BBA 45967

CHARACTERIZATION OF CHLOROPLAST PHOTOSYSTEMS 1 AND 2 SEPARATED BY A NON-DETERGENT METHOD

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(Received February 20th, 1970)

SUMMARY

Class II spinach chloroplasts were fragmented by passage through the French pressure cell (French press), and the fragments were separated by fractional centrifugation. Fragments sedimenting between 1000 × *g* and 10000 × *g* (10K) have a lower chlorophyll *a*/chloropyll *b* ratio and lower P-700 content than whole chloroplasts. Fragments sedimenting between 40000 × *g* and 160000 × *g* (160K) have a much higher chlorophyll *a*/chlorophyll *b* ratio (6.0) and a much higher P-700 content (1 P-700 per 105 chlorophylls) than whole chloroplasts. The chlorophyll and cytochrome contents of the French press fractions are similar to those found in fractions isolated after digitonin disruption.

The 160K fraction performs Photosystem 1 but not Photosystem 2 reactions. The 10K fraction contains both photosystems. Electrophoresis of sodium dodecyl sulfate solubilized 10K and 160K fractions gives further evidence for this distribution of photosystems.

Thin sectioning and freeze fracturing show that the 160K fraction originates from stroma lamellae and the end membranes of grana stacks and contains only 110 Å particles. The 10K fraction originates from the partition regions of grana stacks and contains both 110 and 175 Å particles. This distribution of particles on fracture faces of stroma *versus* grana lamellae is shown to exist in freeze fractured Class I chloroplasts.

These data demonstrate that both digitonin and French press treatments of chloroplasts initially break stroma lamellae and end membranes to yield small vesicles which contain only Photosystem 1.

INTRODUCTION

In 1968 MICHEL AND MICHEL[1] reported that the two photochemical systems of spinach chloroplasts could be partially separated on a density gradient following chloroplast breakage in a French pressure cell. They obtained an enrichment of Photosystem 1 in the light fraction and a small enrichment of Photosystem 2 in a heavier fraction with corresponding high and low chlorophyll *a*/chlorophyll *b* ratios. The

Abbreviations: DCIP, dichlorophenolindophenol; TCIP, trichlorophenolindophenol.

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photochemical activities of these fractions have been further characterized by MURATA AND BROWN[2]. Initially we had difficulty repeating these experiments, and our light fraction yielded chlorophyll *a*/chlorophyll *b* ratios only slightly higher than whole chloroplasts. Recent publications by JACOBI and co-workers[3–5] showed that separation of fractions with high chlorophyll *a*/chlorophyll *b* ratio from sonically treated chloroplasts was possible only with very short sonication times. Longer sonication times obscured this fraction with material of much lower ratio. We reasoned that a similar effect might occur with our French press treated chloroplasts and subsequently developed a relatively mild French press treatment followed by fractional centrifugation. These fractions are highly active in the light reactions and, contrasted with fractions prepared by detergent treatment, are particularly suited for studies on the relationship of the light reactions to the original thylakoid structure.

In this paper we report the chemical, photochemical and ultrastructural characterization of fractions centrifugally separated from a French press homogenate. The yields of chlorophyll, chlorophyll *a*/chlorophyll *b* ratios, chlorophyll difference spectra, cytochrome contents and photochemical activities of fractions separated by our physical techniques are very similar to the results obtained with the chemical technique of digitonin extraction[6,7]. We show by ultrastructural studies that the principal effect of French press treatment on Class II chloroplasts is to break stroma lamellae connecting grana stacks. The released grana stacks constitute the rapidly sedimenting fractions which contain all the Photosystem 2 and some Photosystem 1 activity. The stroma lamellae connecting the stacks and some of the single membranes which terminate the grana stacks (end membranes) yield vesicles exclusively Photosystem 1 in character.

MATERIALS AND METHODS

Preparation of French press fractions

Commercially obtained spinach leaves (150 g) were homogenized 30 sec in a Waring blendor containing 250 ml of 0.05 M potassium phosphate buffer (pH 7.4)–0.01 M KCl–0.5 M sucrose. The slurry was passed through eight layers of cheesecloth and centrifuged for 5 min at 200 × *g*. The precipitate was discarded and the supernatant was centrifuged at 1000 × *g* for 15 min. This crude chloroplast precipitate was resuspended in 20 ml of 0.15 M KCl–0.05 M potassium phosphate buffer (pH 7.4) and consisted predominantly of Class II plastids. The preparation of Class II plastids was then passed once through an Aminco French pressure cell at 6000 lb/inch^2 at approx. 20 ml/min. Further passes through the cell gave only slight increases in yields of the high *g* fractions. With our press, three passes at 12500 lb (the Carnegie technique)[1] never yielded high *g* fractions with a chlorophyll *a*/chlorophyll *b* ratio greater than 3.5. The French press homogenate was then fractionally separated by centrifugation at 1000 × *g*·10 min, 10000 × *g*·30 min, 40000 × *g*·30 min, 160000 × *g*·60 min (designated 1K, 10K, 40K, and 160K fractions). The resulting precipitates were resuspended in 0.05 M potassium phosphate buffer (pH 7.4)–0.01 M KCl. Less than 2% of the total chlorophyll remained in the 160000 × *g* supernatant after this treatment.

Chlorophyll *a* and chlorophyll *b* were determined using the spectrophotometric method of ARNON[8]. P-700 was determined by the ferricyanide method of YAMAMOTO

AND VERNON[9]. $NADP^+$ reductase and purified ferredoxin were prepared following the methods of SHIN *et al.*[10] and TAGAWA AND ARNON[11]. Plastocyanin was purified by the procedure of KATOH *et al.*[12]. Photosystem I reduction of $NADP^+$ from sodium isoascorbate was followed spectrophotometrically in the apparatus described by SAUER AND BIGGINS[13] using the reaction mixture of ANDERSON AND BOARDMAN[6], except that Tricine (0.1 M) was used as buffer. Optimum amounts of ferredoxin, plastocyanin and $NADP^+$ reductase were added to the reaction mixture. Photosystem 2 activity was followed spectrophotometrically using water as reductant and dichlorophenolindophenol (DCIP) as oxidant in the presence of methylamine as described by SAUER AND PARK[14]. The extinction coefficients for DCIP according to ARMSTRONG[15] were used to calculate DCIP reduction rates at various pH levels. Cytochromes were quantitatively determined at room temperature using difference spectra of unextracted material following the methods of BOARDMAN AND ANDERSON[7]. Manganese was determined by standard digestion and atomic absorption techniques. Electrophoresis of sodium dodecyl sulfate solubilized lamellae was conducted according to the method described by CLARKE[16]. The electrolyte was modified to contain 0.2 % sodium dodecyl sulfate.

Electron microscope methods

Fractions were resuspended in buffer containing 3 % glutaraldehyde. Each fraction was completely pelleted in a 1-ml centrifuge tube in a microcentrifuge or in adaptors in the swinging-bucket rotor of a Servall centrifuge so that a 0.5-mm-thick pellet was formed. The total time for fixation and pelleting was 1 h. The pellets were then washed 4 times by replacing the fluid above each with 0.025 M phosphate buffer (pH 7.2) and were post-fixed for 1 h in 2 % O_8O_4 in the phosphate buffer. These procedures were carried out at 0°. After further washing in cold buffer, pellets were dehydrated in ethanol and propylene oxide at room temperature. The end of the centrifuge tube was then cut off and the pellet extruded and embedded in Epon. For sectioning the Epon-embedded pellets were oriented and trimmed so that each section contained a range of material from the top to the bottom of the pellet. Sections were stained with saturated uranyl acetate in 50 % ethanol for 2 h followed by lead citrate according to FISKE[17] for 20 min before examination in a Siemens Elmiskop 1A electron microscope. Freeze fracturing and deep etching of the fractions were carried out as described by PARK AND PFEIFHOFER[18]. Class I chloroplasts for freeze fracturing were prepared by the method of JENSEN AND BASSHAM[19].

Biochemical results

In our initial experiments we prepared the French press homogenate as described under MATERIALS AND METHODS and separated it into fractions using a discontinuous sucrose density gradient as a modification of the Carnegie procedure. Further experiments showed that the fractions were separating on the basis of size rather than density. Therefore we replaced sucrose density gradients with fractional centrifugation as a separation procedure. The absence of sucrose in these fractions was a great advantage for the subsequent analyses and ultrastructural studies. The characteristics of fractions separated by fractional centrifugation are given in Table I.

The small fragments, while depleted in manganese, are greatly enriched in chlorophyll *a* and P-700 compared with the starting material. There is a corresponding

TABLE I

DISTRIBUTION OF CHLOROPHYLLS, P-700 AND MANGANESE IN FRENCH PRESS FRACTIONS

Class II chloroplasts were passed through the French press at 6000 lb/inch^2 and separated into fractions by differential centrifugation. Chlorophylls, P-700 and manganese were determined as described in MATERIALS AND METHODS.

Fraction	*Ratio* $\frac{chlorophyll\ a}{chlorophyll\ b}$	*Chlorophyll* (%)	*Ratio* $\frac{chlorophyll}{P\text{-}700}$	*Ratio* $\frac{chlorophyll}{manganese}$
French press homogenate	2.9	100	423	136
1K + 10K	2.4	67.1	650	127
40K	3.0	23.7	253	—
160K	6.0	7.4	105*	845
160K supernatant	4.7	1.8	—	—

* The range of values observed for this fraction were 100–130.

TABLE II

PHOTOCHEMICAL ACTIVITIES OF FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATION OF A FRENCH PRESS HOMOGENATE

Reaction mixtures and conditions for photochemical assays are described in MATERIALS AND METHODS. Values are expressed as μmoles reduced per mg chlorophyll per h.

Fraction	*DCIP (pH 7.0)*	*$NADP^+$ (pH 7.4)*
Original chloroplasts	174	172
French press homogenate	52	87
1K	100	62
10K	74	75
40K	—	87
160K	0	169

enrichment of manganese and depletion of chlorophyll *a* and P-700 in the 1K and 10K fractions. These data suggest that a Photosystem 1 fraction (160K) has been separated from the French press homogenate. More evidence for such a separation is presented in Table II. There is an immediate decrease in both photochemical activities upon passage through the French press. However, the remaining activities demonstrate stabilities with time comparable to those of the original chloroplast material. The 160K fragments are twice as active in Photosystem 1 activity as the starting material (French press homogenate). They possess no detectable Photosystem 2 activity, whereas the 1K and 10K fractions are enriched in Photosystem 2 activity but are slightly depleted in Photosystem 1 activity. The data in Tables I and II indicate that the 160K fraction is relatively pure Photosystem 1. The 1K and 10K fractions, on the other hand, contain both photosystems though they are relatively enriched in Photosystem 2 and depleted in Photosystem 1 compared with the starting homogenate. Further evidence for this distribution of the two photosystems was obtained by electrophoretic separation of the fractions following solubilization in sodium dodecyl sulfate. The 160K fraction yielded predominantly Complex 1 of THORNBER *et al.*[20] with a chlorophyll *a*/chlorophyll *b* ratio of 8, while the 10K fraction yielded both Complexes 1 and 2.

TABLE III

CYTOCHROMES IN FRACTIONS SEPARATED BY DIFFERENTIAL CENTRIFUGATION OF A FRENCH PRESS HOMOGENATE

Cytochromes were determined by difference spectra as described by BOARDMAN AND ANDERSON[7].

Fraction	*Ratio chlorophyll / total cytochrome b*	*Ratio chlorophyll / cytochrome f*
French press homogenate	109	510
1K	160	635
10K	113*	575
40K	108	425
160K	286**	530
160K supernatant	No detectable cytochromes	

* 10K fraction relatively enriched in cytochrome 559.
** 160K fraction relatively enriched in cytochrome b_6.

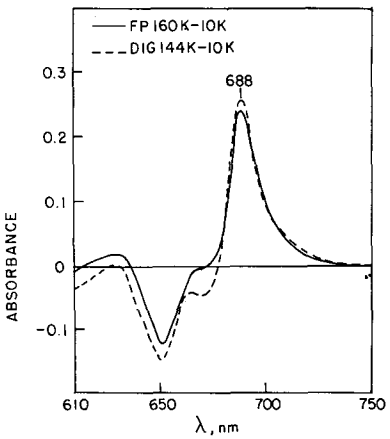


Fig. 1. ———, a difference spectrum of 160K fraction *minus* 10K fraction with absorbances equalized at 678 nm; – – – –, a difference spectrum of 144000 × *g minus* 10000 × *g* digitonin fractions from data of ANDERSON AND BOARDMAN[6].

Total cytochrome *b* in the 160K fraction (Table III) is only 35 % that of the starting material on a chlorophyll basis. The residual cytochrome *b* in this fraction gave a difference-spectrum peak at 563 nm indicating it is primarily cytochrome b_6. The cytochrome *b* content of the 1K and 10K fractions, on the other hand, does not differ greatly from the French press fraction and consists of both cytochrome b_6 and cytochrome 559 in about equal proportions. Our data does not show marked variations of cytochrome *f* abundance to chlorophyll between the various fractions.

The solid line in Fig. 1 is a difference spectrum of the 160K fraction *minus* the 10K fraction with the absorbances equalized at 678 nm with a value of 1.65. The main features of this spectrum are the peak at 688 nm and the trough at 650 nm. This again indicates the relative enrichment of long-wavelength absorption in the 160K fraction. Most remarkable is the extraordinary similarity between these data and those published by ANDERSON AND BOARDMAN[6], in which they give the difference

spectrum of their 144000 × *g* fraction *minus* their 10000 × *g* fraction. The data of ANDERSON AND BOARDMAN are plotted on Fig. 1 as a dashed line. Since they did not give the absorbance of the suspensions on which the difference spectra were performed, these two curves are comparable only in shape, not in magnitude.

Though a number of similarities exist between our French press data and those of ANDERSON AND BOARDMAN, the question remains, is the source of Photosystem I material identical for both treatments? If the same source provides the Photosystem I material in both treatments, we would predict that a French press fraction depleted in Photosystem I (10K) would not yield appreciable additional Photosystem I material upon digitonin extraction. If different sources provide the Photosystem I material isolated in the two treatments, digitonin extraction of the French press 10K fraction would be expected to yield a Photosystem I fraction corresponding to about 10 % of the chlorophyll initially present. This expectation is based on our observation that 30 min incubation of whole chloroplasts with 0.5 % digitonin yielded an average of 10 % of the chlorophyll in Photosystem I fractions with a high chlorophyll *a*/chlorophyll *b* ratio. To test these two possibilities the 10K fraction from a French press homogenate was resuspended in the standard buffer for French press treatment and passed once more through the French press. This material was centrifuged at 10000 × *g* for 30 min. The supernatant contained 3 % of the chlorophyll with a chlorophyll *a*/chlorophyll *b* ratio of 4. The resulting precipitate (10K French press homogenate$_2$) was resuspended in 0.05 M potassium phosphate buffer (pH 7.4)–0.01 M KCl to a chlorophyll concentration of 0.3–0.4 mg/ml. To this 2 % digitonin was added to a final concentration of 0.5 %. This suspension was incubated 30 min with stirring at 1–2°. It was then centrifugally fractionated. The data in Table IV show that digitonin

TABLE IV

DIGITONIN FRACTIONATION OF 10K FRENCH PRESS HOMOGENATE$_2$ FRACTION ACCORDING TO THE PROCEDURE OF ANDERSON AND BOARDMAN[6]

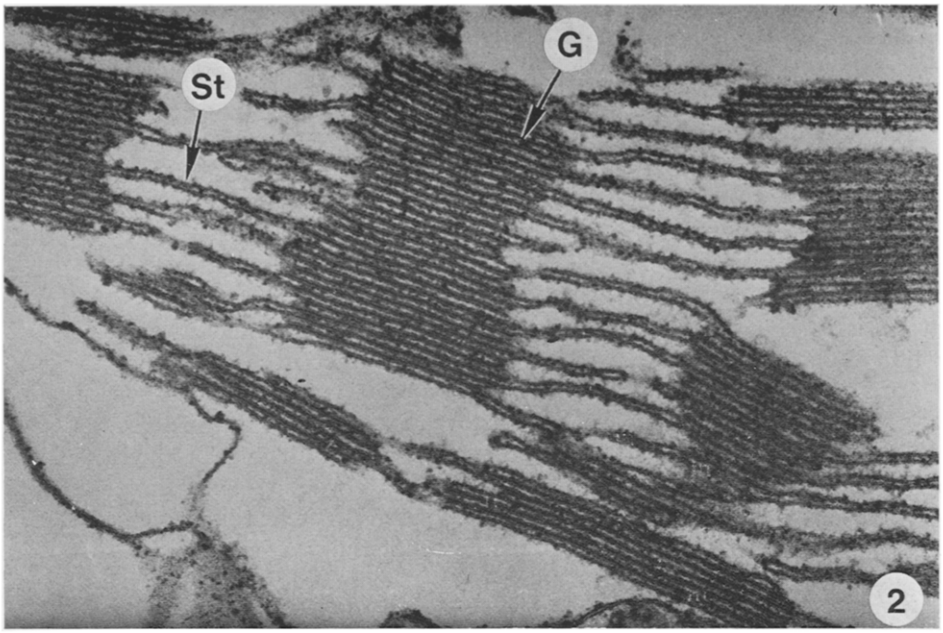
10K French press homogenate$_2$ fraction was prepared by passing the 10K fraction through the French press at 6000 lb/inch^2 and collecting a fraction sedimenting at 10000 × *g*·30 min. Digitonin treatment described in text.

Fraction	*Ratio* $\frac{\textit{chlorophyll a}}{\textit{chlorophyll b}}$	*Chlorophyll* (%)
10K (30 min)	2.2	98.3
50K (30 min)	2.8	1.1
50K supernatant	5.0	0.6

treatment of the 10K French press homogenate$_2$ fraction yields only 0.6 % of the starting material as a Photosystem I fraction. The inability of digitonin treatment of the French press 10K fraction to remove appreciable Photosystem I material in addition to that already removed by the French press supports our initial hypothesis — namely, the source of Photosystem I is the same for both treatments.

Ultrastructural results and interpretation

Thin sections of the Class II spinach chloroplast preparation before and after passage through the French press are compared in Figs. 2 and 3. In Fig. 2 the grana



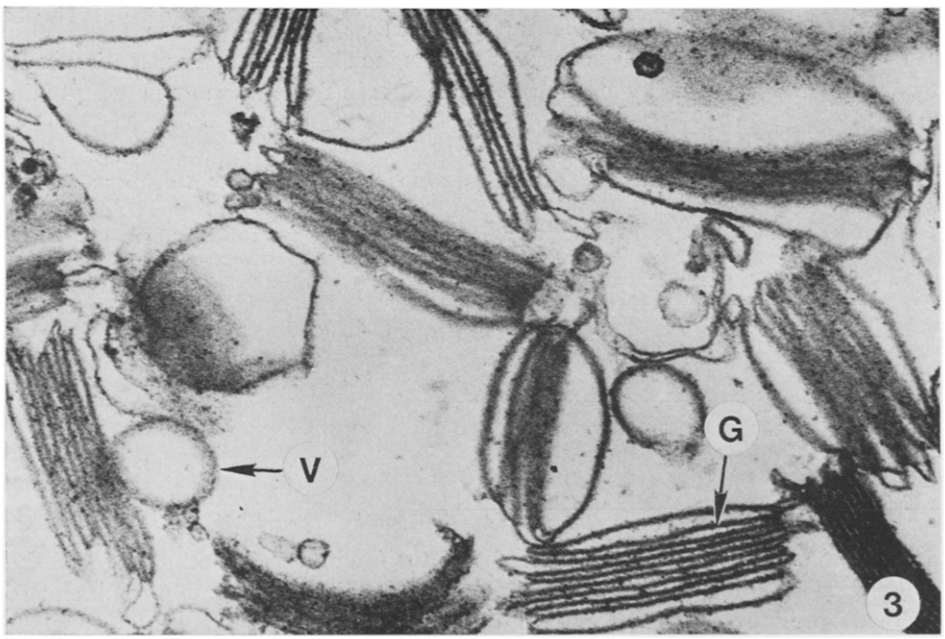


Fig. 2. Portion of a Class II chloroplast before French press treatment showing grana stacks (G) and interconnecting stroma lamellae (St). × 80000.

Fig. 3. Fraction after French press treatment showing grana stacks (G) and vesicles (V). Note absence of interconnecting stroma lamellae. × 66000.

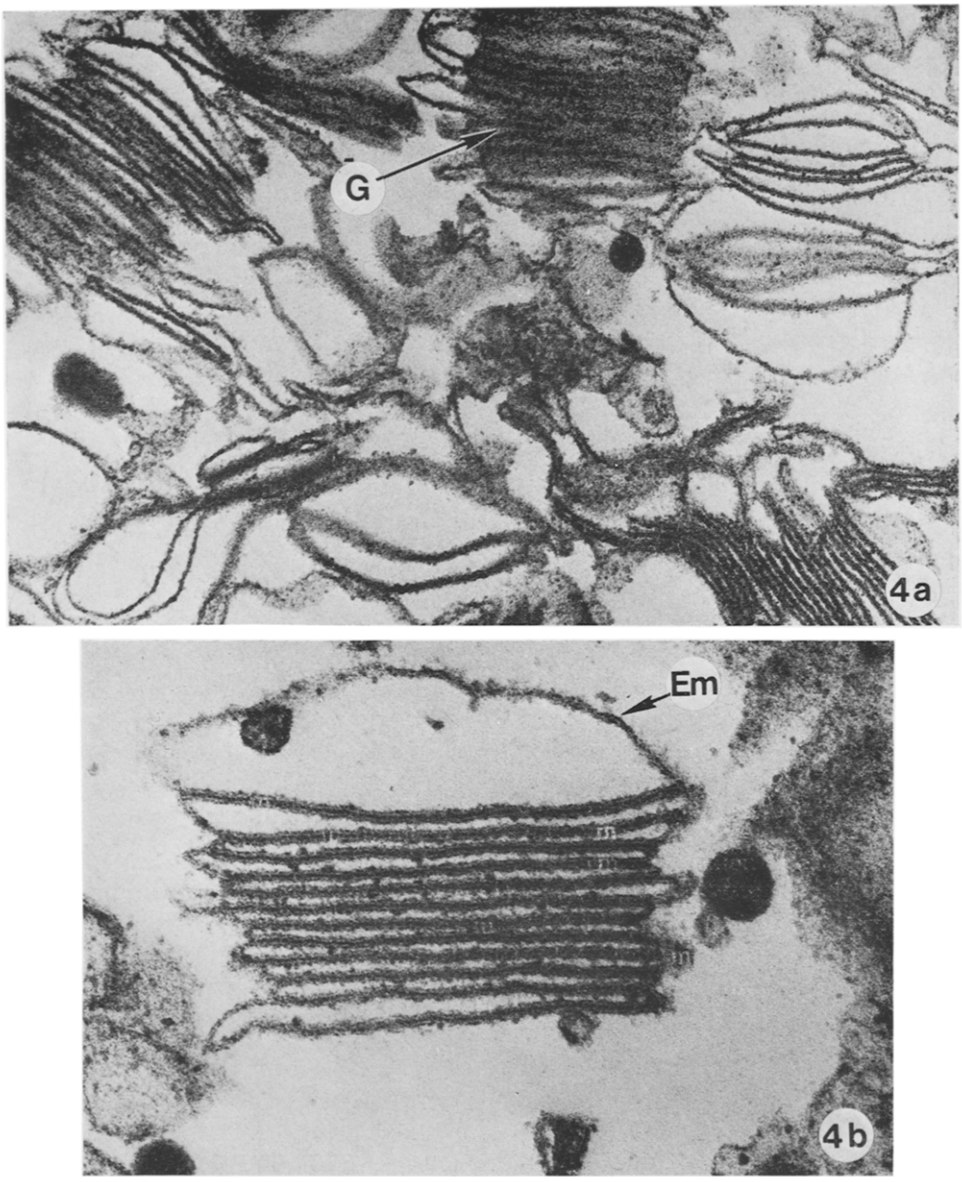


Fig. 4. Sections through 10K fraction. a. Section showing remnants of grana (G) and large vesicles with partitions. × 70000. b. An example of a granum after passage through the French press in which one end membrane (Em) is swollen and the other is absent. × 132000.

stacks within the Class II chloroplasts are interconnected by a network of stroma lamellae. After French press treatment most of the grana stacks are intact. However, the network of stroma lamellae has been destroyed to yield various sized vesicles. Some of these vesicles may also have originated from one of the single membranes which terminate a grana stack. We shall call such terminal single membranes end membranes.

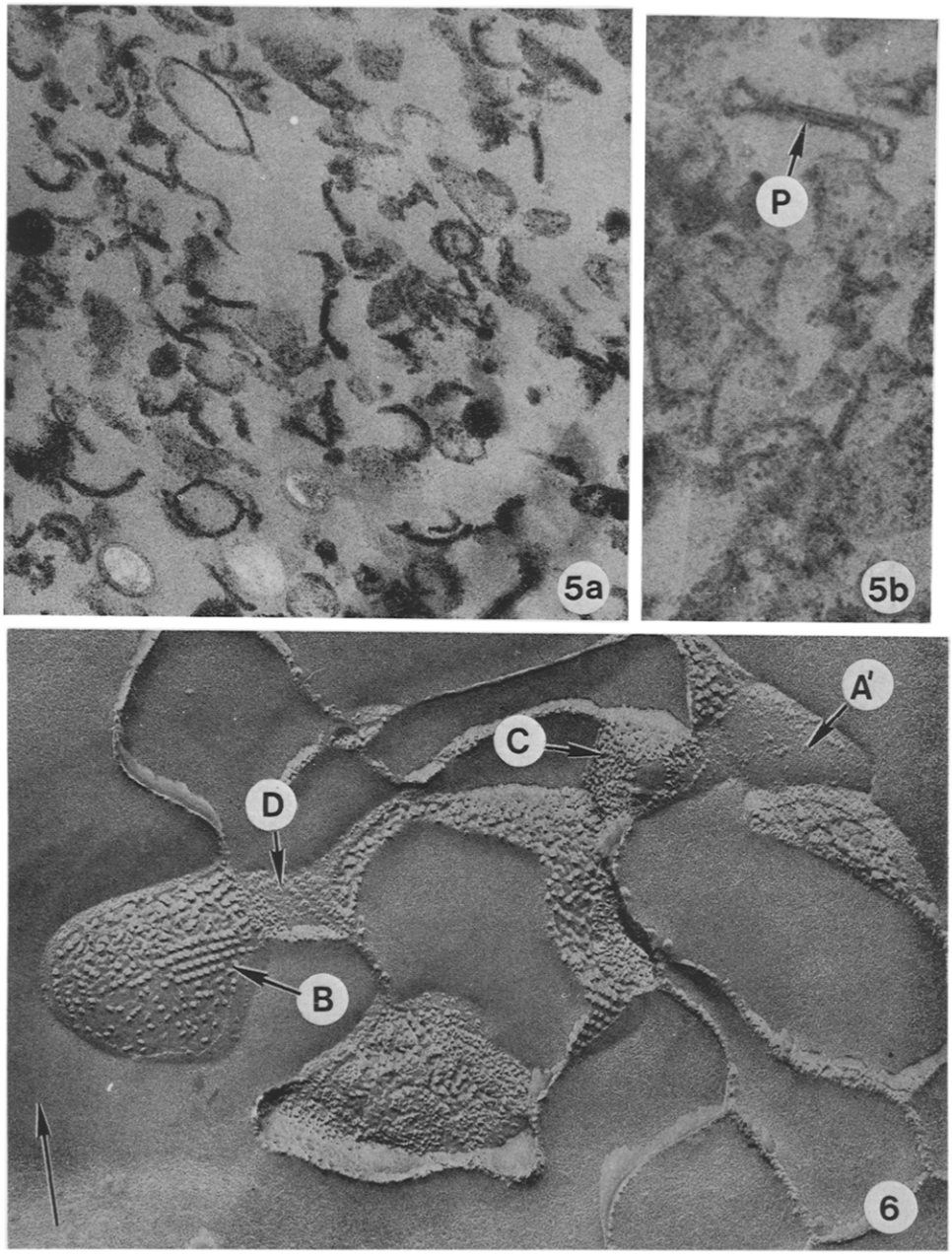


Fig. 5. Sections through 160K fraction. a. Section showing predominance of vesicles of various sizes. × 70000. b. Flattened vesicle with partition-type region (P). × 140000.

Fig. 6. Freeze-fractured and deep-etched 10K fraction showing A′ and D surfaces and B and C faces. × 64000. →, direction of shadow.

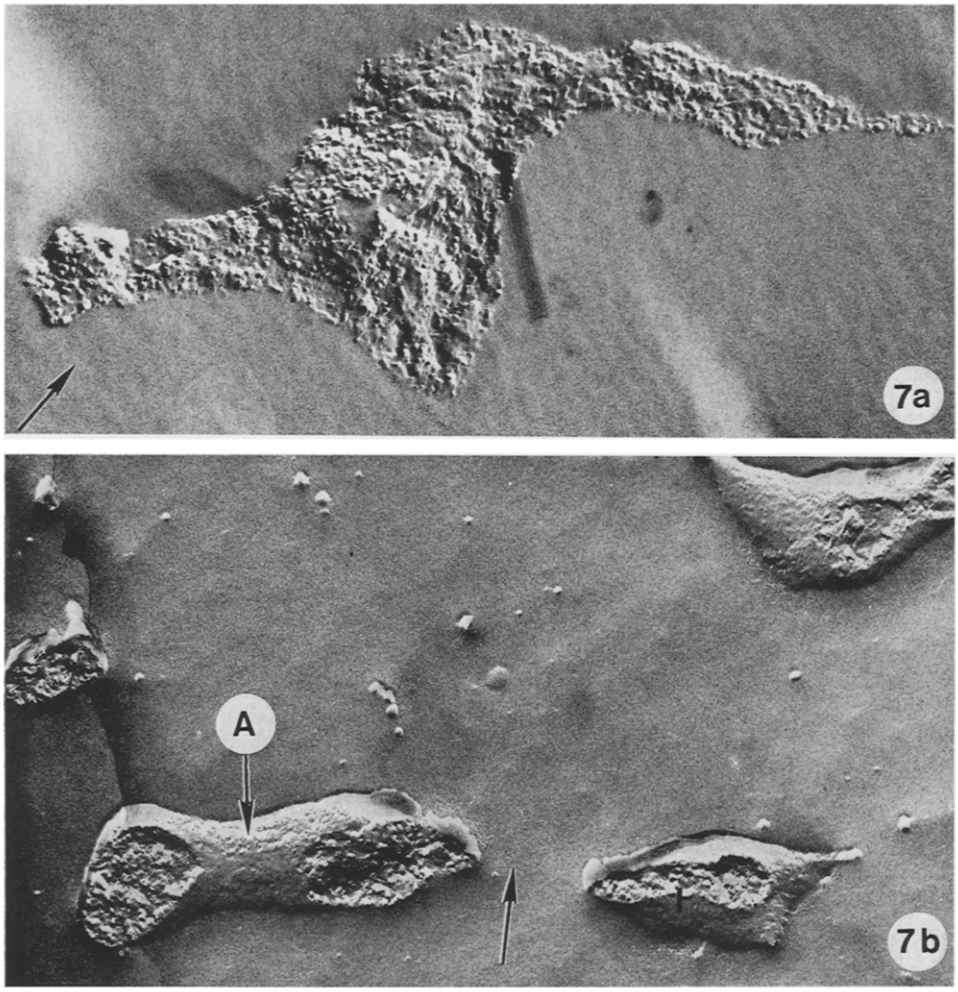


Fig. 7. Freeze-fractured and deep-etched 160K fraction. a. Freeze-fracture face of vesicle showing only 110-Å particles. × 70000. b. Freeze-fractured and deep-etched vesicle showing only 110-Å particles and A type surfaces. × 70000. ——→, direction of shadow.

Thin sections of the 10K and 160K fractions from the French press homogenate are compared in Figs. 4 and 5. The 10K fraction consists primarily of grana stacks while the 160K fraction consists primarily of small vesicles. The vesicles of the 160K fraction are often collapsed (Fig. 5b) yielding small fragments with regions having the appearance of a partition. We believe these are not fragmented partitions but are collapsed vesicles, for the following reasons: (1) The vesicles were pelleted at 160000 × *g*, which might be expected to flatten them. (2) As will be evident from Figs. 6 and 8, they do not contain the large 175-Å particles associated with the spinach grana partitions. Freeze-fractured and deep-etched preparations of the 10K and 160K fractions are compared in Figs. 6 and 7. Both fractions were resuspended in water to allow deep etching. This treatment causes some unfolding of the grana as seen in

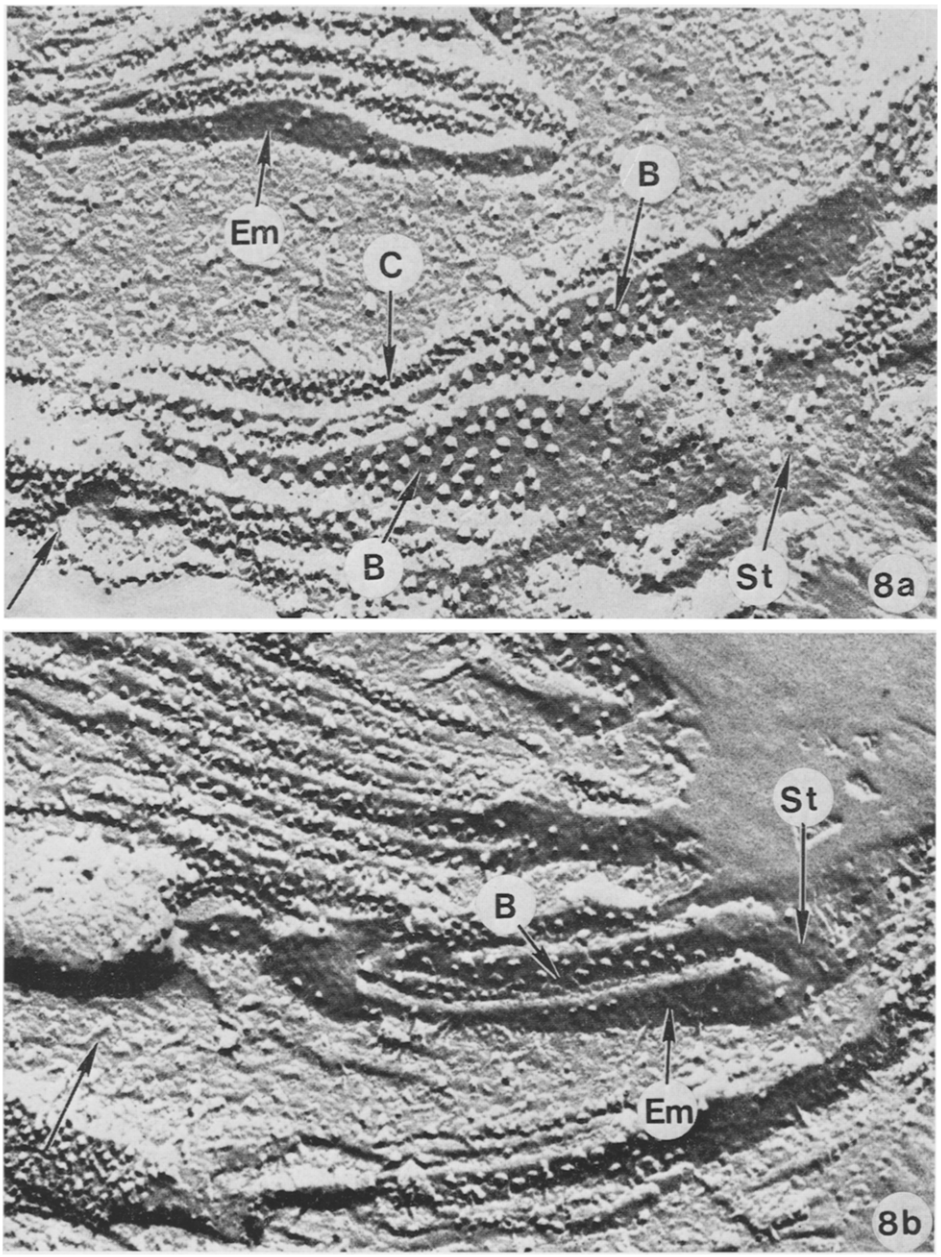


Fig. 8. Freeze-fractured JENSEN AND BASSHAM[19] type chloroplasts. a. Fracture face showing 110-Å and 175-Å particles on the same B face. The 175-Å particles appear only in partition regions while the 110-Å particles appear on a typical interconnecting stroma lamella (St) and end membranes (Em). × 110000. b. End membrane (Em) continuous with a stroma lamella (St) and with partition B face. × 110000. ——→, direction of shadow.

Fig. 6. Nomenclature for the fracture faces and membrane surfaces used here is that given in the model presented in Fig. 1 of ref. 18. The 10K fraction contains both large (175-Å) and small (110-Å) particles on B and C fracture faces respectively, whereas the 160K fraction possesses only 110-Å particles on its fracture planes. Both A′ and D surfaces are seen in the deep-etched regions of the 10K fraction, whereas only A′ surfaces are seen in the 160K fraction. Finally, Fig. 8 shows that two kinds of particles exist on B fracture faces in Class I chloroplasts. In Class I chloroplasts the large (175-Å) B face particle exists only in the partition region of grana stacks. As the B fracture face extends into a stroma lamella or on to an end membrane, only small 110-Å particles are seen. In Fig. 8a two typical B fracture faces in a grana stack are connected by a stroma lamella which has only small particles. In Fig. 8b the folding of an end membrane to form a typical partition region is evident. A similar distribution of fracture face particles in bean chloroplasts was recently reported by REMY[21]. Our earlier observations[18] showed that 175-Å particles can exist in single membranes. However, these observations were made on water-washed material to be used for deep etching. Apparently extensive unfolding of grana stacks in low salt produced the single membrane regions with 175-Å particles. In mature Class I spinach chloroplasts the 175-Å particles appear to be restricted to the partition region.

Since the 160K membrane vesicles contain only 110-Å particles on their fracture planes, we concluded they must have originated from stroma lamellae and end membranes. That the 160K fraction did not originate from fragmentation of the partitions is based on the following observations: the 160K fraction contains only small 110-Å particles on the fracture faces and no D surfaces. If whole grana were being fragmented, small vesicles containing both particle sizes and D surfaces would be obtained. This prediction was verified by suspending the 10K grana fraction in water, passing it through the French press once more and isolating a 160K fraction. When observed by freeze fracturing this fraction contained the expected abundance of large and small particle faces. It could be argued that if whole grana are not being fragmented to yield the small vesicles of the 160K fraction, perhaps just the small particle layer is being stripped from the grana stacks. We believe this is not the case for two reasons. First, we fail to see how mild shearing forces which leave grana stacks intact in an aqueous environment would bring about such splitting. Second, such splitting would give rise to another population of large 175-Å particle vesicles which came from the other side of the membrane. No such population is observed.

It is our conclusion that in mature Class I spinach chloroplasts, stroma lamellae and end membranes are unique and contain only 110-Å particles, whereas the partitions in the grana contain both the 175-Å and 110-Å particles. We further conclude that the 160K fraction from the French press homogenate consists of stroma lamellae vesicles with some end membranes. These vesicles have only Photosystem 1 activity, high chlorophyll *a*/chlorophyll *b* ratio, and high P-700 content. The 10K fraction consists primarily of partitions and has both light reactions and both kinds of particles.

DISCUSSION

A comparison of our results with the digitonin fractionation data of BOARDMAN AND ANDERSON[6,7] is given in Table V. Digitonin solubilizes almost 11 % of the total chlorophyll into a 144K supernatant fraction, whereas the corresponding fraction

TABLE V

COMPARISON OF THE DATA OF ANDERSON AND BOARDMAN[6,7] ON DIGITONIN FRACTIONS AND DATA ON FRENCH PRESS FRACTIONS FROM THIS PAPER

Activities given in μmoles of $NADP^+$ reduced per mg chlorophyll (Photosystem 1) or μmoles DCIP reduced per mg chlorophyll per h (Photosystem 2). Comparison of 160K (144K)–10K difference spectra is given in Fig. 1. Data of ANDERSON AND BOARDMAN are given in brackets.

Fraction	*Ratio* $\frac{\text{chlorophyll } a}{\text{chlorophyll } b}$	*Chlorophyll* (%)	*Chemical or enzymatic property*					
			Photosystem 1		*Photosystem 2*		*Ratio* $\frac{\text{chlorophyll } a + \text{chlorophyll } b}{\text{cytochrome } b}$	*Ratio* $\frac{\text{cytochrome } b}{\text{cytochrome } f}$
			Ascorbate → NADP⁺		*DCIP*	*TCIP*		
Starting material	2.9 (2.8)	100 (100)	87	(18)	52	(81)	109 (118)	4.7 (3.6)
1K	— (2.4)	— (19.0)	62	(14)	100	(139)	— —	— —
10K	— (2.3)	— (46.2)	75	(17)	74	(61)	113 (120)	5.1 (6.1)
1K + 10K	2.4 —	67.1 —	—	—	—	—	— —	— —
40K (50K)	3.0 (4.4)	23.7 (12.3)	87	(70)	—	(0)	— —	— —
160K (144K)	6.0 (5.3)	7.4 (11.7)	169	(123)	0	(0)	286 (390)	1.9 (2.3)
160KS (144KS)	4.7 (3.8)	1.8 (10.8)	—	(103)	—	(0)	— —	— —

from the French press material never exceeds 2 %. Also, the chlorophyll *a*/chlorophyll *b* ratio of the digitonin material is lower than corresponding fractions from the French press. These differences can be explained when the methods of breakage by the two methods are compared. It is our contention that shearing forces in the French press release vesicles from those stroma lamellae and swollen end membranes which extend over a sufficient shear gradient. Small stroma lamellae and unswollen end membranes would not be released readily by the French press. Digitonin would be expected to attack stroma lamellae and end membranes equally, as well as starting to solubilize the grana lamellae. Such solubilization is indicated by the low chlorophyll *a*/chlorophyll *b* ratio of the 144000 × *g* supernatant from digitonin extraction. This mode of action would explain both the increased yield and lower chlorophyll *a*/chlorophyll *b* ratios obtained by the digitonin method compared with the French press method. The cytochrome *b* content of the 160K French press fragment is intermediate between the digitonin fragments prepared by standard and dilution techniques. The P-700 content of the French press fraction is similar to the digitonin fractions except for increased P-700 in the French press 160K fraction[22]. In the final analysis, we are much more impressed by the similarities than the differences in the above data.

An ultrastructural comparison between the French press and digitonin fractions is more difficult to make because digitonin does not preserve the morphological relationships in the grana fraction nearly so well as the French press preparation[23,24]. However, our biochemical evidence on the similarity of French press and digitonin fractions strongly suggests that the rapidly precipitated digitonin fractions also originate from grana regions.

Freeze fracturing data also support our view of thylakoid breakage. We have shown that stroma lamellae and the end membranes appear to possess only the 110-Å particles while both the 110-Å and 175-Å particles are found on the fracture faces of partitions in grana stacks. Thus, the 160K fraction which contains only 110-Å particles and no D surfaces is presumed to come only from stroma lamellae and end membranes, while the 10K fraction with both types of particles and with D surfaces is presumed to come from grana stacks. Though it is tempting to immediately associate 110-Å particles with Photosystem 1 which occurs in both membrane systems and the 175-Å particle with Photosystem 2 which occurs only in the grana, no direct evidence for the functions of these particles exists. Freeze fracturing of digitonin fractions by ARNTZEN *et al.*[24] also shows that the small Photosystem 1 fragments contain only small particles. They have stated that their large-fragment fraction contains a preponderance of large particles, though the areas of large and small particles appear to be about equal in their micrographs. The effect of digitonin on thylakoid fracture faces is unknown and interpretation is increasingly difficult.

Digitonin fractionation as performed by WESSELS[25], ANDERSON AND BOARDMAN[6], ARNTZEN *et al.*[24] and HUZISIGE *et al.*[26], in our view, does not involve removal of Photosystem 1 from a closely associated Photosystem 2 by selective solubilization of external Photosystem 1 particles or splitting along membrane hydrophobic regions. It involves instead breakage similar to that obtained with the French press, namely, the selective breakage of stroma lamellae and some end membranes from the grana to yield a Photosystem 1 vesicle fraction while the lower *g* fractions are grana derived and contain both Photosystems 1 and 2, probably in close physical association. HUZISIGE *et al.*[26] have shown that the heavier digitonin fractions can be treated with

Triton X-100 to yield a fraction enriched in Photosystem 2 activity. However, the relatively high chlorophyll *a*/chlorophyll *b* ratio (2.0) of this fraction suggests that selective destruction of photosystem 1 may be involved as well as enrichment of Photosystem 2. Our model for thylakoid breakage by French press and digitonin treatments leads to several predictions concerning the relationships of the two photosystems.

(1) There are two kinds of Photosystem 1 in spinach chloroplasts, which differ in unit size. One of these, which is located in grana regions, exists in much closer association with Photosystem 2 than the second type of Photosystem 1, which is located in stroma lamellae. Evidence for two unit sizes of Photosystem 1 is obtained from the relative P-700 contents of whole chloroplasts as compared with the 160K and 10K fractions. Whole spinach chloroplasts contain 1 P-700 per 425 chlorophylls. Quantum-yield measurements on the two photosystems by SAUER AND PARK[14] and by KELLY AND SAUER[27] show that the chlorophyll of whole chloroplasts is about equally distributed between the two photosystems. On this basis, the average ratio of P-700 to Photosystem 1 chlorophylls in whole chloroplasts would be 1 P-700 per 212 chlorophylls. However, the 160K fraction which accounts for 10 % of the total chlorophyll (20 % of the Photosystem 1 chlorophyll) contains 1 P-700 per 105 chlorophylls. The remaining 80 % of Photosystem 1 in the grana fraction must be relatively depleted in P-700. By calculation it should contain more than 200 chlorophylls per 1 P-700. The light intensity in the stroma regions will be higher than that in the interior of a grana stack and there appears to be a corresponding decrease in unit size.

JACOBI and co-worker[4,5] have isolated Photosystem 1 fractions from chloroplasts by two different techniques. One fraction is isolated by short sonication (yield < 1 % of total chlorophyll). The large residual fragments (UP 10 fraction accounting for 80 % of total chlorophyll) are then treated with digitonin to obtain a second kind of Photosystem 1 fraction. The two fractions differ in response to added plastocyanin and DCIP; however, these differences may be due to the fact that both fractions were sonicated but only one fraction was treated with digitonin. In any case, the data cannot be meaningfully compared with our 160K and 10K fractions at this time because of the great differences in yield and methods of preparation.

(2) The role of Photosystem 1 in stroma lamellae and end membranes may be largely cyclic photosynthetic phosphorylation. However, we found half saturating amounts of $NADP^+$ reductase in the 160K fraction. This suggests that this fraction also plays a role in electron transport *via* a diffusable electron carrier which interacts with Photosystem 2. In spinach a diffusion path from a granum to the extremities of a stroma lamella probably does not exceed 2 μ. If one assumes the electron carrier has a diffusion coefficient of about 10^{-5} cm^2/sec (within a factor of 2 for most small molecules and ions), the time for the concentration at 2 μ from the granum to become 1/e or 37 % the value at the origin can be calculated from the relation $x_e^2 = 4Dt$, where x_e is the distance traveled, D is the diffusion coefficient, and t is the time in seconds. This time is 10^{-3} sec, a value within the measured dark reaction times for some photosynthetic electron transport reactions[27,28].

This model also explains certain observations in the literature. It is compatible with the observation of WEIER *et al.*[29] that light-dependent tetrazolium reduction by chloroplasts appears to occur only in partition regions. It is also consistent with the cytological and biophysical observation that almost all chlorophyll fluorescence in

chloroplasts at 20° originates from the grana regions and is emitted by Photosystem 2 (refs. 30–32). This would be expected if Photosystem 2 is restricted to grana regions as we have found. The model explains the observation of ANDERSON AND VERNON[33] that digitonin extraction solubilizes fractions with high chlorophyll *a*/chlorophyll *b* ratio only when conditions for stabilization of grana are present. The model is also consistent with the preferential association of the Ca^{2+}-dependent ATPase with the high *g* digitonin fractions[24].

JACOBI has isolated a fraction with a high chlorophyll *a*/chlorophyll *b* ratio from sonicated chloroplasts which accounts for $< 1\%$ of the total chlorophyll[4]. He concludes that this fraction "may come from grana stacks or from the intergrana area and no answer can be given about the original localization"[5]. Our freeze etch data are the first direct demonstration that a fraction with a high chlorophyll *a*/chlorophyll *b* ratio accounting for $> 7.4\%$ of total chlorophyll originates exclusively from stroma lamellae and end grana membranes.

We have not considered in detail the relationship of our data to detergent data other than the digitonin experiments. We anticipate that the extensive work by VERNON *et al.*[34], BRIANTAIS[35] and others on Triton X-100 fractionation is probably related to the kinds of breakage we have observed here. Also, the relationship of our fractions to the electron transport scheme proposed by KNAFF AND ARNON[36] is not clear at this time but is under investigation.

NOTE ADDED IN PROOF (Received June 12th, 1970)

We wish to emphasize that the 6000 lb/inch^2 treatment of chloroplasts in the French pressure cell (needle valve type) refers to the pressure within the cell after correction for the piston diameters of the hydraulic press and the pressure cell. Criteria for optimum breakage are: (1) observation of the French press homogenate in the light microscope to show the absence of Class 2 chloroplasts and the presence of separated grana stacks, (2) 10K fractions with chlorophyll *a*/chlorophyll *b* ratios of 2.3 or lower and (3) 160K fractions with chlorophyll *a*/chlorophyll *b* ratios greater than 4.5.

ACKNOWLEDGEMENTS

The technical assistance of Albert Pfeifhofer is gratefully acknowledged. This work was supported by National Institute of General Medicine Grant GM-13943-04 and the U.S. Atomic Energy Commission.

REFERENCES

1 J. MICHEL AND M. MICHEL, *Carnegie Inst. Year Book*, 67 (1968) 508.
2 N. MURATA AND J. BROWN, *Plant Physiol.*, 45 (1970) 360.
3 G. JACOBI AND H. LEHMANN, *Z. Pflanzenphysiol.*, 59 (1968) 457.
4 G. JACOBI, *Z. Pflanzenphysiol.*, 61 (1969) 203.
5 G. JACOBI AND H. LEHMAN, in H. METZNER, *Progr. Photosynthesis Res.*, 1 (1969) 159.
6 J. M. ANDERSON AND N. K. BOARDMAN, *Biochim. Biophys. Acta*, 112 (1966) 403.
7 N. K. BOARDMAN AND J. M. ANDERSON, *Biochim. Biophys. Acta*, 143 (1967) 187.
8 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
9 H. Y. YAMAMOTO AND L. P. VERNON, *Biochemistry*, 8 (1969) 4131.
10 M. SHIN, K. TAGAWA AND D. I. ARNON, *Biochem. Z.*, 338 (1963) 84.

11 K. TAGAWA AND D. I. ARNON, *Nature*, 195 (1962) 537.
12 S. KATOH, I. SHIRATORI AND A. TAKAMIYA, *J. Biochem. Tokyo*, 51 (1962) 32.
13 K. SAUER AND J. BIGGINS, *Biochim. Biophys. Acta*, 102 (1965) 55.
14 K. SAUER AND R. B. PARK, *Biochemistry*, 4 (1965) 2791.
15 J. MCD. ARMSTRONG, *Biochim. Biophys. Acta*, 86 (1964) 194.
16 J. T. CLARKE, *Ann. N.Y. Acad. Sci.*, 121 (1964) 428.
17 S. FISKE, *J. Microscopy*, 5 (1966) 355.
18 R. PARK AND A. O. PFEIFHOFER, *J. Cell Sci.*, 5 (1969) 299.
19 R. G. JENSEN AND J. A. BASSHAM, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1095.
20 J. P. THORNBER, R. P. F. GREGORY, C. A. SMITH AND J. LEGGETT BAILEY, *Biochemistry*, 6 (1967) 391.
21 R. REMY, *Compt. Rend.*, 268 (1969) 3057.
22 J. M. ANDERSON, D. C. FORK AND J. AMESZ, *Biochem. Biophys. Res. Commun.*, 23 (1966) 874.
23 S. MURAKAMI, S. KATOH AND A. TAKAMIYA, in S. SENO AND E. V. COWDRY, *Intracellular Membranous Structure*, Japan Society for Cell Biology, Okayama, 1965, p. 173.
24 C. J. ARNTZEN, R. A. DILLEY AND F. L. CRANE, *J. Cell Biol.*, 43 (1969) 16.
25 J. S. C. WESSELS, *Biochim. Biophys. Acta*, 65 (1962) 561.
26 H. HUZISIGE, H. USIYAMA, T. KIKUTI AND T. AZI, *Plant Cell Physiol. Tokyo*, 10 (1969) 441.
27 J. KELLY AND K. SAUER, *Biochemistry*, 7 (1968) 882.
28 H. T. WITT, G. DÖRING, B. RUMBERG, P. SCHMIDT-MENDE, U. SIGGEL AND H. H. STIEHL, *Brookhaven Symp. Biol.*, 19 (1966) 161.
29 T. E. WEIER, C. R. STOCKING AND L. K. SHUMWAY, *Brookhaven Symp. Biol.*, 19 (1966) 353.
30 D. SPENCER AND S. G. WILDMAN, *Australian J. Biol. Sci.*, 15 (1962) 599.
31 L. N. M. DUYSENS AND H. E. SWEERS, in S. MIYACHI, *Studies on Microalgae and Photosynthetic Bacteria*, Japanese Soc. Plant Physiologists, Tokyo, 1963, p. 353.
32 N. K. BOARDMAN, S. W. THORNE AND J. M. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 586.
33 J. M. ANDERSON AND L. P. VERNON, *Biochim. Biophys. Acta*, 143 (1967) 363.
34 L. P. VERNON, B. KE, S. KATOH, A. SAN PIETRO AND E. R. SHAW, *Brookhaven Symp. Biol.*, 19 (1966) 102.
35 J. M. BRIANTAIS, *Physiol. Végétale*, 7 (1969) 135.
36 D. B. KNAFF AND D. I. ARNON, *Proc. Natl. Acad. Sci. U.S.*, 64 (1969) 715.