SELECTIVE PRESENTATION OF DNA-REGIONS AND MEMBRANES IN CHLOROPLASTS AND MITOCHONDRIA

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The existence of DNA-containing regions in chloroplasts and mitochondria of plants of varying systematic relationship and developmental stage has been sufficiently proved by electron microscopy (1, 2). So far, however, no study of the exact spatial distribution or of the variation in size and number of these regions per organelle has appeared, even for the more simply constructed chloroplast of higher plants.

Radioautographic studies of plant material (*Beta vulgaris* L. provar. altissima (Döll) Helm) with large and/or small plastids showed (*a*) that chloroplasts of different size (2–25 μ) incorporate varying quantities of thymidine-^{3}H in a form that can be largely removed by DNase, and, when fixation was good, (*b*) that labeling was not diffuse but appeared mostly in centers above "fully differentiated" chloroplasts, the number of centers being correlated with the size of the chloroplast. Genetically this result indicated polyvalency and morphologically a polyenergid organizational stage of the chloroplast (3–5).

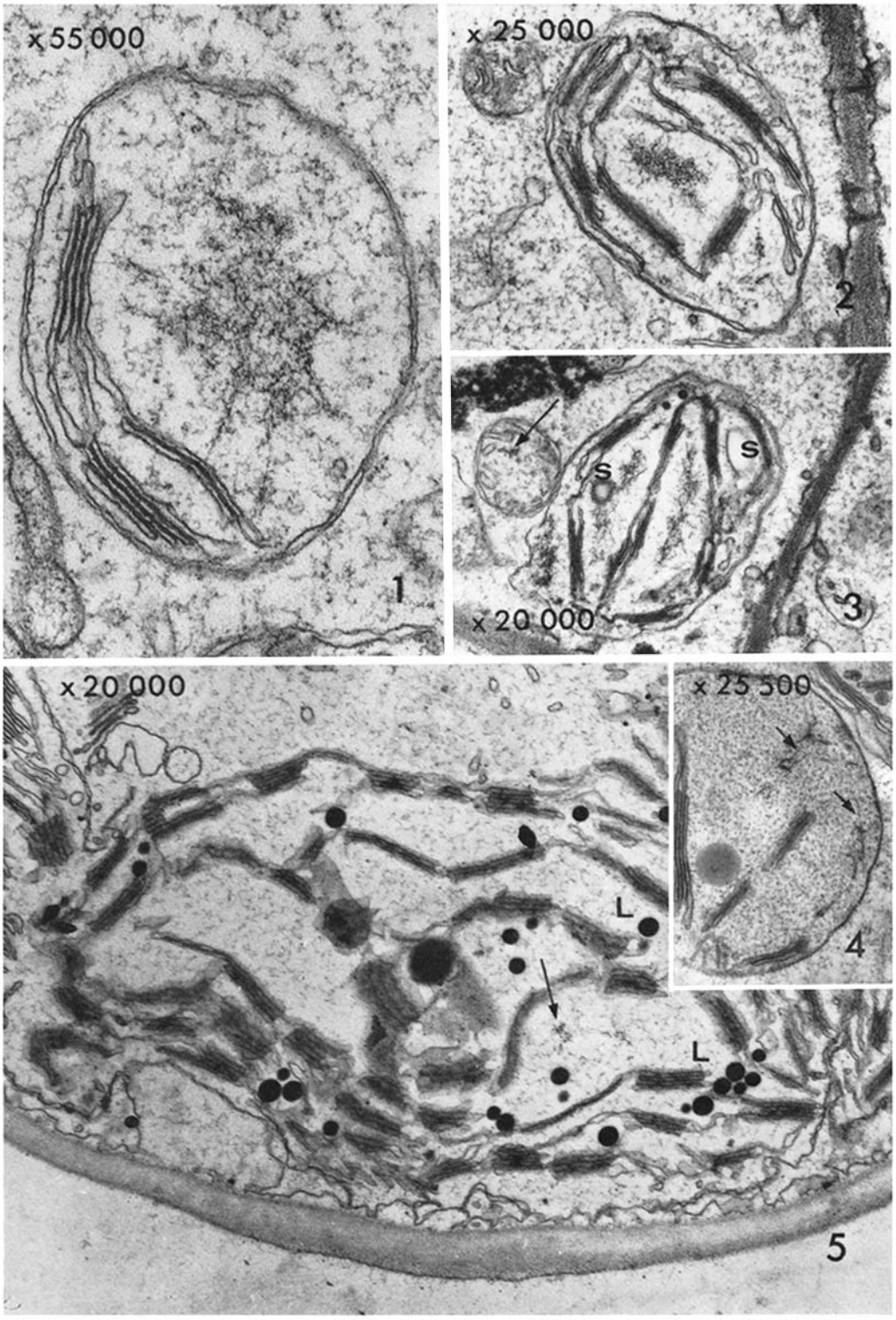
Light-microscopic radioautography alone cannot demonstrate polyenergid organization, since an analysis of the third dimension is not possible. Therefore, it was necessary to verify this result by electron microscopy of serial sections (5). In contrast to mitochondria and very young plastids (i.e., etioplasts), in the case of more or fully developed chloroplasts it is often difficult to demonstrate clearly their DNA-areas in a single section (obviously due to their thin, elongated, or branched form [1, 6]), and it is completely impossible to follow the distribution of DNA within the matrix by means of serial sections, especially at the terminations of the areas (5). For this reason, it was necessary to develop a method that would allow unambiguous identification of DNA-containing structures even in old chloroplasts. By means of this method, the number, distribution, and variation in size of the DNA-centers in chloroplasts of all developmental stages or in chloroplasts of different sizes can be analyzed—at least in our material—by serial sections (5).

DNA-containing structures in organelles are, as a rule, made visible in fixed samples by double staining with uranyl acetate and lead citrate. Thereafter they appear more or less distinctly as low-contrast areas within a high-contrast matrix (Figs. 6, 8). RNase treatment following formaldehyde fixation as used by Kislev et al. (7) gives considerable improvement by decreasing the contrast of the matrix.

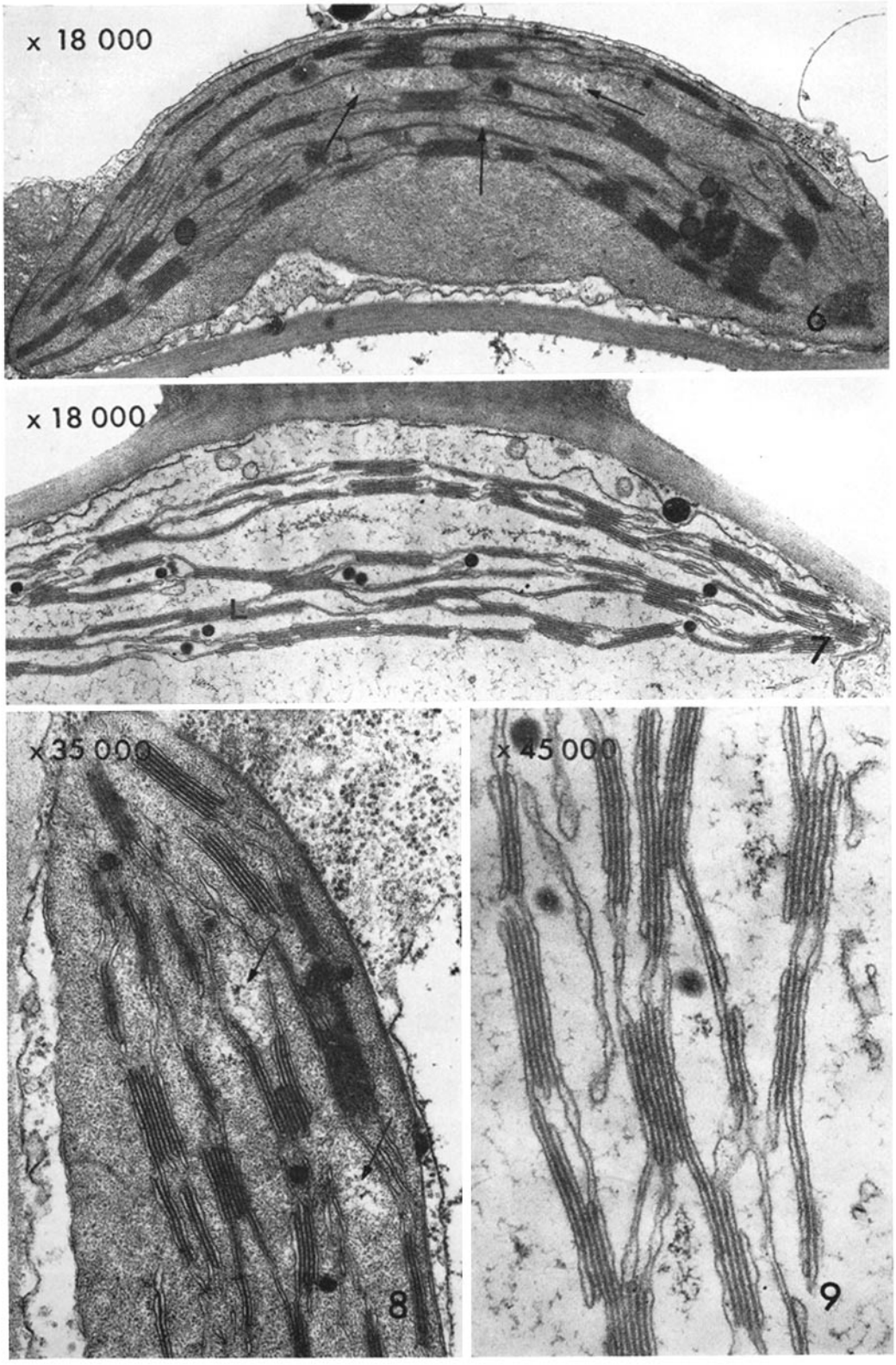
In our own experiments, application of the protease trypsin or Pronase resulted in almost complete digestion of the matrix, leaving chiefly starch, plastid membranes, and DNA structures. The latter could be demonstrated selectively even in old chloroplasts; they are digestible with DNase.

MATERIALS AND METHODS

The plants were cultivated as already described (4) and kept in darkness for 24 hr before fixation. Small strips of about 2 mm in width were cut from both young (1 cm in length) and fully developed leaves (length of the lamina, ~8 cm) and fixed in 4% glutaraldehyde made up in either 0.05 M cacodylate or phosphate buffer, pH 7.0. The fixation time ranged from 2 hr to overnight, but was usually 5 hr when enzyme treatment was to follow. After fixation, the samples were cut into small sections of about 1 mm, transferred to pure buffer for several rinsings, and incubated in trypsin (1 mg/ml) in Tris 0.05 M, pH 8.0, plus 0.03 M $CaCl_2$ or 0.05 M phosphate buffer, pH 7.0, at 37°C for 20 hr, the enzyme solution being changed after 8 hr. Thereafter, the samples were washed several times in the above mentioned cacodylate buffer or acetate buffer plus 3 mM Mg^{++}, pH 5.05. In the latter case, DNase digestion (DNase, Worthington Biochemical Corp., Freehold, N. J., code DPFF) followed (4). Postfixation in 1% OsO_4 in cacodylate buffer, pH 7.0 was carried out at 4°C overnight. Dehydration in alcohol and embedding in Epon 812 (mixture 7:3)(8) followed, according to standard methods. The sections were cut with a diamond knife on a Reichert Ultramicrotome OM-U-2 (Reichert, Vienna, Austria), mounted on carbon-coated copper grids, double stained with saturated aqueous uranyl acetate for 20 min followed by lead citrate for 10 min (9), and examined in a Siemens Elmiskop I A.



FIGURES 1–5 Chloroplasts after trypsin treatment. Fig. 1 shows a chloroplast with a loosely distributed, central DNA, typical of very young plastids. × 55,000. Fig. 2 shows a slightly older plastid showing a central DNA-area. × 25,000. Fig. 3 shows a chloroplast of similar developmental stage; several separate DNA-areas are situated between thylakoid stacks. Note the DNA-area within the adjacent mitochondrion (arrow). *s*, starch. × 20,000. In Fig. 4 is seen a young chloroplast with fibrillar DNA-structures (arrows). × 25,500. Fig. 5 shows a fully developed chloroplast with thylakoids partly in tangential section and also a small DNA-area (arrow). *L*, lipid globule. × 20,000.



FIGURES 6–9 Fully developed chloroplasts. Fig. 6 shows an untreated chloroplast showing three small DNA-areas (arrows) as light areas within a dark-contrasted matrix. × 18,000. Fig. 7 shows a chloroplast after trypsin treatment. Several elongated DNA-areas are visible, by their dark contrast, within the light matrix. *L*, lipid globule. × 18,000. In Fig. 8 the detail of a chloroplast showing two well-defined DNA-areas with fibrillar structure (arrows) is seen. × 35,000. Fig. 9 shows a chloroplast after trypsin treatment, more highly magnified to show three clearly visible DNA-areas. × 45,000.

RESULTS

After trypsin treatment, most of the membranes, especially of chloroplasts, mitochondria, Golgi bodies, and endoplasmic reticulum (ER), are still distinct. While in young cells (small vacuoles) the tonoplast and the plastid envelope remain intact (Figs. 1–4); in old cells they are ruptured or even absent (Figs. 5, 7, 9). The plasmalemma and the nuclear envelope are always fragmented. As is to be expected, the lipid globules and starch grains in chloroplasts and, important in the present context, the DNA content of the nucleus, chloroplasts, and mitochondria (Fig. 3) survive this treatment. The micrographs, which in their "washed out" appearance resemble those of permanganate-fixed samples, demonstrate that the contrast of the DNA-regions is excellent. Moreover, they indicate that an analysis of the three-dimensional arrangement of the membrane systems is possible even when they are cut tangentially (Fig. 5).

Since DNA-areas in chloroplasts are frequently located near the margin of starch grains, they can easily be overlooked in untreated sections. We were not able to detect DNA-containing areas in all the fully developed chloroplasts of the control-embeddings (i.e., without enzyme treatment). However, in all trypsin-treated chloroplasts of *Beta* examined so far, DNA-containing areas could be observed (1, 10).

DISCUSSION

DNA-containing regions in the chloroplast and mitochondrion are known to be similar in appearance to the bacterial or blue-green algal nucleoid (if one considers merely the existence of a fibrillar material and not degree of its order), but different in appearance from the nuclear chromatin. Depending upon fixation, in chloroplasts and mitochondria they appear either as fibrous, corelike aggregates or as cloudy aggregates (1, 2, 7). After trypsin digestion they appear, in chloroplasts, mitochondria, and in the nucleus, to be fluffy, sometimes nodular, but never lumpy. This result could be interpreted as an indication of the presence of some kind of protein component in the DNA-regions of the organelles. This interpretation has already been suggested (11) and is probably supported by the dependency of the DNase reactivity on the kind of nonmetallic fixative used. In treated chloroplasts the DNA-areas generally appear somewhat larger than in untreated ones. On the one hand, it is possible that DNA-areas appear in their full extent only after digestion of the matrix; on the other hand, the removal of spatial limitation may lead to an artificial enlargement of the DNA-area. In comparison with control-chloroplasts, slightly enlarged DNA-centers are observed mainly in young chloroplasts. However, in trypsin-treated chloroplasts there are DNA-areas with completely fibrillar substance, which are identical in size and structure to those of comparable control sections (Fig. 4). Experience shows that also in untreated control sections DNA may occur in fibrillar form, as well as artificially lumped. In the available series of sections of whole chloroplasts, a spatial distortion of the DNA-centers can be definitely excluded due to the compartmentation of the chloroplast by the thylakoids. Whether the particles remaining in the chloroplast matrix after trypsin-treatment are proteinaceous or are ribosomal RNA, or whether the RNA-component is largely dissolved out of the organelle by this treatment, is still unknown and, in any case, is irrelevant to our considerations.

Young chloroplasts always contain, as the literature also shows, few or usually only one well defined center per section (Figs. 1, 2–4) (cf. 6, and also the general observation that Feulgen staining is limited to chloroplasts of young cells). In more developed chloroplasts, on the other hand, a larger number of centers per section is usually obtained, and the centers are generally considerably smaller (Figs. 6, 8, 9). Although there are indications (or assumptions) of a larger number of independent DNA-regions per chloroplast in some cases (cf. especially the studies of 12), the presence of several centers and the number of centers per chloroplast (3–5) can be established only by observations on a complete series of sections of suitable treated material, since what appear as separate centers in any given section actually may be interconnected in other sections of the chloroplast (13). The results that were obtained by applying the protease method, serial sectioning, and semiquantitative evaluation to *Beta vulgaris* chloroplasts will be published shortly (5).

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