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FURTHER EVIDENCE FOR STROMA LAMELLAE AS A SOURCE OF PHOTOSYSTEM I FRACTIONS FROM SPINACH CHLOROPLASTS

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SUMMARY

The mechanism of digitonin action on spinach chloroplasts was investigated by thin sectioning. Evidence is presented which shows that digitonin continues to modify membranes for many minutes after the addition of the fixative glutaraldehyde. However, the action of digitonin can be stopped by simultaneous fixation and dilution of the detergent. Such experiments indicate that the initial action of digitonin is to release stroma lamellae which in turn yield a Photosystem I fraction. This interpretation is further supported by a significant correlation between the chlorophyll *a*/chlorophyll *b* ratio and the ratio of stroma to grana lamellae in spinach chloroplasts.

INTRODUCTION

Recent work from this laboratory showed that spinach chloroplasts broken in a French pressure cell can be separated into two membrane fractions consisting of stroma lamellae and grana lamellae. Assays of these fractions for the two photosystems showed that stroma lamellae contained only Photosystem I while the grana fraction contained both photosystems[1].

ANDERSON AND BOARDMAN[2] have used the detergent digitonin to separate spinach chloroplasts into membrane fractions which are very similar to the French pressure cell fractions in chemical composition, photochemical activity and yield. Both methods yield approx. 10% of the chlorophyll in a fraction possessing only Photosystem I activity and a high chlorophyll *a*/chlorophyll *b* ratio. ARNTZEN *et al.*[3] extended the work of ANDERSON AND BOARDMAN[2] by investigating the ultrastructure of the digitonin fractions by sectioning and freeze etching. They concluded that digitonin had split the chloroplast lamellar membranes longitudinally to yield the fraction having only Photosystem I activity.

The biochemical similarity between the digitonin and French pressure cell fractions has led us to re-examine the ultrastructure of the digitonin fractions to find the extent to which our predictions or the ARNTZEN *et al.*[3] interpretation is correct. We conclude from the work reported here that in the early stages of digitonin treatment the digitonin and French pressure cell are acting in a similar manner

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and that in both methods the Photosystem I fraction is derived from stroma lamellae and not from membrane splitting. Subsequently, selective solublization of Photosystem I from grana lamellae may occur.

Additional support for this distribution of photosystems between grana and stroma lamellae comes from the observation that in spinach chloroplasts there is a significant correlation between a high chlorophyll *a*/chlorophyll *b* ratio and a high proportion of stroma lamellae.

MATERIALS AND METHODS

Chloroplasts were isolated from field grown spinach plants in potassium phosphate buffer (pH 7.4) –0.01 M KCl –0.5 M sucrose and the pellet resuspended in the same buffer without sucrose[1]. For digitonin treatment the chlorophyll concentration was adjusted to 0.3–0.4 mg/ml with buffer and to this 2% digitonin was added (2 times recrystallized) to a final concentration of 0.5%[2]. The incubation with digitonin was carried out in an ice bath with constant stirring. Chlorophyll *a* and chlorophyll *b* were determined using the spectrophotometric method of ARNON[4] and a Cary model 14R spectrophotometer with a scattered transmission accessory.

For electron microscopy of the chloroplast suspension, samples were removed and pelleted as completely as possible in a 1-ml centrifuge tube in an adaptor in the swinging bucket rotor of a Servall centrifuge at 27500 × *g* for 20 min. The resulting pellets were washed 4 times by replacing the fluid above each with phosphate buffer and then post-fixing for 1 h in 2% osmium tetroxide in phosphate buffer. These procedures were carried out in the cold, but the samples were dehydrated in acetone and propylene oxide at room temperature. Then the end of the centrifuge tube was cut off and the pellets were 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 90 min followed by FISKE's[5] lead citrate for 20 min before examination in a Siemens Elmiskop 1A electron microscope.

When leaf pieces were fixed for electron microscopy a similar procedure was adopted except that pellets were replaced by mm squares of spinach leaf tissue.

EXPERIMENTAL AND RESULTS

Digitonin treatments

Time-course studies of photochemical activity of both chloroplast and algae following glutaraldehyde addition show that complete fixation may take up to 1 h (ref. 6). Therefore it is unlikely that the addition of glutaraldehyde will stop the action of digitonin immediately. Two experiments were carried out to reveal the ultrastructural state of the membranes at the time when the digitonin fractions are subjected to differential centrifugation for the separation of Photosystem I. In the first experiment the time course of digitonin action was studied by adding fixative to the incubation mixture. In the second experiment dilution was combined with fixation in an attempt to stop the action of digitonin.

Fixative addition experiment

In this experiment 0.5-ml samples were removed from the chloroplast digitonin incubation mixture at 5, 20, and 60 min after the addition of digitonin. To each was

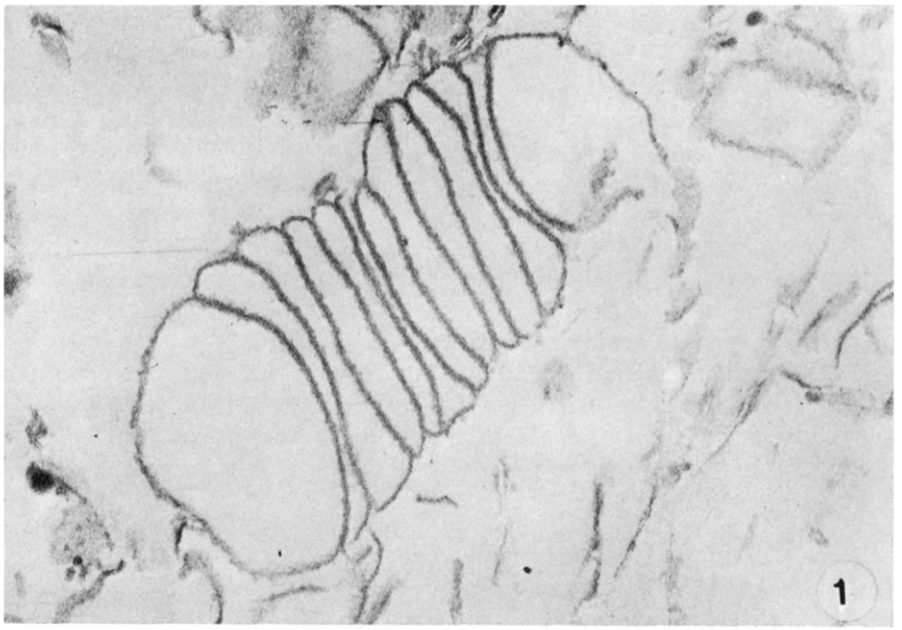

Fig. 1. Section through a pellet from 5-min digitonin incubation followed by glutaraldehyde fixative addition showing a typical swollen grana stack but no interconnecting stroma lamellae (× 100000).

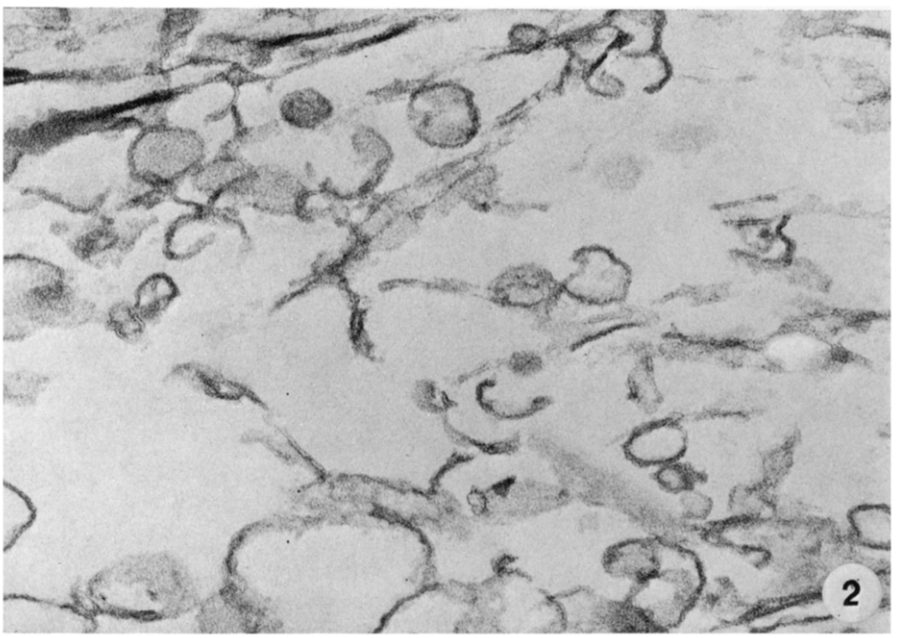

Fig. 2. Section through a pellet from 60-min digitonin incubation followed by glutaraldehyde fixative addition. No organized grana are present (× 100000).

added 0.5 ml of 4 % glutaraldehyde in phosphate buffer. The sample was centrifuged and embedded as described in MATERIALS AND METHODS. Fig. 1 shows a section through a pellet from 5-min digitonin treated chloroplasts. The interconnecting stroma lamellae are absent but grana stacks are still intact although most are swollen. Fig. 2 shows the 60-min digitonin treatment. Grana stacks are absent and all that remains are swollen vesicles, presumably remnants of stroma lamellae and grana lamellae. The 20-min treatment was intermediate between these two and had very few grana stacks. We interpret these pictures to indicate that stroma lamellae are separated from grana during the first few minutes of digitonin action but that prolonged digitonin treatment causes grana to dissociate into vesicles.

Fixative dilution experiment

Chloroplasts were treated with digitonin as described previously[5], and after 5 and 30 min treatment a 1-ml sample was removed and diluted in 100 parts of 0.05 M potassium phosphate buffer (pH 7.4) –0.01 M KCl containing 2 % glutaraldehyde. This material was sedimented at 40000 × *g* in 50-ml centrifuge tubes and the pellets transferred to the 1-ml tubes for treatment as described in MATERIALS AND METHODS. Fig. 3 shows a section through a pellet from the 5-min digitonin treatment which shows intact, and relatively unswollen, grana stacks but no interconnecting stroma lamellae. The 30-min digitonin treatment yielded similar pictures. Both these results indicate that dilution is necessary in addition to glutaraldehyde fixation to minimize the continued action of digitonin.

A 5-min digitonin treatment followed by dilution was sufficient to separate

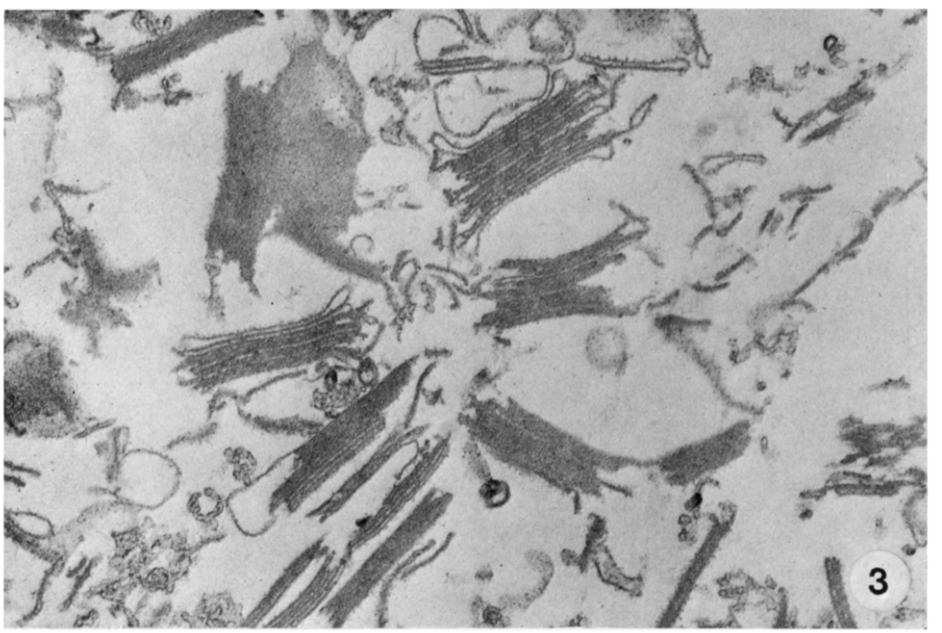

Fig. 3. Section through a pellet from 5-min digitonin incubation followed by glutaraldehyde fixative addition and dilution. Well preserved grana stacks are present but are not interconnected by stroma lamellae (× 45000).

grana and stroma lamellae. In another 5-min fixative dilution experiment the fraction that sedimented between 40000 × *g* and 160000 × *g* was separated for determination of the chlorophyll *a*/chlorophyll *b* ratio. This fraction had a chlorophyll *a*/chlorophyll *b* ratio of 5.0 and represented approx. 5 % of the total chlorophyll in the incubation mixture. No such fraction was obtained from a control in which distilled water replaced digitonin. This additional evidence supports the evidence from electron micrographs that chloroplast fractionation was achieved with a 5-min digitonin incubation followed by dilution. The fact that the interconnecting stroma lamellae are absent (Fig. 3) again implicates them as the source of the high chlorophyll *a*/chlorophyll *b* ratio fraction which has been shown to possess only Photosystem I activity.

Chloroplast structure related to chlorophyll a/chlorophyll b ratio

In our earlier work we showed that the Photosystem I fraction obtained from spinach leaf chloroplasts by the French pressure cell technique represented the stroma lamellae of the intact chloroplasts. This fraction has a high chlorophyll *a*/chlorophyll *b* ratio, and as a consequence one would predict a simple relationship between the ratio of grana and stroma lamellae and the chlorophyll *a*/chlorophyll *b* ratio of intact spinach chloroplasts. Chloroplasts having a high chlorophyll *a*/chlorophyll *b* ratio should yield a higher percentage of the Photosystem I fraction. Young spinach obtained from the field near San Francisco in the month of April 1970 yielded a chloroplast preparation with a chlorophyll *a*/chlorophyll *b* ratio of 3.2. The yield of Photosystem I fraction from these chloroplasts following French pressure cell treatment was 26 % compared to a yield of 10 % from chloroplasts having a chlorophyll *a*/chlorophyll *b* ratio of 2.6.

To establish a correlation between the chlorophyll *a*/chlorophyll *b* ratio and the percentage of stroma lamellae in spinach chloroplasts, use was made of the fact that in a young spinach leaf cell division within the lamina ceases at an early stage of development in the tip region but continues for an extended period at the base of the leaf[7]. This finding suggested that the younger chloroplasts in the basal region may have a higher percentage of stroma lamellae and also a higher chlorophyll *a*/chlorophyll *b* ratio than the leaf as a whole.

TABLE I

RELATIONSHIP BETWEEN CHLOROPHYLL *a*/CHLOROPHYLL *b* RATIO AND RELATIVE PROPORTIONS OF STROMA AND END GRANA LAMELLAE

Results from two experiments on the tip and basal regions of a young spinach leaf, showing a correlation between chlorophyll *a*/chlorophyll *b* ratio and stroma and end grana lamellae length percentages, expressed as percentages of total chloroplast lamellar length. Percentage differences between tip and base significant at 0.1%.

	Tip		*Base*	
	Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
Ratio chlorophyll *a*/chlorophyll *b*	2.7	2.8	3.5	3.5
Stroma lamellae	17.3	9.6	37.8	23.1
Stroma and end grana lamellae	33.8	28.1	58.2	44.1

A young leaf was selected that was obviously dark green at the tip and lighter green at the base. The terminal 0.5 cm was removed and one-half cut into mm squares and fixed for electron microscopy. The other half was extracted in 80 % acetone for chlorophyll determinations. The basal 0.5 cm of the leaf was treated in the same manner.

The results of two such experiments are shown in Table I. The total length of stroma, end grana and grana lamellae were measured on electron micrographs of single chloroplasts at a magnification of 42000 × and the results expressed as a percentage of the total lamellar length. In the first experiment, 5 chloroplasts were measured from each of four different leaf pieces and the results analyzed statistically using a *t* test. In the second experiment, 7 chloroplasts were measured from a leaf tip and 11 from the base. Although some variation was evident, the differences between tip and base were significant at the 0.1 % level. This result indicates that there is a correlation between the chlorophyll *a*/chlorophyll *b* ratio and the percentage of stroma and end grana lamellae in spinach chloroplasts. A higher chlorophyll *a*/chlorophyll *b* ratio is significantly correlated with a greater percentage of stroma and end grana lamellae.

DISCUSSION

The results reported here provide further support for our earlier observation that digitonin and French pressure cell treatments produce similar fractions from spinach chloroplasts[1]. ARNTZEN *et al.*[3] showed that the Photosystem I fraction from digitonin treated spinach chloroplasts contained only small particles on freeze fractured faces, compared with the small and large particles observed by BRANTON AND PARK[8] in intact spinach chloroplasts. SANE *et al.*[1] have also demonstrated small particles in a similar ultracentrifuge fraction following French pressure cell treatment. ARNTZEN *et al.*[3] believe that the chloroplast membranes split longitudinally to produce the small particle fraction. Their conclusion is based on fixed and sectioned digitonin treated spinach chloroplasts and their Fig. 6 resembles Fig. 2 of this paper.

The experiments reported here support the notion that glutaraldehyde fixation of chloroplast membranes is not rapidly achieved in the presence of 0.5 % digitonin. If it were, then the images observed in the 20- and 30-min treatments from the fixative addition and fixative dilution experiments should be similar. The fact that few, if any, vestiges of grana stacks could be observed after 20 min in the fixative addition experiment, while many grana stacks were observed in the fixative dilution experiment, suggests that the mere addition of fixative is not sufficient to stop the action of digitonin. Since ARNTZEN *et al.*[3] added glutaraldehyde to their reaction mixture after incubation (our fixative addition procedure), their electron micrographs probably do not reflect the true state of the membranes at that time, but an advanced stage of digitonin treatment. The differential centrifugation for the preparation of the photochemically active fractions is begun after approx. 30 min incubation in digitonin[2] when the grana stacks are still intact, as shown by the fixative dilution experiment. The resulting rapid removal of the grana fraction leaves stroma lamellae which are subsequently precipitated as the Photosystem I fraction. Longer digitonin treatments (60 min, Fig. 2) show almost complete dissolution of the grana.

Further evidence for the implication of stroma lamellae as the source of the

Photosystem I fraction is provided by WEHRMEYER[9]. He used concentrations of digitonin that are lower than the 0.5 % in 0.3 mg/ml of chlorophyll used for photosystem fractionation. However, his pictures clearly show (Figs. 3–5) that the primary action of digitonin is on the stroma lamellae of spinach chloroplasts. These first swell, and as the digitonin concentration is increased the stroma lamellae finally form round vesicles between the grana stacks and separate from them.

SANE *et al.*[1] provided sectioning, freeze fracturing and deep etching evidence to implicate stroma lamellae and end grana membranes as a source of the Photosystem I fraction produced by the French pressure cell treatment of spinach chloroplasts. Further support is provided from the measurements of chloroplasts from regions of the same leaf having different chlorophyll *a*/chlorophyll *b* ratios. In this case a higher proportion of stroma lamellae, and end grana membranes, was present in the high chlorophyll *a*/chlorophyll *b* ratio area of the leaf. Also French pressure cell fractionation of chloroplasts from high chlorophyll *a*/chlorophyll *b* ratio material produced a higher yield of Photosystem I fraction. This result does not fully explain the findings of OHKI AND TAKAMIYA[10] who obtained a Photosystem I fraction that represented 40 % of the total chlorophyll in the reaction mixture by a modified digitonin treatment. Although they used isolated spinach chloroplasts with a chlorophyll *a*/chlorophyll *b* ratio of 3.0 compared with the ratio of 2.6 to 2.8 of ANDERSON AND BOARDMAN[2] and ARNTZEN *et al.*[3], the high percentage of Photosystem I obtained suggests that membrane splitting of grana may have occurred in addition to solublization of stroma lamellae.

All these results are consistent with the interpretation suggested by SANE *et al.*[1] that both digitonin and French pressure cell treatments of spinach chloroplasts release stroma lamellae as a Photosystem I fraction leaving a grana fraction having both Photosystems I and 2. Further detergent separation of the grana fraction and clarification of the possible precursor relationship of stroma to grana lamellae[11–13] should provide fruitful areas for future research.

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