

# CYTOCHEMICAL STUDIES CONCERNING THE OCCURRENCE AND DISTRIBUTION OF RNA IN PLASTIDS OF *ZEA MAYS*

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## ABSTRACT

The occurrence of RNA in plastids from etiolated and green maize leaves was demonstrated cytochemically, with both the light and the electron microscope. Etiolated leaves were allowed to incorporate tritiated cytidine for several hours and were subsequently fixed in formalin. Radioautographs of leaf sections 2  $\mu$  thick showed silver grains over the regions of the cytoplasm containing plastids. Plastids in these sections appeared intensely basophilic when stained with azure B. Both the basophilia and radioactivity were removable with ribonuclease, clearly demonstrating the occurrence of RNA in these organelles. Examination under the electron microscope of similar plastids which had been fixed in formalin revealed a particulate component in the plastid measuring approximately 170 A in diameter. This particulate component was completely removable with ribonuclease. Thus, it was concluded that RNA occurs in a particulate form in plastids from etiolated leaves. Mature plastids, when stained with azure B, did not appear basophilic under the light microscope. Nevertheless, when formalin-fixed tissues were examined with the electron microscope, the mature plastids were seen to contain particles in the stroma, identical in appearance with those visible in the plastids in etiolated leaves. Osmium tetroxide-fixed tissues were also examined with the electron microscope. Particles similar to those seen in plastids fixed with formalin were observed, although the results obtained with this fixative were variable. It is concluded that plastids from etiolated and green maize leaves contain RNA in a particulate form which resembles ribosomes.

## INTRODUCTION

Conflicting reports exist in the literature concerning the occurrence of RNA in plastids of green plants. Although two different experimental approaches can be employed in the examination of this problem, thus far questions have arisen concerning the validity of the results which have been obtained with both methods. Preparations of isolated chloroplasts have been examined chemically for their nucleic acid content (4, 5, 12, 14, 19, 20, 26, 31, 36). Difficulties have been encountered with this method since the values ob-

tained for both DNA and RNA are low, and the significance of the data is severely limited by the lack of purity of the plastid preparations. Thus some authors consider that the values which have been obtained for RNA and DNA in plastids in this manner may have been obtained through nuclear contamination (15, 37).

Cytochemical procedures may often avoid the problem of contamination, and chloroplasts have been examined cytochemically with the light microscope. At the proper pH value, selective

dye binding has been demonstrated between basic dyes such as azure B and nucleic acids (6). When coupled with proper fixation procedures as well as careful ribonuclease treatment, this method provides an effective means for localizing RNA in tissue sections (35). Nevertheless, cytochemical techniques require care and are limited in their sensitivity. Thus, although numerous reports concerning the cytochemical localization of RNA appear in the literature (3, 22, 23, 32, 34), Littau has carefully reexamined the major portion of this work and has shown that under appropriate conditions and with suitable controls the usual cytochemical procedures are not sufficiently sensitive to show RNA in mature plastids (16).

A more convincing argument for the occurrence of RNA in plastids has been provided in a series of recent reports by Brawerman and Chargaff (1, 2, 27). These authors have shown that a net synthesis of RNA occurs when dark-grown *Euglena* cells are exposed to the light. Furthermore, when such cells are ruptured, the *de novo* synthesis of RNA is found to be associated with the cell fraction which contains the plastids.

One might anticipate that, if RNA were to occur in plastids of higher plants, it would be observed most readily in developing plastids where rapid structural synthesis is occurring. Etiolated leaves provide a favorable system for studying this problem since plastids in such leaves develop to considerable size while remaining proplastid in character. This communication presents some observations on the proplastids of etiolated corn leaves. It was possible to verify that these proplastids do contain large amounts of RNA. Correlated studies with both the light and the electron microscope have allowed the morphological characterization of the RNA which was observed and have permitted similar observations to be extended to mature plastids.

#### MATERIALS AND METHODS

Plastids were examined from 10- to 15-day old etiolated and green maize seedlings (*Zea mays*, pedigree WF9 X M14, Indiana Seed Improvement Association). Etiolated material was obtained by allowing seedlings to develop in complete darkness at 27°C. The seedlings were grown in quartz sand and were supplied with distilled water. Prior to fixation, leaves were allowed to incorporate  $H^3$ -cytidine in the following manner. The leaves to be treated were severed approximately 4 cm from the

tip. The free portion of each leaf was then recut under water and immersed in a solution containing 50  $\mu$ c of uniformly labeled tritiated cytidine (1.25  $\mu$ c/mm, Schwartz BioResearch, Inc., Mt. Vernon, New York). This solution was prepared by adding the cytidine to 0.2 ml of a 0.15 M sucrose solution. The leaves remained in this solution for 5½ hours. Small pieces of tissue were then removed from the leaves and subjected to one of three different fixation procedures for 1 to 2 hours:

1. 10 per cent formalin in 0.2 M phosphate buffer, pH 7.4.
2. 1 per cent  $OsO_4$  in 0.14 M Veronal acetate, pH 7.4.
3. Fixation in the 10 per cent formalin solution followed by 30 minutes in the 1 per cent osmium solution.

In order to localize RNA with the electron microscope it was necessary to treat small pieces of tissue with ribonuclease prior to embedding them in plastic. For this purpose, some of the formalin-fixed material was sectioned at 100  $\mu$  on a freezing microtome. The sections were then incubated for 3 hours in a solution of crystalline pancreatic ribonuclease obtained from Worthington Biochemicals Corporation, Freehold, New Jersey. This was prepared at a final concentration of 0.7 mg/ml and adjusted to pH 6.5 with sodium hydroxide. (This treatment will be designated "formalin-ribonuclease" in all further discussion.) Control tissues were incubated in distilled water adjusted to pH 6.5. Both ribonuclease-treated and control tissues were then extracted with 5 per cent trichloroacetic acid at 0°C for 30 minutes.

All tissues were dehydrated through a graded series of alcohols and embedded in Epon 812 according to the method of Luft (17). Leaf segments fixed in formalin were also embedded in paraffin.

The Epon blocks were sectioned for viewing both with the light and the electron microscope. For light microscopy, sections 2  $\mu$  thick of both formalin and formalin-ribonuclease-treated tissues were cut. These were placed on glass slides which had previously been coated with albumin. Some of these sections were then stained with a modified azure B reaction using a high concentration of dye (12.5 mg/ml) and prolonged incubation (48 hours at 60°C or 1 week at room temperature). This modification was necessary in order for the dye to penetrate into the Epon. Unstained sections were used in the preparation of radioautographs as described later.

For electron microscopy, thin sections were prepared from tissue fixed by all three procedures. These sections were mounted on carbon-coated copper grids for examination under the electron microscope. Sections were stained with a 3 per cent solution of uranyl acetate for 10 to 36 hours. Grids were examined with an RCA 3C electron microscope.

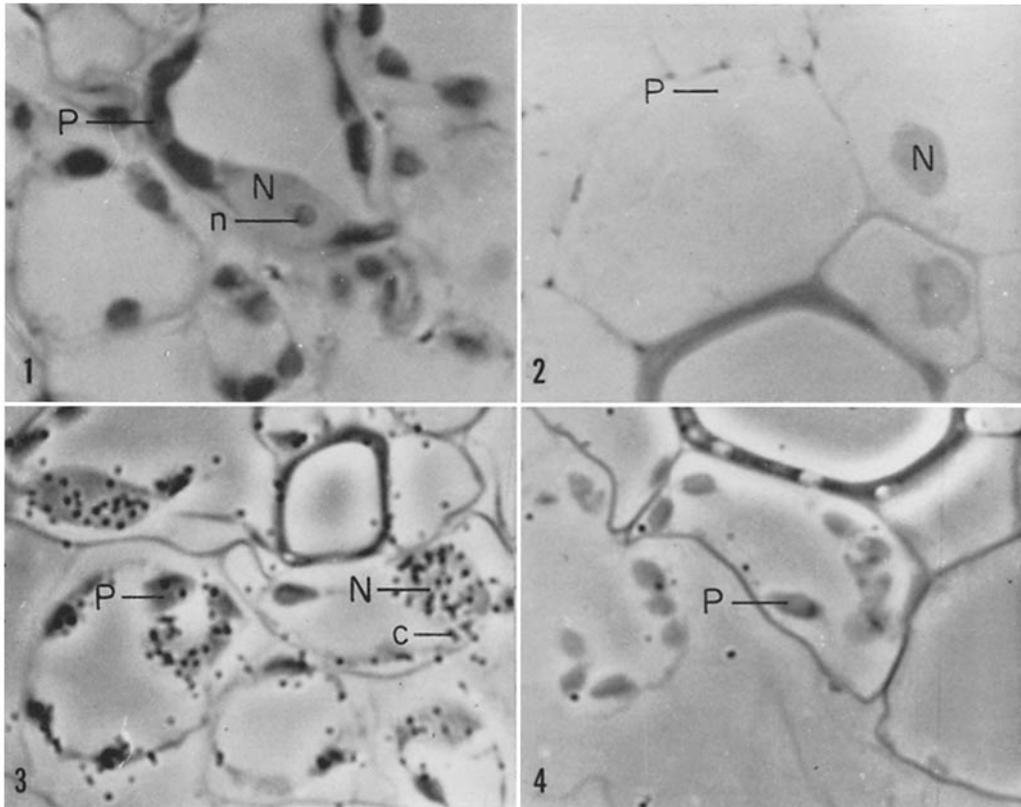


FIGURE 1

Region of a  $2\ \mu$  Epon section from an etiolated corn leaf stained with azure B to show the basophilia of the proplastids (*P*). A nucleus (*N*) appears in the center of the field and the nucleolus (*n*) is also visible.  $\times 1,000$ .

FIGURE 2

Region of a  $2\ \mu$  Epon section through an etiolated corn leaf which was treated with ribonuclease prior to dehydration. The sections were stained with azure B. Note absence of basophilia in proplastids (*P*) and the lack of a visible nucleolus although the DNA of the nucleus (*N*) is still stained.  $\times 1,000$ .

FIGURE 3

Region of an autoradiograph prepared with an unstained Epon section adjacent to the section seen in Fig. 1. The micrograph was taken with phase optics. It can be seen that tritiated cytidine was incorporated into the nucleus (*N*), and cytoplasm (*c*) as well as into the proplastids (*P*).  $\times 1,000$ .

FIGURE 4

Region of an autoradiograph prepared with an unstained Epon section adjacent to the section seen in Fig. 2. Ribonuclease is seen to remove the label from all parts of the cell, including the proplastids (*P*). Photograph taken with phase optics.  $\times 1,000$ .

Etiolated leaves embedded in paraffin blocks were sectioned at  $5\ \mu$  and mounted on slides which had been coated with albumin. Control sections were incubated in distilled water adjusted to pH

6 with sodium hydroxide; additional sections were incubated in ribonuclease, prepared at a final concentration of  $0.2\ \text{mg/ml}$ , and adjusted to pH 6 with sodium hydroxide. (This lower pH was found neces-

sary for azure B staining, since much of the basophilia was extracted from the ribonuclease controls in water at pH 6.5 but not at pH 6.0.) After 1 hour at room temperature several slides from each treatment were stained with azure B according to standard procedures (6). The remaining slides were used for radioautography. Both control slides and those treated with ribonuclease were covered with stripping film (Kodak AH 10) and stored at 4°C for 2 weeks. These slides were then developed in Kodak D-19 (18°C for 6 minutes) and stained with azure B.

Unstained sections, 2  $\mu$  thick, of formalin and formalin-ribonuclease-treated tissues embedded in Epon were coated with nuclear track emulsion (Kodak NTB 2) and exposed for 3 weeks. The coated slides were also developed in D-19 at 18°C for 6 minutes. It should be noted that all micrographs presented in this report concerning formalin and formalin-ribonuclease-treated tissue, for both the light and the electron microscope, are of adjacent sections from the same two Epon blocks.

Green tissue was collected from seedlings which had been raised in the greenhouse or from etiolated material which was allowed to turn green under artificial illumination. Both kinds of plants were supplied with an inorganic nutrient solution. Tissues from these plants were fixed in the same manner as those from the etiolated material except that no attempt was made to incorporate tritiated cytidine into the plastids.

## RESULTS

When paraffin sections of formalin-fixed etiolated leaves were stained with azure B and examined under the light microscope, the chloroplasts appeared intensely basophilic. Radioautographs of these preparations showed a heavy deposition of silver grains over regions of the cytoplasm containing plastids. Treatment with ribonuclease completely eliminated any binding of the dye

with the plastids and also removed all the radioactivity from the sections so that autographs of these tissues were not significantly above background in grain density. Comparable results were also obtained with Epon sections 2  $\mu$  thick. Sections stained with azure B are presented in Figs. 1 and 2. It is clear that proplastids stain intensely with azure B (Fig. 1) and that this basophilia is completely removed with ribonuclease (Fig. 2). Figs. 3 and 4 show autographs of unstained sections taken adjacent to those which were stained with azure B. Few silver grains remain after ribonuclease treatment (Fig. 4) when compared with untreated tissue (Fig. 3) where it can be seen that tritiated cytidine was incorporated into all cellular components. Since the sections shown in Figs. 3 and 4 are 2  $\mu$  thick, and the plastids vary in size from 3 to 5  $\mu$ , it may be presumed that the majority of the plastids are cut during sectioning. It thus seems likely that most grains over the plastids are not due to the surrounding cytoplasm. From these experiments one may conclude that plastids from etiolated maize leaves contain a considerable amount of RNA.

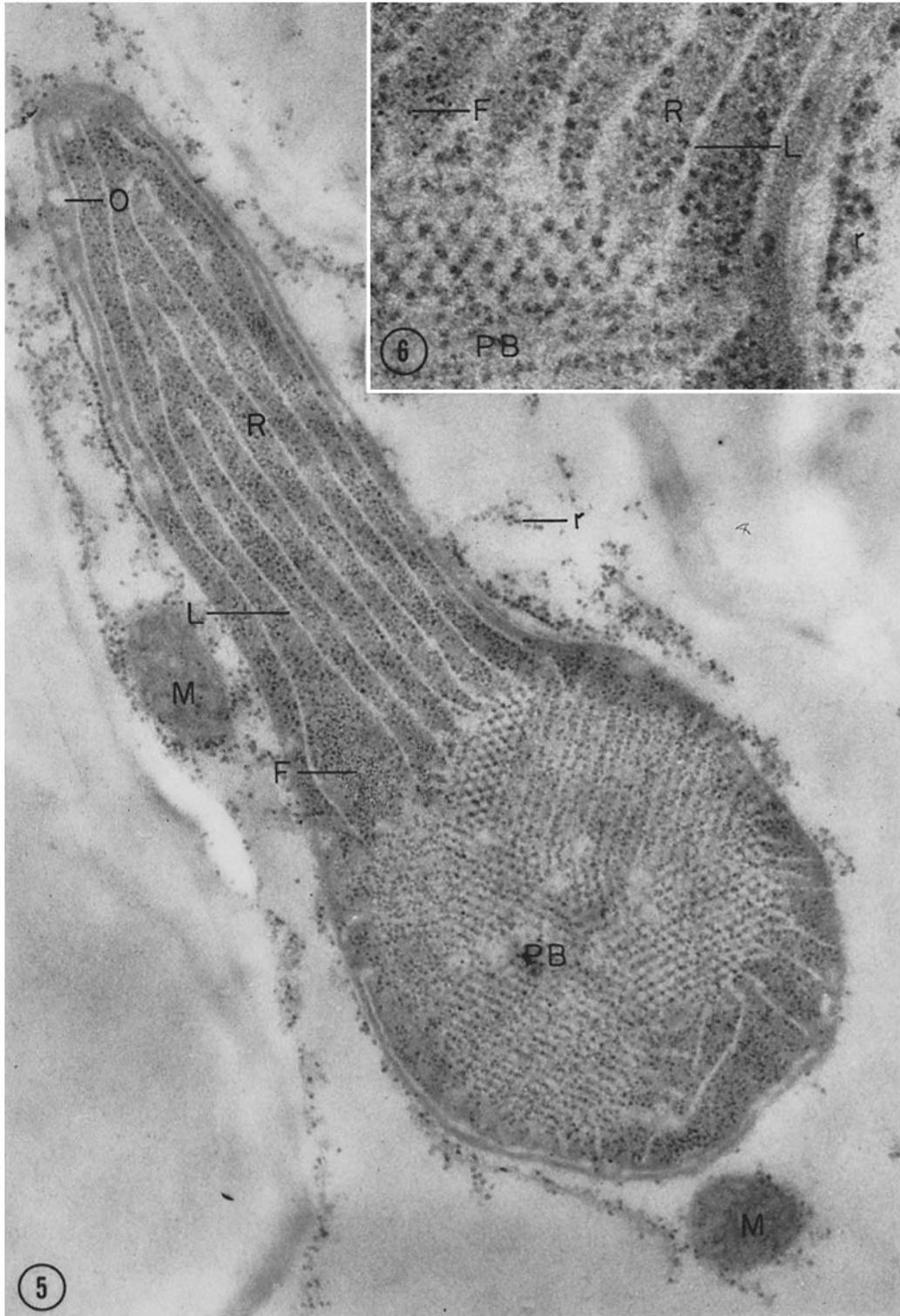
Adjacent thin sections of the formalin-fixed tissues were also examined with the electron microscope. With this fixative, as was expected, the lipid portion of lamellar system of the plastids was not preserved. However, the entire matrix of the plastids was clearly visible and contained a densely packed particulate component which measured approximately 170 A in diameter. Typical plastids are illustrated in Figs. 5 to 7. It can be seen that the particles within the plastid closely resemble the ribosomal particles visible in the surrounding cytoplasm. It was possible to establish that the particles observed within the plastids did in fact contain RNA by examination

FIGURE 5

Proplastid from the same tissue as sections shown in Fig. 1. Since the tissue was fixed in formalin, the lamellae (*L*) do not appear electron-dense. Particles (*R*) within the plastid matrix measure approximately 170 A and resemble the cytoplasmic ribosomes (*r*). Osmiophilic granules (*O*) do not appear electron-dense. Particles 60 A in diameter, resembling phytoferritin (*F*), are visible. The prolamellar body (*PB*) appears less dense than the adjacent plastid matrix but also contains particles measuring approximately 170 A in diameter. Two mitochondria (*M*) are visible.  $\times 46,000$ .

FIGURE 6

An enlarged region of Fig. 5 showing a portion of the prolamellar body (*PB*), phytoferritin (*F*), lamellae (*L*), and the particulate RNA-containing component (*R*). Cytoplasmic ribosome, *r*.  $\times 70,000$ .



of sections from ribonuclease-treated material under the electron microscope. The particulate component of the plastids was completely removed with ribonuclease (Figs. 8 and 9).

Sections from etiolated leaves which had been fixed with osmium tetroxide were also examined under the electron microscope (Figs. 10 and 11). Although the lamellar structure of the plastids was clearly visible with this method of fixation, the particulate nature of the plastid matrix was not so evident as in the sections of formalin-fixed material. A faint particulate component was occasionally visible in unstained plastids, but a prolonged incubation in uranyl acetate was generally necessary to make this component visible. Even after prolonged staining with uranyl acetate, the appearance of the particles within the plastids varied and frequently appeared different from the ribosomes present in the cytoplasm (compare Figs. 10 and 11). It was not possible to make accurate measurements of plastid particles in these preparations since the tissues fixed at different times varied considerably in their preservation of the plastid particulate component.

It was interesting to examine plastids which had been fixed in formalin followed by osmium tetroxide (Fig. 12). Here lamellae, although not completely preserved, were far more prominent than in tissue which had been fixed only in formalin. In addition, the particulate material (*R*) was also quite prominent.

In the course of these investigations a smaller particulate plastid component was also observed in etiolated leaves. These particles have diameters approximating 60 Å and occur either in patches

or scattered throughout the entire plastid (Figs. 5 to 9). The particles were clearly visible in unstained plastids of etiolated leaves, but could not be seen in mature plastids. Recently particles have been described from etiolated plastids by Hyde, Hodge, and Birnstiel (13), as phytoferritin. The particles described here correspond in size with those reported by these authors and probably are the same.

Once the morphological nature of the RNA in the plastids from etiolated leaves was established, observations were extended to plastids from green leaves. By use of the same fixation and staining procedures, it was found that green plastids from both the vascular sheath and the more typical mesophyll cells contained a particulate component identical with that described for etiolated leaves. Chloroplasts from formalin-fixed leaves are shown in Figs. 13 to 15. In the plastids from mesophyll cells, it can be seen that only the protein in the lamellae of the grana and stroma is preserved. Thus, the lamellae are only faintly electron-dense. Particles, 170 Å in size, are clearly visible in the stroma (Figs. 13 and 14). However, they appear reduced in number, and from the fact that such chloroplasts do not appear basophilic under the light microscope one can estimate that the particles must occur in a considerably reduced concentration. In sheath plastids, as previously described (11), no grana are visible. The stroma is again filled with prominent particles identical with those seen in plastids from etiolated leaves (Fig. 15). Chloroplasts from green leaves which were fixed with osmium tetroxide were also examined with the electron microscope. In most

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FIGURE 7

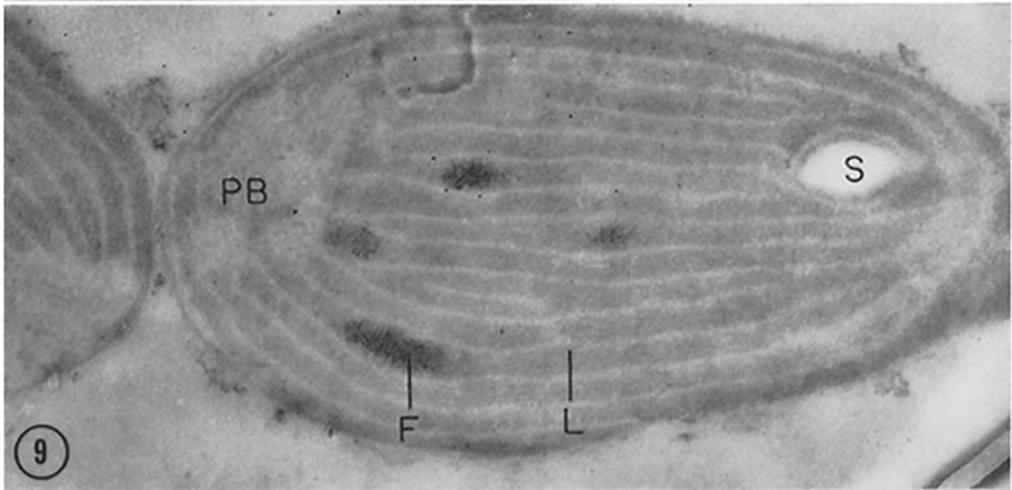
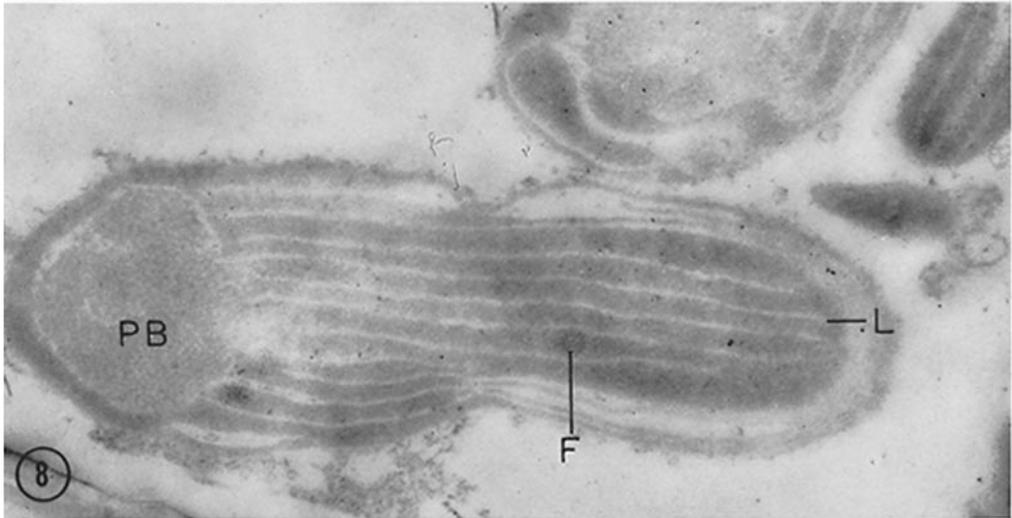
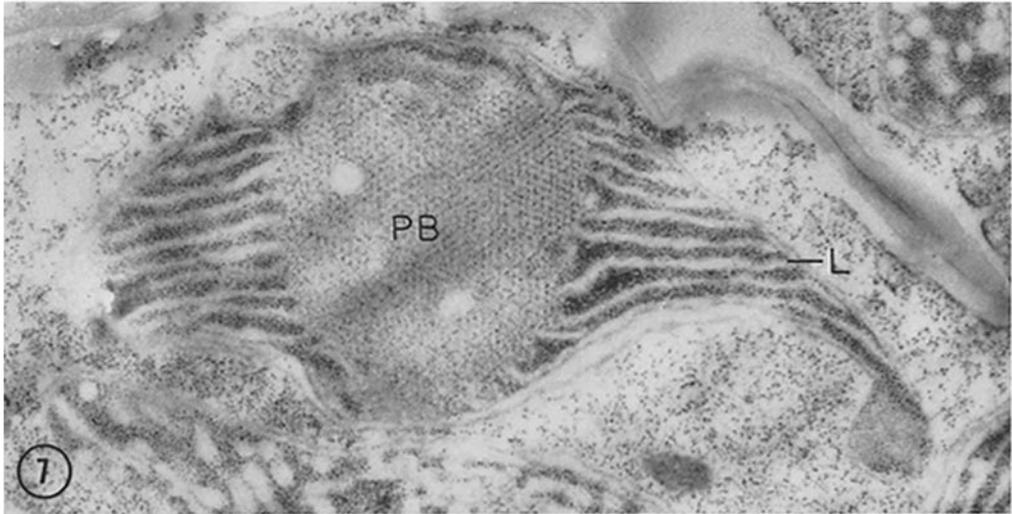
A proplastid similar to that illustrated in Fig. 5. *PB*, prolamellar body; *L*, lamellae.  $\times 23,000$ .

FIGURE 8

Ribonuclease-treated proplastid obtained from the same block as sections illustrated in Fig. 2. The effect of ribonuclease on the particulate component (*R*) previously seen in Figs. 5, 6, and 7 can be observed; although the prolamellar body (*PB*) and lamellae (*L*) are still visible, the particulate material of the plastid matrix cannot be seen. Faintly stained particles are still present in the cytoplasm, presumably reflecting the differential rate at which ribonuclease can attack RNA within plastids as compared with adjacent cytoplasm. Phytoferritin (*F*) is still visible.  $\times 26,000$ .

FIGURE 9

A proplastid which has been treated with ribonuclease. A starch grain (*S*) is visible. *PB*, prolamellar body; *F*, phytoferritin; *L*, lamellae.  $\times 31,000$ .



instances a particulate component was clearly visible in the green plastids prepared in this manner. The morphology of the particles, however, was again observed to vary. In Fig. 16 it can be seen that plastid particles appear quite similar to cytoplasmic ribosomes. However, in Fig. 17, which illustrates a portion of a sheath plastid, particles within the plastid appear less dense and probably are smaller than those visible in the adjacent cytoplasm.

#### DISCUSSION

In view of the controversy recorded in the literature concerning the role of nucleic acids in chloroplast development, a brief summary of several aspects of this problem is relevant here. Chloroplasts, by virtue of their discrete color and form, have lent themselves to extensive genetic analysis (8, 28). Such studies show that, in the course of their development, these organelles may be at least partially independent of the regulating mechanisms of the nucleus. Morphological evidence has proved less definitive. Very early studies of plastids in algae showed that in these organisms chloroplasts divide in phase with cell division and thus provide a continuous lineage of these organelles in successive generations of plants (8). In higher plants, fully formed plastids are found only in fully differentiated cells. As early as 1883, however, Schimper postulated that small colorless plastids were to be found in meristematic tissue and that these organelles could divide and thus

be transmitted from cell to cell (30). This theory was further elaborated by Guilliermond (9). Guilliermond also noted the resemblance between very young plastids and mitochondria under the light microscope.

Recent electron microscopic observations do not seem to support the concept of the continuity of plastid generations in higher plants. Although Menke has reported recently that proplastids can be distinguished from mitochondria in some meristematic tissue (21), it is more generally agreed that both proplastids and mitochondria originate from the same kind of very small vesicles, as described by Mühlethaler (24, 25), and it seems probable that clearly defined organelles only become visible in differentiating cells such as young leaf primordia. In later stages of development, plastids may be able to replicate by division (7), but present techniques are inadequate to show whether plastid continuity is maintained by division of small undifferentiated cytoplasmic vesicles.

In view of the replicative properties of certain nucleic acids found in cell nuclei as well as in viruses, the occurrence of either RNA or DNA within plastids would aid in the visualization of a mechanism by which plastids maintain their autonomy. As mentioned previously, numerous investigations have dealt with this problem. To date, conclusive evidence has been lacking for the higher plants.

Plastids from etiolated maize leaves provided

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FIGURE 10

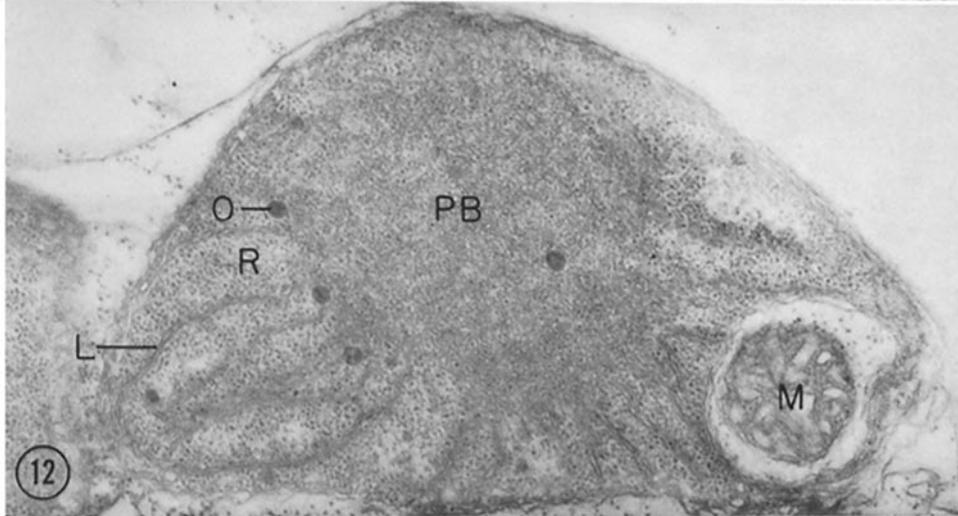
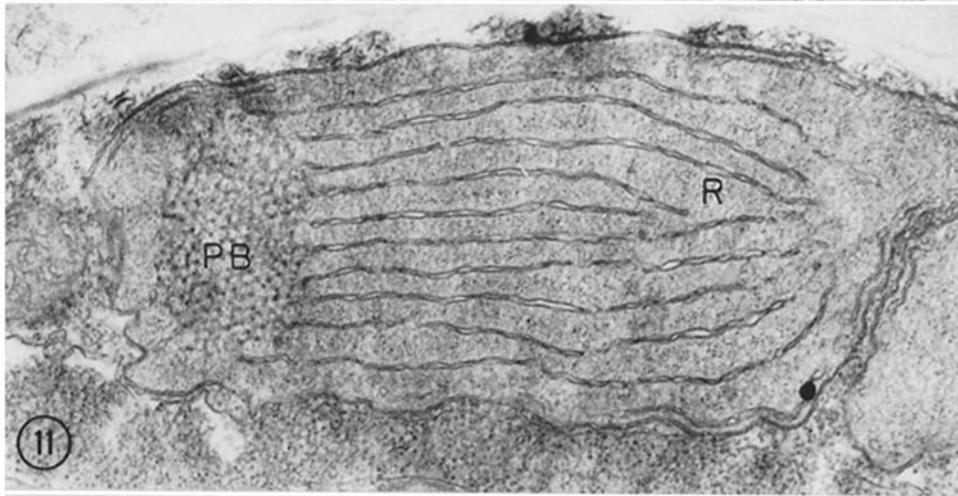
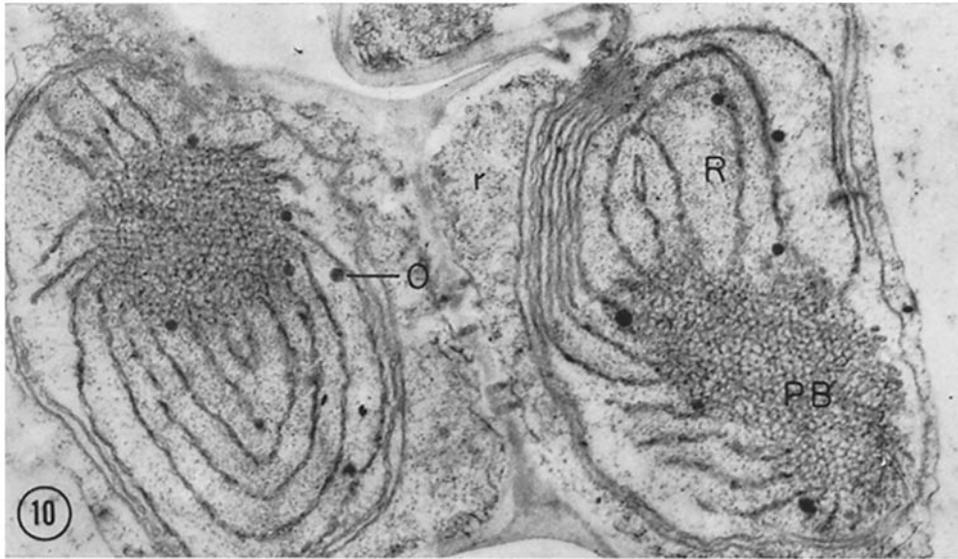
Two proplastids from an etiolated leaf which was fixed with 1 per cent  $\text{OsO}_4$ . Particulate material (*R*) within the plastid appears similar to cytoplasmic ribosomes (*r*). In these sections lamellae are clearly visible. The prolamellar body (*PB*) appears vesicular, and the osmiophilic granules (*O*) appear electron-dense.  $\times 23,000$ .

FIGURE 11

Proplastid similar to that seen in Fig. 10. Note, however, the different appearance of the particulate material (*R*) within the plastid when compared with adjacent cytoplasmic ribosomes or particulate material (*R*) in Fig. 10. *PB*, prolamellar body.  $\times 31,000$ .

FIGURE 12

Proplastid from a leaf which was fixed in 10 per cent formalin followed by  $\frac{1}{2}$  hour in 1 per cent  $\text{OsO}_4$ . Note the presence of particulate material (*R*) within the plastid. Lamellae (*L*) are more visible than in Fig. 5, and osmiophilic granules (*O*) appear moderately dense. A mitochondrion (*M*) appears to be within the plastid in this section, demonstrating the irregular nature of the chloroplast surface. *PB*, prolamellar body.  $\times 35,000$ .



exceptionally favorable material with which to reexamine this problem. Plastids from such leaves are quite large although they still lack the lamellar organization normally associated with green plastids of similar dimensions. Cytochemical examination revealed that they contained large amounts of RNA which could be further characterized as being associated with 170 A particles under the electron microscope. On the basis of these findings it was possible to extend these observations to mature plastids, and here too a particulate, RNA-containing component was visible. In the green plastids, however, the concentration of RNA-containing particles was greatly reduced when compared with plastids from the etiolated leaves.

The precise nature of the RNA-containing particles of maize plastids is not yet clear. However, Lyttleton (18) has recently reported the isolation of ribosomes from spinach chloroplasts. In our material, evidence for the ribosomal nature of the RNA-containing particles is still lacking, since it should be demonstrated that these particles are truly functional in protein synthesis before they can be identified as ribosomes. It is probable that the particles we have described

are similar to those reported by Lyttleton, since chloroplasts have also been reported as able to synthesize their own protein (10, 33).

Since it is not yet obvious that ribosomal RNA can function as a replicating template, it was disappointing to find all the demonstrable nucleic acid in maize plastids as RNA particulates. Attempts to localize DNA in the plastids from etiolated leaves with either the standard or azure A-Feulgen reaction have thus far led to negative results at the light microscope level. The presence of 25 A fibrils possibly representing DNA was recently reported in electron micrographs of *Chlamydomonas* by Ris (29). In corn, uranyl acetate staining of ribonuclease-treated material did not reveal any stainable fibrils in etiolated plastids. In nuclei, DNA-containing structures were clearly visible under similar treatment. Thus, although it is now clear that RNA occurs in maize plastids, the mechanism by which chloroplasts maintain any genetic autonomy is not yet obvious.

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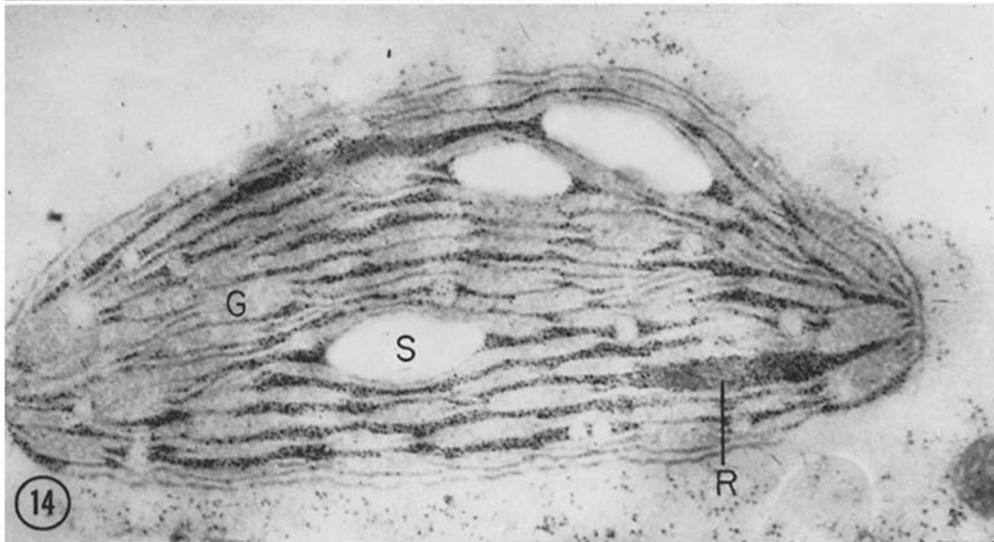
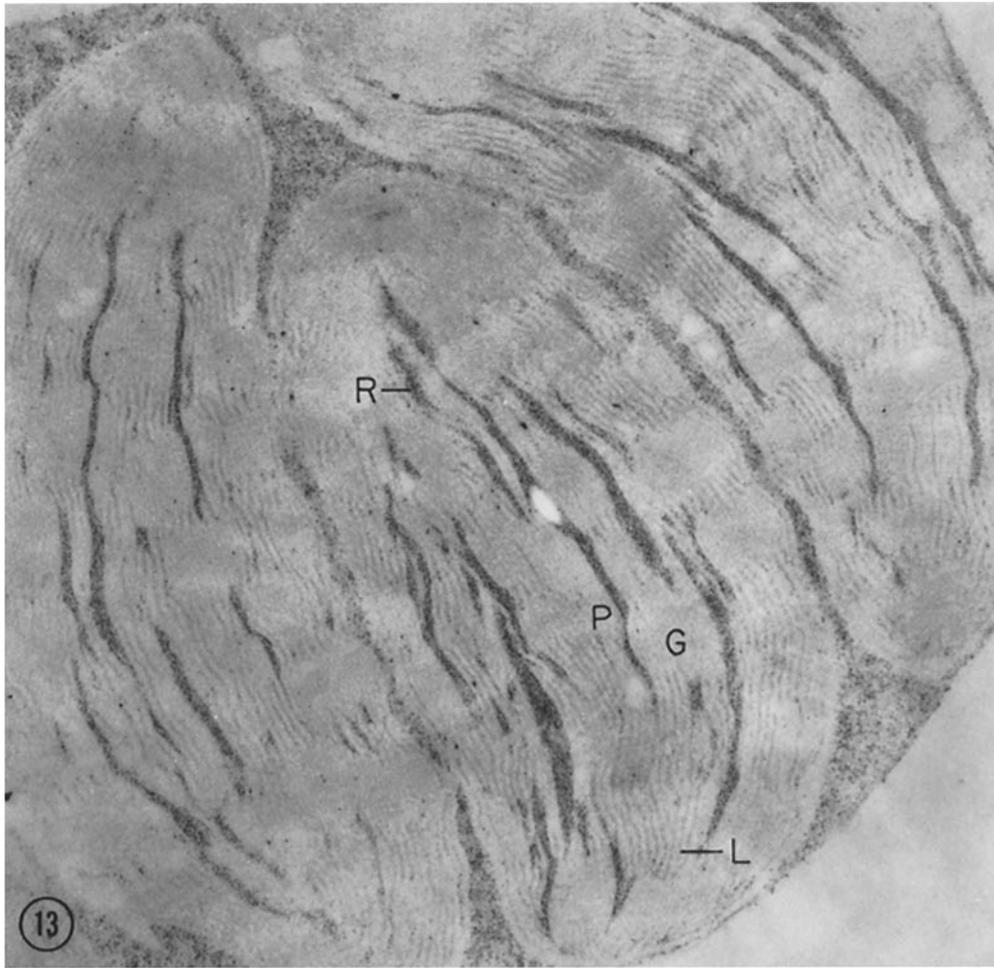
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FIGURE 13

Mature plastid (P) from a mesophyll cell of a leaf fixed with formalin. Note the lack of electron density of lamellae (L) obtained with this fixation procedure. Although grana (G) are still visible, the stroma in these plastids is seen to contain particles (R) similar to those described in plastids of etiolated leaves in Figs. 5 and 7.  $\times 26,000$ .

FIGURE 14

The same as Fig. 13. Note the clear appearance of starch grains (S) and osmiophilic granules (O). G, grana; R, particulate component.  $\times 31,000$ .



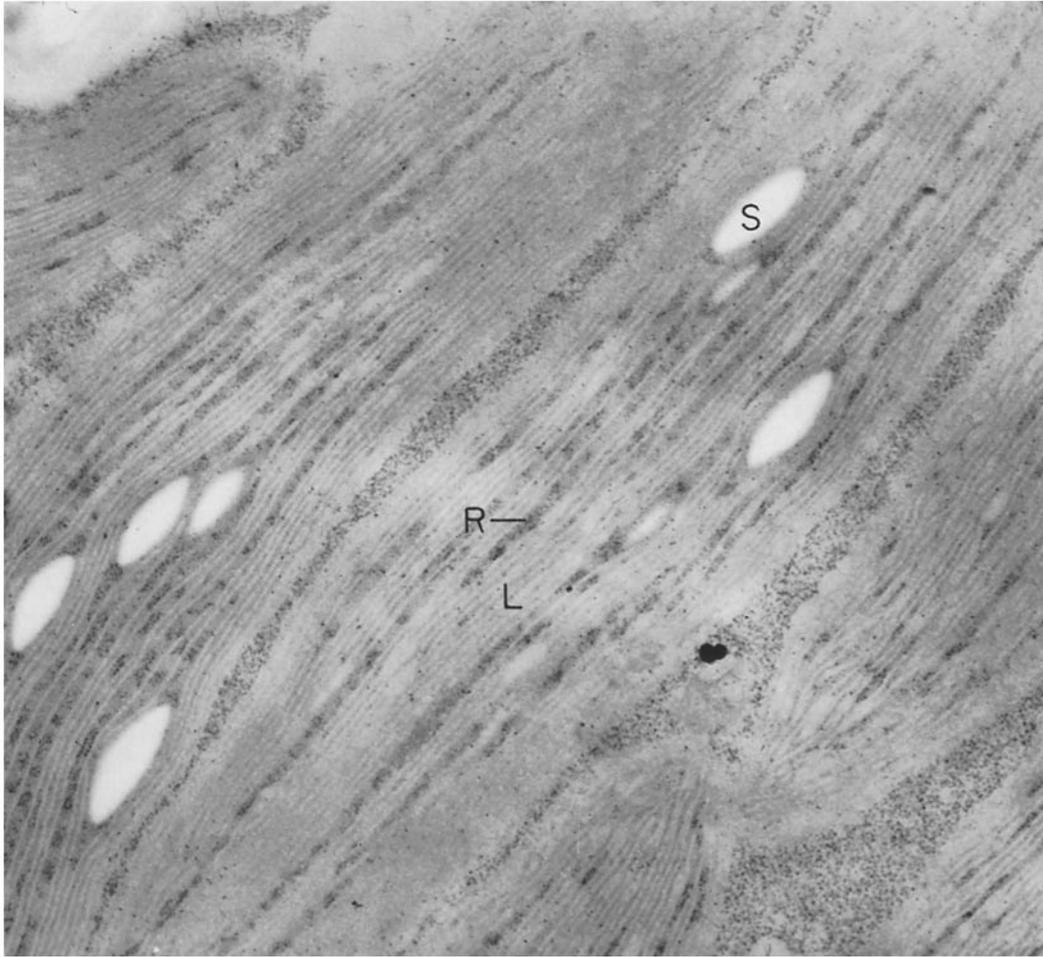


FIGURE 15

Chloroplasts found in the cells of the vascular sheath. The leaf was fixed in formalin. Lamellae (*L*) do not appear electron-dense. Grana are not visible. The particulate component (*R*) is clearly visible in the stroma. *S*, starch grains.  $\times 27,000$ .

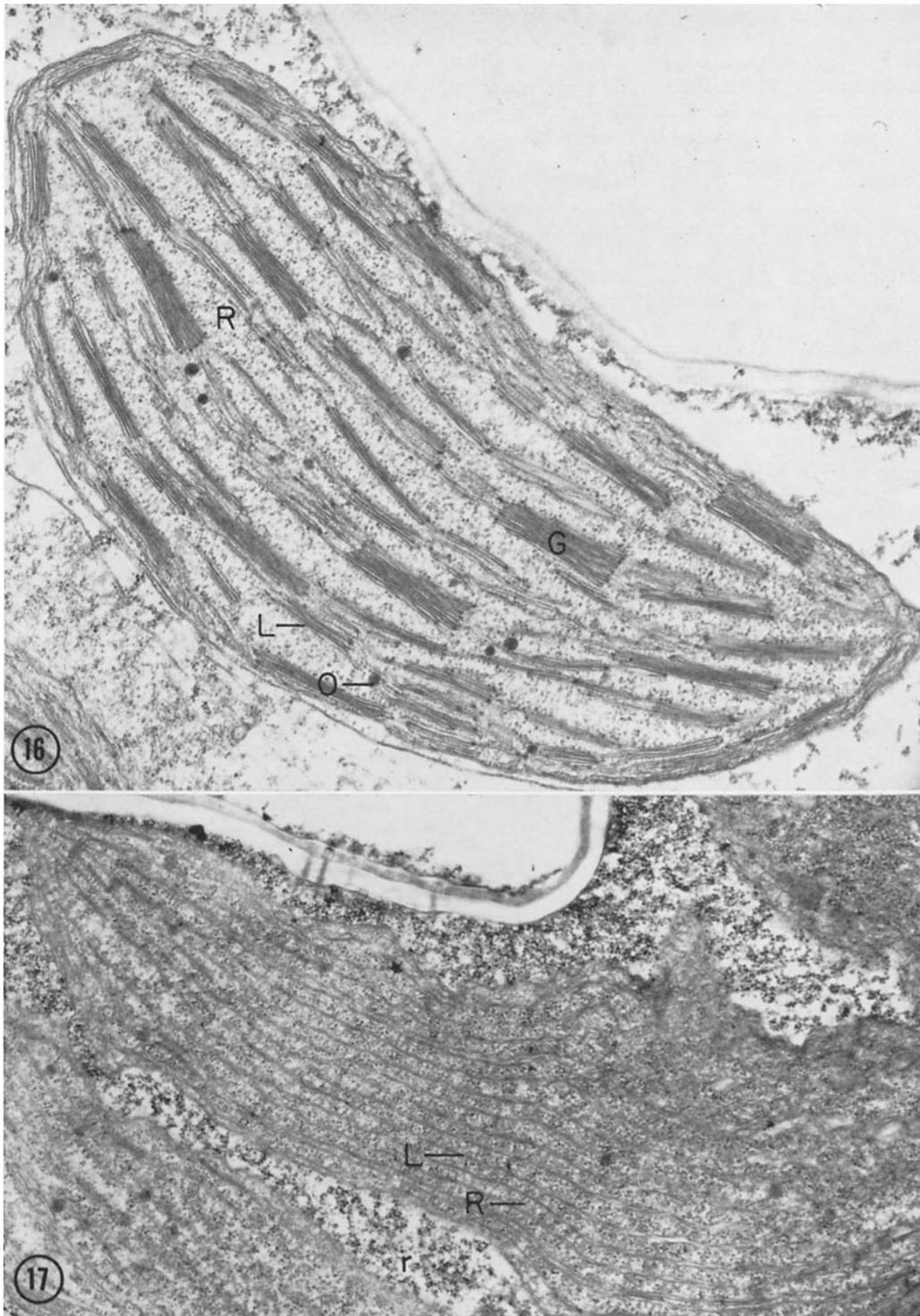
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FIGURE 16

Mature plastid from mesophyll cell of a leaf which was fixed with  $\text{OsO}_4$ . Lamellae (*L*) and grana (*G*) are present. Osmiophilic granules (*O*) appear dense. The particulate component (*R*) is visible throughout the stroma.  $\times 47,000$ .

FIGURE 17

Chloroplast found in a cell of the vascular sheath. The leaf was fixed with 1 per cent  $\text{OsO}_4$ . Lamellae (*L*) extend the entire length of the plastid. No grana are visible. The particulate material (*R*) of the stroma is visible but appears less dense than the surrounding cytoplasmic ribosomes (*r*).  $\times 47,000$ .



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