THE FORM AND SIZE OF MITOCHONDRIAL DNA OF THE RED BEAN, PHASEOLUS VULGARIS*

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Mitochondrial DNA (M-DNA) of a variety of animals ranging from insects to mammals has been shown by electron microscopy to be in the form of a circle with a contour length of 4.5–6.0 μ.[1–12] M-DNA of *Neurospora crassa*[13] and of yeast[14, 15] is of the same order of size as animal M-DNA. Some circular forms of M-DNA have been demonstrated in yeast[15] but not in *N. crassa*. Suyama and Bonner[16] isolated DNA from mitochondrial fractions of a number of monocotyledonous and dicotyledonous angiosperm plants and in each case demonstrated a difference in buoyant density to the respective nuclear DNA. The present report relates the results of experiments to determine the form and size of mitochondrial DNA of the dicotyledonous angiosperm *Phaseolus vulgaris*.

Material and Methods.—Preparation of mitochondria: Etiolated hypocotyls of the red bean, *Phaseolus vulgaris*, were produced by planting seeds in moist vermiculite and allowing growth in the dark at 23°C for 14 days. The hypocotyls were harvested (approximately 1,200 gm), chilled to 0–4°C, washed by shaking 3 times with distilled water, and ground up in a mortar and pestle in a buffer containing 0.3 *M* mannitol, 0.1% bovine serum albumin, 1 mM EDTA (ethylenediaminetetraacetic acid), and 0.05 *M* tris/HCl at pH 7.2.[16] The material was further broken down in a Waring Blendor (20 sec at high speed) and filtered through two layers of muslin. The filtrate was centrifuged at 480 *g* for 10 min. The pellet from this centrifugation contains most of the intact nuclei and is referred to as the crude nuclear fraction. The mitochondria were pelleted from the supernatant by centrifugation at 10,000 *g* for 10 min, resuspended in 10 ml buffer, and beef pancreatic DNase I was added to 50 μg/ml and $MgCl_2$ to 7mM, and the mixture incubated at 37°C for 25 min.[17] The digestion was stopped with 1 ml of 0.4 *M* EDTA, and the mitochondria were washed 3 times with buffer containing 0.02 *M* EDTA. The mitochondria were then either frozen or used directly.

DNA extraction and purification (modified from Marmur[18]): DNA was extracted from the various fractions by digestion with 1 mg/ml pronase (freed from DNase activity by the method of Hotta and Bassel[19]) in a buffer containing 0.015 *M* NaCl, 0.01 *M* EDTA, 0.05 *M* phosphate (pH 8.0), and 0.25% SDS (sodium dodecyl sulfate) at 37°C for 4 hr. The digest was shaken twice with phenol for 60 min at 0°C. Phenol was removed from the aqueous phase by shaking with ether. The ether was blown off with air and the material dialyzed against 0.3 *M* NaCl for 1 hr. Beef pancreatic RNase (Worthington, previously heated to 90°C for 5 min at pH 5.0) was added to 25 μg/ml, and α-amylase to 15 μg/ml and the mixture incubated for 1 hr at 37°C and then dialyzed again against 0.3 *M* NaCl at 23°C for 1 hr. The solution was applied to a column of MAK;[20] the column was washed with 0.3 *M* NaCl until no UV-absorbing material appeared in the effluent; and the DNA was then eluted with 0.75 *M* NaCl.

Equilibrium centrifugation: Buoyant density centrifugation in CsCl gradients according to the method of Meselson *et al.*[21] were carried out in a four-place ANF rotor in a Spinco model E ultracentrifuge at 44,770 rpm for 28 hr at 20°C.

Denaturation and reannealing of DNA: DNA extracted either from mitochondrial fractions or from bacteria was denatured by adding NaOH to a final concentration of 0.1 *M* to a solution containing 10 μg/ml DNA, 0.8 *M* NaCl, 0.05 *M* phosphate (pH 6.7), and 0.001 *M* EDTA, and kept at 23°C for 20 min. The solution was then neutralized with an equal amount of 0.1 *M* HCl in 1 *M* tris (pH 7.2) and heated to 65°C for 1 hr.

Isolation and culturing of bacteria: Immediately after DNase treatment, samples from a mitochondrial fraction were streaked on 6 plates of nutrient agar. Colonies were collected 24 hr later, pooled, and grown in nutrient broth in 2-liter flasks at room temperature for 72 hr. The bacteria were harvested by centrifugation, and the DNA was isolated and purified by the same technique as that described for DNA from the mitochondrial fractions.

Preparation of chloroplasts: Seeds were planted in moist vermiculite and grown in the light for 14 days. Chloroplasts were isolated by the method of Kislev *et al.*[22] Leaves were harvested, chilled to 0–4°C, washed 3 times in distilled water, and ground in sand in a 0.5 *M* sucrose–0.5 *M* tris/HCl (pH 8.0) buffer containing 0.005 *M* EDTA. The suspension was filtered through two layers of muslin and centrifuged for 10 min at 100 *g*. The pellet was removed and is referred to as the crude nuclear fraction. The supernatant was centrifuged at 3,000 *g* for 10 min. The green pellet was collected, resuspended in buffer, placed on a continuous sucrose gradient (68–18% sucrose in 0.5 *M* tris/HCl, pH 8.0), and centrifuged for 60 min at 23,000 *g* in a SW-25 rotor of a Spinco model L centrifuge. The green band was collected. A drop was examined under phase-contrast and appeared to consist entirely of chloroplasts. No nuclei were seen. DNA was extracted from chloroplasts, the crude nuclear fraction, and from a whole cell homogenate as described above except that 1% sodium deoxychlolate was used to lyse the organelles. The chloroplast DNA was purified on MAK. DNA from the crude nuclear fraction and the whole cell homogenate was precipitated by adding one volume of isopropanol, spooled out and redissolved in 1 × SSC.

Electron microscopy: (a) *Sections:* Pellets of mitochondria were fixed in Kellenberger's 1% OS_4 for 30 min, treated with uranyl acetate,[23] dehydrated in a graded series of ethanols, and embedded in Epon.[24] Thin sections were examined in a Siemens Elmiskop I at 11,000 ×. (*b*) *Protein monolayers:* Samples of mitochondria were suspended in a solution containing 0.015 *M* NaCl, 0.01 *M* EDTA, 0.25% SDS, and 1 mg/ml DNase-freed pronase, and incubated at 37°C for 4 hr. Approximately 10 μl of the digest was picked up with a wide-bore (at least 2 mm) pipette and added to 0.1 ml of 1 *M* ammonium acetate containing 0.05% cytochrome *c* and 0.5% formaldehyde.[25] This solution was poured down an inclined glass slide onto a hypophase of 0.3 *M* ammonium acetate containing 0.5% formaldehyde. The surface film was picked up on carbon-coated formvar films supported on 100-mesh copper grids. The grids were then shadowed on a rotary turntable at an angle of about 8° with 10 mg of platinum-palladium wire evaporated from a 23-mil tungsten wire at a mean distance of 10 cm from the grids in a Kinney model KSE-2 evaporating unit. Grids were examined in the electron microscope and methodically searched from one side to the other. The first 27 DNA molecules seen in each preparation which did not run out of view off the grid square, or were not excessively tangled so as to make measuring meaningless, were photographed at original magnifications calibrated with a diffraction grating replica (2,160 lines/mm) to be 10,600. Measurements of molecules were made on positive prints at a magnification of 60,400 with a map measurer. DNA molecules were similarly prepared from other fractions for examination in the electron microscope and similar photographing and measuring.

Results.—The mitochondrial fractions consisted mainly of mitochondria (Fig. 1), although remnants of other cell organelles were present. Less than one bacterial profile per 1,500 mitochondrial profiles was found. The results of buoyant density centrifugation of the various fractions of the dark-grown plants are shown in Figure 2. The DNA from the crude nuclear fraction had a main peak at $\rho = 1.693$ and a shoulder at $\rho = 1.702$. In contrast, the DNA from the mitochondrial fraction has a sharp peak at $\rho = 1.707$, which is close to the value of $\rho = 1.706$ reported by Suyama and Bonner (1966) for the M-DNA of a number of angiosperms including the closely related mung-bean, *Phaseolus aureus*. Renatured M-DNA formed a rather sharp band about 0.003 gm/cm^3 heavier than the

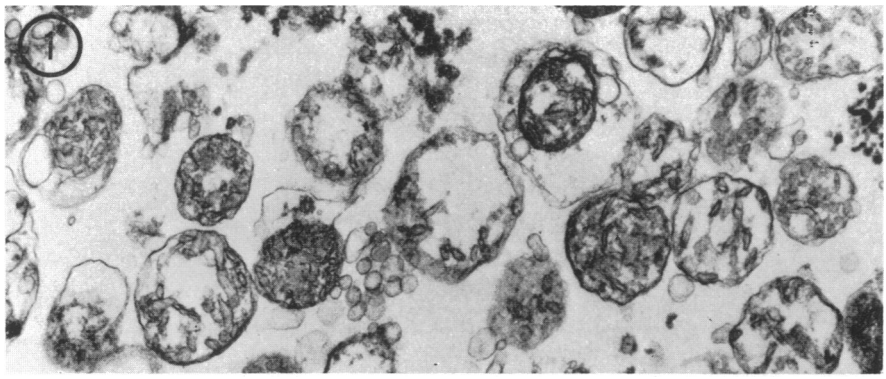

FIG. 1.—A thin section of a pellet of a mitochondrial fraction isolated from hypocotyls of dark-grown *Phaseolus vulgaris*. The fraction consists mainly of mitochondria, but remnants of other cell organelles are present. × 27,000.

native M-DNA, which is in agreement with the results of previous studies on M-DNA's from a variety of animals, and from yeast.[26–29, 12, 14]

Most of the DNA isolated from bacteria cultured from a mitochondrial fraction formed a sharp band in buoyant CsCl at a density of 1.719 gm/cm³. A distinct minor band of $\rho = 1.699$ was also apparent. DNA with a buoyant density similar to that of M-DNA (1.707) was not found. Further, under the conditions used to renature M-DNA, the bacterial DNA that was isolated renatured to only 0.010 gm/cm³ from native density. These findings, together with the low bacterial counts in mitochondrial fractions, make it very unlikely that bacterial contamination accounts for the DNA which we are attributing to mitochondria.

Length measurements of DNA molecules from two separately isolated mitochondrial fractions are summarized in Figure 3. One was frozen and thawed before digestion and preparation for electron microscopy, and the other was digested and prepared directly. Only linear molecules were found (Fig. 4). They range in length from 1 to 62 μ and have a mean length of about 19.5 μ. DNA extracted and purified from a mitochondrial fraction (the same fraction as that from which the molecules represented in the upper histogram in Fig. 3 were prepared) was also examined in the electron micro-

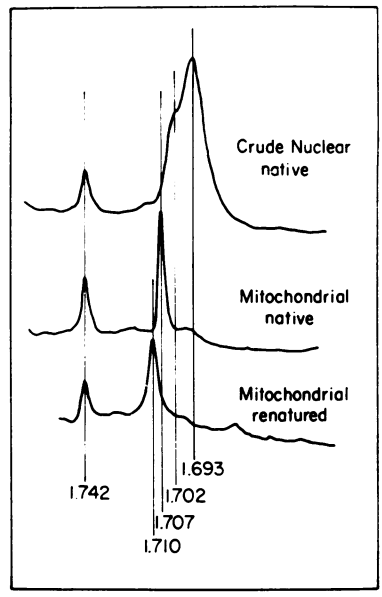

FIG. 2.—Microdensitometer tracings of ultraviolet photographs of CsCl buoyant density gradients of DNA from the various fractions indicated, isolated from hypocotyls of dark-grown *Phaseolus vulgaris*. The reference band to the left is native DNA of SPO1.

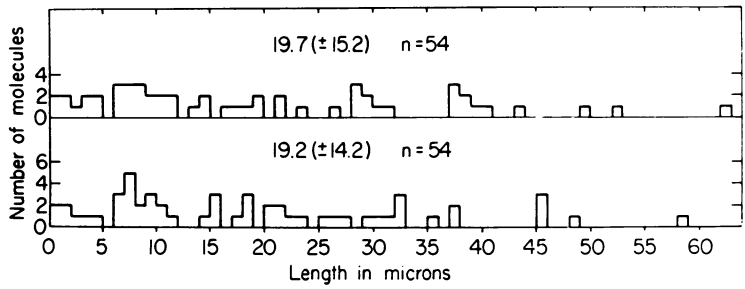

FIG. 3. Frequency distributions of the lengths of DNA molecules from two mitochondrial fractions isolated from hypocotyls of dark-grown *Phaseolus vulgaris*. The mitochondria in each fraction were digested with DNase-freed pronase in the presence of SDS and the digest spread directly. The molecules represented in the upper histogram are from a mitochondrial fraction frozen and thawed before pronase digestion, and the molecules represented in the lower histogram are from a mitochondrial fraction digested with pronase directly after isolation. The molecules are all linear. The mean and standard deviation of each sample are given.

scope. In this preparation (Fig. 5), the longest molecule observed was only 27 μ and the number of shorter molecules was greatly increased. Shearing of the DNA during purification may account for the apparent absence of longer molecules, and the lower mean length.

Although the buoyant density results show clearly that most of the DNA extracted from the mitochondrial fraction is distinct from nuclear DNA, the possibility of a small amount of nuclear contamination remains. Length measurements of the DNA from the crude nuclear fractions corresponding to the mitochondrial fractions from which the DNA in Figure 3 was taken and measured are shown in Figure 6. From the first fraction the longest molecule recorded was only 6 μ, and from the second fraction a few longer molecules were found but they were considerably shorter than many of the M-DNA molecules. The shortness of the nuclear DNA molecules could be due to a cellular DNase which is activated when the cells are ruptured and which enters nuclei but not mitochondria. The possibility that only DNA in intact nuclei is broken into shorter molecules, and that DNA from ruptured nuclei which come into association with mitochondria remain long, is unlikely, as DNA from a mitochondrial fraction not treated with DNase (and presumably contaminated with nuclear DNA) had a mean length of 1.19 μ (range 0.2–5.2 μ, n = 108). Nuclear DNA which contaminates mitochondria may be expected to be further broken down by the DNase treatment prior to digestion of the mitochondria.

In view of the results presented, it seems very unlikely that DNA of nuclear origin could account for more than a few of the shorter molecules of the DNA scored as mitochondrial.

Cells of etiolated hypocotyls contain proplastids which presumably contain the same DNA as the chloroplasts into which they develop on exposure to light. Such DNA must also, therefore, be considered as a possible contaminant of the M-DNA. In Figure 7 the banding positions at equilibrium of DNA's from the

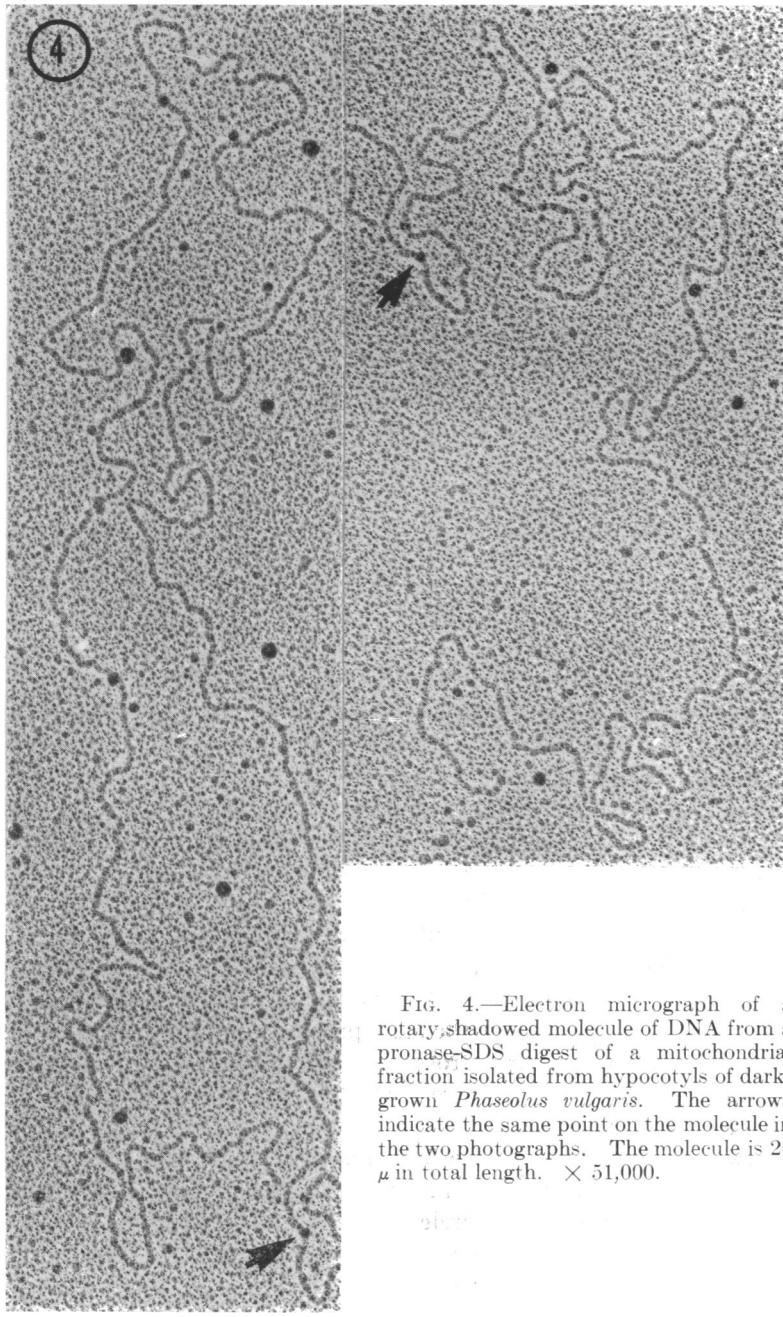

FIG. 4.—Electron micrograph of a rotary-shadowed molecule of DNA from a pronase-SDS digest of a mitochondrial fraction isolated from hypocotyls of dark-grown *Phaseolus vulgaris*. The arrows indicate the same point on the molecule in the two photographs. The molecule is 21 μ in total length. $\times$ 51,000.

various fractions of light-grown leaves in CsCl gradients are compared. Also shown is a tracing of a chloroplast sample which was treated with DNase, then washed free of the enzyme; marker SP01 DNA was added, and the DNA extraction procedure applied as with the other samples. DNA from the crude nuclear

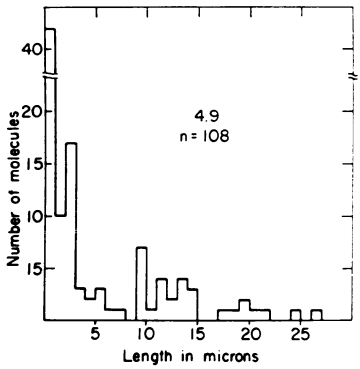

FIG. 5.—Frequency distribution of the lengths of molecules from a sample of purified DNA prepared from the same mitochondrial fraction as that from which the DNA molecules represented in the upper histogram of Fig. 3 were prepared. The mean is given.

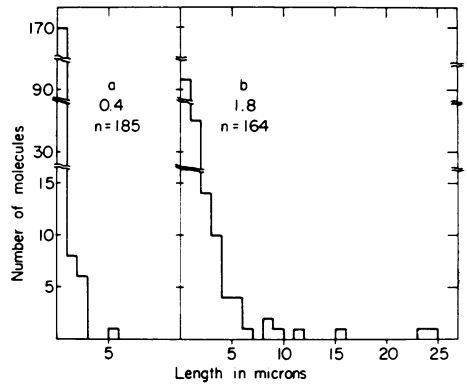

FIG. 6.—Frequency distribution of the lengths of DNA molecules from two crude nuclear fractions. These fractions, *a* and *b*, correspond respectively to the mitochondrial fractions from which the DNA molecules represented in the upper histogram and in the lower histogram of Fig. 3 were prepared. The mean of each preparation is given.

fraction banded similarly to the DNA from the corresponding fraction of the dark-grown material; the main peak was at $\rho = 1.693$ with a shoulder at $\rho = 1.702$. The chloroplast DNA had a peak at $\rho = 1.695$ and a shoulder in the range of $\rho = 1.702$. Much of this DNA could be nuclear contamination. It is clear, however, that chloroplasts do not contain appreciable amounts of DNA with a similar buoyant density to the DNA extracted from the mitochondrial fraction, i.e. 1.707. Further, isolated chloroplasts, unlike isolated mitochondria, appear to be permeable to DNase. Enzyme treatment of the kind used on all mitochondrial fractions produced a complete loss of all UV-absorbing material.

The possibility that proplastids could account for some of the longer molecules of DNA from the mitochondrial fraction is also unlikely from the following. Plants were grown in the dark for 14 days and then before harvesting were set in the light for 2 days to convert the stem proplastids to chloroplasts. A mitochondrial fraction was prepared from the hypocotyls (which had turned pale green by the end of the 2-day light treatment) as before, treated with DNase and then lysed and prepared for electron microscopy. The mean length of the DNA molecules was 18.3 μ, and their range 2–58 μ ($n = 54$).

In order to test whether the DNase treatment of mitochondria could be responsible for our failure to find circles, DNA was prepared for electron microscopy from a mitochondrial fraction which had not been treated with DNase. Not a single circle was found in the more than 5,000 molecules examined.

Rat liver mitochondria were digested and prepared for electron microscopy by the method used to digest and prepare plant mitochondria. The DNA was found to be all circular and, further, 90 per cent of the circles were in the supercoiled form. Also, when rat liver mitochondria and *P. vulgaris* mitochondria were mixed, digested, and prepared for electron microscopy, circles were recovered,

and the same proportion was found to be in the supercoiled form as in a control preparation comprising only rat liver mitochondria.

Discussion and Summary.—DNA extracted from mitochondria of etiolated hypocotyls of *Phaseolus vulgaris* has a buoyant density in CsCl which distinguishes it from that of the plant's nuclear DNA and from DNA isolated from a chloroplast fraction. In electron-microscope preparations the M-DNA molecules appear to be all linear, with a mean length of about 19.5 μ and a range of 1–62 μ. The results indicate that nuclear and chloroplast DNA could account for no more than a few of the smaller molecules attributed to mitochondria. Also, it is very unlikely that bacterial contamination accounts for the DNA we are calling mitochondrial, for the following reasons: (1) Examination of pellet sections indicated less than one bacterium per 1,500 mitochondria in the mitochondrial fractions; (2) we were unable to detect a DNA with a buoyant density similar to that of M-DNA included in the DNA's isolated from bacteria cultured from mitochondrial fractions; and (3) the DNA that was isolated from bacteria renatured to a much smaller extent than did M-DNA (see also Marmur and Doty[30]).

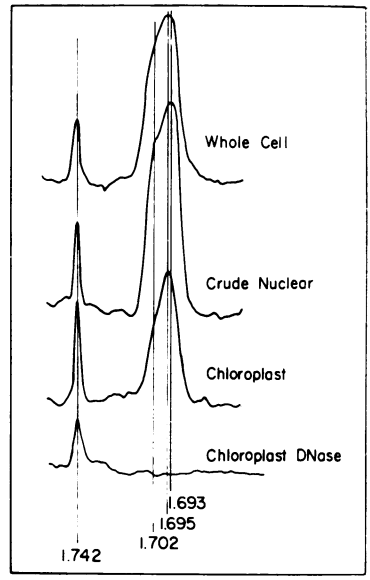

FIG. 7.—Microdensitometer tracings of ultraviolet photographs of CsCl buoyant density gradients of DNA from the various fractions indicated, isolated from leaves of light-grown *Phaseolus vulgaris*. The reference band to the left is native DNA of SPO1.

A molecule of 62 μ has a molecular weight of 119×10^6.[31] This is in general agreement with the estimate of 10^8 daltons for the mean molecular weight of M-DNA from a number of angiosperms made by Bonner and Suyama.[16] If a molecule of this size contains a unique sequence of nucleotides, it could carry about 11 times the genetic information of a single animal M-DNA circle. Alternatively, such a molecule may comprise tandem repeats of a shorter sequence. The different-sized molecules seen in electron-microscope preparations of *P. vulgaris* M-DNA could be the products of breakdown during preparation of the longest molecules, or the length measurements may be a fair estimate of the sizes of the *in situ* M-DNA molecules. The latter situation might be an expression either of heterogeneity of M-DNA molecules in this species or of different degrees of tandem repetition of a basic unit.

In both supercoiled and open circular animal M-DNA molecules, the circularity is dependent upon at least one phosphodiester bond between each sequential nucleotide pair. Supercoiled circles consist of two completely intact polynucleotide strands, while at least one of the strands of the open circular form must contain at least one phosphodiester bond break[32, 28] As all the rat liver M-DNA was circular when isolated by the method used to isolate plant M-DNA,

and as the presence of plant mitochondria in the digesting medium did not effect the percentage of supercoiled molecules, it seems unlikely that *in situ* plant M-DNA is in the form of covalently linked circles.

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