Molecular Size and Conformation of Chloroplast Deoxyribonucleic Acid from Pea Leaves*

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SUMMARY

The molecular size and conformation of chloroplast (ct-) DNA has been studied by renaturation kinetics and electron microscopy. Ct-DNA of buoyant density 1.698 ± 0.001 g per cm³ showed a Tₘ of 84° and a homogeneous melting pattern. Denatured and sheared ct-DNA renatured as a single kinetic class with no suggestion of repeating sequences. Ct-DNA is found to have a molecular weight of 95 × 10⁶ by renaturation rate, assuming a value of 106 × 10⁶ for Tₘ DNA. The lyed chloroplasts are shown to contain 37% of the total DNA in circular molecules of 37 to 42 µ. The remaining DNA is found to be in linear form ranging from 1 to 30 µ. Ct-DNA has been isolated by deproteinization and shown to contain 25% of the DNA in circular form. Supercoiled molecules constitute 30% of the circular molecules. Three percent of the circular DNA is found in circles of dimer lengths. ϕX174 RF II DNA and circularized phage λ DNA have been used as internal standards, and the molecular weight of ct-DNA is calculated to be 91 × 10⁶.

Chloroplast DNA from algae and higher plants has been shown to differ from nuclear DNA in buoyant density and in base composition. Further, ct-DNA, unlike n-DNA, has been shown to readily renature to its native density after denaturation (1). This property has been utilized to calculate the genome size of ct-DNA. Wells and Birnstiel (2) reported that ct-DNA of lettuce was kinetically heterogeneous consisting of “fast” and “slow” renaturing fractions. The fast fraction had a molecular weight of 95 × 10⁶ and constituted 25% of the total ct-DNA; the slow fraction had a molecular weight of 120 × 10⁶. Tewari and Wildman (3) carried out a detailed analysis of ct-DNA from tobacco leaves and showed it to renature as a single homogeneous species with a molecular weight of 114 × 10⁶. No evidence for a species with a molecular weight of 95 × 10⁶ using ϕX174 RF II DNA and λ DNA as standards. Manning et al. (4) have shown ct-DNA from Chlamydomonas to renature homogeneously with kinetic complexity of 194 × 10⁶.

Molecular size and conformation of ct-DNA have also been studied by electron microscopy. Woodcock and Fernández-Morán (5) reported two conformations of DNA in osmotically shocked spinach chloroplasts. “Linear” forms had threads of 20 to 50 A in diameter, and “mesh” forms had diameters of about 15 A. Ct-DNA molecules ranging in length from 40 to 150 µ were observed. However, studies on chloroplasts from envelope-cells of Acetabularia revealed threads of uniform diameter (6), but the maximum length of free lying threads of DNA was only 4 µ. In other studies, strand length of ct-DNA has been reported to range from 200 to 400 µ (7, 8). Molecular weight calculated from these length measurements of ct-DNA will correspond to approximately 90 × 10⁶ to 150 × 10⁶ in spinach chloroplast and 400 × 10⁶ to 800 × 10⁶ in Acetabularia. These values are in great variance to those found by renaturation kinetics (9).

We have now studied ct-DNA from pea leaves by electron microscopy of lysed chloroplasts and of deproteinized DNA. Our results conclusively show that DNA molecules in the chloroplast exist as circular molecules with a contour length of 39 µ. The molecular weight of circular ct-DNA is calculated to be 95 × 10⁶ using ϕX174 RF II DNA and λ DNA as standards. Manning et al. (10) were the first to report circular ct-DNA in E. gracilis. Our results show the occurrence of circular ct-DNA in higher plants and agrees with the molecular weight of 83 × 10⁶ reported for ct-DNA from Euglena gracilis (10). The molecular weight of ct-DNA has also been analyzed by renaturation kinetics and found to be 95 × 10⁶. Renaturation analysis has further shown that ct-DNA from pea leaves exists as a single homogeneous species with no evidence of inter- or intramolecular compositional heterogeneity.

MATERIALS AND METHODS

Isolation of Chloroplast DNA.—In a typical experiment, 1 kg of 12- to 15-day-old pea leaves was homogenized with 4 liters of buffered medium (Medium A) containing 0.3 M mannitol, 0.05 M Tris, pH 8.0, 3 mM EDTA, 0.1% bovine serum albumin, and 1 mM mercaptoethanol. The homogenates were filtered through four layers of cheese cloth and centrifuged for 10 min at 100 X g for 15 min. The supernatant was centrifuged at 100 X g for 15 min and the chloroplast pellet (containing broken nuclei and some mitochondria) was suspended in 80 ml of Medium A containing 10 mM Mg++· DNase I (50 µg per ml) was added and the suspension was incubated for an hour at 4°. At the end of the incubation, 240 ml of a medium (Medium B) containing 0.15 M NaCl and 0.1 M EDTA were added and the mixture was...

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The abbreviations used are: ct-DNA, chloroplast DNA; n-DNA, nuclear DNA; SSC, 0.15 M NaCl-0.015 M sodium citrate.

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centrifuged for 15 min at 1020 \times g. The pellet was washed twice by suspending it in 120 ml of Medium B. The pellet was finally suspended in 10 ml of Medium B, sodium deoxycholate was added to a final concentration of 2%, and the lysed chloroplasts were extracted with an equal volume of phenol buffered with 0.1 M Tris, pH 12.0. The aqueous phase was re-extracted with an equal volume of phenol. Two volumes of 95% alcohol were added to the aqueous phase, the mixture was kept in a freezer overnight, and the precipitate was collected by centrifugation and dissolved in 5 ml of Medium A. The solution was incubated for 2 hours at 37° with 50 g per ml of RNase and 50 units per ml of RNase T1, followed with 5 \mu g per ml of Pronase and a further incubation of 2 hours. At the end of incubation, two phenol extractions were carried out and the aqueous phase was dialyzed against a total volume of 10 liters of SSC with five changes in 24 hours.

Lysis of Chloroplasts for Electron Microscopy—The crude chloroplast fraction was obtained from 100 g of leaves as described above and suspended in 20 ml of Medium A. After DNase treatment, chloroplasts were washed twice with 3 volumes of 0.35 M sucrose 0.1 M EDTA, pH 8.0 (Medium C), and finally suspended in 4 ml of Medium C. An aliquot of 0.05 ml of this suspension was added to 0.05 ml of 4% sodium-sarcosyl, 20 mM EDTA, and 30 mM NaCl and allowed to stand for 30 min. To this mixture, 0.2 \mu g of \phi X RF II DNA was added for marker DNA when desired. The solution was diluted with 1.0 ml of 1 M ammonium acetate containing 0.05% formaldehyde and 0.1 ml of 1 mg per ml of cytochrome c. After centrifugation at 12,000 \times g for 30 min, the supernatant was spread on a hypophase of 0.3 M ammonium acetate containing 0.5% formaldehyde for 15 min, picked up on carbon-coated Formvar mounted on copper mesh (200) grids, and rotary shadowed with 80% Pt:20% Pd wire.

Isolation of DNA for Electron Microscopy—The DNase-treated and washed chloroplasts from 100 g of leaves were suspended in 4 ml of Medium C, and 1 ml of 10% sodium-sarcosyl containing 30 mM NaCl and 20 mM EDTA was added. After 30 min the mixture was gently extracted twice with chloroform-isooamyl alcohol (95:5). The supernatant was dialyzed against 0.15 M NaCl-0.1 M EDTA, pH 8.0, for 24 hours. An aliquot of 0.1 ml of ct-DNA was added to 1.0 ml of 1 M ammonium acetate (pH 5.5) and 0.1 ml of 1 mg per ml of cytochrome c. In the above spreading mixture, 0.2 \mu g of \phi X RF II DNA or 0.1 \mu g of circularized \lambda phage DNA, or both, was included. The solution was spread on 0.3 M ammonium acetate (pH 5.5) for 15 min and prepared as described under “Lysis of Chloroplasts for Electron Microscopy.”

Electron Microscopy—Preparations were examined in a Zeiss EM 9A with a 30-\mu objective aperture. Photographs were taken on 7 cm x 7 cm sheet film at a magnification of 8250 \times which was calibrated with a diffraction grating (Ludl 25,200 lines per inch). Tracings were made from negatives projected at a final magnification of 250,000 \times with a Besseler projector, and these tracings were measured.

RESULTS

Characterization of Chloroplast DNA—The isolated ct-DNA was analyzed in an analytical CsCl centrifugation to determine the purity and the homogeneity of the sample (Fig. 1). The profile of DNA obtained from the 12,000 \times g pellet (after removing the nuclear fraction by centrifuging at 100 \times g for 10 min) without DNase treatment is also presented in Fig. 1. There are two major bands, one at a density of 1.694 g per cm³ and another at a density of 1.697 g per cm³. In addition, a minor band at a density of 1.705 g per cm³ is also present (Fig. 1A). The DNase-treated chloroplast fraction showed only single band at a density of 1.697 g per cm³ (Fig. 1B), identifying this component as ct-DNA. Purified n-DNA was found to band at a density of 1.694 g per cm³. The DNA component banding at a density of 1.706 g per cm³ has been similarly shown to be obtained from DNase-treated mitochondria (Fig. 1C). Purified ct-DNA was denatured in the presence of 0.1 N NaOH, neutralized, and added again in CsCl (Fig. 2). The density of denatured ct-DNA was found to be 1.711 g per cm³ (Fig. 2B). Incubation of denatured ct-DNA at 60° for 3 hours resulted in 90% renaturation of the molecule as evidenced by its banding at a density of 1.690 g per cm³ (Fig. 2B). n-DNA on denaturation increased to a density of 1.710 g per cm³, but incubation for 3 hours at 60° decreases its buoyant density by only 0.003 g per cm³. Thus, the isolated DNA shows characteristic properties of ct-DNA's (9).

Melting Pattern of Chloroplast DNA—Thermal denaturation of ct-DNA was performed in SSC. The melting profile of ct-DNA shown in Fig. 3. The ct-DNA showed a sharp transition with a Tm of 84° ± 0.5°, maximal hyperchromicity of 0.36 (\theta_{max} = (A_{max}/A_{m}) - 1) and a dispersion (\sigma 2%) of 6.6° (A_{n, x} =

![Fig. 1. Photoelectric scans of DNA centrifuged in CsCl of density 1.710 g per cm³ for 18 hours at 28° using a Beckman model E analytical ultracentrifufige equipped with photoelectric scanner. Micrococcus lysodeikticus DNA of density 1.731 g per cm³ was used as a marker. The buoyant density was calculated by the method of Schildkraut et al. (11). A, DNA isolated from 12,000 \times g pellet containing chloroplasts and mitochondria. The picture shows a significant amount of nuclear contamination (DNA of density 1.694 g per cm³). Although the difference in densities between nuclear and chloroplast DNA is only 0.003 g per cm³, the banding pattern clearly shows two components. B, DNA from chloroplast fraction after DNase treatment (see “Materials and Methods”). C, DNA from mitochondrial fraction after DNase treatment.

![Fig. 2. Same as Fig. 1. A, neutral ct-DNA, B, alkali-denatured ct-DNA; C, renatured ct-DNA.](http://www.jbc.org/)

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optical density at 260 nm of denatured DNA; $A_{260}$ = optical density at 260 nm of native DNA at 20°C; $\sigma$ = temperature range in which the increase in optical density takes place. There was no evidence of any other component in the melting pattern. T4 DNA was similarly treated and was found to have a $T_m$ of 84°C, $h_{\text{max}}$ of 0.34, and $\sigma 2^{1/2}$ of 6.6°C. The melting behavior of ct-DNA from Chlamydomonas (4) is in complete contrast to the results obtained in this investigation. The ct-DNA from Chlamydomonas (4) was found to melt over a broad range of temperatures, and within the melting temperature range the cooperative transition of the double helix of the ct-DNA appeared to be heterogeneous. The experiments presented in Fig. 3 were carried out with a temperature rise of 1°C per min. It occurred to us that this rate of rise in temperature might be too fast to observe inter- or intramolecular microheterogeneity, or both, during the melting of DNA. Therefore, the melting pattern of ct-DNA was studied using a temperature rise of 1°C per 10 min. The results of such a melting are presented in a quadratic derivative form in Fig. 4. There appears to be no suggestion of heterogeneity, and the melting patterns of T4 DNA and ct-DNA are very similar. In an effort to investigate the possibility of heterogeneity, the $T_m$ of ct-DNA was determined in SSC/100. There was a drop of 20°C in $T_m$ to 65°C but the molecules still melted sharply with a $\sigma 2^{1/2}$ of 7° and $h_{\text{max}}$ of 0.32. On the other hand, T4 DNA which showed a $\sigma 2^{1/2}$ of 7° in SSC showed a $\sigma 2^{1/2}$ of 10° in SSC/100.

The ct-DNA was heated to 100°C at the rate of 1°C per min and kept at 100°C for 15 min. The temperature of the cuvette in the spectrophotometer was equilibrated to 60°C. Optical density was continuously recorded. The data of this experiment are presented in Fig. 3. Denatured DNA incubated at 60°C showed hypochromicity and in 200 min had regained the optical density corresponding to native DNA. That the hypochromicity observed resulted from actual formation of native base pairs and not stacking of bases is seen in the data where renatured DNA is again melted. A sharp melting of DNA with a $T_m$ of 84.5°C is observed instead of the melting over a broad range of temperatures characteristic of unpaired structures. It is to be noted that even renatured DNA molecules did not show a heterogeneous melting pattern. The sample of DNA was renatured and melted again. The $T_m$ was still 85°C with a $\sigma 2^{1/2}$ of 6° and an $h_{\text{max}}$ of 0.31.

Renaturation Rate and Size of ct-DNA. It has been reported that heat-denatured ct-DNA showed two component kinetics, whereas alkali-denatured DNA gave only one kinetic class (3). We considered the possibility that thermal denaturation is not sufficient to completely eliminate some nucleation sites which would favor faster reassociation during the early part of the renaturation process. All of our renaturation studies have, therefore, been performed on alkali-denatured DNA samples. The ct-DNA was fragmented by passing through a number 27 gauge needle and $k_2$ determined in band centrifugation (12). The alkali-denatured and neutralized samples were added to cuvettes which had been equilibrated to 60°C. The maximum time required for temperature equilibration was 2 min. The kinetics of renaturation using three different concentrations of ct-DNA is plotted in Fig. 5. This plot showed a second order rate. It is seen that no rapidly renaturing component is present. Eighty per cent of the total DNA was found to renature as a single kinetic class. From the slope of the curve in Fig. 5, the renaturation rate constant $k_2$ was calculated according to Wetmur and Davidson (13). Using this method the renaturation of T4 DNA was studied and its $k_2$ was calculated. The $k_2$ values for five different preparations of ct-DNA and T4 DNA, obtained using fragments of 17 to 18S (segmented), are given in Table I. The rate constants for T4 ranged from 11.4 moles⁻¹ s⁻¹ to 12.4 moles⁻¹ s⁻¹, whereas $k_2$ for ct-DNA ranged from 12.9 moles⁻¹ s⁻¹ to 13.8 moles⁻¹ s⁻¹. The molecular weight of ct-DNA was calculated using an analytically determined value for the molecular weight of T4 DNA as a standard. The molecular weight of T4 has been reported to be of the order of 130 x 10⁶ (14). The molecular weight of T4 DNA has been redetermined (15, 16) and found to be about 106 x 10⁶. We have calculated molecular weights of ct-DNA using both values of molecular weights (Table I). The mean molecular weight of ct DNA was found to be 116 x 10⁶ using the T4 value of 130 x 10⁶ and 95 x 10⁶ using a molecular weight of 106 x 10⁶ for T4 DNA. The value of 116 x 10⁶ is in agreement with the molecular weight previously reported for
TABLE II
Renaturation rates at different concentrations of ct-DNA

The renaturation rates were studied at different concentrations of DNA. All the other details are described in the legend of Table I.

<table>
<thead>
<tr>
<th>Concentration of ct-DNA</th>
<th>Rate constant, $k_2$</th>
<th>Mol wt of ct-DNA ($\times 10^{-8}$) calculated from T4 DNA assuming a mol wt of</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu g/ml$</td>
<td></td>
<td>$130 \times 10^6$</td>
</tr>
<tr>
<td>12</td>
<td>12.4</td>
<td>125.8</td>
</tr>
<tr>
<td>18</td>
<td>13.5</td>
<td>115.5</td>
</tr>
<tr>
<td>24</td>
<td>13.7</td>
<td>113.8</td>
</tr>
<tr>
<td>32</td>
<td>12.2</td>
<td>127.8</td>
</tr>
<tr>
<td>38</td>
<td>14.1</td>
<td>110.6</td>
</tr>
<tr>
<td>44</td>
<td>13.3</td>
<td>117.3</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>118.4</td>
</tr>
</tbody>
</table>

TABLE III
Renaturation rate constants of chloroplast DNA at different molecular weights

The renaturation rate constant of ct-DNA using different size fragments. All the other details are described in the legend of Table I.

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Molecular weight calculated by Studier's method (17)</th>
<th>Rate constant, $k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 11 (Obtained by sonicating ct-DNA)</td>
<td>$X 10^{-8}$</td>
<td>0.9</td>
</tr>
<tr>
<td>2. 15 (Obtained by passing DNA through No. 27 gauge needle)</td>
<td></td>
<td>2.8</td>
</tr>
<tr>
<td>3. 28</td>
<td></td>
<td>16.6</td>
</tr>
<tr>
<td>4. 30</td>
<td></td>
<td>20.9</td>
</tr>
<tr>
<td>5. 33 (Preparations 3, 4, and 5 were isolated DNA without fragmentation)</td>
<td></td>
<td>28.2</td>
</tr>
</tbody>
</table>

square root relationship between the second order rate constant and the molecular weight (Table III).

Electron Microscopy of Chloroplast DNA—The chloroplast preparations were lysed and DNA spread as described under "Materials and Methods." The DNA was examined in the electron microscope and was found to contain circular forms. The frequency distribution of lengths of DNA molecules is presented in Fig. 6. Thirty-seven per cent of the total DNA measured was found in the form of circles with contour lengths of 38 to 42 $\mu$. The remaining DNA was in the form of linear molecules that ranged in size from 1 to 30 $\mu$. No linear DNA molecule larger than 31 $\mu$ was found. These circular molecules could only be from chloroplasts since it has already been shown that under our experimental conditions pure ct-DNA is isolated. The nuclear fraction was not found to contain any circular molecules. The circular molecules found in the lysate cannot be of mitochondrial origin because mitochondrial DNA from pea leaves has been found to exist as circular molecules of 30 $\mu$ and a molecular weight of $70 \times 10^6$ (18). No circular molecule of this size was found in the lysed chloroplast fraction.

The ct-DNA from the DNase-treated chloroplast fraction was isolated as described under "Materials and Methods" and studied by electron microscopy. Circular molecules in such a prepara-
FIG. 6. Frequency distribution of the DNA released from lysed chloroplasts. The values expressed are the percentages of the total DNA lengths measured. All of the molecules in one grid square were measured. Thirty-seven per cent of the DNA is found to be circular. ■, circles; □, linear.

FIG. 7. Electron micrograph of a typical DNA molecule isolated from chloroplasts. The molecule is 38.1 μ. Bar length equals 1 μ. The arrows denote marker φX174 RF II DNA, which was isolated by the method of Rush and Warner (19).
Fig. 8. Electron micrograph of a supercoiled ct-DNA molecule. Bar length equals 1 μ.

The ct-DNA obtained after deproteinization was also spread along with φX174 RF II DNA. The distribution of the lengths of circular ct-DNA molecules obtained under these conditions is presented in Fig. 11B. Fourteen ct-DNA molecules ranged in length from 13.0 to 15.1 μ (S.D. ± 0.09 μ). The ct-DNA was also co-spread with circularized λ DNA. Nineteen molecules were found to range from 13.0 to 15.2 μ (S.D. ± 0.7 μ), and fourteen ct-DNA molecules ranged from 37.6 to 39.6 μ (S.D. ± 0.6 μ) (Fig. 11C).

It is clear from length measurements that both ct-DNA and φX174 RF II DNA molecules give different contour lengths when spread under different conditions. However, calculation of the ratio of lengths of ct-DNA and φX174 RF II DNA from lysed chloroplast preparations gave a value of 26.5 and a value of 26.8 was obtained from isolated DNA. The difference in the values is only of the order of 1.1%.

The molecular weight of ct-DNA has been calculated from these ratios by taking φX174 RF II DNA as 3.4 × 10⁶ (28). In the lysed chloroplast preparations, ct-DNA is found to be 90.0 × 10⁶ and 91.2 × 10⁶ in deproteinized DNA. It may be pointed out that the value obtained using isolated DNA is probably more reliable because it was easier to measure molecules in these preparations. In lysed preparations, it was harder to measure lengths accurately because these preparations often resulted in tangled molecules. However, the S.D. ± 1.1 μ in measured length showed reliability and reproducibility in measurements.

DISCUSSION

In sarcosyl-lysed chloroplasts, 37% of the DNA has been found to be in the form of circular molecules of 39 μ. These circular molecules could not be due to non-DNA links since such molecules amounted to 25% of the DNA isolated after detergent, Pronase, and chloroform-isomyl alcohol extraction. The percentage of total DNA obtained as circular molecules should be taken as a minimal value. Our efforts are currently directed towards a much higher yield by utilizing centrifugation of lysed chloroplasts in CsCl gradients containing ethidium bromide. None of the linear molecules observed in chloroplast preparations were found to be larger than the length of the circular molecules, and therefore, we conclude that all of the ct-DNA is present in the chloroplasts as circular molecules of monomer length of 37 to 39 μ. The contour length of circular ct-DNA was not found to be significantly affected by the techniques of isolation as reported by Manning et al. (10). For example, the contour length of ct-DNA was only 36 to 42 μ whether measured in lysed chloroplasts, isolated DNA, or DNA spread after digestion with Pronase. Lysis of pea chloroplast with 2% sodium dodecyl sulfate resulted in the breakdown of all circular molecules, whereas in the case of E. gracilis (10) a similar treatment gave 34% of the DNA in the form of circles. It appears lysis conditions will have to vary in different organisms to demonstrate the circularity of organelle DNA's. The ct-DNA is firmly bound to the membranes of thylakoids (29), and this binding may involve specific sequences of the chloroplast genome. As these molecules are extremely large, the chemical agents to bring about detachment of DNA from the membrane would have to be as gentle as possible to maintain the stability of the circular conformation during isolation. We have come across this problem particularly with isolation of mt-DNA from pea leaves (30), where we had to work out ideal conditions of detergent, Pronase treatment, and solvent extraction for obtaining intact mt-DNA.

Three per cent of the isolated DNA has been found to exist in molecules of dimer lengths. It is difficult to decide between catenated and replicative intermediates in the molecules composed of two circles. However, circular dimers can be unambiguously identified. Fig. 9 shows such a dimer. The contour length of this molecule is found to be 86 μ. This will correspond to a molecular weight of 180 × 10⁶ (see below) which makes it the largest circular molecule yet isolated. The possibility that such large molecules can be isolated intact provides us means to study replicative mechanisms in larger molecules.

The necessity of including an internal standard in molecular weight determinations from contour length measurements of DNA molecules has been discussed. In such experiments, variations arising out of monolayer spreading conditions caused out, and a good agreement can be obtained in the molecular weight values reported by different investigators. For example,
the ratio of contour lengths between circularized λ DNA and φX174 RF II DNA is found to be 9.1 from the data of Fig. 11D. The similar length ratios measured by Davis and Davidson (22) are 9.0 ± 0.1. Reliable data for molecular weights can be obtained by electron microscopy using the well characterized value for φX174 RF II DNA. From the length ratio of 9.1, the molecular weight of λ DNA is calculated to be $31 \times 10^6$ assuming a value of $3.4 \times 10^6$ for φX174 RF II DNA. Strider and Warner\(^2\) have redetermined the molecular weight of φX174 RF II DNA utilizing the CsCl density gradient equilibrium centrifugation technique of Schmid and Hearst (31) and have shown that the correct value for this DNA is probably $3.2 \times 10^6$. Utilizing this value and a ratio of 9.1, the molecular weight of λ DNA is calculated to be $29.2 \times 10^6$; the value is in excellent agreement with the molecular weight of $29.6 \times 10^6$ obtained by sedimentation analysis (22). Therefore, we have used length ratios between ct-DNA and internal standards to determine the molecular weight of ct-DNA. The length ratio between ct-DNA and λ DNA has been found to be 2.85 (Fig. 7C) which yields a molecular weight of $85.5 \times 10^6$ for ct-DNA. The relative length between ct-DNA and φX174 RF II DNA has been found to be 28.8 (Fig. 7), which yields a molecular weight of $85.7 \times 10^6$ and $91.1 \times 10^6$ assuming corresponding values of $3.4 \times 10^6$ and $3.2 \times 10^6$ for φX174 RF II DNA. The mean value for the molecular weight of ct-DNA by electron microscopy is found to be $87.4 \times 10^6$.

The amount of ct-DNA in higher organisms has been reported to range from $0.5 \times 10^{-14}$ g to $1.5 \times 10^{-14}$ g per chloroplast (9). Using the molecular weight of $90 \times 10^6$ and the minimum value of $0.5 \times 10^{-14}$ g, there might be about 30 molecules of DNA per chloroplast. This high frequency of DNA molecules is of con-

\(^2\)W. S. Strider and R. C. Warner, personal communication.
FIG. 10. Electron micrograph of a molecule showing a supercoiled and an open circle. The length of the open circle is 37 μ and the length of the supercoiled circle is 38.2 μ. The arrows designate the point of crossover between the molecules. It is difficult to decide whether the molecule is a true catenane or a replicative intermediate. The possibility of this molecule being two tangled circles is unlikely because there were only four molecules per grid square in this preparation. The other large piece of DNA at the upper right-hand corner is a part of a λ DNA molecule.
agree with that obtained by contour lengths. There is excellent
and molecular size but still contain different genetic information.
inter- and intramolecular heterogeneity. It is possible that
c&DNA
thus no detectable intermolecular base compositional heterogene-
ity is observed. Since there is no heterogeneity in the absorb-
ing pattern of DNA in CsCl centrifugation.
The melting characteristics of ct-DNA have failed to show any
and found it to contain circles (32) of the same size as those
present in pea leaves.
agreement between the molecular weight of $91 \times 10^6$ obtained by
electron microscopy and $94 \times 10^6$ obtained by kinetic considera-
tions. Therefore, ct-DNA molecules having significantly dif-
ferent base sequences are unlikely. It is also apparent from renatu-
ration rates that no repeating sequences are present in the
ct-DNA.

Fig. 11. Length distribution of DNA's. A, chloroplast lysate
co-spread with $\phi X 174$ RF II DNA. Ct-DNA is found to have a
contour length of $39.2 \mu$ (S.D. $\pm 1.1 \mu$) and $\phi X$ of $1.48 \mu$ (S.D. $\pm 0.05$
$\mu$). The ratio between the lengths of two DNA's is 26.5. B, isolated
ct-DNA co-spread with $\phi X 174$ RF II DNA. Ct-DNA is
found to be $37.1 \mu$ (S.D. $\pm 0.7 \mu$) compared to $1.39 \mu$ (S.D. $\pm 0.07$
$\mu$) for $\phi X 174$ RF II DNA. The ratio between the lengths of two
DNA's is 26.8. C, isolated ct-DNA co-spread with circularized
$\lambda$ DNA. Ct-DNA is found to be $38.6 \mu$ (S.D. $\pm 0.6 \mu$), and $\lambda$ DNA is
found to be $13.6 \mu$ (S.D. $\pm 0.2 \mu$). The ratio between the
lengths of two DNA's is 2.85. D, $\phi X 174$ RF II DNA co-spread with circularized
$\lambda$ DNA is found to have contour length of $14.4 \mu$ (S.D. $\pm 0.9 \mu$) and
$\phi X 174$ RF II DNA is $1.53 \mu$ (S.D. $\pm 0.13 \mu$). The ratio between the
lengths of two DNA molecules is 9.11.

considerable interest. The question is whether this represents a
form of redundancy where all of the ct-DNA molecules have the
same base sequences or there are different base sequences in
these molecules.

The melting characteristics of ct-DNA have failed to show any
heterogeneity in the DNA molecules. The hyperchromic be-
havior of DNA over a wide range of temperatures reflects both
inter- and intramolecular heterogeneity. However, heterogene-
ity involved between different molecules results in a broad banding
pattern of DNA in CsCl centrifugation. From Fig. 1 it is seen that the experimental conditions and sensitivity of the
method are such that a difference in the density of 0.003 g per
cm$^3$ between DNA molecules is readily apparent. The banding
pattern of pure ct-DNA shows a sharp peak (Figs. 1 and 2), and
thus no detectable intermolecular base compositional heterogene-
ity is observed. Since there is no heterogeneity in the absorb-
ance-temperature profile of ct-DNA, there is no indication of inter-
and intramolecular heterogeneity. It is possible that
ct-DNA molecules may be of the same over-all base composition
and molecular size but still contain different genetic information.
In such a case, the molecular weight calculated from $k_2$ will not
agree with that obtained by contour lengths. There is excellent

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