STUDIES ON MITOCHONDRIAL STRUCTURE AND FUNCTION IN *PHYSARUM POLYCEPHALUM*

V. Behavior of Mitochondrial Nucleoids throughout Mitochondrial Division Cycle

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ABSTRACT

The fine structure of mitochondria and mitochondrial nucleoids in exponentially growing *Physarum polycephalum* was studied at various periods throughout the mitochondrial division cycle by light and electron microscopy. The mitochondrial nucleoid elongates longitudinally while the mitochondrion increases in size. When the nucleoid reaches a length of approximately 1.5 μm the mitochondrial membrane invaginates at the center of the mitochondrion and separates the mitochondrial contents. However, the nucleoid does not divide even when the mitochondrial sections are connected by a very narrow bridge. Just before division of the mitochondrion, the nucleoid divides by constriction of the limiting membrane of the dividing mitochondrion. After division, one end of the nucleoid appears to be associated with the inner mitochondrial membrane. The nucleoid then again becomes situated in the center of the mitochondrion before repeating these same processes.

It has been reported that in the plasmodium of *Physarum polycephalum* morphological changes in the mitochondria occur throughout the mitochondrial division cycle (15) (Fig. 1). The mitochondrion contains a large, rodlike nucleoid situated in the center of the inner matrix (6, 7, 9, 16, 21) which is composed of a large amount of DNA (10, 11), RNA (9), and protein (9, 11, 14).

Little information is available about the behavior of the mitochondrial nucleoid during the mitochondrial division cycle. The object of the present study was to clarify the morphological steps in the division of the mitochondrial nucleoid. The morphology of the nucleoid at various phases of the mitochondrial division cycle was studied by light microscopy using acid fuchsin and thionine staining techniques and by electron microscopy.

MATERIALS AND METHODS

Culture of Plasmodia

Mitotically synchronized plasmodia of *Physarum polycephalum* were prepared by fusion of microplasmodia with the methods reviewed by Guttes and Guttes (5). Surface plasmodia from the second postfusion mitosis (MII) to the third postfusion mitosis (MIII) were used in these experiments.

Identification of Mitotic Cycle

The length of each portion of the mitotic cycle after fusion was determined by removing small explants from

the plasmodium and examining smears of these pieces stained with azure B stain by a procedure described previously (9).

Fixation and Acid Fuchsin Staining for Light Microscope Observations of Whole Mitochondria

After MII, small explants of plasmodia were harvested at hourly intervals. They were fixed in ice-cold Champy's fluid (4) for 24 h, dehydrated in a graded series of water and water-soluble resin, glycol methacrylate (Oken Shoji Co., Tokyo, Japan) (15), and then embedded in glycol methacrylate. Thin sections (approximately 2 μm) were cut on a Porter-Blum ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) with a glass knife, mounted on glass slides, and dried gently with an alcohol lamp. The sections were covered with a small drop of acid fuchsin containing 1 g of acid fuchsin in 10 ml of aniline water (4), air dried on the slide, and stored at room temperature until analysis. Just before examination, any excess acid fuchsin on the sections was washed out with tap water, and then a drop of glycerin and a cover slip were placed on the sections. The stained sections were examined by oil immersion microscopy. Mitochondria and nuclei were stained brilliant red.

Fixation and Thionine Staining for Light Microscope Observations of Mitochondrial Nucleoids

After MII, small explants of plasmodia were harvested at hourly intervals. They were fixed in ice-cold 1% glutaraldehyde solution (buffered with phosphate to pH 6.8) for 5 min, hydrolyzed for 10 min in 1 N HCl at 45°C, and again fixed in 6% glutaraldehyde solution (buffered with phosphate to pH 6.8) for 3 h. Then they were washed in cold distilled water, dehydrated in a graded series of water and glycol methacrylate, and finally embedded in glycol methacrylate. Thin sections were obtained as described above. The samples were stained with thionine by the method of Schaecher (17). Mitochondrial nucleoids were stained dark blue. The lengths of a mitochondrion and mitochondrial nucleoid and the constriction ratio of a mitochondrion were examined by the method described previously (4). The constriction ratio of a mitochondrion was defined as a/b, where a and b are the minimum and the maximum lengths of the minor axis of a mitochondrion, respectively (Fig. 3). The lengths of a mitochondrion and mitochondrial nucleoid and the constriction ratio of a mitochondrion were determined by examining more than 20 figures.

Fixation for Electron Microscope Observations

Small explants of plasmodium at various times after MII were fixed for 3 h in ice-cold 6% glutaraldehyde

buffered with acetate to pH 6.8, washed in acetate buffer, pH 6.8 for 1 h, and postfixed in 1% OsO_4 for 12 h. They were then dehydrated in a graded series of ethanol and propylene oxide (30 min at each step) and embedded in Epon 812 (9). Ultrathin sections were cut on a Sorvall Porter-Blum ultramicrotome with a glass knife, and the sections were mounted on grids which were coated with Formvar.

Thin sections were stained with saturated uranyl acetate for 1 h and, after observation of the degree of uranyl staining, poststained with lead citrate for 5 min. These sections were examined with a Hitachi 11E electron microscope operated at 80 kV.

RESULTS

Light Microscope Observations

Fig. 2 a and b show representative light micrographs of mitochondria and nuclei during the mitochondrial DNA synthesis period (mS) stained with acid fuchsin (Fig. 2 a) and thionine stain (Fig. 2 b). After staining with acid fuchsin, the mitochondria appear in outline but the nucleoid lying within each mitochondrion cannot be observed. After staining with thionine, the outline of the mitochondria is somewhat obscure but the rodlike nucleoid in the matrix of the mitochondrion can be seen clearly (arrow, Fig. 2 b).
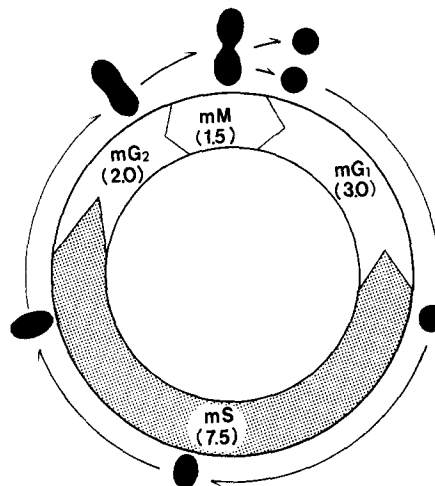
Abbreviations used in figures:

n, nucleus	mM, mitochondrial M
m, mitochondrion	mG_1, mitochondrial G_1
mn, mitochondrial nucleoid	mS, mitochondrial S
v, intramitochondrial vacuole	mG_2, mitochondrial G_2

FIGURE 1   Diagram of the mitochondrial division cycle illustrating the sequence of events in the division of a mitochondrion of *Physarum polycephalum* (15). The duration of each phase is shown in hours.
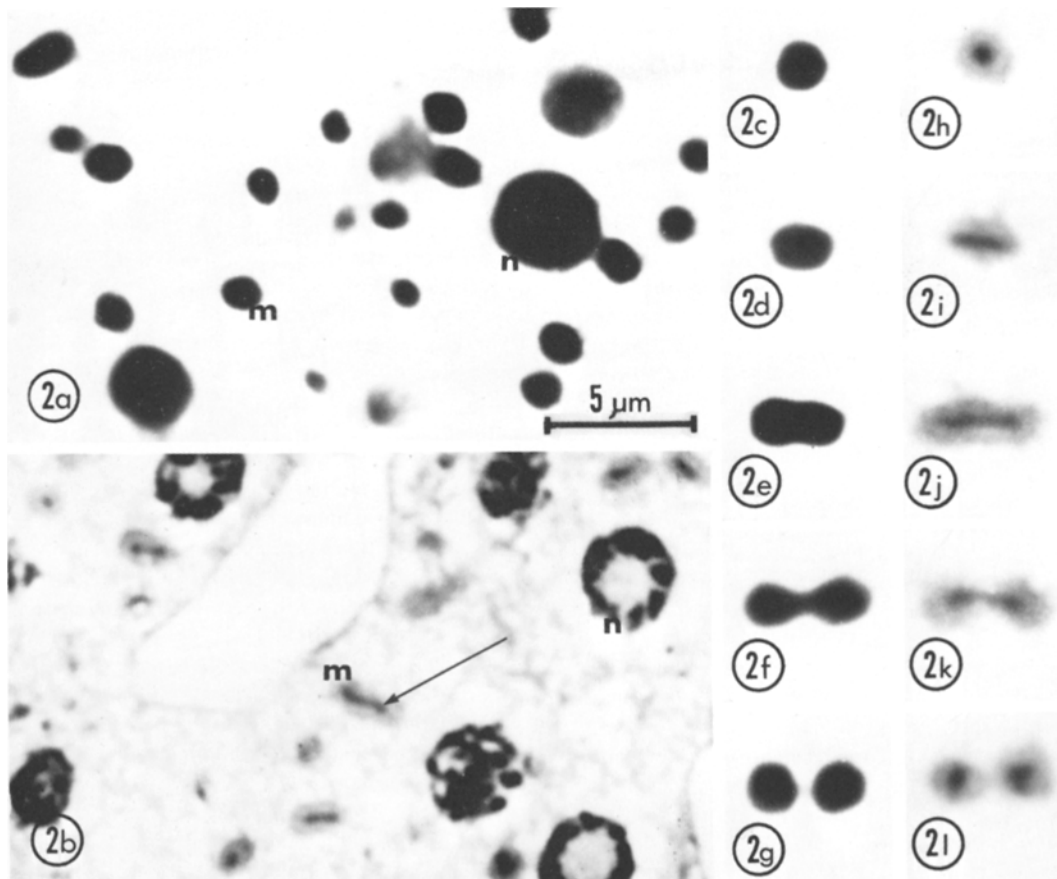
FIGURE 2 a–l   Light micrographs illustrating mitochondria during mitochondrial G₁ (c, g, h, and l), mitochondrial S (d and i), mitochondrial G₂ (e and j), and mitochondrial M (f and k) after staining with acid fuchsin (a and c–g) and thionine (b and h–l). (a, b) × 4,000; (c–l) × 5,000.
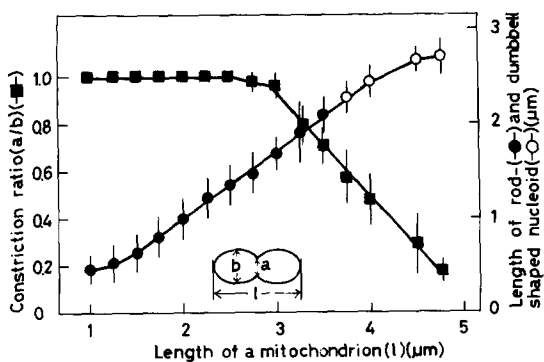

FIGURE 3   Elongation of a mitochondrial nucleoid and changes in the constriction ratio (a/b) of a mitochondrion. a and b are the minimum and the maximum lengths, respectively, of the minor axis and l is the length of the major axis of a mitochondrion as shown at the bottom of the figure.

Fig. 2c–g and h–l demonstrates two series of light micrographs illustrating mitochondria during mitochondrial G₁ (mG₁) (Fig. 2c, g, h, and l), mitochondrial S (mS) (Fig. 2d and i), mitochondrial G₂ (mG₂) (Fig. 2e and j), and mitochondrial M (mM) (Fig. 2f and k) after staining with acid fuchsin (Fig. 2c–g) and thionine (Fig. 2h–l), respectively. The small spherical mitochondria become oval mitochondria (Fig. 2c, d, h, and i). The mitochondrial nucleoid, situated in the center of the mitochondrion, elongates in a direction parallel to the major axis of the mitochondrion during growth of the mitochondrion (Fig. 2h–j). Fig. 3 shows the relationship between the length of the major axis of the mitochondrion and mitochondrial nucleoid and the constriction ratio of a mitochondrion. There seems to be a parallel between the length of the nucleoid and the major axis of the mitochondrion (Fig. 3). When the mitochon-

drion elongates and its major axis reaches approximately 3 μm, the mitochondrial membrane begins to invaginate at the middle of the mitochondrion so that the mitochondrion becomes dumbbell shaped (Fig. 2*f*, *k* and Fig. 3). When the mitochondrion is dumbbell shaped, the nucleoids also become dumbbell shaped but do not divide completely (Fig. 2*k*). After division, one end of the nucleoid often is associated with the limiting membrane of the mitochondrion (Fig. 2*l*). Soon the nucleoid is released from the limiting membrane and becomes situated in the center of the mitochondrial matrix (Fig. 2*h*).

Electron Microscope Observations

Figs. 4*a-d* and 5*a-c* are electron micrographs of mitochondria during mS (Fig. 4*a* and *b*), mM (Figs. 4*c*, *d* and 5*a*, *b*) and mG₁ (Fig. 5*c*), respectively. The fine structure of the mitochondria during mS is similar to that observed in other myxomycetes (18). Instead of having the typical lamellar cristae of higher forms, the mitochondria contain numerous tubular cristae. The central matrix of each mitochondrion is occupied by a nucleoid. The nucleoid is composed of a semi-electron-dense filamentous axial component, which primarily contains DNA, and a peripheral electron-dense component which contains both DNA and RNA (9). Thus, in a longitudinal section of the mitochondrion (Fig. 4*a*), the nucleoid appears elongate or rodlike, while in transverse section it appears tubular (13). The mitochondrial nucleoid elongates during growth of the mitochondrion (Fig. 4*b*). When the nucleoid elongates and reaches a length of approximately 1.5 μm, a depression of the mitochondrial limiting membrane appears in the middle of the mitochondrion (Fig. 4*c* and *d*). In some instances, the nucleoid is bent at the center. In addition, each end of the elongated nucleoid often appears to be closely attached to cristae (arrows in Fig. 4*c*). The nucleoid does not divide completely even when the mitochondrial constriction has proceeded to the point where the mitochondrial sections are connected by a very narrow bridge with the nucleoid in its center (Fig. 5*a*). During the final stages of mitochondrial division, the two daughter mitochondria appear to be connected by a slightly electron-dense limiting membrane, and one end of the nucleoid with its fine inner fibril is associated with the membrane (arrow, Fig. 5*b*) as if the nucleoid is pinched at the middle by the constriction of the limiting membrane. After mitochondrial division, the nucleoid

is released from the limiting membrane and moves to the center of the mitochondrion (Fig. 5*c*). The nucleoid in each daughter mitochondrion has a small rodlike or oval configuration, and the intramitochondrial vacuoles are associated with the nucleoid or the limiting membrane (Fig. 5*c*). Throughout the mitochondrial division cycle, DNA-like fibrils, 30–70 Å in diameter, and somewhat thicker fibrils, 200 Å in diameter, perhaps corresponding to chromatin fibrils, are observed in the peripheral regions of the nucleoid (Fig. 5*b* and *c*). However, it has not been possible to correlate changes in the fine structure of the nucleoid with periods of mitochondrial DNA synthesis. The general relationship between the division of the nucleoid and the division of the mitochondrion are presented diagrammatically in Fig. 6.

DISCUSSION

The mitochondrion of the slime mold *Physarum polycephalum*, like the kinetoplasts of the Bodonidae and Trypanonidae (19), contains a nucleoid which is higher in electron density than the matrix of the mitochondrion, while the mitochondrion of many other organisms contains a nucleoid which is lower in electron density than the matrix of the mitochondria. This difference in the electron density of the mitochondria probably reflects differences in the quantity of DNA per mitochondrion: the mitochondrion of *Physarum* (8) and the kinetoplast-mitochondrion (20) contain 10 and 10³ times more DNA, respectively, than the mitochondria of many other organisms. The nucleoid of *Physarum* offers unique advantages for studying replication and division of the mitochondrial nucleoid because (*a*) its division is semisynchronized (15), and (*b*) changes in the nucleoid are easily observable with the light microscope. The present experiments suggest that the mitochondrial nucleoid elongates longitudinally while the mitochondrion increases in size during mitochondrial S and that it divides by constriction of the mitochondrion. This is similar to what has been reported for the kinetoplast nucleoid (1, 2, 19).

Guttes et al. (6, 7) reported that dumbbell-shaped and ovoid mitochondria of *Physarum* contain two nucleoids. On the basis of electron microscope observations, these workers proposed that the division of the mitochondrion was preceded by the division of the nucleoid, and that the division of the nucleoid is not a passive result of its being pinched into two pieces by the dividing mitochondrion. However, previous experiments (12) sug-
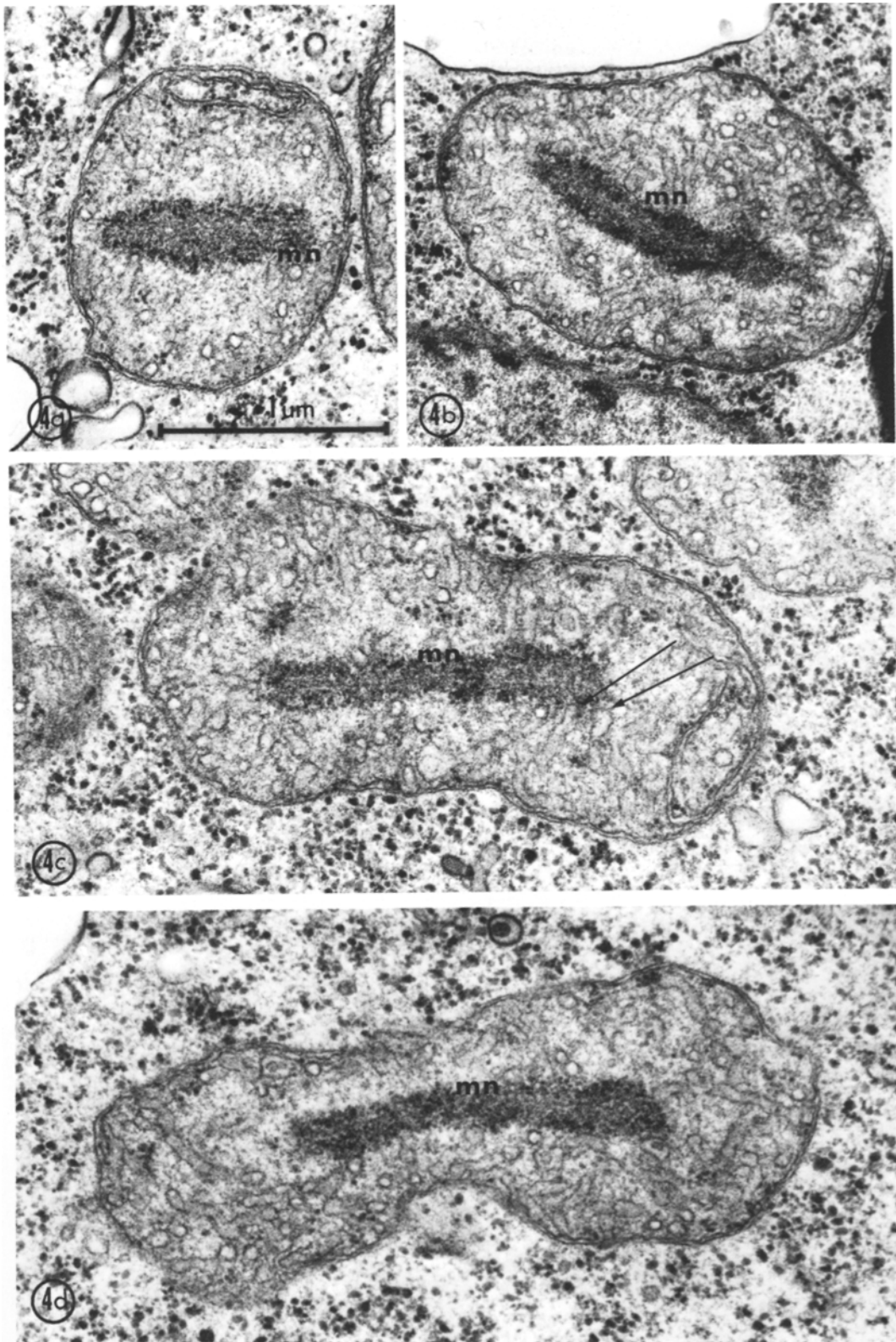
Figure 4a–d Electron micrographs illustrating mitochondria during mS (a, b) and mM (c, d). Each end of the elongated nucleoid appears to be closely attached to cristae (arrows, c). (a) × 36,000; (b) × 34,000; (c) × 35,000; (d) × 35,000.
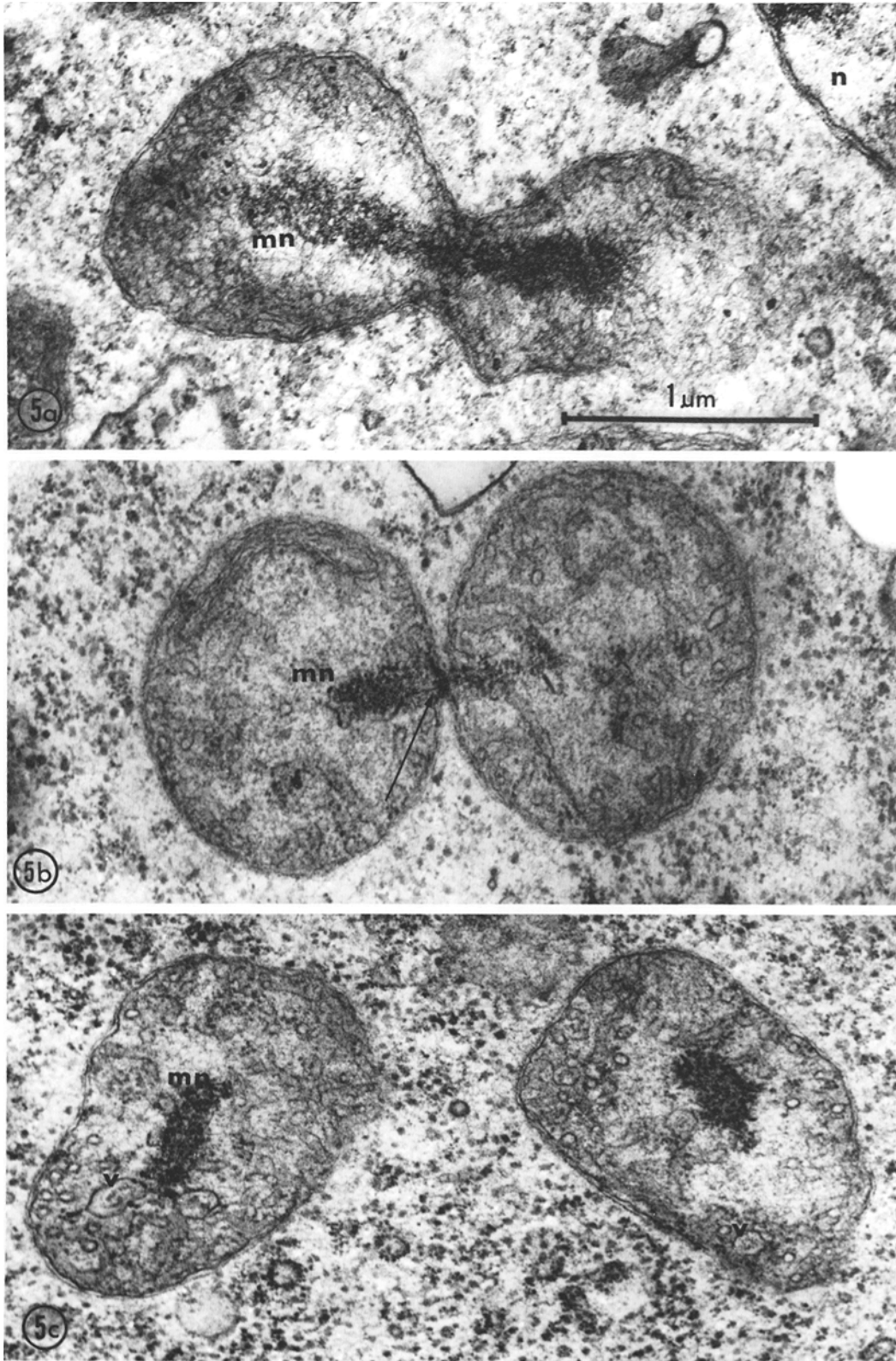
FIGURE 5 Electron micrographs illustrating mitochondria during late mM (*a*, *b*) and mG$_1$ (*c*). One end of the mitochondrial nucleoid with its fine inner fibril is associated with the limiting membrane (arrow, *b*). (*a*) × 38,000; (*b*) × 35,500; (*c*) × 35,000.
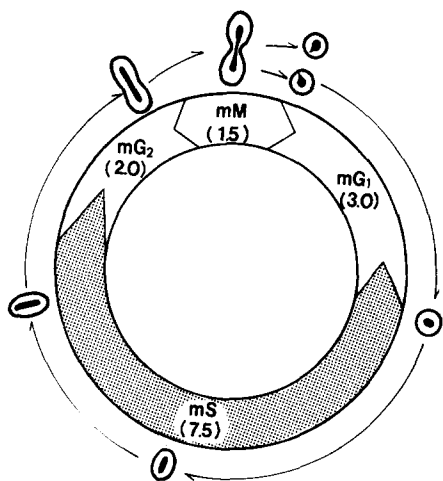
FIGURE 6   Diagram of the mitochondrial division cycle illustrating the sequence of events in the division of a mitochondrial nucleoid. The duration of each phase is shown in hours.

gest that nucleoids in some ovoid and dumbbell-shaped mitochondria are often bent at the middle so that the nucleoid is V shaped. These mitochondria contain not two nucleoids but, rather, only one elongated nucleoid. The present studies of the entire structure of the nucleoid and its behavior during the mitochondrial division cycle suggest that the nucleoid has not divided even when the mitochondrial membrane has invaginated and the mitochondrial segments are connected by only a very narrow bridge (Figs. 2k and 5a). Therefore, the nucleoid must divide just before division of the mitochondrion. This division may occur by the constriction of the membrane of the dividing mitochondrion (Fig. 5a–c). These events in the division of the Physarum mitochondrial nucleoid are similar to events in the division of bacteria or Rickettsiella melolonthae (3). It is well known that the cell membrane-associated mesosome has an important role in the division of the bacterial nucleoid. However, while this apparatus has not been observed in mitochondria, it has been shown that in whole mounted mitochondria the DNA fibers of the nucleoid are bound closely to fragments of cristae (11). In addition, the present results indicate that one end of an elongated nucleoid is associated with the cristae before division (Fig. 4c) but that this association disappears after division (Fig. 5). These results suggest that the membrane of the cristae plays an important role in the division of the Physarum mitochondrial nucleoid.

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