NUCLEIC ACIDS OF CHLOROPLASTS AND MITOCHONDRIA IN SWISS CHARD

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ABSTRACT

Nucleic acids in young leaves of Swiss chard have been studied by light and electron microscope techniques. Leaf DNA has also been characterized by density gradient centrifugation and shown to contain a minor band of higher guanine plus cytosine (GC) content, presumably attributable to chloroplasts. The chloroplasts were faintly stained by the Feulgen reaction; radioautography demonstrated the incorporation of tritiated thymidine in the cytoplasm and in some nuclei. The Feulgen stainability and most of the radioactivity were removable with DNase. Under the electron microscope, both mitochondria and chloroplasts were found to contain filamentous and particulate components within the matrix areas. The morphology of the filamentous component was dependent on the fixation, being partially clumped after OsO_4 or formalin, but finely filamentous after Kellenberger fixation. The filaments were stainable with uranyl acetate, and were extractable with DNase following formalin fixation under conditions in which nuclear DNA was also extracted. The particulate component, after formalin fixation and uranyl staining, was prominent in chloroplasts from young leaves, but was only sparsely distributed in mitochondria. The stainability was removed with ribonuclease. We have concluded that chloroplasts and mitochondria of Swiss chard possess a filamentous component that contains DNA, probably responsible for both cytoplasmic thymidine incorporation and the minor band in CsCl centrifugation. A particulate ribosome-like component that contains RNA is also present.

Mitochondria and chloroplasts share a number of characters not shown by other cell organelles. They contain internal lamellar systems, and are bordered by double membranes. At least in some cases new mitochondria arise from preexisting mitochondria (Manton, 1959; André, 1962; Luck, 1963), and chloroplasts often have been observed to undergo binary fission (Schimper, 1883; Manton, 1959). Undoubtedly related to this mode of reproduction is the fact that both structures display patterns of independent inheritance, as indicated by "petites" in yeast (Ephrussi, 1953; Yotsuyanagi, 1962) or the "iojap" mutant in maize (Rhoades, 1943). The concept that these organelles have arisen in evolution as intracellular parasites or symbionts has been advanced several times in the history of cytology, from the "bioblast" theory of Altmann (1890), to the recent speculations of Ris (1961).

There have been a number of reports of the occurrence of protein synthesis, as well as DNA and RNA, in mitochondria. For a number of years Chèvremont and coworkers (see 1963 for review) have described certain mitotic inhibitors, *e.g.* DNase II, trihydroxymethyl indole, or cold treatment, as producing modified spherical mitochondria that are Feulgen positive and incorporate tritiated thymidine. The kinetoplasts of the parasitic flagellate *Trypanosoma* and related genera have been known to be Feulgen positive for many

years (Bresslau and Scremin, 1924) and to incorporate tritiated thymidine (Steinert *et al.*, 1958). Recently, Steinert (1960) has shown the Feulgen-positive body to lie within a single large mitochondrion near the base of the flagellum. This body somewhat resembles a bacterial nucleus, containing a mass of fine fibrils presumed to be DNA. Similar fine fibrils have been reported in mitochondria of a flagellate (Ris, 1962), chick embryo tissues (Nass and Nass, 1962, 1963 *a* and *b*), and maize root tips (Chrispeels *et al.*, 1963). The presence of ribosome-like particles within mitochondria has been described for rat liver (Rendi, 1959, Watson and Aldridge, 1964), yeast (Mundkur, 1961), and *Tetrahymena* (Swift *et al.*, 1964). Also, biochemical studies have demonstrated the incorporation of labeled amino acids into proteins of isolated mitochondria of *Tetrahymena* (Mager, 1960), and also of rat heart (Craddock and Simpson, 1961) and liver (Roodyn *et al.*, 1961). One certainly cannot, with present evidence, do more than suggest that mitochondria show some of the characteristics expected from a partially autonomous genetic system. That they also contain enzymes known to be under regular nuclear control is obvious.

The reports concerning nucleic acids and protein synthesis in chloroplasts are somewhat similar, although more extensive and probably more convincing. DNA in chloroplasts has been reported by a wide variety of workers, employing cell fractionation (Biggins and Park, 1961; Chun *et al.*, 1963; Leff *et al.*, 1963; Sager and Ishida, 1963; Gibor and Izawa, 1963; Baltus and Brachet, 1963; Kirk, 1963, 1964), or light microscope and electron microscope techniques (Ris and Plaut, 1962; Ris, 1962). The presence of RNA in chloroplasts, occurring, at least in part, as a ribosome-like particulate fraction, now seems well established. We have discussed the literature concerning this component in a previous paper (Jacobson *et al.*, 1963). Evidence for protein synthesis in preparations of isolated chloroplasts has been described by Stephenson *et al.*, (1956). The incorporation of C^{14}-adenine and $C^{14}O_2$ into RNA of enucleate *Acetabularia* has been attributed to autonomous synthesis of chloroplast RNA (Naora *et al.*, 1960).

In the present paper, evidence is presented for the occurrence of DNA and RNA in the mitochondria and chloroplasts of Swiss chard. We have employed simple techniques for nucleic acid localization, using radioautography, and electron microscope cytochemistry with formalin fixation, nuclease extraction, and staining with uranyl acetate (Swift, 1963; Swift *et al.*, 1964). We have also described two fractions of DNA, utilizing cesium chloride centrifugation (Chun *et al.*, 1963). One fraction, with a higher content of guanine plus cytosine, appears to be associated with the chloroplasts.

MATERIALS AND METHODS

Seeds of Swiss chard (*Beta vulgaris* var. cicla), variety Broad White Ribbed (Vaughan Seed Company, Chicago) were sown in washed quartz sand and supplied with nutrient solution 1a (Hoagland and Arnon, 1950), with 2 ppm of ferric iron added as sequestrene iron chelate (Geigy Chemical Corp., Yonkers, New York). After 10 to 14 days, the plants were transferred to liquid culture using the same nutrient solution (in one series the iron was omitted, to produce iron-deficient plants). Some of the plants were then grown at 25–30° on a 20-hour photoperiod at a light intensity of 1,000 foot candles. These plants are designated as "light plants." Other groups of plants were transferred to a dark room maintained at 25–30° after they had developed three pairs of leaves while growing in the light. These plants are designated "dark plants." Thus leaves with either mature or immature plastids were available for study. From the "light plants" pale green young leaves were selected no more than 1 cm in length. Samples from "dark plants" were taken 10 to 14 days after they had been placed in darkness. Only the fourth or fifth pair of leaves were selected; they varied from 1.5 to 4 cm in length, and were white to pale green in color. Similar small white leaves were collected from light plants grown in iron-deficient medium.

Radioautography

Leaves from green, etiolated, and iron-deficient plants 1 to 3 cm in length were harvested. The base of the petiole was cut and recut under water and immersed in a solution containing 50 microcuries per ml of H^3-thymidine (specific activity 6.05 C/mM, Schwartz Bioresearch, Mount Vernon, New York). After 1, 4, 8, or 24 hours under continuous illumination, small pieces were fixed for 1, 2, or 4 hours in 10 per cent formalin contained in 0.2 M phosphate buffer at pH 7.5 at 4°. The samples were dehydrated and imbedded in paraffin.

Sections 6 μ thick were mounted on albumen-coated slides, deparaffinized, and treated as follows: (1) Controls in 5 per cent trichloroacetic acid (TCA) at 5° for 15 minutes; (2) Nuclease controls in 0.003 M magnesium chloride solution for 1 hour followed by cold TCA as above; (3) DNase treatment (as described below for electron microscopy) for 1, 2, and

3 hours at 24° or 35°, in some cases following pretreatment with hot water (80° for 10 minutes); (4) RNase treatment for 1 hour; (5) Hot TCA extraction (90° for 30 minutes); (6) Hot water control (80° for 30 minutes). All nuclease extractions were followed by cold TCA, 5° for 15 minutes.

All slides were coated with Kodak AH-10 stripping film and stored for 2 weeks at 4°. At the end of this period, they were developed in Kodak D-19 at 18° for 6 minutes. Some of the slides were stained through the emulsion with cold azure B for 15 minutes.

Electron Microscopy

Tissues were fixed in either: (1) 10 per cent formalin in 0.2 M phosphate buffer at pH 7.4 or 7.6 for 1, 2, or 4 hours at 4°; (2) Kellenberger *et al.*, (1958) fixative for 1 hour at 4° followed by treatment for 1 hour in 0.5 per cent uranyl acetate; or (3) 1 per cent osmium tetroxide in 0.1 M phosphate buffer for 1 hour at 4°. Formalin-fixed tissues were transferred directly to 50 per cent, and then 70 per cent ethanol to remove some lipids and facilitate penetration of reagents. They were then transferred to water, in which frozen sections 100 μ thick were prepared with a cryostat microtome. These sections were manipulated in one of four ways: (1) Directly dehydrated; (2) Treated with ribonuclease (Worthington, Freehold, New Jersey, crystalline, 1 mg per ml adjusted to pH 6.5 with dilute NaOH); (3) Treated with deoxyribonuclease (Worthington, crystalline, 0.2 mg per ml in 0.003 M magnesium sulfate adjusted to pH 6.5 with dilute NaOH); or (4) Treated with 0.003 M magnesium sulfate at pH 6.5 but without enzyme, to serve as controls for the nuclease treatments. Both treated and control sections were immersed in a large excess of reagent at 25° for 1, 2, or 3 hours before being rinsed with water, and 5 per cent trichloroacetic acid at 4° for 30 minutes to remove hydrolyzed materials. Treatments of 1 hour were usually adequate; prolonged treatment produced some tissue damage. Sections were subsequently rinsed with cold water several times, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in Epon 812 (Luft, 1961). Completeness of the nuclease extractions was checked with azure B or the Feulgen reaction (Swift *et al.*, 1964). Embedded tissues were sectioned with glass knives, mounted on carbon-coated grids and stained with a 3 per cent solution of uranyl acetate at pH 3.8 for 1 hour. Sections of OsO_4-fixed material were stained in lead hydroxide (Karnovsky, 1961) for 5 minutes.

DNA Extraction and Density Gradient Centrifugation

Young etiolated or green Swiss chard leaves were harvested in 100-to-200-gm lots, chilled to 5°, and ground with washed sand and 1 ml of sucrose-Tris buffer (0.5 M sucrose; 0.5 M Tris-HCl at pH 8.0; 0.005 M ethylenediamine tetraacetic acid [EDTA]) per gm of tissue, at 1–5°. From the homogenate either of two fractions was prepared: (*a*) a "gross particulate" fraction; or (*b*) a "chloroplast" fraction.

The *gross particulate* fraction was obtained by filtering the suspension through two layers of cheesecloth and collecting the pellet after centrifugation at 37,000 *g* for 15 minutes. The pellet was resuspended in 3 to 6 ml of saline EDTA (0.15 M NaCl and 0.1 M EDTA at pH 8.0).

The *chloroplast* fraction was obtained by filtering the suspension through 2 to 3 layers of muslin and centrifuging the filtrate for 10 minutes at 100 *g*. The supernatant was recentrifuged for 10 minutes at 3,000 *g*, and the green pellet was resuspended in 10 ml of sucrose-Tris buffer (0.5 M sucrose, 0.05 M Tris-HCl, pH 8.0). Three-ml aliquots of this suspension were put onto continuous sucrose gradients (68 to 18 per cent sucrose in 0.05 M Tris-HCl, pH 8.0) which were then centrifuged for 60 minutes at 23,000 RPM in the SW-25 rotor of a Spinco Model-L ultracentrifuge. The green band of chloroplasts in the middle of the tube was collected and diluted with 2 ml of 0.05 M Tris-HCl, pH 8.0, per 10 ml of plastid suspension. Chloroplasts were collected by centrifugation for 15 minutes at 45,000 RPM in the No. 50 rotor of a Spinco Model-L ultracentrifuge, and the pellet was resuspended in 3 to 6 ml saline EDTA for DNA extraction. This fraction contained no intact nuclei under phase microscopy, but small organelles, apparently mitochondria or fragments of chloroplasts or nuclei, were present.

The gross particulate and chloroplast fractions were treated separately with 1 per cent sodium dodecyl sulfate to lyse organelles, and the procedure of Marmur (1961), as modified by Chun *et al.*, (1963) for the isolation of DNA was followed, except that the incubation with RNase was for 1 hour at 37°.

The isolated DNA was analyzed by centrifugation in a CsCl density gradient, using a Spinco Model-E analytical ultracentrifuge. The CsCl solution had a mean density of 1.7 gm/cm^3, and 2 to 5 μg of plant DNA, plus 0.3 μg SP01 phage DNA used as a density reference (ρ = 1.740 gm/cm^3), were used in each ultracentrifuge run. Ultraviolet absorption photographs of the centrifuge cell were taken after 17, 24, and 36 hours of centrifugation at 44,770 RPM at 25°C. Photographs of the cell at equilibrium were scanned with a Joyce-Loebel recording microdensitometer (Mark III-B). Densities of DNA bands were calculated according to Vinograd and Hearst (1962), from runs in which there was no more than 2 μg of DNA per band.

Although a few bacteria were present in the homogenate, maximum estimates of bacterial cell number were 2×10^5 cells per preparation, far below the minimum number (10^8) estimated by Vinograd and

Hearst (1962) to be necessary to contribute sufficient DNA to be seen in a CsCl gradient.

RESULTS

LIGHT MICROSCOPY AND RADIOAUTOGRAPHY: The distribution of DNA in young leaves was studied with the Feulgen reaction and by radioautography with tritiated thymidine. There was a very faint but detectable stain in chloroplasts, evident with both the basic fuchsin and azure A Feulgen reactions (Fig. 1). The stain was removable with DNase under conditions that also abolished nuclear staining. In tissues fixed for 1 hour, stainable components were largely removed after 2 hours in DNase, and completely extracted after 3 hours. Tissues fixed for 2 or 3 hours required 3 hours in DNase, or a pretreatment of 10 minutes in water at 80° followed by 2 hours in DNase, for complete extraction of all Feulgen-positive material.

Leaves labeled with tritiated thymidine gave strongly positive autographs in cytoplasm and some nuclei after a 2-week exposure. Many nuclei in the mature leaf contained the 4C and 8C amounts of DNA, as indicated by their larger volume and more intense Feulgen stainability (Fig. 1). It is probable that the labeled nuclei were undergoing DNA synthesis in the production of these larger cells. Other nuclei were completely unlabeled. Cytoplasmic labeling was quite uniform, in all cells of the tissue, whether the nucleus was labeled or not. It was not possible to tell whether the label was limited to chloroplasts and mitochondria, but these organelles were always present in the labeled areas. Vacuolar contents were unlabeled (Figs. 2 and 3). Tissues extracted with hot trichloroacetic acid sufficiently to abolish all Feulgen stainability before coating with emulsion were negative, except for occasional clusters of grains outside the epidermal cells at the surface of the leaf, probably attributable to radioactivity in the proteins of adhering bacteria. Tissues ex-

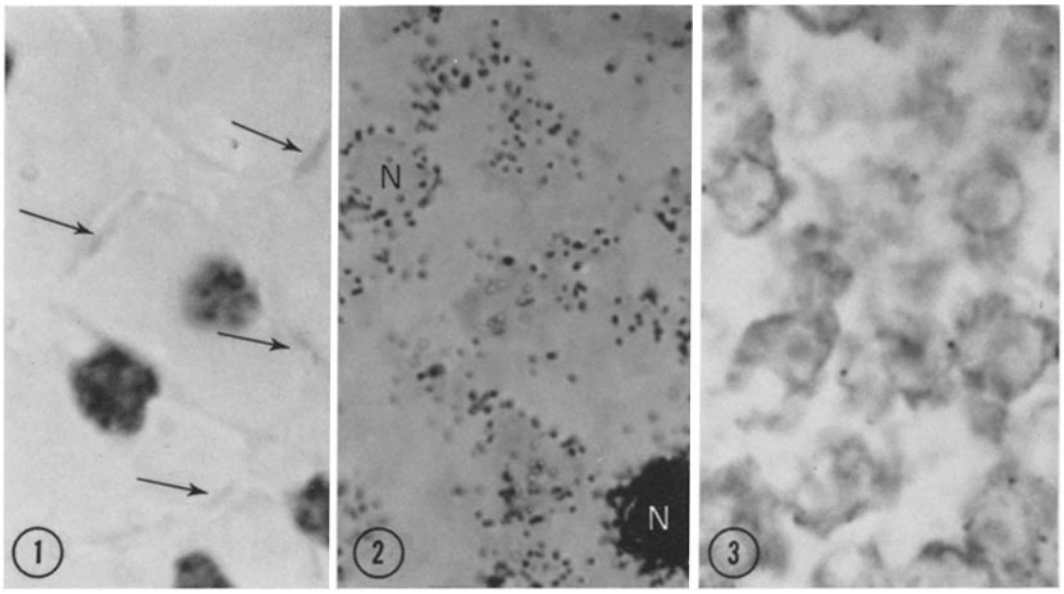


FIGURE 1 Portion of a young Swiss chard leaf stained with the azure-A Feulgen reaction. The largest nucleus is in DNA class 4C, and others are in class 2C. Chloroplasts (arrows) are faintly Feulgen positive. × 2,200.

FIGURE 2 Radioautograph with tritiated thymidine, showing one unlabeled and one strongly labeled nucleus (*N*) and cytoplasmic label. × 2,200.

FIGURE 3 Similar section to that shown in Fig. 2, except that tissue sections were extracted with hot water and DNase before stripping. The tissue contains only a slight and barely significant label. × 2,200.

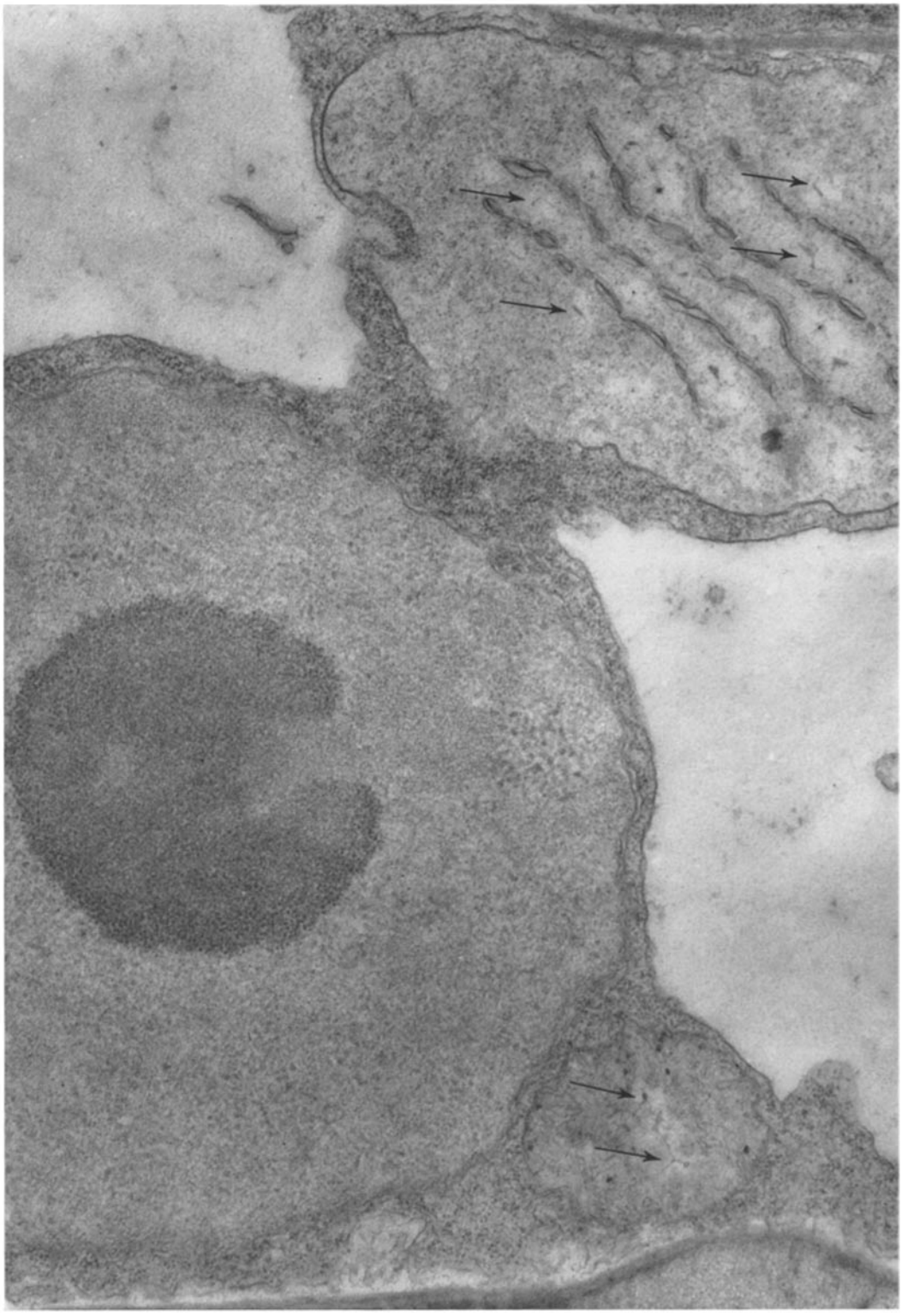


FIGURE 4 Portion of an iron-deficient leaf, fixed in osmium tetroxide and stained with lead hydroxide. Small filamentous inclusions in chloroplast (above) and mitochondrion (below) are shown by arrows. × 26,000.

tractable with DNase showed a significant reduction in grain density, but some nuclear and cytoplasmic label remained, possibly because of incomplete nuclease extraction, or the presence of some label in a non-nucleic acid, hot TCA-extractable component. In slides treated with cold TCA, ribonuclease, or hot water alone, the label was present.

As in studies on etiolated maize leaves (Jacobson *et al.*, 1963), chloroplasts in both dark- and light-grown young leaves were strongly stained with azure B. This basophilia was removable with RNase. Basophilia could not be ascribed to mitochondria, because of their small size, and the stainability of the surrounding cytoplasm.

ELECTRON MICROSCOPY: In the course of a study on the effects of iron deficiency on Swiss chard, a large number of inclusions were noticed inside the mitochondria and chloroplasts of young light-grown leaves. After fixation in buffered osmium tetroxide, these inclusions characteristically contained a thickened central region, often somewhat rectangular in outline, from 10 to 100 mμ across, with a few fine radiating filaments. These inclusions were similar in both chloroplasts and mitochondria, and occupied small clear areas in the matrix, between the lamellae or cristae (Figs. 4 and 5).

This component was then studied in a wide variety of leaves, grown in complete medium under various conditions of illumination. It was evident in the majority of chloroplasts and in some mitochondria from all the leaves investigated, although its detailed structure was dependent upon the fixative used. It appears probable that the chloroplasts and mitochondria not containing obvious inclusions were merely sectioned so as to miss these regions, but no careful studies of serial sections have yet been made.

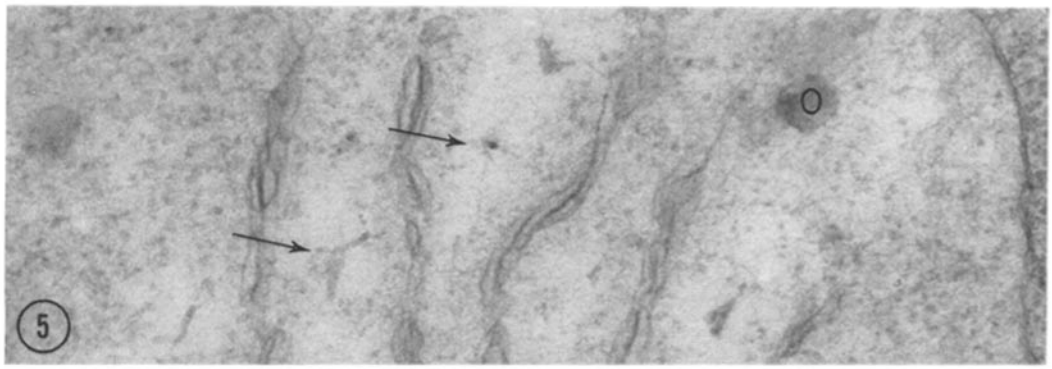
After Kellenberger fixation, the structures were finely filamentous, down to about 5 mμ in width, extending across the clear areas (Figs. 6 and 7). In some cases, the filaments were more or less straight for distances of half a micron, possibly stretched during preparation, since chloroplasts were somewhat swollen after this fixation.

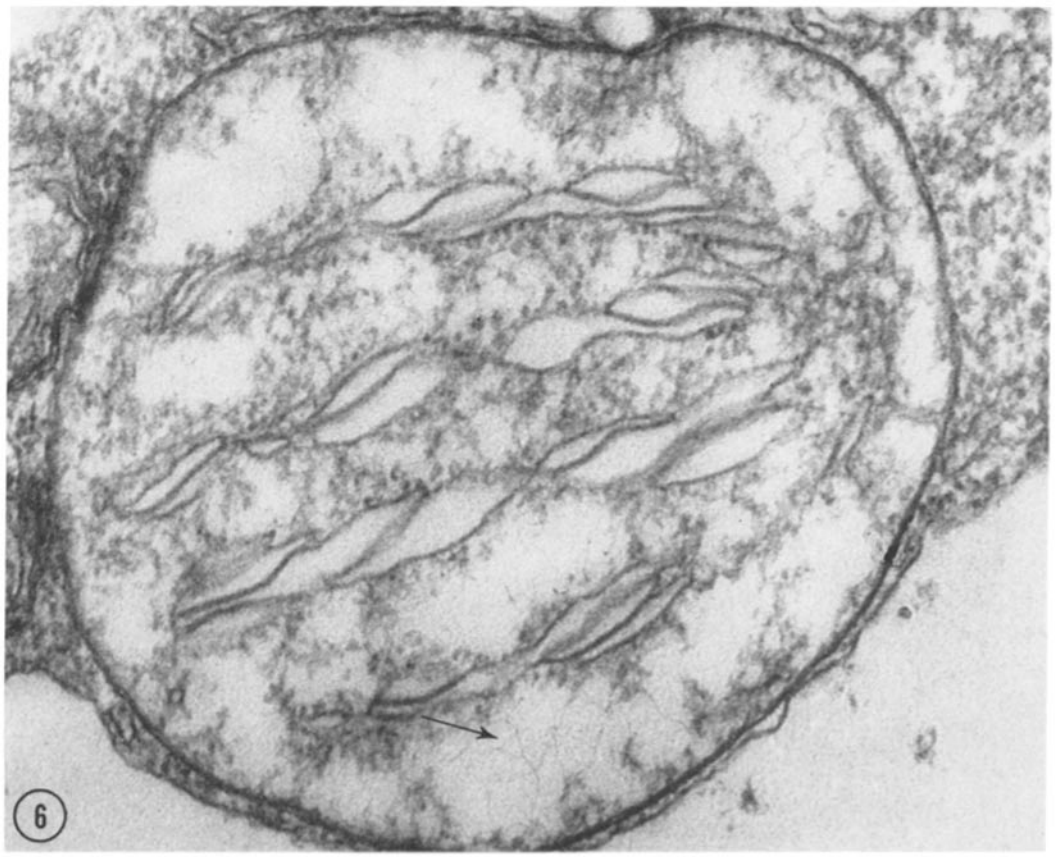
After formalin fixation, the filamentous material was irregularly clumped. It bound uranyl ions strongly, making it evident in stained but not in unstained Epon sections (Figures 8, 11, 14 to 16). In addition to the filamentous material, numerous small ribosome-like particles about 15 mμ in diameter were present in the chloroplast matrix similar to those observed earlier in maize (Jacobson *et al.*, 1963). These particles usually were absent from the immediate vicinity of the filaments. In mitochondria they occurred more sparsely, and in many cases appeared to be poorly defined or absent (Figs. 15 to 17). In addition, both chloroplasts and mitochondria exhibited a diffuse stainability with uranyl ions, particularly in the matrix regions. In the immediate vicinity of the filaments, however, this diffuse stainability was reduced, forming areas of very low electron density in which the filaments occurred. The lamellar regions were also faintly visible as lines of low density. The chloroplasts from light-grown plants also contained starch granules, which were recognizable by their ovoid shape and complete electron lucidity (Fig. 11). Clusters of spherical lucid particles up to 100 mμ across probably represented lipid materials, extracted during tissue dehydration (Figs. 19 and 20), and were doubtless identical with the osmiophilic droplets seen in leaves fixed with osmium tetroxide (Figs. 4 and 5). Also, most chloroplasts contained clusters of small electron-opaque particles, probably phytoferritin (Hyde *et al.*, 1963; Jacobson *et al.*, 1963). These particles possessed an intrinsic electron density, and were clearly evident in unstained sections (Fig. 19). Chloroplasts were thus almost always easily distinguished from mitochondria by their larger size,

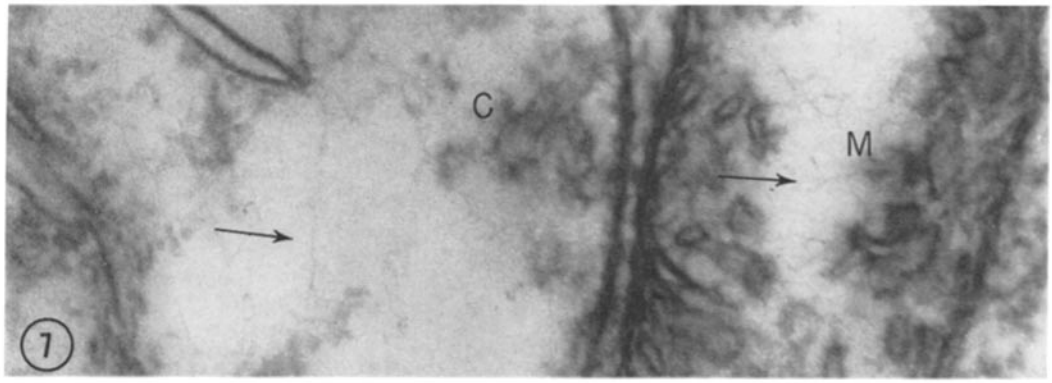
FIGURE 5 Enlargement of chloroplast region from Fig. 4, showing filamentous inclusions (arrows) and an osmiophilic droplet (*O*). × 56,000.

FIGURE 6 Chloroplast from a pale green, light-grown leaf, fixed in Kellenberger's fixative. Fine filaments are present in some portions of the matrix (arrow). × 65,000.

FIGURE 7 Same as Fig. 6, showing portion of a chloroplast at left (*C*) and of a mitochondrion at right (*M*). Note the fine filaments in the matrix areas (arrows). × 81,000.







lamellar structure, and the frequent presence of starch and ferritin. Also, in most cases mitochondria were clearly recognizable by their smaller size and the characteristic negative outline of cristae formed by the denser matrix (Figs. 15 and 17). In the rest of the cell, nuclei with strongly staining chromatin and nucleoli were evident, and the cytoplasm was filled with numerous ribosomes (Fig. 8).

In tissues treated with DNase, the staining of chromatin areas was markedly depressed, indicating that nuclear DNA had been extracted and thus was no longer available to bind uranyl ions. The staining of nucleolus and ribosomes, however, was unchanged (Fig. 10). The filamentous component was also clearly absent from the clear areas of mitochondria and chloroplasts, although the ribosome-like particles of the chloroplast matrix were still evident (Figs. 12 and 21).

In tissues treated with RNase, the nucleolus appeared merely as a lighter hole in the darkly staining chromatin, and the ribosomes and also the particles of mitochondria and chloroplasts were absent (Fig. 9). Under these conditions, the filamentous material of chloroplasts and mitochondria was clearly evident, and the extent of the network of filaments was traceable in the interlamellar regions of the chloroplasts. The outlines of chloroplasts and mitochondria were still evident from the diffuse background staining, and the clusters of phytoferritin were also clearly visible (Figs. 13, 18 to 20).

DNA DENSITY GRADIENT CENTRIFUGATION: The density distribution of DNA from a "gross particulate" and a "chloroplast" fraction is shown in Fig. 22. Band *m* at the extreme left is the reference marker of SPO1-phage DNA, with a density of 1.740 gm/cm^3. The gross particulate fraction (Fig. 22 *A*) shows a major band and a minor band of highest density. In the chloroplast fraction (Fig. 22 *B*) the minor band is markedly increased, and accounts for 30 to 40 per cent of the total DNA. The densities and calculated base ratios for the major, probably nuclear band (*n*), and the minor, presumably chloroplast band (*C*), are given in Table I.

A "chloroplast" fraction was heated for 10 minutes at 100° in 0.01 Tris-HCl at pH 7.5 before centrifugation, in an attempt to determine whether both bands represented double-stranded DNA. Only a single, apparently asymmetric band was obtained (Fig. 22 *C*). This fails to provide conclusive evidence for or against the double-strandedness of band *C*, although evidence for double-stranded satellite bands in chloroplast preparations has been obtained by Chun *et al.*, (1963) and Leff *et al.*, (1963).

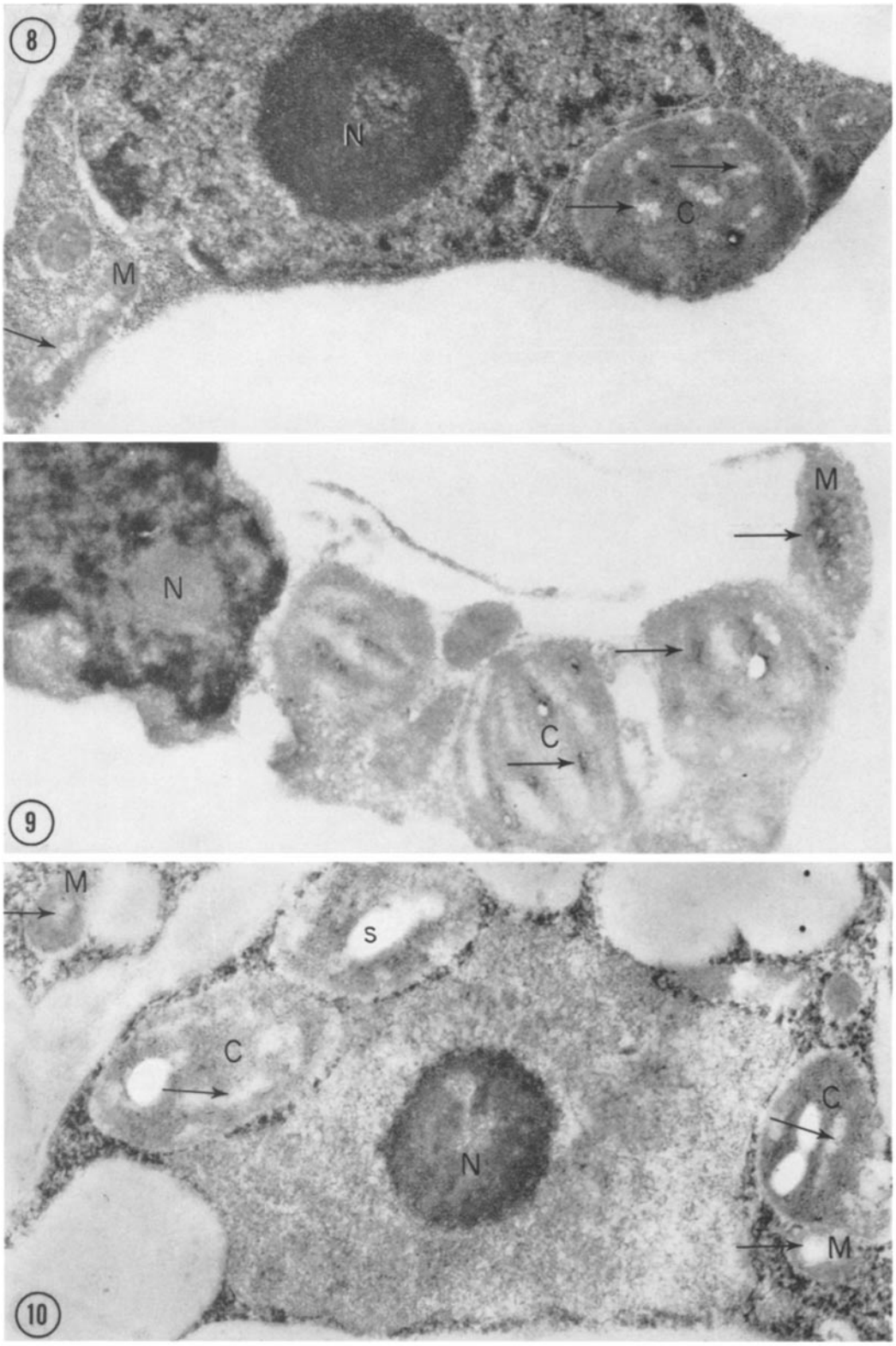
DISCUSSION

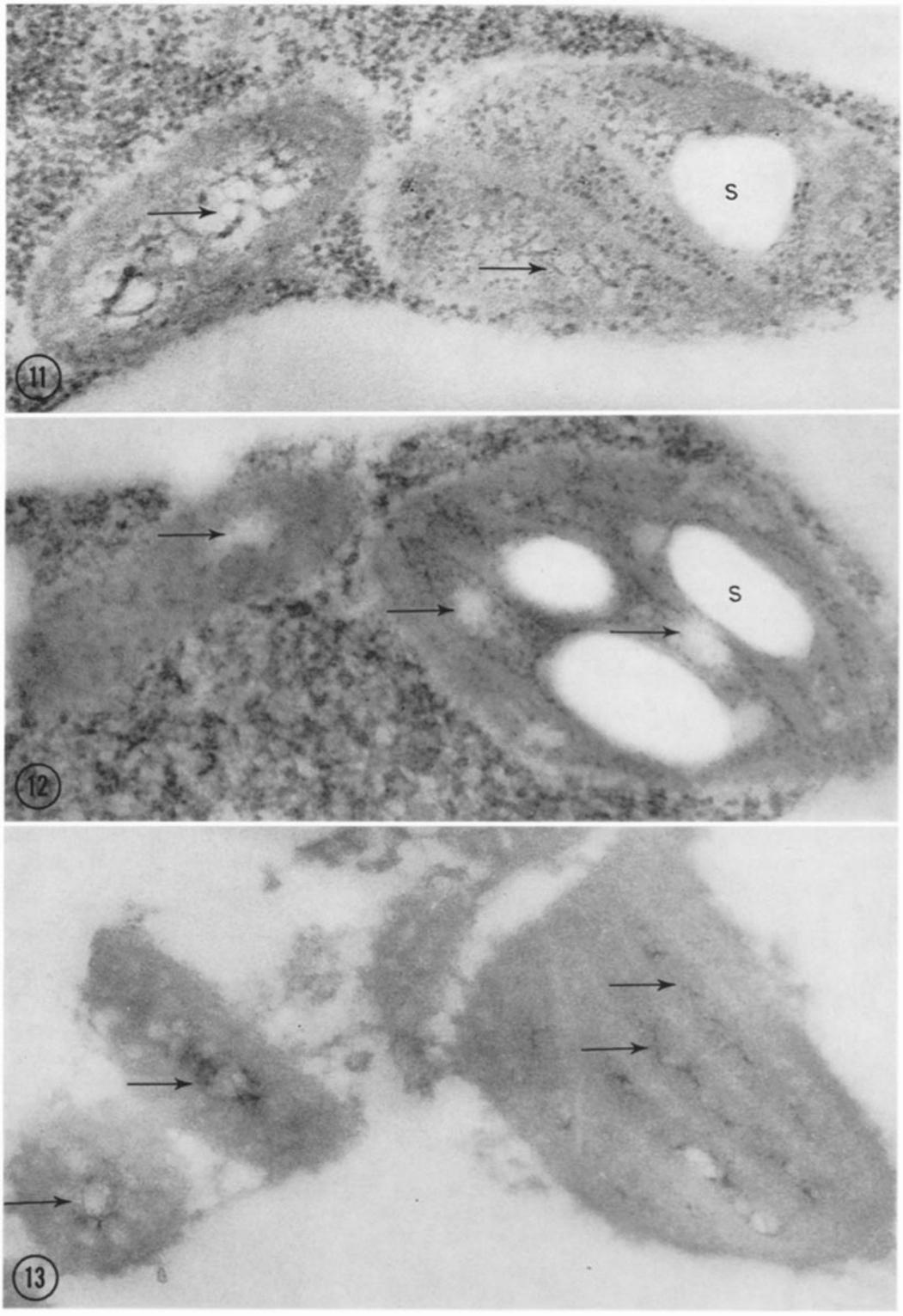
From our observations, we have concluded that there is a DNase-extractable cytoplasmic component in Swiss chard leaves which incorporates tritiated thymidine and is probably related to the minor DNA band shown in CsCl density gradients of the same tissues. Our electron microscope studies indicate that many, if not all, chloroplasts of the Swiss chard plants examined possess DNA-like filamentous material in the interlamellar areas,

FIGURE 8 Control. Portion of a pale green, light-grown leaf (the same leaf as in Figs. 6 and 7), fixed in formalin and kept in water as nuclease control for the extracted tissues shown in Figs. 9 and 10; uranyl stained. Note the mitochondria (*M*) at left (shown enlarged in Fig. 16), chloroplast (*C*) at right, and prominent nucleolus (*N*) within the nucleus. Filaments are visible at arrows. × 24,000.

FIGURE 9 RNase extracted. Similar to Fig. 8, except that the leaf was dark grown, and the RNA has been removed with RNase, so that staining in nucleolus (*N*) and cytoplasm is reduced. Nuclear chromatin and filaments (arrows) of chloroplasts (*C*) and mitochondrion (*M*) still stain. × 25,000.

FIGURE 10 DNase extracted. Portion of the same leaf shown in Fig. 8, after removal of DNA with DNase. The strong characteristic staining of chromatin areas has been markedly reduced, although the RNA staining of nucleolus (*N*) and cytoplasm is still evident. The filamentous component is absent from matrix areas of chloroplasts and mitochondria (arrows). Starch grains (*s*) are visible as lucid areas within the chloroplasts. × 24,000.





FIGURES 11 TO 13 Mitochondria at left, and chloroplasts at right, without extraction (Fig. 11), DNase extraction (Fig. 12), and RNase extraction (Fig. 13). The tissues are similar to those shown in Figs. 8, 10, and 9, respectively. Filamentous areas are shown by arrows, and starch grains by *s*. Fig. 11, × 60,000; Fig. 12, × 48,000; Fig. 13, × 52,000.

surrounded by ribosome-like particles in the matrix. Similar filaments were present in the matrix areas of mitochondria, but ribosome-like particles were usually sparse and indistinct. More clearly defined RNase-removable particles have been observed, however, in *Tetrahymena* mitochondria (Swift *et al.*, 1964). After osmium tetroxide fixation, the filamentous component appeared clumped into block-like structures. After Kellenberger fixation, which involves complexing with uranyl ions before dehydration, it was more disperse and finely fibrous. This material thus behaved somewhat like bacterial nuclei in its fixation properties (Kellenberger *et al.*, 1958).

TABLE I

Density and Base Ratios of DNA from the Major, Probably Nuclear Band (N) and Minor, Presumably Chloroplast Band (C) of Swiss Chard Leaves

Means and variation are given from three preparations.

Band	Density in gm/cm³	Moles guanine and cytosine*
		per cent
N	1.689 ± 0.002	31
C	1.700 ± 0.002	42

* Computed from $\rho = 0.100$ (GC) + 1.658 (Rolfe and Meselson, 1959); this assumes that all DNA is two-stranded and contains no unusual bases.

The DNase-removable component we have described bears a strong resemblance to the filaments described by Chrispeels *et al.* (1963) from mitochondria of maize root apices, by Ris and Plaut (1962) from chloroplasts of maize and *Chlamydomonas*, by Ris (1962) from mitochondria of *Micromonas*, and by Nass and Nass (1962, 1963 *a* and *b*) from mitochondria of chick embryo tissues. All of these workers have suggested that the fibers contained DNA on the basis of uranyl stainability. Nass and Nass also used DNase extraction, and showed that the enzyme treatment caused removal of the filaments from mitochondria. Treatment was carried out on tissues fixed in osmium tetroxide, however, in which the action of DNase may be irregular and somewhat inhibited by bound osmium (Swift, 1955).

A number of authors have found cytochemical evidence for the existence of DNA in chloroplasts, using the Feuglen reaction, methyl green, or acridine orange (Chiba, 1951; Metzner, 1952; Ris and Plaut, 1962), while others have been unable to find positive evidence (Littau, 1958). Incorporation of H^3-thymidine into chloroplasts of *Spirogyra* has been reported (Stocking and Gifford, 1959), but DNase extractions were not used to demonstrate that the label was in DNA. A DNase-extractable label from H^3-thymidine was found associated with chloroplasts of *Nicotiana* by Wollgiehn and Mothes (1963). In our material, chloroplasts were Feulgen positive in very young but not older leaves. The staining, with either the azure A or basic fuchsin Feulgen reactions, was always faint, but DNase extractable. The weak reaction is to be expected, since electron micrographs demonstrate that the filamentous component is dispersed into numerous bodies throughout the matrix area, rather than being clumped into one central mass. Radioautography with tritiated thymidine is a more sensitive method for DNA localization, provided sufficient uptake of precursor is obtained. In our material, the cytoplasmic label was distinct, indicating a considerable incorporation into a DNase-extractable component.

Although there now appears to be little doubt that DNA occurs in the chloroplasts of a wide variety of plants, and apparently also in mitochondria of some plant and animal tissues, the biological importance of this finding is far from clear. It is possible that mitochondria and chloroplasts may contain a certain episomal virus-like DNA component, capable of reproduction within the matrix areas. It is also possible that the filaments represent storage or may be fortuitous products of mitochondrial enzymes, without further biological function.

A much more compelling theory, however, is that mitochondria and chloroplasts, while still dependent on the nucleus, maintain a partial genetic autonomy. Such a situation could have arisen if these organelles represent ancient symbiotes, as suggested by Ris (1961), still bearing a remnant of their genetic mechanism, yet now built so thoroughly into the economy of the cell that they are indispensable to it. This we can call the bioblast theory, after Altmann (1890) who was among the earliest cytologists to suggest that mitochondria, plastids, and possibly other organelles were autonomous elements. It is also possible, on the other hand, that the development of mitochondria and chloroplasts in evolution involved a splitting of the genome of the cell into two separate, but dependent portions (as, for example, in the

macro- and micronuclei of ciliates), the cytoplasmic fraction being necessary to control *in situ* the morphogenesis and replication of mitochondria and chloroplasts. The similarity between the filamentous DNA component of bacteria and blue-green algae and those filamentous components shown here, together with the absence of mitochondria and chloroplasts from these primitive cells, would lend support to the concept of symbiote origin, rather than formation from the splitting of the genome.

The theory that mitochondrial and chloroplast DNA is genetically active would also explain the occurrence of ribosome-like particles, and the apparent independent ability to synthesize proteins, as the translation equipment for the genotype. It would help to explain cases of presumed mitochondrial inheritance in yeast petites (Ephrussi, 1953) and in male sterility in maize (Rhoades, 1950). The evidence for chloroplast inheritance from maize (Rhoades, 1943) and *Chlamydomonas* (Sager and Ramanis, 1963) would also support such a theory, as well as RNA synthesis in *Acetabularia* independent of the nucleus (Naora *et al.*, 1960), and the particulate nature of UV-induced bleaching in *Euglena* (Lyman *et al.*, 1961; Gibor and Granick, 1962).

If certain cell organelles, however, possess genetic mechanisms of their own, then this raises a large number of new questions. Is the filamentous component present in all chloroplasts and mitochondria, and is it required for their replication? In the trypanosomes, the filamentous DNA-containing kinetoplast is present in only one mitochondrion at the centriolar end of the cell; other mitochondria apparently lack DNA (Steinert, 1960). In many organisms both mitochondria and chloroplasts appear to "differentiate," forming clearly different structures in different cell types of the same organism. Does the DNA component play a role in this process? Also, in cells which fail to divide, such as those of the mature leaf, or the vertebrate central nervous system, does mitochondrial replacement continually occur, and if so, does it involve DNA synthesis? For answers to these broad questions, evidence is needed from a wide variety of biochemical, genetic, and cytochemical techniques.

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FIGURE 14 Chloroplast from unextracted nuclease control (same tissue as in Fig. 8), showing lamellar areas (*L*) and ribosome-like particles (*R*) in matrix. Filamentous regions are shown at arrows. × 73,000.

FIGURE 15 Mitochondria from same tissue as Fig. 14, showing filamentous regions (arrows). × 80,000.

FIGURE 16 Same as Fig. 15. This is an enlargement of Fig. 8, with the nucleus (*N*) at left. × 72,000.

FIGURE 17 Mitochondrion from an unextracted dark-grown leaf, showing scattered particulate areas in the matrix. Same leaf as Fig. 9. × 60,000.

FIGURE 18 Same leaf as Fig. 17, but after RNase extraction, showing portion of a nucleus (*N*) and two mitochondria containing filamentous regions (arrows). × 56,000.

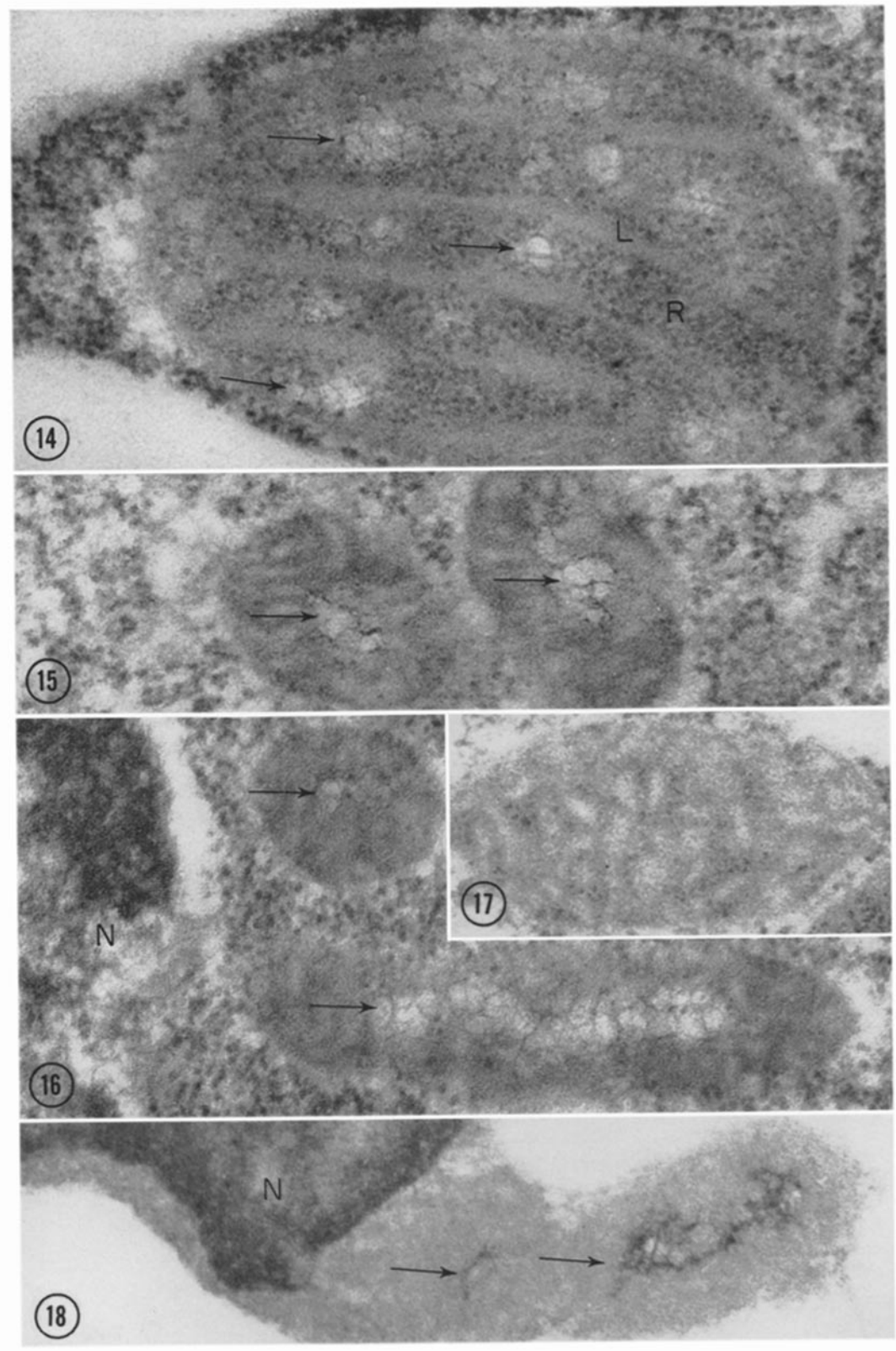
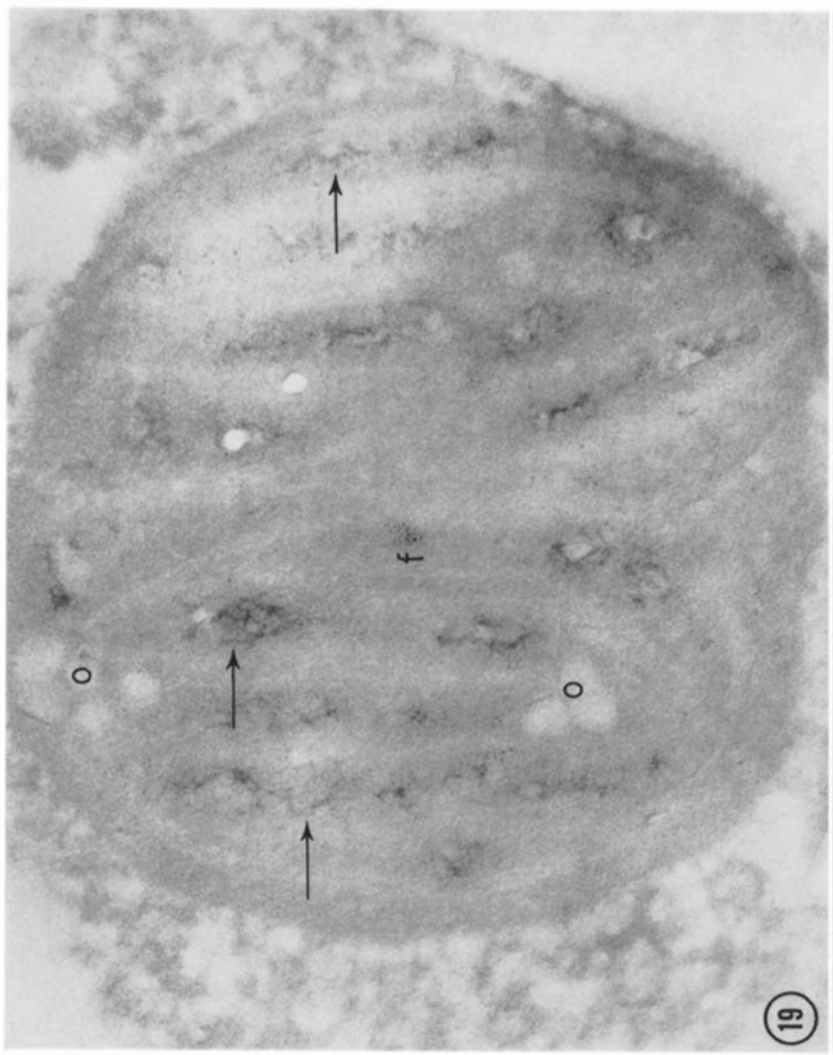
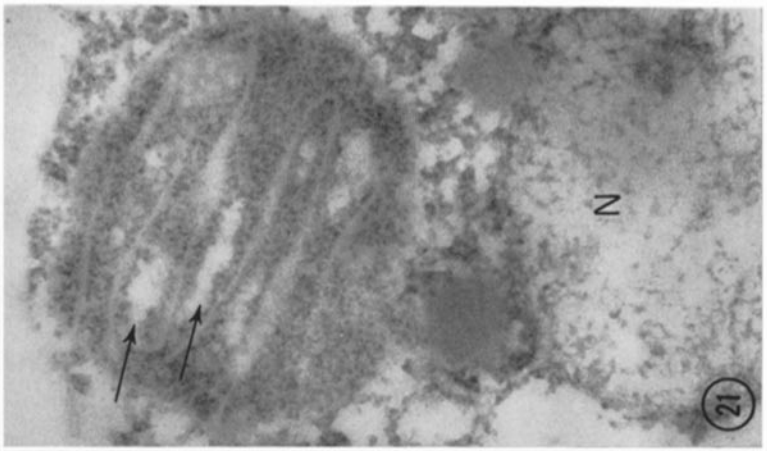


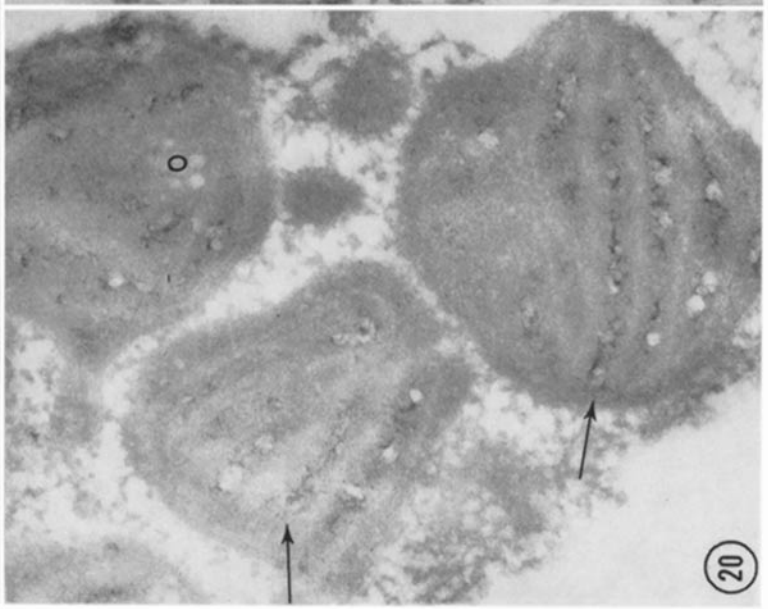
FIGURE 19 Chloroplast from a RNase-extracted dark-grown leaf (the same leaf as shown in Figs. 9, 17, and 18), showing uranyl-stainable filaments (arrows), ferritin granules (*f*) and the light holes left by extracted osmiophilic droplets (*o*). × 71,000.

FIGURE 20 Same as Fig. 19. × 49,000.

FIGURE 21 Same leaf as Fig. 19, but DNase-extracted. Staining in the nucleus (*N*) and filamentous areas (arrows) has been reduced, but ribosomes and particles of the chloroplast matrix still stain. × 49,000.







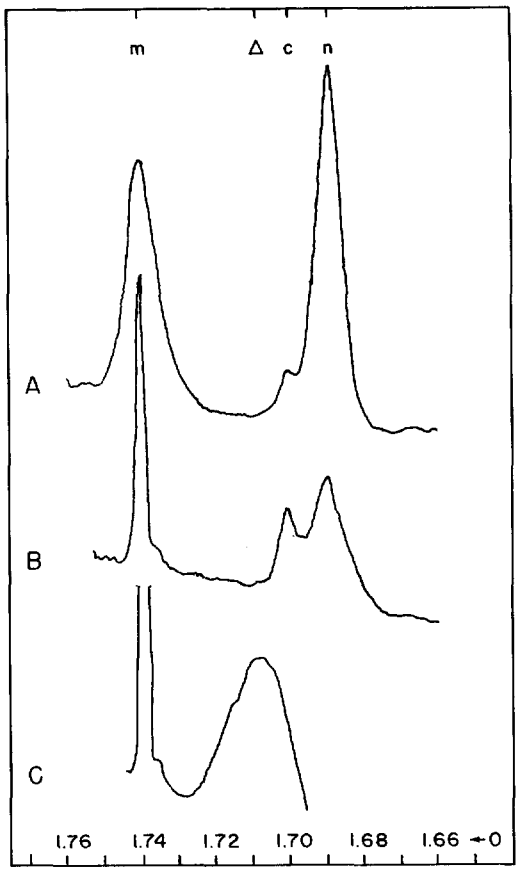


FIGURE 22 DNA preparations from Swiss chard fraction, photographed at equilibrium in an analytical ultracentrifuge with ultraviolet light, and graphed with a recording densitometer. A. "Gross particulate" fraction. B. "Chloroplast" fraction. C. "Chloroplast" fraction after heating to 100° for 10 minutes. *m* = marker band of SP01-phage DNA (ρ = 1.740 gm/cm^3). *c* = minor, presumably chloroplast band, *n* = major, presumably nuclear band, Δ = position of heated DNA maximum.

REFERENCES

ALTMANN, R., "Die Elementarorganismen" und ihre Beziehungen zu den Zellen, Veit und Comp., Leipzig, 1890.

ANDRÉ, J., Contribution à la connaissance du chondriôme, *J. Ultrastruct. Research*, 1962, suppl. **3**, 1.

BALTUS, E., and BRACHET, J., Presence of deoxyribonucleic acid in the chloroplasts of *Acetabularia mediterranea*, *Biochim. et Biophysica Acta*, 1963, **76**, 490.

BIGGINS, J., and PARK, R. B., The nucleic acid content of *Spinacea oleracea* chloroplasts, *Univ. Calif. Radiation Lab. Report*, UCRL-9900, 1961, 39.

BRESSLAU, E., and SCREMIN, L., Die Kerne der Trypanosomen und ihr Verhalten zur Nuclealreaktion, *Arch. Protistenk*, 1924, **48**, 509.

CHÈVREMONT, M., Cytoplasmic deoxyribonucleic acids, *in* Cell Growth and Cell Division, (Harris, R. J. C., editor), New York, Academic Press, Inc., 1963, 323.

CHIBA, Y., Cytochemical studies on chloroplasts. I. Cytologic demonstration of nucleic acids in chloroplasts, *Cytologia* (Tokyo), 1951, **16**, 259.

CHRISPEELS, M. J., VATTER, A. E., and HANSON, J. B., On the development of cristae mitochondriales and E. R. during growth and differentiation of the roots of *Zea mays*, *J. Plant Physiol.*, 1963, **38**, suppl., XV.

CHUN, E. H. L., VAUGHN, M. H., and RICH, A., The isolation and characterization of DNA associated with chloroplast preparations, *J. Molec. Biol.*, 1963, **7**, 130.

CRADDOCK, V., and SIMPSON, M. V., Amino acid-activating enzymes in rat liver mitochondria, *Biochem. J.*, 1961, **80**, 348.

EPHRUSSI, B., Nucleo-cytoplasmic Relations in Microorganisms, Oxford, Clarendon Press, 1953.

GIBOR, A., and GRANICK, S., Ultraviolet-sensitive factors in the cytoplasm that affect the differentiation of *Euglena* plastids, *J. Cell Biol.*, 1962, **15**, 599.

GIBOR, A., and IZAWA, M., The DNA content of the chloroplast of *Acetabularia*, *Proc. Nat. Acad. Sc.*, 1963, **50**, 1164.

HOAGLAND, D. R., and ARNON, D. I., Circular 347, *California Agr. Exp. Station*, 1950.

HYDE, B. B., HODGE, A. J., KAHN, A., and BIRNSTIEL, M. L., Studies on phytoferritin. I. Identification and localization, *J. Ultrastruct. Research*, 1963, **9**, 248.

JACOBSON, A. B., SWIFT, H., and BOGORAD, L., Cytochemical studies concerning the occurrence and distribution of RNA in plastids of *Zea mays*, *J. Cell Biol.*, 1963, **17**, 557.

KARNOVSKY, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.

KELLENBERGER, E., RYTER, A., and SECHAUD, J., Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 671.

KIRK, J. T. O., The deoxyribonucleic acid of broad bean, *Biochem. J.*, 1963, **88**, 45P (abs.).

KIRK, J. T. O., DNA-dependent RNA synthesis in chloroplast preparations, *Biochem. and Biophys. Research Comm.*, 1964, **14**, 393.

LEFF, J., MANDEL, M., EPSTEIN, H. T., and SCHIFF, J. A., DNA satellites from cells of green and aplastidic algae, *Biochem. and Biophys. Research Comm.*, 1963, **13**, 126.

LITTAU, V. C., A cytochemical study of the chloroplasts in some higher plants, *Am. J. Bot.*, 1958, **45**, 45.

LUCK, D. J. L., Formation of mitochondria in *Neurospora crassa*, A quantitative radioautographic study, *J. Cell Biol.*, 1963, **16**, 483.

LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.

LYMAN, H., EPSTEIN, H. T., and SCHIFF, J. A., Studies of chloroplast development in *Euglena*. I. Inactivation of green colony formation by UV light, *Biochim. et Biophysica Acta*, 1961, **50**, 301.

MAGER, J., Chloramphenicol and chlortetracycline inhibition of amino acid incorporation into proteins in a cell free system from *Tetrahymena pyriformis*, *Biochim. et Biophysica Acta*, 1960, **38**, 150.

MANTON, I., E. M. observations on a very small flagellate; the problem of *Chromulina pusilla* Butcher (= *Micromonas pus.*), *J. Mar. Biol.*, 1959, **38**, 319.

MARMUR, J., Procedure for the isolation of deoxyribonucleic acid from microorganisms, *J. Molec. Biol.*, 1961, **3**, 208.

METZNER, H., Cytochemische Untersuchungen über das Vorkommen von Nucleinsaüren in Chloroplasten, *Biol. Zbl.*, 1952, **71**, 257.

MUNDKUR, B., Electron microscopical studies of frozen dried yeast. II. The nature of basophile particles and vesicular nuclei in *Saccharomyces*, *Exp. Cell Research*, 1961, **25**, 1.

NAORA, H., NAORA, H., and BRACHET, J., Studies on independent synthesis of cytoplasmic ribonucleic acids in *Acetabularia mediterranea*, *J. Gen. Physiol.*, 1960, **43**, 1083.

NASS, M., and NASS, S., Fibrous structures within the matrix of developing chick embryo mitochondria, *Exp. Cell Research*, 1962, **26**, 424.

NASS, M., and NASS, S., Intramitochondrial fibers with DNA characteristics. I. Fixation and electron staining reaction, *J. Cell Biol.*, 1963*a*, **19**, 593.

NASS, S., and NASS, M., Intramitochondrial fibers with DNA characteristics. II. Enzymatic and other hydrolytic treatments, *J. Cell Biol.*, 1963*b*, **19**, 613.

RENDI, R., On the occurrence of intramitochondrial ribonucleoprotein particles, *Exp. Cell Research*, 1959, **17**, 585.

RHOADES, M. M., Genic induction of an inherited cytoplasmic difference, *Proc. Nat. Acad. Sc.*, 1943, **29**, 327.

RHOADES, M. M., Gene induced mutation of a heritable cytoplasmic factor producing male sterility in maize, *Proc. Nat., Acad. Sc.*, 1950, **36**, 634.

RIS, H., Ultrastructure and molecular organization of genetic systems, *Canad. J. Genet. and Cytol.*, 1961, **3**, 95.

RIS, H., Ultrastructure of certain self-dependent cytoplasmic organelles, Fifth International Congress of Electron Microscopy, New York, Academic Press, Inc., 1962, **2**, XX-1.

RIS, H., and PLAUT, W., Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas*, *J. Cell Biol.*, 1962, **13**, 383.

ROLFE, R., and MESELSON, M., The relative homogeneity of microbial DNA, *Proc. Nat. Acad. Sc.*, 1959, **45**, 1039.

ROODYN, D. B., REIS, P. J., and WORK, T. S., Protein synthesis in mitochondria. Requirements for the incorporation of radioactive amino acid into mitochondrial protein, *Biochem. J.*, 1961, **80**, 9.

SAGER, R., and ISHIDA, M. R., Chloroplast DNA in *Chlamydomonas*, *Proc. Nat. Acad. Sc.* 1963, **50**, 725.

SAGER, R., and RAMANIS, Z., The particulate nature of nonchromosomal genes in *Chlamydomonas*, *Proc. Nat. Acad. Sc.*, 1963, **50**, 260.

SCHIMPER, A., Über die Entwicklung der Chlorophyllkorner und Farbkorper, *Bot. Zeit.*, 1883, **41**, 105, 121, 137, 153.

STEINERT, G., FIRKET, H., and STEINERT, M., Synthèse d'acide desoxyribonuclèique dans le corps parabasal de *Trypanosoma mega*, *Exp. Cell Research*, 1958, **15**, 632.

STEINERT, M., Mitochondria associated with the kinetonucleus of *Trypanosoma mega*, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 542.

STEPHENSON, M. L., THIMANN, K. V., and ZAMECNIK, P. C., Incorporation of C^{14} amino acids into proteins of leaf disks and cell-free fractions of tobacco leaves, *Arch. Biochem. and Biophysics*, 1956, **65**, 194.

STOCKING, C. R., and GIFFORD, E. M., Incorporation of thymidine into chloroplasts of *Spirogyra*, *Biophys. and Biochem. Research Comm.*, 1959, **1**, 159.

SWIFT, H., Cytochemical techniques for nucleic acids, *in* The Nucleic Acids, (Chargaff and Davidson, editors), New York, Academic Press, Inc., 1955, **2**, 51.

SWIFT, H., Cytochemical studies on nuclear fine structure, *Exp. Cell Research*, 1963, suppl. **9**, 54.

SWIFT, H., ADAMS, B., and LARSEN, K., Electron microscope cytochemistry of nucleic acids in *Drosophila* salivary glands and *Tetrahymena*, *J. Roy. Micr. Soc.*, 1964, **83**, 161.

VINOGRAD, J., and HEARST, J., Equilibrium sedimentation of macromolecules and viruses in a density gradient, *Fortschr. Chem. org. Naturstoffe*, 1962, **22**, 372.

WATSON, M. L., and ALDRIDGE, W. G., Selective electron staining of nucleic acids, *J. Histochem. and Cytochem.*, 1964, **12**, 96.

WOLLGIEHN, R., and MOTHES, K., Über DNS in den Chloroplasten von *Nicotiana rustica*, *Naturwissenschaften*, 1963, **50**, 95.

YOTSUYANAGI, Y., Études sur le chondriôme de la levure. II. Chondriômes des mutants à deficience respiratoire, *J. Ultrastruct. Research*, 1962, **7**, 141.