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### Publication Date

1970-02-01

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

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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 x g and 10,000 x g (10K) have a lower chl a/chl b ratio and lower P-700 content than whole chloroplasts. Fragments sedimenting between 40,000 x g and 160,000 x g (160K) have a much higher chl a/chl b ratio (6.0) and a much higher P-700 content (1 P-700/105 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 dodecylsulfate solubilized 10K and 160K fractions gives further evidence for this distribution of photosystems.

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Abbreviations: chl a, chlorophyll a; chl b, chlorophyll b; DCIP, dichlorophenolindophenol; TCIP, trichlorophenolindophenol; cyt, cytochrome.

Thin sectioning and freeze fracturing show that the 160K fraction originates from stroma lamellae and the end membranes of grana stacks and contains only 110A particles. The 10K fraction originates from the partition regions of grana stacks and contains both 110A and 175A particles. This distribution of particles on fracture faces of stroma versus grana lamellae is shown to exist in freeze fractured class 1 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.

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#### INTRODUCTION

In 1968 the Michels 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<sup>1</sup>. 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  $\text{chl a/chl b}$  ratios. The photochemical activities of these fractions have been further characterized by Murata and Brown<sup>2</sup>. Initially we had difficulty repeating these experiments, and our light fraction yielded chl a/chl b ratios only slightly higher than whole chloroplasts. A recent publication by Jacobi<sup>3</sup> indicated that separation of fractions with high chl a/chl 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 fraction sedimenting at high  $g$  values has a high P-700 content and high photosystem 1 activity. The more rapidly sedimenting fraction has high photosystem 2 activity, reduced photosystem 1 activity and reduced P-700 content. The yields of chlorophyll, chl a/chl 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<sup>4,5</sup>. That digitonin extraction and French press breakage are releasing the same fraction with high chl a/chl b ratio is indicated by extracting the French press heavy fraction with digitonin. This extract yields only small amounts of photosystem 1 material. We have found 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. We conclude that non appressed membranes (stroma lamellae and end membranes of grana stacks) in spinach chloroplasts are primarily photosystem 1 membranes, while the appressed membrane regions (partitions) of the grana stacks contain both photosystems. As a consequence, the thylakoid system is completely surrounded by photosystem 1. Further, freeze fracturing shows that the photosystem 1 membranes contain only 110 A particles, whereas the partitions with both photochemical activities contain both 175 A and 110 A particles.

#### 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  $K(PO_4)$  pH 7.4, 0.01 M KCl, 0.5 M sucrose. The slurry was passed through 8 layers of cheesecloth and centrifuged for 5 min at 200 x g. The precipitate was discarded and the supernatant was centrifuged at 1000 x g for 15 min. This crude chloroplast precipitate was resuspended in 20 ml of 0.15 M KCl, 0.05 M  $K(PO_4)$  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 1500 lb/in<sup>2</sup> at approximately 20 ml/min. Further passes through the cell gave only slight increases in yields of the high g fractions. With our press, 3 passes at 12,500 lbs (the Carnegie technique)<sup>1</sup> never yielded high g fractions with a chl a/chl b ratio greater than 3.5. The French press homogenate (FP)

was then fractionally separated by centrifugation at 1000 x g 10 min, 10,000 x g 30 min, 40,000 x g 30 min, 160,000 x g 60 min (designated 1K, 10K, 40K, and 160K fractions). The resulting precipitates were resuspended in 0.05 M  $K(PO_4)$  pH 7.4, 0.01 M KCl. Less than 2% of the total chlorophyll remained in the 160,000 x g supernatant after this treatment.

Chl a and chl b were determined using the spectrophotometric method of Arnon<sup>6</sup>. P-700 was determined by the ferricyanide method of Yamamoto and Vernon<sup>7</sup>. NADP reductase and purified ferredoxin were prepared following the methods of Shin et al.<sup>8</sup> and Tagawa and Arnon<sup>9</sup>. Plastocyanin was purified by the procedure of Kato et al.<sup>10</sup>. Photosystem 1 reduction of NADP from Na isoascorbate was followed spectrophotometrically in the apparatus described by Sauer and Biggins<sup>11</sup> using the reaction mixture of Anderson and Boardman<sup>4</sup>, 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 DCIP as oxidant in the presence of methylamine as described by Sauer and Park<sup>12</sup>. The extinction coefficients for DCIP according to Armstrong were used to calculate DCIP reduction rates at various pH levels<sup>13</sup>. Cytochromes were quantitatively determined at room temperature using difference spectra of unextracted material following the methods of Boardman and Anderson<sup>5</sup>. Manganese was determined by standard digestion and atomic absorption techniques. Electrophoresis of sodium dodecylsulfate (SDS) solubilized lamellae was conducted according to the method described by Clark<sup>14</sup>. The electrolyte was modified to contain 0.2% SDS.



### 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 hr. 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 in 2% osmium tetroxide in the phosphate buffer. These procedures were carried out at 0°C. 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 hr followed by Fiske's<sup>15</sup> lead citrate 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<sup>16</sup>. Class I chloroplasts for freeze fracturing were prepared by the method of Jensen and Bassham<sup>17</sup>.

### BIOCHEMICAL RESULTS

In our initial experiments we prepared the French press homogenate as described under 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  $M_n$ , are greatly enriched in chl a and P-700 compared with the starting material. There is a corresponding enrichment of  $M_n$  and depletion of chl a and P-700 in the 1K and 10K fraction. These data suggest a separation of the two photosystems has occurred. 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 (FP). 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 SDS. The 160K fraction yielded predominantly Thornber's<sup>18</sup> complex 1 with a chl a/chl b ratio of 8, while the 10K fraction yielded both complexes 1 and 2.

Total cyt b in the 160K fraction is only 35% that of the starting material on a chlorophyll basis. The residual cyt b in this fraction gave a difference spectrum peak at 563 nm indicating it is primarily cyt b<sub>6</sub>. The cyt b content of the 1K and 10K fractions, on the other hand, does not differ greatly from the FP fraction and consists of both cyt b<sub>6</sub> and cyt 559 in about equal proportions. Our data does not show marked variations of cyt f abundance to chlorophyll between the various fractions.

The solid line in Figure 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<sup>4</sup>, in which they give the difference spectrum of their 144,000 x g fraction minus their 10,000 x g fraction. The Anderson-Boardman data is plotted on Figure 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 1 material identical for both treatments?

If the same source provides the photosystem 1 material in both treatments, we would predict that a French press fraction depleted in photosystem 1 (10K) would not yield appreciable additional photosystem 1

material upon digitonin extraction. If different sources provide the photosystem 1 material isolated in the 2 treatments, digitonin extraction of the French press 10K fraction would be expected to yield a photosystem 1 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 1 fractions with a high chl a/chl b ratio. To test these two possibilities the 10K fraction from an FP homogenate was resuspended in the standard buffer for French press treatment and passed once more through the French press. This material was centrifuged at 10,000 x g for 30 min. The supernatant contained 3% of the chlorophyll with a chl a/chl b ratio of 4. The resulting precipitate (10K FP<sub>2</sub>) was used for digitonin extraction. The data in Table IV show that digitonin treatment of the 10K FP<sub>2</sub> fraction yields only 0.6% of the starting material as a photosystem 1 fraction. The inability of digitonin treatment of the French press 10K fraction to remove appreciable photosystem 1 material in addition to that already removed by the French press supports our initial hypothesis--namely, the source of photosystem 1 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 Figures 2 and 3. In Figure 2 the grana 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.

Thin sections of the 10K and 160K fractions from the FP homogenate are compared in Figures 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 160,000 x g, which might be expected to flatten them. 2) As will be evident from Figures 6 and 8, they do not contain the large 175 A particles associated with the spinach grana partitions.

Freeze fractured and deep etched preparations of the 10K and 160K fractions are compared in Figures 6 and 7. Both fractions were resuspended in water to allow deep etching. This treatment causes some unfolding of the grana as seen in Figure 6. Nomenclature for the fracture faces and membrane surfaces used here is that given in the model presented in Figure 1 of reference 16. The 10K fraction contains both large (175 A) and small (110 A) particles on B and C fracture faces respectively, whereas the 160K fraction possesses only 110 A 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, Figure 8 shows that two kinds of particles exist on B fracture faces in class I chloroplasts. In class I chloroplasts the large (175 A) 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 A particles are seen. In Figure 8a two typical B fracture faces in a grana stack are connected by a stroma lamella which has only small particles. In Figure 8b the folding of an end membrane to form a typical partition region is evident. Our earlier observations<sup>16</sup> showed that 175 A 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 A particles. In mature class I spinach chloroplasts the 175 A particles appear to be restricted to the partition region.

Since the 160 K membrane vesicles contain only 110 A 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 A 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 A 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 A particles, whereas the partitions in the grana contain both the 175 A and 110 A particles. We further conclude that the 160K fraction from the FP homogenate consists of stroma lamellae vesicles with some end membranes. These vesicles have only system 1 activity, high chl a/chl b ratios, and high P-700 content. The 10K fraction consists primarily of partitions and has both light reactions and both kinds of particles.

#### DISCUSSION

The similarity of our French press fractions to those prepared by digitonin extraction is most striking. A comparison of our results with Boardman and Anderson's data is given in Table V. Digitonin solubilizes almost 11% of the total chlorophyll into a 144K supernatant fraction, whereas the corresponding fraction from the French press material never exceeds 2%. Also, the chl a/chl 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 all stroma lamellae and end membranes equally, as well as starting to solubilize the grana lamellae. Such solubilization is indicated particularly by the low chl a/chl b ratio of the 144,000 x g supernatant from digitonin extraction. This mode of action would explain both the increased yield and lower chl a/chl b ratios obtained by the digitonin method compared with the French press method.

The cyt 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 FP fraction is similar to the digitonin fractions except for increased P-700 in the French press 160K fraction<sup>19</sup>. 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 FP preparation. The published sectioned material by Murakami<sup>20</sup>, and Arntzen et al.<sup>21</sup> both show that the high chl a/chl b fraction contains small vesicles while the low chl a/chl b fraction contains interconnected thylakoids which have been highly modified by the presence of digitonin and have no readily apparent relationship to intact thylakoids. Our evidence for the similarity of French press and digitonin action, presented in Tables IV and V and in Figure 1, strongly suggests that the modified thylakoids in the rapidly precipitated digitonin fractions also originated from grana regions.



Are the fractions obtained from the French press and digitonin treatment of spinach thylakoids properly termed particles? With the possible exception of the digitonin 144K supernatant, the answer appears to be an emphatic no. Both treatments yield an assortment of membrane fragments usually appearing in vesicle form. These vesicles demonstrate a complete range of sizes from modified grana stacks to very small fragments of the single membranes making up stroma lamellae. This range of fragment sizes precludes the name particle, which implies a fixed size, for all fractions with the possible exception of the very small fragments in the 144K supernatant from digitonin extractions.

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 A particles while both the 110 A and 175 A particles are found on the fracture faces of partitions in grana stacks. Thus, the 160K fraction which contains only 110 A 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 A particles with photosystem 1 which occurs in both membrane systems and the 175 A particle with photosystem 2 which occurs only in the grana, other interpretations are possible. In particular, we do not know whether such large particles might result from purely physical factors in appressed membranes and might have nothing to do with either photosystem. Freeze fracturing

of digitonin fractions by Arntzen et al.<sup>21</sup> also show 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 report. All freeze fracturing of detergent treated membranes is subject to the criticism that the hydrophobic regions of the membrane have been greatly modified and that corresponding changes in fracture faces might be expected.

Anderson and Boardman interpreted digitonin action on thylakoids as solubilization of external particles from a membrane system<sup>4</sup>. Arntzen et al.<sup>21</sup> have interpreted the action of digitonin primarily as splitting along membrane hydrophobic regions in a way analogous to that which occurs during freeze fracturing. Though such processes may become operative during advanced stages of digitonin solubilization of thylakoids, our data support a different mechanism. That this mechanism is equally applicable to digitonin and French press fractionation is supported by the great chemical and enzymatic similarities of fractions obtained by the two processes. The mechanism of fractionation for the French press material appears to be selective breakage and removal of stroma lamellae and some end membranes from the grana. These membranes are primarily photosystem 1 in character. The residual grana with their partition regions contain both photosystems 1 and 2. Digitonin fractionation as performed by Wessels<sup>22</sup>, Anderson and Boardman<sup>4</sup>, and Huzisige et al.<sup>23</sup>, in our view, does not involve removal of photosystem 1 from a closely associated photosystem 2 by selective solubilization. It involves instead the selective breakage

of stroma lamellae and end membranes to yield a photosystem 1 vesicle fraction. The lower g digitonin fractions are grana derived and contain both photosystems 1 and 2, probably in close physical association. Huzisige and his coworkers<sup>23</sup> have shown that these heavier fractions can be treated with Triton X-100 to yield a fraction enriched in photosystem 2 activity. However, the relatively high chl a/chl b ratio (2.0) of this fraction suggests that selective destruction of photosystem 1 may be involved as well as enrichment of photosystem 2.

The model we have proposed for the distribution of photosystems 1 and 2 in spinach chloroplasts yields a number of predictions concerning the relationships of the two systems. Some of these are supported by our data and some are still untested experimentally. These predictions and the evidence supporting them are as follows:

1. There are two kinds of photosystem 1 in spinach chloroplasts. One of these, in grana regions, exists in close association with photosystem 2. The second type exists in stroma lamellae and is not in close physical association with photosystem 2. Evidence for the two kinds of photosystem 1 in this study 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-700/425 chlorophylls. Quantum yield measurements on the two photosystems by Sauer and Park<sup>12</sup> and by Kelly and Sauer<sup>24</sup> 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-700/212 chlorophylls. However, the 160K fraction which accounts

for 10% of the total chlorophyll (20% of the photosystem 1 chlorophyll) contains 1 P-700/105 chlorophylls. The remaining 80% of photosystem 1 in the grana fraction is relatively depleted in P-700 and both by calculation and observation contains about 1 P-700/300 chlorophylls. Our evidence on P-700 distribution supports the notion that the two kinds of photosystem 1 have very different unit sizes. The unit within the grana has 2.5-3 times more chlorophyll per P-700 trapping site than the corresponding unit in the stroma lamellae. This is particularly interesting since the light intensity in the stroma regions will be higher than that in the grana stack and there appears to be a corresponding decrease in unit size.

2. If the photosystem 1 sites in stroma lamellae and end membranes are involved in electron transport as well as photosynthetic phosphorylation, they must be connected via a diffusion pathway to photosystem 2 in the grana stacks. Though no direct evidence for diffusion carriers exists, the feasibility of such a path can be considered. In spinach a diffusion path from a granum to the extremities of a stroma lamella probably doesn't exceed 2 microns. If one assumes the carrier has a diffusion coefficient of about  $10^{-5}$  cm<sup>2</sup>/sec (within a factor of 2 for most small molecules and ions), the time for the concentration at 2 microns 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 reactions times for some photosynthetic electron transport reactions<sup>24,25</sup>. Thus, the

existence of such a path is possible though no direct evidence exists for it at present.

What does the model explain besides the digitonin data? It indicates why excessive breakage by sonication will contaminate an initially released fraction with high chl a/chl b ratio with material of a lower ratio. Continued sonication will break grana as well as stroma lamellae. Jacobi has already offered this interpretation to explain his data<sup>3</sup>.

It is compatible with the observation of Weir, Stocking and Shumway<sup>26</sup> 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 most chlorophyll fluorescence in chloroplasts at 20°C originates from the grana regions and is emitted by photosystem 2<sup>27-29</sup>. This would be expected if photosystem 2 is restricted to grana regions as we have found. The extent to which fluorescence is enhanced by chlorophyll associated with partitions versus non appressed membranes is a topic for future investigation. The model is also consistent with association of the Ca<sup>++</sup> dependent ATPase with the high g digitonin fractions<sup>21</sup>.

The preparation of non detergent treated fractions of grana or stroma lamellae allows a new approach to certain other problems. Primary among these is an investigation of the origin and significance of grana. A comparison of the lipids and proteins in these fractions should give an indication of the factors which are important in influencing partition formation. These fractions should also be useful for low angle X-ray diffraction studies since the fraction containing

only the smaller particles can be compared with the fraction containing both sizes.

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.<sup>30</sup>, Briantais<sup>31</sup> and others on Triton X-100 fractionation is probably related to the kinds of breakage we have observed here, but at present we have no data either for or against such a relationship. In conclusion, non detergent thylakoid breakage by the French press gives us fractions similar to those obtained by digitonin breakage, but with the added advantage that they can be ultrastructurally related to the intact thylakoid system.

#### 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.

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TABLE I

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

Class II chloroplasts were passed through the French press at 1,500 lb/in<sup>2</sup> and separated into fractions by differential centrifugation. Chlorophylls, P-700 and manganese were determined as described in METHODS.

Fraction	Chl a/b	% Chl	Chl/P-700	Chl/Mn
FP	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 super-natant	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 METHODS.

Fraction	$\mu$ Moles reduced/mg chl per h	
	DCIP (pH 7.0)	NADP <sup>+</sup> (pH 7.4)
Original chloroplasts	174	172
FP homogenate	52	87
1K	100	62
10K	74	75
40K	-	87
160K	0	169

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<sup>5</sup>.

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Fraction	Chl/total cyt b	Chl/cyt f
FP	109	510
1K	160	635
10K	113*	575
40K	108	425
160K	286**	530
106K supernatant	No detectable cytochromes	

---

\* 10K fraction relatively enriched in cyt<sub>559</sub>

\*\* 160K fraction relatively enriched in cyt. b<sub>6</sub>

TABLE IV  
DIGITONIN FRACTIONATION OF 10K FP<sub>2</sub> FRACTION ACCORDING TO ANDERSON  
AND BOARDMAN<sup>4</sup> PROCEDURE

10K FP<sub>2</sub> fraction was prepared by passing the 10K fraction through  
the French press at 1,500 lb/in<sup>2</sup> and collecting a fraction sedi-  
menting at 10,000 x g, 30 min.

---

Fraction	Chl a/chl b	Percent chl
10K	2.2	98.3
50K	2.8	1.1
50K supernatant	5.0	0.6

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TABLE V

COMPARISON OF ANDERSON BOARDMAN<sup>4,5</sup> DATA ON DIGITONIN FRACTIONS (IN BRACKETS) AND DATA ON FRENCH PRESS FRACTIONS FROM THIS PAPER

Activities given in  $\mu$ moles of NADP reduced/mg chl/hr (photosystem 1) or  $\mu$ moles DCPIP reduced/mg chl/hr (photosystem 2). Comparison of 160K (144K) -10K difference spectra is given in Figure 1.

Fraction	Chl a/chl b	Percent chl	Chemical or enzymatic property						
			Photosystem 1		Photosystem 2		Chl a + chl b		$\frac{\text{cyt b}}{\text{cyt f}}$
			ASC $\rightarrow$	NADP <sup>+</sup>	DCIP	TCIP	cyt b		
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 -	-	-	-	-	- -	- -	
50K (40K)	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)	- -	- -	

FIGURE LEGENDS

Fig. 1. Solid line is a difference spectrum of 160K fraction minus 10K fraction with absorbances equalized at 678 nm. Dashed line is a difference spectrum of 144,000 x g minus 10,000 x g digitonin fractions from data of Anderson and Boardman<sup>4</sup>.

Fig. 2. Portion of a class II chloroplast before French press treatment showing grana stacks (G) and interconnecting stroma lamellae (St). X 80,000.

Fig. 3. Fraction after French press treatment showing grana stacks (G) and vesicles (V). Note absence of interconnecting stroma lamellae. X 66,000.

Fig. 4. Sections through 10K fraction:

(a) Section showing remnants of grana (G) and large vesicles with partitions. X 70,000.

(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. X 132,000.

Fig. 5. Sections through 160K fraction:

(a) Section showing predominance of vesicles of various sizes. X 70,000.

(b) Flattened vesicle with partition-type region (P). X 140,000.

Fig. 6. Freeze-fractured and deep-etched 10K fraction showing A' and D surfaces and B and C faces. X 64,000.

(direction of shadow → )

**Fig. 7. Freeze-fractured and deep-etched 160K fraction:**

(a) Freeze fracture face of vesicle showing only 110 A particles. X 70,000.

(b) Freeze fractured and deep-etched vesicle showing only 110 A particles and A type surfaces. X 70,000.

(direction of shadow → )

**Fig. 8. Freeze-fractured Jensen and Bassham<sup>17</sup> type chloroplasts:**

(a) Fracture face showing 110 A and 175 A particles on the same B face. The 175 A particles appear only in partition regions while the 110 A particles appear on a typical interconnecting stroma lamella (St) and end membranes (Em). X 110,000.

(b) End membrane (Em) continuous with a stroma lamella (St) and with partition B face. X 110,000.

(direction of shadow → )

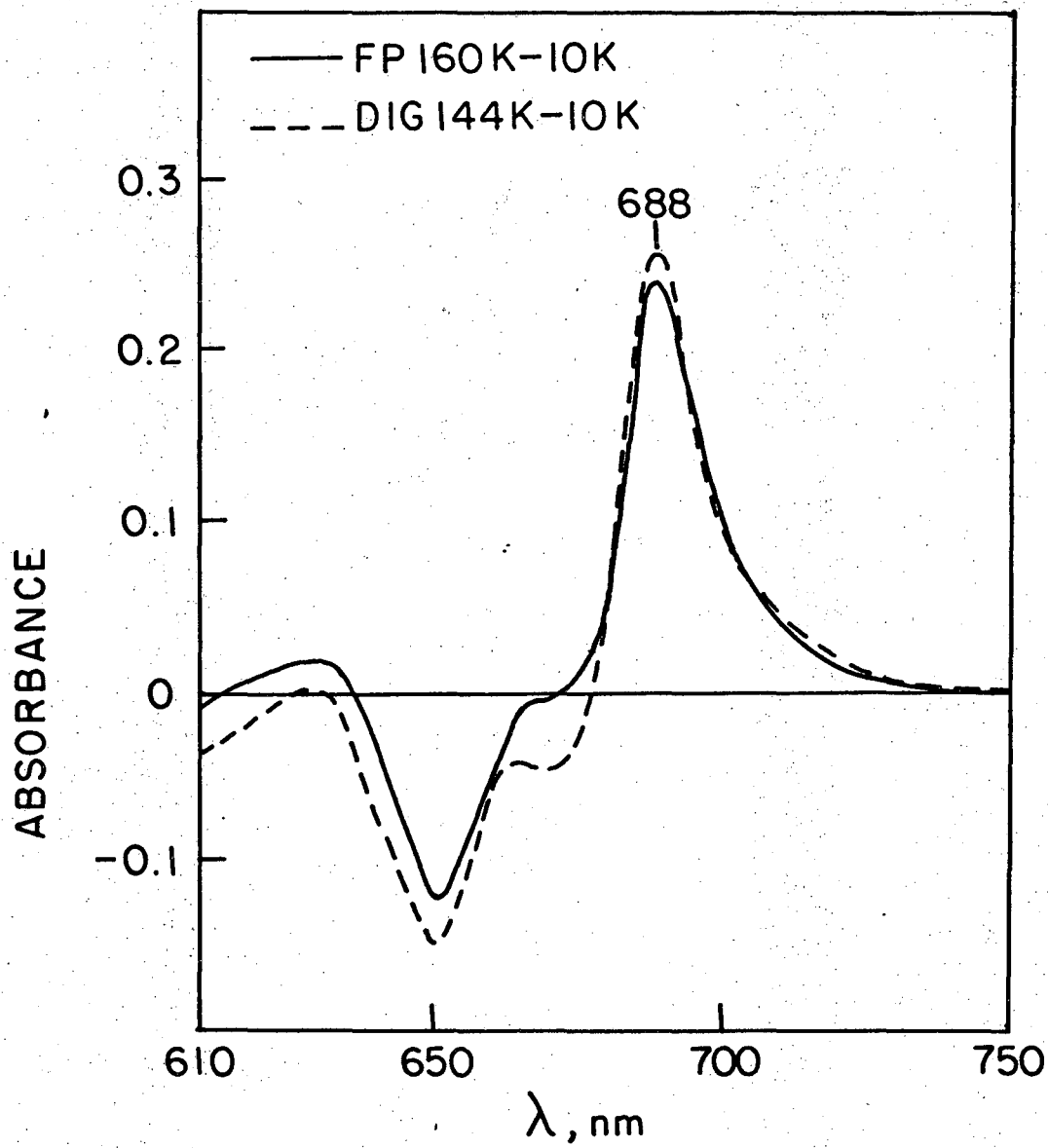


Fig. 1

XBL 701-5026



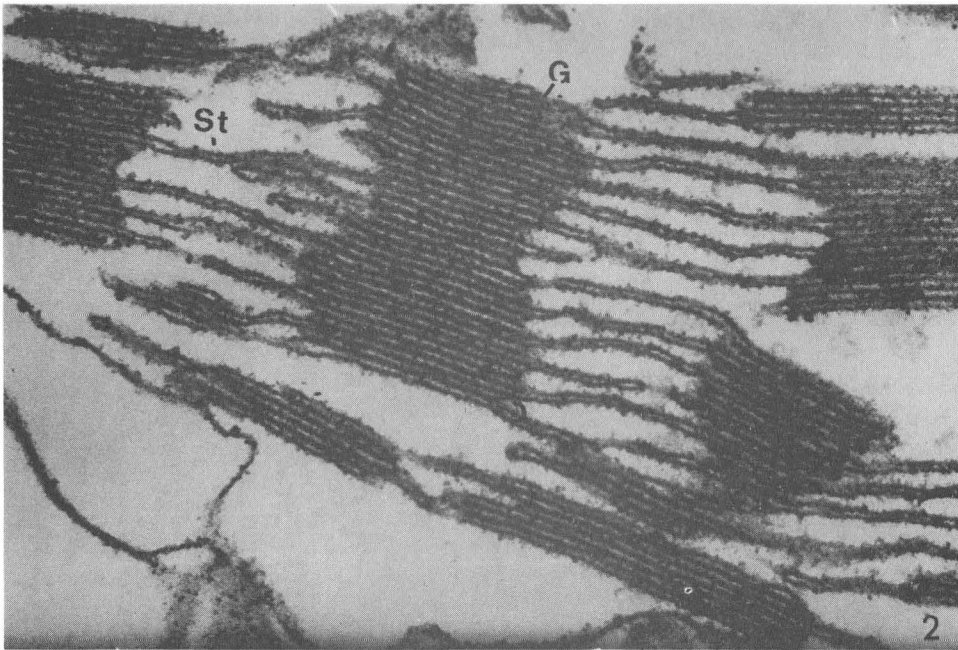


Fig. 2.

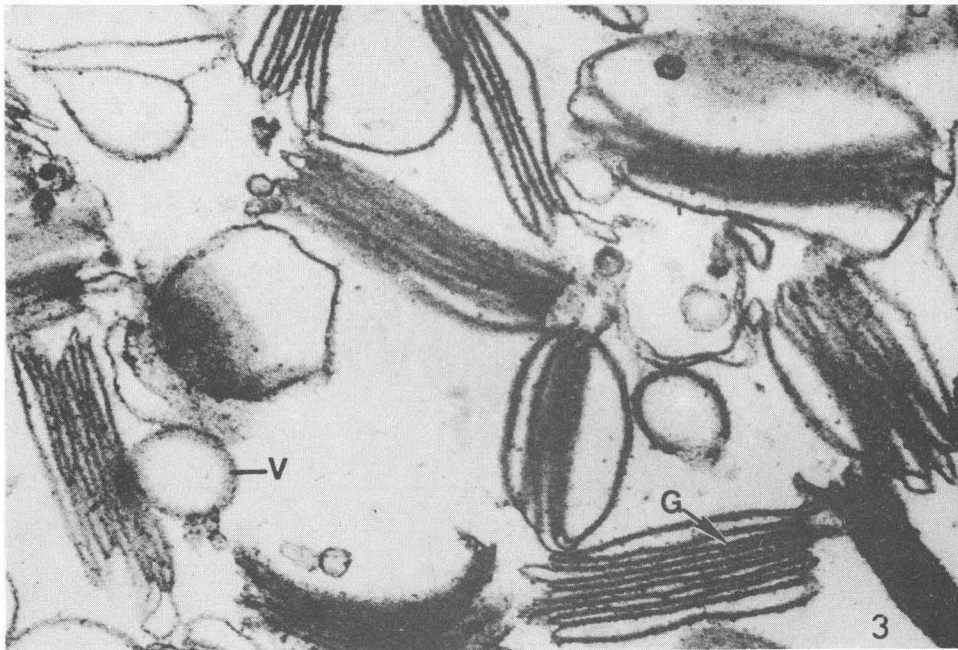


Fig. 3

XBB 704-1755



Fig. 4 (a)

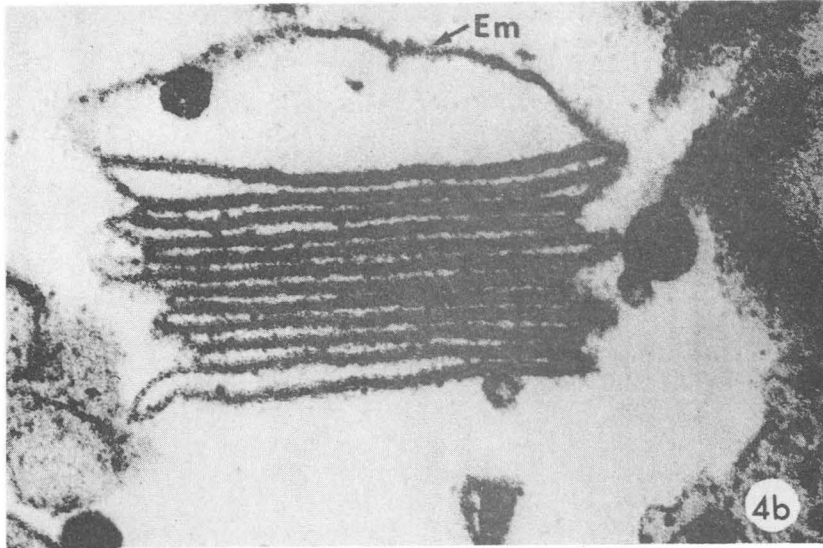


Fig. 4(b)

XBB 704-1756

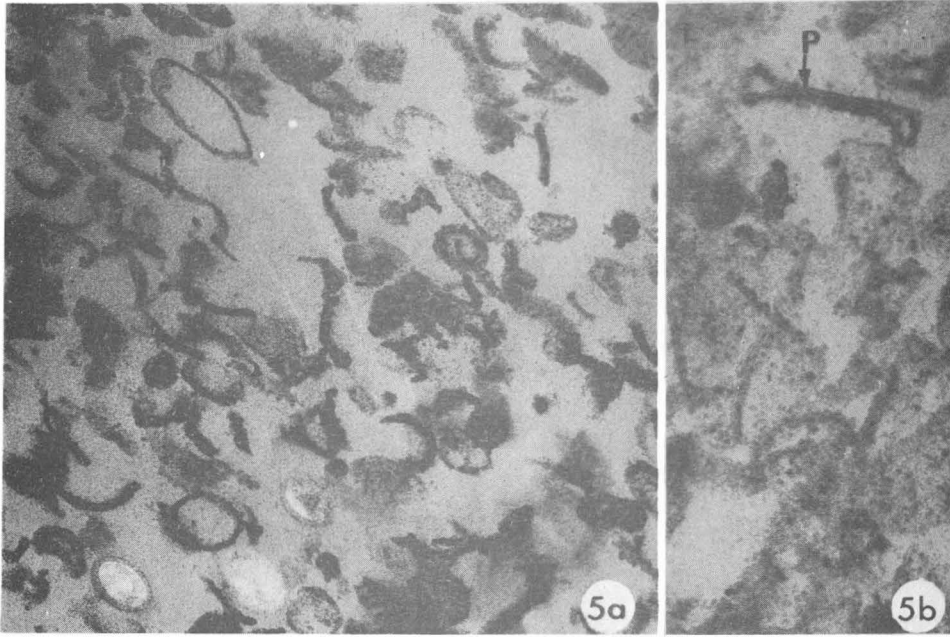


Fig. 5 (a) (b)

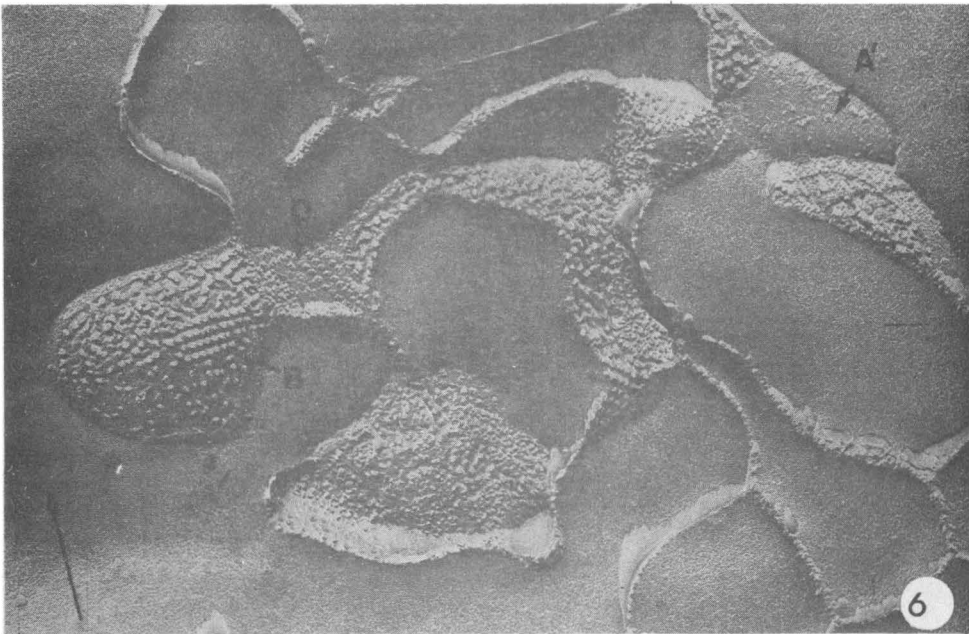


Fig. 6

XBB 704-1757

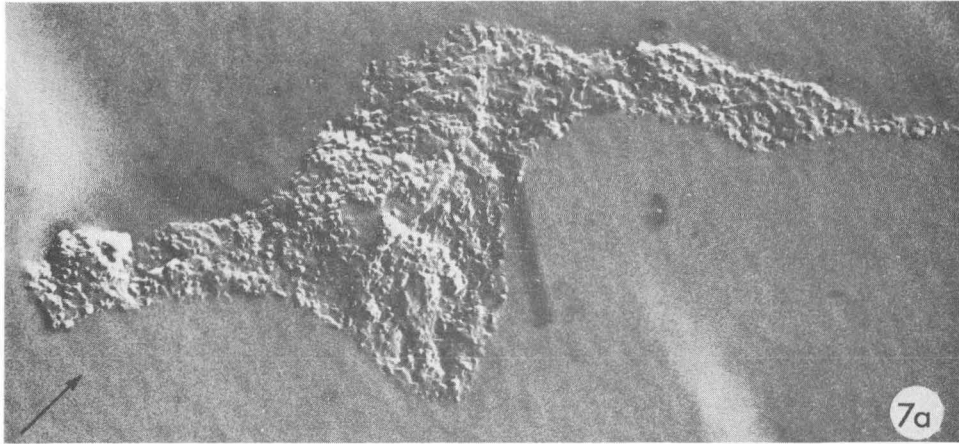


Fig. 7 (a)

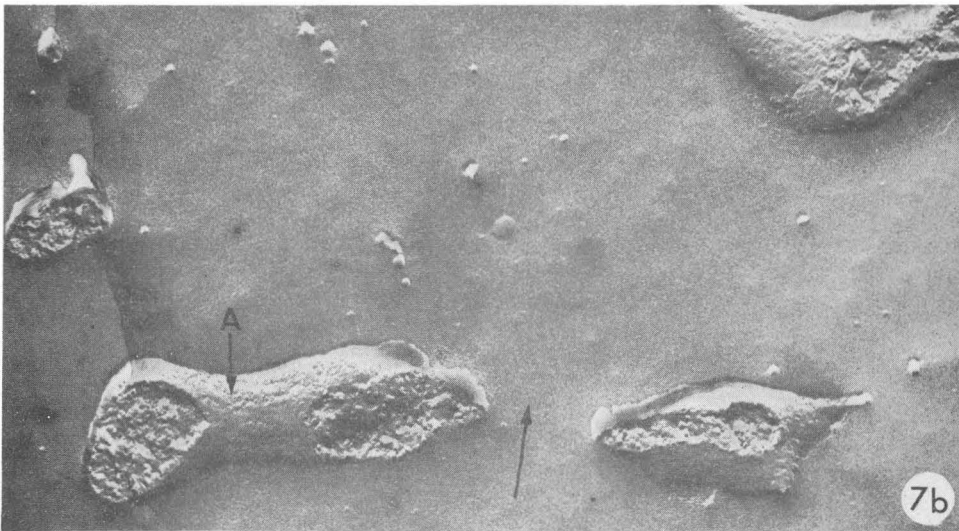


Fig. 7 (b)

XBB 704-1758

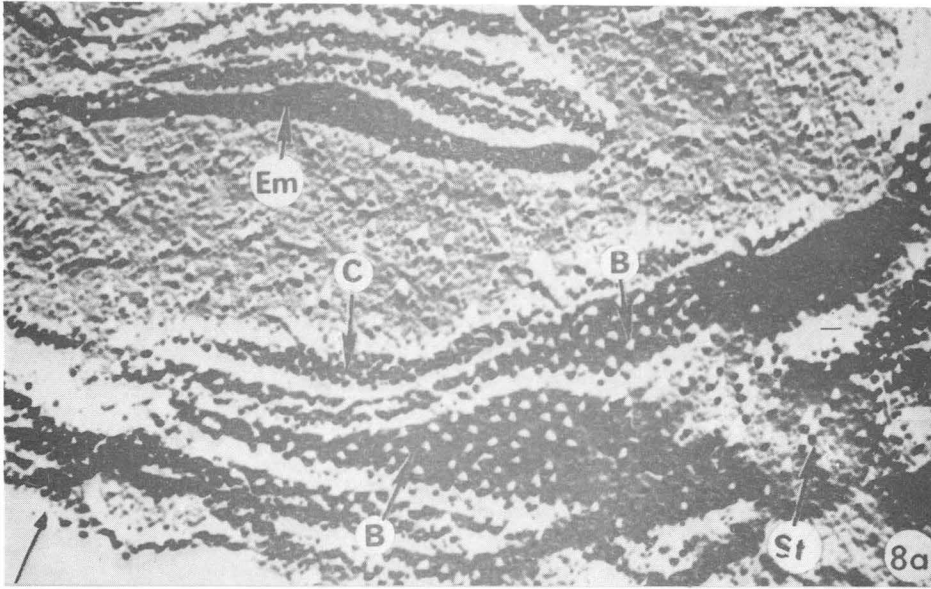


Fig. 8. (a)

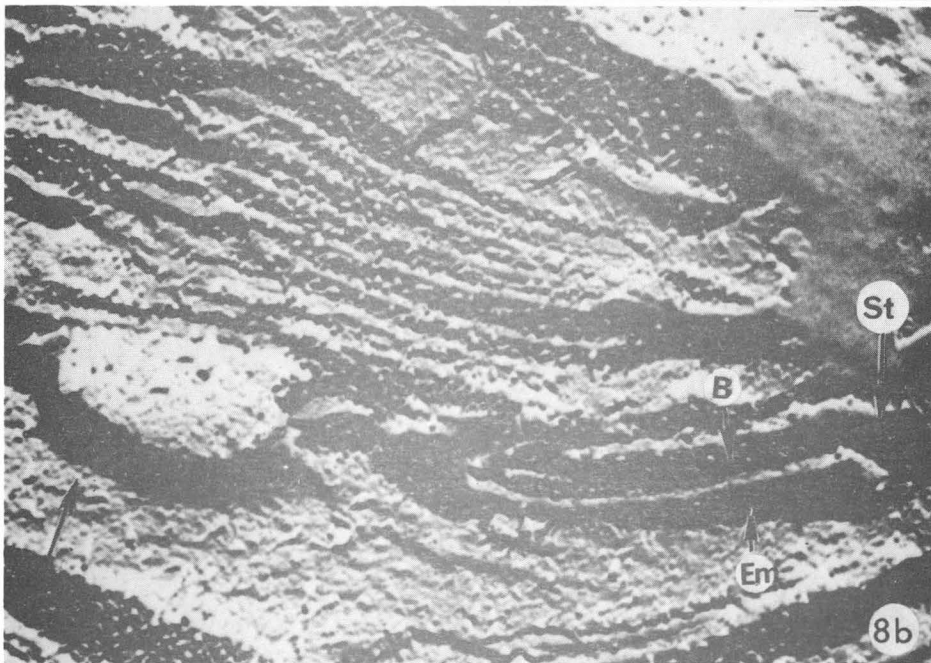


Fig. 8 (b)

XBB 704-1759

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