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SEPARATION OF SUBCHLOROPLAST MEMBRANE PARTICLES BY COUNTER-CURRENT DISTRIBUTION

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SUMMARY

Counter-current distribution in an aqueous Dextran-polyethylene glycol two-phase system has been used to fractionate membrane fragments obtained by press treatment of Class II chloroplasts. By the counter-current distribution technique membrane particles are separated according to their surface properties such as charge and hydrophobicity.

The fractions obtained were analysed with respect to photochemical activities, chlorophyll and *P*-700 contents. The Photosystem II enrichment after counter-current distribution was better than that obtained by differential centrifugation of the disrupted chloroplasts. However, the best separation of Photosystem I and II enriched particles could be achieved if differential centrifugation was combined with the counter-current distribution technique.

Each centrifugal fraction could be further separated into Photosystems I and II enriched fractions since the Photosystem II particles preferred the dextran-rich bottom phase while the Photosystem I particles preferred the polyethylene glycol-rich top phase. By this procedure it was possible, without the use of detergents, to obtain vesicles which were more enriched in Photosystem II as compared to intact grana stacks.

The partition behaviour of undisrupted Class II chloroplasts and the Photosystem I centrifugal fraction was the same. This similarity indicates that the membrane which is exposed to the surrounding polymers by the Class II chloroplasts is the Photosystem I rich membrane of the stroma lamellae.

INTRODUCTION

Several attempts have been made to disintegrate and fractionate the chloroplast lamellae [1–5]. The membrane fragments obtained have usually been separated with methods utilizing size and density differences. The mixture of membrane fragments after disintegration is, however, so complex that it is important to complement centrif-

Abbreviations used: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Cl_2Ind, 2,6-dichlorophenol-indophenol.

ugation with methods that utilize properties of the particles other than size and density. One such method is partition in aqueous two-phase systems [6] which separates mostly according to differences in the surface properties of particles. Counter-current distribution of cells and cell organelles in such systems have recently been reviewed [7]. In chloroplast studies the method has been used for separation of different classes of chloroplasts [8] and in studies of developing etioplasts [9–10].

In the present study we used counter-current distribution in order to separate membrane fragments after mechanical disruption of chloroplasts. The results showed that the chloroplast membrane fragments could be separated into subfractions by counter-current distribution, since the Photosystem II particles preferred the dextran rich bottom phase while the Photosystem I particles preferred the polyethylene glycol-rich top phase. It was also possible to get a partial separation of Photosystem II and Photosystem I from the grana region without the use of detergents. The correlation of partition behaviour to the membrane surfaces of different parts from the thylakoid lamellae will be discussed.

MATERIALS AND METHODS

Polyethylene glycol 4000 was obtained from Union Carbide, New York, N.Y., U.S.A. Dextran-500, batch No. 5996, was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Preparation of Yeda press fractions

Spinacia oleracea L. was grown at 18 °C with a light period of 12 h (40 w · m^{-2}, Atlas 65/80 W and Philips TL 65/80 W/32 RS in the proportion of 1 : 2). Before harvesting, the spinach was kept in dark for 48 h to reduce the starch content.

Samples of 25 g leaves were homogenized for 5 s at maximum speed in a Turmix blender containing 100 ml of preparation medium (50 mM sodium phosphate buffer, pH 7.4/10 mM NaCl/500 mM sucrose). The slurry was filtered through four layers of Nylon mesh (25 μm) and centrifuged for one min at 1000 $\times g$. The pellet was resuspended in 15 ml of preparation medium and centrifuged for 10 min at 1000 $\times g$. The pellet, which consisted of 30–60 % Class I chloroplasts (as revealed from phase contrast microscopy), was treated with 15 ml of 50 mM sodium phosphate buffer, pH 7.4/10 mM NaCl/50 mM sucrose for 30 min and centrifuged for 10 min at 1000 $\times$ g. The obtained Class II chloroplasts were washed twice with preparation medium, and finally resuspended in 50 mM sodium phosphate buffer, pH 7.4/150 mM NaCl to a concentration of 200 μg of chlorophyll/ml, and passed through a Yeda press [11] (Yeda Research and Development Co., Rehovot, Israel) at approximately 6 ml/min. The pressure (100 kg/cm^2) was obtained from a nitrogen, high pressure, gas tube, The Yeda press homogenate was centrifuged 30 min at 10 000 $\times g$. The pellet was resuspended in 50 mM sodium phosphate buffer, pH 7.4/150 mM NaCl to the same concentration as above, and passed once more through the Yeda press. This homogenate and the preceeding supernatant was fractionally separated by centrifugation. The centrifugation scheme was similar to that described by Sane et al. [5] except that 80 000 $\times g$ for 60 min was used and that each pellet was washed in 50 mM sodium phosphate (pH 7.4)/25 mM NaCl/100 mM sucrose.

Counter-current distribution

The counter-current distribution experiments were carried out in a two-phase system containing 6.1 % (w/w) Dextran-500, 6.1 % (w/w) polyethylene glycol 4000, 10 mmol sodium phosphate buffer (pH 7.4)/kg, 5 mmol NaCl/kg and 20 mmol sucrose/kg. A phase system was prepared by mixing 91.5 g of 20 % (w/w) Dextran-500, 45.75 g of 40 % (w/w) polyethylene glycol 4000, 15 g of 0.2 M sodium phosphate buffer (pH 7.4), 15 g of 0.1 M NaCl, 2.05 g sucrose and finally making up to a weight of 300 g with distilled water. The mixture was shaken at 2 °C and allowed to separate at the same temperature overnight in a funnel. After separation the two phases were collected and stored separately while the interphase was discarded.

A sample system was prepared by mixing 4.58 g 20 % (w/w) Dextran, 2.29 g 40 % (w/w) polyethylene glycol, 5.13 g water and 3 ml of chloroplast membranes suspended in 50 mM sodium phosphate buffer (pH 7.4)/25 mM NaCl/100 mM sucrose. The final composition of the sample system (15 g) after addition of chloroplast material was the same as for the 300 g system. The amount of chloroplast material in each sample system corresponded to about 1200 μg of chlorophyll. The volume ratio top:bottom of this system is 1.19 : 1. For the counter-current distribution a volume ratio of 1 : 1 was desired, therefore 1.3 ml of pure bottom phase was added to the sample system.

The automatic thin-layer counter-current distribution apparatus [12, 13] was used. To a plate with 120 chambers numbered 0–119, equal amounts, 0.63 ml of pure top and bottom phase were added to each of the chambers 10–119 using an automatic syringe. The capacity of the bottom chamber was 0.7 ml. Each of the chambers 0–9 were charged with material from the sample system (1.26 ml), which was shaken before use. After each transfer the plate was shaken for 40 s followed by a settling time of 8 min before a new transfer was made. The interphase was kept stationary during the transfers [6, 12].

After 110 transfers the fractions were diluted with 1.26 ml 50 mM sodium phosphate buffer, pH 7.4/25 mM NaCl/100 mM sucrose, in order to break the phase system. The diluted fractions were collected in test tubes and the absorbance at 680 nm was measured and plotted against tube number.

For chlorophyll *a*/*b* ratio determination, photoactivity measurements, electron microscopy and electrophoresis, 15 tubes were pooled (0–15, 16–30, etc.) and the chloroplast membranes were spun down at the required speed. The pellets were resuspended in 50 mM sodium phosphate buffer, pH 7.4/25 mM NaCl/100 mM sucrose.

Chlorophyll determination and photochemical activities

Chlorophylls *a* and *b* were determined according to Arnon [14]. Light induced changes in absorbance were measured on a Schimadzu MPS-50L spectrophotometer with an attached illumination source supplying red light (600–800 nm) of saturating intensity.

The rate of $NADP^+$-reduction was followed at 337 nm using a medium containing 100 μmol sodium phosphate buffer (pH 7.4), 2.6 μmol NaCl, 1.0 μmol $NADP^+$, 1.7 μmol ADP, 5.0 μmol $MgCl_2$, 0.75 μmol Cl_2Ind, 28.8 μmol iso-ascorbate, 0.025 μmol DCMU and finally saturating amounts of ferredoxin (40 μg) and ferredoxin-NADP reductase in a total volume of 2.6 ml. The bleaching of Cl_2Ind was

measured as the decrease in absorbance at 597 nm. The assay medium contained 115 μmol sodium phosphate buffer (pH 5.8), 4.6 μmol NaCl and 0.02 μmol Cl_2Ind, in a total volume of 2.6 ml. The amounts of chloroplast membranes added during the activity measurements corresponded to 10 μg of chlorophyll.

P-700 was determined by the photochemical method, using the instrumentation described by Öqvist [15]. The assay medium contained in a final volume of 1 ml: 17.5 μmol sodium phosphate buffer, pH 7.4, 2.5 μmol NaCl, 10 μmol sucrose, 0.05 μmol methylviologen, 0.01 μmol Cl_2Ind, 20 μmol iso-ascorbate, 3 % Dextran-500 and 3 % polyethylene glycol 4000 (polymers from the phase system).

Electron microscopy

Chloroplast membranes suspended in buffer were fixed with 3 % glutaraldehyde. After 2 h they were pelleted, washed with buffer and post-fixed with 2 % OsO_4 for 2 h. The pellets were stepwise dehydrated in ethanol and finally in propylene oxide before embedding in Epon. All procedures except embedding were done at 2 °C. Sections were cut and finally stained with uranyl acetate and lead citrate (aqueous solutions).

RESULTS AND DISCUSSION

The fragmentation of chloroplasts by passage through the Yeda press, yielded a great variety of membrane particles, which were separated by fractional centrifugation. By this procedure it was possible to get a separation into Photosystem I-enriched particles and Photosystem II-enriched particles in a similar way as described by Sane et al. [5] (Table I).

This conventional fractionation procedure utilizes differences in size and density

TABLE I

CHLOROPHYLL DISTRIBUTION, *P*-700 CONTENTS AND PHOTOCHEMICAL ACTIVITIES OF FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATION OF A YEDA PRESS HOMOGENATE

Class II chloroplasts suspended in 0.15 M sodium chloride/0.05 M sodium phosphate buffer (pH 7.4) were passed through a Yeda press at 100 kg/cm² and separated by fractional centrifugation. Cl_2Ind-reduction was measured with water as electron donor and $NADP^+$-reduction was measured with Cl_2Ind-isoascorbate as electron donor. Activities were measured about 12 h after the start of the preparation and are expressed as μmol per mg chlorophyll per hour. *P*-700 was measured photochemically. Less than 0.5 % of the total chlorophyll was found in the 80K supernatant.

Fraction	Chlorophyll		$NADP^+$-reduction	Cl_2Ind-reduction	Chlorophyll / *P*-700
	%	*a*/*b*			
Class II chloroplasts	100	3.2	53	63	–
Yeda press homogenate	100	3.2	38	60	560
1000 × *g*, 10 min	7	2.9	22	43	–
10 000 × *g*, 30 min	73	2.8	21	53	850
40 000 × *g*, 30 min	15	4.4	50	11	380
80 000 × *g*, 60 min	5	6.2	65	0.3	270

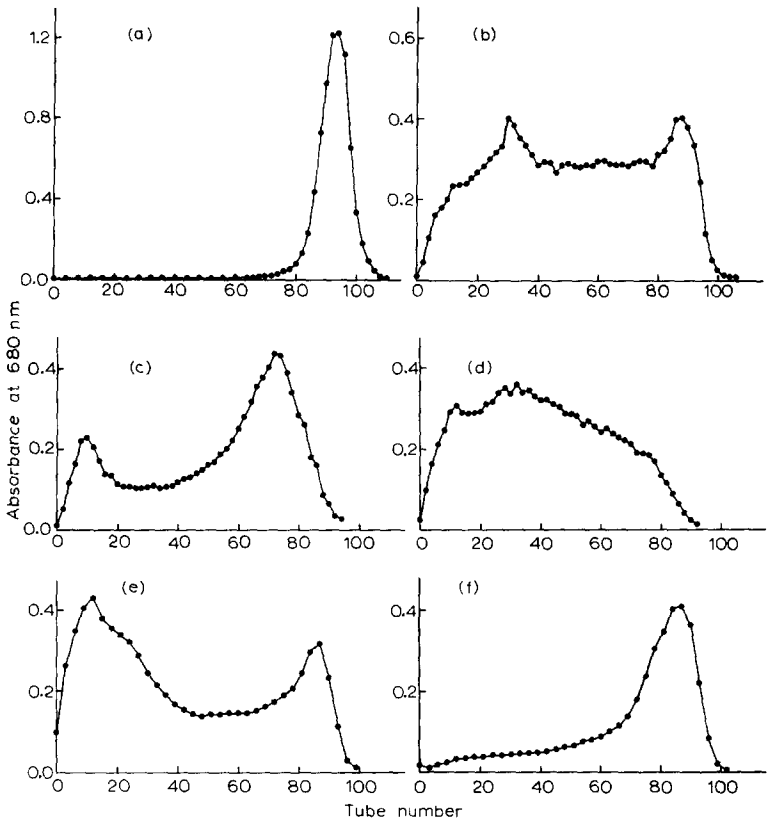

Fig. 1. Counter-current distributions diagrams of (a) class II chloroplasts, (b) Yeda press-treated class II chloroplasts and (c–f) centrifugal fractions of a Yeda press homogenate: (c) 1K (1000 × *g*, 10 min), (d) 10K (10 000 × *g*, 30 min), (e) 40K (40 000 × *g*, 30 min), (f) 80K (80 000 × *g*, 60 min). Counter-current distribution were performed in aqueous Dextran-polyethylene glycol two phase system using 110 transfers.

of the membrane particles. Theoretical considerations and several experimental studies have demonstrated that counter-current distribution separates according to surface properties [6]. Therefore, this separation technique was applied to the Class II chloroplasts, the Yeda press homogenate and centrifugal fractions (Fig. 1a–f).

The Class II chloroplasts gave a distinct peak at the end of the counter-current distribution train indicating a fairly homogeneous population of chloroplasts (Fig. 1a). In contrast, the Yeda press-treated chloroplasts were distributed all over the counter-current distribution train indicating a considerable heterogeneity induced by the press treatment (Fig. 1b). Apparently new membrane surfaces have been exposed, which could be discriminated by the phase system.

When counter-current distribution was applied to the centrifugal fractions (1K, 10K, 40K and 80K), all of them could be further fractionated (Fig. 1c–f). Both the 10K and 40K membrane fractions were distributed all over the counter-current distribution train, showing that these centrifugal fractions were very inhomogeneous in respect to their surface properties. Even the 80K centrifugal fraction

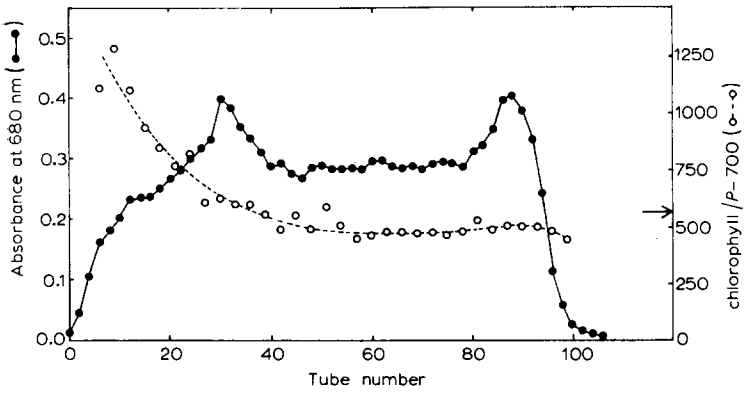

Fig. 2. Counter-current distribution diagram of Yeda press-treated class II chloroplasts. The distribution curve was obtained by measuring the absorbance at 680 nm and determining *P* 700 photochemically. Arrow indicates the chlorophyll to *P* 700 ratio of the material before distribution.

TABLE II

CHLOROPHYLL *a*/*b* RATIOS AND PHOTOCHEMICAL ACTIVITIES OF MATERIAL FROM COUNTER-CURRENT DISTRIBUTION OF YEDA PRESS-TREATED CLASS II CHLOROPLASTS

Cl_2Ind-reduction was measured with water as electron donor and $NADP^+$-reduction was measured with Cl_2Ind-isoascorbate as electron donor. The activities were measured about 36 h after the start of the preparation and are expressed as μmol reduced per mg chlorophyll and h. Tube number intervals in Table II refer to the counter-current distribution diagram in Fig. 2. The reference is material before distribution.

Yeda press homogenate	Reference sample	Tube numbers					
		0–15	16–30	31–45	46–60	61–75	76–90
Chlorophyll *a*/*b*	3.2	2.6	2.6	3.0	3.2	3.4	3.6
Cl_2Ind-red	10	15	12	11	10	7.5	6.4
$NADP^+$-red	45	17	36	48	51	54	57

(Fig. 1f) could be further fractionated although most of the material was found in a main peak at nearly the same position as for the Class II chloroplasts. The peak to the left in the 1K counter-current distribution diagram (Fig. 1c) is mainly due to light scattering from starch granulaes which have high affinity for the Dextran-rich bottom phase.

Efforts were made to see if the behaviour of the thylakoid membrane fragments in counter-current distribution could be correlated to variations in chlorophyll composition or in photochemical activities. As seen from the counter-current distribution of the Yeda press homogenate (Fig. 2 and Table II) the first 15 tubes showed low chlorophyll *a*/*b* ratio, high chlorophyll *P*-700 ratios, high Cl_2Ind reduction and low $NADP^+$-reduction compared to the unfractionated material. This pattern is gradually altered along the counter-current distribution diagram so that the material in the last part showed enrichment in Photosystem I properties. It should be noted that the

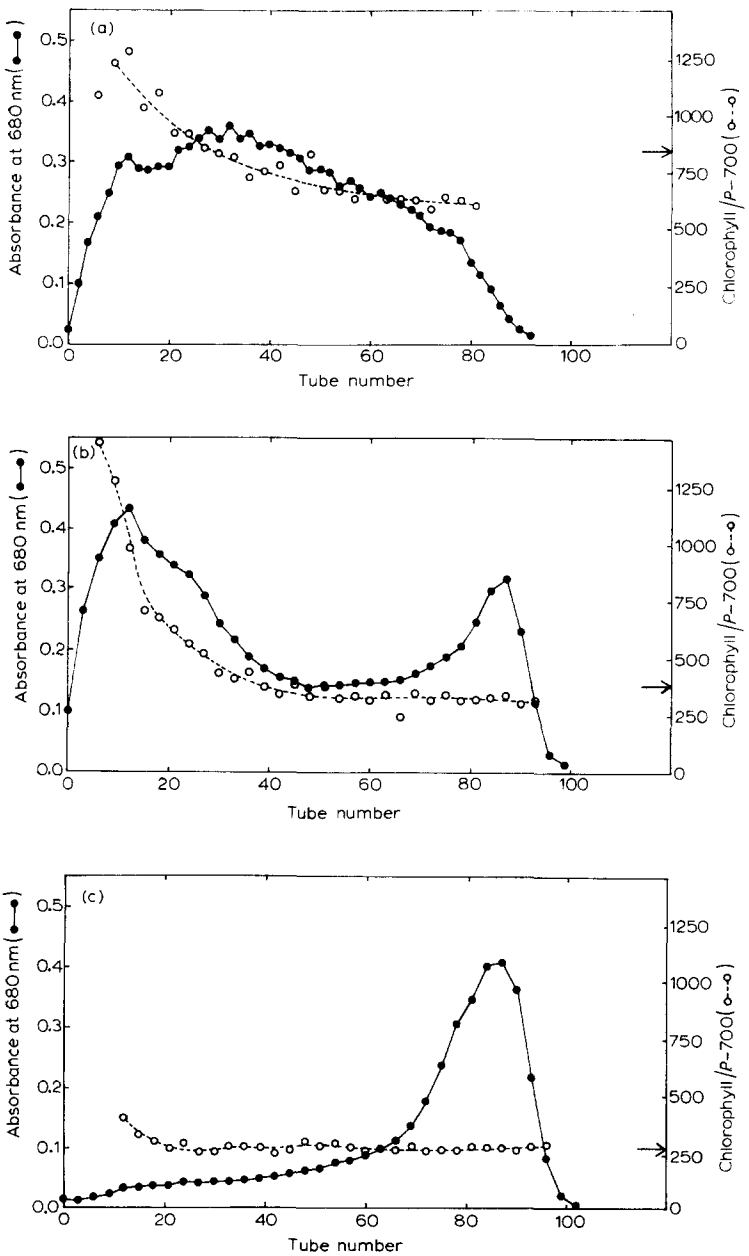

Fig. 3. Counter-current distribution diagrams of (a) 10K, (b) 40K and (c) 80K centrifugal fractions. The distribution curves were obtained by meas uring the absorbance at 680 nm and determining *P*-700 photochemically. Arrows indicate the chlorophyll to *P*-700 ratio of the particular centrifugal fraction before distribution.

TABLE III

CHLOROPHYLL *a/b* RATIOS AND PHOTOCHEMICAL ACTIVITIES OF MATERIAL FROM COUNTER-CURRENT DISTRIBUTION OF YEDA PRESS CENTRIFUGAL FRACTIONS

Cl_2Ind-reduction was measured with water as electron donor and $NADP^+$-reduction was measured with Cl_2Ind-isoascorbate as electron donor. The activities were measured about 36 h after the start of the preparation and are expressed as μmol reduced per mg chlorophyll per hour. Tube number intervals in Table IIIa, b and c refer to the counter-current distribution diagrams in Fig. 3a, b and c, respectively. The reference is undistributed material from the particular centrifugal fraction partitioned.

(a)

10K	Reference sample	Tube numbers					
		0–15	16–30	31–45	46–60	61–75	76–90
Chl *a/b*	2.8	2.4	2.6	2.7	2.9	2.9	3.0
Cl_2Ind-red	29	30	33	29	29	24	16
$NADP^+$-red	24	16	22	29	38	32	–

(b)

40K	Reference sample	Tube numbers				
		0–15	16–30	31–50	51–70	71–90
Chl *a/b*	4.4	2.9	3.5	4.7	5.5	6.1
Cl_2Ind-red	10	24	14	8.5	3.5	3.2
$NADP^+$-red	38	29	54	63	77	73

(c)

80K	Reference sample	Tube numbers				
		0–30	31–60	61–75	76–90	91–105
Chl *a/b*	6.2	4.5	5.8	6.5	6.3	5.8
Cl_2Ind-red	0	0	0	0	0	0
$NADP^+$-red	73	67	80	76	76	67

separation of Photosystem II-enriched particles from the Yeda press homogenate using the counter-current technique was better than that obtained by fractional centrifugation of the same material. The best separation of Photosystem I- and II-enriched membrane particles was achieved when counter-current distribution was combined with fractional centrifugation (Fig. 3 and Table III). Both in the 10K and 40K counter-current distribution experiments Photosystem II-enriched material was partitioned to the extreme left part of the diagram. Material found to the right in the 40K counter-current distribution diagram had almost the same Photosystem I enrichment as the 80K centrifugal fraction.

Sodium dodecyl sulphate gel electrophoresis (mainly according to Weber and Osborne [16]) revealed that the 23 kdalton protein was predominant in the lower number fractions while the three proteins around 60 kdalton were enriched in the higher fractions from each counter-current distribution diagram of disrupted chloro-

plasts. Since the 23 kdalton protein and the 60 kdalton proteins pertain to Photosystem II and Photosystem I, respectively [17, 18], this further supports the interpretation outlined above.

The Yeda press homogenate and the centrifugal fractions could each be fractionated on the basis that the surfaces of the Photosystem II particles preferred the Dextran-rich bottom phase while the surface of the Photosystem I particles preferred the polyethylene glycol-rich top phase (Tables II-III). This partition behaviour could be due to differences in charge and hydrophobicity as well as the specific interactions between the membranes and the polymers.

Studies on the effect of salts on partition of proteins in two-phase systems have shown that the salts create an electrical potential difference between the phases and this affects the partition of charged particles [19]. In the present study the salts added to the phase system make the upper phase positive relative to the lower phase. In addition to electrical factors, differences in hydrophobicity between the polymers influence the partition behaviour, since polyethylene glycol is more hydrophobic than Dextran [20]. In the absence of electrical factors, particles which are partitioned to the upper phase are more hydrophobic than those partitioned to the bottom phase. Taking these considerations into account, the Photosystem II-enriched particles are less hydrophobic and/or less negatively charged than the Photosystem I-enriched particles, since the best Photosystem II enrichment was found in the beginning of the counter-current distribution train. However, specific interactions between the membrane particles and the polymers might complicate the interpretation outlined above. We are now studying these factors more in detail by using phase polymers carrying covalently attached charged and hydrophobic groups.

It should be stressed that the further fractionation of the centrifugal fractions was obtained without addition of detergents as described in other work [5, 21] and that the particles were not degraded and devoid of membrane characters as is the case after detergent treatment. Electron micrographs from the 10K counter-current distribution experiment (Fig. 4) show that the fractions consisting mainly of vesicles (0–15) were more enriched in Photosystem II properties than those consisting of a great portion of intact grana stacks [31–45]. Sane et al. [5] have suggested that the grana contains both Photosystem I and II in a close physical association. Our results indicate that it is possible to obtain vesicles which are more enriched in Photosystem II as compared to intact grana stacks. This must mean that although there is a close association between the photosystems in the grana region, press treatment yields grana vesicles with different enrichment of Photosystems I and II. The surfaces of these grana vesicles differ in such a way that they can be separated by counter-current distribution. Isolation of the grana Photosystem II is therefore not only a disintegration problem but also a problem of separation of the vesicles obtained by disintegration.

The 80K centrifugal fraction seems to be the only membrane population which is fairly homogeneous in respect to surface properties. Most of the material from this fraction was found in a main peak to the right in the counter-current distribution diagram (Fig. 1f). The 80K fraction consisted of small Photosystem I vesicles probably originating from the stroma lamellae similar to the 160K fraction described by Sane et al. [5]. The 40K and 80K Photosystem I particles seem to have the same surface properties since they were found in the same position in the counter-current diagram.

Fig. 4. Electron micrographs from the 10K centrifugal fraction (10K) and samples from different parts of the 10K counter-current distribution diagram (tube numbers 0–15, 31–45 and 75–90).

As can be seen in Fig. 1a the undisrupted Class II chloroplasts were distributed almost to the same part of the counter-current distribution train as the Photosystem I membrane particles. This similarity indicates that the membrane surfaces which is exposed to the surrounding polymers by the Class II chloroplasts is the Photosystem I-rich surface of the stroma lamellae. In contrast, the partition behaviour of the Photosystem II-enriched particles was quite different from that of the Class II chloroplasts, indicating that Photosystem II-rich membranes are not accesible from the outside. This is in agreement with Briantis and Picaud [22] who used antibodies against Photosystem I- and II-enriched particles.

The results from this work show that counter-current distribution alone or in combination with centrifugation is a useful tool for separation of disrupted chloroplasts into fractions enriched in Photosystems I or II. It might also be possible to use phase partition for determining and comparing the membrane properties of different fractions from the thylakoid lamellae. Such a study is in progress.

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REFERENCES

1 Boardman, N. K. (1971) Methods in Enzymology 23A, 268–276
2 Vernon, L. P. and Shaw, E. R. (1971) Methods in Enzymology 23A, 277–289
3 Jacobi, G. (1971) Methods in Enzymology 23A, 289–296
4 Michel, J.-M. and Michel-Wolwertz, M.-R. (1970) Photosynthetica 4 (2), 146–155
5 Sane, P. V., Goodchild, D. I. and Park, R. B. (1970) Biochim. Biophys. Acta 216, 162–178
6 Albertsson, P.-Å. (1971) Partition of Cell Particles and Macromolecules, 2nd edn., Almqvist and Wiksell, Stockholm
7 Albertsson, P.-Å. (1974) Methods in Enzymology 31A, 761–769
8 Larsson, C., Collin, C., and Albertsson, P-Å. (1971) Biochim. Biophys. Acta 245, 425–438
9 Treffry, T. (1974) Eur. J. Biochem. 43, 349–351
10 Treffry, T. and Albertsson, P.-Å. (1974) Proc. 3rd Intern. Congr. Photosynth, pp. 1661–1666, Rehovot, Israel
11 Shnegour, A. and Avron, M. (1970) FEBS Lett. 8, 164–166
12 Albertsson, P.-Å. (1965) Anal. Biochem. 11, 121–125
13 Albertsson, P.-Å. (1970) Sci. Tools, 17, 53–56
14 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
15 Öqvist, G. (1974) Physiol. Plant. 30, 30–37
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
17 Klein, S. M. and Vernon, L. P. (1974) Photochem. Photobiol. 19, 43–49
18 Nolan, W. G. and Park, R. B. (1975) Biochim. Biophys. Acta 375, 406–421
19 Johansson, G. (1974) Mol. Cell. Biochem. 4(3), 169–180
20 Albertsson, P.-Å. (1970) Adv. Prot. Chem.24, 309–341
21 Arntzen, C. J., Dilley, R. A., Peters, G. A. and Shaw, E. R. (1972) Biochim. Biophys. Acta 256, 85–107
22 Briantis, J.-M. and Picaud, M. (1972) FEBS Lett. 20, 100–104