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Development of the photosynthetic unit in lettuce

(membrane peptide/freeze-fracture/chloroplast)

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ABSTRACT **A comparative study of polypeptide composition and freeze-fracture morphology of chloroplast membranes isolated from different, but photosynthetically active, regions of a romaine lettuce shoot is presented. Chloroplasts prepared from outer dark-green leaves possess a fully developed light-harvesting chlorophyll-protein complex, have low chlorophyll *a*:*b* ratios and display fracture faces similar to those found in other higher plant chloroplast membranes; chloroplasts from leaves more to the interior of the shoot, have a much lower content of chlorophyll, show high chlorophyll *a*:*b* ratios, are depleted in components of the light-harvesting chlorophyll–protein complex, and exhibit extensive modifications of their fracture faces. We have also re-examined the freeze-fracture morphology of chloroplast membranes from a barley *b*-deficient mutant that lacks the light-harvesting chlorophyll–protein complex. A tentative interpretation of our findings suggests correlating the assembly of the light-harvesting chlorophyll–protein complex into the chloroplast membranes with the appearance of large freeze-fracture B face particles in the stacked interior-membrane region, and differentiation of typical fracture faces.**

Freeze-fracture of the internal lamellae of mature chloroplast reveals the presence of a large number of particles associated with the hydrophobic interior of these membranes. In grana lamellae (1), these particles partition in a specific way between the two membrane halves to yield two morphologically distinguishable complementary fracture faces: a B_s face, covered with a sparse number of particles averaging 150 A in diameter, and a C_s face, containing densely packed smaller particles about 100 Å in diameter.

The nature and physiological role of the chloroplast intramembranous particles is unknown, at present. There is some evidence suggesting that the particles are made up wholly or largely of protein (2), and over the last few years a number of proposals have appeared in which the freeze-fracture particles are associated with functional components of chloroplast membranes. A recent model and one that has received the widest, if tacit acceptance, implies an identification of the small C-face particles and large B-face particles with photosystems I and II, respectively (3). This model, based mainly on the study of particle sizes found in subchloroplast fragments enriched in photosystems I or II cannot, however, be reconciled with a number of observations (4), and appears too simplistic to accurately depict the true relationship of the particles to the function of chloroplast membranes. The realization, in the past few years, that the pigment-protein complexes constitute the largest part of the photosynthetic lamellar mass suggested a new possibility, namely, that the intramembranous particles are a morphological expression of these complexes. This possibility was first suggested by Thornber *et al.* (5) in a purely speculative way, and was recently favorably argued by Anderson (4) from her extensive literature review on this subject. In this work, we present some data bearing on the relationship of the light-harvesting chlorophyll–protein complex with the intramembranous particles, and discuss also the significance of clearly differentiated fracture faces, as seen in mature chloroplast lamellae, to the physiological activities of these membranes.

MATERIALS AND METHODS

Lettuce leaves (*Lactuca sativa*, L. var. *romana*, Hort.) used in this work were obtained from local stores. Barley plants (*Hordeum vulgare*, L.) were grown at 25° under artificial illumination (2.15×10^4 lux) with an 8 hr photoperiod.

CO_2 Uptake Experiments. Carbon dioxide uptake measurements were performed with detached leaves, by using a leaf-chamber apparatus described in detail elsewhere (6). Carbon dioxide at a concentration of 900 ppm was supplied to the leaf in air flowing at a rate of 966 liters/hr; the measurements were done at a temperature of 22° ± 3° and at a light-intensity of about 5.38×10^4 lux, which was found to be saturating, under these conditions, for leaves from the outer region of the lettuce head.

Chloroplast Isolation. Chloroplasts for peptide-analysis were isolated according to Sane *et al.* (1); after isolation, chloroplasts were twice washed with 0.15 M KCl in 0.05 M potassium phosphate buffer at pH 7.4, and then by a 15 min treatment with 10 mM pyrophosphate (7) to release attached ribulose diphosphate carboxylase protein. Chloroplasts for freeze-fracture observations were isolated according to Jensen and Bassham (8), and immediately frozen in the isolation medium.

Extraction and Solubilization of Protein. The chloroplast membrane protein fraction was prepared as before (9), and dissolved in 0.0625 M Tris·HCl (pH 6.8), 5% 2-mercaptoethanol (vol/vol) and 2% sodium dodecyl sulfate (10) by heating in a boiling-water bath for 2 min.

Gel Electrophoresis. The electrophoretic method employs a discontinuous sodium dodecyl sulfate/Tris/glycine buffer system (10), and was described in detail previously (9). Staining, destaining, densitometric tracings, and molecular-weight estimations were performed as before (9).

Freeze-Fracture. Freeze-fracture was carried out at −110° in a Balzer apparatus as described before (9).

RESULTS

The leaves from successive nodes of a lettuce shoot show a continuous gradient in their size and color, from the large dark-green outer leaves to the small whitish inner leaves close to the meristematic tip of the shoot. In this work, we used leaves from three different regions of the lettuce shoot, namely: (*i*) an outer region, comprising dark-green leaves, with total chlorophyll content of 3–3.5 mg/dm^2 and low chlorophyll *a*:*b* ratios (2.3–2.7); (*ii*) a region immediately beneath the outer part, containing light-green leaves, with a chlorophyll content of 0.7–1 mg per dm^2 and chlorophyll *a*:*b* ratios of 3–4; and (*iii*) a region more to the interior of the shoot, containing yellow-green leaves, with chlorophyll content of 0.2–0.3 mg/dm^2 and

Table 1. Chlorophyll content and photosynthetic rates of leaves from outer and inner regions of a romaine lettuce shoot

Leaf type	Chlorophyll content (mg/dm^2)	Chlorophyll $a:b$ ratio	CO_2 uptake rates (μmol of $CO_2 \cdot mg^{-1}$ chlorophyll$\cdot hr^{-1}$)
Outer	3.0	2.5	270
Inner	0.2	6.0	1200

CO_2 exchange measurements were done at 5.38×10^4/lux in air containing 0.09% CO_2.

high chlorophyll $a:b$ ratios of 5–6. Hereafter, we will refer to leaves from these three parts of a lettuce head as *outer*, *middle*, and *inner* leaves, respectively, but note that *inner leaves* do not refer to leaves close to the center of the head, but rather to leaves occurring midway between this center and the outer portion. Studies were performed either with whole detached leaves or isolated chloroplasts.

CO_2 uptake

CO_2 uptake measurements were done with whole detached outer and inner leaves of the lettuce shoot. The main characteristics of these two leaf-types, as well as their CO_2 fixation rates, are summarized in Table 1. The rates measured for outer leaves agree with expected values for fully developed mature leaves of dicotyledonous plants under the experimental conditions used here (11). The 4- to 5-fold increase in the CO_2 uptake rate of inner leaves, expressed per mg of chlorophyll, compared with outer ones, is comparable with data obtained with leaves equally depleted in their chlorophyll content from a variety of other sources (12).

Because the CO_2 exchange measurements reported here were performed at a saturating light-intensity for outer leaves, the rates of CO_2 uptake measured for these leaves are maximum values under these particular conditions; however, the values found for inner leaves do not necessarily reflect maximum obtainable rates and careful light-saturation studies are needed.

Peptide analysis

Polypeptide profiles of chloroplast membranes from inner, middle, and outer regions of the lettuce shoot are shown in Fig. 1A, B, and C. The polypeptide pattern of chloroplast membranes from inner leaves (Fig. 1A) reveals about 25 discrete components, with four peptide bands being present in relatively large amounts. Some of these major bands have been identified with components of peripheral proteins associated with the photosynthetic process (13). The 34,000 peptide is partially solubilized with a CF_1 protein fraction, but its relationship with this peripheral enzyme is not clear. The physiological role of most of other peptide components is not known; some of them must certainly represent functional components of the photosynthetic electron transport chain, their identification constituting a major goal of present chloroplast membrane research. The polypeptide profile of chloroplast membranes from middle leaves (Fig. 1B), though qualitatively similar to that of chloroplast membranes from inner leaves, shows a few, significant, differences from the latter. Major among these differences is the substantial increase in the 25,000 peak as well as an enrichment of 27,500 and 23,000 peptides; also, the ratio of 19,000 to 20,000 peptides is increased in these membranes. However, the 34,000 band, a major component of the inner leaf chloroplast membranes is somewhat reduced in chloroplasts of middle leaves whereas the 32,000 band becomes a more prominent component here. Larger components ($\geq$ 50,000) are present in comparable amounts in the two types of chloroplasts. The polypeptide profile of chloroplast membranes from outer leaves (Fig. 1C) is apparently strikingly different from those of chloroplast membranes of the two other leaf types (Fig. 1A and B). However, a closer examination shows that only a few major changes in peptide relative amounts occurred and no new peptides were assembled into the membrane. Particularly prominent among these changes is the enrichment of these membranes in the 25,000 peptide which now accounts for about 25% of the total membrane protein. The 27,500 and 23,000 peaks are also substantially increased and the ratio of 34,000 to 32,000 peptides, which was decreased in chloroplasts from middle leaves, is further reduced here.

Recent studies in our laboratory have shown that the 27,500, 25,000, and 23,000 peptides are the major components of the light-harvesting chlorophyll–protein complex in lettuce chloroplasts (unpublished work); studies by Thornber and Highkin on chlorophyll–protein complexes from a chlorophyll deficient mutant and normal barley plants (14) also led to the identification of a similar group of peptides with the light-harvesting chlorophyll–protein complex (9). This complex, in normal chloroplasts, contains about 50% of total organelle chlorophyll and is the major location of chlorophyll *b*; the assembly of such a complex into the photosynthetic membranes, then, brings about a substantial enrichment of these membranes in chlorophyll and, in particular, chlorophyll *b*, which agrees with our data in Table 1.

Freeze-fracture

A freeze-fracture image of chloroplast membranes from lettuce outer leaves is presented in Fig. 2A; as typical for mature chloroplast membranes from higher plants, one sees a clear differentiation of fracture faces, with their characteristic particle sizes and arrangements. The large B-face particle averages 150 Å in the stacked regions of chloroplast internal lamellae, and about 100 Å in the single stroma lamellae; the smaller C-face particle, characterized by their closer packing, ranges in size from 70 to 100 Å.

Chloroplast membranes from inner lettuce leaves showed a wide heterogeneity in their freeze-fracture images, the most representative situations being presented here (Fig. 2B and C). The vast majority of membrane areas examined looked similar to that of Fig. 2B: fracture faces could not be differentiated and the particles were all small in size, ranging from 60 to 100 Å in diameter. A relatively small number of areas exhibited rather well differentiated fracture-faces (Fig. 2C), but again, the sizes of the particles were smaller than expected for typical faces. Chloroplast membranes from middle leaves (Fig. 2D) exhibited a fracture pattern very similar to that of chloroplast membranes from inner leaves; however, areas of well differentiated fracture faces were more frequent and the size of their particles closer to those found in mature chloroplast membranes. The chloroplast membranes from both inner and middle leaves contained, in addition, small patches where full differentiation of particle sizes and fracture faces occurred.

Fig. 3A shows a typical freeze-fracture image of chloroplast membranes from wild-type barley plants; the alternating B and C faces, with their unique sets of particles, very closely resemble the pattern of chloroplast membranes from outer leaves of lettuce (Fig. 2A). After our initial conservative view of membrane structure in barley chlorophyll *b*-deficient mutant

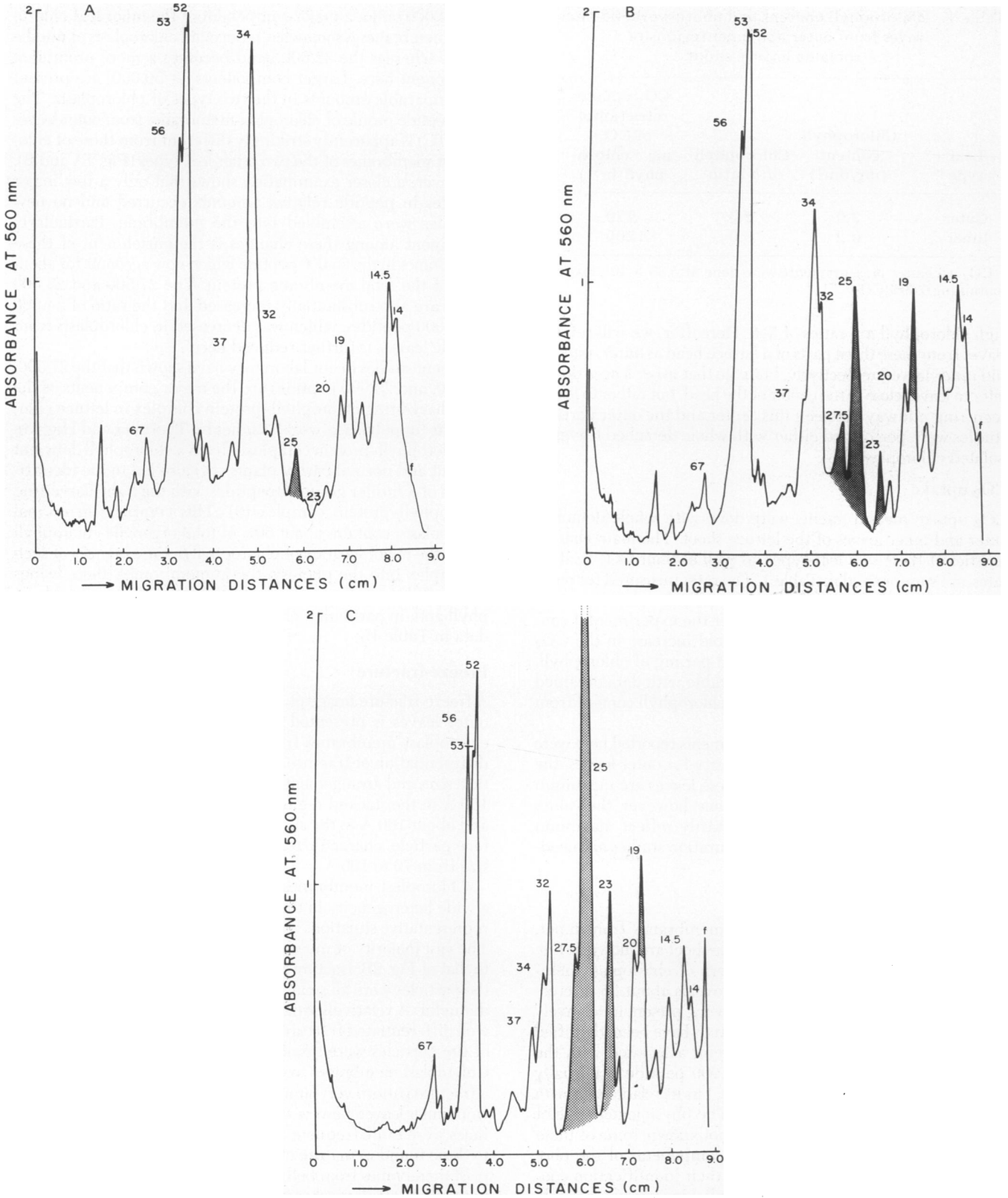

FIG. 1. Densitometric tracings of chloroplast membrane proteins from inner (A), middle (B), and outer (C) leaves of a romaine lettuce shoot. Proteins were solubilized in 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and electrophoresed in 9% acrylamide containing 0.1% sodium dodecyl sulfate. Shaded areas indicate those peptides largely enriched in chloroplasts from middle and outer leaves. Values are molecular weight $\times 10^3$.

chloroplasts (9), we re-examined, in more detail, these membranes and found a considerable heterogeneity in their freeze-fracture appearances (Fig. 3B and C). At least two more situations are evident and common in the chlorophyll *b*-deficient mutant chloroplast membranes. One (Fig. 3B) shows fracture-faces with different densities in particles but only

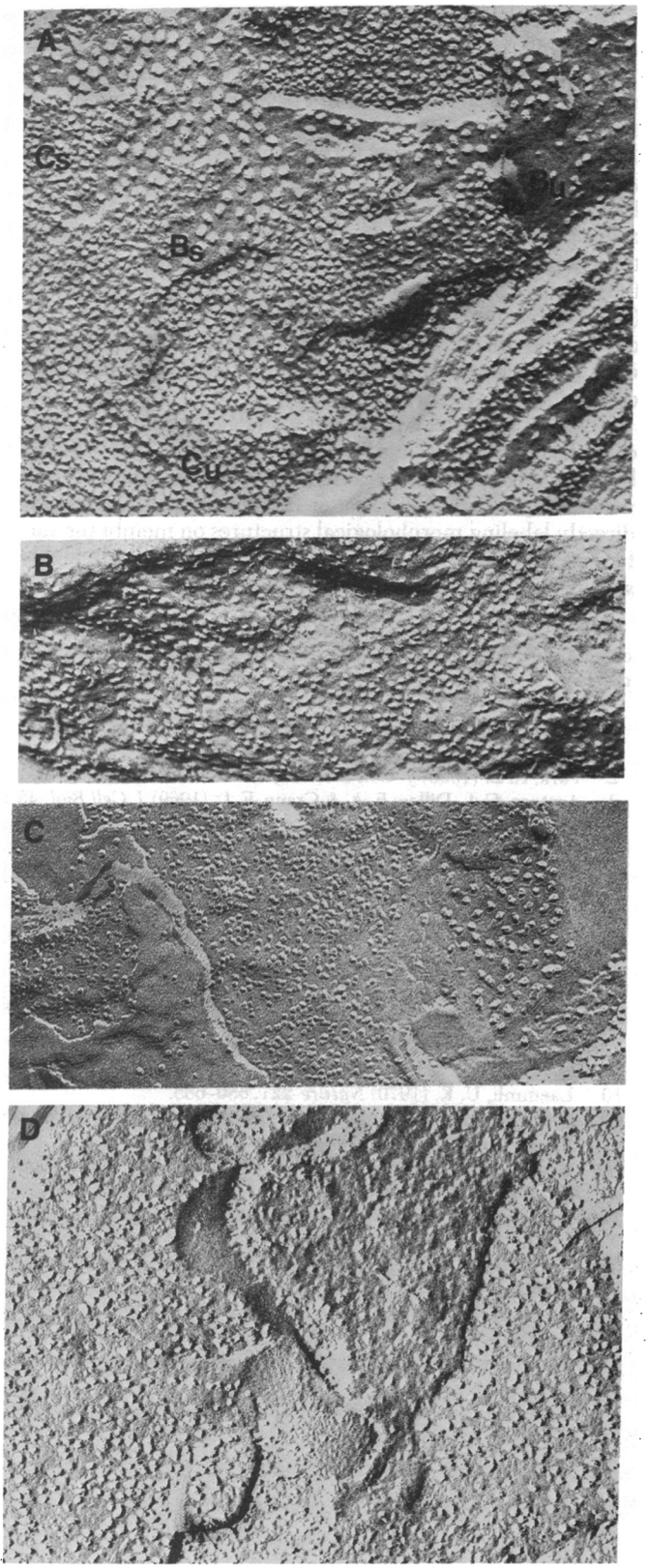

FIG. 2. Freeze-fracture of chloroplast membranes from outer (A), inner (B and C), and middle (D) leaves of a romaine lettuce shoot. ×100,000. After freeze-fracture, B faces occur on the interior portion of the thylakoid membrane, and C faces occur on the outer portion of the thylakoid membrane. The subscripts (s) and (u) respectively refer to stacked and unstacked regions of interior membrane system of chloroplasts.

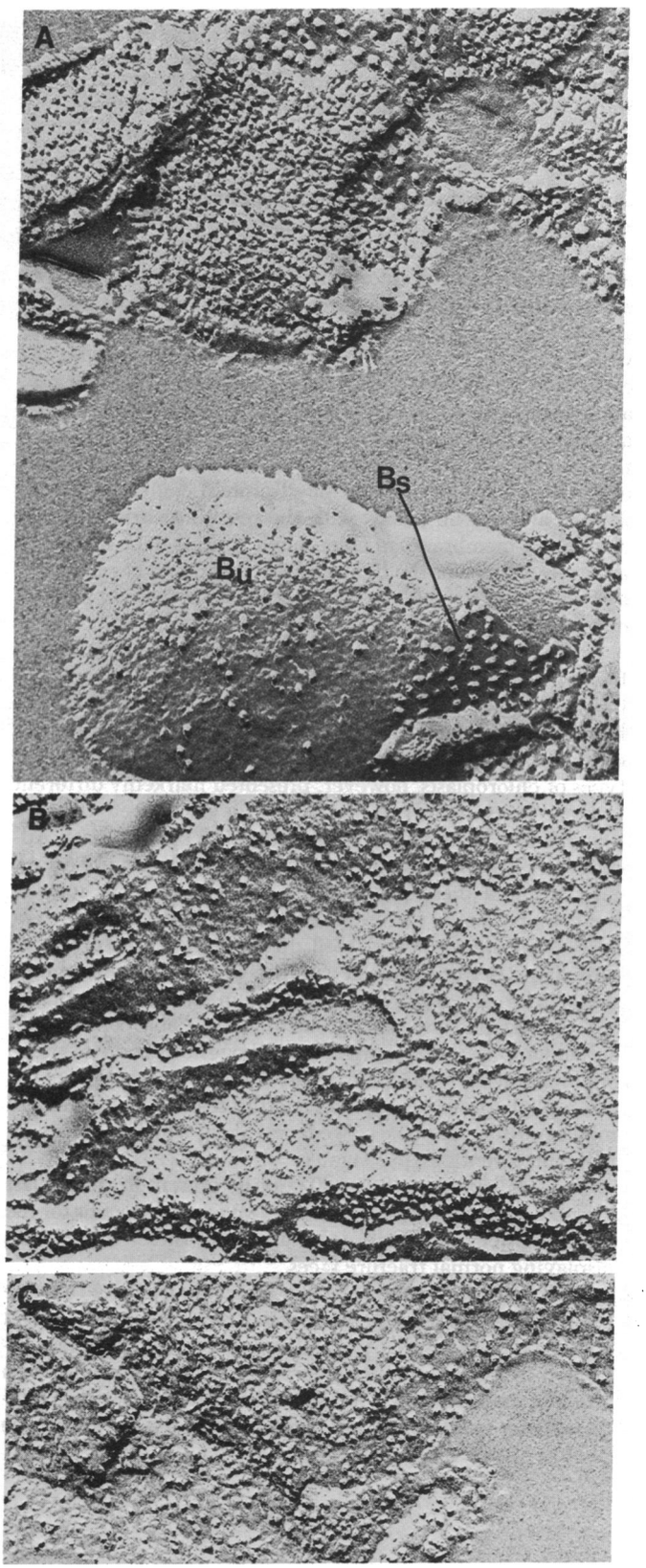

FIG. 3. Freeze-fracture of chloroplast membranes from wild-type (A) and a chlorophyll deficient mutant (B and C) of barley. ×100,000. See Fig. 2 legend for nomenclature.

minor differences in the sizes of these particles; the second, and probably the most prevalent (Fig. 3C) shows no clear differences in particle sizes or densities, allowing no distinction of

fracture faces. The particles average about 100 Å in diameter and, thus, this description of chloroplast membranes from the chlorophyll *b*-deficient mutant is similar to those for membranes from inner and middle leaves of the lettuce shoot.

DISCUSSION

Our data show that the leaves from the different regions of the lettuce shoot used in this work are photosynthetically competent. Taken alone, the much higher rates of CO_2 uptake, when expressed per unit of chlorophyll, exhibited by inner leaves compared with outer leaves are not an absolute indication of a reduction in photosynthetic unit size since more than one variable may be involved (15). However, when these rates are considered in conjunction with chlorophyll content per unit area and peptide composition, they are most easily explained if we assume the photosynthetic unit of inner leaves is smaller than that of outer leaves. The assembly of the peptide components of the light-harvesting chlorophyll-protein complex (27,500, 25,000, and 23,000 peaks) with the resulting enrichment of chloroplast membranes in chlorophyll appears to be related to an increased size of the light-gathering antenna of photosynthetic unit and not with the basic, electron carrier components of this unit.

Freeze-fracture examination of chloroplasts from the different regions of the lettuce shoot also revealed some interesting intramembrane features. Chloroplasts from outer leaves exhibited fracture faces resembling those described for a variety of other higher plant chloroplast membranes. The two other types of chloroplasts, however, presented markedly different fracture faces, mainly characterized by less discrete fracture planes and a more uniform distribution of particle sizes. Additionally, the cleaving plane appeared to travel more erratically through these membranes, as if the discrete zone of weakness present in mature frozen membranes, was not present here. The appearance of well differentiated fracture faces caused by a defined plane of weakness in the frozen membrane seems to be correlated with the insertion, into the membrane, of the 25,000 and associated group of hydrophobic peptides (16) and chlorophyll. The pigments are thought by some (4) to be organized in the membrane as part of the boundary lipid of the chlorophyll–protein complexes and this hydrophobic interface might provide the plane of weakness along which the cleaving plane preferentially propagates.

The small amount of 25,000 peptide present in chloroplast membranes from inner and middle leaves may account for the small number of membrane areas in these chloroplasts displaying normal fracture faces.

The conclusion from both inner and outer leaves of romaine lettuce as well as from the chlorophyll *b*-deficient mutant and wild-type barley is that well differentiated fracture faces exhibited by normal mature chloroplast membranes are not an absolute requirement for the functional integrity of photosynthetic membranes, but are related to the growth of the photosynthetic unit.

The simplest interpretation of our data is that large particles in differentiated mature membranes arise by the addition of the components of the light-harvesting chlorophyll–protein complex to an intermediate sized particle, already present in the photosynthetic membranes. Though the nature and physiological role of the original B-face particle remains unknown, an intriguing possibility is that this original particle may contain PSII-components; the close apposition of the light-harvesting chlorophyll–protein complex is in accordance with the role of this complex in capturing and funneling excitation energy to the internal PSII reaction center as well as adjacent PSI centers (17). The recent proposal (18) that the large particles physically contact the small C-face particles suggested to represent PSI-units in adjacent membranes, would provide another spatial configuration for energy spillover.

Though this model is consistent with data presently available on thylakoid structural and functional relationships, it is a model based largely on inference rather than direct observation. Until we develop reliable chemical or immunological methods for directly labeling morphological structures on membrane surfaces or fracture faces, such models as this must remain highly speculative.

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