THE FINE STRUCTURE OF CHLOROPLAST STROMA FOLLOWING ALDEHYDE OSMIUM-TETROXIDE FIXATION

B. E. S. GUNNING, Ph.D.

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts. Dr. Gunning's present address is Department of Botany, The Queen's University of Belfast, Belfast, Northern Ireland

ABSTRACT

Markedly improved fixation of leaf tissues is obtained by means of a glutaraldehyde (or acrolein)-osmium tetroxide procedure, as compared with the results of potassium permanganate or osmium tetroxide fixation methods. The procedure has proved useful in all species so far examined. Chloroplasts are particularly well preserved. In this paper details of components of the ground-substance of *Avena sativa* plastids are presented. They include the following:—(i) The "stromacentre" is an area of aggregated fibrils, each 85 A in diameter, and of uncertain length. Individual fibrils may be composed of subunits. The whole aggregate is usually up to 1 μ in diameter, and is visible in thin sections in the light microcope. It is present at all stages of plastid development, and, under conditions of rapid synthesis in the plastid, it may be up to 2 μ in diameter. Evidence that it is proteinaceous is presented. Osmiophilic globules are often associated with it. (ii) Areas which resemble bacterial and blue-green algal nucleoplasms, containing fibrils approximately 30 A wide. These regions are smaller than the stromacentre and, like that structure, they occur in all stages of plastid development. Unlike it, there are several such areas per chloroplast. (iii) Particles which have some of the morphological and staining characteristics of ribosomes. Present at all stages of development, they are approximately two-thirds the size of the cytoplasmic ribosomes. They can occur in groups, thus resembling polyribosomes. (iv) The remaining material is granular, and may include dissociated portions of stromacentre material. The validity of the observations and their significance is discussed.

INTRODUCTION

As yet, comparatively little is known of the detailed relationships of structure and function in chloroplasts, and one of the major gaps in our knowledge concerns the characterisation and recognition by electron microscopy of components of the ground-substance, or stroma. It is unfortunate that conventional procedures of fixation with osmium tetroxide and potassium permanganate fixation in general should have failed to preserve adequately this non-membranous part of the chloroplast, for it is thought to house many enzyme systems—notably those concerned with carbohydrate metabolism and protein synthesis (Smillie, 1963).

By contrast, the value of other fixation techniques is shown in the recent studies of Ris and Plaut (1962) on *Chlamydomonas* chloroplasts, and of Jacobsen, Swift, and Bogorad (1963) on *Zea*

plastids. The former authors demonstrated the presence of fibrils thought to contain DNA, using Kellenberger's fixation procedure, and Jacobsen *et al.* employed formaldehyde to fix "RNA in a particulate form which resembles ribosomes." Both of these nucleic acid-containing components occur in the ground-substance of the plastids.

The present work shows that the glutaraldehyde method of fixation introduced by Sabatini *et al.* (1963) is capable of preserving the ground-substance of chloroplasts. Several species have been examined, but, in view of the presence of features of special interest, only plastids from *Avena* will be considered. Some of the results have been abstracted elsewhere (Gunning, 1963).

MATERIALS AND METHODS

For comparative purposes, both phosphate-buffered osmium tetroxide and potassium permanganate solutions were used as fixatives (see captions to Figs. 1 and 2). However, most of the illustrations are of material fixed in 1.5 to 6.0 per cent (usually 2.5 per cent) glutaraldehyde in 0.1 M potassium phosphate buffer at pH 7.0. The tissue consisted of 1 to 2 mm^2 portions of the first leaves of *Avena sativa* (var. Victory) seedlings grown in vermiculite for 8 to 10 days under Sylvania "Grolux" fluorescent lamps. After 3 to 20 hours in glutaraldehyde at 0°, the tissue was thoroughly rinsed in cold buffer to remove excess aldehyde. Fig. 5 shows the granular deposit that occurred if the tissue was *not* rinsed prior to the final step, which was postfixation for 3 to 16 hours in 2 per cent osmium tetroxide in the same buffer solution, also at 0°.

In certain experiments the tissue was dehydrated and embedded immediately after fixation in glutaraldehyde (*e.g.*, Fig. 3). In some other experiments, the initial fixative was 10 per cent acrolein in xylol, and this was followed by rinses and postfixation in oxmium tetroxide as above (*e.g.*, Fig. 4).

The tissue was dehydrated in a graded series of acetone solutions, transferred to propylene oxide, and embedded in a 1/1 mixture of Epon 812 and NMA (Nadic methyl anhydride), with 0.5 per cent DMP-30 (Tridimethylaminomethyl Phenol) (M. C. Ledbetter, personal communication). Sections were cut on either the Porter-Blum or the Huxley microtome, using a diamond knife, and stained with lead (Millonig, 1961), or uranyl acetate followed by lead citrate (Reynolds, 1963).

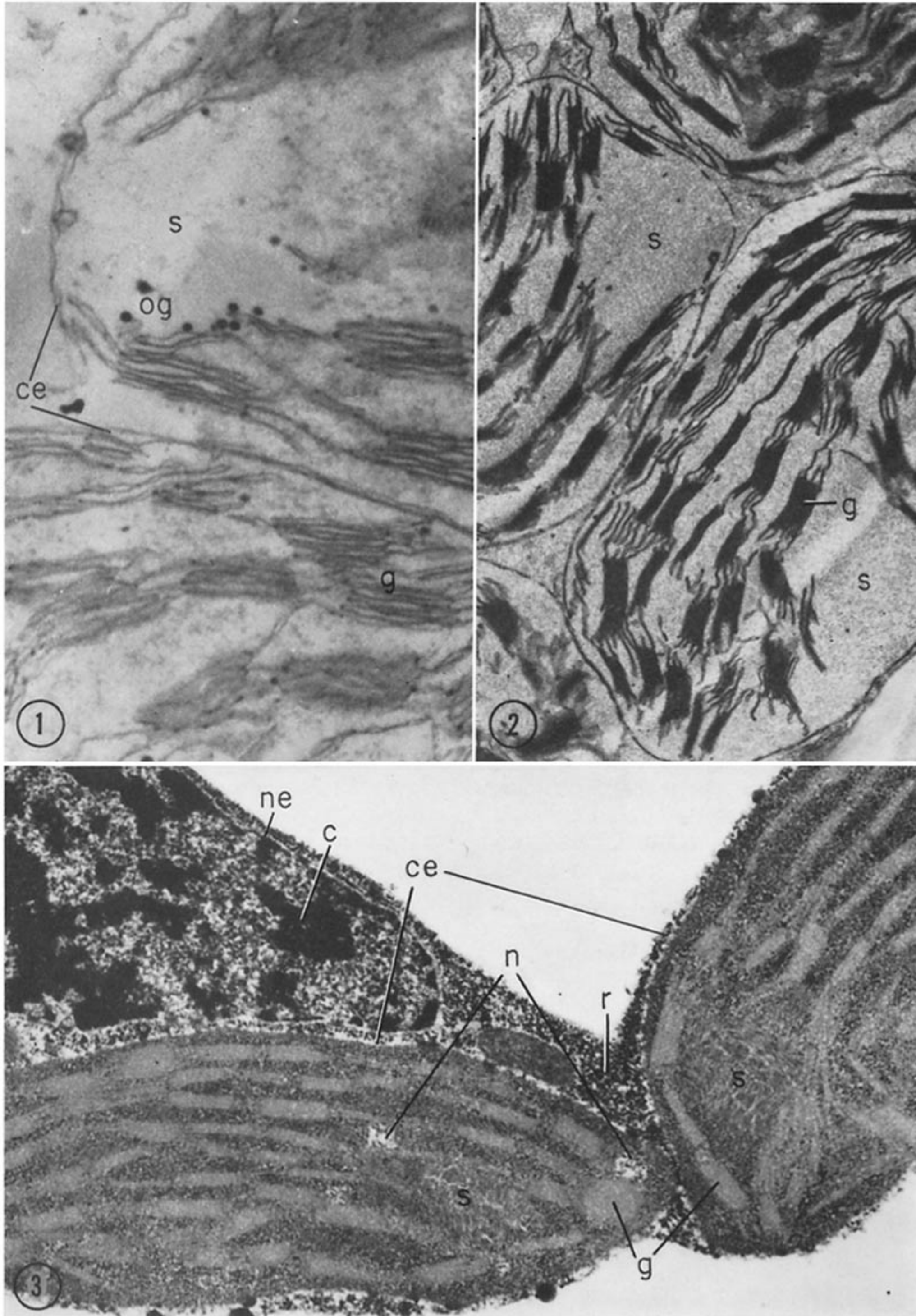
Key to Labelling

ce, plastid envelope
g, granum
s, stromacentre
cr, aggregates of plastid "ribosomes"
is, intercellular space
pm, plasma membrane
v, vacuole
ne, nuclear envelope
r, cytoplasmic ribosomes
th, thylakoid
og, osmiophilic globule
n, nucleoplasm-like region
p, pro-lamellar body
cw, cell wall
t, tonoplast
m, mitochondrion
c, chromatin

FIGURE 1 Parts of two chloroplasts in an *Avena* mesophyll cell. The tissue was fixed in phosphate-buffered 2 per cent osmium tetroxide at pH 7.0 for 22 hours at 0°. The chloroplast envelope (*ce*) and the internal membranes are distorted and the grana (*g*) appear loose. Most of the stroma of the chloroplast, and the surrounding cytoplasm, have been extracted. There is a noticeably clear space (*s*), free of membranes, in one chloroplast; around its margin there are a number of osmiophilic globules (*og*). × 41,000.

FIGURE 2 Section through a group of chloroplasts in mesophyll of a leaf fixed in 2 per cent potassium permanganate for 1 hour at room temperature. The grana (*g*) are compact, and the ground-substance inside and outside the chloroplasts is uniformly granular. Membrane-free regions are illustrated (*s*). × 19,000.

FIGURE 3 Portion of a mesophyll cell fixed in glutaraldehyde without postfixation in osmium tetroxide. Section stained heavily with uranyl acetate and lead citrate. The stromacentre regions (*s*), the chloroplast and nuclear envelopes (*ce* and *ne*), the grana (*g*), and other chloroplast membranes are not so heavily stained as are the chromatin (*c*) and the granules in the cytoplasm (*r*) and in the chloroplast ground-substance. There are two "nucleoplasm-like" (see text) regions (*n*) in one of the chloroplasts. × 22,000.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RESULTS

General

Components of the chloroplast ground-substance are not preserved by the osmium tetroxide and permanganate fixation procedures used in this study (Figs. 1 and 2). Nevertheless, distinct regions of the chloroplast which are not traversed by membranes are occasionally seen, and osmium tetroxide fixation shows that osmiophilic globules are associated with these "empty" spaces in the chloroplast. The spaces are usually approximately 1 μ in diameter, whereas the chloroplast is usually approximately 5 μ; hence many sections do not include these well defined clear areas—see, *e.g.*, the photomicrograph shown in Fig. 6 (of a 0.5-μ section seen in the light microscope).

Where glutaraldehyde fixation is used without any posttreatment in osmium tetroxide (Fig. 3), the chloroplast membranes show up in low density, whilst a more heavily stained granular background represents the stroma. This conforms essentially to the description by Jacobsen *et al.* (1963) of formaldehyde-fixed *Zea* preparations. Formaldehyde did not preserve the stroma in oats, whereas acrolein was as successful as glutaraldehyde (Fig. 4). In oats, acrolein or glutaraldehyde fixation permits recognition of two distinct regions in the stroma, in addition to the general granular background. One of these regions has the appearance of an aggregated mass of fibrils, and when an osmium tetroxide postfixation treatment is included (Fig. 4), osmiophilic globules are often found near its margins. Its dimensions and the association with globules suggest strongly that it is the counterpart of the clear areas illustrated in Figs. 1 and 2 and described above. Presumably, the fibrils are not preserved during fixation in osmium tetroxide or permanganate. For convenience, and without any functional implications, this mass of fibrils will be referred to as the "stromacentre." The second distinct region is smaller and more or less circular in section, with a diameter of 0.2 to 0.4 μ, and consists of a network of fine fibrils traversing a region of stroma from which most of the granular ground-substance is excluded. For reasons which will become apparent later, such regions will be described as resembling bacterial nucleoplasm.

These components of the *Avena* chloroplast stroma will now be considered in more detail.

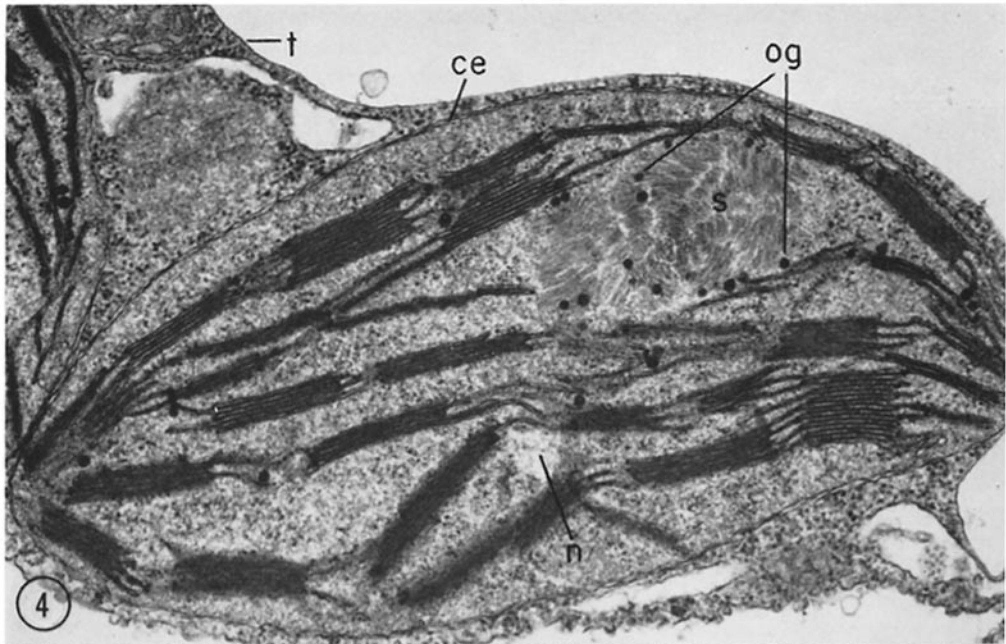
The Stromacentre

Fig. 6 shows stromacentres as observed in 0.5-μ sections in the light microscope, and Fig. 7 shows one at high magnification in the electron microscope. It consists of bundles of fibrils which are seen in either transverse or oblique section, or in side view. The three-dimensional picture is uncertain; other methods of preparation will have to be used to decide whether the stromacentre consists of a large number of relatively short fibrils packed together, or of a smaller number of very long fibrils wound much as in a ball of wool. Since the fibrils pass out of the plane of the section, it is not possible to estimate their length, but stretches of up to 1200 A have been measured. In transverse section, each fibril is some 80 to 85 A in

FIGURE 4 Chloroplast after fixation in acrolein and osmium tetroxide. There is very little cytoplasm between the tonoplast (*t*) and the chloroplast envelope (*ce*) over much of the inner surface of the chloroplast. The stromacentre (*s*) is associated with osmiophilic globules (*og*). A "nucleoplasm-like" region (*n*) is included in the section. × 27,000.

FIGURE 5 The result of insufficient rinsing between the two stages of fixation. An electron-opaque precipitate forms and is visible both before and after staining the section. It is especially concentrated in the intra-thylakoid (*th*) spaces, but occurs also in mitochondria (*m*), the chloroplast envelope membranes (*ce*), the cytoplasm and the ground-substance of the chloroplasts, and in the stromacentre (*s*) and associated osmiophilic globules (*og*). × 62,000.

FIGURE 6 Light micrograph of mesophyll cells in a 0.5-μ-thick section (Epon, as for the electron micrographs) stained with Azur II and methylene blue (Richardson *et al.*, 1960). Chloroplasts and a very tenuous layer of cytoplasm line the walls of the cells (*v*, vacuoles; *is*, intercellular space). Grana are visible in the chloroplasts; the stromacentres (*s*) take up very much less stain. × 4,000.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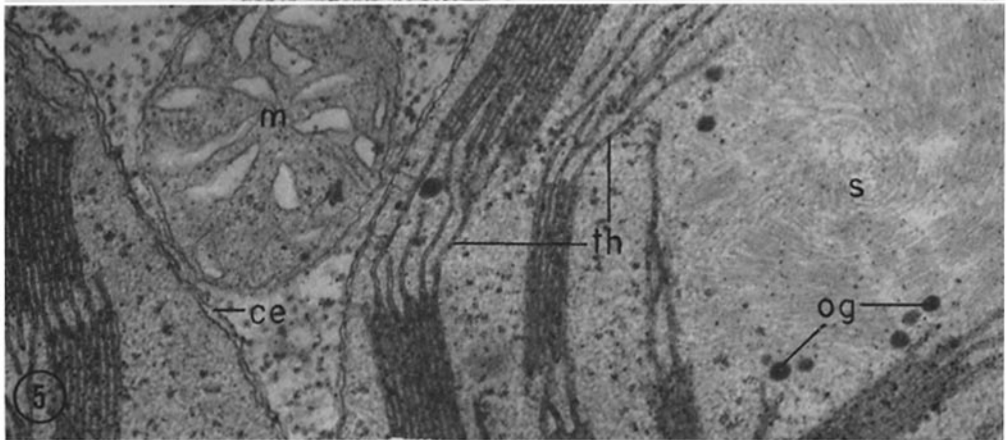
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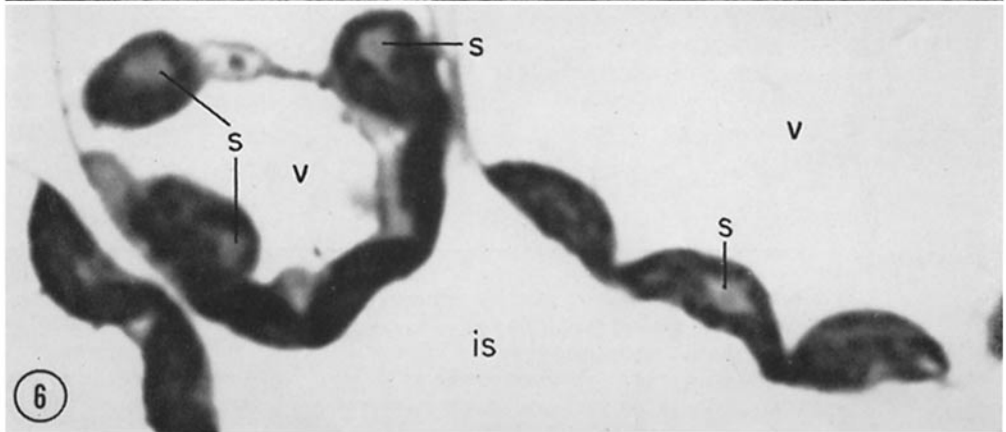
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diameter. The whole aggregate is approximately 1 μ in diameter, though glancing sections include much smaller portions. The inclusion of two separate stromacentre regions in a section of a single chloroplast is rare.

High magnification views of the individual fibrils sometimes suggest the presence of subunits. Examples are shown in the longitudinal and transverse views inserted in Figs. 7 and 10. Whatever its molecular organisation, the stromacentre is a markedly homogeneous region. It has no limiting membrane, yet it is not penetrated by other components of the stroma, and there is no continuity apparent between stromacentre fibrils and chloroplast membranes. It is this homogeneity that renders the stromacentre visible at the light microscope level (Fig. 6), and histochemical and autoradiographic procedures are now being used to study its composition.

There is negative evidence that the stromacentre is proteinaceous. Thus, if it were lipoidal in nature, it would be expected to be much more osmiophilic. Similarly, it would presumably become densely stained with uranyl acetate if it contained nucleoprotein. However, as shown in Fig. 3, the cytoplasmic ribonucleoprotein particles and the chromatin have a much greater affinity for this stain than has the stromacentre. Again, it is not stained by a periodic acid–Schiff procedure that does stain cellulose cell walls and starch grains. Starch grains are but rarely encountered in chloroplasts of oat leaves grown in the present conditions of light intensity, and when they *are* present, they resemble starch grains as seen after normal osmium tetroxide fixation. The stromacentre is, therefore, unlikely to be lipid, nucleoprotein, or polysaccharide.

Further information comes from examination of primordial and etiolated leaf tissues. No plastid developmental stages examined so far have been found without stromacentres. They are present in reduced size in leaf primordium proplastids (Figs. 8 to 10), prior to the formation of any extensive membrane system. This, together with the fact that they occur in completely etiolated plastids (Fig. 11), would indicate that they are not a direct product of photosynthesis. However, they have not been found in proplastids in oat root meristem and cell extension zones. The largest stromacentres so far observed (up to 2 μ diameter) have been in plastids in leaves that were grown in darkness and then illuminated (Figs. 12 and 13). In such conditions there is extensive and rapid production of chloroplast membranes and, after 10 hours in light, grana are clearly recognisable (Fig. 14). By this time, the prolamellar body has degenerated (Gunning, in preparation), and a large number of osmiophilic globules appears in its place, possibly as a breakdown product of its remaining lipoprotein membrane. At the same time, osmiophilic globules gather at the periphery of the stromacentre, so that it appears very much as in mature chloroplasts (Fig. 14; compare Fig. 4). Osmiophilic globules are not always present in mature chloroplasts, but when they are, the association between them and the stromacentre is clear. Possibly the physical properties of the stromacentre-stoma interface are such as to attract or retain lipid globules.

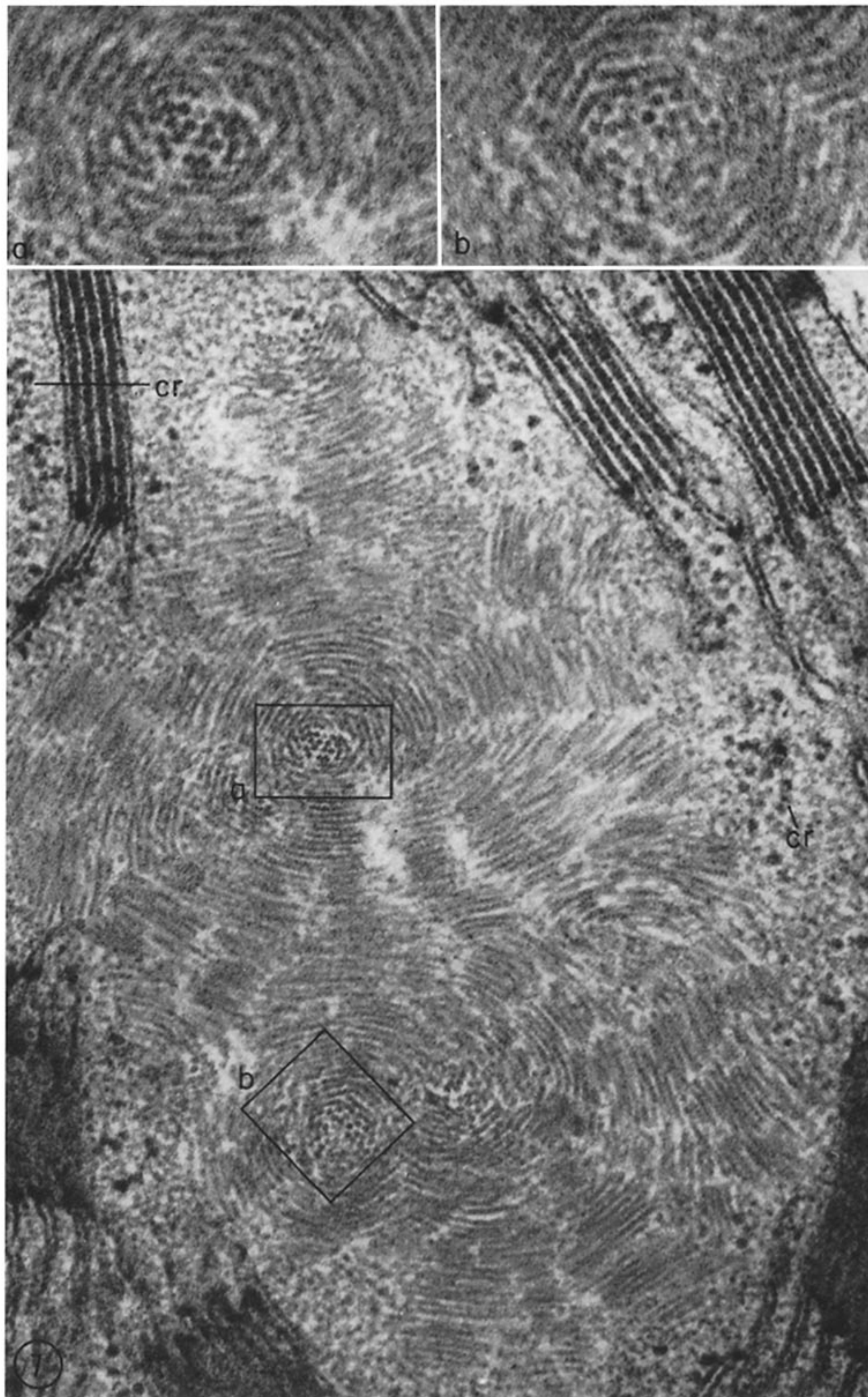
Regions Resembling Bacterial Nucleoplasm

Like the stromacentres, these regions can be found at all stages of development-mature chloroplasts (Figs. 3 and 4); stages between etiolated and green (Fig. 13); and proplastids (Fig. 8). Unlike the stromacentres, several such regions may be present within one section of a single plastid (Figs. 3 and 15). Inserts in Fig. 15 illustrate the network of fine fibrils, each 25 to 30 A thick, traversing the "nucleoplasm-like" areas. Occasional particles lie amongst the fibrils, but at a much lower density than in the surrounding stroma.

The Ground-Substance

In the above descriptions, the ground-substance lying between the lamellae, and surrounding the specialised areas, has simply been referred to as

FIGURE 7 Stromacentre in a mature, green, chloroplast. The stromacentre fibres are viewed from various angles. Two areas showing cross-sections are enlarged in the insets, where the fibres are seen not to be solid and homogeneous. Chloroplast membranes lie around the margin of the stromacentre, and the ground-substance between the membranes merges into the stromacentre fibres. Aggregates of "ribosomes" (*cr*) are present, as well as individual particles. × 122,000. Insets (Figs. 7*a* and *b*), × 264,000.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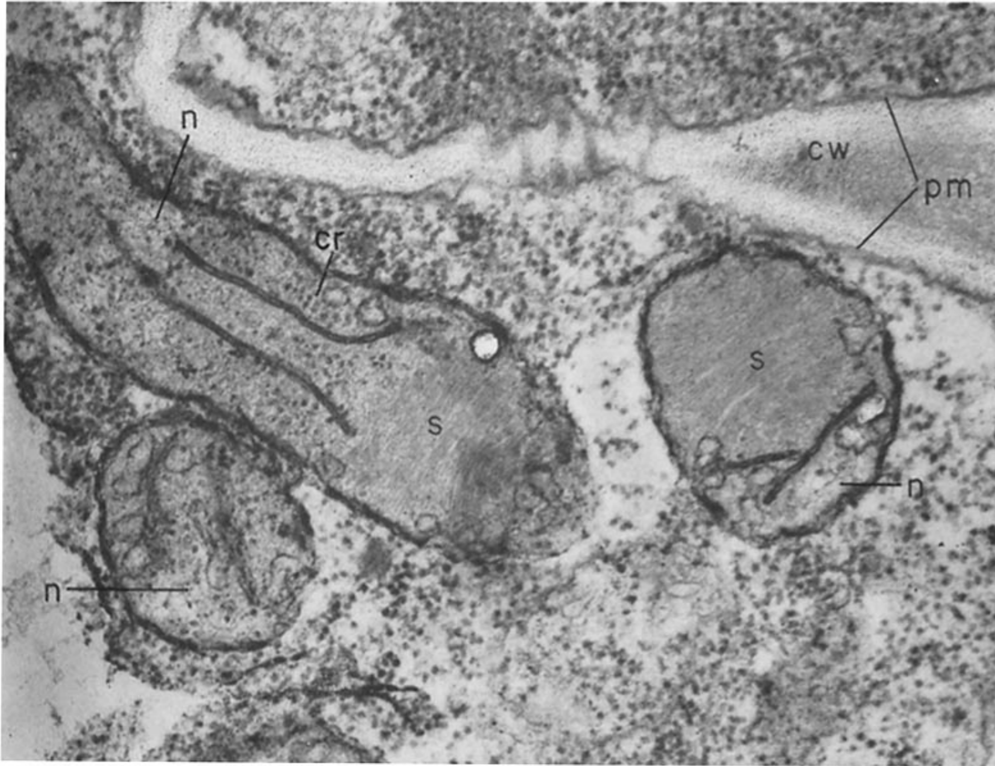


FIGURE 8 Section through a leaf primordium close to the apical meristem of the oat seedling. There are three proplastids, two of which are sectioned through a stromacentre (*s*). Nucleoplasm-like regions (*n*) are present. One of several aggregates of "ribosomes" is indicated (*cr*). The cell wall (*cw*) is lined by the three-layered plasma-membrane (*pm*). × 59,000.

"granular." In fact, there is more than one category of granule present. Figs. 3, 4, 7 to 12, 14, and 15 all illustrate the presence of particles which have the appearance of ribosomes, and which take up uranyl acetate stain. They occur at all stages, and clearly increase in numbers during plastid development. However, as seen in Figs. 8 to 10, 12, and 15, the chloroplast "ribosomes" are only about two-thirds the size of those lying in the cytoplasm outside the chloroplast. They are frequently seen in groups (Figs. 7, 8, 10, and 15) as well as scattered uniformly throughout the ground-substance. They are excluded only from the stromacentres, "nucleoplasm-like" regions, and intra-thylakoid (Menke, 1962) spaces. They lie in finer granular material which does not have a high affinity for uranyl acetate, and which may include different types of particle. It is possible that stromacentre material occurs in an unaggregated form in this ground-substance.

DISCUSSION

General surveys carried out using the glutaraldehyde-osmium tetroxide acid method of fixation show that it is reliable and can be applied to a wide range of tissues, both animal (Sabatini *et al.*, 1963) and plant (Ledbetter and Gunning, 1963). It is likely that it will prove especially useful in the study of mature plant cells, where osmium tetroxide may be an unsuccessful fixative. This is certainly the case in the present work, in which the large and highly hydrated oat mesophyll cells (see Fig. 13) could not be examined in detail by the conventional methods. However, after acrolein or glutaraldehyde treatment, all of the usual components of cells were observed, in several cases with evident improvement in fixation. One component —the stromacentre—was not recognised as a definite structure until it was seen in a well preserved form (after glutaraldehyde fixation). Only in the light of this knowledge could it be detected in

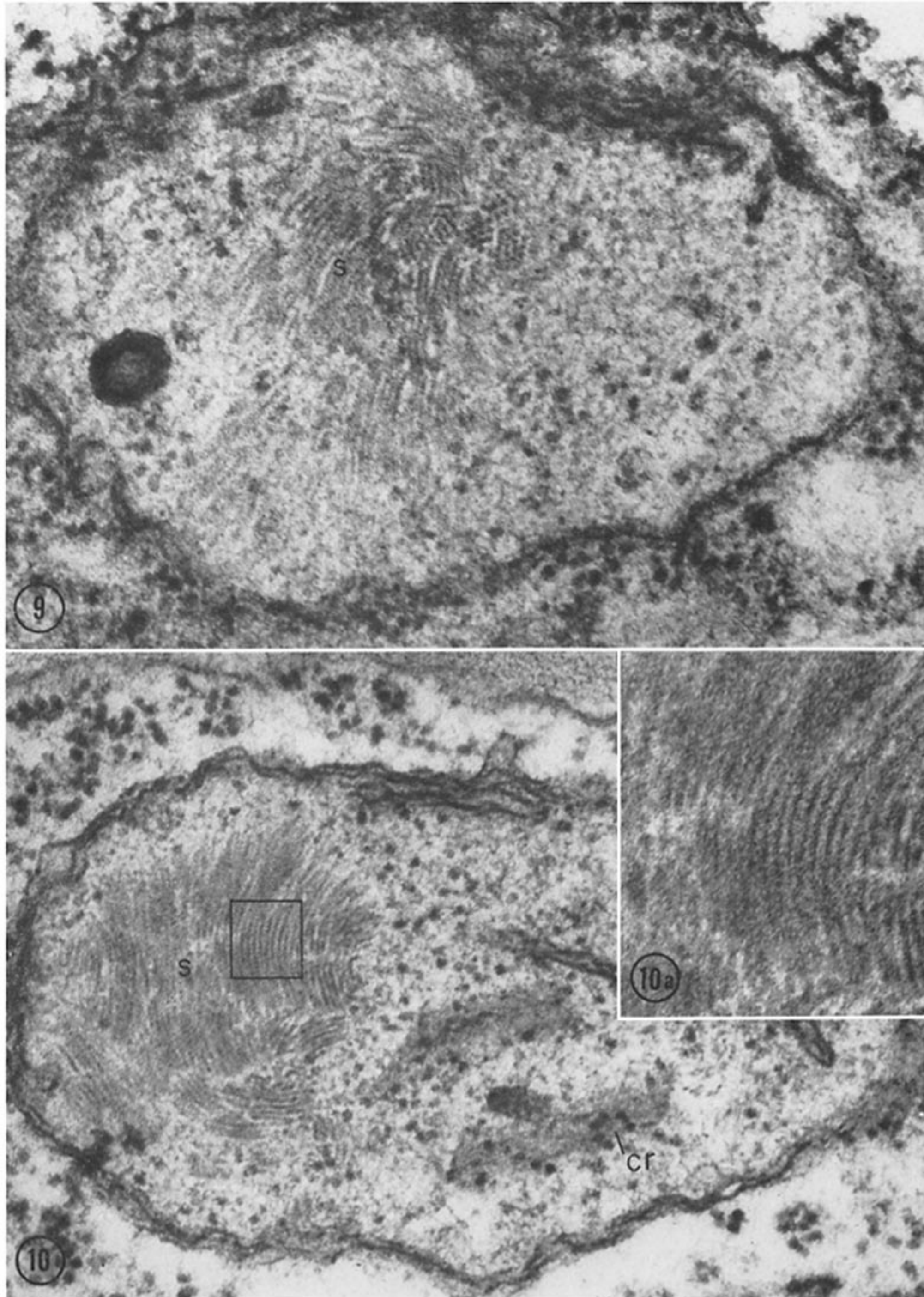
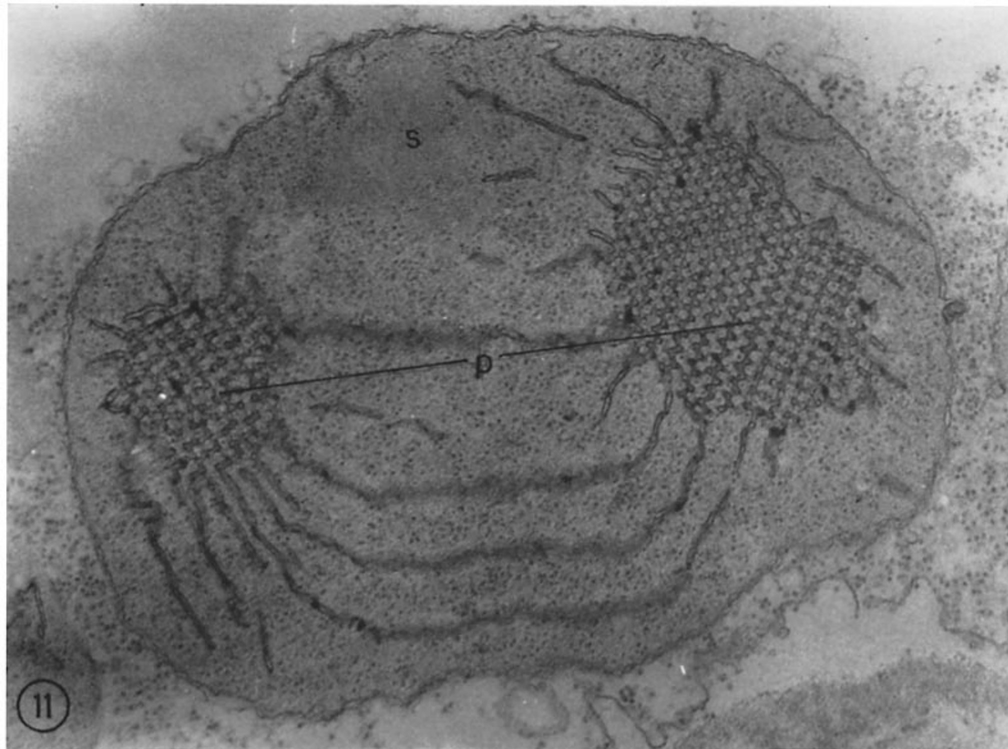


Figure 9 Proplastid in a leaf-primordium cell. Many of the stromacentre (*s*) fibres are seen in cross-section. Particles which have stained to the same extent as the cytoplasmic ribosomes are present within the proplastid. × 124,000.

Figure 10 As Fig. 9, but the majority of the stromacentre fibres are seen in side view. The inset (Fig. 10 *a*) shows an enlarged view of some fibres (rectangle), with suggestions of longitudinal periodicity. An aggregate of "ribosomes" is marked (*cr*). These stained particles within the proplastid are clearly smaller than those in the cytoplasm. × 92,000. Inset, × 196,000.



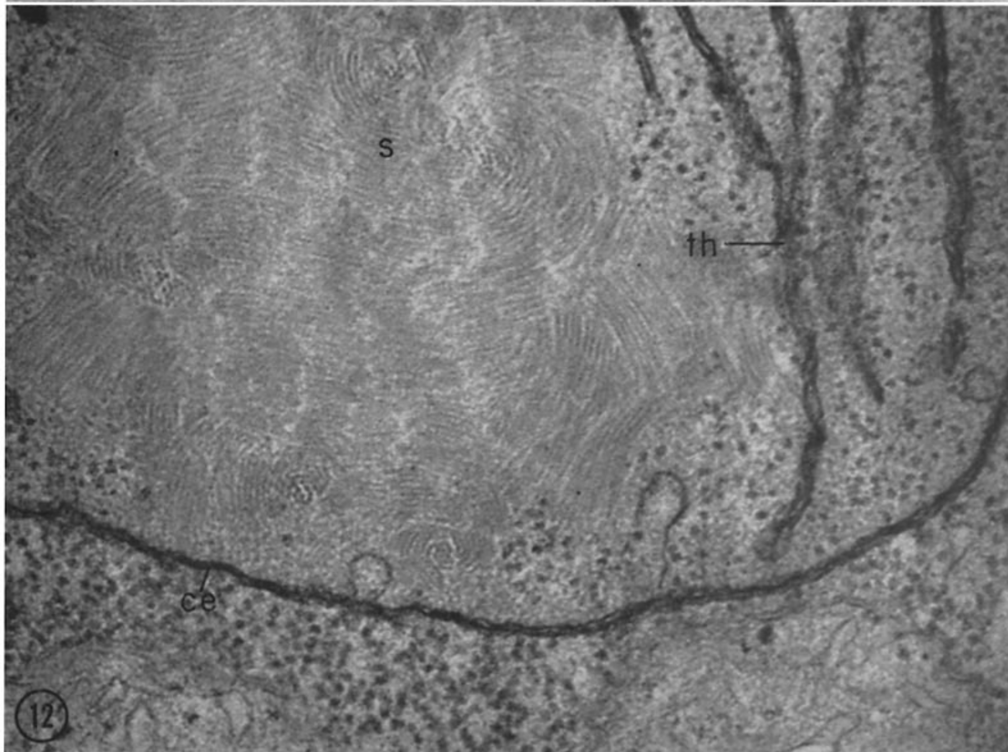


FIGURE 11 Plastid in an etiolated leaf. The stromacentre (*s*) is very different in morphology from the prolamellar bodies (*p*P. × 38,000.

FIGURE 12 Part of a plastid in the mesophyll of a leaf grown in darkness and fixed after 2 hours' illumination. Some membranes (*th*), but no grana, are present. The stromacentre (*s*) has the same fine structure as in mature leaves. Plastid "ribosomes" are smaller than those outside the plastid envelope (*ce*). ×81,000.

its incompletely preserved forms in chloroplasts fixed in permanganate or osmium tetroxide.

The efficacy of the glutaraldehyde-osmium tetroxide procedure in preserving chloroplasts may also be judged from the fact that they retain their pigment throughout dehydration and infiltration, and even when polymerised in Epon they still appear green in transmitted light, and exhibit the characteristic red fluorescence of chlorophyll. If the postfixation treatment is not carried out, the pigment is lost during dehydration.

There is now considerable biochemical evidence for the occurrence of DNA in chloroplasts (Chun *et al.*, 1963; Kirk, 1963; Sager and Ishida, 1963). Ris and Plaut (1962) have related Feulgen reactivity to the presence of 25-A fibrils in certain regions of *Chlamydomonas* chloroplasts, and they point out that they are very similar to the fine fibrils of bacterial and blue-green algal nucleoplasms. In oats, as in *Chlamydomonas*, it seems justified to describe regions containing such fibrils as the nucleoplasm of the chloroplast. It is not yet clear whether the fibrils occur outside the nucleoplasm-like regions in the oat chloroplasts (perhaps obscured by the granules of the ground-substance), or whether they are confined to them, perhaps embedded in a matrix of electron-transparent material which excludes the surrounding granules. Autoradiography of 0.5-μ sections of oat leaves supplied with tritiated thymidine shows the presence of label within the chloroplasts (Gunning, unpublished), but it remains to be seen whether the electron microscope will show the silver grains to be associated with the nucleoplasm-like areas.

In their discussion, Ris and Plaut (1962) attributed the genetic properties and autonomy in reproduction of chloroplasts to their possession of "nucleoplasm." In this connection, it is of considerable interest that oat plastids as well as possessing "nucleoplasm" contain what appears to be a distinctive particle which, like the larger type found outside the chloroplast envelope, has morphological and staining properties which seem to justify the use of the term "ribosome." There is, however, no direct evidence that such particles participate in protein synthesis in the plastid. Jacobsen *et al.* (1963) found that the ribosome-like particles in *Zea* vascular sheath plastids are smaller than those in the cytoplasm, but that this size difference does not apply to mesophyll plastids. Other evidence that plastid ribosomes are distinctive has been obtained (Lyttleton, 1962; Brawerman, 1963); on the other hand, Mikulska *et al.* (1962) found chloroplast ribosomes from *Clivia* and *Chenopodium* to be the same as those derived from the cytoplasm.

The groups of "ribosomes" seen in oat plastids (Figs. 7, 8, 10, and 15) may represent polysomes, or groups of ribosomes engaged in protein synthesis (Warner *et al.*, 1962). If so, the existence of plastid "messenger" RNA may be postulated. Taken along with the evidence for the presence of DNA, the cytological evidence for "nucleoplasm," and evidence that plastids produce specific proteins (Smillie, 1963), this suggests that plastids might house their own coding and protein-synthesising system.

The available evidence on the chemical nature of the stromacentre has been presented, and the most useful hypothesis is that it represents an aggregate of protein molecules. Present at all stages of development, it might be interpreted as a store or pool—it is certainly augmented during plastid development, and becomes very prominent when an etiolated leaf is illuminated. Its absence from root-tip proplastids may imply some indirect relation to the photosynthetic process. On the other hand, the stromacentre does not appear to be a constant feature of chloroplasts. It has not been found in glutaraldehyde-osmium tetroxide–fixed chloroplasts in *Spirodela* (Ledbetter, personal communication), *Phleum pratense*, *Phaseolus sp.*, *Mimosa pudica*, *Pisum sativum*, *Pelargonium sp.*, and a moss. This restriction in distribution can, at least, be taken as an indication that the stromacentre is not a general artefact of aldehyde fixation.

Whatever the nature and function (if any) of the stromacentre, it should prove to be useful in cyto- and histochemical work on plastid reactions, providing as it does a recognisable aggregate of molecules in the ground-substance. Work along such lines is in progress, and it should be pointed out that, although the stromacentre may not be widely distributed in plants, the sort of reactions that produce it and break it down may be widespread. Any conclusions that are drawn need not necessarily be restricted in application to the genus *Avena*. Indeed, aggregates of the stromacentre type may be more common than at present suspected. Buvat (1958) has described a somewhat different structure in *Phajus wallichii* leucoplasts, and further examples may come to light now that a routine method for fixing the chloroplast stroma is available.

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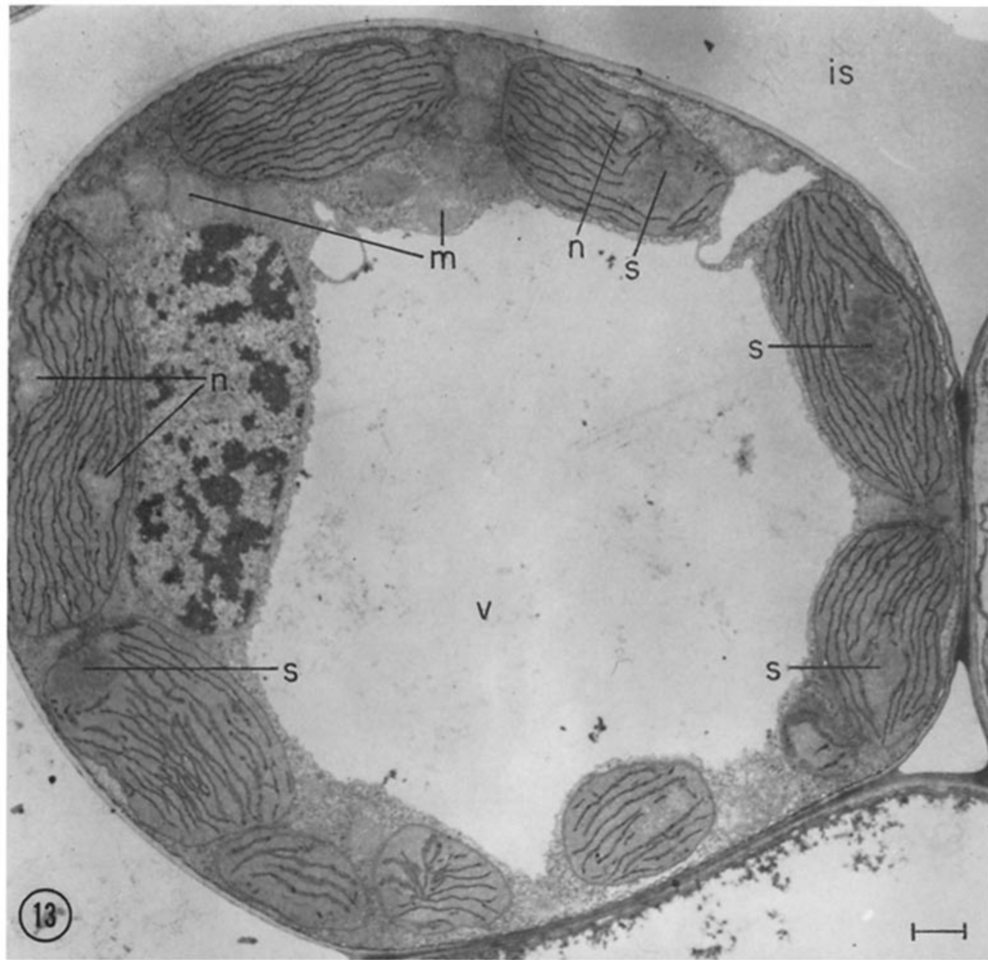
FIGURE 13 Mesophyll cell in a leaf grown in darkness and fixed after 2 hours' illumination. The large proportions of the vacuole (*v*) and intercellular spaces (*is*) in this tissue are evident. Membranes, but no grana (as in Fig. 12), are present in the plastids, and the stromacentres (*s*) are very well developed. "Nucleoplasm-like" areas (*n*) may be distinguished. Some mitochondria (*m*) are present. × 6,600.

FIGURE 14 Parts of two plastids in a dark-grown leaf fixed after 10 hours' illumination. Grana (*g*) have been formed, and the prolamellar body is apparently degenerating (right plastid) and giving place to a number of osmiophilic globules. At the same stage, globules appear in association with the stromacentres (*s*) as, *e.g.*, in the left plastid. × 58,000.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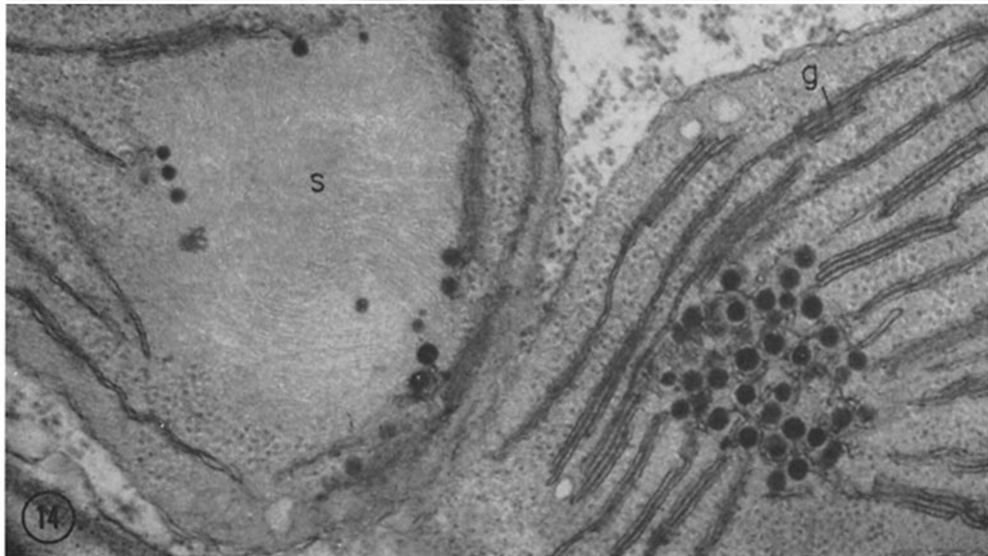
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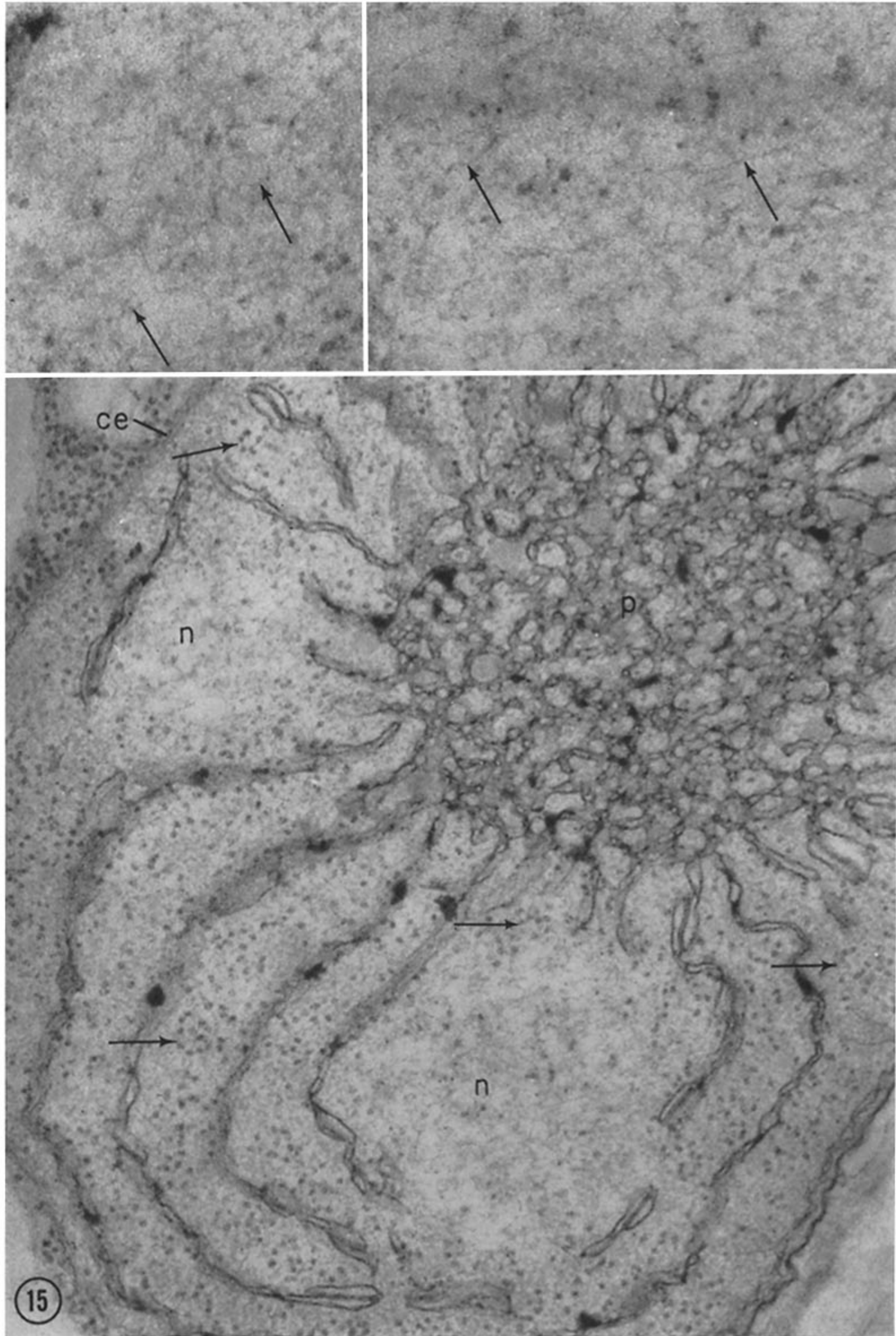
FIGURE 15 Plastid in a dark-grown leaf, fixed after 4 hours' illumination. The prolamellar body (*p*) is not so ordered and "crystalline" as in the completely etiolated plastids (Fig. 11). Two nucleoplasm-like regions (*n*) are sectioned and shown at higher magnification in the insets, where arrows indicate some of the approximately 30-A-wide fibrils. Arrows in the main part of the figure point to poly-"ribosomes." Cytoplasmic ribosomes outside the envelope (*ce*) are larger. × 69,000. Insets, × 160,000.


ce
n
p
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