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Short Communication

A Site With an Affinity for Heavy Metals on the Thylakoid Membranes of Chloroplasts[1]

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In experiments designed to localize adenosine triphosphatase activity on cellular membranes of the pea tendril we have localized a non-enzymic, discrete deposition of lead, osmium and tungsten at specific sites on the thylakoid membranes of chloroplasts. These reactive sites may be exposed to the stain or partially blocked depending on the nature of the aldehyde employed for preliminary fixation of the tissue.

We have noted several published electron micrographs of chloroplast fine structure in which similar dense staining at these sites is evident. With few exceptions, the authors have ignored these deposits and their possible significance, undoubtedly owing to their very sporadic appearance in glutaraldehyde-fixed tissue. In pea tendrils fixed with hydroxyadipaldehyde, such deposits appear consistently in the chloroplasts and the degree of staining with heavy metals at these sites is appreciably magnified. The present report describes some characteristics of this reaction.

Materials and Methods

Segments of tissue from the unbranched tendril arising at the fifth internode of light grown pea plants (*Pisum sativum* L. var. Alaska) were used for all experiments. The material was fixed in a solution of either 3 % (v/v) glutaraldehyde or 3 % (v/v) hydroxyadipaldehyde in 0.075 M cacodylate buffer (pH 7.2) containing 8 % (w/v) sucrose and depending upon the experiment, with or without 0.001 M $MgSO_4$ and 0.01 % $CaCl_2$. Fixation took place at 2 to 4° for 30 min with glutaraldehyde or 45 min with hydroxyadipaldehyde. Both aldehydes were purified before use by filtration through layers of calcium carbonate and Norit A activated charcoal to remove acid and polymer impurities. Following fixation, the tissue was washed overnight (*ca.* 18 hr) in 2 changes of cacodylate buffer and then incubated for 2 hr at pH 7.2 in the Wachstein-Meisel (WM) medium (8) containing 1 mM lead nitrate. After incubation, the tissue was rinsed briefly in the cacodylate buffer and post-fixed in 1 % (w/v) osmic acid for 60 to 90 min at 2 to 4°. Dehydration was effected with acetone and the material was embedded in Epon 812. Thin sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome and stained briefly (4 min) with Reynold's lead citrate. The grids were examined in a JEM 6C electron microscope operating at an accelerating voltage of 50 kv. Any deviations from this basic procedure are mentioned where appropriate in the text.

Results

Following brief fixation with hydroxyadipaldehyde, the degree of structural integrity retained in cellular components was somewhat variable. In general, the chloroplast was adequately preserved, although in some instances the envelope was either ruptured or lost.

When the tissue was incubated in the WM medium in the presence of 1 mM lead nitrate, small but very distinct, electron-dense deposits were present at the lateral edges of the thylakoid membranes (Fig. 1). Glancing sections through the grana or

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surface views of the thylakoids indicated that the spots were discretely localized at intervals along the margins of the discs (Fig. 2). The deposits varied a little in size from sample to sample, but within a single chloroplast they were remarkably uniform in their dimensions. Owing to their size and location they were clearly distinguishable from the relatively much larger osmiophilic globules in the stromal matrix.

As long as lead was present in the incubation medium, the deposits were obtained irrespective of the presence or absence of ATP, Mg^{2+}, or Ca^{2+}, in various combinations or alone. Increasing the duration of fixation to 1.5 hr had no inhibitory effect. If the pea plants were grown in the dark for 24 hr to eliminate the effects of photophosphorylation and any possible residual traces of endogenous ATP, the spots were still distinctly visible. No change in this reaction was evident if *p*-nitrophenyl phosphate was substituted for ATP in the incubation medium, or if the tissue was pretreated for 30 min with 5×10^{-7} M dichlorophenyl-dimethylurea and incubated in the presence of the inhibitor.

Deposits were also obtained under conditions where lead was added to the fixative and the incubation step was omitted. However, if lead was absent from the fixation or incubation media, the deposits, although still occasionally evident, were much smaller and fainter. For reasons discussed below, we believe that this weaker reaction represents the attachment of osmium to the reactive sites on the thylakoid membranes.

If the tissue was fixed in hydroxyadipaldehyde, dehydrated in ethanol, and then incubated for 2 hr in an alcoholic solution of phosphotungstic acid (2% w/v), some of the chloroplasts demonstrated the negative staining expected. However, others appeared to be positively stained and showed clear indications of dense deposits at the same sites, in this case, presumably of tungsten salts (Fig. 3).

When glutaraldehyde was the fixative employed, the grana were generally devoid of any deposits (Fig. 4). Only in very exceptional cases did we find accumulations of lead on the thylakoid margins in such material. When present, they occurred in the presence of ATP, uridine triphosphate, or inosine triphosphate, and were associated with heavy depositions of lead within the cells or in situations where the chloroplast envelope was ruptured (Figs. 5, 6). In view of the rarity of such examples and the results obtained with hydroxyadipaldehyde fixation under control conditions it appears extremely unlikely that the deposits reflect the activity of any enzyme.

In the micrographs presented here, the thin sections were stained briefly (4 min) with Reynold's lead citrate. However, when staining was omitted the deposits were still visible in hydroxyadipaldehyde-fixed chloroplasts, indicating that lead in the staining solution did not contribute to the reaction. Furthermore, when sections of glutaraldehyde-fixed tissue were stained for as long as 20 min each in uranyl acetate and/or Reynold's lead citrate, the thylakoid membranes were free of any such localized sites of stain deposition.

Discussion

Very few publications mention the accumulation of stains at the lateral edges of the thylakoid membranes. Significantly, several micrographs that demonstrate these deposits were published prior to the advent of glutaraldehyde as a tissue fixative. Sun (6) described 20 to 30 osmiophilic globules around the boundary of the grana discs in chloroplasts of *Cycas* cells fixed only with Dalton's chromic acid-osmic acid fixative. More recently, Thompson (7), Blumenthal-Goldschmidt and Poljakoff-Mayber (2), and Spurr and Harris (5) described the presence of lipid globules at the corners of the grana compartments or along the margins of the grana. However, in each case, the tissue was either fixed in osmic acid alone, or following a brief fixation with glutaraldehyde, was treated with osmic acid for extended periods (variously described as overnight or 18–24 hr). In a report by Ljubesic (3), the electron micrographs include 1 chloroplast fixed in osmic acid alone which displays the reaction, and others treated with osmic acid following preliminary fixation with glutaraldehyde or formaldehyde that are completely free of any similar deposits on the grana. Ohad, Siekevitz, and Palade (4) have obtained prominent deposits of a similar nature in the chloroplast of *Chlamydomonas reinhardi* fixed overnight in osmic acid, either alone or after glutaraldehyde. Their interpretation was that the dense spots indicated the irregular thickening of the disc rims. We have never encountered these deposits in chloroplasts when the tissue was fixed with glutaraldehyde and post-fixed in osmic acid for 3 hr or less. From the reports mentioned above and our more detailed observations, it is apparent that while glutaraldehyde generally inhibits the accumulation of electron-dense deposits at these sites on the grana, extended osmication may result in some reaction.

It would seem very unlikely that the deposits represent the staining of lipid globules. No evidence in the literature suggests that glutaraldehyde may act specifically to screen particular lipids from the action of osmium or lead. Furthermore, the deposits that we have obtained do not always present the normal globular appearance of osmiophilic globules in the chloroplast stroma or cytoplasm; they are also extremely uniform in size and distribution within a chloroplast. It is equally improbable that any enzyme activity is involved in their appearance. A more likely possibility is the chelation of metal ions to negatively charged sites on the grana membranes. The evidence that ferredoxin in chloroplasts is the most reducing substance so far isolated from living cells (1) suggests that the very negative redox

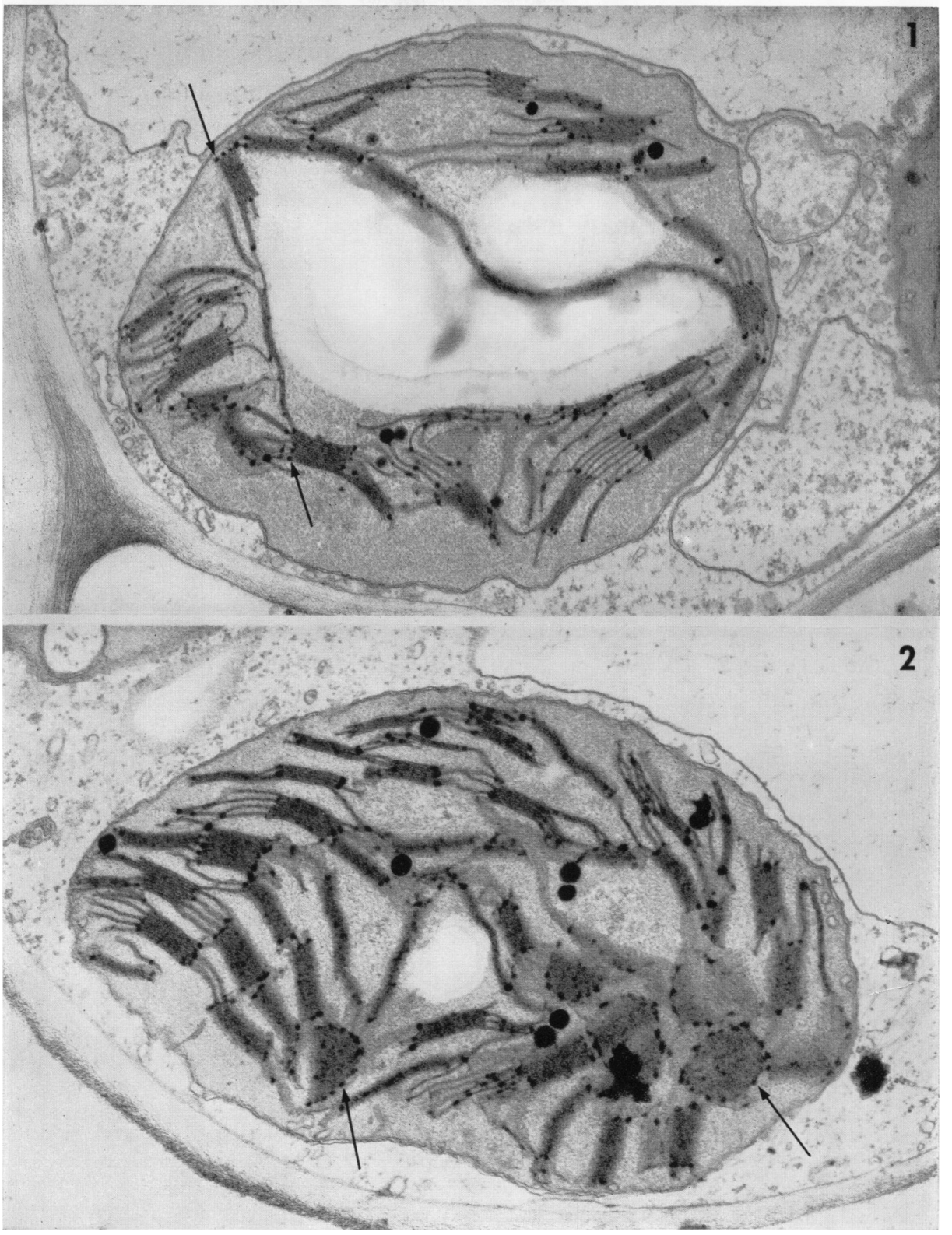

FIG. 1. Chloroplast from hydroxyadipaldehyde-fixed cells of the pea tendril. The material was incubated in the WM medium containing 1 mM lead nitrate. Arrows indicate dense deposits at the lateral edges of the thylakoid membranes. × 26,300

FIG. 2. Material treated as in Fig. 1. The dense deposits are present around the margins of grana discs seen in surface view or sectioned obliquely (arrows). × 30,700

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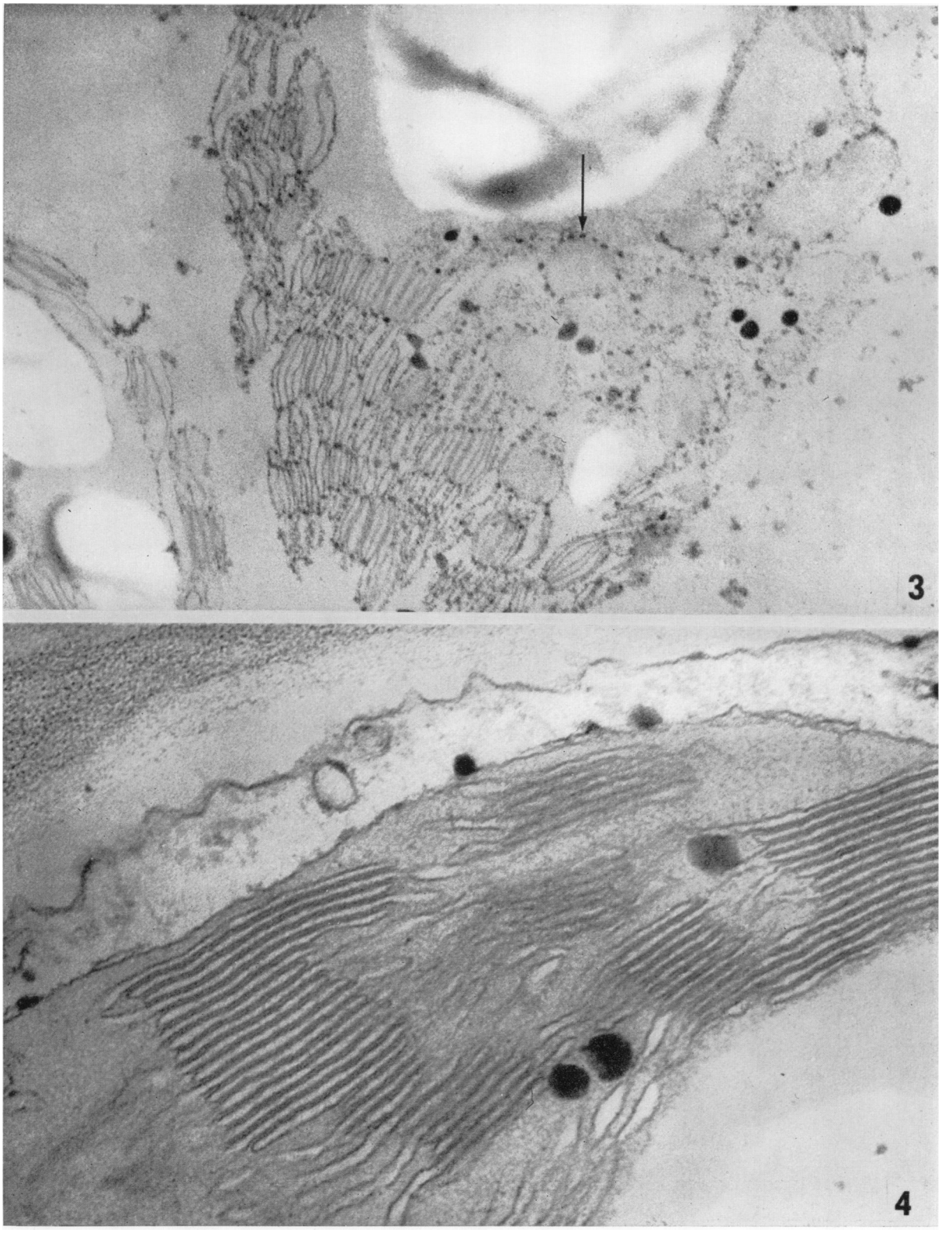
FIG. 3. Chloroplast from hydroxyadipaldehyde-fixed cells, dehydrated in ethanol, and incubated in alcoholic phosphotungstic acid. The membranes appear positively stained and dense deposits are visible at the edges of the grana (arrow). × 32,100

FIG. 4. Chloroplast from glutaraldehyde-fixed cells incubated in the WM medium containing 1 mM lead nitrate. The grana membranes are free of any deposits. The chloroplast envelope is intact. ×96,750



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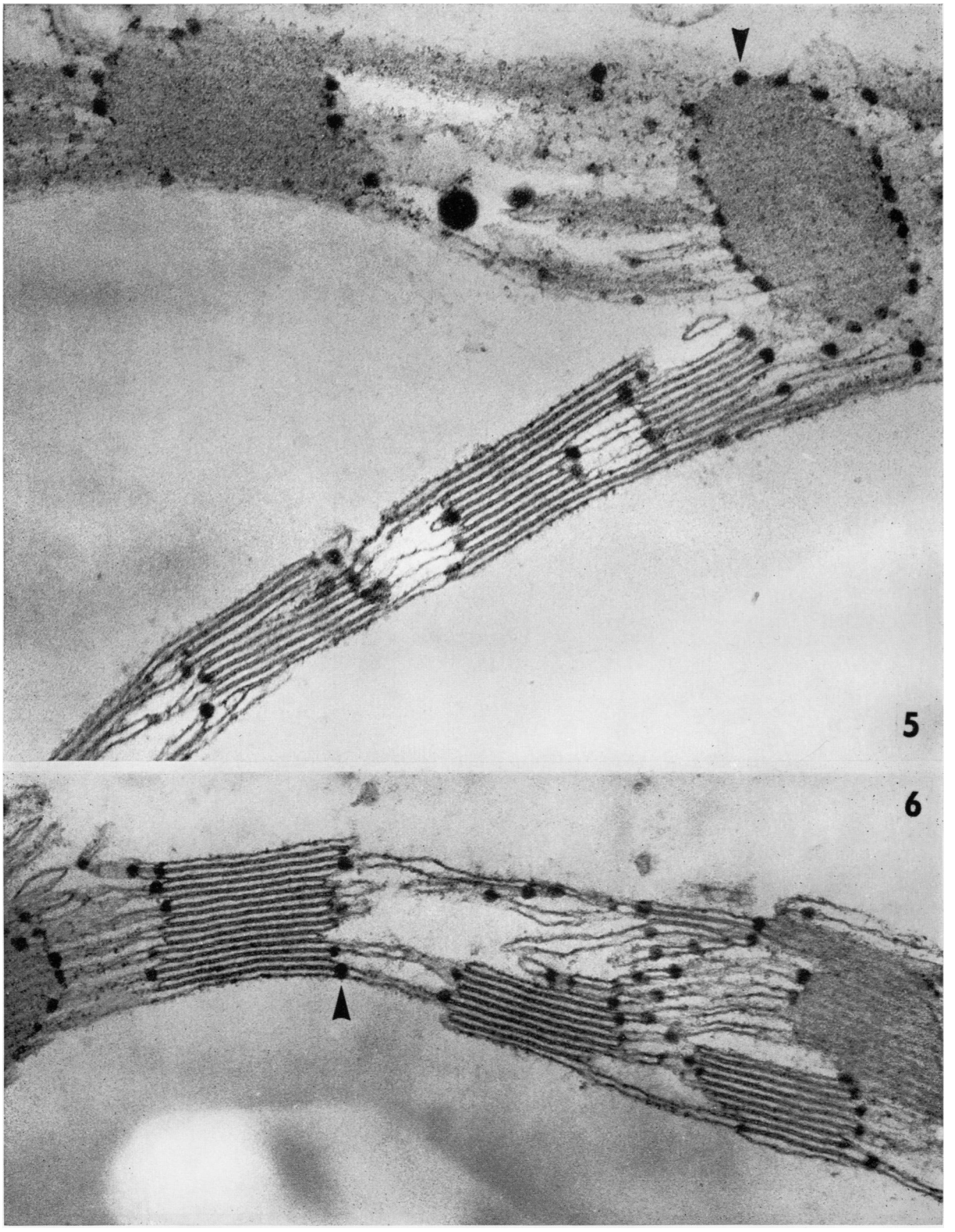

FIG. 5 and 6. Material prepared as in Fig. 4. The chloroplast envelope (not seen) was ruptured. Dense deposits at the lateral margins of the thylakoid membranes are seen both in surface views (pointer in Fig. 5) and sections through the grana (pointer in Fig. 6). Fig. 5. × 87,750, Fig. 6. × 78,750.

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potential of at least some electron carriers in the chloroplast may suffice for the precipitation of lead or other positively charged ions. It is intriguing that whereas hydroxyadipaldehyde exposes the reactive sites on the grana to the heavy metals that we have tested, glutaraldehyde effectively blocks the reaction. Too little is known regarding the action of either aldehyde to permit an explanation. Nevertheless, the indications of localized sites on the grana membranes with a differential affinity for metal ions may prove of some significance in the study of chloroplast activity.

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