Biochimica et Biophysica Acta, 449 (1976) 525–535

BBA 47206

ISOLATION OF PHOTOSYSTEM II ENRICHED MEMBRANE VESICLES FROM SPINACH CHLOROPLASTS BY PHASE PARTITION

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(Received May 5th, 1976)

SUMMARY

Partition in an aqueous Dextran-polyethylene glycol two-phase system has been used for the separation of chloroplast membrane vesicles obtained by press treatment of a grana-enriched fraction after unstacking in a low salt buffer.

The fractions obtained were analysed with respect to chlorophyll, photochemical activities and ultrastructural characteristics. The results reveal that the material partitioning to the Dextran-rich bottom phase consisted of large membrane vesicles possessing mainly Photosystem II properties with very low contribution from Photosystem I. Measurements of the H_2O to phenyl-*p*-benzoquinone and ascorbate-Cl_2Ind to $NADP^+$ electron transport rates indicate a ratio of around six between Photosystem II and I.

The total fractionation procedure could be completed within 2–3 h with high recovery of both the Photosystem II water-splitting activity and the Photosystem I reduction of $NADP^+$.

These data demonstrate that press treatment of low-salt destabilized grana membranes yields a population of highly Photosystem-II enriched membrane vesicles which can be discriminated by the phase system. We suggest that such membrane vesicles originate from large regions in the native grana membrane which contain virtually only Photosystem II.

INTRODUCTION

Many different disintegration procedures have been used in fractionation studies of the chloroplast lamellae, but the separation has usually been obtained by traditional centrifugation methods [1, 2]. An alternative method is counter-current distribution in aqueous Dextran-polyethylene glycol two-phase systems which

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea; Cl_2Ind, 2,6-dichlorophenol-indophenol; PPBQ, phenyl-*p*-benzoquinone.

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separate particles according to differences in membrane surface properties, such as charge and hydrophobicity, rather than size and density [3, 4].

In a recent paper [5], we showed by this technique that press-treated chloroplasts could be separated into Photosystem I- and II-enriched fractions. Membrane vesicles, probably originating from grana unstacked during the isolation procedure, were partitioned to the dextran-rich bottom phase, showing a higher Photosystem II enrichment than purified grana fractions.

In the present communication, we report a rapid non-detergent method for the isolation of highly Photosystem II-enriched membrane vesicles with improved yield and purity. The method, including low salt unstacking of a grana-enriched fraction, followed by aqueous two-phase partition, could be performed with relatively small activity losses with regard to both the Photosystem II water splitting reaction and the Photosystem I reduction of $NADP^+$.

Membrane vesicles comparable in size to grana discs, possessing mainly Photosystem II properties, were isolated in the dextran-rich bottom phase. These membranes constituted at least 20 % of the chloroplast lamellae on a chlorophyll basis. These data show that our non-detergent treatment is sufficient to partly dissociate the grana photosystems from each other into membrane vesicles with different surface properties which can be discriminated by the phase system. Based upon these results we discuss possible arrangements of the photosystems in the grana region.

METHODS

Spinach chloroplasts, prepared as described earlier [5], were osmotically broken up in 50 mM sodium phosphate buffer, pH 7.4/10 mM NaCl/50 mM sucrose and centrifuged 5 min at $2000 \times g$. To reduce the contamination of envelope and stroma material the pellet was washed twice in the same buffer and finally suspended in 150 mM NaCl/50 mM sodium phosphate buffer, pH 7.4.

Such washed class II chloroplasts were fragmented by passage through a Yeda press at a nitrogen pressure of 10 MPa (100 kg/cm^2). The fractionation of this material is summarized in Fig. 1. Stroma lamellae were removed from fast sedimenting grana by centrifugation for 30 min at $40\,000 \times g$. The grana-enriched fraction so obtained was unstacked by suspension in low salt medium, 10 mM sodium phosphate buffer, pH 7.4/5 mM NaCl/100 mM sucrose, and passed twice through the Yeda press. The direct isolation of the 40K fraction without prior centrifugation at $1000 \times g$ for 10 min and $10\,000 \times g$ for 30 min was choosen to increase the yield of Photosystem II vesicles [5] and to get a more rapid isolation procedure.

This material designated 40K ls (low salt) was either separated analytically by the thin-layer counter-current apparatus as described earlier [5], or preparatively by a few step two-phase partition. In both types of experiment, a two-phase system of the following composition was used: 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.

For the preparative method, 5 ml of the 40K ls fraction (800 μg chlorophyll/ml) was added to 20 g of a polymer mixture to yield a two-phase system of the described composition. The phase system was mixed, and centrifuged for 3 min at $1500 \times g$ to facilitate phase separation. The top phase was removed and the bottom phase, con-

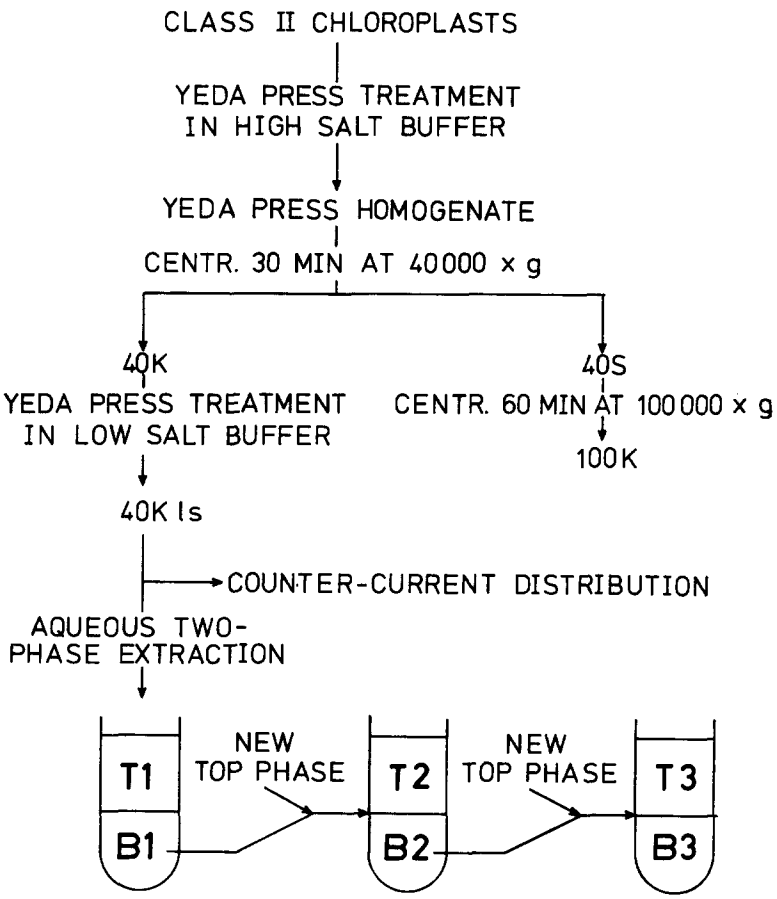

Fig. 1. Scheme for the fragmentation and fractionation of chloroplasts lamellae.

taining mainly Photosystem II enriched material, was washed twice with 15 ml new top phase. In the last step, the material at the interface was removed together with the top phase. For activity measurements, the material was removed from the bottom phase by adding new top phase in which 25 % of the polyethylene glycol was replaced by trimethylaminopolyethylene glycol [6]. The total isolation procedure was completed within 2–3 h, including 20–30 min for the two-phase extraction. All operations were performed at 2–3 °C.

Chlorophyll *a* and *b* were determined according to Arnon [7]. Fluorescence spectra were recorded at liquid nitrogen temperature using the instrumentation of Öquist [8]. The *P*-700 content was estimated chemically (Table I) using an Acta CIII spectrophotometer with an external recorder. Manganese was determined by flameless atomic absorption (at 279.5 nm) using a Varian Techtron AA6 supplied with a CRA-63. $NADP^+$ reduction was measured spectrophotometrically either with water or ascorbate-Cl_2Ind as electron donor. Oxygen evolution was measured polarographically with phenyl-*p*-benzoquinone as electron acceptor. This hydrophobic redox substance was shown, by inhibition studies with KCN (not published), to be a

TABLE I

CHLOROPHYLL, *P*-700 AND MANGANESE CONTENTS OF FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION AND AQUEOUS TWO-PHASE PARTITION OF PRESS DISRUPTED CHLOROPLAST LAMELLAE

Subchloroplast fractions were obtained and designated as illustrated in Fig. 1. Chlorophyll *a*/*b* was determined according to Arnon and chlorophyll fluorescence spectra was recorded at −196 °C. *P*-700 was estimated by the oxidized minus reduced absorbance difference at 700 nm, by adding 0.8 mM $K_3Fe(CN)_6$ or 1.7 mM ascorbate to a chloroplast sample containing 15–20 μg chl/ml. Manganese was estimated by atomic absorbtion. Less than 0.5 % of the chlorophyll was found in the 100K supernatant.

Fraction	Chlorophyll				
	%	*a*/*b*	F735/F685	*P*-700	Mn
Yeda press homogenate	100	3.2	2.5	430	61
40K ls	93.8	3.1	2.3	460	110
T1	77.4	3.2	2.7	410	103
B1	16.4	2.5	1.3	1150	67
T2	7.5	3.0	1.9	740	83
B2	8.9	2.4	1.0	>1200	73
T3	2.8	2.6	1.3	—	—
B3	6.1	2.3	1.1	>1200	50
100K	6.2	6.7	6.9	210	266

better electron acceptor for Photosystem II than *p*-benzoquinone, Cl_2Ind and $K_3Fe(CN)_6$. Samples for electron microscopy were thin-sectioned and stained as described in the legend to Fig 2.

RESULTS

It is well known that suspension of chloroplast lamellae in a low salt buffer leads to unstacking of grana [9]. In the present study a medium containing 10 mM sodium phosphate buffer, pH 7.4/5 mM NaCl/100 mM sucrose, was used. This ionic strength was low enough to cause unstacking of class II chloroplasts (Fig. 2). The 40K fraction, enriched in grana after removal of stroma lamellae (Table I and III), were suspended in this low salt buffer and passed through the Yeda press two more times. This press treatment in low salt medium yielded mainly unstacked membrane vesicles as shown by electron micrographs (Fig. 4).

After such a destabilization of grana, no further fractionation of the photosystems could be achieved by differential centrifugation (Table II). In contrast, counter-current distribution of these vesicles showed that they were very inhomogeneous with respect to their surface properties, yielding two well resolved fractions (Fig. 3). The material distributed to the first main peak, because of a high affinity for the dextran-rich bottom phase, showed low chlorophyll *a*/*b* and F735/F685 ratios. This Photosystem II-enrichment was gradually altered along the counter-current distribution train, so that the material under the second main peak, preferring the top phase, was slightly enriched in Photosystem I properties.

Because of the extreme differences in partition behaviour of the main popula-

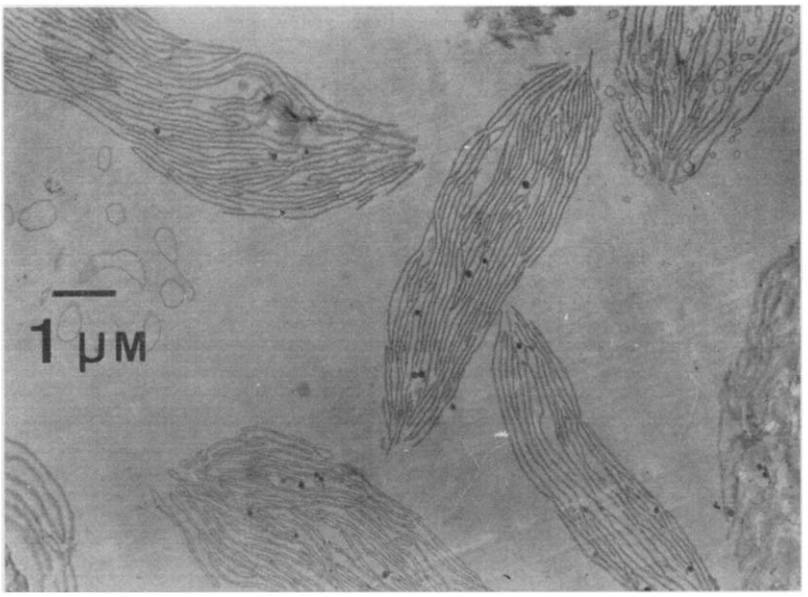

Fig. 2. Electron micrograph of chloroplasts unstacked by suspension in low salt medium, 10 mM sodium phosphate buffer, pH 7.4/5 mM NaCl/100 mM sucrose. The sample was fixed in 3 % glutaraldehyde, washed with buffer and post-fixed in 2 % OsO_4 for 2 h. The dehydration was performed stepwise in ethanol and further in propylene oxide before embedding in Epon. Sections were cut and finally stained with uranyl acetate and lead citrate.

TABLE II

CHLOROPHYLL DISTRIBUTION AFTER DIFFERENTIAL CENTRIFUGATION OF THE LOW-SALT TREATED GRANA FRACTION (40K ls)

Values should be compared to those obtained after aqueous two-phase partition of the same material.

Fraction	Chlorophyll	
	%	*a*/*b*
40K ls	100	3.1
1 000 × *g*, 30 min	8	2.9
2 000 × *g*,	6	3.0
3 000 × *g*,	10	2.9
4 000 × *g*,	9	3.0
5 000 × *g*,	5	3.0
10 000 × *g*,	31	3.0
20 000 × *g*,	16	3.1
40 000 × *g*,	11	3.3
80 000 × *g*, 90 min	4	3.7

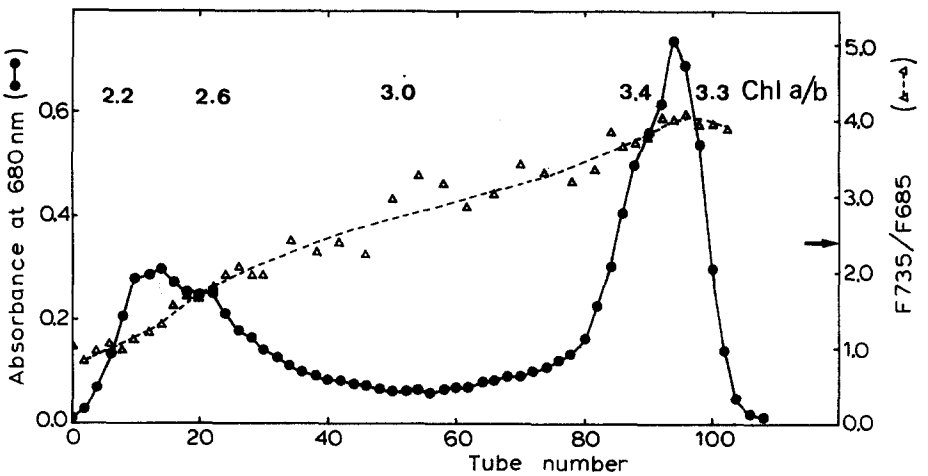

Fig. 3. Counter-current distribution diagram of the low salt treated grana fraction (40K ls). The experiment was carried out in an aqueous dextran-polyethylene glycol two-phase system at 2 °C. After 110 transfers using a shaking time of 30 s followed by a settling time of 8 min, the fractions were diluted with low salt buffer and collected in test tubes. The absorbance at 680 nm was measured and plotted against the tube numbers. Spectra of chlorophyll fluorescence was recorded at −196 °C. Arrow indicates F735/F685 of the material before distribution.

TABLE III

PHOTOCHEMICAL ACTIVITIES OF FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF A YEDA PRESS HOMOGENATE

The specific activities are expressed as μmol O_2 produced or μmol $NADP^+$ reduced per mg chlorophyll and hour. Values within brackets are the percentage of the total activity in each centrifugal fraction calculated from the total activities of the class II chloroplasts. Assay of Photosystem II: oxygen evolution was measured with a Clarce type of electrode in a medium containing 0.4 μmol phenyl-*p*-benzoquinone, 70 μmol sodium phosphate buffer (pH 6.5), 12 μmol NaCl and chloroplast material corresponding to 46 μg chlorophyll, in a total volume of 2.3 ml. Assay of Photosytem I: $NADP^+$ reduction was followed at 337 nm using a spectrophotometer equipped with an illumination source, in 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 ascorbate, 25 nmol DCMU, saturating amounts of ferredoxin, ferredoxin-$NADP^+$ reductase and plastocyanin and chloroplast material corresponding to 10 μg chlorophyll, in a total volume of 2.6 ml. Assay of complete electron transport: as for Photosystem I, except that the pH was 6.5 and that DCMU, ascorbate (Asc) and Cl_2Ind were omitted.

Fraction	Photosystem II activity ($H_2O \rightarrow$ PPBQ)	Photosystem I activity (Asc-$Cl_2Ind \rightarrow NADP^+$)	Complete electron transport chain ($H_2O \rightarrow NADP^+$)
Class II chloroplasts	103 (100)	136 (100)	160 (100)
Yeda press homogenate	65 (64)	133 (98)	58 (36)
40K ls	68 (62)	88 (61)	29 (17)
100K	12 (0.7)	196 (8.9)	2 (0.1)

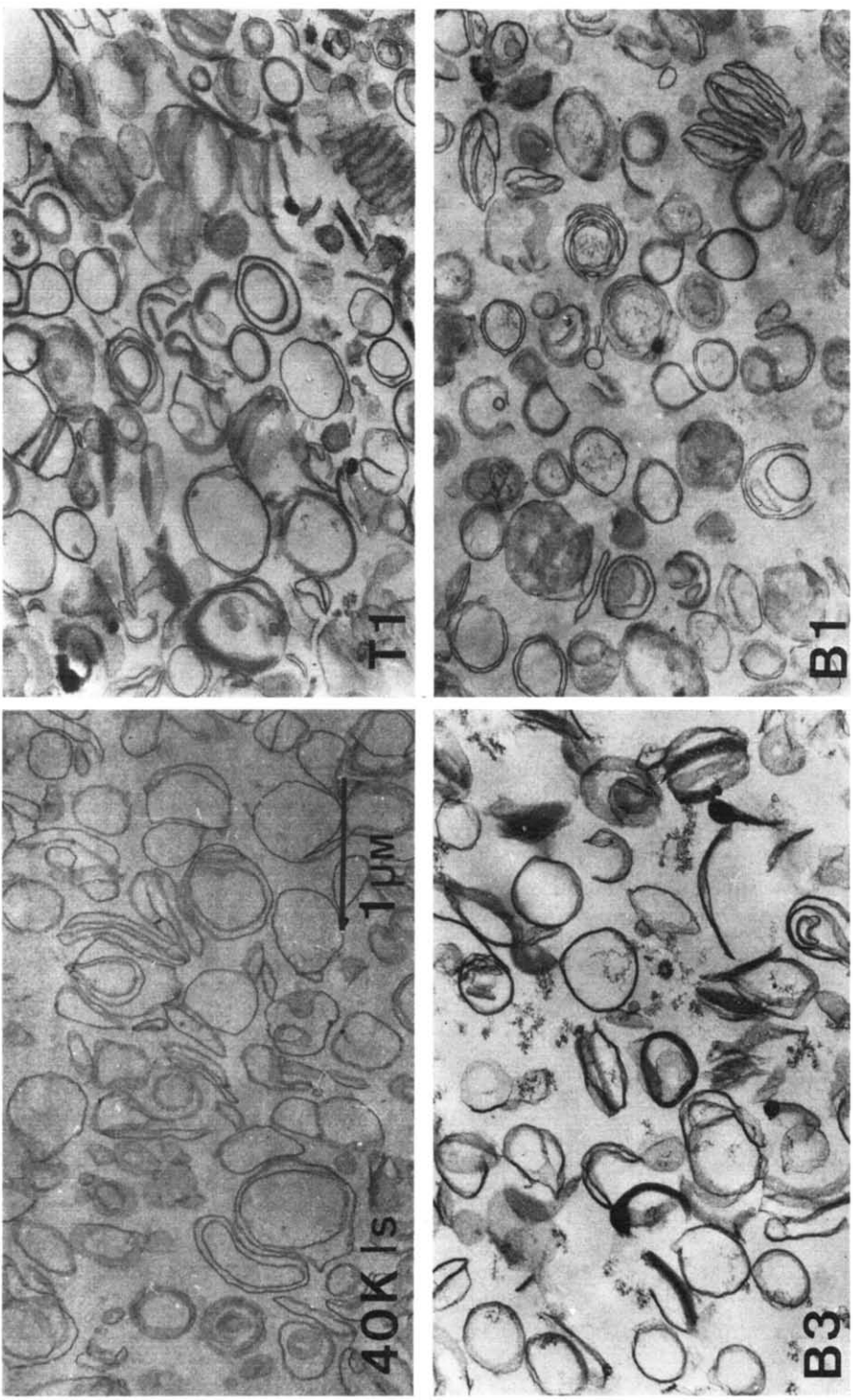

Fig. 4. Electron micrographs of the 40K ls, T1, B1 and B3 fractions.

tions, a three-step phase partition (Fig. 1) was sufficient to yield about the same resolution (Table I) as after a 110 transfer counter-current distribution experiment (Fig. 3). Simply by one partition step, 16 % of the material was recovered in the bottom phase (Table I) with low chlorophyll *a*/*b* and F735/F685 ratios, high manganese content and hardly detectable amounts of *P*-700. This Photosystem II enrichment was further improved by extraction of the bottom phase (B1) two more times with pure top phase. This three step phase partition required only 20–30 min, which made analysis possible after a total isolation procedure of 2–3 h. This is a big improvement, compared to the time required for 110 transfer counter-current distribution, especially for fast declining activities such as the water splitting reaction of photosystem II. Another advantage is that large amounts of material can be separated by simply scaling up the phase volumes.

It should be pointed out that during the two-phase partition the top phases which were removed (T1-T3) were not repartitioned with fresh bottom phase as is the case during counter-current distribution. That explains why only 6 % of the partitioned material can be found in the B3 fraction (Table I) while around 20 % is distributed to the left peak after the counter-current distribution (Fig. 3).

From the photochemical activities, after the phase partition (Table IV), the following points are of special interest: (a) the ratio between the percent total Photosystem II activity and the percent total Photosystem I activity in the B3 fraction is about six, confirming the high Photosystem II enrichment of these membranes; (b) there are no losses of activity during the two-phase extraction, leading to around 100 % recovery of total activity of both photosystems in each partition step. This important observation in combination with the relatively low activity losses during the Yeda press treatments (Table III) indicates that the observed Photosystem-II enrichment does not originate from selective destructive effects; (c) high rates of water splitting activity remain throughout the complete isolation procedure and there is no need for addition of diphenyl carbazide for measuring photosystem II activity; (d) the specific Photosystem I activity of the B3 fraction is of the same order as for the complete electron transport from water to $NADP^+$. It seems therefore reasonable to assume that the contaminating Photosystem I in some way is associated with Photosystem II and not due to free Photosystem I particles.

No clear ultrastructural differences between the membrane material partitioned to the top or bottom phase could be observed from electron microscopy studies (Fig. 4). All fractions consisted of comparatively large membrane vesicles in which the size of the vesicles resembled that of a swollen grana disc. Further, the thickness of the membranes did not appear to be less than around 7 nm.

DISCUSSION

By utilizing differences in surface properties for separation of unstacked membrane vesicles, two main fractions can be isolated where the membrane population preferring the dextranous bottom phase shows a prominent Photosystem II enrichment. In contrast, differential centrifugation utilizing particle size differences does not yield any separation of the photosystems after destabilization of grana [2]. This is also confirmed when our 40K ls fraction was subjected to differential centrifugation, as revealed by the chlorophyll *a*/*b* ratios presented in Table II. Therefore mechanical

disintegration procedures have so far been limited to removal of Photosystem I-rich stroma lamellae from fast sedimenting grana stacks containing both photosystems.

Fractions of similar Photosystem II enrichment to that obtained in the present study, have required detergent solubilization followed by density gradient centrifugation [10–12]. The D II particle of Arntzen et al. [10], prepared in such a way, shows similar properties as our B3 particles, but is devoid of water splitting capacity.

Photosystem II fractions completely uncontaminated by Photosystem I activity can be obtained by detergent fractionation [13–15] but often in low yield and with considerable loss of activity. It is also difficult to make interpretations from such studies about the native thylakoid membrane system since detergents solubilize membrane components into lipid protein-detergent comicelles [16] leaving a modified membranous residue.

In our opinion, high recovery of total activity in combination with preservation of the membrane structure are necessary prerequisites when conclusions regarding the native thylakoid system will be drawn from fractionation studies.

Since the 40K fraction was obtained from the Yeda press homogenate without prior centrifugation at $1000 \times g$ for 10 min and $10\,000 \times g$ for 30 min, it would contain some unbroken material as well as contaminating stroma lamellae. This contamination does not affect the Photosystem II enrichement since unbroken material and stroma lamellae have a high preference for the top phase [5]. It could therefore be argued that our Photosystem II enrichment is simply a result of removal of stroma lamellae and grana end membranes. This cannot, however, be the only explanation, since that would restrict nearly all grana Photosystem I to the end membranes and still make up about the same amount of chlorophyll compared to Photosystem II. Moreover, a conventional press 10K fraction (Chlorophyll *a*/*b* 2.8, Chlorophyll/*P*-700 = 850), where most of the stroma lamellae and unbroken material have been removed by centrifugation, could be further fractionated by the described method into vesicles with predominantly Photosystem II properties (unpublished results).

We also think it unlikely that the separation of the photosystems is achieved by membrane splitting [17, 18], since the thickness of the membranes in the vesicles appears normal.

We do instead claim that the present non-detergent isolation of about 20 % of the chloroplast lamellae as large membrane vesicles ($\phi \cong 300$ nm), with predominantly Photosystem II properties shows that press treatment is sufficient to part grana Photosystem I and Photosystem II from each other. This speaks against a model where the grana membrane possesses both photosystems in similar amounts and in close physical association to each other. Our results suggest instead that in the native grana membrane there exist membrane regions of mainly Photosystem II character and that such a membrane region is the origin of our B3 vesicles.

It is tempting to speculate that such a Photosystem II membrane region could participate in complete electron transport by cooperation with Photosystem I on an adjacent membrane in a grana stack. This would provide one explanation for the higher quantum yield obtained when the thylakoids are closely stacked [19]. Recently Staehlin [20] from the geometrical arrangement of freeze fracture particles proposed an interaction between Photosystem I and Photosystem II of adjacent membranes in the grana region. Furthermore, studies on microsomal membranes have revealed that reducing capacity can be transmitted from one membrane to another when they come into contact [21].

TABLE IV

PHOTOCHEMICAL ACTIVITIES OF FRACTIONS OBTAINED BY TWO-PHASE PARTITION OF THE LOW SALT TREATED GRANA FRACTION (40K ls)

Activities were measured as described in Table III. The specific activities are expressed as μmol O_2 produced or μmol $NADP^+$ reduced per mg chlorophyll and hour. Values within brackets are the percentage of the total activity in each fraction calculated from the total activity of the 40K ls fraction. Asc: ascorbate.

Fraction	Photosystem II activity ($H_2O \rightarrow$ PPBQ)	Photosystem I activity (Asc-Cl_2Ind $\rightarrow$ $NADP^+$)	Complete electron transport chain ($H_2O \rightarrow NADP^+$)
40K ls	68 (100)	88 (100)	29 (100)
T1	63 (76)	104 (97)	37 (107)
B1	94 (24)	44 (8.2)	26 (16)
T2	90 (11)	71 (6.1)	25 (6.5)
B2	103 (14)	32 (3.2)	21 (6.4)
T3	100 (4.1)	38 (1.2)	—
B3	110 (10)	27 (1.9)	21 (4.4)

The Photosystem II rich regions could also cooperate with neighbouring regions rich in Photosystem I by laterial diffusion of redox molecules. Alternatively, the Photosystem II rich regions could be self supporting electron transport systems where the remaining Photosystem I units are coupled to the six times as many Photosystem II units in a mosaic pattern.

Although there is an enrichment of Photosystem II in the bottom phase, the material partioned to the top phase contains about 75 % of the total Photosystem II activity present in the 40K ls fraction (Table IV). Whether this means that the grana material in the top phase is a mixture of vesicles containing either mainly Photosystem I or mainly Photosystem II, or represents vesicles containing both photosystems in about equal amounts can not be judged at the present stage. If the former is the case, there must be some differences in surface properties between Photosystem II enriched vesicles preferring the bottom phase and those preferring the top phase. Such differences could be caused by losses of extrinsic proteins influencing the partition behaviour, or be due to differences in the sidedness of the membrane vesicles. A mixture of vesicles containing either Photosystem I or Photosystem II would be in agreement with a model where grana photosystems are located to different regions. On the other hand, if the material partitioned to the top phase represents vesicles containing both photosystems in about equal amounts, such a model would be less plausible.

Whatever the explanation for the origin of the B3 particles is, they should provide an interesting material for further studies of Photosystem II. Using these membrane particles (prepared rapidly with a high yield), the structure and function of Photosystem II can be studied without much influence from Photosystem I and still be in a membranous state.

ACKNOWLEDGEMENTS

We wish to thank Miss Agneta Persson for skilful technical assistance and Miss Katarina Wallgren for sectioning and staining preparations for electron microscopy. We are also grateful to Dr G. Öquist (Department of Plant Physiology, University of Umeå) for valuable discussions and for providing laboratory facilities. The manganese determinations were kindly performed by Dr Erik Lundberg (Department of Analytical Chemistry, University of Umeå). This work was supported by a grant from the Swedish Natural Science Research Council.

REFERENCES

1 Boardman, N. K. (1970) Annu. Rev. Plant Physiol. 21, 115–140
2 Park, R. B. and Sane, P. V. (1971) Annu. Rev. Plant Physiol. 22, 395–430
3 Albertsson, P.-Å. (1971) Partition of Cell Particles and Macromolecules, 2nd edn, Almqvist and Wiksell, Stockholm
4 Albertsson, P.-Å. (1974) Method Enzymology 31 A, 761–769
5 Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 423, 122–132
6 Johansson, G., Hartman, A. and Albertsson, P.-Å. (1973) Eur. J. Biochem. 33, 379–386
7 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
8 Öquist, G. (1974) Physiol. Plant 31, 55–58
9 Izawa, S. and Good, N. E. (1966) Plant Physiol. 41, 544–552
10 Arntzen, C. J., Dilley, R. A., Peters, G. A. and Shaw, E. R. (1972) Biochim. Biophys. Acta 256, 85–107
11 Boardman, N. K. (1972) Biochim. Biophys. Acta 283, 469–482
12 Gazanchyan, R. M., Abilow, Z. K., Aliev, Z. Sh. and Gasanov, R. A. (1975) Photosynthetica 9, 268–276
13 Huzige, H., Usiyama, T., Kikuti, T. and Azi, T. (1969) Plant. Cell. Physiol. 10, 441–455
14 Vernon, L. P., Shaw, E. R., Ogawa, T. and Raveed, D. (1971) Photochem. Photobiol. 14, 343–357
15 Wessels, J. S. C., van Alphen-van Waveren, O. and Voorn, G. (1973) Biochim. Biophys. Acta 292, 741–752
16 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
17 Arntzen, C. J., Dilley, R. A. and Crane, F. L. (1969) J. Cell Biol. 43, 16–31
18 Arntzen, C. J. and Briantais, J. M. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 51–113, Academic Press, New York
19 Sun, A. S. K. and Sauer, K. (1972) Biochim. Biophys. Acta 256, 409–427
20 Staehelin, A. (1975) Biochim. Biophys. Acta 408, 1–11
21 Archakov, A. J., Karyakin, V. and Skulachev, V. P. (1975) FEBS Lett. 60, 244–246