

STUDIES ON MITOCHONDRIAL STRUCTURE AND FUNCTION IN *PHYSARUM POLYCEPHALUM*

III. Electron Microscopy of a Large Amount of DNA Released from a Central Body in Mitochondria by Trypsin Digestion

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Biochemical studies have revealed that isolated mitochondria of *Physarum polycephalum* contain 10 times more DNA (1) than the mitochondria of many other organisms (2-6). It has also been observed with the light and the electron microscopes that the mitochondria contain an elongated chromosome-like body, situated in a central portion of the inner matrix (7-11), which is Feulgen positive (11) and undergoes division similar to bacterial nucleoids (10). Cytochemical studies of ultrathin-sectioned mitochondria have indicated that the central body contains DNA (9, 11) as first described for the slime mold *Didymium nigripes*

by Schuster (12). These data suggest that a large amount of the DNA in the mitochondria may be condensed in the central body. Furthermore, with [³H]uridine electron microscope autoradiography it has been shown that the central body and its peripheral region synthesize RNA which corresponds to approximately 6% of that in the nucleus (11). In addition, the synthesis of RNA occurs nonrandomly on the rodlike body, in spite of the fact that the DNA is distributed homogeneously throughout the body (13). These findings suggest that there may be a mechanism by which a large amount of the DNA is organized in the central

body and that its activity is regulated selectively.

Initially this problem has been approached from a cytological and cytochemical standpoint. This paper demonstrates morphologically the presence of a large amount of DNA released from the central bodies of the mitochondria by enzymatic digestions after osmotic shock.

MATERIALS AND METHODS

Surface plasmodia after the second mitosis (MII) were used in these experiments and were cultivated using a modification of the methods reported by Daniel and Rusch (14) and reviewed by Guttes and Guttes (15). Three plasmodia 4 cm in diameter during G_2 phase were scraped from filter paper in a Petri dish into 30 ml of the control medium containing 0.25 M sucrose, 0.01 M CaCl_2 , 0.01 M Tris buffer (pH 7.0 with HCl), and 0.1% (wt/vol) Triton X-100 (16). The suspension was homogenized for 30 s at 3,000 rpm in a 50-ml Waring blender cup. The foam was removed with a pipette. The homogenate was filtered by gravity through two thicknesses of nylon meshes with pores of $31 \mu\text{m}$ (NBC Ind., Japan). 10 ml of the homogenate was layered over a discontinuous sucrose density gradient with sucrose concentrations ranging from 0.5 to 1.5 M, the difference between two adjacent sucrose solutions being 0.25 M. The tubes were centrifuged at 0°C for 30 min at 1,500 rpm (300 g) in a swinging bucket rotor with a Kokusan H-103 centrifuge. The mitochondria banded at the border between the 0.75 and 1.00 M sucrose layers. The mitochondrial fraction was then collected and the mitochondria were sedimented for 10 min at 3,000 rpm. The mitochondria were washed once by resuspending in 10 ml of 0.25 M sucrose solution followed by recentrifugation. The pellet was resuspended in 0.5 ml of distilled water. Isolated mitochondria were stained with a 0.2% aqueous solution of azure B and examined under the light microscope. Preparation of ultrathin sections of the plasmodia before isolation of the mitochondria or of the mitochondrial pellet was done by the procedure employed previously (11).

Spread mitochondria preparations were made according to the procedures described previously (17). The mitochondria spread on the air-water surface were picked up on a carbon-coated grid, fixed in 0.2% glutaraldehyde in pH 6.8 phosphate buffer for 5 min at 4°C , and stained in 2% (wt/vol) phosphotungstic acid or in 0.02% (wt/vol) uranyl acetate for 10 min at room temperature.

Enzymatic digestions were done on the mitochondria spread on the grid. The grids were transferred from fixative to acetate buffer at pH 6.8, rinsed in this buffer, and extracted at 34°C in: (a) 1 mg DNase (Sigma Chemical Co., St. Louis, Mo., electrophoretically pure, beef pancreas)/1.0 ml 0.1 M acetate buffer containing 0.003 M magnesium acetate at pH 5.5 for 20 min, (b) 1 mg RNase (Sigma Chemical Co., five times crystallized,

bovine pancreas)/1.0 ml 0.1 M acetate buffer at pH 6.5 for 20 min, (c) 1 mg trypsin (Nutritional Biochemical Corporation, three times crystallized)/1.0 ml 0.1 M phosphate buffer (pH 6.8) for 20 min, or (d) 1 mg pepsin (ICN Nutritional Biochemicals Div. International Chemical & Nuclear Corp., Cleveland, Ohio, three times crystallized)/100 ml 0.1 M HCl for 30 min. When double or triple digestions were done with DNase and trypsin or DNase, RNase, and trypsin, the specimens were washed with 0.1 M acetate buffer at pH 7.0 before treatment with the second or third enzyme. Controls were incubated in the same solutions without enzyme. Then the samples were dehydrated in a graded series of ethanol and water and dried in air. They were shadowed with platinum-palladium alloy *in vacuo* at an angle of 8° . Electron micrographs were made with a Hitachi-11 E electron microscope.

RESULTS

Fig. 1 *a* is a light micrograph of mitochondria isolated from the plasmodium during G_2 phase. Almost all of the mitochondria contain a central body which has been stained with azure B. Figs. 1 *b* and *c* are electron micrographs of thin sections of the nonisolated (Fig. 1 *b*) and isolated mitochondria (Fig. 1 *c*), respectively. The morphology of the isolated mitochondria is very similar to that of nonisolated mitochondria, except that the internal matrix of the body is slightly less dense. These mitochondria contain numerous tubular cristae with diameters of about 500 \AA (short arrow in Fig. 1 *b*). In addition, the central matrix between the cristae is occupied by an electron-dense rod-shaped structure (long arrows in Fig. 1 *b, c*).

Fig. 2 *a* illustrates several bumpy branch-shaped structures, averaging about $500\text{--}600 \text{ \AA}$ in diameter, and a centrally located electron-dense body, released from isolated mitochondria by osmotic shock and shadowed with platinum-palladium. Figs. 2 *b* and *c* are higher magnification micrographs of a portion of the branch-shaped structures, negatively stained with phosphotungstic acid (Fig. 2 *b*) and positively stained with uranyl acetate (Fig. 2 *c*), respectively. Since the diameter of the branch-shaped structures is very similar to that of the tubular cristae in thin section (Fig. 1 *b*), and since elementary particle-like particles, approximately 90 \AA in diameter, can be seen on the surface of the branch-shaped structures (arrows in Figs. 2 *b, c*), the branch-shaped structures may correspond to the cristae in thin section. An electron-dense rod-like body of irregular shape is intimately associated with several cristae near the

center of the spread cristae. A few fibers can be seen extending from the periphery of the body and often one side is specifically attached to a fragment of the crista (long arrows in Fig. 2 *a*). The individual fibers range from 70 to 200 Å in diameter. Occasionally, as shown by the short arrows in Fig. 2 *a*, a single bumpy fiber of varying diameter can be seen.

After trypsin digestion (Fig. 3 *a, b*), the appearance of the cristae is similar to that of the control preparation except that the surface of the cristae is slightly smooth, and the centrally located electron-dense body disappears and numerous fine filaments remain (Fig. 3 *a*). Fig. 3 *b* is a higher magnification micrograph of a portion of Fig. 3 *a* (arrow in Fig. 3 *a*). The filaments are about 70 Å wide, which is slightly finer than the filaments in the control (Fig. 3 *b*). A few free fiber ends are found at the periphery of the fine filaments. The connections of the fine fibers to cristae fragments

can no longer be seen. It seems likely that this trypsin-resistant filament consists of DNA since it can be extracted with DNase (Fig. 4 *a, b*) though not with RNase. These results indicate that a large amount of DNA has been released from an intramitochondrial body by trypsin treatment. This release of DNA is higher with trypsin digestion than with pepsin treatment. Pepsin treatment was less effective in digesting the electron-dense body than trypsin, although the presence of a small number of DNA fibers represented a partial emergence from the electron-dense body.

Fig. 4 *a* shows an electron micrograph of a spread mitochondrion after DNase digestion. Fig. 4 *b* is a higher magnification micrograph of a portion of Fig. 4 *a*. The appearance of the cristae is similar to that of the control preparation, and a few semielectron-dense bag-like bodies, 0.1–0.2 μm in diameter, are seen to be associated with cristae. On the other hand, neither the electron-dense

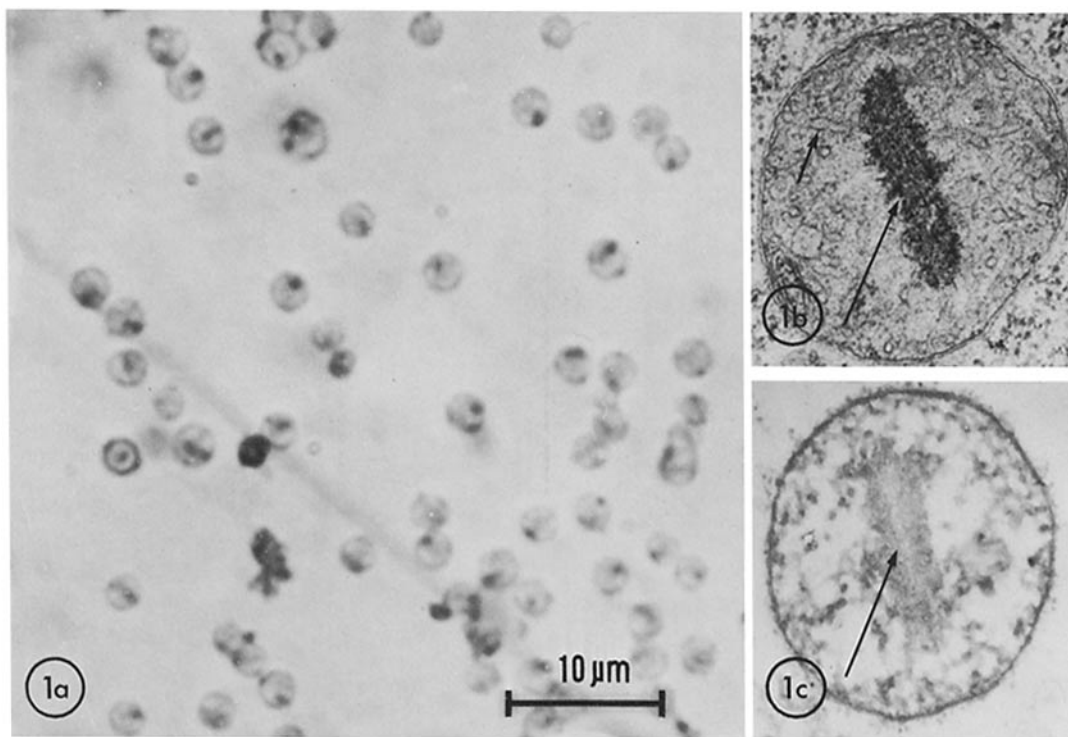


FIGURE 1 A light micrograph of isolated mitochondria (Fig. 1 *a*); thin-section electron micrographs of nonisolated (Fig. 2 *b*) and isolated mitochondria (Fig. 1 *c*). Almost all of the mitochondria contain a central body which stains with azure B (Fig. 1 *a*). The body (long arrows in Figs. 1 *b* and *c*) occupies the central matrix and is surrounded by numerous tubular cristae (short arrow in Fig. 1. *b*) of the mitochondria. Fig. 1 *a*, $\times 2,100$; Fig. 1 *b*, $\times 21,500$; Fig. 1 *c*, $\times 20,000$.

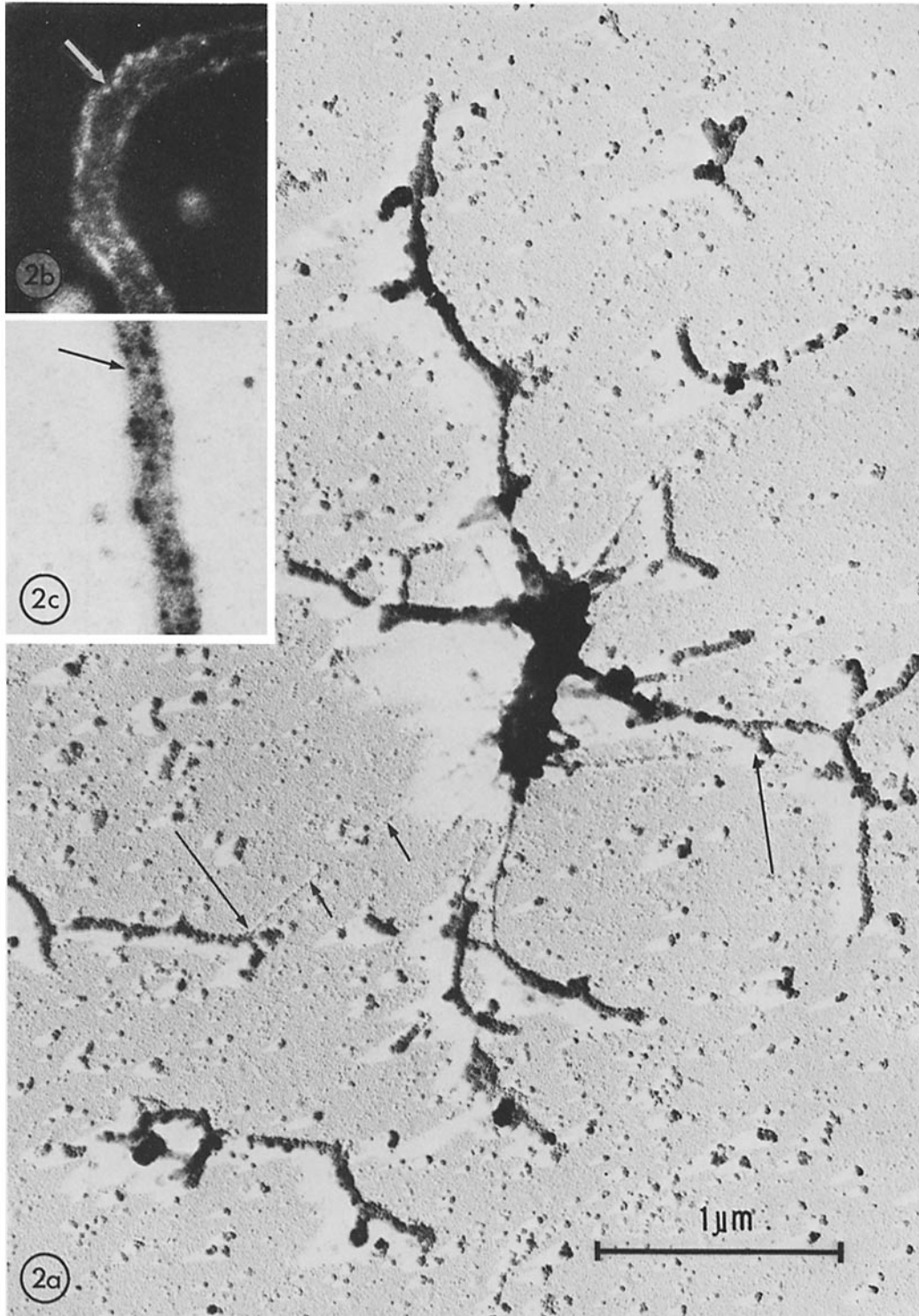


FIGURE 2 Electron micrographs of surface-spread and shadowed mitochondrion (Fig. 2 *a*) and a portion of phosphotungstic acid- (Fig. 2 *b*) or uranyl acetate-stained cristae (Fig. 2 *c*) in a control preparation. In Fig. 2 *a*, several tubular cristae and a centrally located electron-dense rodlike body can be seen. Some of the cristae are branched out from portions of other cristae. A few fibers differing in thickness appear at the periphery of the body and often one side is associated with a fragment of the tubular cristae as shown by the long arrows in Fig. 2 *a*. In addition, a single bumpy fiber of varying diameter can be seen (short arrows in Fig. 2 *a*). At higher magnification, elementary particle-like particles can be seen on the surface of the cristae (arrows in Figs. 2 *b* and *c*). Fig. 2 *a*, $\times 36,500$; Fig. 2 *b*, $\times 122,000$; Fig. 2 *c*, $\times 99,000$.

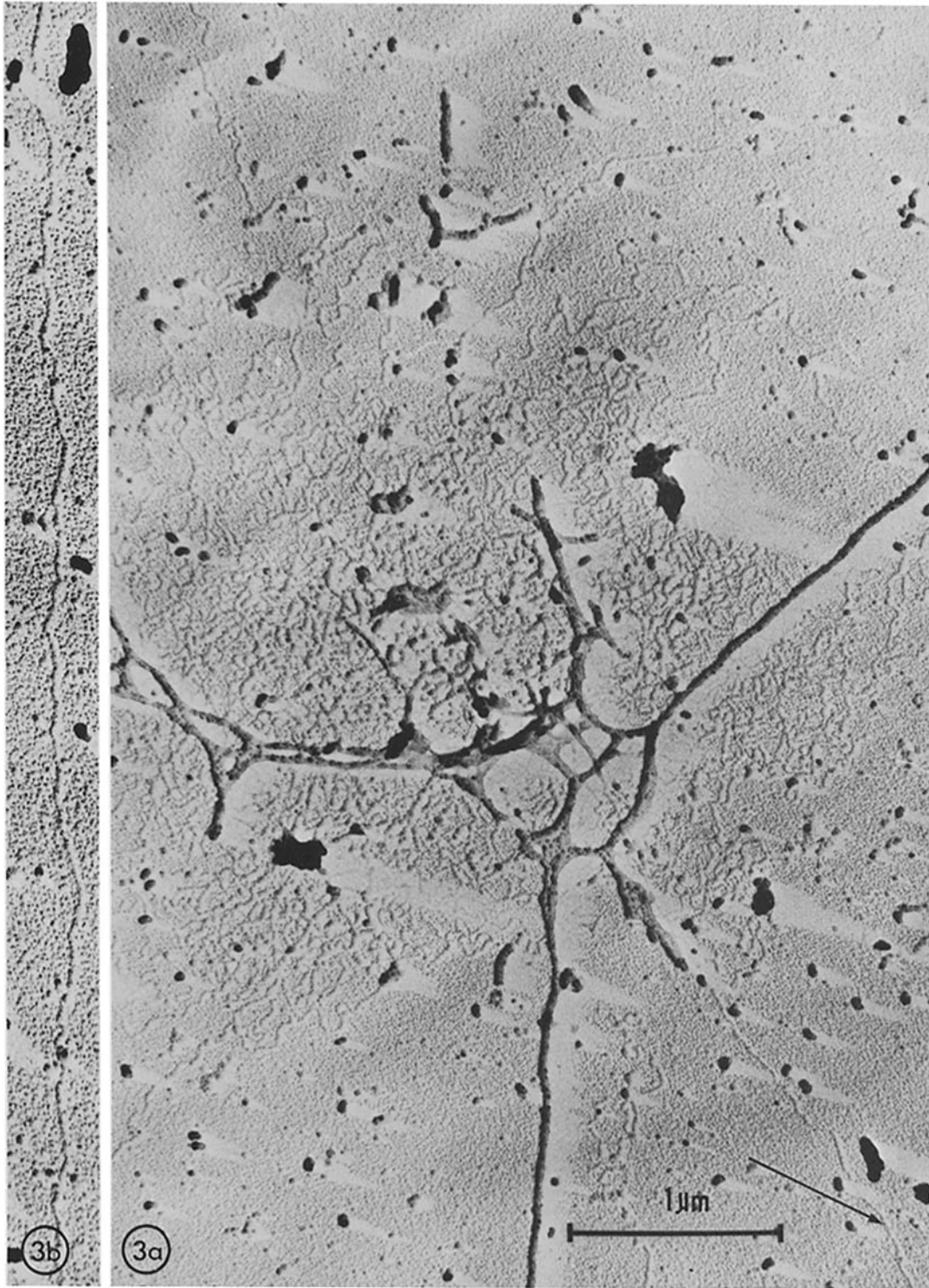


FIGURE 3 Electron micrographs of surface-spread and shadowed mitochondria after trypsin treatment. Fig. 3 *b* shows a higher magnification micrograph of a portion of Fig. 3 *a* (arrow in Fig. 3 *a*). The appearance of the cristae is similar to that of the control preparation, whereas the centrally located electron-dense body disappears and numerous fine filaments can be seen in the central background (Fig. 3 *a*). The filaments are approximately 70 Å wide (Figs. 3 *a, b*). Fig. 3 *a*, $\times 33,000$; Fig. 3 *b*, $\times 49,500$.

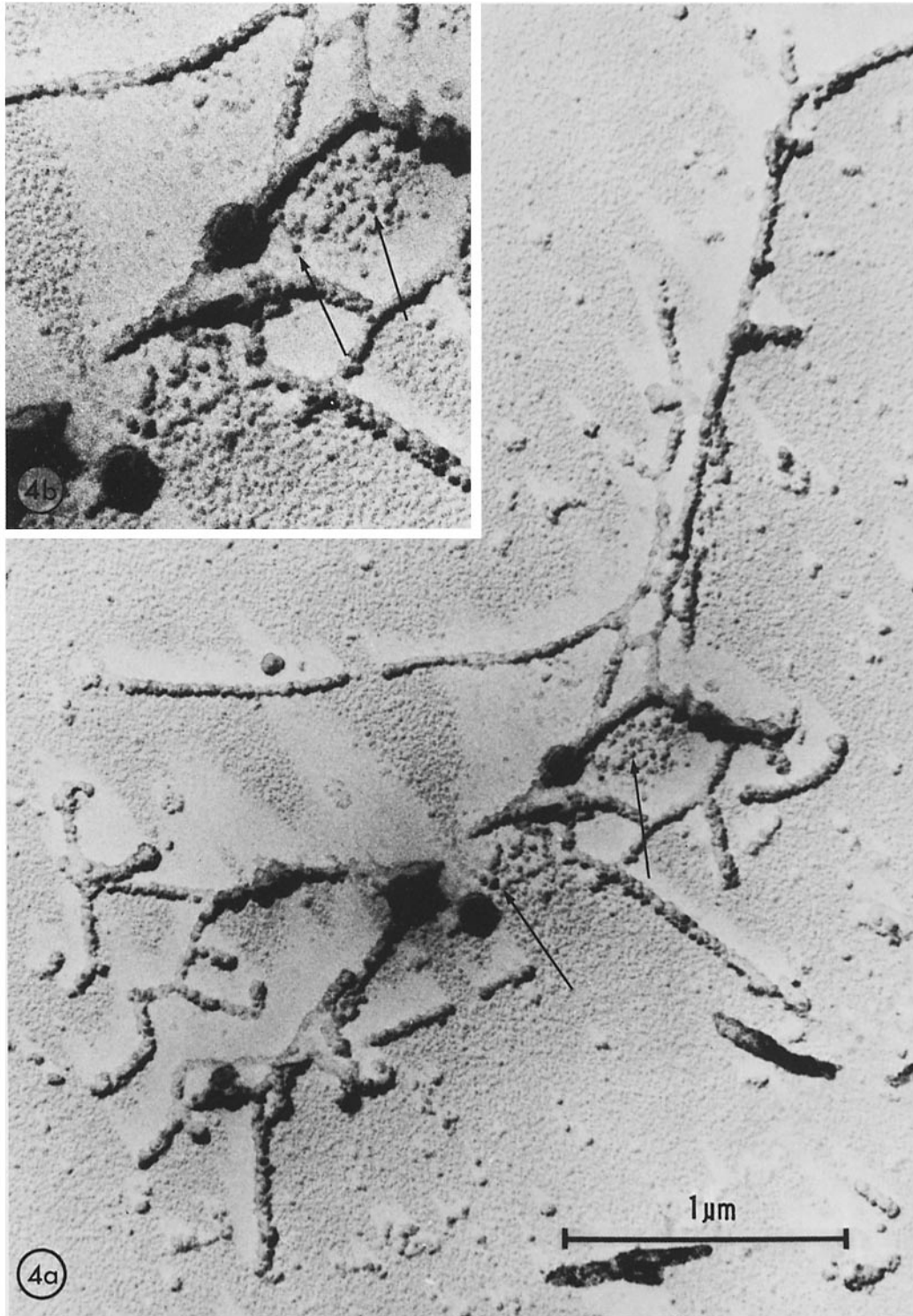


FIGURE 4 Electron micrographs of surface-spread and shadowed mitochondria after DNase treatment. Fig. 4 *b* shows a higher magnification micrograph of a portion of Fig. 4 *a*. A number of fine filaments disappear completely and numerous fine granules remain (arrows in Fig. 4 *b*). Fig. 4 *a*, $\times 43,000$; Fig. 4 *b*, $\times 66,900$.

central body which is present in the control preparation nor the large number of DNA-like filaments which appear in the trypsin-digested preparation are present after DNase digestion, and a granular substance appears in the central area of the background (arrows in Fig. 4 b). These granules were digested completely by RNase and trypsin treatment after DNase treatment. This suggests that, in addition to DNA, protein and RNA occur in the central body.

DISCUSSION

The mitochondria of *P. polycephalum* contain approximately 10^{-3} pg of DNA (1), which corresponds to approximately 60 molecules of mitochondrial DNA since a molecule of mitochondrial DNA of *P. polycephalum* is approximately 12 μ m long (18). Evans and Suskind (18) suggested that the mitochondrial DNA of *P. polycephalum* is linear. This does not conflict with the data presented here since the ends of the DNA released from a central body by trypsin digestion probably represent the ends of several different DNA molecules.

It was difficult in *P. polycephalum* to visualize the organization of the mitochondrial DNA by using only osmotic shock. Since it was possible to liberate a relatively large number of DNA molecules from the central body by trypsin digestion, it seems likely that protein plays an important role in organizing the DNA in the central body of *Physarum* mitochondria. The biochemical properties of this protein are not clear. It is not known how mitochondrial DNA is held in its supercoiled configuration. However, the undigested filaments released by osmotic shock often were irregular and the diameter of the fibers varied from 200 to 70 Å, whereas the trypsin-resistant filaments always had a diameter similar to that of the thinnest fibers in the undigested preparations. Mohberg and Rusch (19) have shown by using biochemical techniques that *Physarum* mitochondria contain almost as much basic protein as do the nuclei. Recently, it has been shown with the ammoniacal silver reaction for histones that the central body in the mitochondria of *P. polycephalum*, like the nuclei, exhibits many ammoniacal silver reaction products.¹ This may suggest that any histone-like protein of the central body is intimately linked

¹ Kuroiwa, T. 1974. Mitochondrial nucleoid staining with ammoniacal silver. Manuscript in preparation.

with the mitochondrial DNA filaments rather than being a basic protein coat over a mass of DNA filaments.

Studies are in progress to further characterize the trypsin-sensitive protein species in the intramitochondrial body of *P. polycephalum* using various biochemical techniques.

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BIBLIOGRAPHY

1. KUROIWA, T. 1973. Isolation of nuclei and mitochondria of *Physarum polycephalum*. Proceedings of the 38th Annual Meeting of the Botanical Society of Japan. 72.
2. NASS, S., M. M. K. NASS, and U. HENNIX. 1965. Deoxyribonucleic acid in isolated rat-liver mitochondria. *Biochim. Biophys. Acta.* **95**:426.
3. SUYAMA, Y., and J. R. PREER, JR. 1965. Mitochondrial DNA from protozoa. *Genetics.* **52**:1051.
4. NASS, M. M. K. 1966. The circularity of mitochondrial DNA. *Proc. Natl. Acad. Sci. U. S. A.* **56**:1215.
5. NASS, M. M. K. 1969. Advances, problems, and goals. Studies of size and structure of mitochondrial DNA related to biogenesis and function of this organelle. *Science (Wash. D. C.)*. **165**:25.
6. BAXTER, R. 1971. Origin and continuity of cell organelles. Topical Volume in Developmental Biology. W. Beerman, editor. Springer-Verlag, Berlin. **2**:46.
7. GUTTES, S., E. GUTTES, and R. HADEK. 1966. Occurrence and morphology of a fibrous body in the mitochondria of the slime mold *Physarum polycephalum*. *Experientia (Basel)*. **22**:452.
8. NICHOLS, T. J. 1972. The effects of starvation and light on intra-mitochondrial granules in *Physarum polycephalum*. *J. Cell Sci.* **10**:1.
9. STOCKEM, W. 1968. Über den DNS- und RNS-gehalt der mitochondrien von *Physarum polycephalum*. *Histochemie.* **15**:160.
10. GUTTES, E., S. GUTTES, and R. VIMLA DEVI. 1969. Division stages of the mitochondria in normal and actinomycin-treated plasmodia of *Physarum polycephalum*. *Experientia (Basel)*. **25**:66.
11. KUROIWA, T. 1973. Studies on mitochondrial structure and function in *Physarum polycephalum*. I. Fine structure, cytochemistry, and ³H-uridine autoradiography of central body in mitochondria. *Exp. Cell Res.* **78**:351.
12. SCHUSTER, F. L. 1965. A deoxyribose nucleic acid

- component in mitochondria of *Didymium nigripes*, a slime mold. *Exp. Cell Res.* **39**:329.
13. KUROIWA, T. 1973. Studies on mitochondrial structure and function in *Physarum polycephalum*. II. Behavior of RNA synthesized in central body of mitochondria as revealed by electron microscopic autoradiography. *J. Electron Microsc.* **22**:45.
 14. DANIEL, J. W., and H. O. RUSCH. 1961. The pure culture of *Physarum polycephalum* on a partially defined soluble medium. *J. Gen. Microbiol.* **25**:47.
 15. GUTTES, E., and S. GUTTES. 1964. Mitotic synchrony in the plasmodia of *Physarum polycephalum* and mitotic synchronization by coalescence of microplasmidia, *In Methods in Cell Physiology*. D. M. Prescott, editor. Academic Press, Inc., New York. **I**:43.
 16. MOHBERG, J., and H. P. RUSCH. 1971. Isolation and DNA content of nuclei of *Physarum polycephalum*. *Exp. Cell Res.* **66**:305.
 17. KUROIWA, T. 1973. Fine structure of interphase nuclei. VI. Initiation and replication sites of DNA synthesis in the nuclei of *Physarum polycephalum* as revealed by electron microscopic autoradiography. *Chromosoma (Berl.)*. **44**:291.
 18. EVANS, T. E., and D. SUSKIND. 1971. Characterization of the mitochondrial DNA of the slime mold *Physarum polycephalum*. *Biochim. Biophys. Acta.* **228**: 350.
 19. MOHBERG, J., and H. P. RUSCH. 1970. Nuclear histones in *Physarum polycephalum* during growth and differentiation. *Arch. Biochem. Biophys.* **138**:418.