*THE CIRCULARITY OF MITOCHONDRIAL DNA**

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Communicated by Britton Chance, August 29, 1966

Studies of the last few years have demonstrated that a small amount of DNA is located specifically in one or more central regions of mitochondria[1-4] representing cell types of all vertebrate and invertebrate phyla.[4] A DNA differing in compositional[5-12] and metabolic[6, 8, 9] properties from nuclear DNA can be isolated from purified mitochondria of many organisms. A rapidly expanding body of literature, already too voluminous to cite fully in publications other than a review, deals with mitochondrial DNA, RNA, protein synthesis, mitochondrial "self-duplication," and genetic determinants (cf. refs. 4 and 13).

Pertinent to the present paper are the observations that mitochondrial and nuclear DNA may not merely differ in guanine plus cytosine content (a difference which may be small[9, 12] or undetectable in some mammalian species[10]) but also in their renaturation properties. Heat- or alkali-denatured DNA's of mitochondria from various mammalian cell types[10-12] and yeast[10] renature under conditions in which nuclear DNA remains denatured.

We have initiated a series of studies that bear on the problem of mitochondrial duplication during the cell cycle of cultured mammalian cells. Mitochondria of L cells contain double-stranded DNA which differs from nuclear DNA in base composition and renaturation properties.[12] The latter resemble the unique renaturation characteristics originally described for circular polyoma virus DNA.[14] The experiments reported here demonstrate that mitochondrial DNA of L cells, unlike nuclear DNA from animal sources, also has a circular configuration.

Materials and Methods.—Cell fractionation: Mitochondria were isolated from logarithmically growing mouse fibroblasts (L cells) cultured in suspension in Eagle's medium supplemented with 10% fetal calf serum. The procedure was based on that of Borst,[15] except that cells were broken with a Dounce homogenizer in 0.10 *M* sucrose–1 mM EDTA instead of water, and a treatment of mitochondria with 10 μg per ml deoxyribonuclease in 0.25 *M* sucrose–5 mM $MgCl_2$ for 40 min at 2°C was included to remove residual nuclear DNA.[6, 12] Mitochondria were further purified by centrifugation in a linear sucrose gradient (1.03–1.91 *M* sucrose–1 mM EDTA–2 mM Tris buffer pH 7.4). The final fraction of mitochondria was rich in cytochrome *c* oxidase activity[16] and consisted of typical mitochondria with few to numerous cristae as revealed by electron microscopy.

Isolation of DNA: DNA was extracted from detergent-treated mitochondria or nuclei with freshly distilled phenol essentially as described.[5] The procedure included treatment with both pancreatic and T1 ribonucleases and centrifugation in a cesium chloride gradient. The sedimentation profiles showed a single uniform peak for the DNA of mitochondrial preparations. The details on the isolation and properties of mitochondrial and nuclear DNA's are presented elsewhere.[12]

Chemical analyses: DNA was measured as described previously[6] or, more routinely, by the fluorometric method of Kissane and Robins[17] with minor modifications. After the final extraction with ether, the dried residue was extracted twice for 1 hr with 10% NaCl at 100°C. The nucleic acids were precipitated with 2 vol of ethanol at −10°C, dried, and analyzed[17] for deoxyribose content using deoxyadenosine as standard. Protein was determined as previously described.[6]

Counting of mitochondria: Dilute solutions of mitochondria were counted by phase-contrast microscopy in a Petroff-Hauser bacterial counter following the methods of Allard *et al.*[18] Mitochondria were suspended in 2 *M* sucrose to reduce Brownian movement.

Spreading of purified DNA: The method described by Freifelder and Kleinschmidt[19] was chosen in order to preserve single-stranded regions of DNA if present. Mitochondrial or nuclear DNA was freshly diluted from a tenfold stock solution (in saline-citrate) with 1 *M* ammonium acetate–0.5% formaldehyde to contain 2 μg DNA per ml or less.

Spreading of DNA released by osmotic shock: The procedure described by Kleinschmidt *et al.*[20] for the osmotic release of viral DNA was followed. Freshly isolated mitochondria were diluted with ice-cold 4 *M* ammonium acetate to contain approximately 5×10^8 particles per ml. Cytochrome *c* was added to a final concentration of 0.01%. The preparation was spread on ice-cold double-distilled water. Ten μg per ml pancreatic deoxyribonuclease (electrophoretically purified, ribonuclease-free, Worthington) and 5 mM $MgCl_2$ were added to some preparations prior to spreading.

Rotary shadowing and electron microscopy: The monolayers were picked up with carbon-coated Formvar films supported by copper grids, dehydrated with ethanol, and immediately rotary-shadowed with platinum-iridium (80:20) at an angle of 6–8° in a Siemens evaporating unit VBG 500. Micrographs were taken with a Siemens Ia electron microscope. The magnification (×10,000) was calibrated with replicas of diffraction gratings mounted on copper grids (2160 lines per mm and 28,600 lines per inch).

To measure the length of DNA strands, photographic plates were printed at a magnification of 10 times after assuring negligible distortion of prints in both directions during drying. The strands were traced in duplicate with a map-measuring device.

Results.—Electron microscopy of purified DNA molecules: Phenol-extracted mitochondrial DNA was found to have a cyclic configuration. The majority of DNA molecules consisted of partially twisted ring structures with one to several points of crossing. A smaller percentage of molecules was divided between completely untangled ring forms and highly twisted ones which were too tangled to measure. A few molecules had rosette patterns similar to phage DNA prepared by osmotic shock,[21] but lacked visible free ends. A few linear strands which were of the same length or shorter than the ring molecules were observed mainly in older preparations and were presumed to be broken pieces of circular DNA. Measurement of the contour lengths of 63 circular molecules with no or few crossings were taken from three different preparations of DNA. The results followed a Gaussian distribution, as summarized in the histogram of Figure 1, with a mean molecular length of 4.74 ± 0.02 μ. An illustration of two untwisted circular molecules is given in Figure 2*a* and *b*.

On the other hand, nuclear DNA that was prepared by the same procedures as mitochondrial DNA consisted of linear molecules only, ranging mostly from 10 to 15 μ in length, with some smaller fragments present. No circular forms were observed. Nuclear DNA is illustrated in Figure 3.

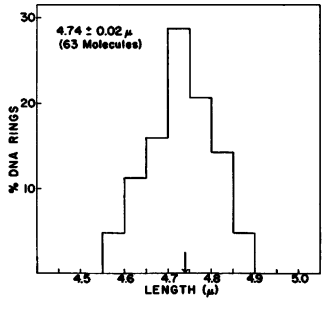

FIG. 1.—Length distribution of purified mitochondrial DNA. Mean length and standard deviation are indicated.

Electron microscopy of DNA molecules released by osmotic shock: The finding that purified mitochondrial DNA is circular necessitated further experiments to test whether DNA exists in circular form inside the mitochondrial organelle or whether the ring structures possibly formed by joining of cohesive ends of linear molecules.[22] Freshly isolated mitochondria were therefore disrupted osmotically on a film of cytochrome *c* so that released DNA would be adsorbed immediately to the protein film with little chance for gross molecular alterations. Approxi-

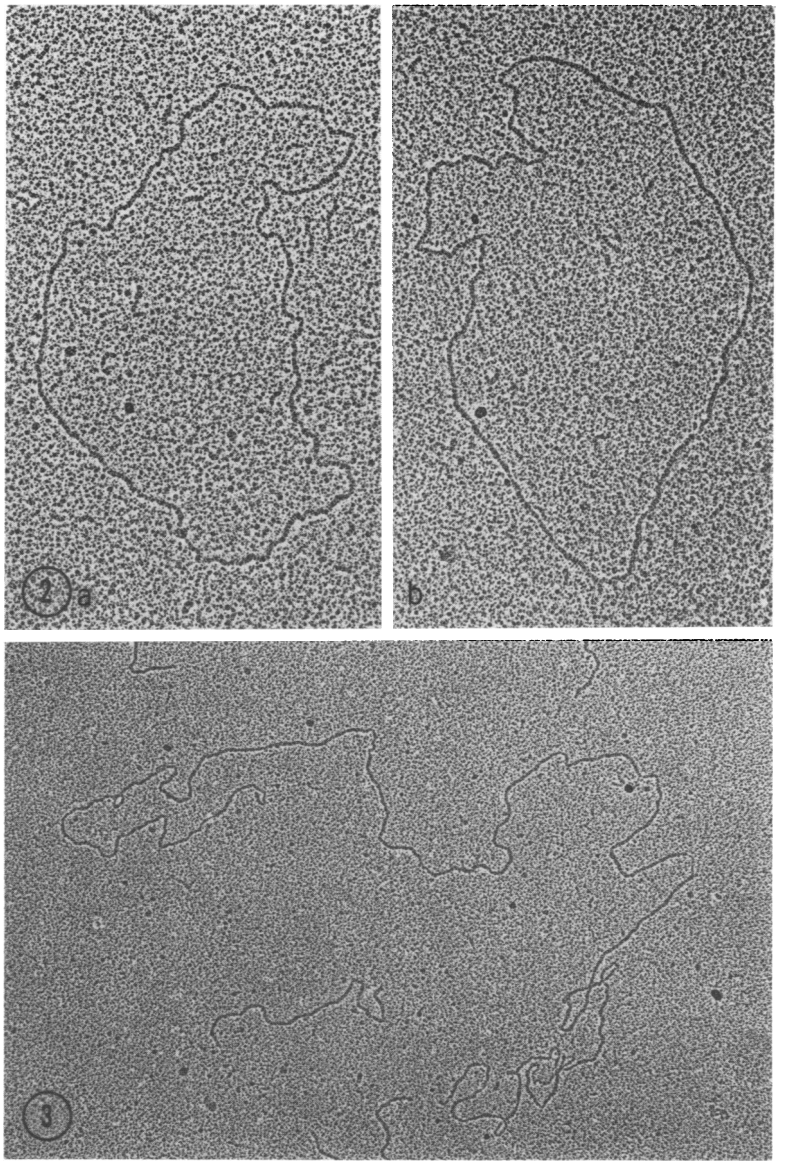

FIG. 2.—Electron micrographs of purified mitochondrial DAN showing circularity. The evaporation of metal on samples shown in Figs. 2 and 3 was heavier than that in Figs. 4–6, resulting in higher contrast but coarser background. ×38,400.

FIG. 3.—Electron micrograph of purified nuclear DNA showing linear conformation. ×24,000.

mately 10–20 per cent of the organelles released their DNA content to various degrees. It was consistently observed that strands emerged from mitochondrial fragments in pairs, with no free ends visible, either ending in a tangled configuration, as shown in Figure 4, or making contact with looplike DNA fibers stemming from neighboring mitochondria. Many strands were seen to remain attached at one or more points along their length to small fragments of membrane.

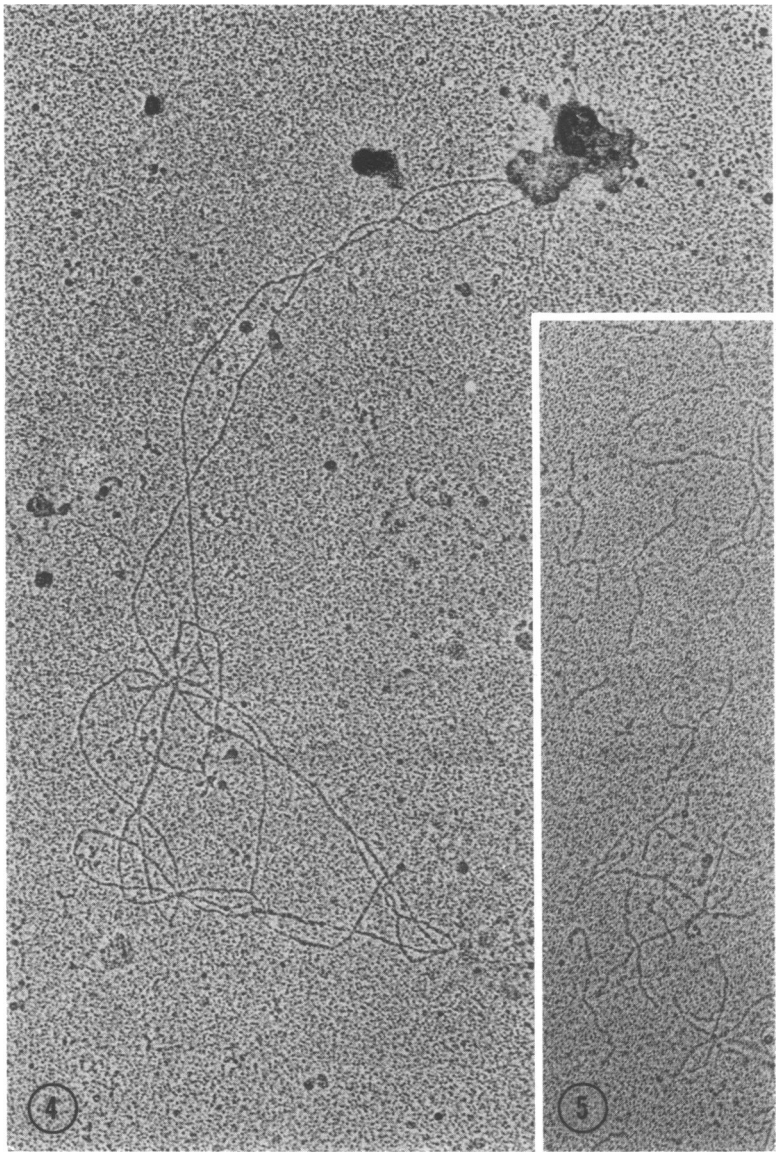

FIG. 4.—Electron micrograph of DNA extruded from osmotically disrupted mitochondrion. Fragment of mitochondrion is seen in upper right. DNA measures about 10 μ, with no free ends visible. This structure is included in Table 1, line 2. ×56,000.

FIG. 5.—Same preparation as that of Fig. 4, except that DNA is degraded by treatment with deoxyribonuclease. ×56,000.

In order to verify that the extruded strands were actually DNA, mitochondrial preparations were mixed with deoxyribonuclease and then lysed by osmotic shock as before, allowing the enzyme to contact the adsorbed fibers for 3–5 min. Figure 5 shows that under these conditions the long, endless strands were indeed reduced to small linear fragments. Ribonuclease did not disrupt the fibers.

Numerous isolated molecules were observed in mitochondrial preparations near

fragments of membrane and appeared as moderately to highly twisted ring structures as shown in Figure 6*a* and *b*. Although most of the tangled structures were difficult to measure, an estimate of their mean contour lengths is presented in Table 1. It can be seen that circular molecules of a basic size, measuring 5.24 ± 0.25 μ, are present in mitochondria. These data are compatible with the results obtained with highly purified DNA. Moreover, multiple amounts of the basic complement of DNA were found to be associated with apparently single ruptured mitochondria, suggesting that two to six molecules of DNA may be contained in a single mitochondrion. Some of the 10-μ aggregates of fibers could actually be resolved into two 5-μ circular structures.

TABLE 1

MEAN LENGTH OF MITOCHONDRIAL DNA PREPARED BY OSMOTIC SHOCK

DNA	No. molecules or aggregates of molecules	Length (μ)	Length / 5.24
Individual twisted ring molecules	30	5.24 ± 0.24	1.0
Complex tangled structures, no free ends visible	9	10.7 ± 1.2	2.0
	3	14.2 ± 0.3	2.7
	3	21.6 ± 0.8	4.1
	1	31.5	6.0

DNA and protein content of mitochondria: Mitochondria were found to contain 8.0 (± 0.7) × 10^{-11} mg of protein per organelle. The mean DNA content of nucleic acid extracts of mitochondria obtained by both methods[6, 17] was 1.1 ± 0.2 μg per mg of protein. The same amount of DNA was digested by treatment of lipid-extracted mitochondria with deoxyribonuclease. The DNA values appeared to vary slightly, depending on the conditions of cell growth. The mean DNA content per mitochondrion was estimated to be 8.8 × 10^{-17} gm. Mitochondrial DNA per cell constitutes 0.2 per cent of total cell DNA.

Discussion.—The data presented provide direct evidence for the circularity of mitochondrial DNA of osmotically disrupted mitochondria. The low percentage of linear fragments in purified DNA and the virtual absence of free ends in lysed preparations argues for a ring structure as the primary (but not necessarily only) form of mitochondrial DNA *in vivo*. The molecules were preserved like duplex molecules with no detectable single-stranded regions (cf. ref. 19).

The narrow distribution of measured lengths of phenol-extracted DNA obtained from several preparations reflects uniformity of molecular size and precision of the technique. DNA obtained by osmotic shock was more tangled than purified DNA, which resulted in more variable measurements. The difference between lengths of 4.74 ± 0.02 μ and 5.24 ± 0.24 μ appears to be due to differences in technique. Caro[21] observed 10–25 per cent shortening of phenol-extracted phage DNA as compared with DNA obtained by osmotic shock. Although variations in technique seem to affect the lengths to some degree,[20, 21] the high accuracy of this method in determining molecular weight and molecular weight heterogeneity is generally acknowledged.[23] Assuming the B configuration of DNA and a mass per unit length of 1.92 × 10^6 daltons per μ,[24] the molecular weights were estimated to be 9.1 × 10^6 and 10.0 × 10^6 for the two types of DNA preparations. The mean molecular weight of total DNA per mitochondrion was estimated from chemical determina-

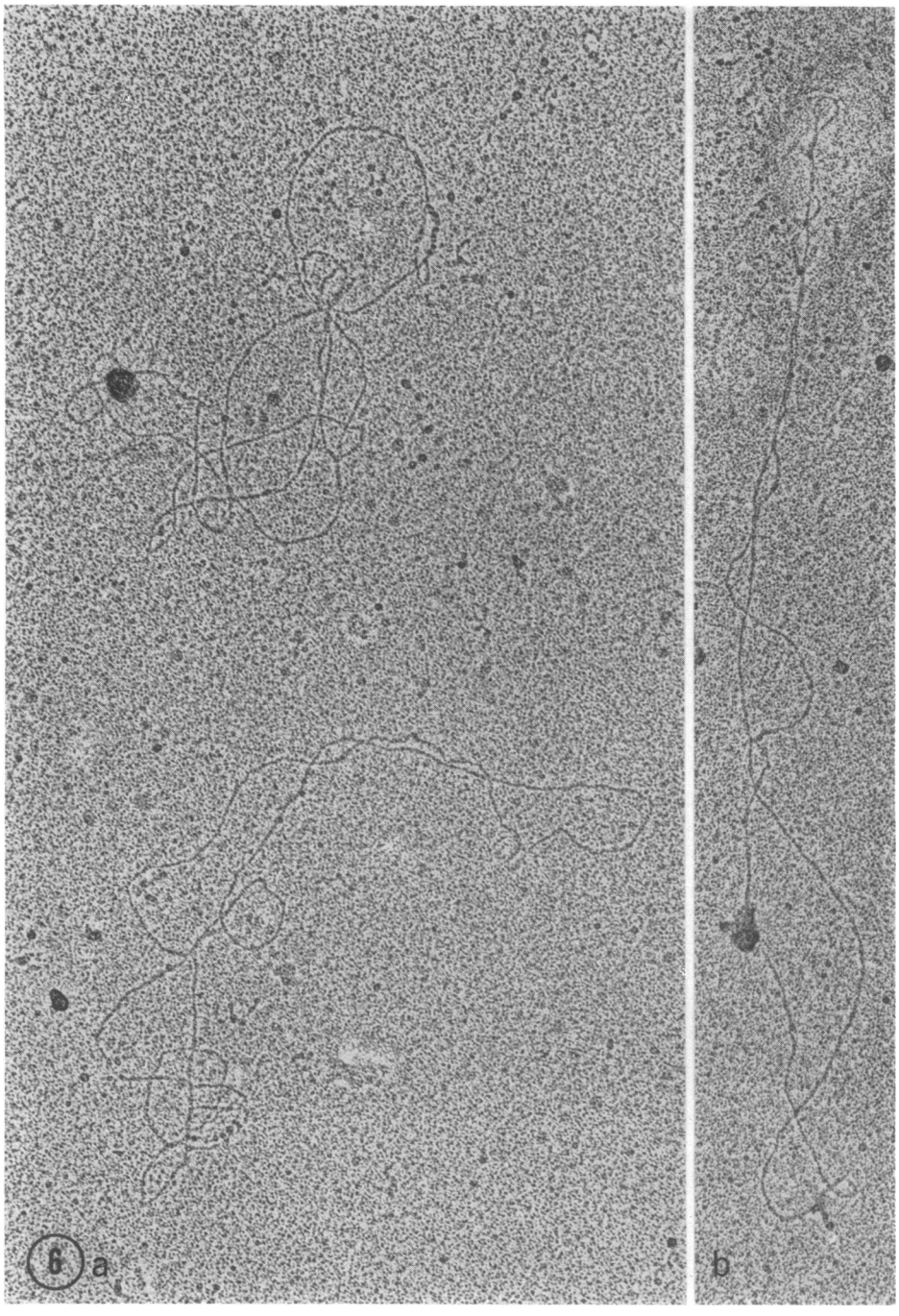

FIG. 6—Electron micrographs of three twisted circular DNA molecules, about 5 μ in length, extruedd from osmotically disrupted mitochondria. These molecules are included in Table 1, line 1. (*a*) ×66,500; (*b*) ×61,750.

tions (8.8×10^{-17} gm DNA) to be 53×10^6, which allows an average number of five to six ring molecules per mitochondrion. Estimates of the DNA content of other types of mitochondria were 1.0×10^{-16} gm[6] and 1.9×10^{-16} gm or 14 molecules of molecular weight 8.7×10^6 (ref. 12) for rat liver, and 3.7×10^{-16} gm for *Tetrahymena*.[7]

The technique of preparing DNA by osmotic shock illuminated the problem of the number of DNA rings per mitochondrion, since all molecules are expected to be adsorbed to the protein film in close proximity to a ruptured mitochondrion if lysis was complete. Although the degree of lysis was difficult to judge, it was apparent that at least two to six or possibly more ring molecules were associated with remnants of a mitochondrion, which is in good agreement with chemical measurements. It is probable that the DNA content per mitochondrion is variable depending on the type of tissue and conditions of growth. The highly branched mitochondria of chick cardiac cells, for example, contain DNA fibers in each branch.[4] Moreover, the size of the organelles is quite variable. Lark[25] reported that the DNA content of *E. coli* 15T$^-$ is greater at rapid growth rates than at slow rates. A similar situation may apply to mitochondria as well.

Circular DNA had not previously been clearly demonstrated in animal cells.[26] The circles reported in pig sperm DNA[27] seemed to be scarce and highly heterogeneous in length, 0.5–16.8 μ; the possibility that mitochondria of the sperm midpiece contributed this DNA was not discussed. The DNA of *Neurospora* mitochondria was reported to be linear and of varying lengths;[5] a length of 6.6 μ was given for one molecule. The possibility of fragmentation of originally circular molecules should be considered, however, since increasing proportions of linear fragments were seen in our cyclic DNA samples after shearing or prolonged storage. Although circularity of genetic linkage maps has been nothing new to the microbial geneticist, electron microscopic techniques have provided direct evidence that the DNA of a number of viruses may assume a circular structure during some part of the viral replication cycle, e.g., the DNA's of ϕX174, polyoma, and papilloma viruses. The DNA's of bacteriophages T2 and lambda exist in linear form, but can be induced artificially to assume ring forms. The molecular weights range from 1.6 to 130×10^6 (cf. ref. 23).

Since the ease of renaturation appears to be characteristic of circular viral DNA,[14] and since purified mitochondrial DNA's of yeast[10] and several mammalian species[10–12] have similar properties, these findings suggest that circularity of mitochondrial DNA may be a more general phenomenon. However, in these as well as in recent preliminary studies on ring-shaped DNA of avian liver mitochondria,[28] the possibility of artificial ring closure caused by the isolation procedures could not be excluded. The present work shows that the DNA of L-cell mitochondria can be circular *in vivo*. The cyclic appearance of clumped DNA seen in thin sections of atypical ascites tumor mitochondria[3] may also relate at least partly to circularity of individual DNA fibers.

The circular DNA molecules observed thus far in electron micrographs did not show evidence of branching as seen in autoradiographs of the bacterial chromosome,[29] which appears to be circular. Therefore, the possibility that newly replicated DNA is resistant to isolation is being investigated. The frequently seen attachment of pieces of membrane to DNA may be fortuitous but may also represent

sites where replication is initiated. The association of mitochondrial DNA with cristae has been described previously.[1, 4]

The fact that evidence for circular DNA has been found only in viruses and bacteria but not in nuclei of eukaryotic cells brings the mitochondrion a large step closer to a possible microbial origin during the evolution of the cell (cf. refs. 2 and 4). Analyses of the conformation of other types of cytoplasmic DNA, especially chloroplast DNA, should also prove to be of interest.

Summary.—Evidence has been presented that mitochondrial DNA of L cells exists in circular form either when extruded from osmotically lysed organelles or extracted by phenol. In contrast, nuclear DNA is linear. Mitochondrial DNA appears to be membrane-bound at one or more sites. The molecular weight of mitochondrial DNA was estimated by electron microscopic and by chemical methods. Two to six ring molecules of molecular weight 10×10^6 may be present in a single mitochondrion. Some of the implications of these findings were discussed.

* This investigation has been supported by USPHS grant 1-PO1-AI07005. The skilled technical assistance of Miss Anneke Theunissen and Mr. John R. W. Hobbs is acknowledged.

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