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THE CHLOROPLAST NUCLEOID IN *OCHROMONAS DANICA*

I. THREE-DIMENSIONAL MORPHOLOGY IN LIGHT- AND DARK-GROWN CELLS

SARAH P. GIBBS, D. CHENG AND T. SLANKIS
Department of Biology, McGill University, Montreal H3C 3G1, Canada

SUMMARY

The 3-dimensional structure of the plastid nucleoid was determined from serial sections of the plastid of dark-grown, greening, and light-grown cells of *Ochromonas danica*. In light-grown and greening cells, the chloroplast nucleoid forms a continuous cord or ring which closely follows the rim of each lateral lobe of the chloroplast and is continuous across the top and bottom of the bridge connecting the 2 chloroplast lobes. The nucleoid always lies just inside the chloroplast girdle bands where they loop around the rim of the plastid. It was demonstrated by electron-microscopic autoradiography of greening cells labelled with [^{3}H]-thymidine that all the plastid DNA is localized in this peripheral ring-shaped nucleoid. In the proplastid of dark-grown cells, the nucleoid also forms a ring-shaped structure lying just inside the single girdle thylakoid, although frequent irregularities, such as gaps, are present. It is postulated that the girdle bands determine the shape of the chloroplast nucleoid, possibly by having specific attachment sites for the plastid DNA molecules. A survey of the literature shows that a peripheral ring-shaped chloroplast nucleoid is a characteristic feature of the 5 classes of algae whose chloroplasts possess girdle bands, namely the Raphidophyceae, Chrysophyceae, Bacillariophyceae, Xanthophyceae, and Phaeophyceae, and has never been observed in plants whose plastids lack girdle bands.

INTRODUCTION

In electron micrographs of sections of chloroplasts of most kinds of plants, the chloroplast DNA is seen to be localized in a number of separate electron-translucent areas which appear to be distributed at random throughout the chloroplast. In the few cases where the 3-dimensional organization of these scattered DNA areas has been studied by serial sections, in *Beta vulgaris* (Herrmann & Kowallik, 1970; Kowallik & Herrmann, 1972) and in the dinoflagellate, *Prorocentrum micrans* (Kowallik & Haberkorn, 1971), it has been shown that most of these DNA regions, or nucleoids, are separate bodies and are not connected to each other by strands of DNA.

In 5 closely related classes of algae, the Raphidophyceae, Chrysophyceae, Bacillariophyceae, Xanthophyceae, and Phaeophyceae, an entirely different pattern of chloroplast DNA organization is observed. In these algae, the chloroplast DNA is seen to be present at each end of the chloroplast just inside the girdle bands of thylakoids which loop around the rim of the chloroplast (Gibbs, 1970). We postulated (Gibbs, 1968) that in 3 dimensions, the chloroplast nucleoid of these algae would have the form of a continuous cord or ring encircling the rim of the chloroplast.

This was confirmed from an analysis of serial sections for a phaeophycean alga, *Sphacelaria*, by Bisalputra & Bisalputra (1969). In the present study, we have employed serial sections to determine the 3-dimensional structure of the plastid nucleoid in dark- and light-grown cells of the chrysophycean alga, *Ochromonas danica*. We have also used electron-microscopic autoradiography to demonstrate that chloroplast DNA is localized only in the regions of the chloroplast identified on morphological grounds as chloroplast nucleoid.

MATERIALS AND METHODS

Culture conditions

Stocks of *Ochromonas danica* Pringsheim were obtained from the Culture Collection of Algae at Indiana University (Culture no. 1298). All experimental cultures were grown at 29 °C in complete medium (Aaronson & Baker, 1959) either in the dark or under a bank of fluorescent and incandescent lamps adjusted to give a light intensity of 3890–4850 lux (360–450 ft-c.) at the culture surface. Under these conditions dark-grown cells show exponential growth up to 3×10^6 cells/ml, whereas light-grown cells grow exponentially up to 1 to 2×10^7 cells/ml. Cell counts were made with a haemocytometer or with a Coulter Counter, model ZB_1, equipped with a 100-μm aperture.

Ultrastructural studies

Cultures. Studies on the fine structure and 3-dimensional morphology of the chloroplast nucleoid were made on the following types of cultures: light-grown cultures in either the exponential or stationary phase of growth, dark-grown cultures in either the exponential or stationary phase of growth, and greening cultures. In the greening experiments, dark-grown cultures in the exponential phase of growth were placed in the light and allowed to green for various intervals before being fixed for electron microscopy.

Fixation and embedding. No one fixative gave satisfactory preservation of both the chloroplast DNA and other chloroplast components. Thus all cultures were fixed by 2 different techniques. One sample of cells was fixed by a standard glutaraldehyde-osmium tetroxide method. These cells were fixed in 1·25 or 2·5 % glutaraldehyde, either in Palade's (1952) acetate-veronal buffer or in 0·05 or 0·1 M sodium phosphate buffer, pH 7·2–7·4, usually for 30 min to 2 h at either room temperature or 4 °C. After 3 washes in the same buffer, the cells were postfixed in 1 % osmium tetroxide, either in Palade's acetate-veronal buffer or in 0·1 or 0·15 M sodium phosphate buffer, pH 7·2–7·4, for 1–2 h at room temperature or 4 °C. A second sample of cells was fixed by a modified Ryter & Kellenberger (1958) method. These cells were fixed for 2·5 h at room temperature in a solution of 2 % osmium tetroxide and 0·1 % $CaCl_2$ in acetate-veronal buffer, pH 7·3, to which 0·1 % tryptone was added at time of use. After 3 rinses in acetate-veronal buffer, the cells were postfixed in an aqueous 0·5 % uranyl acetate solution, pH 4·5, for 2–3 h at room temperature. Cells fixed by both methods were dehydrated in a graded ethanol series, followed by propylene oxide, and embedded either in Epon 812 (Luft, 1961) or in Spurr's (1969) low-viscosity epoxy resin. All sections were stained with lead citrate (Venable & Coggeshall, 1965) or lead tartrate (Millonig, 1961) or occasionally with uranyl acetate followed by lead citrate. Sections were viewed with a Philips EM 200 electron microscope.

Serial sections. Serial sections were made of the dark-grown, greening, and light-grown cells fixed by the glutaraldehyde-osmium tetroxide method, and also of dark-grown cells fixed by the Ryter & Kellenberger method. Blocks were trimmed to give a rectangular face approximately $0·2 \times 0·05$ mm, and unbroken ribbons of sections (10–45 sections long) were cut using a Dupont diamond knife and a Porter-Blum MT-2 ultramicrotome. The ribbons of sections were picked up on Formvar-coated single-slot grids. Consecutive sections of a given cell were photographed in sequence with missed sections being indicated by a blank frame on the film.

Model building. Since the 3-dimensional structure of the plastid nucleoid in dark-grown cells

is less regular than in light-grown cells, models of serial sections through 10 different proplastids of cells fixed by Ryter & Kellenberger method were constructed. Prints were made of each section, usually at a magnification of 51 200 times, and the chloroplast envelope and the thylakoids were traced on transparent plastic sheets with a fine black felt-tip pen. The DNA areas were shaded in red. The tracings were cut out, arranged in order, and correctly aligned with each other by matching main membrane patterns. Where a section had been missed, a blank plastic sheet was inserted. The resultant stack of plastic sheets was securely taped together, and holes were drilled through the stack on a drill press. The tracings were threaded sequentially on copper wires with pieces of transparent Tygon tubing inserted between each sheet to represent the thickness of the section. The path of the proplastid nucleoid could be easily followed in these models.

Electron-microscopic autoradiography

A culture of greening cells which had been in the light for 5·5 h and contained $5{\cdot}1 \times 10^6$ cells/ml was collected by centrifugation at 800 *g* and resuspended at an 8-fold concentration in 5 ml of growth medium containing 50 μCi/ml of [*Me*-^{3}H]thymidine (The Radiochemical Centre, Amersham; sp. act., 14·9 Ci/mmol). The cells were incubated in the light for 18 h in the presence of isotope. Because the cells had been concentrated, they did not divide, but chloroplast development, as measured by chloroplast volume, chlorophyll per cell, and thylakoid formation, continued normally. After rinsing in growth medium, the cells were fixed in 2·5 % glutaraldehyde in 0·1 M potassium phosphate buffer, pH 7·2, for 3 h at 4 °C, rinsed for 1·5 h in 4 changes of cold buffer, and postfixed in 2 % osmium tetroxide in 0·1 M potassium phosphate buffer for 2 h at 4 °C. After blocking in agar, the cells were dehydrated in ethanol and propylene oxide and embedded in Araldite (Glauert & Glauert, 1958). Silver sections were picked up on collodion-coated nickel grids and coated with Ilford L-4 nuclear emulsion by the loop method of Caro & van Tubergen (1962). The grids were stored over Drierite at 4 °C for 14 months and developed in D-19 for 5 min at 18 °C. Sections of cells were photographed at random and micrographs printed at a final magnification of 16000 times. In ascribing a silver grain to one organelle or another, an arbitrary rule was made that a grain belonged to an organelle if over half of the grain fell within the organelle. However, since the diameter of a section of chloroplast nucleoid (0·25 μm) is smaller than the length of a silver grain (0·3–0·5 μm), this rule could not be applied to the chloroplast nucleoid. Instead a grain was counted as originating in the nucleoid if it overlapped any part of the nucleoid. If, however, a grain just touched the edge of the nucleoid, it was counted as belonging to the remainder of the chloroplast. To determine grains/unit area, each micrograph was traced in its entirety on high-quality tracing paper; then the individual cell organelles were cut out, collected together, weighed, and their areas calculated.

RESULTS

Fine structure of the chloroplast nucleoid

Light-grown cells of *Ochromonas danica* contain a single large chloroplast which consists of 2 flattened lateral lobes, which are frequently bilobed at their posterior end, and which are connected to each other by a broad bridge dorsal to the cell's nucleus. Fig. 1 is a cross-section through a lateral lobe of the chloroplast in a cell fixed by the standard glutaraldehyde-osmium tetroxide method. With this fixation the matrix material is very well preserved, so well preserved that the chloroplast ribosomes are almost completely obscured (cf. fig. 7 in Smith-Johannsen & Gibbs, 1972). At each end of the chloroplast section there is an area of very low electron density which lies just inside the girdle bands of thylakoids (*gb*) where they loop around the rim of the chloroplast. Since these areas of low electron density are the regions of the chloroplast where DNA-like fibrils are observed and where electron-microscopic autoradiography

shows the chloroplast DNA to be localized (see below), they will be referred to as sections of the chloroplast nucleoid. In electron micrographs of glutaraldehyde and osmium tetroxide-fixed cells, it is usually possible to see a few clumped fibres of DNA in sections of the nucleoid. For example, DNA-like fibrils can be seen in the left-hand nucleoid in Fig. 1, in the uppermost nucleoid of Fig. 2 and in the upper section of the proplastid nucleoid in Fig. 4.

However, to visualize clearly the DNA fibrils of the chloroplast nucleoid, it is necessary to fix the cells by the method of Ryter & Kellenberger (1958). Fig. 3 is a cross-section of the chloroplast nucleoid in a greening cell fixed by this method. Numerous fine DNA fibrils are present. Most of the fibrils are single, measuring 2·5–4·0 nm in diameter, although at places several fibrils appear to be intertwined with each other. Chloroplast ribosomes are, as a rule, excluded from the interior of the chloroplast nucleoid, although an occasional cluster of ribosomes may be found within the nucleoid (Fig. 3, arrow). Numerous chloroplast ribosomes are present along the inner face of the chloroplast nucleoid (right side of Fig. 3; left side of figs. 2 and 3 in Gibbs, Mak, Ng & Slankis, 1974). Sometimes a DNA fibril appears to make contact with a cluster of ribosomes at the nucleoid's periphery (Fig. 3, double arrow).

Three-dimensional shape of the chloroplast nucleoid in light-grown cells

Since in every section cut perpendicularly to the plane of a chloroplast lobe, an electron-translucent area is seen at each extremity of the chloroplast just inside the girdle bands, it was postulated that the chloroplast nucleoid had the shape of a continuous cord which encircled the rim of the chloroplast (Gibbs, 1968). An analysis of more than 60 sequences of serial sections, of 10–30 sections each, through different regions of light-grown chloroplasts indicates that this is the case for most chloroplasts. Fig. 6 is a series of 10 consecutive sections through the posterior extremity of one lobe of a light-grown chloroplast. In Fig. 6A, the nucleoid (*n*) is sectioned at each extremity of the chloroplast lobe. In Fig. 6B and C, the nucleoid is also sectioned in what appears to be the middle of the chloroplast lobe (arrows). Actually, however, the chloroplast is about to branch into 2 lobes at this point, so what appears to be a centrally located piece of chloroplast nucleoid is actually a peripherally located piece. In Fig. 6D and E, the apparently centrally located section of chloroplast nucleoid splits into 2, and in Fig. 6F, the chloroplast has split into 2 lobes, each with 2 peripherally located nucleoid regions. By Fig. 6H, the 2 nucleoid areas of the lower chloroplast lobe have joined each other; in other words, the section is cut parallel to the chloroplast nucleoid. Fig. 6I is also cut parallel to the chloroplast nucleoid of the lower lobe. Fig. 6J has passed beyond the nucleoid region of the lower lobe and cuts through the membranes of either the girdle thylakoids or the chloroplast envelope. In the subsequent section (not shown) the lower lobe of the chloroplast was no longer present. Many series of sections similar to this have been observed.

A few series of sections through the bridge region of the chloroplast were also obtained. Fig. 7 is a representative one. Since this series consisted of 17 consecutive serial sections, it was necessary to leave out some of the unessential sections in the

montage. The number and location of the omitted sections are indicated by the alphabetical lettering. Fig. 7A is a section through the two lobes of the chloroplast just ventral to the bridge region. The nucleus is still present between the posterior ends of the 2 lobes. In Fig. 7C, the 2 chloroplast lobes are first joined by a narrow bridge. In Fig. 7F–K, the bridge becomes deeper along its anterior-posterior axis. At the same time, as the width of each bridge section decreases, the 4 nucleoid areas move closer to each other (Fig. 7G–L). In Fig. 7M, the 2 nucleoid areas at the top of the chloroplast bridge become one, and in Fig. 7O, the 2 nucleoid areas at the bottom of the bridge merge. Fig. 7O is the last section of this series, but presumably the next section would have cut tangentially through the membranes behind both sections of the nucleoid and by a section or two later, the chloroplast bridge would have completely disappeared from the section.

These 2 series of sections, in conjunction with the numerous others studied, give a clear picture of the 3-dimensional structure of the chloroplast nucleoid. The chloroplast nucleoid is a continuous cord or ring which closely follows the rim of each flat lateral lobe of the chloroplast and is continuous across the top and bottom of the chloroplast bridge. It should be emphasized that in 3 dimensions the entire rim of the 2 chloroplast lobes and their connecting bridge is completely enclosed by girdle bands (Figs. 6, 7) and the chloroplast nucleoid lies just inside these girdle bands at the chloroplast rim.

Occasionally, in some light-grown cells, a chloroplast section is seen which has 2 sets of girdle bands at one of its extremities. In such sections, an electron-translucent DNA area is usually seen lying just inside each set of girdle bands (Fig. 2). Whenever we found such an image, serial sections were photographed in order that we could determine the 3-dimensional structure of the chloroplast nucleoid in these anomalous chloroplasts. In several cases, the single nucleoid was split in 2 when the second set of girdle bands appeared with one part of the nucleoid continuing throughout the entire series and the other ending abruptly as a blind spur. In one series, the second set of girdle bands completely interrupted the nucleoid in such a way that in the whole chloroplast, the nucleoid presumably had the shape of a ring with 2 overlapping free ends. In the remaining 10 series obtained, the 2 sets of girdle bands with 2 parallel nucleoids continued for the entire length of the series. In 3 dimensions, the nucleoid of these chloroplasts could have been a ring with a blind spur or an overlapping ring as described above, or possibly a continuous ring with an open loop. The important thing, however, about these irregularities in nucleoid structure is that they are never observed except when there are 2 sets of girdle bands at the chloroplast extremity instead of the normal single set.

Three-dimensional shape of the plastid nucleoid in dark-grown and greening cells

Sections of proplastids of dark-grown cells typically contain a fenestrated single thylakoid, the girdle thylakoid (Fig. 4, *gt*). In the least-differentiated proplastids, the girdle thylakoid tends to be single and centrally located as it is in the proplastid illustrated in Fig. 4. In more differentiated proplastids, the girdle thylakoid tends to lie closer to the periphery of the proplastid and also to have a second thylakoid

appressed to it over parts of its circumference. A few internal thylakoids or thylakoid bands may also be present. The chloroplast DNA in dark-grown proplastids is always found lying just inside the girdle thylakoid (Fig. 4). Usually the proplastid nucleoid is sectioned twice suggesting that even in proplastids the nucleoid has the shape of a ring. To verify this, a number of models were made of series of sections through entire or almost entire proplastids. Fig. 5 is a model constructed from one of these series. The DNA region is shaded in dark grey (the black dots are the eyespot granules). It can be seen that in this proplastid the nucleoid has the shape of a continuous cord encircling the periphery of the proplastid. Although the nucleoids of all proplastids examined (30 series) had this same basic shape, many more abnormalities were observed than in the chloroplasts of light-grown cells. Often the nucleoid had a gap in it for a number of sections. Sometimes a region of the nucleoid would be separated from the rest of the nucleoid by gaps on both sides. Sometimes the nucleoid in one lobe of a proplastid had the form of an open ring, but in the other lobe was a solid rod filling the entire area enclosed by the centrally located girdle thylakoid.

These irregularities of nucleoid structure of dark-grown proplastids are lost early in chloroplast development. A solid rod rapidly opens out into a peripheral ring as a chloroplast lobe expands and the girdle thylakoid moves peripherally. Gaps in the chloroplast nucleoid are almost never observed in cells which have had more than 6 h of light. As the chloroplast grows, the peripherally located nucleoid must, of course, become much longer and presumably also increase in volume. These quantitative aspects of nucleoid development are the subject of the following paper (Gibbs *et al.* 1974).

Evidence that chloroplast DNA is localized only in the peripheral ring-shaped nucleoid

Two kinds of evidence indicate that chloroplast DNA is localized only in the nucleoid region of the chloroplast, which is identified in glutaraldehyde and osmium tetroxide-fixed cells by its very low electron density. First of all, in both dark- and light-grown cells fixed by the Ryter & Kellenberger method which preserves DNA fibrils, all DNA-like fibrils are found in the regions of the plastid which would appear electron-translucent after glutaraldehyde-osmium fixation. Since most of the dense material of the chloroplast matrix is lost during Ryter & Kellenberger fixation, if any DNA fibrils were present in the chloroplast matrix, they should be clearly visible. An occasional single fibril is sometimes found in the chloroplast matrix, but never the network of fibrils characteristic of plastid DNA.

Secondly, an electron-microscopic autoradiographic study was made of greening cells of *Ochromonas* which were labelled with [*Me*-^{3}H]thymidine for 18 h in the light and then fixed by the glutaraldehyde-osmium method. Digestion of aldehyde-fixed cells with deoxyribonuclease established that virtually all the cell grains arose from labelled DNA (Gibbs & Poole, 1973). Table 1 gives the results obtained. It can be seen that the chloroplast nucleoid is very heavily labelled, whereas the concentration of grains over the remainder of the chloroplast is only slightly above the background level. These data show that at most only a very small percentage of the total chloroplast DNA could be located outside the chloroplast nucleoid. Since grains which

just touched the nucleoid were not counted as nucleoid grains, but could very well have arisen from radioactive DNA within the nucleoid, it is most likely that all chloroplast DNA is localized in the peripheral ring-shaped nucleoid.

Table 1 also indicates that the nucleolus as well as the non-nucleolar regions of the nucleus contains DNA and that all the extrachloroplastic cytoplasmic DNA is localized in the mitochondria.

Table 1. *Distribution of grains among different cell organelles after labelling greening cells with [^3H]thymidine for 18 h*

Cell organelle	Per cent of total cell grains*	Grains/100 μm^2 of organelle sectioned
Nucleoplasm	23	106
Nucleolus	1	28
Chloroplast		
Chloroplast nucleoid	19	972
Remainder of chloroplast	5	7
Mitochondria	16	33
Remaining cytoplasm	16	8
Digestive vacuoles	3	12
Leucosin vacuole	17	5
Background	—	4

* Determined from 245 random cell sections containing 1238 grains.

DISCUSSION

Classes of algae in which the chloroplast DNA is organized in a peripheral ring-shaped nucleoid

Although this is only the second publication to demonstrate by means of serial sections that the chloroplast nucleoid may have the form of a peripheral ring-shaped cord, a survey of the literature on the ultrastructure of algae shows that the presence of a peripheral ring-shaped chloroplast nucleoid is a characteristic feature of 5 closely related classes of algae, namely the Raphidophyceae, Chrysophyceae, Bacillariophyceae, Xanthophyceae, and Phaeophyceae. These 5 classes of algae also differ from all other groups of algae in having one or more bands of thylakoids, the girdle bands, called girdle lamellae by many authors, which loop around the rims of their chloroplasts.

The presence of a ring-shaped chloroplast nucleoid is best documented for members of the Phaeophyceae. Bisalputra & Bisalputra (1969) have shown by serial and tangential sections that the chloroplasts of the brown alga, *Sphacelaria*, have a circular peripheral nucleoid. In a later paper, Bisalputra & Bisalputra (1970) mention that they have evidence that the chloroplast DNA in *Ectocarpus*, *Pylaiella*, *Sorocarpus*, and *Dictyota* is also located in a peripheral ring. The fact that the chloroplasts of *Dictyota dichotoma* have a ring-shaped nucleoid is confirmed by fig. 5 in Evans & Holligan (1972). In this figure, a number of chloroplasts are cut in the plane of the

nucleoid and the nucleoids can be seen to be present for varying distances around the rims of these chloroplasts. Similarly, in a published micrograph of another brown alga, *Zonaria farlowii* (Neushul & Dahl, 1972, fig. 7), several chloroplasts which are sectioned in the plane of the chloroplast nucleoid are seen to have an electron-translucent DNA area extending approximately half-way round the chloroplast. Thus, there is little doubt that at least 6 species of brown algae have a ring-shaped chloroplast nucleoid. In addition, micrographs of a variety of other species of brown algae show that electron-translucent areas in which DNA-like fibrils can often be seen are present at each end of the chloroplast lying just inside the girdle band. In 3 dimensions, these areas probably form a continuous ring. Such peripheral DNA areas can be seen in the published micrographs of *Himanthalia lorea* (Berkaloff, 1963), *Giffordia* (Bouck, 1965), *Alaria esculenta* (Evans, 1966), *Laminaria digitata* (Evans, 1966), *Ishige foliacea* (Yokomura, 1967), *Sargassum horneri* (Yokomura, 1967), *Egresia menziesii* (Bisalputra & Bisalputra, 1967*b*), *Leathesia difformis* (Cole & Lin, 1968), *Fucus vesiculosus* (McCully, 1968), *Ascophyllum nodosum* (Evans, 1968), *Pelvetia canaliculata* (Evans, 1968), *Halidrys siliquosa* (Evans, 1968), *Cystoseira tamariscifolia* (Evans, 1968), and *Himanthalia elongata* (Evans, 1968).

In the case of *Egregia menziesii*, Bisalputra & Bisalputra (1967*b*) claim that each chloroplast contains 2 separate DNA areas, one at each end of the chloroplast. To prove this assertion, they published 3 sections of the same chloroplast, one through the membranes of the chloroplast tip external to the chloroplast nucleoid (fig. 5), one through the nucleoid at the chloroplast tip (fig. 6), and a third section some distance from the chloroplast tip in which the authors claim no chloroplast DNA is present (fig. 7). However, inspection of this micrograph (fig. 7, Bisalputra & Bisalputra, 1967*b*) clearly shows that at the top half of the chloroplast, there are 2 sets of girdle bands each with a DNA area lying just inside it. The other extremity of the chloroplast is not included in the micrograph, but had it been, the chloroplast nucleoid would doubtless have been sectioned. Thus, at present there is no evidence for any brown alga not having a ring-shaped chloroplast nucleoid.

The present paper has shown by serial sections that the chloroplast of one member of the Chrysophyceae, *Ochromonas danica*, has a single continuous cord-like nucleoid encircling its rim. Although the chloroplasts of other Chrysophycean species have not been studied by serial sections, inspection of the published micrographs of other species shows that electron-translucent nucleoid areas are present at each extremity of the chloroplast just inside the girdle bands in *Sphaleromantis tetragona* (Manton & Harris, 1966), *Chrysococcus rufescens* (Belcher, 1969), *Olisthodiscus luteus* (Leadbeater, 1969, and personal observation), and *Ochromonas tuberculatus* (Hibberd, 1970). Presumably, therefore, these species also have a single ring-shaped chloroplast nucleoid.

Although girdle bands have been observed in the chloroplasts of a wide variety of chrysophycean species (Gibbs, 1970), Hibberd (1971) has recently shown that the chloroplast of the chrysophycean alga, *Chrysamoeba radians*, does not possess girdle bands. Unfortunately, it is not possible to ascertain from Hibberd's (1971) published micrographs where the chloroplast DNA of this species is localized, but since the chloroplast bands extend all the way to the ends of the chloroplast and terminate

abruptly just inside the chloroplast envelope, there is no room at the rim of the chloroplast for a peripheral nucleoid of the size of that in *Ochromonas danica*. Thus, we suspect that in this exceptional chrysophyte which lacks girdle bands, the chloroplast DNA will prove to be scattered throughout the chloroplast. In fact, in the Haptophyceae, a class of algae which has only recently been separated from the Chrysophyceae, girdle bands are not present in the chloroplast, and in the published micrographs of 4 species belonging to this class, *Prymnesium parvum* (Manton & Leedale, 1963), *Chrysochromulina chiton* (Manton, 1966, 1967), *Hymenomonas carterae* (Pienaar, 1969), and *Chrysochromulina acantha* (Leadbeater & Manton, 1971), electron-translucent areas containing fine DNA-like fibrils can be seen scattered at random throughout the chloroplast.

Among the xanthophycean species, Bisalputra & Bisalputra (1970) noted that the chloroplasts of *Ophiocytium*, *Botrydium*, and *Tribonema* all appeared to possess a single ring-shaped nucleoid. Published micrographs of *Ophiocytium majus* (Hibberd & Leedale, 1971), *Botrydium granulatum* (Falk, 1967), and *Tribonema viride* and *T. vulgare* (Falk & Kleinig, 1968; Massalski & Leedale, 1969) show clearly that electron-translucent areas containing DNA-like fibrils lie just inside the girdle bands, so no doubt a ring-shaped chloroplast nucleoid is present in these species. Several species of the Xanthophyceae, e.g. *Bumilleria sicula*, *Bumilleriopsis filiformis*, and *Pseudobumilleriopsis pyrenoidosa*, lack girdle bands, but unfortunately in the few published micrographs of these species it is not possible to tell where the chloroplast DNA is located (Massalski & Leedale, 1969; Deason, 1971). However, in species belonging to the closely related class, the Eustigmatophyceae (Hibberd & Leedale, 1970), whose chloroplasts characteristically lack girdle bands, electron-translucent DNA regions can be seen scattered throughout the chloroplasts, e.g. in *Polyedriella helvetica* and *Pleurochloris commutata* (Hibberd & Leedale, 1972).

In the Bacillariophyceae, Drum (1969) observed that in *Amphipleura rutilans*, a region containing DNA fibrils followed the plastid rim. Likewise, a ring-shaped chloroplast nucleoid can be inferred to be present in *Nitzschia palea* (Drum, 1963), *Amphipleura pellucida* (Stoermer, Pankratz & Bowen, 1965,) *Lithodesmium undulatum* (Manton & von Stosch, 1966; Manton, Kowallik & von Stosch, 1968, 1969, 1970), *Navicula pelliculosa* (Coombs, Lauritis, Darley & Volcani, 1968), and in *Biddulphia levis* (Heath & Darley, 1972) because electron-translucent areas containing fine fibrils of DNA are seen to be present at each end of the chloroplast just inside the girdle band.

A ring-shaped chloroplast nucleoid is also apparently present in the very small class, the Raphidophyceae. In sections of chloroplasts of *Vacularia virescens* (Mignot, 1967; Heywood, 1972), a finely fibrillar clear area is always seen at each end of the chloroplast inside the girdle band.

In summary, it appears from the present literature that algae belonging to the Raphidophyceae, Chrysophyceae, Bacillariophyceae, Xanthophyceae, and Phaeophyceae possess chloroplasts which characteristically have both girdle bands and a peripheral ring-shaped nucleoid which encircles the chloroplast rim just inside these girdle bands. In the exceptional species of the Chrysophyceae and Xanthophyceae

whose chloroplasts lack girdle bands, the location of the chloroplast DNA is not yet known, but it is unlikely that it will be found to form a peripheral ring. In surveying the literature, we have to date found only one alga whose chloroplasts appear to have typical girdle bands, but whose chloroplast DNA appears to be scattered throughout the chloroplast. Van Valkenburg (1971) has shown that the chloroplasts of the silicoflagellate, *Dictyocha fibula*, contain typical girdle bands, but in her published micrographs electron-translucent areas containing DNA-like fibrils are found lying between the central lamellar bands rather than inside the peripheral girdle bands. The silicoflagellates are bizarre organisms which are sometimes classified by themselves, but are usually grouped with the Chrysophyceae (Prescott, 1968). The presence of chloroplasts with girdle bands but without a peripheral ring-shaped nucleoid may prove to be another one of their aberrant features.

Organization of chloroplast DNA in other classes of algae and in vascular plants

The chloroplast DNA in all the other classes of algae appears to be scattered at random throughout the chloroplast. In published micrographs of algae belonging to the Rhodophyceae (Gantt & Conti, 1965; Bisalputra & Bisalputra, 1967*a*; Yokomura, 1967; Brown & Weier, 1968; Ramus, 1969; McBride & Cole, 1969; Lichtlé & Giraud, 1969; Evans, 1970; Wehrmeyer, 1971; Seckbach, 1971; McDonald, 1972; Scott & Dixon, 1973; Chamberlain & Evans, 1973; and Gordon & McCandless, 1973), Cryptophyceae (Wehrmeyer, 1970; Gantt, Edwards & Provasoli, 1971; and Lucas, 1970), Dinophyceae (Dodge, 1967; Dodge, 1968; Babillot, 1970; Schmitter, 1971; Kowallik & Haberkorn, 1971; and Dodge & Crawford, 1971), Haptophyceae (references above), Eustigmatophyceae (references above), Euglenophyceae (personal observations on *Euglena gracilis*), Prasinophyceae (Oschman, 1966; Belcher, 1968; and Manton, 1969), and the Chlorophyceae (references too numerous to cite), one can clearly see that electron-translucent areas containing DNA-like fibrils lie scattered in the stroma of the chloroplast. The 3-dimensional organization of these scattered DNA areas has only been studied in one dinoflagellate, but presumably in all these classes of algae, the chloroplast contains a number of separate nucleoids. In the dinoflagellate alga whose chloroplast has been studied by serial sections, *Prorocentrum micans*, Kowallik & Haberkorn (1971) have observed discrete DNA areas which have no connexions with neighbouring DNA areas. On the basis of their studies, Kowallik & Haberkorn estimate that each of the cell's 2 large multilobate chloroplasts contains between 80 and 100 separate nucleoids. Presumably in species of algae with smaller chloroplasts, many fewer nucleoids are present.

It should be emphasized that in none of these groups of algae which have scattered DNA areas in their chloroplasts have true girdle bands been observed (Gibbs, 1970). Occasionally a chloroplast is seen in which a band of thylakoids by chance encircles one of its ends in the plane of section, as has been observed in some of the chloroplasts of the symbiotic dinoflagellate, *Endodinium chattonii* (Taylor, 1971). Such chloroplasts, however, cannot be considered to possess girdle bands. In most of the red algae studied (e.g. all the references given above except Gantt & Conti's (1965) paper on *Porphyridium cruentum*), the chloroplasts have one or more concentric single

thylakoids which encircle their periphery more or less completely. Some authors have called these concentric single thylakoids girdle thylakoids, but they are, of course, morphologically very different from the girdle bands of the Chrysophyceae and related groups of algae. The chloroplast DNA in these red algae can be seen lying between the central straight thylakoids as well as between the peripheral concentric thylakoids.

Recently 2 dinoflagellates, *Glenodinium foliaceum* (Dodge, 1971) and *Peridinium balticum* (Tomas & Cox, 1973) have been discovered to possess both a dinoflagellate, or mesokaryotic, nucleus and a eukaryotic nucleus. As Tomas & Cox pointed out, both the eukaryotic nucleus and all the cell's chloroplasts belong to an endosymbiont. These chloroplasts have typical girdle bands and DNA-like fibrils which are invariably found just inside these girdle bands. Thus, there is no doubt that these chloroplasts could not belong to the dinoflagellate host, but must belong to an alga belonging to one of the 5 classes of algae which possess girdle bands and ring-shaped chloroplast nucleoids.

The chloroplasts of all vascular plants also appear to have their DNA located in scattered electron-translucent areas. Except for very small proplastids which may have a single central DNA area (Sprey, 1968), the chloroplasts of vascular plants probably contain a number of separate nucleoids. Herrmann & Kowallik (1970) have studied the chloroplasts of *Beta vulgaris* by means of serial sections and have shown clearly that whereas some very small plastids have only a single nucleoid, larger plastids have a number of nucleoids. Furthermore, the larger the chloroplast, in general, the more nucleoids it contains (Kowallik & Herrmann, 1972). The question of whether fully differentiated chloroplasts have more DNA than undeveloped proplastids will be discussed in the following paper (Gibbs *et al.* 1974).

What determines the ring-shaped structure of the chloroplast nucleoid?

When we first postulated that the chloroplast of *Ochromonas danica* contained a single ring-shaped nucleoid (Gibbs, 1968), we thought that this might be the result of the chloroplast possessing a single large circular molecule of DNA. However, we have now shown that the chloroplast of green cells of *Ochromonas* contains at least ten molecules of DNA which segregate from each other at chloroplast division (Gibbs & Poole, 1973). Thus, in *Ochromonas*, the single ring-shaped chloroplast nucleoid possesses a number of DNA molecules which are probably distributed along its length. It is unlikely therefore that the shape of the nucleoid is directly related to its DNA content, but rather it appears that the peripheral ring-shaped structure of the nucleoid is a consequence of the presence of girdle bands. We have shown in this paper that even in the dark-grown proplastid where the girdle bands are reduced to a single girdle thylakoid, the nucleoid regions are always found directly inside this girdle thylakoid. In those proplastids where the single girdle thylakoid is centrally located, the plastid nucleoid forms a small centrally located ring-shaped structure. In those proplastids where the girdle thylakoid lies just inside the plastid's rim, the nucleoid forms a peripherally located ring.

A second reason we believe that the presence of girdle bands determines the

structure of the chloroplast nucleoid is that in those anomalous chloroplasts which have 2 separate sets of girdle bands looping around a region of the chloroplast's rim, DNA is usually present inside both sets of girdle bands. In 3 dimensions, we have shown that sometimes the second DNA area is a blind-ending spur which projects from the ring where the second set of girdle bands originates. In one case, though, the second set of girdle bands completely interrupted the ring-shaped nucleoid, so that a broken ring with 2 overlapping free ends was formed. These abnormalities in nucleoid structure are apparently the direct consequence of abnormalities in the structure of the girdle bands.

The third type of evidence that the presence of a ring-shaped nucleoid is dependent on the presence of girdle bands is the fact summarized above that a ring-shaped chloroplast nucleoid is only present in those algae which possess girdle bands. We are not aware of any exceptions to this rule.

A plausible way that the presence of girdle bands could give rise to a ring-shaped nucleoid is for the chloroplast DNA molecules to have specific attachment sites to the membrane of the inner thylakoid of the innermost girdle band in the region where it loops around the chloroplast's rim. In Ryter & Kellenberger-fixed cells of *Ochromonas*, DNA fibrils often appear to come in contact with the membrane of the adjacent thylakoid. However, these apparent points of contact may not be attachment sites at all, although both Bisalputra & Burton (1969, 1970) in their studies on *Sphacelaria* and Kowallik & Haberkorn (1971) in their study of *Prorocentrum* state that they are. Thus, for the present, the suggestion that the ring-shaped structure of the chloroplast nucleoid is determined by specifically located DNA attachment sites must remain a plausible but unproven theory.

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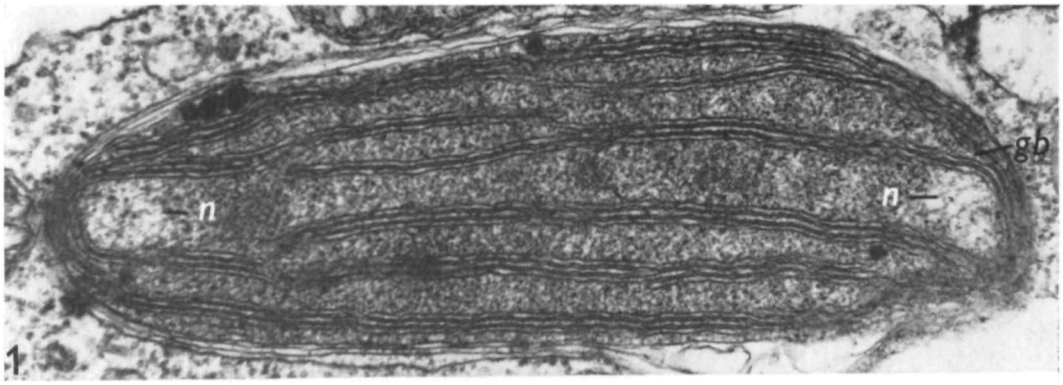
Fig. 1. Cross-section of a lobe of the chloroplast of a log-phase greening cell of *Ochromonas*. The DNA-containing region of the chloroplast, the chloroplast nucleoid (*n*), is sectioned twice and appears as electron-translucent regions of the chloroplast matrix which lie at each end of the chloroplast just inside the girdle bands (*gb*). 30 h light. $2{\cdot}5 \times 10^6$ cells/ml. Glutaraldehyde-osmium fixation. × 47400.

Fig. 2. End of a chloroplast lobe of a log-phase greening cell showing an infrequently observed organization of the girdle bands and the chloroplast nucleoid. Here two sets of girdle bands encircle the end of the chloroplast and a DNA area is present just inside each set. Characteristic DNA fibrils are just discernible in the upper nucleoid section. 30 h light. $2{\cdot}5 \times 10^6$ cells/ml. Glutaraldehyde-osmium fixation. × 39000.

Fig. 3. Cross-section of the chloroplast nucleoid in a log-phase greening cell fixed by the Ryter & Kellenberger method. Fine DNA fibrils are clearly visible. The chloroplast ribosomes are, as a rule, excluded from the chloroplast nucleoid and are seen at the border of the nucleoid, being most abundant at the interior edge of the nucleoid (right). However, at the arrow, a row of ribosomes (presumably a polysome) is seen to extend into the nucleoid. At the double arrows, a DNA fibril can be seen to be closely associated with a peripheral cluster of ribosomes. 24 h light. $1{\cdot}9 \times 10^6$ cells/ml. × 78400.

Fig. 4. Longitudinal section of one lobe of a proplastid of a log-phase dark-grown cell. A single interrupted girdle thylakoid (*gt*) is present. At this early stage of proplastid differentiation, the girdle thylakoid is located a considerable distance from the chloroplast periphery. The chloroplast DNA is present in 2 electron-translucent areas (*n*) which lie just inside the girdle thylakoid. The lower nucleoid region extends upwards towards the upper nucleoid region, but does not quite meet it in this section. Fine DNA fibrils are visible in the upper nucleoid area. $1{\cdot}1 \times 10^6$ cells/ml. Glutaraldehyde-osmium fixation. *e*, eyespot granule; *nu*, nucleus. × 45000.

Fig. 5. Model of an entire proplastid of a dark-grown cell. This proplastid was one of two in a large premitotic cell in which the original single proplastid had already divided into 2 daughter proplastids which had migrated a short distance away from each other over the surface of the nucleus (cf. Slankis & Gibbs, 1972). The chloroplast envelope and the girdle thylakoid are represented by black lines. The striations represent the girdle thylakoid where it is cut obliquely. The dark grey shaded areas (red in the original model) are the regions where DNA fibrils were present. The black granules at the top of the model are the granules of the eyespot. It can be seen that the DNA areas form a single continuous nucleoid which encircles the periphery of the proplastid. $6{\cdot}6 \times 10^6$ cells/ml. Ryter and Kellenberger fixation. × 22000.


n
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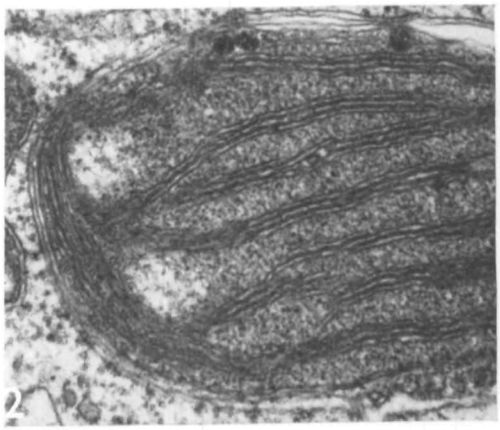
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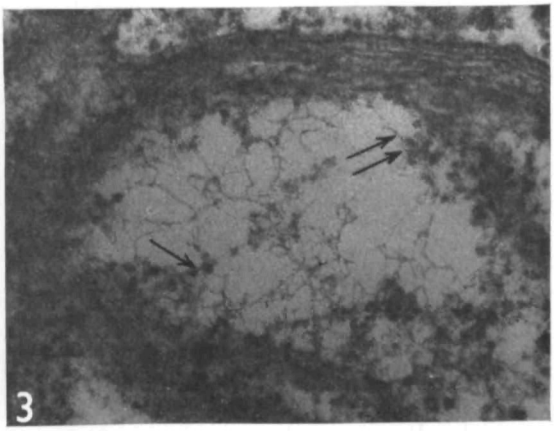
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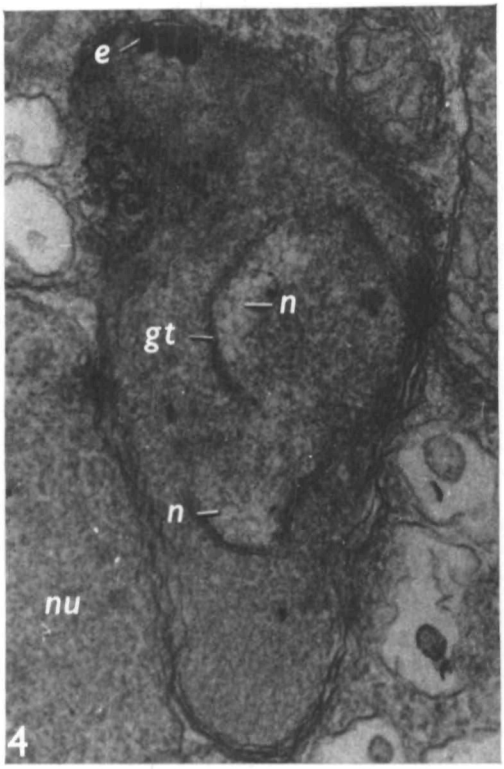

e
n
gt
n
nu
4


5

Fig. 6A-J. Consecutive serial sections through the posterior end of a chloroplast lobe of a log-phase light-grown cell. In Fig. 6A, the chloroplast nucleoid (*n*) is sectioned at each extremity of the chloroplast. In Figs. 6B and C, the chloroplast nucleoid is sectioned a third time (arrows) in what would appear to be the centre of the chloroplast lobe. However, since the section in Fig. 6C is only 3 sections removed from the section in which the chloroplast splits into 2 lobes (Fig. 6F), the apparently centrally located piece of the chloroplast nucleoid is in fact located at the periphery of the chloroplast at the point where it is about to branch into 2 lobes. In Fig. 6D and E, the 'central' nucleoid splits into two and in Fig. 6F, the chloroplast itself splits into 2 lobes, each having 2 peripherally located nucleoid sections. In Fig. 6G, the 2 peripheral sections of the chloroplast nucleoid of the lower lobe approach each other and in Figs. 6H and I join each other. In Fig. 6J, the chloroplast nucleoid of the lower lobe has passed out of the plane of the section, the section cutting tangentially the membranes of either the girdle bands or the chloroplast envelope (arrow). $8{\cdot}2 \times 10^6$ cells/ml. Glutaraldehyde-osmium fixation. × 14600.

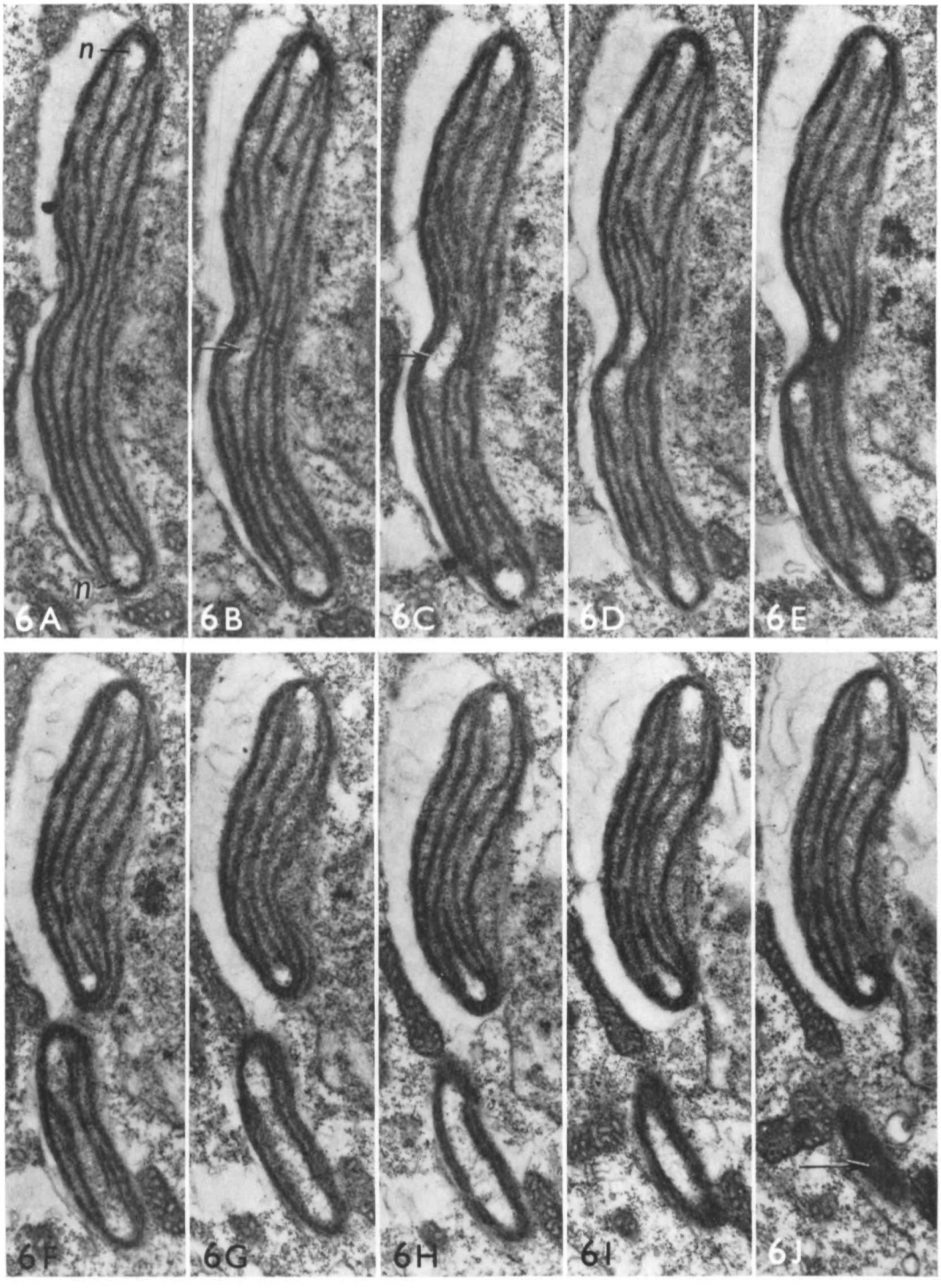
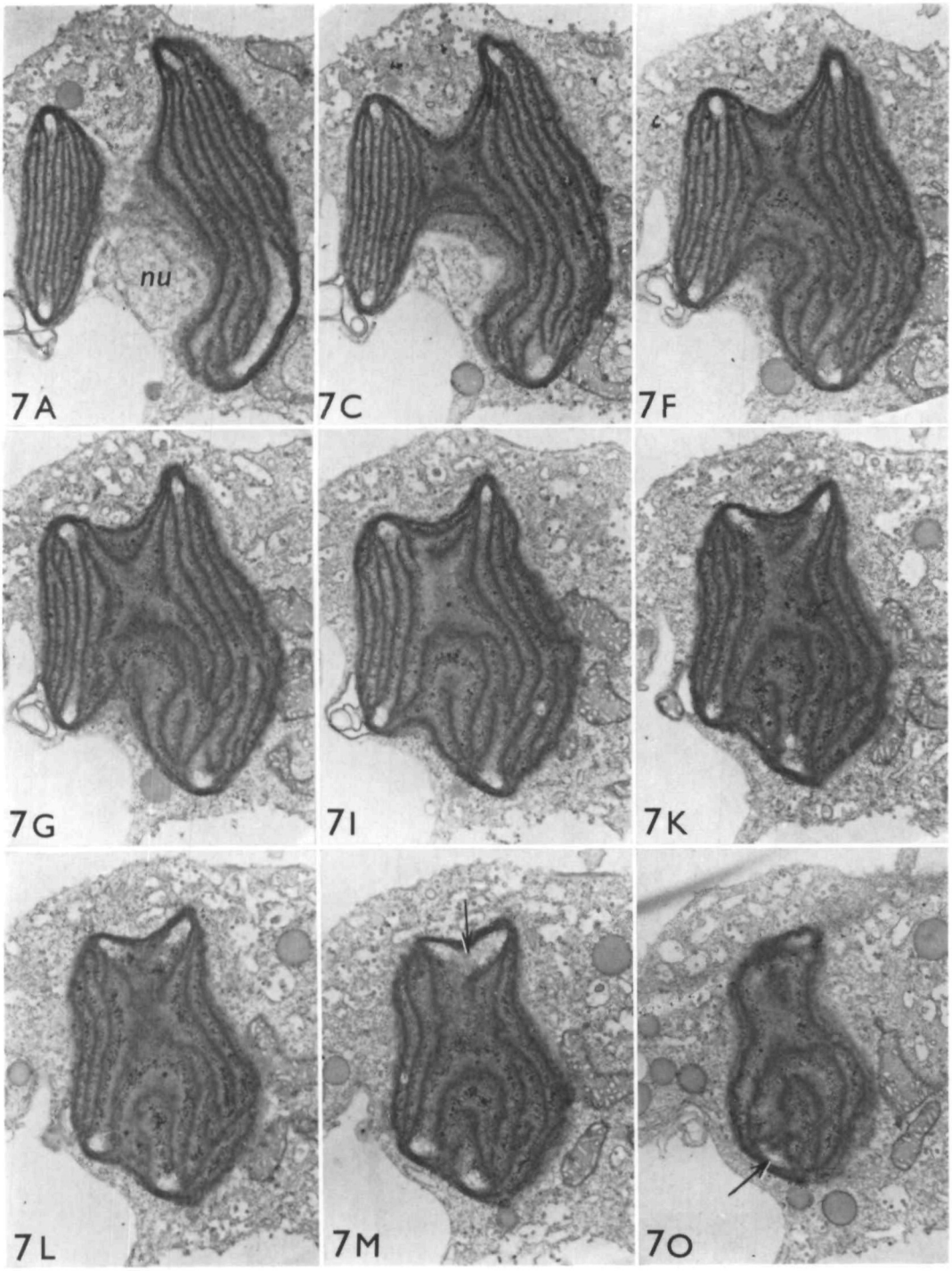

n
n
6A
6B
6C
6D
6E
6F
6G
6H
6I
6J

Fig. 7A-O. Selected serial sections through the bridge region of the chloroplast of a log-phase greening cell. This figure demonstrates that the chloroplast nucleoid which encircles the periphery of one lateral lobe of the chloroplast joins the nucleoid encircling the other lateral lobe at the top and bottom of the bridge which connects the 2 lobes, thus forming a single continuous nucleoid. To economize on space, non-essential sections have been omitted. The missing sections are indicated by the missing letters of the alphabet. In Fig. 7A, the 2 main chloroplast lobes are observed lying on each side of the nucleus (*nu*). By Fig. 7C, the 2 chloroplast lobes are joined by a narrow bridge which lies dorsal to the anterior region of the cell's nucleus. In Fig. 7F–L, the bridge broadens in the anterior-posterior plane of the cell. The 4 nucleoid areas, which appear as electron-translucent spots at this low magnification, are seen gradually to approach each other and in Fig. 7M, the 2 top nucleoid areas join (arrow). Finally, in Fig. 7O, the 2 lower nucleoid areas join (arrow). 24 h light. $2{\cdot}0 \times 10^6$ cells/ml. Glutaraldehyde-osmium fixation. ×9100.


nu
7A
7C
7F
7G
7I
7K
7L
7M
7O