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Isolation and Bicarbonate Transport of Chloroplast Envelope Membranes from Species of Differing Net Photosynthetic Efficiency

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ABSTRACT

A three-phase discontinuous sucrose gradient yielded two fractions of chloroplast envelope membranes from spinach (*Spinacia oleracea* L.), sunflower (*Helianthus annuus* L.), and maize (*Zea mays* L., mesophyll and undifferentiated chloroplasts). These species were selected to represent plants with fast photorespiration and slow net photosynthesis, fast photorespiration yet fast net photosynthesis, and slow photorespiration and fast net photosynthesis, respectively. Buoyant densities were 1.08 and 1.11 g cm^{-3}. The light fraction contained primarily single (incomplete) membrane vesicles and the heavy fraction double (complete) ones. Enzymic, chemical, and electron microscopic examination of the complete envelope membranes showed a lack of microbial, microsomal, mitochondrial, and lamellar membrane contamination as well as stromal contamination. Envelope membranes for all species examined were found to contain 2 to 4% of the total chloroplast protein and yields of about 0.2 to 0.4 mg of protein were obtained from 40 g leaves. An Mg^{2+}-dependent nonlatent ATPase, a marker enzyme for chloroplast envelope membranes, had the following activities (μmoles of phosphate released/ hr^{-1} mg $protein^{-1}$): spinach, 77; sunflower, 163; old maize, 126; and young maize, 87. Bicarbonate transport was directly correlated with levels of ATPase activity in spinach and sunflower envelope membranes. Transport of HCO_3^- with sunflower envelope membranes approached that of young maize.

Plant species can be conveniently divided into groups that have slow (C_3 species) or fast (C_4 species) rates of net photosynthesis. These differences in photosynthetic efficiency result largely from the extent of CO_2 lost through photorespiration (28). For example, leaves of certain photosynthetically efficient species, such as maize, have low rates of photorespiration as opposed to plants with less efficient photosynthesis like spinach with high rates of photorespiration. In addition, both groups have distinctly different morphological characteristics (2).

A very few exceptions to this classification exist. Sunflower leaves have the morphology and photorespiratory rates indicative of species with less efficient photosynthesis, yet their photosynthetic rates approach those of the more photosynthetically efficient species (28). Clearly, sunflower has some mechanism to compensate for a fast photorespiration.

One site of this greater efficiency in sunflower may reside in the chloroplast envelope membrane, which regulates the uptake of metabolites required for photosynthesis, including CO_2 (7, 20, 21, 25). Studies with chloroplasts involving transport and release of permeants in the presence of anions have indicated the presence of specific carriers ("translocators") in the envelope membrane. These translocators facilitate the exchange of phosphate, dicarboxylic acids, and ATP (7, 25). A comparison of the biochemical properties of chloroplast envelope membranes from species with different photosynthetic rates might show differences in the permeability properties and help elucidate the operations of translocators. To this end chloroplast envelope membranes from leaves of the less efficient species, spinach and broad bean, have been isolated and partially characterized in several laboratories (4, 11, 12, 19, 22). No descriptions of the properties of isolated chloroplast envelope membranes from more efficient species, sunflower or maize, have previously been reported. This report describes the preparation and establishes the purity of isolated chloroplast envelope membranes from sunflower and maize and shows large differences in the activities of Mg^{2+}-dependent nonlatent ATPase among chloroplast envelope membranes of plant species with different rates of net photosynthesis. These activities were compared with differences in the transport of bicarbonate.

MATERIALS AND METHODS

Plant Material and Growing Conditions. Spinach (*Spinacia oleracea* L., var. Viroflay, Asgrow Seed Co.), maize (*Zea mays* L., hybrid 595S, Agway Inc.), and sunflower (*Helianthus annuus* L., hybrid 896 [cmsHA 89 × RHA 266], a gift of G. Fick, United States Department of Agriculture at North Dakota State University) were grown in the greenhouse in vermiculite. Nutrient solution was added twice weekly.

Chloroplasts. Intact mesophyll chloroplasts were isolated from 10-g batches of freshly harvested, rinsed leaves (4- to 6-weeks-old) for preparations from spinach, sunflower, and mature maize (6, 8, 27). Undifferentiated intact chloroplasts were prepared from 4- to 6-day-old leaves of young maize (15). CO_2 fixation was determined as described by others (6, 8, 15, 27) and Chl was determined in 80% acetone (3).

Chloroplast Envelope Membranes. Intact chloroplasts for each species were prepared from 40, 80, or 120 g of leaves as above. Buoyant densities of incomplete and complete envelope membranes (22) were determined on a continuous sucrose gradient. Their properties were sufficiently similar so that preparations from each of the species could be prepared by the same procedure developed for isolating spinach chloroplast envelope membranes (22). Conditions were slightly modified only because a centrifuge head with larger volume capacity was used.

Each pellet of intact chloroplasts (derived from 10 g leaves) was suspended in 3 ml of hypotonic medium (Tricine buffer, pH 7.6, 50 mM). A 5-cc hypodermic syringe with a 10 cm 14-gauge

cannula was useful for suspending the chloroplasts. These suspensions (about 0.50 to 0.80 mg of Chl/ml) were maintained at 4 C for 20 min with occasional swirling, whereupon they were pooled and homogenized by making three complete passes in a Ten Broeck glass homogenizer. The homogenized suspension was mixed with 1.4 M sucrose in 50 mM Tricine buffer (pH 7.6) to yield a suspension containing 350 mM sucrose. The suspension was purified on a three-phase discontinuous sucrose gradient in a Beckman Model L preparative centrifuge. The sucrose gradient, buffered as described above, consisted from top to bottom of 16 ml of the osmotically shocked suspension of chloroplasts (derived from four chloroplast pellets) in 350 mM sucrose, 6 ml of 672 mM sucrose, and 9 ml of 876 mM sucrose. Tubes of the above sucrose gradient were centrifuged in a swinging bucket rotor (type SW 25.1) at 4 C for 90 min at 23,000 rpm (75,000g at R_{max}), after which time two pale bands, visible as milky white bands with back lighting, were present at the two interfaces. Lower bands containing complete envelope membranes were carefully removed without turbulence.

Electron Microscopy. Chloroplast envelope membranes were fixed with 2% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, and postfixed in 1% osmium tetroxide in the same buffer and embedded in Spurr resin as described previously (22). Sections cut with a diamond knife were stained with uranyl acetate and lead citrate and examined with a Zeiss EM9 S-2 electron microscope.

Enzyme Assays. Ribulose bisphosphate carboxylase and carbonic anhydrase activities were assayed by the procedures of Paulsen and Lane (16) as modified by Murai and Akazawa (13) and Rickli *et al.* (23), respectively. NAD(P)H:Cyt *c* oxidoreductase activity was measured by the method of Douce *et al.* (5). The Mg^{2+}-dependent ATPase activity was measured by the procedure of Nelson *et al.* (14). Inorganic phosphorus was determined with molybdate reagent (9) and protein with Folin reagent (10).

Transport. Conditions for transport experiments were as described previously (21). The final medium contained the following: 50 mM Tricine (pH 7.6), 330 mM sorbitol, 3 mM Mn^{2+}, 3 mM Mg^{2+}, 2 to 4 μg of envelope membrane protein, and various concentrations of $H^{14}CO_3^-$. Transport was terminated after 20 sec by collecting the membrane vesicles on a Millipore filter with vacuum filtration.

RESULTS AND DISCUSSION

Intact chloroplasts from the various species differed in their ability to fix CO_2. Rates observed in terms of μmoles of CO_2 fixed mg protein^{-1} hr^{-1} were as follows: for chloroplasts of spinach, 75 to 100; sunflower, 3 to 5; maize (mesophyll), 0; undifferentiated maize chloroplasts, 10 to 12. These rates are similar to those reported by others (6, 8, 15, 27).

Sunflower chloroplasts were intact, based upon appearance under a light microscope with phase optics and their having a protein/Chl ratio of 14.1. This ratio is the same as that observed for intact chloroplasts of other species (28). Rates of ribulose bisphosphate carboxylase activity were 50 to 60 μmoles of CO_2 fixed mg protein^{-1} hr^{-1}. Although this activity is about one-third that expected for spinach (28), low activity or envelope membrane damage would not appear to account for the low rates of CO_2 fixation observed with sunflower chloroplasts. Losses of ribulose bisphosphate carboxylase probably resulted from enzymic inhibition rather than leakage because addition of supernatant fractions from centrifuged sunflower chloroplasts caused 60 to 70% inhibition to the ribulose bisphosphate carboxylase activity in spinach chloroplasts. Inhibition by phenolics was suspected, but isolation of sunflower chloroplasts in the presence of phenolic chelators, such as PVP 40, activated charcoal, or Dowex resin failed to improve rates of CO_2 fixation.

Buoyant densities of incomplete and complete envelope membranes from the species examined were similar to those reported previously (22) for spinach (Table I) as determined on a continuous sucrose gradient at equilibrium. Therefore, the method of isolating the spinach chloroplast envelope membranes was attempted on the other species (22).

Two envelope membrane bands were observed as before (22) at the two interfaces of the three-phase discontinuous sucrose gradient. The upper band consisted of a layer of incomplete membranes (single membrane vesicles) and the lower band was formed by the complete envelope membranes (double membrane vesicles), as defined previously for spinach chloroplast envelope membranes (22). Incomplete chloroplast envelope membrane layers were quite distinct for sunflower and spinach, but not for maize. Both young and older maize chloroplasts produced a heavy, cloudy yellow protein layer in the upper portion of the sucrose gradient, which at times appeared to merge into the layer of incomplete envelope membranes. At all times, the lower layer of complete envelope membranes was sharply defined. Electron micrographs of complete envelope membranes are shown in Figure 1 and are similar in size and appearance to those reported previously for spinach (19, 22).

Estimates of the percentages of complete envelope membranes in the lower layer were obtained from a minimum of 30 electron micrographs. Results are shown in Table I. Protein yields, percentages of total chloroplast protein, and volumes for complete envelope membranes are also shown in Table I. Recoveries of applied chloroplast protein on the sucrose gradient were always greater than 90%. The envelope membrane protein would appear to contain 2 to 4% of the total chloroplast protein. Sunflower, like spinach (22), yielded high percentages of complete envelope membranes in the lower layer, unlike preparations from young or older maize leaves (Table I). Upper layers of incomplete envelope membranes from maize were more intensely yellow in color and contained more protein than those found from spinach or sunflower. The more vigorous grinding required to obtain mesophyll chloroplasts from mature, fibrous maize leaves and the fragility of undifferentiated chloroplasts of young maize leaves may give rise to more damaged chloroplasts than is found with spinach or sunflower preparations. Deliberate

Table I. *Characterization of Isolated Chloroplast Envelope Membranes*

Average values for at least three separate preparations of isolated chloroplast envelope membranes.

	Spinach	Suflower	Maize	
			Mesophyll	Undifferentiated
Buoyant density (g cm^{-3})[1]				
Incomplete	1.08[4]	1.08[4]	1.08	1.07
Complete	1.11[3]	1.11	1.11	1 .11
Per cent complete[2]	75[4]	70	40	25
Volume (ml)[2]	3.5	3.7	3.7	3.5
Protein (mg)[2]	0.40	0.31	0.21	0.23
Percentage of total chloroplast protein[3]	2.7	4.3	1.6	2.2
ATPase activity[2] (μmoles Pi released hr^{-1} mg protein^{-1})	77(80[4])	163	126	87

[1] As determined with single (incomplete) and double (complete) membrane vesicles on a continuous sucrose gradient.

[2] Data for complete membranes were only from 40 g of leaves.

[3] Combined yield of complete and incomplete envelope membranes isolated with a three phase discontinuous sucrose gradients.

[4] Ref. 22.

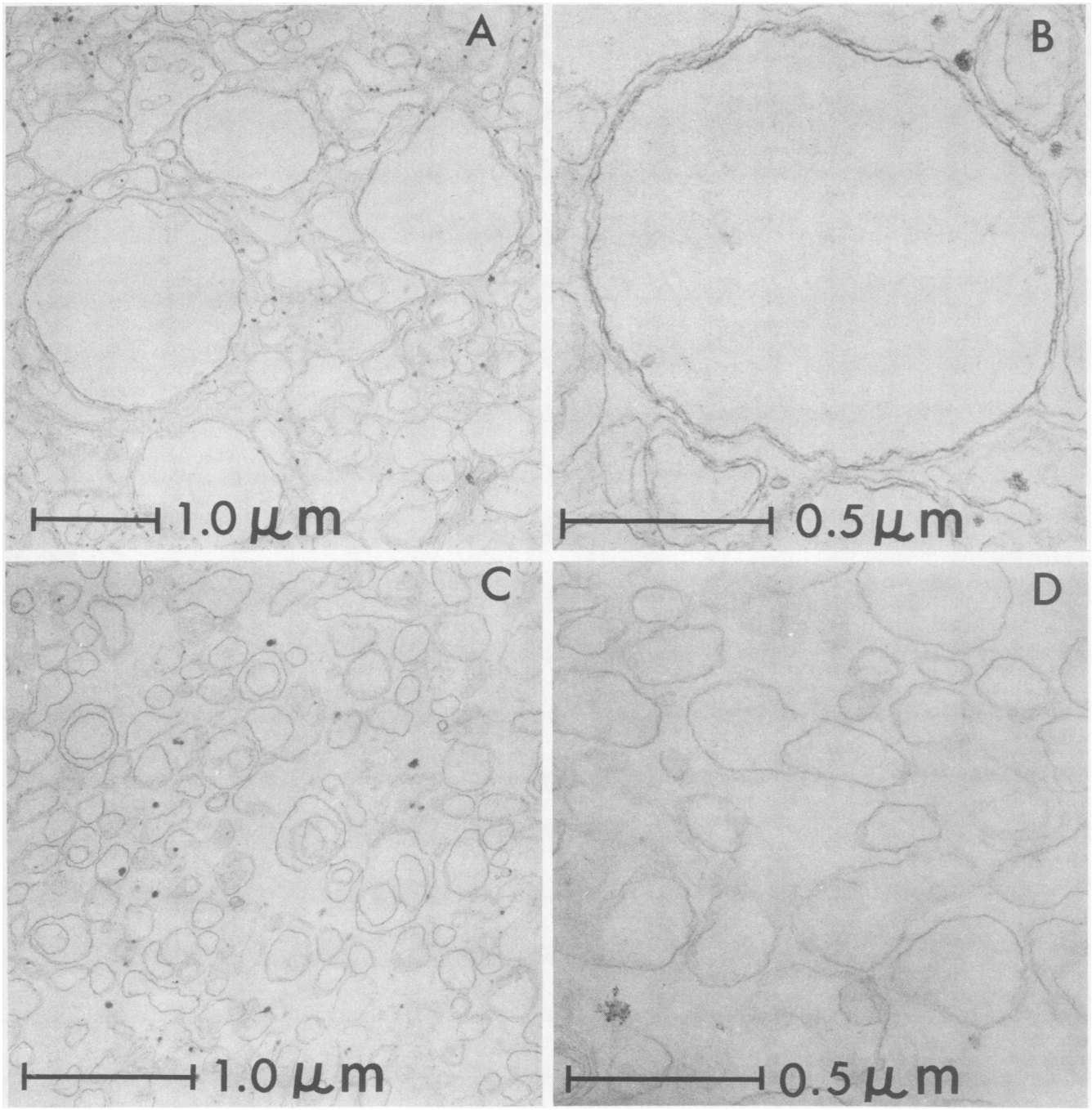

FIG. 1. Representative fields of complete envelope membranes from mesophyll chloroplasts of sunflower (A and B) and maize (C) as well as undifferentiated chloroplasts of maize (D). A typical enlargement of a chloroplast envelope membrane from sunflower with a 75 to 100 Å spacing between the two membranes comprising the envelope membrane is seen in B.

excessive grinding of sunflower or spinach leaves as observed before (19) resulted in an increase in the proportion of incomplete envelope membranes.

Chloroplast envelope membranes were essentially free of bacterial and fungal contamination, as verified by light and electron microscopy and by plating on nutrient agar. Approximately 1 bacterium per 100,000 envelope membranes was observed when preparative solutions were Millipore filtered (0.22 μm) to remove possible contaminating microorganisms.

Enzyme assays were used to determine levels of contamination by the chloroplast stroma or by membranes arising from mitochondria or microsomes. Assays of ribulose bisphosphate carboxylase activity, located in the stroma of mesophyll chloroplasts of spinach, sunflower, and undifferentiated maize chloroplasts (2, 15, 28), showed less than 1% contamination by stromal material for these chloroplast envelope membranes. With envelope membranes of mesophyll maize chloroplasts, similar levels of ribulose bisphosphate carboxylase activity were observed, indicating no contamination from stromal contents of mesophyll or bundle-sheath chloroplasts, although the presence of ribulose bisphosphate carboxylase in mesophyll chloroplasts is subject to question (2, 18). Assays for NAD(P)H:Cyt *c* oxidoreductase indicated less than 1% contamination of any envelope membranes by membranes from mitochondria or microsomes (5).

No detectable carbonic anhydrase activity was present in assays with 0.1 mg of envelope membrane protein for all the above chloroplast envelope membranes. With spinach, and probably sunflower, carbonic anhydrase is present in the stroma of the chloroplast (17), again suggesting no stromal contamination. Unless it is loosely bound to the envelope membrane, carbonic anhydrase is also not associated with either the maize mesophyll or undifferentiated chloroplast envelope membrane. The exact location of this enzyme in maize is uncertain. Some investigators have reported it in mesophyll cells, where it is thought to be in the cytoplasm and there is some evidence that up to 30% of the activity may be associated with chloroplasts (1, 2, 18). A report, based on histochemical staining, shows that carbonic anhydrase is present in both mesophyll and bundle-sheath chloroplasts of maize (24).

Lamellar membrane contamination was determined from protein/Chl ratios. Lamellar membranes, isolated as pellets at the bottom of the sucrose gradients, were found to have protein/Chl ratios of 4.2, 5, and 6.6 for chloroplasts of sunflower, older maize, and younger maize, respectively. From protein/Chl ratios of the envelope membranes, it was calculated that for sunflower, older maize, and young maize, envelope membranes were at least 97, 99, and 90% pure with respect to lamellar membrane contamination. A value of 98% was reported previously for spinach (22). In undifferentiated chloroplasts of maize, the lamellae are less developed (15), and the membranes have a buoyant density closer to that of the chloroplast envelope membrane.

As with spinach chloroplast envelope membranes, a nonlatent, Mg^{2+}-dependent ATPase activity was observed for the envelope membranes from the other species (Table I). In spinach chloroplast envelope membranes this ATPase is of sufficient activity to serve as a marker enzyme (22). Similar or higher activities in envelope membranes of mesophyll chloroplasts from sunflower or maize and of undifferentiated maize chloroplasts (Table I) also establish this ATPase as a marker enzyme for these envelope membranes.

Sunflower chloroplast envelope membranes possess levels of ATPase activity twice that found previously and now for spinach (Table I). If these ATPase function in membrane transport, the transport capacity of the sunflower chloroplast envelope membrane might be potentially greater than that of spinach. This may be related to the differences in photosynthetic efficiency between these two C_3 species, as yet only demonstrated in leaves and not isolated chloroplasts. ATPase activities were also higher for envelope membranes of undifferentiated and mesophyll maize chloroplasts when compared with those of spinach (Table I). Levels of ATPase are less certain for maize with their lower percentages of complete envelope membranes, since losses of ATPase activity have occurred with damaged spinach chloroplast envelope membranes (19, 22).

Bicarbonate transport studies were conducted with the envelope membranes (Fig. 2) of spinach, sunflower, and young maize chloroplasts having the ATPase activities shown in Table I. With sunflower and spinach, two C_3 plants differing in their photosynthetic efficiency, the HCO_3^- transport capabilities showed trends that paralleled their ATPase activities at 5 mM or higher HCO_3^- concentrations (Fig. 2). In fact the HCO_3^- transport of sunflower, a C_3 species with the photosynthetic efficiency of a C_4 species, approached that of young maize. However, the young maize envelope membranes had ATPase levels only slightly higher than those of spinach (Table I).

Werdan *et al.* (26) recently reported that for intact chloroplasts a stromal pH of 8.1 is optimal for CO_2 fixation and that pH differentials between the stroma and external medium decrease with increasing external HCO_3^- concentrations up to 20 mM. At this pH, HCO_3^- is the predominant species and its extrachloroplastic effect upon stromal pH suggests passage through the membrane of HCO_3^- and subsequent conversion to CO_2 rather than direct passage of CO_2. Although the concentrations of HCO_3^- that were most effective on transport here (Fig. 2), and in the experiments of Werden *et al.* (26) appear higher than would be expected on a physiological basis, localized high concentrations of HCO_3^- may conceivably result in *in vivo* at the envelope membrane surface from transitory binding of HCO_3^- prior to membrane passage.

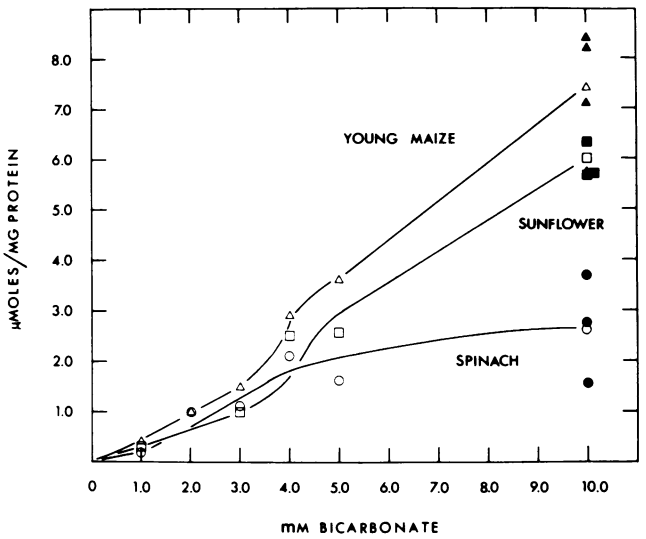

FIG. 2. Effect of external HCO_3^- concentration upon HCO_3^- transport by isolated envelope membranes of young maize (△), sunflower (□), and spinach chloroplasts (○). Open symbols represent averages of at least three experiments, and closed symbols represent individual values from separate experiments carried out with different preparations. Transport was terminated at 20 sec.

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