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EFFECTS OF CATIONS ON THE ADHESION BETWEEN MEMBRANE VESICLES OBTAINED BY DIGITONIN FRACTIONATION OF SPINACH CHLOROPLASTS

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The heavy fraction obtained by digitonin treatment of stacked spinach chloroplasts, suspended in media with different ionic composition, was examined by electron microscopy. In the presence of 5 mM $MgCl_2$ the thylakoid fragments adhere to one another in a 'stacked configuration,' while, in the presence of 10 mM NaCl, mainly only single 'unstacked' vesicles are present, which, upon addition of 5 mM $MgCl_2$, completely revert to the stacked configuration. As previously reported (Chow, W.S. and Barber, J. (1980) Biochim. Biophys. Acta 593, 149–157), no difference in fractionation of chlorophyll between light and heavy fractions was seen after a second digitonin treatment of this fraction suspended in media containing different cation concentrations. From these results it was concluded: (1) that for the unstacking process the movement of proteins or complexes from the stromal to the granal lamellae is not required. Upon lowering the screening by cations of the surface negative charges, the membranes separate from one another; (2) that, under these conditions, as in others (Jennings, R.C., Gerola, P.D., Garlaschi, F.M. and Forti, G. (1980) FEBS Lett. 115, 39–42), digitonin fractionation is not a tool to investigate the degree of membrane stacking.

Introduction

It is well known that thylakoid membranes at physiological pH values carry a net negative charge [1–5]. Barber and co-workers [6,8,9] and Duniec and co-workers [7] have proposed that the screening by cations of this negative surface charge is involved in grana formation and in other cation-influenced phenomena. They have also recently proposed [10–14] that, in unstacked chloroplast membranes, upon increasing the cation concentration of the suspension medium, a lateral movement of proteins and complexes occurs, which leads to the formation of membrane zones with low negative charge density (which adhere to one another to form grana stacks) and high negative charge density zones (the stroma lamellae). In agreement with the hypothesis that the negative charges in certain membrane areas regulate the degree of stacking, we have demonstrated [15–17] that at pH 5.4 (a pH value well above the isoelectric point of chloroplast membranes [1–5]) protons cause thylakoid stacking which is morphologically and biochemically similar to that observed at higher pH values in the presence of metal cations.

In a recent paper, Chow and Barber [18] demonstrated that digitonin treatment of the 'granal fraction' obtained by digitonin fractionation of stacked chloroplasts is not sensitive to the ionic composition of the suspension medium. They concluded that the membranes of the granal fraction are unable to unstack when suspended in low salt media, and this was interpreted to indicate that a lateral movement of

Abbreviations: PS, photosystem; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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protein complexes carrying a high negative charge density (perhaps PS I complexes) from stromal to granal membranes is required for the unstacking process.

However, doubts exist as to the precise interpretation of digitonin fractionation data in terms of membrane stacking [16]. This has led us to reexamine the effect of the ionic composition of the medium on the granal fraction. While, by digitonin analysis, we have obtained similar results to those of Chow and Barber [18], electron microscope studies have revealed that the granal fraction reversibly unstacks upon changing the ionic composition of the suspension medium.

Materials and Methods

Freshly harvested spinach leaves were homogenized, in the presence or absence of 5 mM $MgCl_2$, in 30 mM Tricine buffer (pH 8) containing 0.4 M sucrose and 10 mM NaCl. The homogenate was filtered through eight layers of muslin and centrifuged at 1500 $\times g$ for 5 min. The pellet was then washed in a hypotonic medium and resuspended in a small volume. The washing and resuspending medium were varied, depending on the procedure of the subsequent digitonin fractionation. When digitonin treatment was performed as reported by Boardman and Anderson [19], the washing medium contained 10 mM NaCl, 30 mM Tricine (pH 8) ± 5 mM $MgCl_2$, and the resuspension medium was the same as the homogenizing medium. When digitonin fractionation was performed as reported by Chow and Barber [18], the washing medium contained 10 mM KOH, 10 mM Hepes (pH 7.5, HCl) ± 5 mM $MgCl_2$, and the resuspension medium was 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl) ± 5 mM $MgCl_2$.

Digitonin fractionation was performed following two different procedures, respectively reported by Boardman and Anderson [19] (first procedure) and by Chow and Barber [18] (second procedure). First procedure: chloroplasts were resuspended in 10 mM NaCl, 30 mM Tricine (pH 7.4) ± 5 mM $MgCl_2$ at 400 μg Chl/ml, and digitonin (2%, w/v) was added to 0.5% final concentration. Treatment with digitonin was performed with agitation at 0°C for 30 min, when the chloroplast membrane/detergent mixture was diluted 3-fold with the same buffer, maintaining the $MgCl_2$ concentration unchanged.

Second procedure: chloroplasts were suspended either at 300 μg Chl/ml or at 100 μg Chl/ml in 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl) ± 10 mM NaCl, ± 5 mM $MgCl_2$, and digitonin (2% w/v) was added to 0.4% final concentration. Treatment with digitonin was done with agitation at room temperature for 2 min, when the chloroplast membrane-detergent mixture was diluted 20-fold with cold medium containing 0.1 M sorbitol, 1 mM KOH and 1 mM Hepes (pH 7.5, HCl).

Independently of the digitonin treatment procedure, separation of the heavy and the light membrane fractions was achieved by centrifugation at 10 000 $\times g$ for 30 min. Chlorophyll was extracted with 80% acetone, and Chl *a/b* ratios were determined according to the equations of MacKinney [20]. Sample preparation for electron microscopy was performed as previously reported [15].

Results

Digitonin fractionation of stacked chloroplasts has been reported in the literature as a method to separate the grana (in the heavy fraction) from the stromal membranes (in the light fraction) [21,22]. The two fractions obtained are respectively enriched in Chl *b* and in Chl *a* as compared to the chloroplasts [19]. In Table I the chlorophyll yields and the Chl *a/b* ratios of the light and the heavy fractions obtained by digitonin fractionation according to the method of Boardman and Anderson [19] or that of Chow and Barber [18] are reported. As can be seen the chlorophyll yields and the Chl *a/b* ratios of the fractions are different: the heavy fraction obtained when digitonin treatment of stacked chloroplasts was performed according to the method of Boardman and Anderson [19] has a higher chlorophyll yield and is less enriched in Chl *b* than that obtained following the procedure reported by Chow and Barber [18].

By comparing Figs. 1a and 2a it is possible to see that differences in the membrane structure of the heavy (granal) fraction accompany the differences in the Chl *a/b* ratio. In the first case (Fig. 1a) it is possible to observe membrane areas which adhere to one another (highly contrasted lines), connected at the sides by single, poorly contrasted membranes, in the usual configuration of thylakoid grana. These observations support the earlier reported conclusion [21,

TABLE I

DIGITONIN FRACTIONATION OF STACKED AND UNSTACKED CHLOROPLAST MEMBRANES

(A) Digitonin treatment was performed according to the method of Boardman and Anderson [19]; (B) digitonin treatment was performed as reported by Chow and Barber [18] at a chlorophyll concentration of 300 μg/ml (see Materials and Methods).

Cation composition of the incubation medium	Heavy fraction		Light fraction	
	Chlorophyll yield (%)	Chl *a*/*b* ratio	Chlorophyll yield (%)	Chl *a*/*b* ratio
A				
$MgCl_2$ (5 mM), NaCl (10 mM)	79.7	2.58	20.3	7
$MgCl_2$ (0 mM), NaCl (10 mM)	26.4	2.7	73.6	2.9
B				
$MgCl_2$ (5 mM), NaCl (0 mM)	48.1	2.18	51.9	3.68
$MgCl_2$ (0 mM), NaCl (10 mM)	13.1	3.07	86.9	3.07

22] that the digitonin treatment of stacked chloroplasts, when performed at 0°C according to the method of Boardman and Anderson [19], fractionates the thylakoid membranes mainly by separating the integrana lamellae from the grana (including partitions, margins and some end membranes) which constitute the heavy fraction.

A different situation is seen in Fig. 2a. When digitonin treatment is performed as reported by Chow and Barber [18], very few grana, with a lower number of appressed vesicles, are observed in the heavy fraction, which mainly consists of single highly contrasted lines. The fact that they are lines and not vesicles and that they are highly contrasted has led us to conclude that they are formed by two membranes adhering together along the full length. A different action of digitonin was then hypothesized. Under these conditions, digitonin should fractionate not only by separating the intergrana lamellae from the grana, but also, partially, by separating the margins and the end membranes from the partitions, which, upon resealing, form inside-out vesicles, perhaps by a mechanism similar to that described by Andersson et al. [23]. These inside-out vesicles, under stacking conditions, should appear as highly contrasted lines. This hypothesis would imply that, if the membranes of this fraction were to separate from one another, only single vesicles should appear, which, upon 'restacking,' should reform the structures which appear as highly contrasted lines. In fact, in Fig. 2b (granal fraction suspended under unstacking conditions) mainly poorly contrasted vesicles are present and, upon readdition of 5 mM $MgCl_2$ (Fig. 2c), the same 'highly contrasted line' configuration reappears.

As was observed in the granal fraction obtained according to the method of Chow and Barber (Fig. 2), the granal fraction obtained using the procedure of Boardman and Anderson [19], when resuspended under unstacking conditions (Fig. 1b), is mainly formed by poorly contrasted vesicles. The heavy fraction obtained from initially unstacked chloroplasts when resuspended in the presence of 10 mM NaCl (Fig. 1d) is also formed mainly by poorly contrasted vesicles. Although some 'grana-like' structures are present, these vesicles are poorly contrasted and very few contact points can be seen, thus demonstrating that these structures are formed from unstacked membranes. When to the unstacked granal fraction (Fig. 1b) 5 mM $MgCl_2$ was added, the same stacked configuration depicted in Fig. 1a reappeared (Fig. 1c). Chow and Barber [18], examining the heavy (granal) fraction by means of a subsequent digitonin fractionation, reached the conclusion that even in the absence of divalent cations these membranes are unable to unstack. As this conclusion is not in agreement with the present results we attempted to repeat their experiment.

In Table II are reported chlorophyll yields of the heavy fractions obtained by a subsequent digitonin treatment of the granal fraction and, for comparison, by digitonin treatment of the previously unfractionated thylakoids. As reported by Chow and Barber

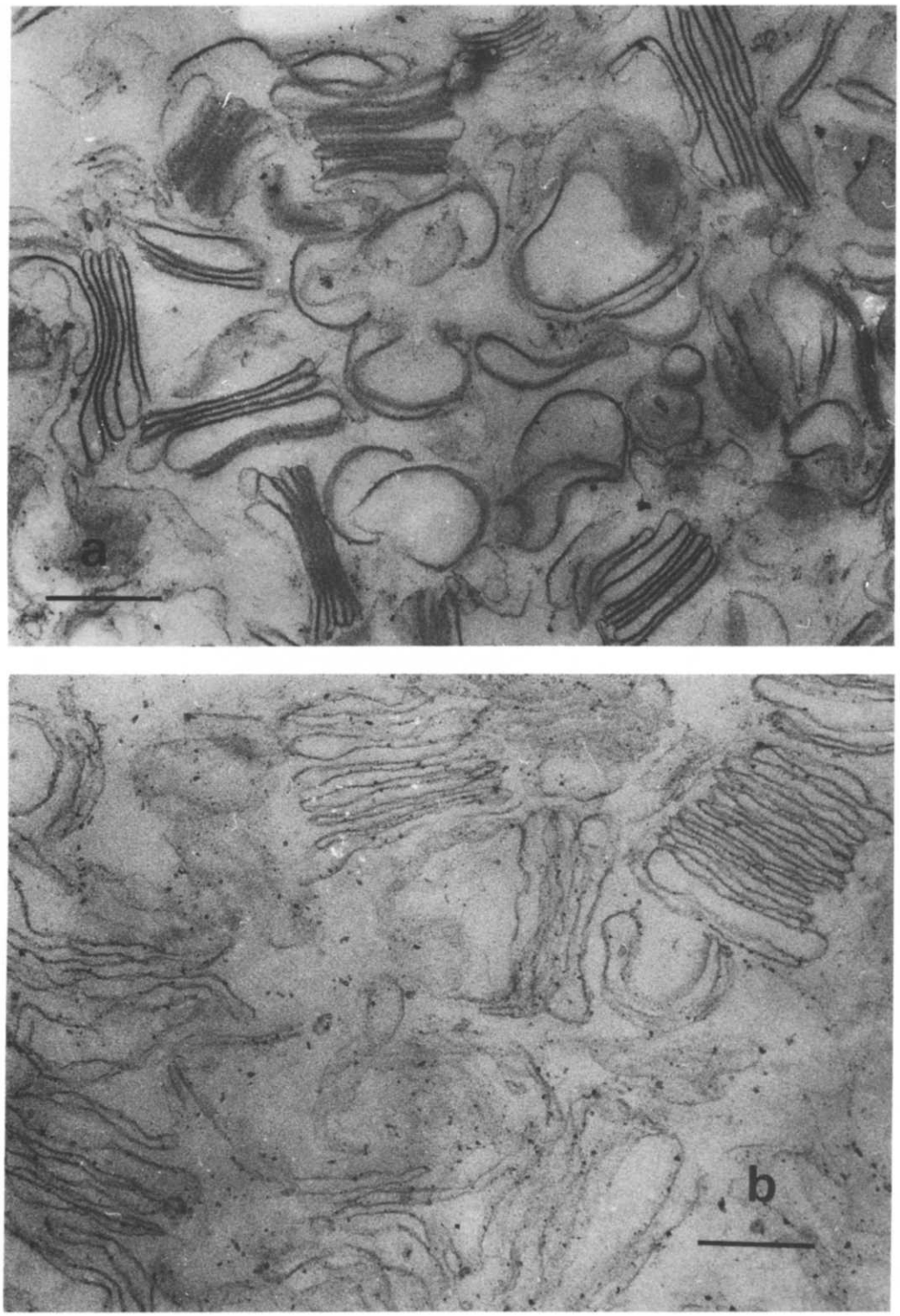


Fig. 1. Electron micrographs of the heavy fractions obtained by digitonin treatment of stacked or unstacked chloroplasts and suspended in media with different cation concentrations. Digitonin treatment was performed according to the method of Boardman and Anderson [19] (see Materials and Methods). (a–c) The heavy fraction obtained from stacked chloroplasts was washed

and resuspended in 10 mM NaCl, 30 mM Tricine (pH 8) in the presence (a) or absence (b) of 5 mM $MgCl_2$. To an aliquot of (b) 5 mM $MgCl_2$ was added (c). (d) The heavy fraction obtained from unstacked chloroplasts was washed and resuspended in 10 mM NaCl, 30 mM Tricine (pH 8). Bar = 0.5 μm.

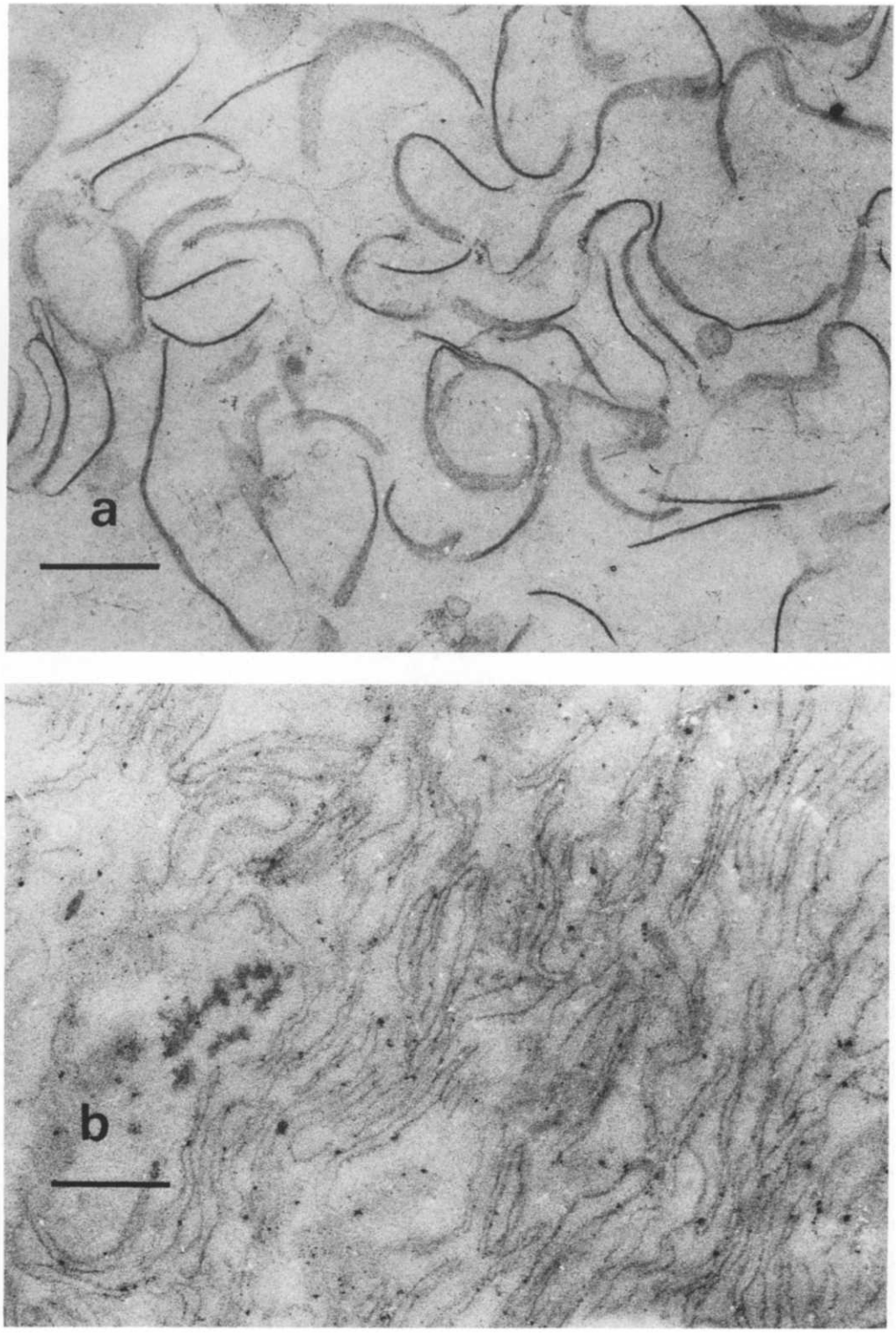
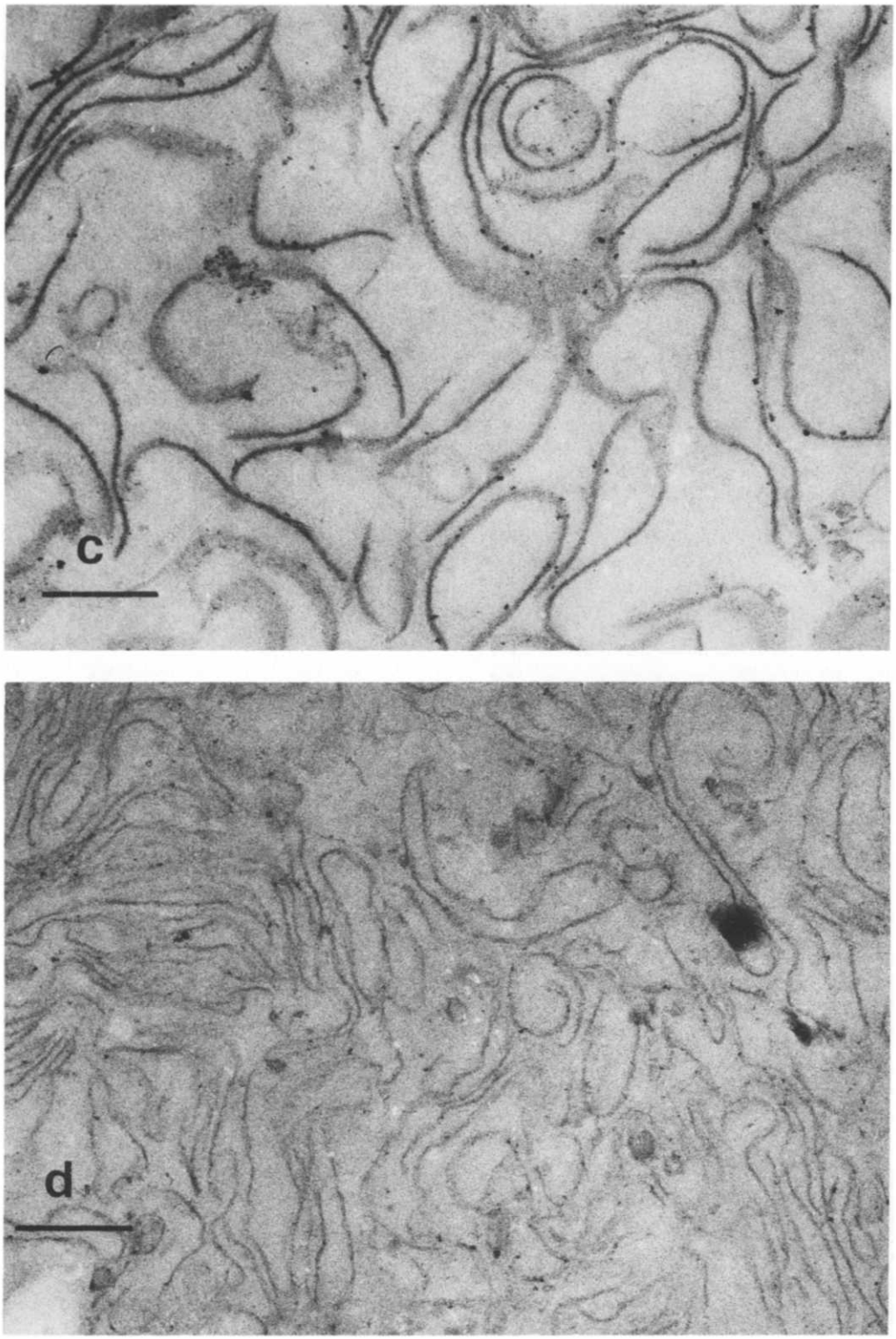


Fig. 2. Electron micrographs of the heavy fractions obtained by digitonin treatment of stacked or unstacked chloroplasts and resuspended in media with different cation composition. Digitonin treatment was performed according to the method of Chow and Barber [18] at a chlorophyll concentration of 300 μg/ml (see Materials and Methods). (a–c) The heavy fraction obtained



from stacked chloroplasts was resuspended in 0.1 M sorbitol, 1 mM KOH, 1 mM Hepes (pH 7.5, HCl) in the presence of 5 mM $MgCl_2$ (a) or 10 mM NaCl (b). To an aliquot of (b) 5 mM $MgCl_2$ was added (c). (d) The heavy fraction obtained from unstacked chloroplasts was suspended in 0.1 M sorbitol, 1 mM KOH, 10 mM NaCl, 1 mM Hepes (pH 7.5, HCl). Bar = 0.5 μm.

TABLE II

CHLOROPHYLL YIELD OF THE HEAVY FRACTION OBTAINED BY DIGITONIN FRACTIONATION OF THE GRANAL FRACTION AND OF WHOLE THYLAKOIDS

(A) Digitonin treatment was performed according to the method of Boardman and Anderson [19]; (B) digitonin fractionation was performed as reported by Chow and Barber [18] at a chlorophyll concentration of 100 μg/ml (see Materials and Methods). The heavy fractions, before digitonin treatment, were washed and resuspended as reported in Figs. 1 and 2.

	Whole thylakoids		Granal fraction	
A Ionic composition of the suspension medium	5 mM $MgCl_2$ 10 mM NaCl		5 mM $MgCl_2$ 10 mM NaCl	0 mM $MgCl_2$ 10 mM NaCl
Chlorophyll yield (%)	85.7		95.2	91.9
B Ionic composition of the suspension medium	5 mM $MgCl_2$ 0 mM NaCl	0 mM $MgCl_2$ 10 mM NaCl	5 mM $MgCl_2$ 0 mM NaCl	0 mM $MgCl_2$ 10 mM NaCl
Chlorophyll yield (%)	18.1	4.7	35.4	35.2

[18], when the granal fraction was subjected to digitonin treatment, the same chlorophyll yields were observed in the heavy fraction, independently of the ionic composition of the suspension medium of the granal fraction and of the procedure of digitonin fractionation. The chlorophyll yield of the heavy fractions is always higher than that obtained by digitonin fractionation of stacked chloroplasts.

Discussion

The capacity of the granal fraction of thylakoids obtained by two different digitonin fractionation procedures to undergo the normal stacking-unstacking changes upon varying the ionic composition of the suspension medium was studied both by electron microscopy and by digitonin analysis. As reported by Chow and Barber [18], no effect of the ionic composition of the suspension medium on the granal fraction was observed by means of digitonin fractionation studies. However, the electron microscope analysis revealed that the membranes of the granal fraction, which are stacked in the presence of divalent cations, unstack when resuspended in unstacking conditions. That these membranes are able to unstack in the absence of divalent cations is clearly demonstrated and does not depend on the hypothesis mentioned in Results that the heavy fraction, prepared according to the method of Chow and Barber [18] in the presence of divalent cations, consists mostly of inside-out vesicles.

Chow and Barber [18] have also reported that the membranes of the granal fraction unstack when suspended in the presence of linolenic acid. In this article we have demonstrated that the unstacking of the granal fraction can be reversed by addition of 5 mM $MgCl_2$. Thus, the involvement of fatty acids or of other aging phenomena in the unstacking process can be excluded.

In 1976, Staehelin [24] demonstrated that the thylakoid unstacking process can be kinetically separated into two steps. At first the grana membranes unstack but 'the overall organization of the grana and stroma lamellae is retained together with the normal distribution of particles between the different areas'. Only later, a reorganization of the lamellae into large, roughly parallel folds and a randomization of the particles along the membranes occur. The observation presented here, that isolated grana unstack on removal of divalent cations, is consistent with this idea inasmuch as particle exchange with stroma lamellae is not possible in this case. The partition zones carry sufficient negative charges which, when not screened by cations, cause membrane unstacking and the movement of negatively charged molecules from stromal to granal membranes is not required. The presence of negative charges on the partition membranes was demonstrated by Itoh [25]. He estimated the isoelectric point in the close vicinity of the primary electron acceptor of PS II (which is mainly located in the partitions [26–28]) to be 5.2. Interestingly, this value is close to the pH value (pH 5.4) required for stacking in the absence of cations [15, 16].

Digitonin fractionation of broken chloroplasts has been used as a quick and easy method to evaluate grana formation [10,12,29,30] and a close correlation was observed between this technique and salt-induced chlorophyll fluorescence changes [8,30,31]. However, we have pointed out previously [16] that digitonin fractionation is not always according to granal and nongranal membrane regions. The present data support and extend these observations. As the mechanism of action of digitonin is unknown it is difficult to explain why the digitonin method seems to work under some conditions and not in others. We would only emphasize that, in our opinion, it is rather dangerous to utilize digitonin fractionation studies as indicative of grana formation without checking with the electron microscope.

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