leaf material after the initial grind is then blended further in the Omnimixer (2–4 min) to ensure that the remaining mesophyll cells are stripped from the strands of bundle sheath cells. It is essential to monitor the material by light microscopy at this stage, to be sure that the bundle sheath strands are relatively free of mesophyll cells. This is a subjective assessment and it is impossible to estimate accurately the degree of mesophyll contamination from the small amount of the residue examined. We believe, however, that the contamination is less than 10%.

In developing the differential grinding procedure, the aim was to isolate intact bundle sheath chloroplasts as well as intact mesophyll chloroplasts. At present, however, we have been unable to release the chloroplasts from the strands of bundle sheath cells of *S. bicolor* without fragmenting the chloroplasts.

BISHOP *et al.*[4] reported that the Hill oxidants, DCIP, ferricyanide and cytochrome *c* were reduced by both mesophyll and bundle sheath chloroplasts of *S. bicolor* and *Z. mays*. With ferricyanide as oxidant, the mesophyll chloroplasts were more active than the bundle sheath chloroplast fragments by a factor of 2.5 for *Z. mays* and 5.2 for *S. bicolor*. With DCIP as oxidant the corresponding factors were 1.7 and 2.5. Mesophyll chloroplasts of *Z. mays* were more active than the bundle sheath chloroplasts when cytochrome *c* was used as the oxidant in the absence of ferredoxin, while the mesophyll chloroplasts of *S. bicolor* were more active than the bundle sheath chloroplasts by a factor of 2.4.

However, the rates of reduction reported by BISHOP *et al.*[4] were low for both mesophyll and bundle sheath chloroplasts. For example, Hill activities of sorghum bundle sheath chloroplasts varied from 14 μmole oxidant reduced/mg chl per h with cytochrome *c* to 40 with DCIP. Sorghum mesophyll chloroplasts gave activities of 34 with cytochrome *c*, 71 with DCIP and 162 with ferricyanide. Since the Photosystem II activities of the bundle sheath chloroplast fragments were quite significant compared to those of mesophyll chloroplasts, BISHOP *et al.*[4] concluded that Photosystem II was present in the bundle sheath chloroplasts. The Photosystem II activities obtained in the present study for bundle sheath chloroplast fragments from *S. bicolor* are comparable to those of BISHOP *et al.*[4], but our sorghum mesophyll chloroplasts gave Hill activities which were many fold higher than those reported by BISHOP *et al.*[4]. In view of our own experience, it seems likely that the mesophyll chloroplasts prepared by BISHOP *et al.*[4] were greatly inhibited.

The low Photosystem II activities of sorghum bundle sheath chloroplasts are certainly consistent with the composition of these chloroplasts. The striking similarity of sorghum bundle sheath chloroplasts with the Photosystem I subchloroplast fragments from spinach indicates that the bundle sheath chloroplasts are highly deficient in the pigment assemblies of Photosystem II. We consider that the grinding procedure used to obtain the bundle sheath chloroplast fragments was not responsible for spe-

cifically releasing Photosystem I fragments from the bundle sheath chloroplasts. A large proportion (approx. 95 %) of the chlorophyll of the preparation at the bundle sheath cell stage is recovered in fragments after grinding in the mill. Secondly, the fluorescence spectrum at 77 °K of intact bundle sheath cells, still attached to lengths of vascular tissue, was very similar to the spectrum of the bundle sheath chloroplast fragments[2]. DOWNTON *et al.*[13] treated leaf sections of *Sorghum sudanense* with tetranitro blue tetrazolium chloride, which acts as a Hill oxidant. Photoreduction of the dye was observed in the mesophyll chloroplasts but not in the bundle sheath chloroplasts.

The photosynthetic unit is conveniently defined as the minimum number of chlorophyll molecules associated with the transport of an electron from OH^- to $NADP^+$, rather than the original definition of EMERSON AND ARNOLD[14] which was based on the number of chlorophyll molecules required for the evolution of 1 molecule of O_2. Spinach chloroplasts contain 1 mol of P700 and 1 mole of cytochrome *f* for every 400 or so moles of chlorophyll. Thus, the photosynthetic unit size of a C_3 plant such as spinach is 400–450 chlorophylls, with the chlorophyll approximately equally divided between the two Photosystems, *i.e.* just over 200 chlorophylls in each Photosystem[15]. These is little doubt that sorghum mesophyll chloroplasts have a similar composition to spinach chloroplasts and their fragmentation pattern on incubation with digitonin is identical. It is concluded therefore that sorghum mesophyll chloroplasts which show both Photosystem I and Photosystem II activities possess a photo. synthetic unit size similar to that of spinach chloroplasts. The same is true for the mesophyll and bundle sheath chloroplasts of *A. spongiosa* both of which have Photosystem I and Photosystem II activities[2].

In contrast, the bundle sheath chloroplast fragments of *S. bicolor* possess a photosynthetic unit with about half the number of chlorophyll molecules as compared to mesophyll and spinach chloroplasts. We have previously shown that these chloroplasts have good Photosystem I activity, but are incapable of photoreducing $NADP^+$ with water as the electron donor[2]. We suggested that these chloroplasts were highly deficient in the pigment assemblies of Photosystem II since the low-temperature fluorescence bands of Photosystem II were almost absent, as was cytochrome *b*-559, the cytochrome located in Photosystem II (ref. 2). The present finding of a much smaller photosynthetic unit in the agranal bundle sheath chloroplasts is consistent with our conclusion that these chloroplasts are deficient in the pigment assemblies of Photosystem II. The fragmentation studies with digitonin support the view that the photosynthetic unit size of Photosystem I in the bundle sheath chloroplasts of *S. bicolor* is the same as for Photosystem I in the mesophyll chloroplasts.

As mentioned in INTRODUCTION, BLACK AND MAYNE[5] concluded from measurements of P700 and chl *a*/chl *b* ratios that C_4 plants either had a more active Photosystem I or a smaller photosynthetic unit. More recently, EDWARS AND BLACK[16] have separated intact mesophyll and bundle sheath cells from crabgrass. The chl *a*/chl *b* ratio of the bundle sheath cells was 4.5 compared with 3.0 for the mesophyll cells. The bundle sheath cells were about half as active as the mesophyll cells in the Hill reaction, they contained twice as much P700 per mole of chlorophyll, and their fluorescence properties indicated that they were enriched in Photosystem I. The bundle sheath chloroplasts of crabgrass are not completely agranal, but the development of grana is poor. It is possible that in both structure and function the bundle

sheath chloroplasts of crabgrass represent an intermediate condition between the agranal state as in maize and sorghum and the granal form, as in *Atriplex spongiosa*.

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After submission of this manuscript, SMILLIE *et al.*[17] reported that the photoreduction of $NADP^+$ by maize bundle sheath chloroplasts was stimulated by plastocyanin, and the rates obtained in the presence of plastocyanin were comparable to those obtained with maize mesophyll chloroplasts. They imply that Photosystem II was fully present in the maize bundle sheath chloroplasts. However, the rates for $NADP^+$ photoreduction were low compared with the rates of TCIP and ferricyanide photoreduction reported in this present paper for sorghum mesophyll chloroplasts. We have also obtained high rates of photoreduction of TCIP and ferricyanide with maize mesophyll chloroplasts when isolated by the procedure described in this present paper. In the presence of methylamine, the bundle sheath chloroplasts of maize showed only about 17% of the activity of maize mesophyll chloroplasts. We consider from this result and from compositional data that the maize bundle sheath chloroplasts contain more Photosystem II than sorghum bundle sheath chloroplasts, but they are deficient in Photosystem II when compared with maize mesophyll chloroplasts.

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