CIRCULAR DNA MOLECULES ASSOCIATED WITH CHLOROPLASTS OF SPINACH, *SPINACIA OLERACEA*

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INTRODUCTION

Results of cytochemical studies (Sagan and Scher, 1961; Ris and Plaut, 1962; Kislev et al., 1965) and of studies involving cell fractionation and buoyant density analyses (Sager and Ishida, 1963; Ray and Hanawalt, 1964; Brawerman and Eisenstadt, 1964; Edelman et al., 1964; Wells and Birnstiel, 1969) have shown that chloroplasts of microorganisms and of higher plants contain DNA which is distinct from that of the nuclear DNA of these organisms. We have recently presented evidence that chloroplast DNA of the protozoan flagellate, *Euglena gracilis* is in the form of circular molecules about 40 μ in contour length (Manning et al., 1971).

Evidence presented in the present report indicates that circular DNA molecules, approximately 44 μ in contour length, are associated with chloroplasts of the dicotyledonous angiosperm, *Spinacia oleracea* (spinach). Also, circular molecules approximately 43 μ in contour length were obtained from chloroplast fractions of the monocotyledonous angiosperm, *Zea mays* (corn).

MATERIALS AND METHODS

Isolation of Chloroplasts

Chloroplasts of spinach (*S. oleracea*) were isolated by the procedure described previously for *E. gracilis* (Manning et al., 1971), with the following modifications. Leaves were harvested from intact growing plants and immediately chilled to 4°C and washed twice with distilled water. The midribs were removed and 500 g of the remaining tissues were immersed in 500 ml of a buffer containing 0.37 M sucrose, 10 mM Tris·HCl, 50 mM EDTA (sodium ethylenediamine-

tetraacetate), pH 7.6. The leaves were homogenized for 10 sec at high speed in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), the homogenate was filtered through 10 layers of cheesecloth, and the filtrate was centrifuged at 3000 *g* for 10 min at 4°C. The pellet contained most of the chloroplasts and some broken and unbroken cells. The chloroplasts were isolated free of the contaminating cellular material by sucrose flotation centrifugation (Manning et al., 1971). Finally, the purified chloroplasts were suspended in an equal volume of 0.15 M NaCl, 0.1 M EDTA, 50 mM Tris·HCl (pH 9.0) and stored at −20°C.

Isolation of DNA

DNA was isolated from spinach chloroplasts and whole cells, *Micrococcus lysodeikticus*, and bromouracil-labeled *Escherichia coli* for analytical density gradient centrifugation as described by Richards et al., (1971).

Spinach chloroplast DNA and corn (*Z. mays*) chloroplast DNA were prepared for electron microscopy in a manner designed to minimize shearing. 2 ml of thawed, packed chloroplasts were added to 2 ml of 0.79 M sucrose, 0.15 M NaCl, 0.1 M EDTA, 50 mM Tris·HCl (pH 9.0). Sodium dodecyl sulfate (SDS) (0.5%), pronase (1 mg/ml, Grade B, Calbiochem, Los Angeles, Calif., and freed from DNase activity by the method of Young and Sinsheimer [1967]), and sodium deoxycholate (1%) were added and the mixture was gently rocked overnight at 4°C. $NaClO_4$ (5 M) was added to 1 M final concentration, and the solution was rocked for 30 min at 4°C with 0.5 vol of chloroform:isoamyl alcohol (24:1) and 1 vol of phenol saturated with 0.15 M NaCl, 0.1 M EDTA, 50 mM Tris·HCl (pH 9.0). The mixture was centrifuged at 1000 *g* for 15 min at 4°C. The aqueous phase was removed and dialyzed overnight against 0.15 M NaCl, 15 mM sodium citrate, 0.5 mM EDTA, pH 8.0, in the cold, and then prepared directly for electron microscopy.

Whole cell DNA was prepared for electron microscopy from spinach leaves by first immersing the leaves in the same buffer in which the thawed chloroplasts were placed, homogenizing in a Waring blender for 10 sec at high speed, and isolating the DNA by the minimum shear procedure described for chloroplast DNA.

DNA was isolated from lambda sus $O_{29}CI857$ bacteriophage by the method described by Thomas and Abelson (1966) and circularized by the procedure of MacHattie and Thomas (1964).

Denaturation and Annealing of DNA

DNA extracted from whole cells or from chloroplasts was denatured by adding 1.0 N NaOH to a final concentration of 0.2 N to a solution containing 5 μg of DNA, 0.15 M NaCl, and 15 mM sodium citrate. The mixture was allowed to stand for 15 min at 25°C and 37% formaldehyde was added to a final concentration of 2%. The mixture was then neutralized by adding 1.0 M KH_2PO_4 to a final pH of 7.1. DNA to be annealed was denatured in a similar way, but formaldehyde was omitted. The solution was neutralized with KH_2PO_4 and the DNA (6 μg/ml for both chloroplast DNA and for whole cell DNA) was annealed by incubating for 200 min at 60°C.

Analytical Cesium Chloride Density Gradient Centrifugation

Density gradient centrifugations were performed in the Spinco model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) as described previously (Richards et al., 1971), using either *M. lysodeikticus* DNA or bromouracil-labeled hybrid DNA from *E. coli* as a density marker. The density of the DNA was determined by the method of Schildkraut et al. (1962).

Chloroplast Lysates and Digests

Crude lysates of chloroplast fractions were used to make rotary-shadowed preparations for electron microscopy. These were prepared (*a*) by suspending chloroplasts in a solution containing 15 mM sodium chloride, 10 mM EDTA (pH 8.0), and 2% SDS, and leaving at room temperature (≃23°C) for 30 min, and (*b*) by incubating chloroplasts at 37°C for 4 hr in the same solution as that used in (*a*), but with 1 mg/ml of pronase (freed from DNase activity as before) and 0.25% SDS (Wolstenholme and Gross, 1968).

Electron Microscopy

SECTIONS: Pellets of chloroplasts were fixed in Kellenberger's 1% osmium tetroxide for 12 hr, treated with uranyl acetate (Ryter et al., 1958), dehydrated in a graded series of ethanolic solutions and embedded in Epon (Luft, 1961). Thin sections were cut on an LKB Ultrotome III microtome (LKB Instruments, Inc., Rockville, Md.) with a diamond knife and picked up on uncoated 400-mesh copper grids.

PROTEIN MONOLAYERS: Approximately 10 μl of a lysate, digest, or deproteinized DNA was added to 0.1 ml of 1 M ammonium acetate containing 0.05% cytochrome *c* and 0.5% formaldehyde (Freifelder and Kleinschmidt, 1965). 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 onto carbon-coated formvar films supported on 100-mesh copper grids and shadowed as previously described (Wolstenholme and Gross, 1968). Grids were examined, and micro-

graphs were made with a Siemens Elmiskop 101 electron microscope.

Estimates of the percentage of the total length of DNA in the form of circles in preparations of chloroplast DNA and of whole cell DNA were made from data obtained in the following way. Grids were first chosen on which the DNA was in low enough concentration that the molecules did not overlap each other. The number of molecules in the form of circles was first determined by examining at least 4000 molecules in each preparation. The mean length of linear molecules in each preparation was next determined. Grid squares were methodically searched from one side to the other and *all* of the linear molecules seen were photographed at an original magnification of ×6000 (calibrated with a diffraction grating replica [2160 lines/mm]). The molecules were measured on positive prints at a magnification of × 30,000 with a map measure. The mean lengths of circular molecules, both open and highly twisted, were determined by photographing at an original magnification of ×10,000 and measuring on positive prints at a magnification of ×65,000.

RESULTS

The results of cesium chloride equilibrium density centrifugations of whole cell DNA and of DNA from a chloroplast fraction of spinach are shown in Figs. 1 and 2. Whole cell DNA formed a single symmetrical band at a buoyant density of 1.694 g/cm³. Chloroplast DNA also formed a single band at a buoyant density only 0.002 g/cm³ denser than whole cell DNA. These values are in close agreement with the buoyant densities of spinach whole cell and chloroplast DNA reported by Whitfeld and Spencer (1968), Bard and Gordon (1969), and Wells and Birnstiel (1969). Upon alkali denaturation, whole cell DNA increased in buoyant density by 0.018 g/cm³ and chloroplast DNA by 0.011 g/cm³. After neutralization and annealing under the same conditions for 200 min, however, whole cell DNA showed no change in density while chloroplast DNA returned to within 0.004 g/cm³ of the native density. The respective behaviors for the two DNAs are again in agreement with previous reports (Whitfeld and Spencer, 1968; Bard and Gordon, 1969) and suggest that the DNA from the chloroplast fraction does not include substantial amounts of contaminating nuclear DNA.

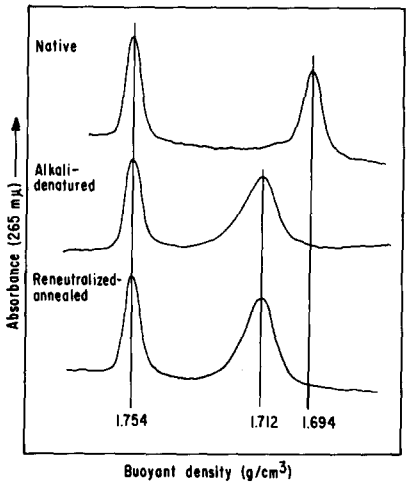

FIGURE 1 Densitometer tracings of ultraviolet photographs of cesium chloride equilibrium density gradients of *S. oleracea* whole cell DNA after the treatments indicated. The reference band (ρ = 1.754 g/cm³) to the left is *E. coli* bromouracil-labeled hybrid DNA.

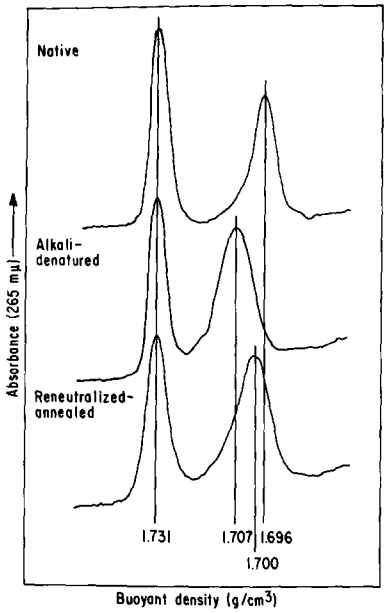

FIGURE 2 Densitometer tracings of ultraviolet photographs of cesium chloride equilibrium density gradients of *S. oleracea* chloroplast DNA after the treatments indicated. The reference band (ρ = 1.731 g/cm³) to the left is native DNA of *M. lysodeikticus*.

When deproteinized DNA from a chloroplast fraction was examined in the electron microscope, it was found to contain open circular molecules with a mean contour length of 43.8 μ (Figs. 3 and 5) and highly twisted circular molecules with a mean contour length of 42.2 μ (Figs. 4 and 5). The lower value obtained for the latter may be related to the twisted nature of the molecules. Data con-

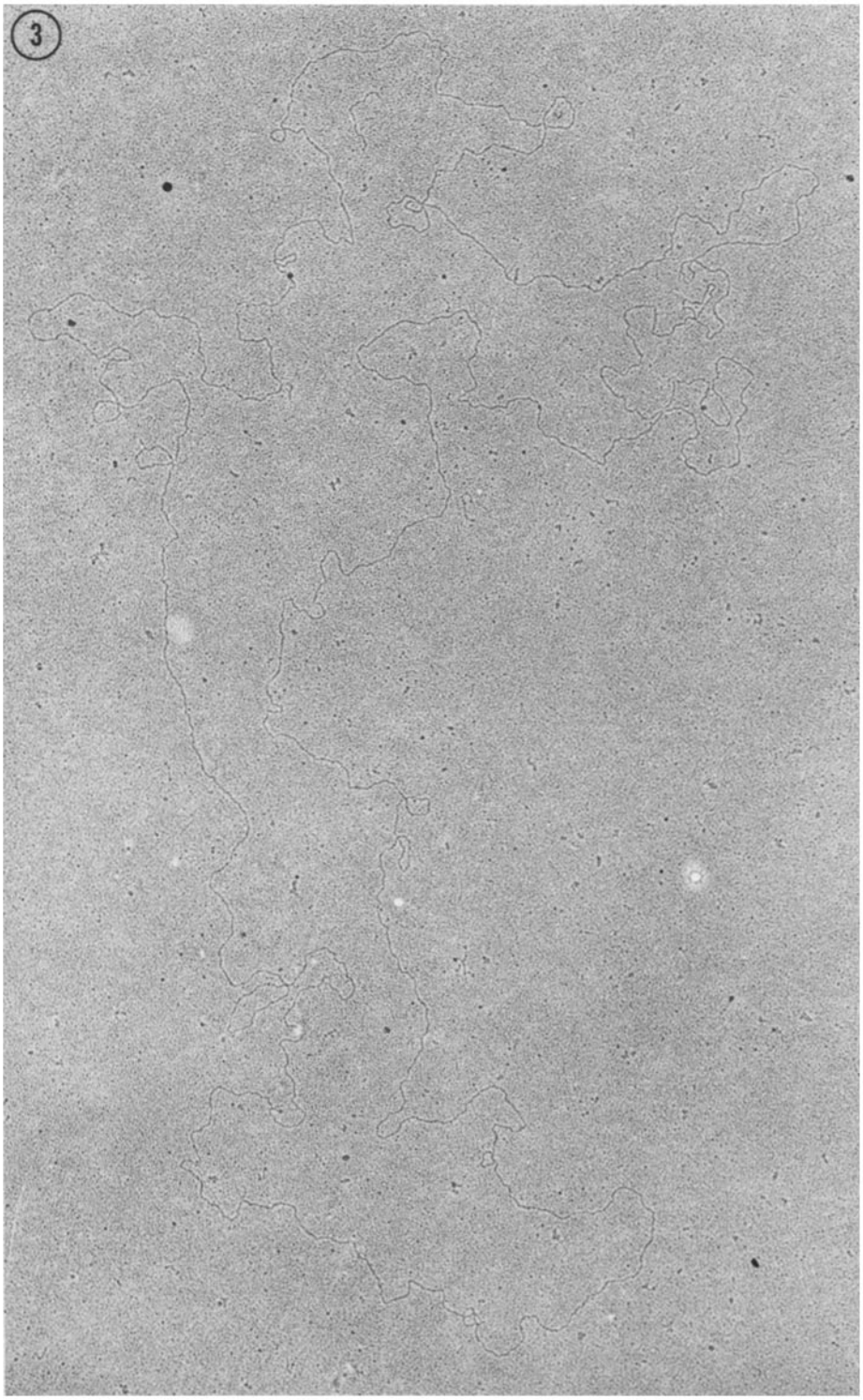

FIGURE 3 Electron micrograph of a rotary-shadowed circular molecule of DNA from a deproteinized preparation of chloroplasts of *S. oleracea*. The molecule is 44 μ in total length. × 27,700.

cerning the frequencies of these two molecular forms in chloroplast DNA and whole cell DNA are given in Table I. Highly twisted circular molecules were approximately twice as frequent as the open circular molecules in the chloroplast DNA preparation and were the only form of circular molecules found in whole cell DNA.

Approximately 7.6% of the DNA from the chloroplast fraction was represented by 42–45 μ circles. The remaining DNA was in the form of linear pieces 1–78 μ in length. Only 9.7% of this DNA was in pieces longer than the length of a circle. In contrast to the finding for DNA from a chloroplast fraction, only about 0.7% of the DNA

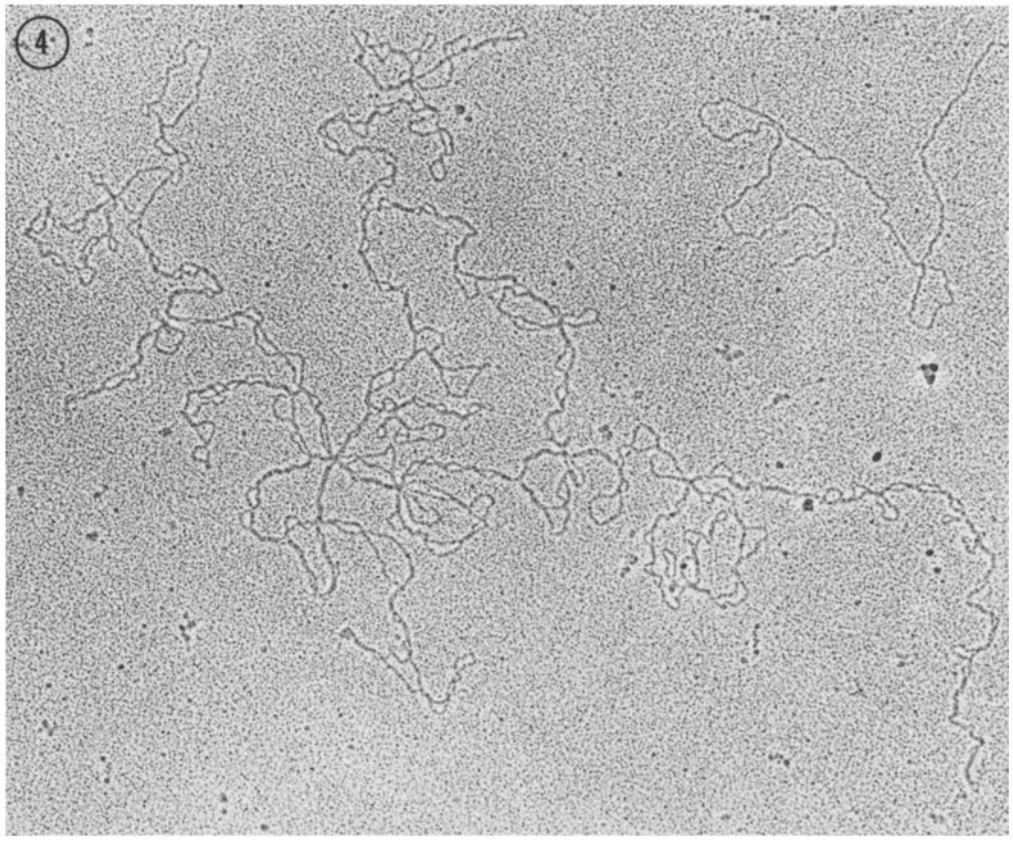

FIGURE 4 Electron micrograph of a rotary-shadowed highly twisted circular molecule of DNA from a deproteinized preparation of chloroplasts of *S. oleracea*. The molecule is 42 μ in total length. × 43,300.

TABLE I

Data Concerning Estimates of the Percentage Length of DNA as Circular Molecules in Preparations of Chloroplast and of Whole Cell DNA

The mean length of open circular molecules and of twisted circular molecules used to make these estimates were obtained from the data presented in Fig. 5 for deproteinized DNA

	No. of molecules examined	No. of circular molecules found		Linear molecules		Length of DNA as circular molecules
		Open circles	Twisted circles	Mean length	No. of molecules measured	
				μ		%
Chloroplast DNA	4088	17	35	6.63	178	7.57
Whole cell DNA	5000	0	4	5.03	174	0.67

isolated from whole cells was found to be in the form of large circles. These findings, together with the buoyant density data, are consistent with the interpretation that the circles are of chloroplast origin. The following observations are also in agreement with this view. In the light microscope, the freshly isolated chloroplast fractions appeared to comprise only intact chloroplasts. In thin sections of the chloroplast fractions examined extensively in the electron microscope, only intact chloroplasts and broken membranes were seen. Mitochondria, bacteria, or other contaminating organisms were never found.

Open circular molecules with a mean contour length of about 44 μ were also found in SDS lysates and pronase-SDS digests of a spinach chloroplast fraction (Fig. 5).

It is known that the mass per unit length of DNA prepared by the protein monolayer technique is influenced by the salt concentration of both the hypophase and the spreading solution (Inman, 1967; Lang, 1970). Therefore, in order to make an estimate of the molecular weight of a molecule from its length, it is necessary to compare this length with that of a molecule whose molecular weight has been determined using independent techniques. This was done for the circular DNA molecules in chloroplast fractions by spreading circular lambda bacteriophage DNA molecules, using conditions identical to those used for chloroplast DNA. A comparison was then made between the mean contour lengths found for 10 bacteriophage DNA molecules from each of three different preparations (14.8 μ; SE = $\pm$0.05 μ; n = 30) and the mean lengths of the circular chloroplast molecules. A ratio of mean lengths of 1.00:2.96 was found for lambda circles and chloroplast circles, respectively. A mean value of 30 $\times$ 10^6 daltons has been reported from sedimentation and electron microscope studies for the molecular weight of lambda bacteriophage DNA (Freifelder, 1970). A molecular weight of 89 $\times$ 10^6 daltons is therefore indicated for the spinach circular chloroplast DNA molecule. (The technique which would have involved mixing lambda and chloroplast DNA, and comparing the lengths of the respective molecules from the same grid square [see for example, Lang, 1970] was not used as some triple length molecules of 44 μ were present in our lambda preparation.)

When a preparation of DNA from a chloroplast fraction of corn was examined in the electron microscope, circular molecules were also found

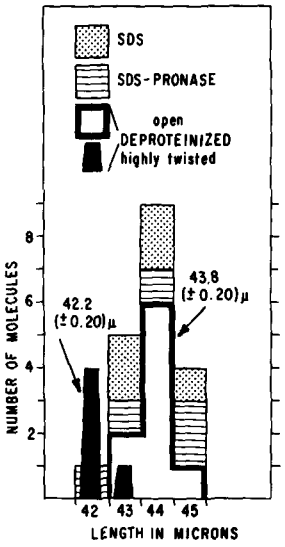

FIGURE 5 Frequency distribution of the lengths of circular DNA molecules from a chloroplast fraction of *S. oleracea*. The DNA was prepared directly for electron microscopy after the treatments indicated. The mean and standard error on the left are for the supercoiled molecules from the deproteinzed sample. The mean and standard error on the right are for the remaining molecules, which are all open and which represent five molecules from the SDS lysate, five from the SDS-pronase digest, and 10 from the deproteinized DNA preparation.

with a mean contour length of 42.4 μ (SE = 0.42 μ; n = 5).

DISCUSSION

The results presented are consistent with the interpretation that the observed circular DNA molecules of 44 μ originate in the spinach chloroplasts. It appears that at least 7.6% of the chloroplast DNA is in this form. It is not clear from our data whether the smaller linear pieces of DNA from the chloroplast fraction represent broken circles. It is also not clear whether the linear molecules found in the same preparation which had lengths greater than 45 μ represent nuclear DNA contamination or a second form of chloroplast DNA. Woodcock and Fernández-Morán (1968) examined DNA molecules from osmotically disrupted chloroplasts of spinach. They did not report the finding of circular molecules. The molecules which they measured ranged in length from 1 μ to 30 μ and had a modal length of 3–5 μ.

As was argued for the circular chloroplast DNA molecules of *E. gracilis* (Manning et al., 1971), it

seems unlikely from our data that the continuity of the circular molecules is dependent on non-DNA links. If such links existed, they must be stable to detergent, pronase, phenol, and chloroform–isoamyl alcohol, and they must be relatively short and/or have a diameter close to that of the DNA double helix.

Highly twisted circular molecules have been found in preparations of *E. gracilis* chloroplast DNA (Manning and Richards, unpublished observations) and in preparations of almost all circular DNA molecules from a variety of viruses (see review of Thomas and MacHattie, 1967), bacterial plasmids and sex factors (see review of Helinski and Clewell, 1971), and mitochondria (see reviews of Swift and Wolstenholme, 1969; Wolstenholme et al., 1971). Whenever examined, these molecules have been found to be covalently closed structures as defined by Vinograd and Lebowitz (1966); that is, phosphodiester bonds are found throughout the length of the two nucleotide strands of the molecule. Our present finding that spinach chloroplast DNA (and probably corn chloroplast DNA) includes circular molecules of approximately 44 μ, together with our previous finding of circular molecules of a comparable size in *E. gracilis* chloroplast DNA (Manning et al., 1971), has a clear evolutionary implication. It appears that a quantity of information has been preserved in chloroplast DNA throughout the evolution of organisms which have led from a common ancestor to the present day chloroplast-containing protozoans and monocotyledonous and dicotyledonous angiosperms. Qualitative differences in this information are indicated by differences in buoyant density, at least between *E. gracilis* (ρ = 1.685 g/cm^3) and spinach (ρ = 1.696 g/cm^3) chloroplast DNAs.

It is of interest in this regard that a relatively stable molecular size has also been found for circular mitochondrial DNA of metazoan animals (for references and review see Wolstenholme et al., 1971). The mitochondrial DNA from all these organisms examined, which range from nematode worms to man, is in the form of a circle with a species-specific contour length of 4.5–6.0 μ. Real differences in both mean contour lengths and in buoyant densities suggest that in this case both qualitative and quantitative differences have occurred during evolution (Wolstenholme and Dawid, 1968).

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