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**Invited Article**

# The Nuclei of Cellular Organelles and the Formation of Daughter Organelles by the "Plastid-Dividing Ring"

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It has been established that organelles, such as mitochondria and plastids, contain organelle-specific DNA and arise from the division of pre-existing organelles (e.g., Possingham and Lawrence, 1983). We propose that organelle DNAs, such as mitochondrial DNA and plastid DNA are not naked in organelles *in situ* but are organized in each case to form an "organelle nucleus" with basic proteins (Kuroiwa, 1982). The concept of organelle nuclei has changed our ideas about the division of organelles. Thus, the process of organelle division must be composed of two main events: division of the organelle nucleus and organellekinesis (divison of the other components of the mitochondrion or plastid). The latter term has been adopted as an appropriate analogue of cytokinesis.

We were the first to identify the plastid-dividing ring (PD-ring), which is located in the cytoplasm close to the outer envelope membrane at the constricted isthmus of dividing chloroplasts in the red alga *Cyanidium caldarium*. The PD-ring is about 60 nm in width and 25 nm in thickness, and is a circular bundle of actin-like, fine filaments, each about 4-5 nm in diameter. Since cytochalasin B, an inhibitor of polymerization of actin filaments, inhibits the formation of the PD-ring and, thus, prevents subsequent division of chloroplasts, the PD-ring is thought to be a structure that is essential for the division of plastids (plastidkinesis).

The behavior of the PD-ring during a cycle of chloroplast division can be classified into the following four stages on the basis of morphological and temporal differences. The chloroplast growth stage: the small, spherical chloroplast increases in volume and becomes a football-like structure, while the PD-ring from the previous division disappears. Formation of the PD-ring: the somewhat electron-dense body (see below) is fragmented into many, somewhat electron-dense granules, which are aligned along the equatorial region of the chloroplast and fine filaments are formed from the somewhat electron-dense granules in the equatorial region. The fine filaments of the PD-ring align themselves according to the longest axis of their overall domain, i.e., circumferentially. Contraction stage: a bundle of fine filaments begins to contract and generates a deep furrow. Conversion stage: after chloroplast division, the remnants of the PD-ring are converted into somewhat electron-dense bodies. Similar events occur during the second cycle of chloroplast division. Since similar structures are observed extensively in the

Abbreviations: PD-ring, plastid-dividing ring; DAPI, 4′-6-diamidino-2-phenylindole; pt-nucleus, plastid nucleus; pt-DNA, plastid DNA; cp-DNA, chloroplast DNA; cp-nucleus, chloroplast nucleus; mt-DNA, mitochondrial DNA; mt-nucleus, mitochondrial nucleus; ISC, initiation of synchronous culture; SEG, somewhat-electron dense granule; SEB, somewhat-electron dense body.

plastids of algae, moss and higher plants, the PD-ring appears to be an essential structure for the division of plastids in plants.

Key words: Actin-like filaments — *Cyanidium caldarium* — Plastid division — Plastid DNA — Plastid nucleus (nucleoid) — Plastid-dividing ring — Plastidkinesis.

Proplastids are organelles found in cells with virtually no color in all eukaryotic plants and are believed to be the source of several types of plastid, such as chloroplasts, amyloplasts, elaioplasts and chromoplasts. Muhlethaler and Frey-wyssling (1959) suggested that proplastids arise from smaller organelles termed "plastid initials" (0.002 $\mu$m to 0.05 $\mu$m in diameter) which are present in leaf buds and leaf meristems of a number of higher plants. It was also suggested that "plastid initials" are derived either from invaginations of the nuclear envelope (Muhlethaler and Bell, 1962) or from small bodies derived from degenerating plastids (Maltzahn and Muhlethaler, 1962). With new and improved methods for fixation for electron microscopy, it is now possible to distinguish clearly between most of the organelles in meristematic tissue and, significantly, there have been no recent observations of "proplastid initials" (Possingham and Lawrence, 1983). Possingham and Lawrence appear to be basically correct in their assessment that the theory of formation of various plastids from the "plastid initial" can be abandoned and that plastids cannot arise *de novo* but are formed only by the division of pre-existing plastids.

With the advance of 4′-6-diamidino-2-phenylindole (DAPI)-epifluorescence microscopy, the number of copies per plastid of the plastid genome can easily be counted. Such studies have shown that there are at least two types of proplastid (Kuroiwa *et al.*, 1981). One type of plastid is characterized by the presence of 1 to 2 copies of the plastid genome per plastid of about 1 $\mu$m in diameter and by the presence of a single plastid nucleus (pt-nucleus; synonymous with nucleoid) per plastid. The other is characterized by several copies of the plastid genome per plastid of about 2 to 3 $\mu$m in diameter and about 2 to 5 pt-nuclei per plastid. Herein, the latter will be reformed to as "proplastids" to distinguish them from the morphologically less complex and smaller "proplastid precursors" characteristic of the former type.

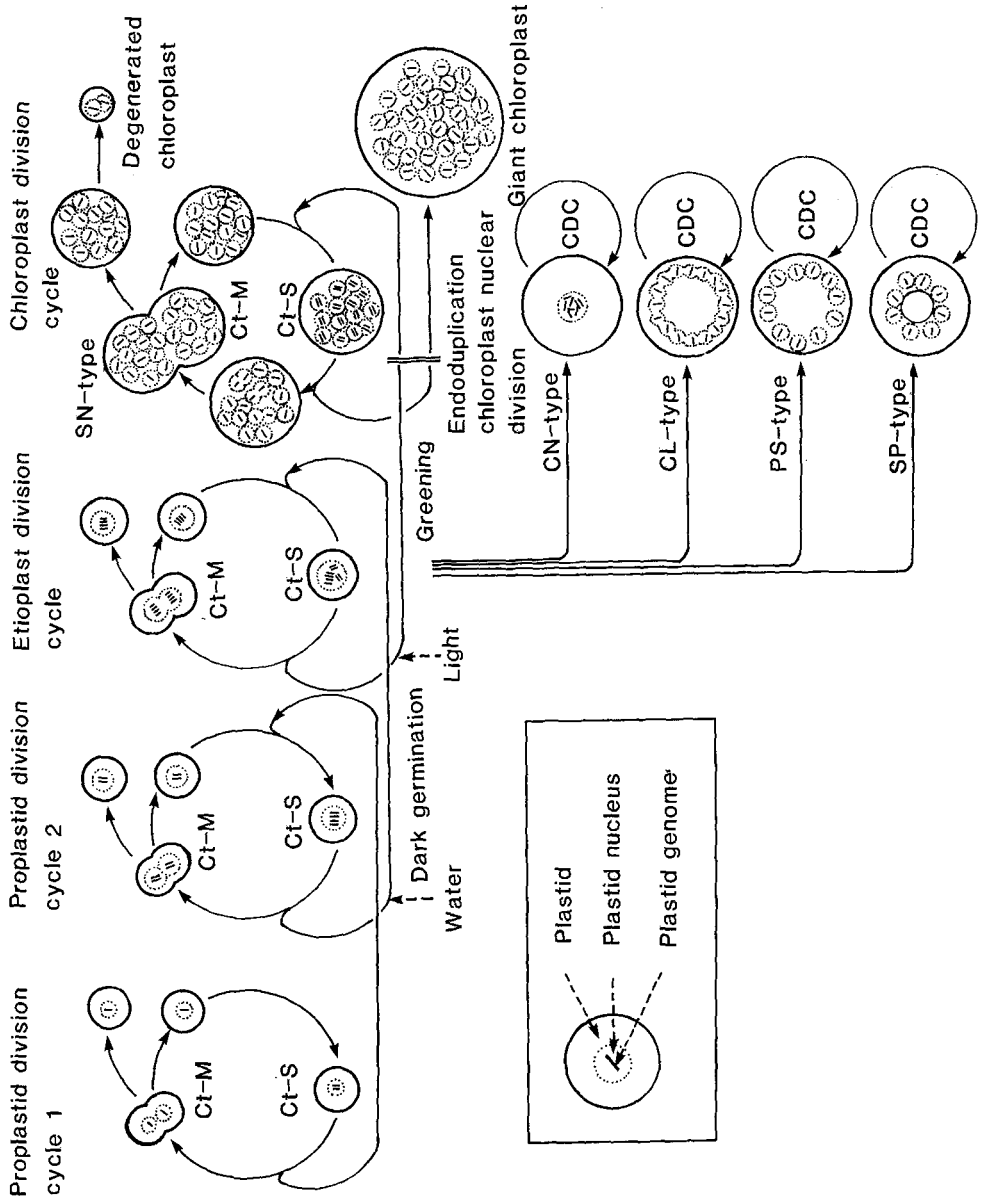
Proplastids, which arise from proplastid precursors, are about 2 to 3 $\mu$m in diameter and contain plastid DNA (pt-DNA), as do other plastids. Generally, it has been assumed that the pt-DNA is naked and is not associated with histone-like proteins but is organized in a prokaryotic manner, since the DNA is found in one or more regions of the plastid that are not bounded by a nuclear membrane. Recently, it has been proven by biochemical techniques (Nemoto *et al.*, 1988; 1989) that proplastid DNA is associated with proplastid DNA-binding proteins to form plastid nuclei (pt-nuclei) similar to bacterial nuclei. The proplastids in cultured tobacco cells divide into daughter proplastids soon after the synthesis of proplastid DNA (Yasuda *et al.*, 1988).

The proplastid precursor in higher plants contains only one small, spherical pt-nucleus. When the proplastid precursors develop into proplastids and furthermore etioplasts during growth in the dark, the pt-nucleus become cup-shaped, with concomi-

tant synthesis of pt-DNA and is often found near starch grains or the prolamellar body (Kuroiwa *et al.*, 1981) (Fig. 1). In etioplasts of monocotyledons, the pt-nucleus becomes a ring-shaped structure (Sellden and Leech, 1981; Hashimoto, 1986; Miyamura *et al.*, 1986). Once etioplasts have been illuminated, the pt-nuclei begin dividing into 20–30 small, spherical pt-nuclei, which are distributed individually into mature chloroplasts (Fig. 1). Kuroiwa *et al.* (1981) showed qualitatively, by staining with DAPI, that in *Brassica* the DNA content per plastid increases markedly during the division cycle of proplastids and the development from proplastids to etioplasts, but the DNA content increases only slightly after illumination, even though the cp-nucleus divides into small, scattered cp-nuclei. Miyamura *et al.* (1986) carefully examined the fluorescence intensity of each pt-nucleus using a video-intensified microscope photon-counting system (VIMPICS) and showed clearly that the number of copies of pt-DNA increased approximately 8-fold during the division cycle of plastids and during differentiation of the proplastid to the etioplast, and the number of copies reached a plateau when the chloroplasts began to engage in photosynthesis. As a result of the division of young chloroplasts, a 10-fold increase in the number of chloroplasts per cell occurs in spinach leaves and a two-to three-fold increase occurs in wheat leaves; the process is, therefore, important in determining the photosynthetic potential of the mature leaf (Leech, 1976). Plastids of both epidermal and palisade cells of *Phaseolus vulgaris* also divide at all stages of plastid development, but division ceases soon after the plastids become mature (Whatley, 1980).

The number, size, shape and distribution of pt-nuclei differ among classes of eukaryotic algae and land plants and change markedly during the development of chloroplasts (Fig. 1) (Kuroiwa *et al.*, 1981: Coleman, 1985). Most plants can be classified into one of five groups: SN-type, CN-type, CL-type, PS-type and SP-type, based on differences in the shape, size and distribution of the pt-nuclei in their mature chloroplasts. The differences in nuclear patterns in chloroplasts are explained by the pattern of distribution of the replicated pt-DNA in mature chloroplasts, which is distributed randomly throughout the chloroplast (SN-type); fused to form spherules in the central area of the chloroplast (CN-type); fused to form a circle along the periphery of the chloroplast (CL-type); distributed along the periphery of the chloroplast (PS-type); or gathered around the pyrenoid in the chloroplast (SP-type). Land plants and the green algae *Nittella axilliformis* and *Chlamydomonas reinhardtii* are of the SN-type. The red alga *Cyanidium caldarium* RK 1 is typical of the CN-type. Almost all of the brown algae are of the CL-type while the red algae are of the PS-type and the green alga *Bryopsis plumosa* is of the SP-type. Regardless of differences in distribution and organization of pt-DNA in plastids, all plastids multiply basically by binary fission of pre-existing plastids.

In addition to differentiation from proplastids to chloroplasts, the proplastids are also able to differentiate into functional plastids of various forms, such as amyloplasts, elaioplasts and chromoplasts (Possingham and Lawrence, 1983), which contain basically the same genome as proplastids (Nemoto *et al.*, 1988). Possingham (1980) briefly summarized the function of these plastids as follows: "the chlorophyll-containing


Proplastid division cycle 1
Ct-M
Ct-S
Proplastid division cycle 2
Ct-M
Ct-S
Dark germination
Water
Etioplast division cycle
Ct-M
Ct-S
Light
Greening
Chloroplast division cycle
SN-type
Degenerated chloroplast
Ct-M
Ct-S
Endoduplication chloroplast nuclear division
Giant chloroplast
CN-type
CDC
CL-type
CDC
PS-type
CDC
SP-type
CDC
Plastid
Plastid nucleus
Plastid genome

chloroplasts of green leaves are a specialized form for photosynthesis, and they perform the fixation of carbon by using the energy of the sun and synthesize amino acids as precursors of various proteins. The amyloplasts of storage tissues and pollen grains are plastids filled with starch. Their function in tissues, such as cotypledons, endosperm, and tubers is to synthesize starch as a reserve material. Elaioplasts are plastids largely filled with oil. They are found in the epidermal cells of some monocotyledonous families such as Liliaceae and Orchidaceae. Chromoplast is the term used for carotenoid-containing plastids responsible for the yellow, orange, and red colors of fruits, flower petals, and some roots. Therefore, they are perhaps the most important organelles for the existence of mankind, as they are the ultimate source of most of our past and present fuel and of our current food supplies".

It has been established that proplastids and these functionally differentiated organelles contain plastid-specific DNA (Chiba, 1951 ; Iwamura, 1962 ; Ris and Plaut, 1962 ; Sager and Ishida, 1963 ; Shinozaki *et al.*, 1986 ; Ohoyama *et al.*, 1986) and arise from the division of pre-existing plastids (Schimper, 1883 ; Kusunoki and Kawasaki, 1936 ; Green, 1964 ; Manton, 1959 ; Kiyohara, 1962 ; Possingham and Saurer, 1969 ; Nishibayashi *et al.*, 1982 ; Possingham and Lawrence, 1983). How do plastids divide and differentiate ? We cannot yet answer this simple question at the molecular level. The plastids are believed to be devoid of mitotic apparatus. This review aims to summarize the information that is currently available on the mechanism of chloroplast division, with special emphasis on the physical apparatus involved in the division. The information will be used to generate a proposed model, at the cellular and molecular level, of plastid division.

## Mitochondrial Nuclei and Plastid Nuclei

Chiba (1951) demonstrated the presence of nucleic acids in chloroplasts in *Tradescantia fluminensis*, *Selaginella Savatieri* and *Rhoeo discolor* using Feulgen staining. Then Ris and Plaut (1962) showed clearly that the chloroplasts of *Chlamydomonas reinhardtii* include two DNA-containing areas by fluorescence micros-

Fig. 1. A diagram of chloroplast nuclear events during the development and division of chloroplast. A proplastid in land plants develops into a chloroplast, with replication of the chloroplast genome, through least four different division cycles : proplastid division cycle 1 ; proplastid division cycle 2 ; the etioplast division cycle, and the chloroplast division cycle. Nuclear division in the plastid takes place prior to plastid-kinesis. Most plants can be classified into one of five types : SN-type, CN-type, CL-type, PS-type or SP-type, based on differences in shape, size and the distribution of cp-nuclei in their mature chloroplasts. The differences in chloroplast nuclear patterns are explained by the patterns of distribution of the replicated chloroplast genomes in mature chloroplasts, which are ; distributed randomly throughout the chloroplast (SN-type) ; fused to form spherules in the central area of the chloroplast (CN-type) ; fused to form a circle along the periphery of the chloroplast (CL-type) ; distributed along the periphery of the chloroplast (PS-type) ; or gathered around the pyrenoid in the chloroplast (SP-type). (Kuroiwa *et al.*, 1981 ; reproduced by permission of Plant Cell Physiol.)

copy after acridine-orange staining and electron microscopy. Iwamura (1960) presented biochemical evidence to suggest that the subcellular fractions, including the chloroplasts, of *Chlorella* contain nucleic acids. In 1963, Sager and Ishida suggested that a satellite peak of DNA isolated from *Chlamydomonas reinhardtii* corresponded to cp-DNA obtained by isolating a chloroplast fraction. Since 1963, the molecular biology of nucleic acids and proteins in mitochondria and chloroplasts has advanced considerably (e.g., Rabinobitz and Swift, 1970) and it has been demonstrated that the molecular weights of cp-DNA from various plants are approximately 150 kd and that about 120 genes are located on the chloroplast genome. Anderson *et al.* (1981) determined the complete nucleotide sequence of the human mitochondrial genome which has 16569 base pairs and encodes about 30 genes. Recently, Shinozaki *et al.* (1986) and Ohoyama *et al.* (1986) determined the complete base sequences (about $1.5 \times 10^6$ bases) of cp-DNA from the chloroplasts of both *Nicotiana tabacum* and *Marcantia polymorpha*, respectively. It has generally been believed that mitchondria and chloroplasts contain naked mitochondrial DNA (mt-DNA) and cp-DNA, respectively. However, we were never convinced that the several copies of these organelle genomes exist naked in small organelles and function naked in transcription and replication because such DNAs are located compactly in a specific area of the organelles. Since 1973, we have been working to elucidate the organization of mitochondrial genomes and plastid genomes *in situ* in organelles and have proposed the concept of "organelle nuclei" using, as a model, the mitochondrion of the slime mould *Physarum polycephalum* (Kuroiwa, 1973; 1974; 1982). *Physarum* mitochondria are characterized by electron-dense mitochondrial nuclei (mt-nuclei) with a large amount of mitochondrial DNA (mt-DNA), as can be seen in mitochondria of all Trypanosomatidae. Initially, we showed by cytochemical techniques and isolation of pure, intact oragnelle nuclei that mt-DNAs are not naked but are packed and organized to form organelle nuclei by association with specific basic proteins including "mitochondriamin" (Kuroiwa, 1973; 1982; Kuroiwa and Hizume, 1974; Kuroiwa *et al.*, 1976; Suzuki *et al.*, 1982). Yoshida *et al.* (1978) obtained the folded cp-DNA complex from spinach using a method that included several deproteinization steps, but without using CsCl or intercalating dyes. The structure of the looped and folded cp-DNA was maintained even after several, very drastic, deproteinization steps, such as treatment with diethylpyrocarbonate, SDS, chloroform and low levels of proteinase K. By contrast, treatments with pronase or trypsin of the cp-nuclei *in situ* in *Nitella axilliformis* (Kuroiwa *et al.*, 1981) revealed the presence of trypsin- and pronase-sensitive cp-DNA-organizing proteins. Similarly, pt-DNAs, which form a typical, ring-shaped pt-nuclei of the CL-type in the chloroplasts of the brown alga *Ectoculpus indicus*, are organized with trypsin sensitive proteins to from pt-nuclei (Kuroiwa and Suzuki, 1982). Hallick *et al.*, (1976), Briat *et al.*, (1979), Briat *et al.*, (1982), Briat *et al.*, (1984), Lebrun *et al.*, (1986) and Reiss and Link (1985) all isolated transcriptionally active plastid chromosomes or DNA-protein complexes. Hansmann *et al.* (1985) isolated pt-nuclei from chloroplasts and chromoplasts of *Narcissus pseudonarcissus* on a column of Sepharose 4B after treatments with Triton X-100 and suggested that chloroplast and

chromoplast DNA molecules are organized by specific, basic proteins. Recently Nemoto *et al.* (1988) isolated the proplastid nuclei from the proplastids of *Nicotiana tabacum*. The isolated proplastid nuclei recondensed completely after treatment with pronase and NaCl. These data support the hypothesis that organelle DNAs, such as the DNA from mitochondria and plastids are not naked but are organized to from "nuclear structures" by associations with specific proteins (Kuroiwa, 1974 ; Kuroiwa and Hizume, 1974 ; Kuroiwa *et al.*, 1976 ; Kuroiwa and Suzuki, 1981 ; Kuroiwa, 1982) and that a plant cell contains three different types of nuclei, namely, the cell nucleus, the mt-nucleus and the pt-nucleus (Kuroiwa, 1982).

The definition of an organelle nucleus has been given in detail elsewhere (Kuroiwa, 1982). However, certain important points deserve to be repeated here. In the late-nineteenth century, investigators first observed that several rod-shaped bodies are always found between the poles of dividing plant and animal cells, and Waldeyer named these objects "chromosomes". Since then "chromosome" has been used to denote a gene-containing body which appears in metaphase during cell division in many eukaryotes. With the advances in the molecular biology of the gene, the term "chromosome" has been used in a functional sense, rather than in the original, strictly morphological sence. Therefore the circular DNAs of bacteria and chloroplasts are called "bacterial chromosomes" and "chloroplast chromosomes". "Genome" also is used to denote structurally and functionally a minimum chromosomal set by which eukaryotic and prokaryotic organisms, and organelles such as mitochondria and chloroplasts, can function and/or proliferate. With regard to the nucleus, ever since its discovery by Brown in 1835 in a plant cell, the cellular nucleus has been recognized as an important organelle that contains genetic substances in eukaryotes. The term "nucleus" has been extended by many microbiologists to bacterial cells (Mason and Powlson, 1956 ; Robinow, 1956 ; Ryter, 1968) and *Chlamydia* (Matsumoto, 1981) and is used to denote, structurally and functionally, the chromosome-containing area in prokaryotic cells, although the "nucleus" need not be surrounded by a nuclear membrane. Of course, we know that rod-shaped chromosomes of cell nuclei also are not surrounded at the metaphase by a nuclear envelope. Thus the definition of the organelle nucleus does not depend on the existence of a nuclear envelope. The area containing the DNA filament, which appears in the electron-lucent region of mitochondria and plastids, has been given several names, which include the term "nucleoid". However, since mitochondrial chromosomes are located not only in the electron-lucent area but also in the mitochondrial matrix, the term "nucleoid" means a part of the mass of the organella genome. In addition, the term has also often been used to designate an electron-dense inclusion, without any DNA, in microbodies (Tsukada *et al.*, 1966 ; DeDuve and Baudhuin, 1966 ; Usuda *et al.*, 1988). In this review, thus the terms "organelle nucleus", "mt-nucleus" and "pt-nucleus" are used to describe the genome-containing area organized by associations with basic proteins in mitochondria and plastids.

The mitochondria of *Physarum* have a synchronous division cycle in which mitochondrial nuclear division slightly precedes the division of the mitochondrial

matrix (mitochondriokinesis). Kuroiwa *et al.* (1978) proposed a four-phase cycle of mitochondrial division, consisting of a mitochondrial M phase (mM), a mitochondrial G1 phase (G1), a mitochondrial S phase (mS), and a mitochondrial G2 phase (G2). The duration in hours of the four phases in *Physarum polycephalum* were mM, 1.5 hr; mG1, 3.0 hr; mS, 7.5 hr; and mG2, 2.0 hr, and the generation time of mitochondria was 14 hr. The duration of the mitochondrial generation time of *P. polycephalum* was slightly longer than those of *Trypanosoma mega* and *Neurospora crassa*, which lasted 6.6 hr and 8.5 hr (Hansley and Wagner 1967; Cosgrove and Skeen, 1970), respectively. In the case of plastids, the generation time of plastids in *Nitella axillaris* and *Spinacia* is about 24 hr (Green, 1964) and 30 hr, respectively, but the various phases, such as plastid G1, S and G2 can not be clearly delineated (Possingham and Lawrence, 1983). In multiplastidic cells, since the division of plastids is not synchronized, it is difficult to identify the duration of each of the sub-periods of the division cycle.

The concept of autonomy and continuity of mitochondria and plastids led to the idea that it might be possible to culture these organelles *in vitro*. The combined data suggest that chloroplasts of some lower plants may be able to survive and perhaps divide over periods of several days outside the plant cell. Evidence of division of isolated plastids from higher plants (Ridley and Leech, 1970; Kameya and Takahashi, 1971) is less convincing, although it does appear that the dumbbell stage of chloroplast division can be observed and possibly induced in isolation media (Possingham and Lawrence, 1983). The fact that isolated chlorolasts can divide led to the hypothesis that the structure of chloroplasts includes all the machinery required for their division.

## Application of the Concept of Organelle Nuclei to Nuclear Division in Organelles

The concept the mt-DNA is not naked and is not distributed throughout the entire mitochondrion but is organized with histone-like, basic proteins into a rod-shaped or spherical plastid nucleus, in the central region, changed out ideas about organelle division. The division of organelles, such as mitochondria and plastids, is composed of two main processes: organelle nuclear division and organellekinesis (divison of the other components of the organelle). In fact, since *Physarum* mitochondria contain an electron-dense mitochondrial nucleus, it is possible to observe the two separate processes quite clearly.

It is of great interest to us to elucidate the structural basis of the mechanism of organellekinesis. As described in previous studies (Kuroiwa *et al.*, 1977), the mode of mitochondrial divison of *Physarum polycephalum* under ordinary conditions is very simple. A spherical mitochondrion elongates, becomes ovoid, and grows into a dumbbell shape. The mitochondria then divide somewhat synchronously during mid-S to form pairs of spherical daughter mitochondria. At the same time, each dumbbell-shaped mt-nucleus also divides and separates into two daughter mitochondria (Fig. 2a). When a small explant of a plasmodium was exposed to cytochalasin B at 50 $\mu$g/m$l$ for 3 hr prior to fixation, a large number of mitochondria subsequently

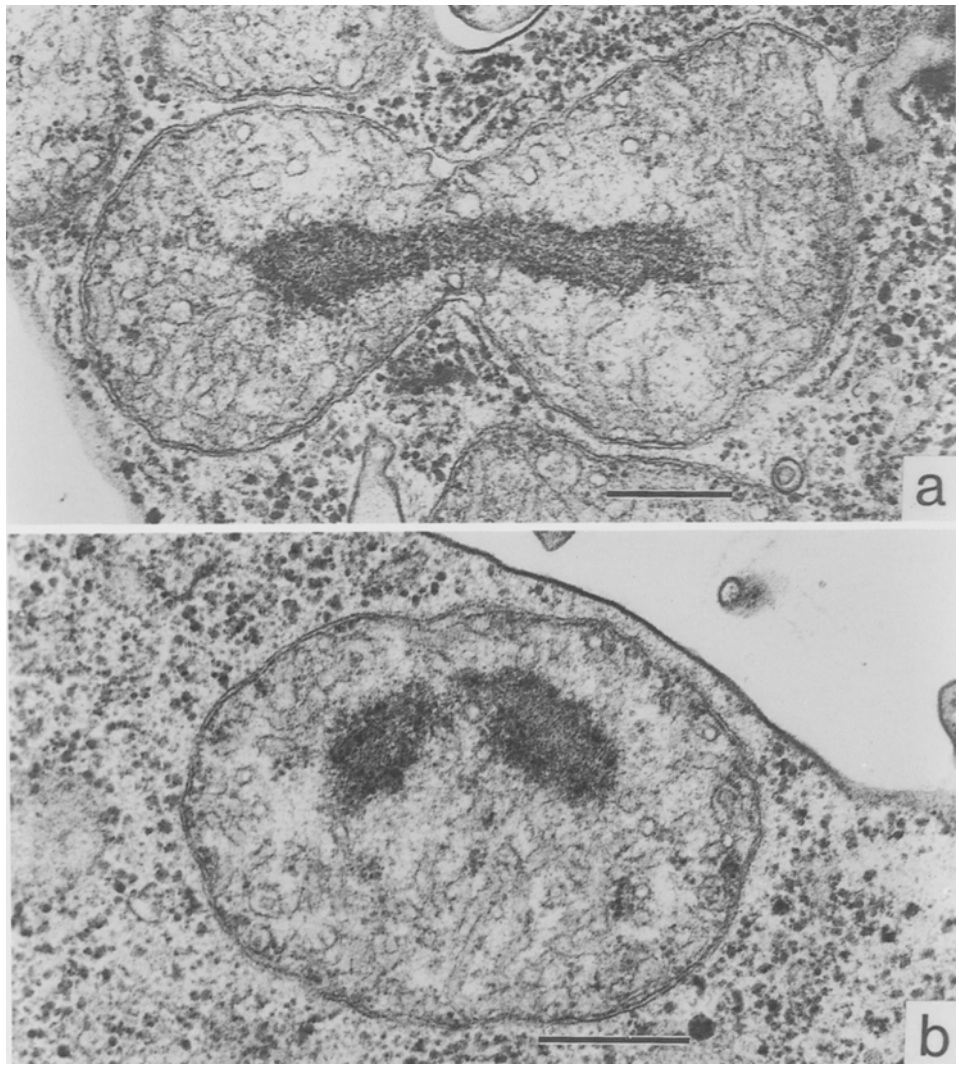



Fig. 2. Electron micrographs of mitochondria in plasmodia fixed immediately after incubation for 3 hr in control nutrient medium (a) or in a nutrient medium that contained cytochalasin B at 50 $\mu$g/m$l$ (b). The micrographs depict the normally dividing mitochondrion (a) with one rod-shaped mt-nucleus. The cytochalasin B treated mitochondrion appears as a large sphere and contains a V-shaped mt-nucleus. Scale bar represents 0.5 $\mu$m. (Kuroiwa and Kuroiwa, 1980 ; reproduced by permission of Birkhauser Verlag).

exhibited a large spherical or ovoid configuration. They contained a V-shaped mt-nucleus or a pair of mt-nuclei (Fig. 2b) (Kuroiwa and Kuroiwa, 1980). Cytochalasin B is well known as a specific inhibitor of microfilament function. Therefore, the simplest explanation for the absence of dumbbell-shaped mitochondria is that the cytochalasin B disrupted the function of the contractile proteins so that the mitochondrion failed to lengthen. It is likely that actin-like filaments are involved in

mitochondriokinesis. However, we have been unable, as yet, to discern any fine structures that are intimately related to mitochondriokinesis. Probably only a few actin-like filaments, which cannot be visualized under the electron microscope after fixation by conventional methods, may be important for mitochondriokinesis.

## Discovery of the PD-Ring

Since plastids are considerably larger than mitochondria and contain more highly developed membrane systems, a bulkier apparatus may be required for plastidkinesis than is required for mitochondriokinesis. On the basis of morphological aspects of the daughter plastids, the mode of plastid division can be classified into three types, namely, spherical (S-type) (Fig. 3a), ellipsoidal (E-type) (Fig. 3b) and irregular (I-type) (Fig. 3c). The S-type is seen in lower eukaryotes such as *Cyanidium caldarium* RK-1. The E-type is seen extensively in land plasts and the I-type is seen in red and brown algae. Frequently, in one species, 2–3 types can be seen at different developmental stages or in different tissues of the plants. The PD-rings have been found in

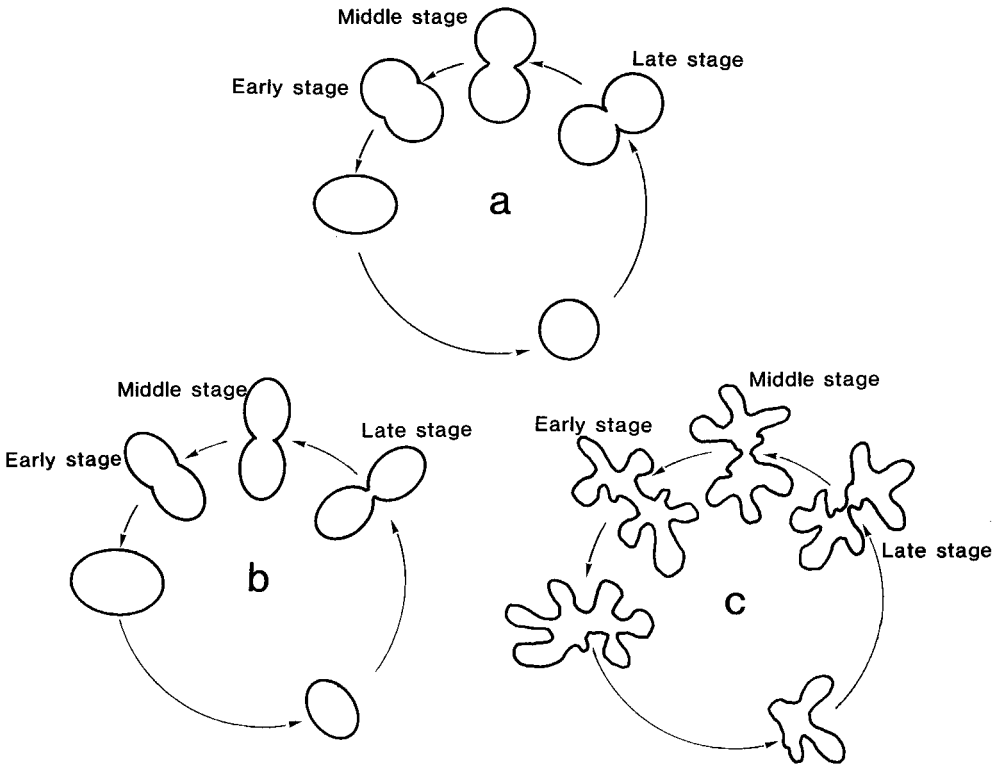



Fig. 3. The three representative modes of division of plastids observed in various plants. On the basis of morphological aspects of the daughter plastids, the mode of plastid division can be classified into three types, namely spherical (S-type) (a), ellipsoidal (E-type) (b) and irregular (I-type) (c). The S-type is seen in lower eukaryotes including *Cyanidium caldarium* RK-1. The E-type is seen extensively in land plants.

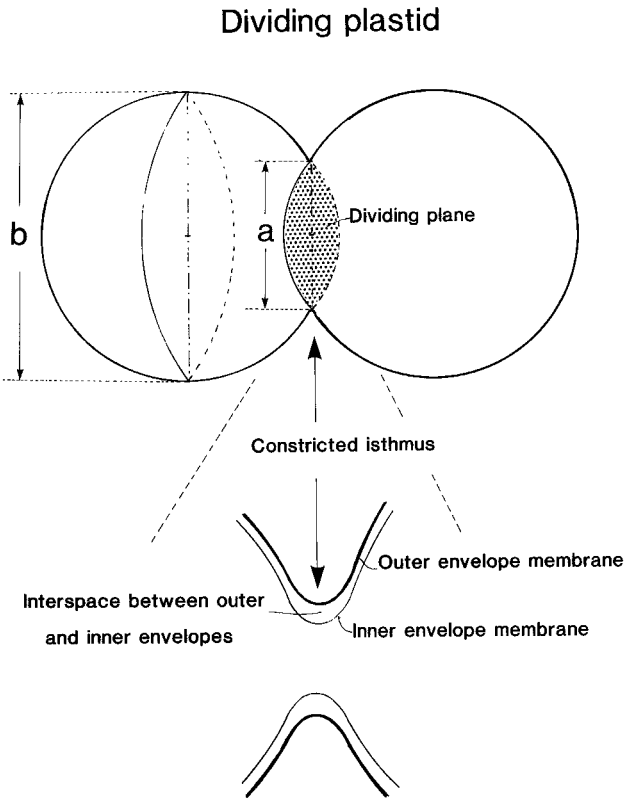



Fig. 4. Schematic representation of a dividing plastid. To avoid a confusion of terms related to the dividing plastid, several basic terms are illustrated. a and b show the diameter of dividing plane and the diameter of daughter plastid, respectively.

both S- and E-type division, as described below. To avoid any confusion of terms in descriptions of the dividing plastid, several basic terms are illustrated in Fig. 4. The division stage can also be conveniently divided into three phases, the early, middle and late periods of the plastid division cycle, according to the ratio of the diameter of the dividing plane (a) to diameter of daughter plastids (b). Plastids during early, middle, and late phases of division have constriction ratios (a/b) of $1 > a/b \geq 1/2$, $1/2 > a/b \geq 1/4$, and $1/4 > a/b$, respectively (Fig. 4).

Suzuki and Ueda (1975) and Luck and Jordan (1980) reported the appearance of electron-dense material, which they considered to be evidence of "baffles" and a "septum", respectively, at the constricted isthmus between daughter proplastids and daughter amyloplasts, at the late phase of plastid division in *Pisum sativum* and *Hyacinthiodes non-scripta*. Similar electron-dense deposits have been observed at the narrow neck of dividing chloroplasts and have been described as "fuzzy plaques" in *Triticum aestivum*, *Atriplex semibaccata* and *Sesamum indicum* var. glauca (Leech *et al.*, 1981). Fuzzy plaques of electron-opaque material were frequently, but not always, seen covering or displacing the membranes of the isthmus (Leech *et al.*, 1981).

These observations were recorded from single thin sections. Subsequently, Chaly and Possingham (1981) surveyed the deposits located at the constricted isthmus between daughter chloroplasts in various plants, such as *Pisum sativum*, *Phaseolus vulgaris*, *Lycoperisicon esculentum*, *Lactuca sativa*, *Citrullus lanatus*, *Hordeum valgare*, *Zea mays*, *Triticum aestivum* and *Pinus radiata*, using serial sections and they concluded that electron-dense deposits, which are parts of an annulus, are located in the interspace between the outer and inner envelope membranes at the constricted isthmus of dumbbell-shaped proplastids of these plants. To explain the formation of the "fuzzy plaque" or "annulus", Leech *et al.* (1981) and Possingham and Lawrence (1983) applied to plastids the theoretical model of cell division proposed by Greenspan (1977): namely, that the internal fluid flow generated by surface changes leads to a concentration of material in the equatorial region and to formation of an annulus.

Table 1. Observations of electron-dense materials at constricted isthmus of dumbbell-shaped plastid and a plastid-dividing ring in various preparations of plant material

| Species | Tissue | Plastid | Stage | Name | Distribution | Reference |
|---|---|---|---|---|---|---|
| Higher plants | | | | | | |
| *Pisum sativum* | Root tip | Proplastid Amyloplasts | Late | Septum | Plastids | Suzuki & Ueda (1975) |
| *Hyacinthiodes non-scripta* | Pollen Meiosis | Plastid (Proplastid) | Late | Septum | Plastids | Luck & Jordan (1980) |
| *Triticum aestivum* | Root tip | Proplastid | Late | Fuzzy plaque | Plastids | Leech *et al.* (1981) |
| *Sesamum indicum* | Root tip | Proplastid | Late | Annulus | Plastids (Interspace) | Chaly & Possingham (1981) |
| *Pisum sativum* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Phaseolus vulgaris* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Lycoperisicon esculentum* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Lactuca sativa* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Citrullus lanatus* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Hordeum vulgare* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Zea mays* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Triticum aestivum* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Pinus radiata* | 〃 | 〃 | 〃 | 〃 | 〃 | 〃 |
| *Avena sativa* | First leaf | Proplastid Chloroplasts | Late | PD ring doublet | Cytoplasm Matrix | Hashimoto (1986) |
| Moss | | | | | | |
| *Funaria hygrometrica* | Protonema | Chloroplast Amyloplast | Late | PD ring | Cytoplasm | Tewinkel & Volkmann (1987) |
| Red alga | | | | | | |
| *Cyanidium caldarium* RK-1 | Single cell | Chloroplast | Early Middle Late | PD ring | Cytoplasm | Mita *et al.* (1986) Mita & Kuroiwa (1988) |

They suggested that the electron-opaque material might be present, but diffuse, at earlier stages of division and only be visible when sufficiently concentrated within a narrow constriction.

Common features of these earlier observations can be summarized briefly as follows: electron-dense deposits appear only at the narrowly constricted isthmus of the dumbbell-shaped plastid just before separation of daughter plastids and are never seen at the early stage of plastid division; electron-dense deposits are located inside the plastids themselves and the timing of appearance of dumbbell-shaped plastids is obscure because, within each multiplastidic cell, the plastids do not divide in synchrony.

The conflicting suggestions of previous authors can be resolved by our identification of a novel cellular structure, which we have called the PD-ring, in the red alga *Cyanidium caldarium* (Mita *et al.*, 1986). The PD-ring is not located inside the plastids but is observed in the cytoplasm close to the outer envelope membrane which is seen around the constricted isthumus of dividing chloroplasts (Mita *et al.*, 1984; Mita and Kuroiwa, 1988). Similar PD-rings have been observed in the moss *Funaria hygrometrica* (Tewinkel and Volkmann, 1987) and in higher plants such as *Avena sativa* (Hashimoto, 1986) and *Nicotiana tabacum* (Fig. 17c). In higher plants, there is a definite discrepancy with respect to the distribution of electron-dense deposits between the observations of Hashimoto (1986) and earlier authors which will be discussed below. The plants in which the electron dense deposit, the annulus or the PD-ring has been observed thus far are summarized in Table 1.

## The PD-ring of the Red Alga Cyanidium caldarium

It was advantageous for our analyses of the apparatus involved in chloroplast division to use synchronous cultures of the unicellular alga *C. caldarium*, which contains a cell nucleus, a mitochondrion and a chloroplast (Figs. 5 and 8) (Nagashima and Fukuda, 1981). Synchronous cultures can be initiated from young cells. These young cells are small daughter cells with volumes of approximately 4 $\mu m^3$ and are obtained by Percoll density-gradient centrifugation of seven-day-old cultures. The number of cells increases stepwise 44 hr and 60 hr after initiation of the incubation and finally reaches a value of about four times the initial number (Mita and Kuroiwa, 1988). The increase in number at the second step is markedly greater than that at the first step. The basic life cycle of *C. caldarium* is shown in Figs. 5 and 15 and is derived from data described previously (Mita *et al.*, 1986). If we start with an examination of young cells, we see that each cell contains a cell nucleus of about 0.1 $\mu m^3$ in volume and a chloroplat of about 1 $\mu m^3$ in volume. Growth of mother cells takes place up to 50 hr after initiation of synchronous culture (ISC) and these growing cells are mostly four-endospore cells after the second endospore divisions. The relative proportions of various types of cell, such as young cells (I), single cells (II), two-endospore cells (III) and four-endospore cells (IV) were examined at different times after ISC (Fig. 5). The divisions of the chloroplast, the cell nucleus, the

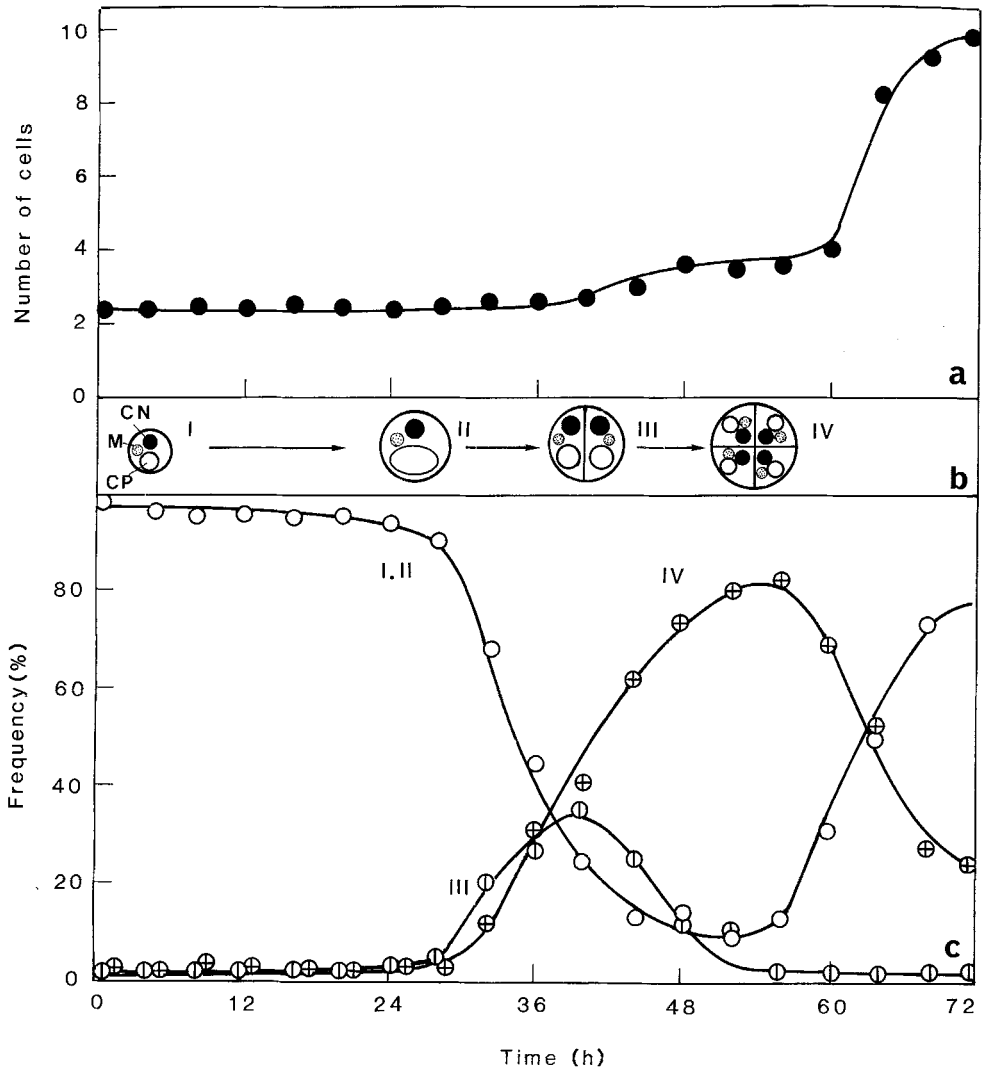



Fig. 5. Changes in cell number ($\times 10^7$ cells/m$l$) (a) and a schematic representation (b) and frequencies (%) (c) of four different types of cell: young cell (I) and single cells (II), two-endospore cells (III) and four-endospore cells (IV), at various times after the initiation of synchronous cultures of *Cyanidium caldarium* prepared by Percoll density-gradient centrifugation. One mother cells contain a cell nucleus (CN), a mitochondrion (M) and a chloroplast (CP) and divides synchronously to form four daughter endospores after two endospore divisions. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

mitochondrion and the cell occur in that order. The cells containing two endospores increase in number just before appearance of the cells that contain four endospores. These increases occur at the same times as the decrease in number of young cells and of single-endospore cells, about 20 hr before the increase in cell number. The levels of cp-DNA increase soon after ISC and reach 4 times the initial value before the first

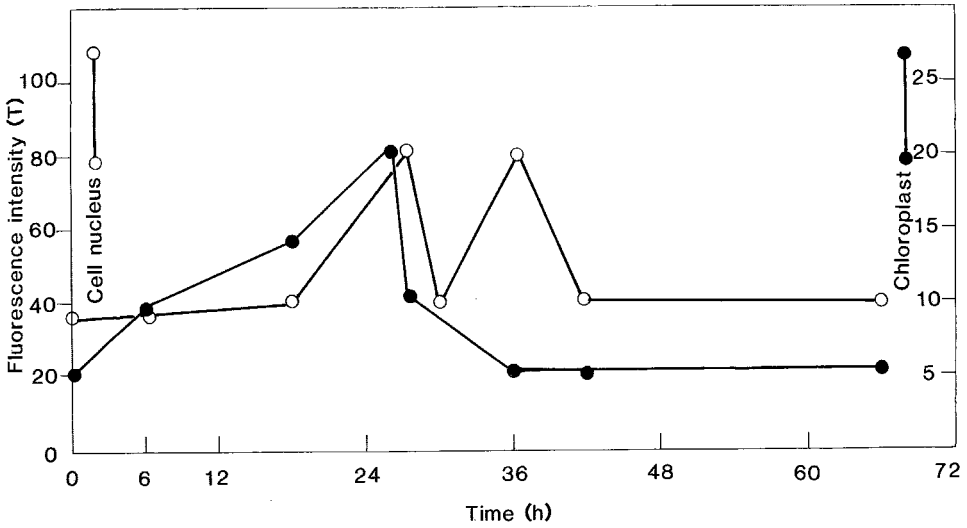



Fig. 6. Change in DNA content of a plastid nucleus and cell nucleus. DNA content of the plastids was determined from fluorescence images of the specimens analyzed with a photonic microscope system and is expressed in arbitrary units of "T". T corresponds to fluorescence intensity of T4 phage. The young cell increases in DNA content soon after ISC and reaches about 4 times its initial volume. The DNA in the chloroplast decreases stepwise after each endospore division, while the DNA in the cell nucleus is duplicated during each endospore-division cycle. (Figure courtesy of Drs. Mita and Kuroiwa).

division of chloroplasts. The DNA in the chloroplast decreases stepwise after each endospore division, while the DNA in the cell nucleus is duplicated during each endospore-division cycle (Fig. 6). Since the components, including pigments and cp-DNA, are not synthesized during the second endospore-division cycle, the chloroplasts decrease in volume to half of the origdnal volume of chloroplasts immediately after ISC (Mita and Kuroiwa, 1988). This series of events demonstrates how the young cells grow, develop, and mature and the mother cell divides to form new young cells.

In the control preparation (Fig. 7a, b), when the mother cell was excited with ultraviolet light after DAPI staining, four spherical, cell nuclei and four irregulary shaped ct-nuclei, emitting blue-white fluorescence, could be seen (Fig. 7a). When the same field of the cell was excited with green light, four chloroplasts, emitting red fluorescence, could be clearly seen in the areas where ct-nuclei were located (Fig. 7b). These results indicate that the untreated mother cell is composed of four discrete endospore cells, each of which contains a cell nucleus and a chloroplast. The mitochondrial nucleus is very small and, thus, it was only rarely observed when cells were squashed with relatively high pressure on glass slides. When cells were treated throughout two sequential endospore divisions, with cremart, an inhibitor of the assembly of tubulin, each mother cell was seen to contain one cell nucleus (Fig. 7c) and four chloroplasts as predicted (Fig. 7d). By contrast, when the cells were treated

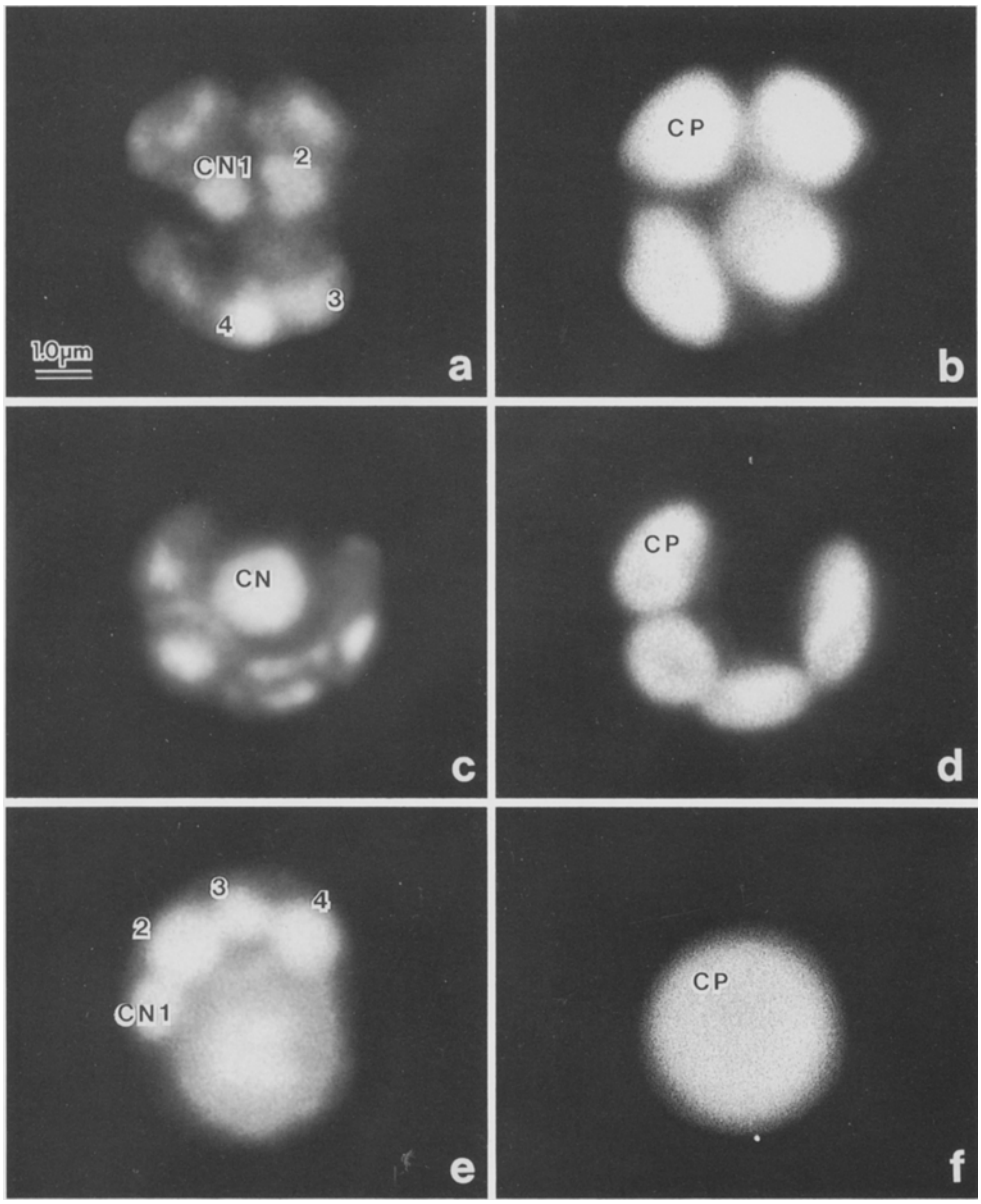



Fig. 7. Epifluorescence photomicrographs illustrating cell nuclei (CN1-CN4) and the chloroplast (CP) in mother cells fixed immediately after incubation for 30 hr without inhibitors (a, b); and with cremart at 10 $\mu$g/m$l$ (c, d). Fluorescence photomicrographs with UV (a, c, e) and green lights (b, d, f) were taken in the same field for each treatment. In the control (a, b), one mother cell is composed of four discrete endospore cells, each of which contains a cell nucleus and a chloroplast. When the cells were treated with cremart through two sequential endospore divisions, the mother cell contains one cell nucleus and four chloroplasts (c, d). By contrast, when the cells were treated with cytochalasin B, the mother cell contains four cell nuclei and one large chloroplast (e, f). Scale bar represents 1 $\mu$m. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

throughout two sequential endospore divisions with cytochalasin B, an inhibitor of the polymerization of actin into filaments, each mother cell was seen to contain four cell nuclei (Fig. 7e) and one large chloroplast (Fig. 7f). The results suggest that microtubules are not involved in the division of the chloroplast, but are involved in the division of the cell nucleus, while polymerized actin filaments are not involved in division of the cell nucleus, but play an important role in the division of chloroplasts (plastidkinesis) and in cytokinesis.

Leonard and Rose (1970) compared the effects of chloramphenicol and cycloheximide on the growth and division of chloroplasts in spinach leaf discs. They showed that chloroplasts division continues in discs treated with chloramphenicol, a drug that inhibits protein synthesis in chloroplasts, but that no chloroplast division occurred in the discs treated with cycloheximide, a drug that inhibits cytoplasmic synthesis of proteins. However, cell size was significantly reduced by cycloheximide, which is known to be a relatively nonspecfic inhibitor. When the protonema of the moss *Funaria hygrometrica* Sibth were exposed to cytochalasin B at a concentration of 20 $\mu$g/m$l$, there was no clear indication of whether or not actin is necessary for constriction of plastids (Tewinkel and Volkmann, 1987).

## Staining of Actin Bundles around the Chloroplast

When cells of *C. caldarium* were stained with rhodamin-conjugated phalloidin, a fluorescent dye specific actin, a ring which emitted orange-colored fluorescence appeared faintly in the equatorial region of the dividing chloroplast, which itself emitted a red color in gently squashed preparations (Mita and Kuroiwa, 1988). However, the result could not exclude completely the possibility that the ring may correspond to a cytoplasmic contractile ring for cytokinesis. There is no information available about the staining of actin bundles around the chloroplast in land plants and algae except for these data on *C. caldarium*.

## Formation of the PD-Ring around the Chloroplast

There are several reports of electron-microscopic observations of the PD-ring, namely, in the red alga, the moss, and higher plants as described above. However, since the moss and higher plants have multiplastidic cells and the divisions of the plastids are not synchronized, it is difficult to examine the behavior of plastids during a division cycle. Thus, sequences of ultrastructural changes that occur as the chloroplasts of land plants divide have not been described in detail. However, in synchronized cells of the monoplastidic alga *C. caldarium*, it is possible to observe events in detail, from the formation of the PD-ring during the early stages to the disappearance of the PD-ring during the late stage. Fig. 8 shows representative epifluorescence and electron micrographs of a young cell soon after ISC. The cell contains one cell nucleus, one rod-shaped mt-nucleus and one cp-nucleus (Fig. 8a). The images in Fig. 8a strongly support the three nuclear theory of cell that plant cells contain three

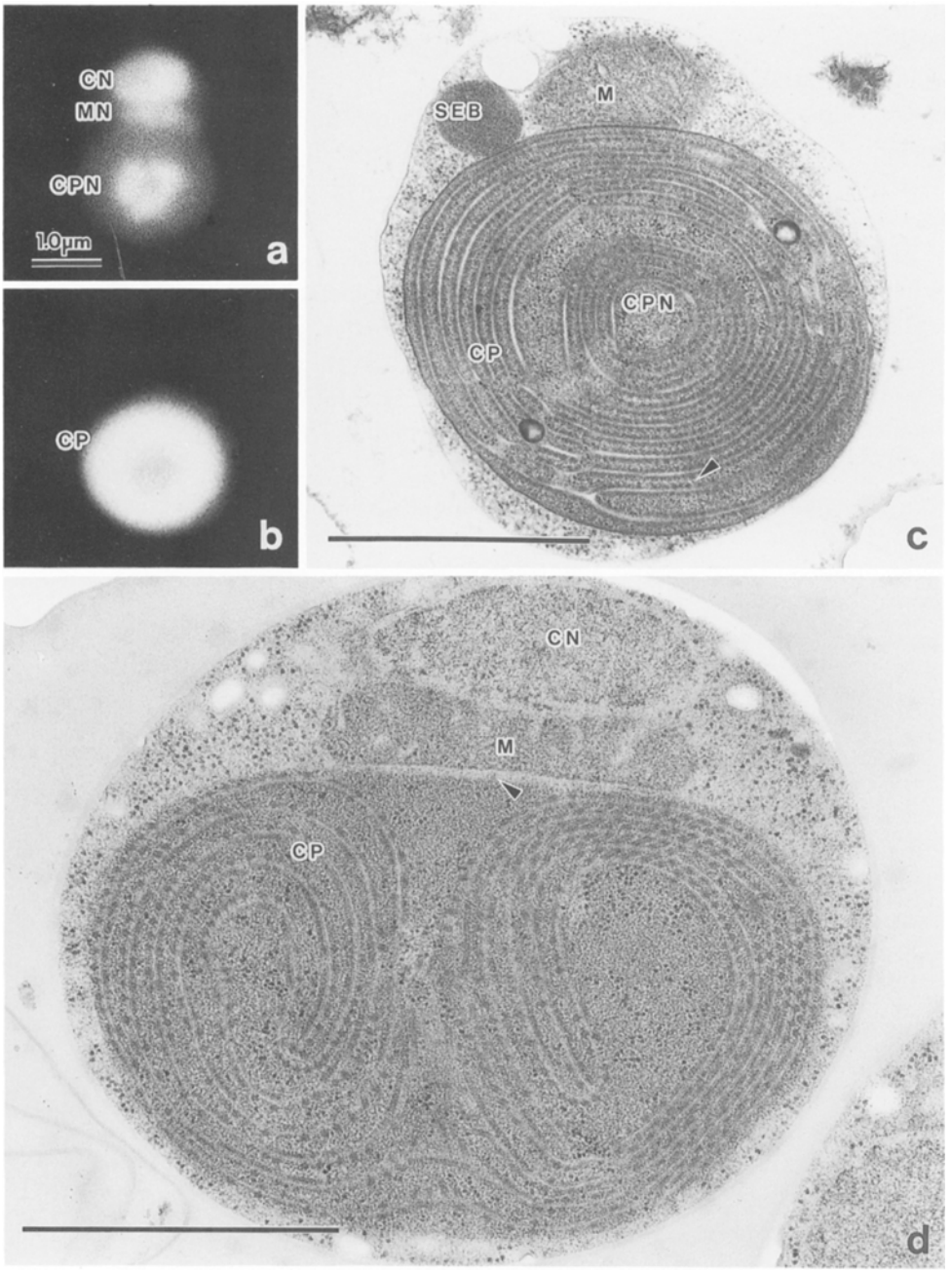
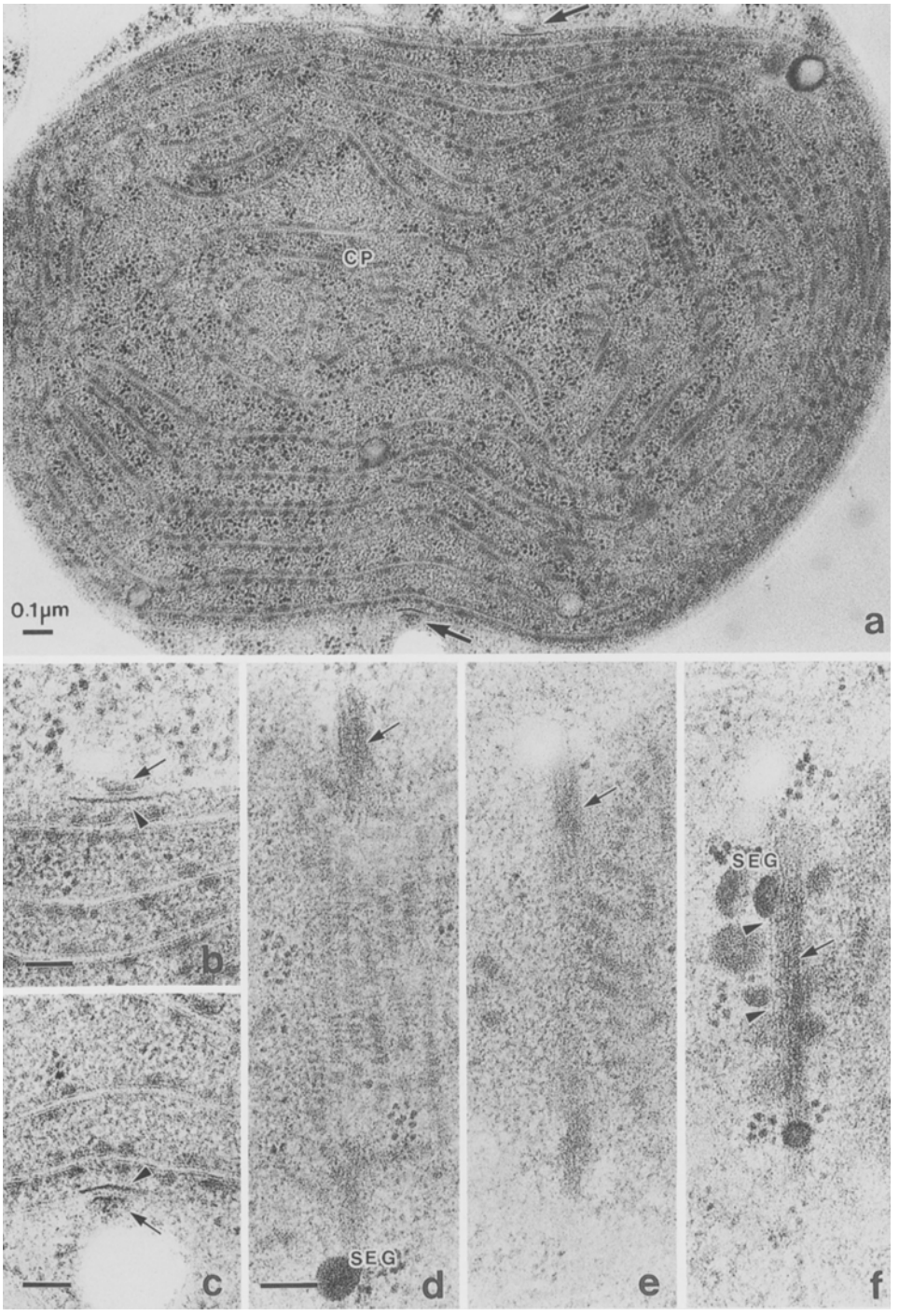



Fig. 8. Epifluorescence photomicrographs (a, b) and electron micrographs of intervening sections (c, d) of a cell nucleus (CN), a chloroplast (CP), a cp-nucleus (CPN), a mitochondrion (M), a mt-nucleus (MN), and a SEB, in cells fixed 4 hr (a–c) and 24 hr (d) after ISC. (a) and (b) are images of nuclei (a) and chloroplast (b) taken in the same field under excitation by UV (a) or green light (b) after DAPI staining. An arrow shows electron dense deposit which is a part of the PD-ring. Scale bars show 1.0 $\mu$m. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

different types of nuclei, namely, the cell nucleus, the mitochondrial nucleus and the plastid nucleus (Kuroiwa, 1982). The cp-nucleus is located in the central region of the spherical chloroplast (Fig. 8a, b). Under the electron microscope, the spherical chloroplast is seen to contain concentric, circular, thylakoid membranes with phycobilisomes of about 30 nm diameter. In addition to the cell nucleus, in the cell cytoplasm, a somewhat electron-dense body (SEB) and a mitochondrion are also visible (Fig. 8c). When the cell, the cell nucleus, and the chloroplast of *C. caldarium* increase in volume about three-fold, two-fold and 3.5-fold, respectively, the shape of the chloroplast changes from a spherule to a football-like structure and concentric, circular thylakoid membranes in the chloroplast begin to separate into two parts (Fig. 8d). At that time, the initial sign of the formation of the PD-ring appears at the equatorial area of the chloroplast: many somewhat electron-dense granules (SEGs), each 40–90 nm in diameter (Fig. 9d-f), and electron dense deposits appear in the cytoplasm close to the outer envelope membrane and begin to be distributed at the equatorial region of the chloroplast (Figs. 8d and 9a-c). As judged from the density of SEGs, the SEG appears to be derived from the somewhat electron-dense body (SEB). The PD-ring is made up of SEG. When the PD-ring was serially sectioned in a direction perpendicular to the plane of division, a portion of the PD-ring was seen as a bar, about 60 nm in width, at the edge of the PD-ring. The bar consisted of fine filaments, each about 5 nm in diameter, which were aligned parallel to the longitudinal direction of the bar (Fig. 9f). This arrangement indicates that the PD-ring is a bundle of fine filaments. Often the end of a fine filament appears to coincide almost exactly with the edge of the SEG, suggesting that fine filaments are made from the SEG. At higher magnification, the filaments can be seen to be rough-surface and small protruding nodes are seen to be associated with the filaments (Fig. 9f). These fine filaments are probably organized into bundles, which form in several parts of the equatorial region, as shown in the second division of the chloroplast, and then are coalesced with each other to form a complete, closed ring structure.

## Contraction of PD-Ring

By the time the PD-ring of *C. caldarium* starts to contract, the small SEGs have completely disappeared. When sequential thin sections are cut through the constricted isthmus of a dividing chloroplast of the alga during the middle phase of division, it appears that the electron-dense deposits at the bridge between daughter chloroplasts are distributed as a close ring or belt-like structure, 60 nm wide and 50 nm thick, along the outside of the outer envelope of the chloroplast (Mita and Kuroiwa, 1988). The PD-ring is a bundle of fine filaments, but it is difficult to discern any individual fine filaments. After succeeding contractions, the PD-ring becomes thicker and more electron-dense than at the early stage of the division of the chloroplast. As a belt of microfilaments, the PD-ring has an overall width of about 60 nm, a size that corresponds very precisely to the furrow, as if the PD-ring generates the constricting force that separates the daughter chloroplasts. Even though the division of the chloroplast


CP
0.1μm
a
b
c
SEG
d
e
SEG
f

has almost reached its final stage, nuclear division and mitochondrial division have not yet started (Fig. 10). At the final stage, the width of the deposits that make up the constricted PD-ring appears to be somewhat greater than that of the deposits at the early and middle stages of chloroplast division (Fig. 10a). However, the width of the PD-ring does not deviate very much from the cited value of 60 nm, regardless of the stage of division of the steepness of the walls of the furrow between the daughter chloroplasts, nor does it vary much among chloroplasts of vastly different volumes. The chloroplast division of the S-type, as shown in Fig. 3, is characterized by the steepness of the wall of the furrow at the constricted isthmus. Mita *et al.* (1986) suggested that the PD-ring may generate the contractile force that is involved in the division of the chloroplast. At higher magnification, the cross sections of the PD-ring clearly reveal that the PD-ring is located on the cytoplasmic side of the outer envelope (Fig. 10b). Inside the inner envelope, some electron-dense deposits can also be seen but their width and thickness do not change from the early stage of chloroplast division until it is complete (arrow heads in Figs. 9c and 10b, c). Similar events can be seen in the second division of the chloroplasts. The PD-ring, when seen in sections cut in a direction parallel to the plane of division, is clearly made up of a circular belt which appears to be composed of tightly packed, fine filaments (Fig. 10c).

### Conversion of the PD-Ring to the Somewhat Electron-Dense Body

At the final stage of chloroplast division in *C. caldarium* cells, the electron density of the PD-ring increases markedly and what is left of the ring remains as a conspicuous, electron-dense structure between daughter chloroplasts which are each approximately 3.5 $\mu m^3$ in volume. When the chloroplast has ceased dividing, a centriole-like plaque with microtubules develops outside the cell nucleus and an electron-dense deposit beneath the cell membrane also appears, suggesting the beginning of the division of the cell nucleus and of the cytokinesis required for the first endospore division (double arrow heads in Fig. 11a). Soon after the chloroplast has divided, the remnants of the PD-ring can be seen, associated with a slender part of the balloon-like SEB (arrow heads in Fig. 11b). Since the PD ring disappears completely before initiation of the second endospore division, it is possible that it is converted into the SEB at the time

Fig. 9. Electron micrographs of the intervening region (a-c) and the edge (d-f) of the PD-ring (arrows) sectioned serially in a direction perpendicular to the plane of division of the chloroplast, in a cell fixed 24 hr after initiation of a synchronous culture. Somewhat electron-dense, flat deposits appear opposite one another on either side of the constricted isthmus of the dividing chloroplast (arrows in a). At higher magnification, the deposits are seen to be located in the cytoplasm. The margins of the inner matrix beneath the inner envelope also become electron-dense (arrowheads in b and c). In the three serial sections of the edge of the PD-ring, the dense deposits appear as a bar about 60 nm wide (arrows in d-f). Fine filaments are aligned parallel to the longitudinal direction of the bar (d-f). Small, SEGs, 30-70 nm in diameter, appear along the bundle of fine filaments and the SEGs can be seen to be associated with the fine filaments (arrowheads in f). Scale bars represent 0.1 $\mu m$. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

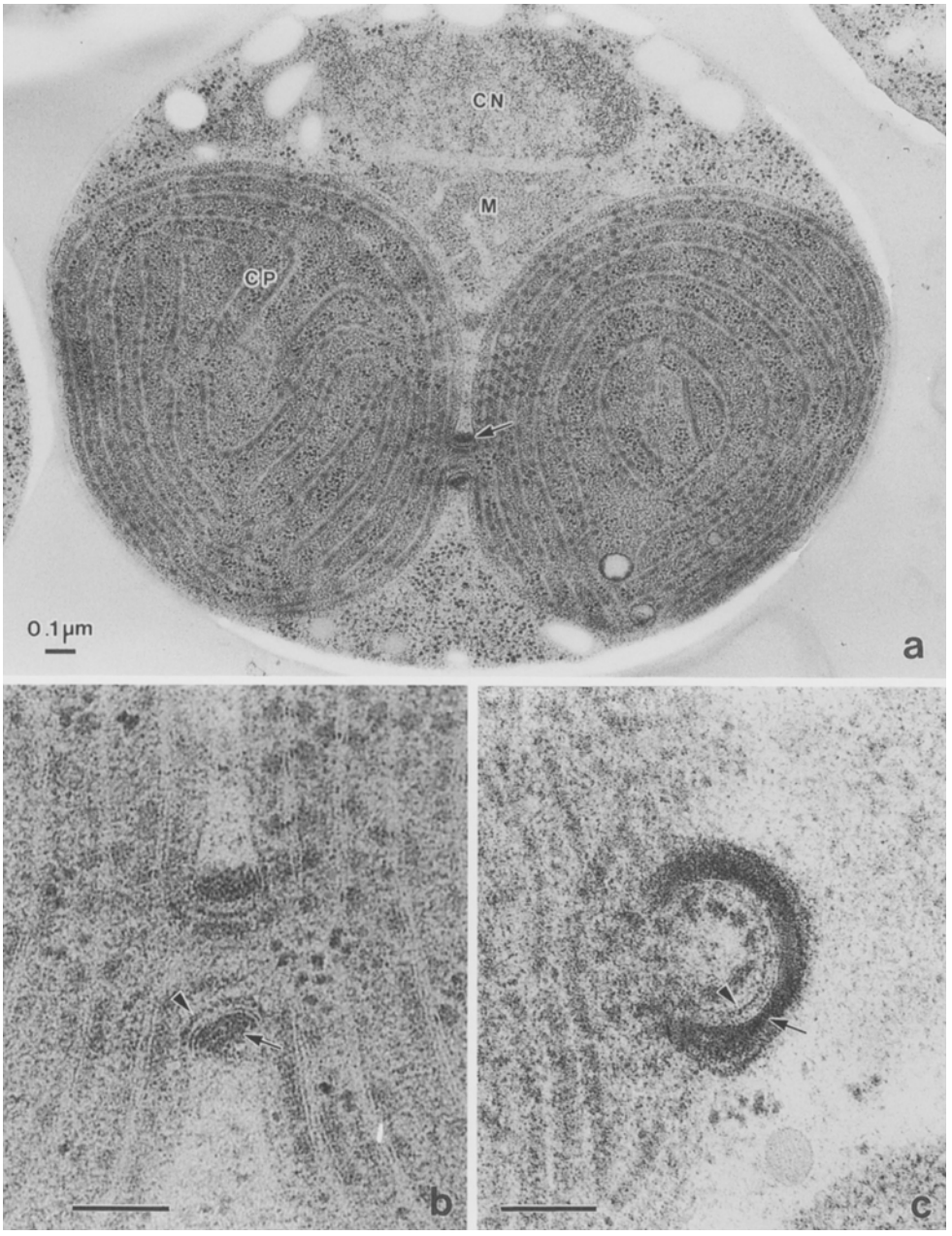



Fig. 10. Electron micrographs of sections cut in directions perpendicular to (a, b) and parallel to (c) the plane of division at the constricted isthmus of the dividing chloroplast, in cells fixed 32 hr after ISC. When the chloroplast is progressively pinched, the PD-ring becomes more-electron dense and is seen to increase in thickness (arrow in a). At higher magnification, the PD-rings can be seen clearly to be located on the cytoplasmic side of the outer envelope membrane (arrows in b and c), while characteristics of the inner electron-dense belt (arrowheads in b and c) do not change from those observed at the early phase of the division. Scale bars represent 0.1 $\mu$m. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

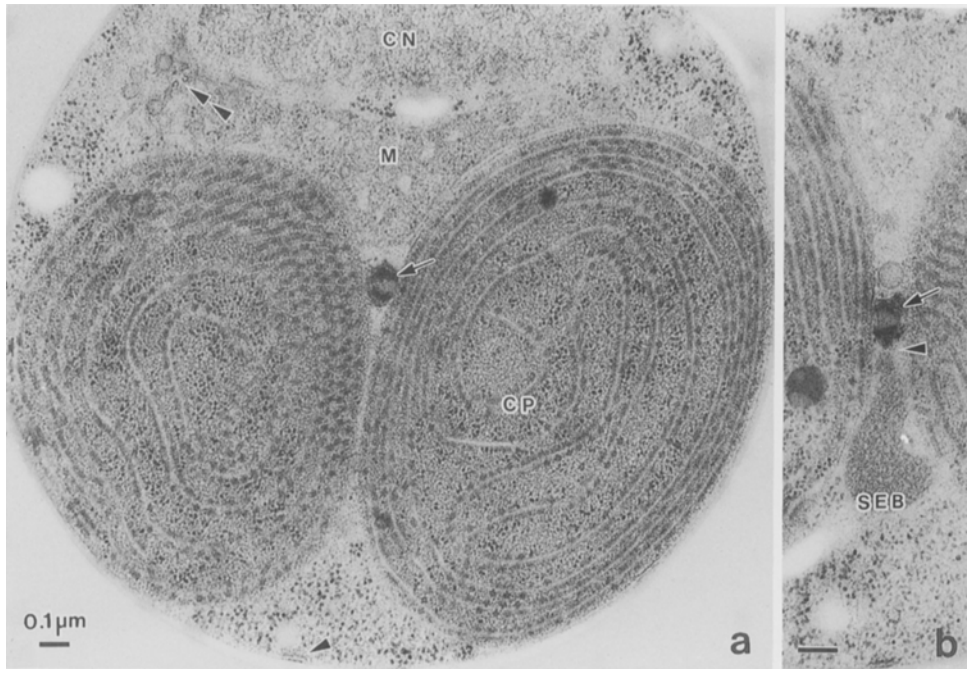



Fig. 11. Electron micrographs of the PD-rings of chloroplasts sectioned in a direction perpendicular to the plane of division of the chloroplast in cells fixed 32 hr after ISC. The electron-dense PD-ring appears on the tenuous bridge between daughter chloroplasts (arrows). The centriole-like plaque (double arrowheads) at the pole of the cell nucleus and the electron-dense deposit (arrowhead) beneath the cell membrane, both of which are required for cytokinesis, are now visible. Soon after this point in time, the chloroplast divides completely and the association between the PD-ring and the balloon-like, SEB is seen (arrowhead in b). Scale bars represent 0.1 $\mu$m. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

of this association. At the very least, the association suggests that there is an interaction between the PD ring and SEB.

### The Size of the PD-Ring Depends on the Volume of the Chloroplast

Is there a relationship between the amount of material that constitutes the PD-ring, and the volume of the chloroplast? In *C. caldarium*, the mother cell divides into four daughter cells after the second endospore division (Fig. 5). Therefore, two chloroplast divisions during the second endospore-division cycle occur to generate four daughter chloroplasts. The volume of dumbbell-shaped chloroplasts, which appear during the second endospore division, is approximately half that of the original volume at the first endospore division, since the components of the chloroplasts, including cp-DNA, are not synthesized after the first endospore division (Fig. 6). Therefore the alga provides a very convenient system for the examination of the relationship between the size of the PD-ring and the volume of the chloroplast.

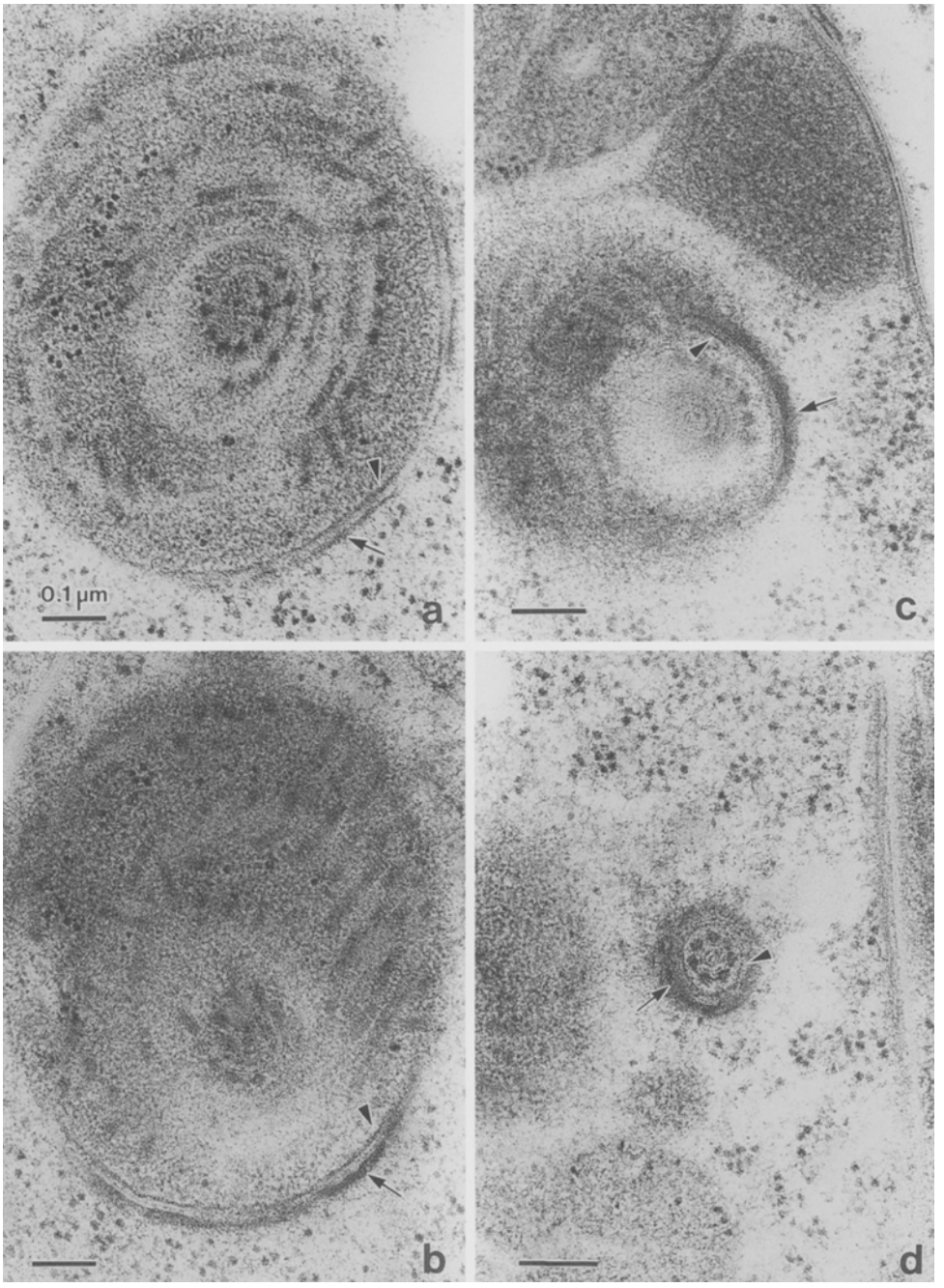



Fig. 12. Electron micrographs illustrating the circular PD-rings sectioned in directions parallel to the planes of division at the constricted isthmus of the dividing chloroplasts, in two-endospore cells fixed 40 hr (a, b) and 44 hr (c, d) after ISC. In the second chloroplast division, fragments of the PD-ring fuse to form a circular ring which then increases in thickness with the advance of the chloroplast division (arrows), while the inner matrix belts do not change in thickness (arrowheads). Scale bars represent 0.1 $\mu$m. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

At the second endospore division of the cell, the flattened deposits indicative of the cross section of the PD-ring appear again on the cytoplasmic side on each constricted isthmus between daughter chloroplasts (Mita and Kuroiwa, 1988). However, the width and thickness of the PD-ring appear to be smaller than those of the PD-ring present during the first chloroplast division. With the progression of the chloroplast divison, the PD-ring, which appears at the constricted isthmus of the dividing chloroplast in each of the two-endospore cells, thickens and its electron density increases, so that the PD-ring resembles the ring that was involved in the first chloroplast division. From the various sections that were cut in a direction parallel to the planes of division of the dividing chloroplasts at the early (Fig. 12a, b), and middle (12c) and late (12d) phases of the second chloroplast division, one can clearly visualize the process of contraction of the PD-ring. At the early phase, parts of the PD-ring are absent in a few regions, suggesting that fragments of the PD-ring have been deposited in various parts of the equatorial region of the chloroplast and then fused to form a circular ring (Fig. 12a). The increases in electron density and thickness of the PD-ring take place as the contraction of PD-ring progresses (arrows in Fig. 12a-d) but the thickness and electron density of the inner belts within the inner envelopes do not change (arrow heads in Fig. 12a-d). There is a very interesting relationship between the size of the PD-ring and the diameter of the plane of division of dividing chloroplasts at various stages of chloroplast division. The width of the PD-ring does not change after the initial stage of contraction but the thickness increases constantly during the contrac-

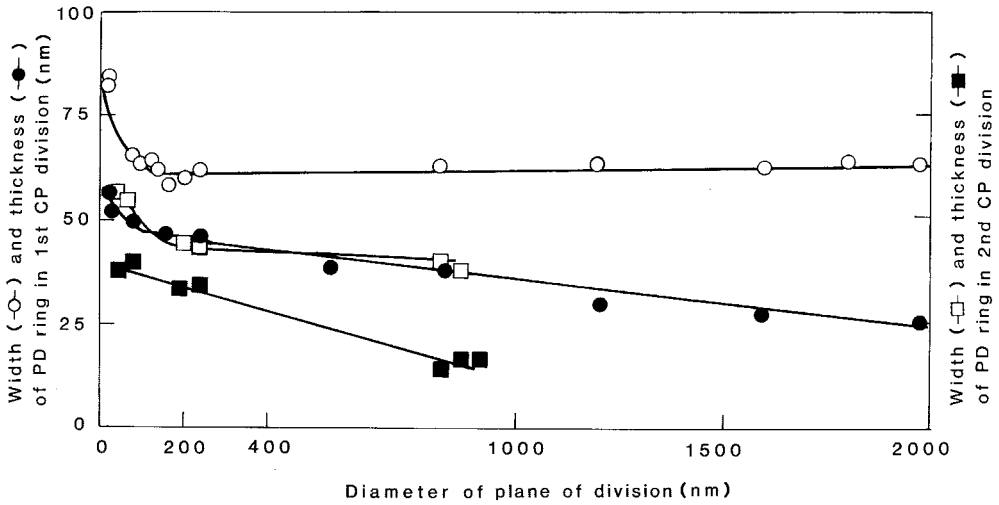



Fig. 13. Changes in the width and the thickness of the PD-ring during the first and second chloroplast divisions. The width of the PD does not deviate far from a value of approximately 60 nm through the first chloroplastkinesis while the thickness of the ring increases constantly. However, at the final step of the division, both width and thickness increase rapidly. Similar events occur during the second chloroplastkinesis except that the width and the thickness are about half of those of the ring involved in the first chloroplastkinesis. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

tion (Fig. 13). These results suggest that the actual physical components of the PD-ring remain constant during the contraction. The volume of the PD-ring during the second division of the chloroplast are about half as large as those during the first division. Since the early dividing chloroplasts are about 1.7 $\mu m^3$ in volume, or about half the size of those involved in the first division, the size or volume of the PD-ring appears to be intimately related to the volume of the dividing chloroplast. Judging from these results, we can assume that the mode of the second chloroplast division is basically similar to that of the first division and that there is a positive relationship between the sizes of chloroplasts and the sizes of their respective PD-rings.

## Components of the PD-Ring

Since the PD-ring is a very small part of the chloroplasts, and since the PD-ring appears only briefly during division of the chloroplasts and is ssociated with the outer envelope membrane at the constricted isthmus of the dumbbell-shaped chloroplasts, proplastids, amyloplasts and etc., it is not possible, at the present time, to examine the components of the PD-ring biochemically by isolation of the PD-ring. Therefore, we tried to identify the components of the PD-ring using cytochemical and cyto-physiological techniques (Mita and Kuroiwa, 1988). As described above, we propose that the main components of the PD-ring are actin-like filaments, on the basis of the following pieces of evidence. (1) Cytochalasin B, an inhibitor of the formation of actin filaments, inhibited the division of chloroplasts without inhibiting division of the cell nucleus (Fig. 7). (2) The ring-like structure around the chloroplasts can be stained with rhodamin-conjugated phalloidin (Mita and Kuroiwa, 1988). (3) Fine

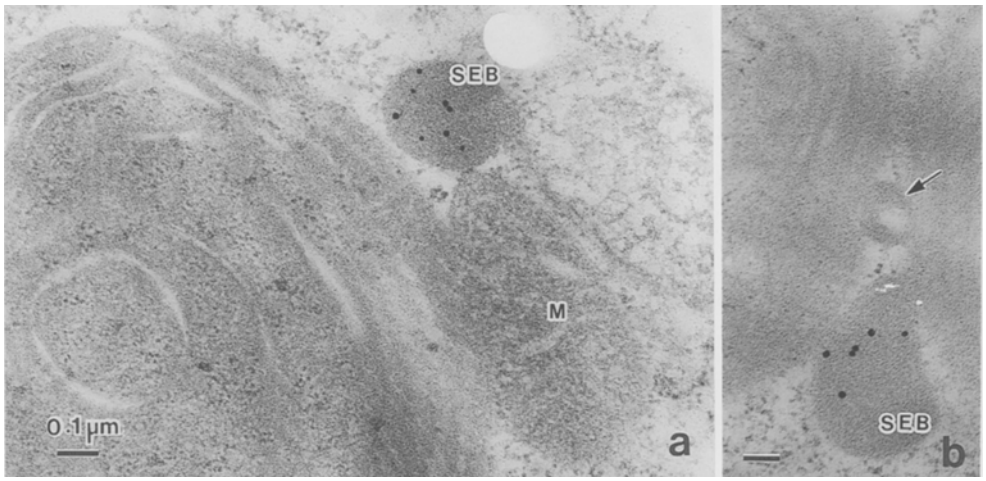



Fig. 14. Immuno-gold electron micrographs of SEB and the PD-ring of a chloroplast sectioned longitudinally in a cell fixed 24 hr (a) and 38 hr (b) after ISC. Gold particles do not appear over the PD-ring (arrow) but are seen over the SEBs. Scale bars represent 0.1 $\mu m$. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

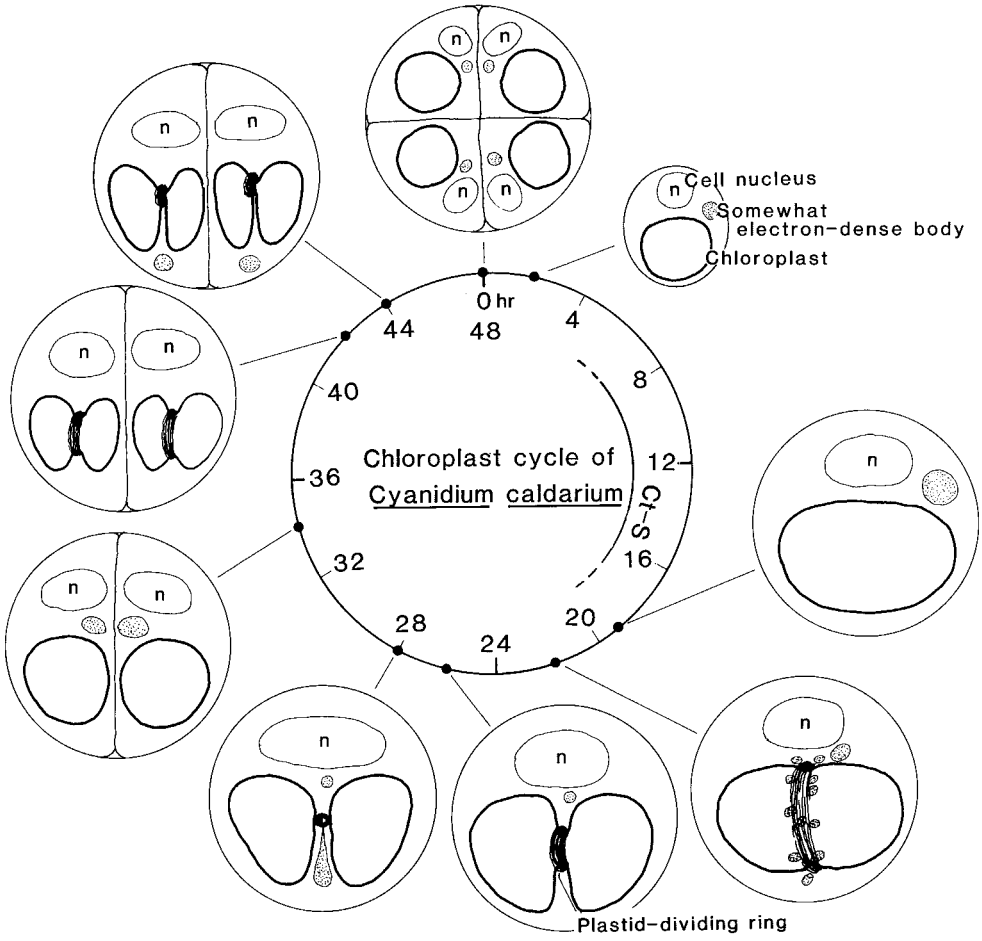



Fig. 15. Diagram of the life cycle of *Cyanidium caldarium* depicting a possible sequence of events by which the PD-ring is formed, becomes aligned, causes contraction, and is eventually disassembled. The process of chloroplast division can be classified into the following four stages on the basis of morphological and temporal differences. The chloroplast growth stage: the small, spherical chloroplast increases in volume and becomes a football-like structure. Formation stage of the plastid-dividing ring: the SEB is fragmented into many SEGs, which are aligned along the equatorial region of the chloroplasts and fine filaments are formed from the SEGs in the equatorial region. The fine filaments of the PD-ring align themselves according to the longest axis of their overall domain, i.e., circumferentially. Contraction stage: a bundle of fine filaments begins to contract and generates a deep furrow. Conversion stage: after chloroplast division, the remnants of the PD-ring are converted into SEBs. Similar events occur during the second cycle of chloroplast division. Ct-S represents chloroplast DNA synthetic stage. (Mita and Kuroiwa, 1988; reproduced by permission of Springer-Verlag).

filaments, which are observed in the PD-ring, are very similar in diameter to actin filaments (Fig. 9). However, our proposal is not supported by the results of an immuno-gold staining experiment: a few gold particles, which show localization of actin, appeared on the SEGs and none on the PD-ring (Fig. 14). However, the interpretation of the data obtained by immuno-gold electron microscopy is very difficult. The absence of immuno-gold particles on the PD-ring may be the result of one of the following possibilities. (1) The PD-ring may not contain actin. (2) The amount of actin contained in the PD-ring may be too small to be identified quentitavely by the present application of the immuno-gold technique. (3) The hypothetical actin filaments in the PD-ring may not react qualitatively with the antibody raised against actin from chicken gizzard. Thus, we have concluded that the PD-ring contains actin-like material. The cycle of formation and conversion of the PD-ring during the life cycle of *C. caldarium* is summarized in Fig. 15.

## The PD-Ring in Land Plants

The monoplastidic cell *C. caldarium* is very useful for observations of the behavior of the PD-ring during its life cycle, as described above. By contrast, in the plastids of land plants, it is difficult to decide whether or not the dumbbell-shaped plastids in higher plants are dividing plastids, because dumbbell-shaped plastids are not always dividing plastids in living cells. Whatlay (1980) pointed out that, in *Phaseolus*, conventional dumbbell-shaped plastids are found alongside amoeboid plastids, and that without additional information about changes in numbers of plastids that could be correlated with changes in the shape of plastids, one cannot conclude that either the dumbbell- or the amoeba-shaped plastids are indeed dividing. Thus, it is difficult to observe the behavior of the PD-ring during the division cycle of plastids in multiplastidic land plants. This problem is the basis for the discrepancies in the observed distribution in the PD-ring in higher plants (Fig. 16).

Suzuki and Ueda (1975) were the first to report the appearance of electron-dense material, which they considered to be a septum, at the constricted isthmus between daughter proplastids or daughter amyloplasts, after the middle phase of the division of plastids in *Pisum sativum* (Fig. 16a). Similar electron-dense deposits have been observed at the constricted isthmus of dumbbell-shaped chloroplasts of *Hyacinthiodes non-scripta* (L) (Luck and Jordan, 1980). Leech *et al.* (1981) have described such electron-dense materials as "fuzzy plaques" in the dividing chloroplasts *Triticum aestivum*, *Atriplex semibaccata* and *Sesamum indicum* var. gluca (Leech *et al.*, 1981) (Fig. 16b). Subsequently, Chaly and Possingham (1981) reported that electron-dense deposits, which are parts of an annulus, were located in the interspace between the outer and inner envelope membrane at the constricted isthmus between daughter proplastids of various higher plants (Fig. 16c). As described above, common features of these earlier observations can be summarized briefly as follows: electron-dense deposits about 20 nm to 25 nm in diameter appear only at the constricted isthmus of the dumbbell-shaped plastid just before separation of daughter plastids and are never

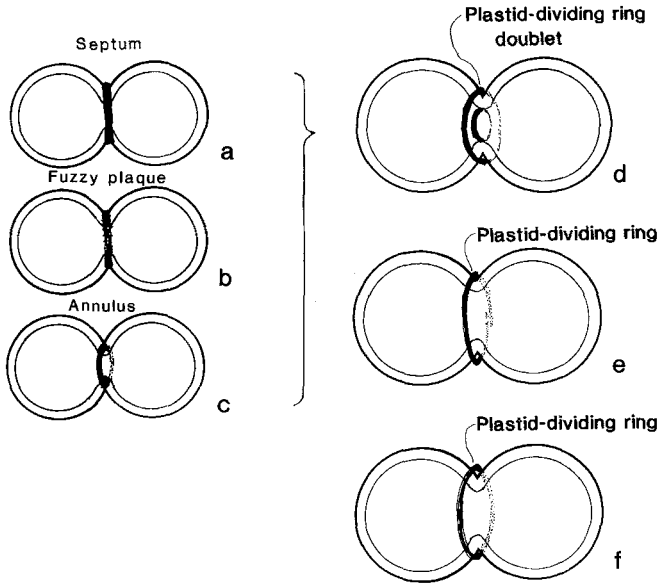



Fig. 16. Electron-dense deposits appear at the constricted isthmus of a dividing plastid during the late phase of plastid division in higher plants, which Suzuki and Ueda (1975) and Luck and Jordan (1980) considered to be a septum (a) and which Leech *et al.* (1981) described as a fuzzy plaque (b). Subsequently, Chaly and Possingham (1981) reported that electron-dense deposits, which are parts of an annulus, are located in an interspace between the outer and inner envelopes at the narrow bridge between daughter proplastids of various higher plants (c). However, the interpretations of these previous observations were corrected initially by Hashimoto (1986). He reported the presence of an electron-dense "double" ring structure (PD-ring doublet) around the narrow neck of dividing plastids in *Avena sativum*. The inner and outer rings of the doublet coat the inside (stromal side) of the inner envelope and the outside (cytoplasmic side). In the moss *Funaria hygorometrica* (e) (Tewinkel and Volkmann, 1987) and the red alga *C. caldarium* (f) (Mita *et al.* 1986; Mita and Kuroiwa, 1988), the PD-ring can be seen to be located in the cytoplasm at the constricted isthmus of the dividing plastid.

seen at the early and middle phases of plastid division; furthermore, electron-dense deposits are located inside the plastids themselves. By contrast, Hashimoto (1986) observed the presence of an electron-dense "double" ring structure (PD-ring doublet) around the constricted isthmus of dividing plastids of *Avena sativum* using a serial thin-sectioning technique. The inner and outer rings of the doublet were reported to coat the inside (stromal side) of the inner envelope membrane and the ouside (cytoplasmic side), respectively. Hashimoto also reported that the ring structures could be recognized by electron microscopy at the final stage of plastid division (Figs. 16d and 17a, b). There are discrepancies in the interpretations of the localization of the PD-ring at the constricted isthmus obtained from different and from the same species of higher plants. Therefore, we also examined carefully the localization of the PD-ring of higher plants using the serial thin-sectioning technique. We recognized the localization of the PD-ring in proplastids of *Nicotiana tabacum* to be similar to that reported by Hashimoto (Fig. 17c). The discrepancies between various authors on

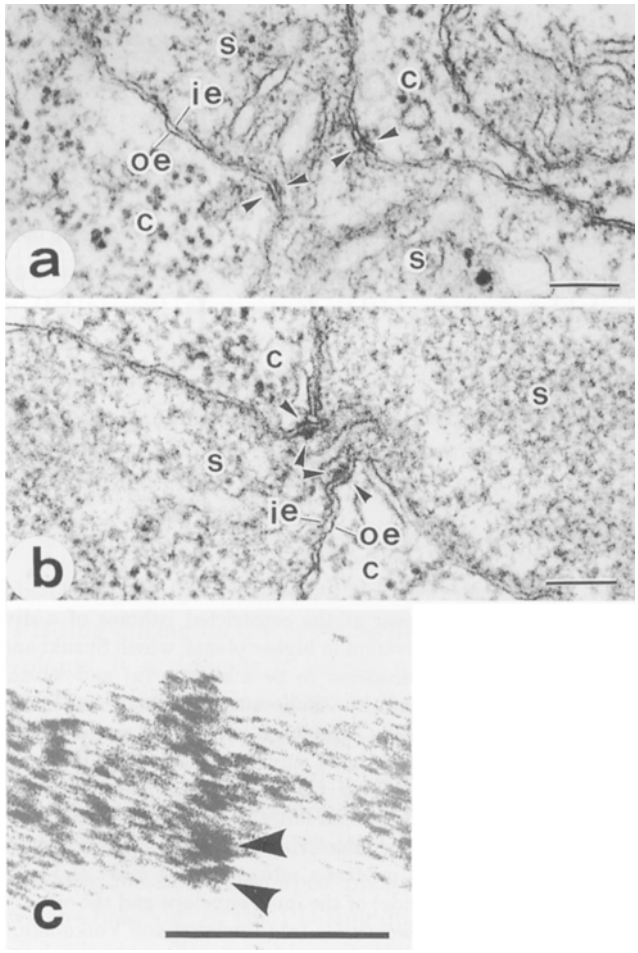



Fig. 17. Sections cut through the mid section of the constricting neck region of dividing proplastids in the region 1-2 mm above the base of the first leaves of *Avena sativum* (a, b) and in a cultured cell of *Nicotiana tabacum* (c). Numbers of a pair of dense deposits (arrowheads) can be seen on the inside of the inner envelope membrane and on the outside of the outer envelope membrane, respectively, on either side of the neck. *ie*, Inner envelope ; *oe*, outer envelope ; *s*, stromal space ; *c*, cytoplasmic space. Scale bar represents 0.1 $\mu$m. (Photographs courtesy (a and b) by Dr. Hashimoto ; reproduced by permission of Dr. Hashimoto and Springer-Verlag).

the distribution of the PD-ring in higher plants can be explained as follows. There are three possibilities to explain why Hashimoto first noticed the PD-ring doublet at the constricted isthmus of dividing plastids during the late stage of plastid division of a higher plant. When the diameter of the dividing plane at the constricted isthmus of the dumbbell-shaped plastid becomes less than about 250 nm, the PD-ring appears (Hashimoto, 1986). Investigators other than Hashimoto observed parts of the PD-ring formed around a dividing plane of less than about 100 nm in diameter while Hashimoto examined serial thin sections of a PD-ring of less than 250 nm in diameter.

The thickness of thin sections is about 70 nm. Therefore, when the dividing planes of 100 nm and 250 nm in diameter are cut perpendicular to the dividing plane, they are cut into 2 to 3 and 4 to 5 thin sections, respectively (Fig. 18). Thus, when the radius of the dividing plane is larger, information obtained from thin sections, cut at the intervening region of the dividing plane, becomes clearer (Fig. 18a-d). Furthermore, the thin sections prepared by Hashimoto were thinner than those of other investigators. In thinner sections, it is easy to observe the distribution of the PD-ring on the envelopes. The last explanation for discrepancies in the various reported observations is the most likeky, namely, that the dividing plane is not a flat disk. Rather, only a part of the disk is flat (Fig. 18e, f). Therefore, even when a dividing plane of less than 100 nm in diameter is cut perpendicular to the plane, since the PD-ring is not bent, it is easy to see the distribution of the deposits of the PD-ring doublet. "Septum" and "fuzzy plaque" and "annulus" must correspond to the images that arise when the PD-ring is cut at its edges (Fig. 18c-3, d-3) and in the region near the central area of the PD-ring (Fig. 18c-2, d-2). In conclusion, the PD-ring doublet is a common structure appearing in dividing plastids of angiosperums (Fig. 18) and gymnosperms.

In the chloronema and caulonema tip cells of the moss *Funaria hygrometrica* Sibth., Tewinkel and Volkmann (1987) observed a distinct filamentous structure similar to the PD-ring in the plane of division outside the plastid, but close to the envelope, after three-dimensional reconstruction from electron micrographs (Fig. 16e). The PD-ring also appeared clearly around the narrow isthmus of dividing chloroplasts and amyloplasts during the late phase of plastid division. The cross-sectioned filamentous structures were 10–40 nm in width and 10–15 nm thick and ran parallel to the outer envelope at a distance of about 10 nm. In addition, although no electron micrographs were provided to support their illustrations, they described their findings as follows, "A three-dimensional reconstruction of the constricted plastids shows the arrangement of the filamentous structures. Frequently, a semicircular structure was observed. Sometimes a split end occurred. Further arrangements included two parallel semicircles or two structures with a crossing point. Some plastids were surrounded by two independent structures on opposite sides of the constricted region. Helical structures were reconstructed, too" (Tewinkel and Volkmann, 1987). However, the function of the various filamentous structures has not yet been identified. Often the ends of the structures were directed into the cytoplasm, but no closed PD-ring structure was observed, as in the case of *C. caldarium*. As judged from the dimensions of the PD-ring, the filamentous structures in plastids of *Funaria hygrometrica* appear to correspond to the PD-rings observed in the chloroplasts of *C. caldarium* (Fig. 16e, f). Therefore, the filamentous structures of the moss must consist of finer subfilaments.

## Basic Structure, Significance and Phylogeny of the PD-Ring

In addition to the PD-ring in the cytoplasm of *C. caldarium*, an inner ring-like electron-dense belt also appeared inside the inner envelope membrane (Figs. 9c and

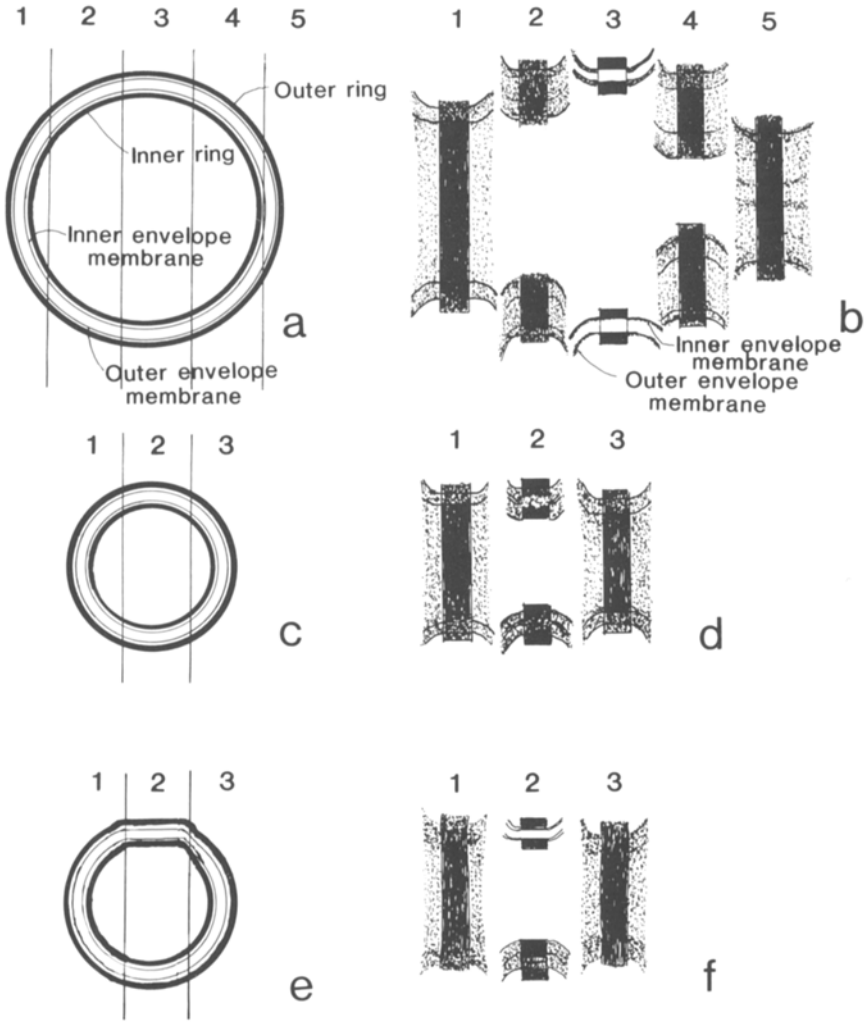



Fig. 18. Schematic representation of thin sections about 70 nm in thickness which were cut perpendicular to the dividing planes when they were about 240 nm (a) and 100 nm (c and e) in diameter and their hypothetical electron-microscopic images (b, d and f). The discrepancies among results from various laboratories on the distribution of the PD-ring in higher plants can be explained from these diagrams. There are three possibilities to explain why Hashimoto first noticed the PD-ring doublet at the constricted isthmus of dividing plastids during the late phase of plastid division. When the diameter of the dividing plane at the constricted isthmus of a dumbbell-shaped plastid is less than about 250 nm, the PD-ring appears (Hashimoto, 1986). Earlier investigators, with the exception of Hashimoto, observed parts of the PD-ring formed around a dividing plane of less than about 100 nm in diameter while Hashimoto examined serial thin sections of the PD-ring of less than 250 nm in diameter. Since the thickness of thin sections is about 70 nm, the dividing planes of 100 nm and 250 nm in diameter, which were sectioned perpendicular to the dividing plane, were cut into 2 to 3 and 4 to 5 thin sections, respectively (a–d). Thus when the radius of the dividing plane is larger, information obtained from thin sections becomes clearer (a–d). Furthermore, the thin sections examined by Hashimoto might be thinner than those of

12a-d). However, the thickness of the belt did not change during plastid division while the cytoplasmic PD-ring increased in thickness with the progressive invagination of the isthmus. Therefore, we cannot exclude the possibility that the inner electron-dense belt arises from the pressure of the depression of the envelope (Mita and Kuroiwa, 1988). If there is an inner matrix ring, it may play an important role in the selection of a specialized, equatorial region on the plastid inner membrane rather than acting as a generator of contractile force. A cytoplasmic PD-ring was also observed around the plastid of the algae *Chlorella* sp. (Ueda *et al.*, personal communication), *Pylaminomonas* sp., (Hori *et al.*, personal communication), the moss *Funaria hygrometrica* (Tewinkel and Volkeman, 1987) during the plastid division but no inner matrix ring was observed. In the fern, nobody tried to observe a PD-ring. In angiosperms and a gymnosperm, there are two rings, an outer and an inner ring, which are observed at the constricted isthmus of dividing plastids, such as proplastids, amyloplasts and chloroplasts (Fig. 17). An outer ring is located in the cytoplasm and an inner ring is in the matrix of the plastid. The outer cytoplasmic PD-ring and the inner matrix PD-ring were observed in plastids of *Avena sativum* and *Nicotiana tabacum*. A similar PD-ring doublet was observed in plastids of *Cycas* sp. (Hori *et al.*, personal communication). Probably, the cytoplasmic PD-ring could be observed in plastids in algae, moss and ferns while the PD-ring doublet could be found in all seed plants if one searched carefully for them.

The PD-ring of the alga can be seen throughout whole process of division of the plastid, while the PD-ring in land plants appears only during the late phase of plastid division. The PD-ring in the red alga is essential for the initiation of the invagination of the envelope membranes at the equatorial region of the plastid and for the cutting of the constricted isthmus between daughter plastids. The PD-ring in land plants may not be essential for initiation of the invagination of the plastid but may only be required for cutting of the narrow bridge of the dumbbell-shaped plastid. This hypothesis is supported by the different modes of division of the red alga and land plants (Fig. 3). The chloroplast of the unicellular alga *C. caldarium* is tightly packed in the small cell and, thus, the plastid must be divided by a steep invagination formed by the PD-ring, to make it look as if a strong contractile force operates on the membrane. By contrast, the plastid in the moss and angiosperms does not form the deep invagination during the early and middle phases of plastid division and the PD-ring appears only during the late phase. The slope of invagination at the constricted isthmus is shallow, as shown in Fig. 3, and the width of the outer ring of the

other investigators. In thinner sections, it is easy to observe the distribution of the PD-ring on the envelopes. Finally, the most likely possibility is that the dividing plane is not a flat disk but that a part of the disk only becomes flat (e and f). Therefore, even when the dividing plane, less than 100 nm in diameter, was cut perpendicular to the plane, since the PD-ring was not bent, it was easy for Hashimoto to see the distribution of the deposits of the PD-ring doublet. "Septum", "fuzzy plaque", and "annulus" must correspond to the images that arise when the PD-ring is cut at its edges (c-3 and d-3) and in the region near the central area of the PD ring (c-2 and d-2).

PD-ring doublet does not always correspond to the width of the invagination. There are two explanations for this phenomenon. One is that a motive force separated from that of the PD-ring operates in the formation of a gentle invagination during the early and middle phases of chloroplast division; while during the late phase, the components of the PD-ring are concentrated to form the PD-ring which then promotes the division of the plastid. A second explanation is that the PD-ring or the components of the PD-ring are related to the contraction of the invagination throughout the entire process of division. The apparent absence of the PD-ring during the early and middle phases of the plastid division may be due to elongation or diffusion of a small amount of an actin-like component on the surface of the outer membrane at the equatorial region of the dividing plastid. Certainly the PD-ring in *C. caldarium* is lower in electron density and thickness during the early phase of plastid division but increases in density and thickness during the middle phase and finally becomes very thick during the late phase. Thus, the PD-ring is easily visible. This sequence can also apply to the PD-ring of land plants. The level of components of the PD-ring in land plants may be lower than that of the PD-ring of the red alga. Therefore, during the early phase it is difficult to see the PD-ring structure, but when the PD-ring is compacted during the late phase, it becomes visible. It is difficult, at present, to determine which is the best explanation. But the latter explanation may be better and more reasonable than the others. However, we can conclude that the cytoplasmic PD-ring is a structure that is essential for plastid division from lower eukaryotes to higher plants, although Leech *et al.* (1981) reported that "fuzzy plaques" of electron-opaque material can frequently, but not always, be seen covering or displacing the membranes of the isthmus. It has been suggested that the force for division must originate within the plastid, since division appears to occur in isolated organelles (Possingham, 1980; Leech *et al.*, 1981). However, the finding of the cytoplasmic PD-ring suggests a strong involvement of cytoplasmic elements in plastid division.

## Mechanism of Formation and Contraction of the PD-Ring

Based on the above mentioned observations, a model can be proposed which suggests a possible molecular mechanism for the division of plastids, the division of plastid nuclei and plastidkinesis. There are several processes related to the mechanisms of plastid division, as follows.

1. Transfer of information for plastid division from cell nucleus or cytoplasm to the plastids.
2. Initiation of synthesis of pt-DNA in plastids.
3. Synthesis of RNA and proteins in plastids.
4. Synthesis of components for the PD-ring in the cytoplasm.
5. Termination of DNA synthesis in plastids.
6. Physical separation of synthesized pt-DNA in plastids.
7. Accumulation of components and formation of the PD-ring at the equatorial region of plastids.

8. Initiation of contraction of the PD-ring.
9. Termination of contraction of the PD-ring.
10. Conversion or disappearance of the PD-ring.
11. Termination of plastidkinesis.

Since there are two types of division cycles of the plastid, namely, with and without the duplication of DNA (Kuroiwa *et al.* 1989), the processes numbered 2, 5 and 6 may not be essential for plastidkinesis. The second chloroplast division in *C. caldarium* occurs without DNA synthesis (Fig. 6) but the PD-ring does appear (Figs. 12. 13 and 15). Chloroplast division is not always synchronous under identical cytoplasmic conditions, as shown in living *Nitella* (Green, 1964) and other multiplastidic organisms (Possingham and Lawrence, 1983). Therefore, it is as if the plastids appear to be autonomous. However, the transfer of information for the division of plastids must be produced in the cell nucleus although there is no direct evidence for this except for the fact that actin genes are encoded in the cell nuclear genome. In *Nicotiana tabacum*, division of mature chloroplasts, which have already ceased to divide, can be induced by the fusion of protoplasts derived from mature leaves and protoplasts derived from cultured cells (Kamata *et al.*, 1989). The cultured cells contain proplastids and cell nuclei which divide prosperously. Thus it is not unreasonable to assume that "plastid division-inducing factors (PDIF)" exist in the cytoplasm of cultured cells or young leaf cells in which plastids divide prosperously. On the other hand, the components of the PD-ring are actin-like proteins and must be encoded on cell nuclear genes. Thus mRNA for actin-like protein is synthesized in the cell nucleus and then moves from the nucleus to the cytoplasm. The synthesized actin-like protein moves from the rough-surfaced ER to the SEB which acts as a storage body of actin-like proteins, as shown in *C. caldarium*, and these bodies form the fine filaments of the PD-ring on the surface of the plastid immediately before division of plastids. It is well known that the actin filaments are polymerized from subunits in the periacrosomal region of sperm only when needed. We consider the the dramatic example of the control of conversion of G to F actin seen in the sperm of many species during fertilization of the egg. At that time, within seconds, long (90 $\mu$m) processes containing actin filaments form from the G actin and extend outward from the periacrosomal region at the tip of the sperm toward the plasma membrane of the egg (Darnel *et al*, 1986). In plant cells, the actin-like protein might polymerize explosively into fine filaments on the equatorial region of the plastid. The molecular mechanism of the contraction of the PD-ring may be analogous to that of the contractile ring which has been observed in the cytokinesis of animal cells. Strong evidence from animal cells suggests that actin-myosin interactions in the contractile ring power cytokinesis in the separation of daughter cells during the last stage of mitosis. Actin, $\alpha$-actinin and myosin have been identified in the contractile ring at the narrow bridge between the two separating animal cells. As the ring of actin filaments contracts, the neck of the cleavage furrow is narrowed, and eventually the daughter cells are separated. However, in detail, the mechanism of animal cytokinesis is different from that of plastidkinesis In cytokinesis, as the actin ring tightens, the thickness of the

ring remains constant. The phenomenon could, however, explain the fact that disassembly of the ring occurs together with contraction. By contrast, the PD-ring increases in thickness as the contraction progresses. In fact, when actin-like filaments were seen at the edge of the PD-ring (Fig. 9f), the filaments were rough. The sliding of actin-like filaments and an unidentified myosin-like substance may power the contraction of the PD-ring.

## Other Cytoplasmic Factors Related to Plastid Division

Associations between various cytoplasmic organelles and dividing plastids have been repeatedly noted. In studies of proplastids, Chaly and Possingham (1981) suggested an association between the constricted proplastid profile and the nucleus. They suggested that the association might indicate nuclear involvement in proplastid replication. A similar association was noted by Whatley (1974) in studies of the single plastid found in meristematic regions of *Isoetes*. In the unicellular alga *C. caldarium*, since the cell nucleus, the mitochondrion and chloroplast are aligned in tandem, the dividing chloroplast is never close to the cell nucleus. Therefore, the cell nucleus is not directly associated with plastidkinesis in this organism.

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