Kinetic Complexity of Chloroplastal Deoxyribonucleic Acid and Mitochondrial Deoxyribonucleic Acid from Higher Plants

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1. Chloroplasts and mitochondria were isolated by aqueous and non-aqueous cell-fractionation techniques. In a variety of higher plants the mitochondrial DNA bands in a caesium chloride gradient at 1.706 g.cm^{-3} , whereas chloroplastal DNA has a buoyant density of 1.697 g.cm^{-3} . 2. In total cellular DNA of moderate molecular weight, the chloroplastal DNA is found within the Gaussian distribution of the nuclear DNA and is not resolved as a satellite. 3. Both chloroplastal DNA and mitochondrial DNA from lettuce renature rapidly. 4. The kinetic complexity of mitochondrial DNA is $> 10^8$ daltons. 5. Chloroplastal DNA is made up from fast and slow renaturing sequences with kinetic complexities of 3×10^6 and 1.2×10^8 daltons respectively. 6. From the discrepancy between analytical and kinetic complexity it is concluded that chloroplastal DNA is extensively reiterated.

It is well established that chloroplasts and mitochondria contain DNA (Iwamura, 1966; cf. Borst, Kroon & Ruttenberg, 1967). In buoyant-density gradients, DNA fractions from these organelles can often be detected as satellites to the main nuclear-DNA band, and in *Euglena* a satellite-DNA band has been unequivocally assigned to each of these organelles (Brawerman & Eisenstadt, 1964; Edelman, Schiff & Epstein, 1965; Ray & Hanawalt, 1965; Edelman, Epstein & Schiff, 1966).

In higher plants a satellite-DNA band with a buoyant density in the range $1.703\text{--}1.707 \text{ g.cm}^{-3}$ has been attributed by some workers to chloroplasts (Chun, Vaughan & Rich, 1963; Shipp, Kieras & Haselkorn, 1965; Green & Gordon, 1967), whereas other workers have attributed this DNA satellite band to plant mitochondria (Suyama & Bonner, 1966; Wells & Birnstiel, 1967; Wolstenholme & Gross, 1968).

Lyttleton & Petersen (1964), working on tobacco, suggested that chloroplastal DNA might be very similar to nuclear DNA in buoyant density. This contention was substantiated by the observations by Suyama & Bonner (1966), who recorded, for DNA from *Brassica* chloroplastal fractions, a buoyant density of 1.695 g.cm^{-3} , close to that of nuclear DNA (1.692 g.cm^{-3}) and distinct from that of mitochondrial DNA (1.706 g.cm^{-3}). We have reported that a similar situation obtains for the mitochondrial-DNA, chloroplastal-DNA and

nuclear-DNA fractions of spinach, lettuce, broad bean and sweet pea (Wells & Birnstiel, 1967). In all higher plants we have examined, the mitochondrial DNA forms a satellite band well separated from the nuclear-DNA band in a caesium chloride density gradient. The chloroplastal DNA, however, bands in such close proximity to the nuclear DNA that, in unfractionated total cellular DNA of moderate molecular weight ($1 \times 10^6\text{--}5 \times 10^6$ daltons), chloroplastal DNA is found within the band of the more abundant nuclear DNA, and so is not detected as a satellite band. The reports of Whitfield & Spencer (1968) and Wolstenholme & Gross (1968) are in agreement with our findings. We have now further characterized the chloroplastal DNA and mitochondrial DNA fractions by studying the rate at which these two DNA components renature. Our work is based on the elegant experiments and theoretical elaborations of Britten & Kohne (1967), Wetmur & Davidson (1968) and Thrower & Peacocke (1968) who have studied the kinetics of DNA renaturation in detail and have demonstrated that the rate of renaturation of DNA is related to its base-sequence complexity.

METHODS

Non-aqueous preparations of chloroplasts. The methods used were essentially those of Biggin & Park (1961) and Stocking (1959). Leaves were carefully washed and the midribs were removed. The leaves were then freeze-dried with a 10-105 Unitrap (The VirTis Co. Inc., New York, N.Y., U.S.A.) and when dry were ground in a china vibratory ball mill (Griffin and George Ltd., Wembley, Middx.) to

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give a fine powder, which was suspended in light petroleum (b.p. 60–80°) and passed through a sieve with a 37 μ m. mesh. The suspension was then adjusted to the appropriate specific gravity by the addition of carbon tetrachloride and centrifuged (Biggin & Park, 1961). Under these conditions the chloroplast fraction pelleted in the solvent mixture of specific-gravity range 1.30–1.35 (Biggin & Park, 1961). The fraction pelleting in the solvent of specific-gravity range 1.00–1.25 was also investigated.

Aqueous preparations of mitochondria and chloroplasts. Mitochondrial fractions were prepared by the procedure of Suyama & Bonner (1966). To prepare chloroplasts the leaves were carefully washed and the midribs were removed. All further steps were carried out at 0–4°. The material was homogenized in a Waring Blendor with 5l. of medium/kg. fresh wt. of leaves. The homogenization medium consisted of (final concentrations) 0.3 M-mannitol, 0.05 M-tris-HCl buffer, 3 mM-EDTA, 0.05% (w/v) cysteine and 0.1% (w/v) bovine serum albumin, pH adjusted to 7.2 (Suyama & Bonner, 1966). The homogenate was filtered through four layers of cheesecloth and then centrifuged at 400 rev./min. for 10 min. in a Griffin-Christ Universal Junior III preparative centrifuge (Griffin and George Ltd.). The pellet consisted largely of cell debris that had not been trapped by the cheesecloth. The supernatant was carefully removed and centrifuged at 2000 rev./min. for 15 min. in the Griffin-Christ centrifuge. The resulting pellet, designated the 'crude chloroplast fraction', was resuspended in the homogenization medium (3 $\times$ volume of pellets) and made 8 mM with respect to $MgCl_2$. Deoxyribonuclease (50 μ g./ml.) was added and the suspension kept in ice for 30 min. Then 3 vol. of saline-EDTA (0.15 M-NaCl–0.1 M-EDTA, pH 8.0) was added to the suspension and the chloroplasts were sedimented by centrifugation at 2000 rev./min. for 15 min. in a Servall HB 4 rotor.

The chloroplasts were washed in excess of saline-EDTA (as above) and sedimented, and the pellet was resuspended in homogenization medium (1 ml./100 g. original fresh wt. of leaves). This suspension was designated the 'chloroplast fraction'.

Aqueous preparations of nuclei. Leaves were homogenized as for the chloroplast preparation in a modified homogenization medium that contained 3 mM- $CaCl_2$ and was free of EDTA. The homogenate was centrifuged at 2000 rev./min. for 15 min. in the Griffin-Christ centrifuge. The resulting pellet contained a mixture of nuclei, chloroplasts and cell debris. The nuclei were purified by pelleting through a 2 M-sucrose–3 mM- $CaCl_2$ medium at 25 000 rev./min. for 60 min. in a Spinco L2 ultracentrifuge SW25.1 rotor (Chauveau, Moulé & Rouiller, 1956).

Preparation of DNA. The chloroplastal, mitochondrial or nuclear suspensions were made 2% (w/v) with respect to sodium dodecyl sulphate and 0.2 M with respect to tris, pH 8.5. The lysate was kept at room temperature (15°) for 10 min. Then 1.5 vol. of chloroform–3-methylbutan-1-ol (24:1, v/v) was added and the mixture shaken for 10 min. A volume of water-saturated phenol equivalent to that of the lysate was added and the whole mixture agitated for a further 10 min. The mixture was then centrifuged at 8000 rev./min. for 10 min. in a Servall preparative centrifuge (HB 4 rotor) and the nucleic acids were precipitated from the aqueous layer by the addition of 2 vol. of ethanol. After storage at –20° for 24 hr. the precipitate was recovered by centrifugation and dissolved in a small volume of

0.1 $\times$ SSC* (1 ml./kg. original fresh wt.), which was made up to 1 $\times$ SSC when the precipitate had fully dissolved. The DNA was purified by the addition of 50 μ g. of deoxyribonuclease-free ribonuclease/ml. (Marmur, 1961) and incubated for 1 hr. at 37°. Then 400 μ g. of Pronase/ml. was added and the mixture was incubated for a further 3 hr. at the same temperature.

The DNA was then pelleted in a saturated NaCl solution in a Spinco 40 rotor at 38 000 rev./min. for 18 hr. at 15°. The pellet was resuspended in 4 ml. of 0.1 $\times$ SSC and analysed by isopycnic CsCl-density-gradient centrifugation in a Spinco model E ultracentrifuge. To obtain DNA free from contaminating u.v.-absorbing material, 5.1 g. of CsCl was added to the DNA solution, which was then centrifuged at 42 000 rev./min. for 48 hr. at 25° (Flamm, Bond & Burr, 1966) in an MSE 50 centrifuge (2410 angle rotor) (Measuring and Scientific Equipment Ltd., London S.W.1). The centrifuge tube was pierced and fractions (1 g.) were collected. The position of the DNA band on the gradient was determined by measuring the E_{260} of the fractions. The fractions containing DNA were pooled and 3 vol. of 0.1 $\times$ SSC was added. The DNA was quantitatively pelleted by centrifugation at 50 000 rev./min. for 18 hr. at 15° in a Spinco L-2 ultracentrifuge (50 rotor). A 1 kg. (fresh wt.) sample of leaves gave values of 150–250 μ g. for chloroplastal DNA and 30–50 μ g. for mitochondrial DNA.

Denaturation and renaturation of DNA. The increase in E_{260} when the DNA was heated was followed in a Beckman DB spectrophotometer. For the renaturation studies native DNA was treated ultrasonically with a Dawe Soniprobe 1130A (Dawe Instruments Ltd., London W.3). The DNA was denatured by treatment at 100° in 0.1 $\times$ SSC for 5 min. and then made up to 1 $\times$ SSC. The molecular weight of the single-stranded DNA was estimated by band sedimentation in alkaline saline (Studier, 1965). The progress of renaturation was followed by observing the decrease in E_{260} at a temperature 25° below the 'melting' temperature, T_m , of the DNA. Both the half-life, $t_{1/2}$, i.e. the time taken to recover half the hypochromicity (Britten & Kohne, 1967), and the second-order renaturation constant, k_2 (Wetmur & Davidson, 1968), were calculated from the hyperchromicity-recovery curves.

RESULTS

Purity of cell fractions. Light-microscopic examination of the chloroplastal or mitochondrial fractions did not show any contaminating nuclei or

* Abbreviation: SSC, 0.15 M-NaCl–0.015 M-sodium citrate, pH 7.2.

EXPLANATION OF PLATE I

Electron micrograph of chloroplastal fractions prepared from lettuce leaves. The chloroplastal fractions were fixed in ice-cold 6.25% glutaraldehyde for 2 hr. and were further fixed with 2% osmium tetroxide in veronal-acetate buffer, pH 7.4, for 1.2 hr. at 0–4°. The pellets were dehydrated in ethanol series containing uranyl acetate (Mollenhauer, 1964) and embedded in Epon-Araldite mixture (Shankar Narayan, Muramatsu, Smetana & Busch, 1966). Post-staining of sections was done with both uranyl acetate and lead citrate. The measure line is 1 μ m.

nucleoli. Electron-microscopic examination of chloroplastal fractions (Plate 1) showed them to be essentially free of contaminating nuclei and mito-

chondria. At least 50% of the plastids possessed an intact outer membrane. The mitochondrial fractions showed a considerable degree of contamination by chloroplast fragments, but the DNA of these fragments is presumably lost during the deoxyribonuclease treatment.

Isopycnic centrifugation of DNA from non-aqueous cell fractions. DNA from the unfractionated spinach leaves banded in caesium chloride gradients at a buoyant density of 1.694 g.cm.⁻³ with a satellite band at 1.706 g.cm.⁻³ (Fig. 1a). DNA from the chloroplast fraction yielded a single component of buoyant density 1.697 g.cm.⁻³, which was distinct from both the nuclear DNA (1.694 g.cm.⁻³) and satellite DNA (1.706 g.cm.⁻³) (Fig. 1b). DNA prepared from the fraction of specific gravity 1.00–1.25, which is low in chloroplast content, yielded a band in caesium chloride gradients with a buoyant density in the range of the satellite DNA (1.706 g.cm.⁻³) (Fig. 1c). Similar results were obtained with leaves from beet, sweet pea and lettuce. A small but consistent difference in buoyant density between nuclear DNA and chloroplastal DNA was found for all preparations (Table 1).

Isopycnic centrifugation of DNA from aqueous cell fractions. DNA prepared from lettuce mitochondrial or chloroplastal fractions that had not been treated with deoxyribonuclease was heavily contaminated with nuclear DNA (Figs. 2a and 2b). Under these conditions the chloroplastal satellite DNA could only be detected when the DNA was of high molecular weight (> 20 × 10⁶ daltons). After treatment of the fraction with deoxyribonuclease the mitochondrial DNA or chloroplastal DNA formed unimodal bands in caesium chloride gradients (Figs. 2d and 2e) at buoyant densities of 1.697 and 1.706 g.cm.⁻³ respectively. Similar values were obtained from bean leaves (Table 2). The DNA removed by the deoxyribonuclease treatment was shown to be coincident in buoyant density with that obtained from nuclei (Fig. 2c). The buoyant-density values for the DNA of chloroplastal fractions were identical with those obtained from the non-aqueous preparations. The buoyant-density value for the mitochondrial fraction agreed well

Fig. 1. Microdensitometer tracing of the photographic record obtained after analytical CsCl-density-gradient centrifugation of DNA from non-aqueously prepared spinach organelle fractions. Marker *Micrococcus lysodeikticus* DNA is at 1.731 g.cm.⁻³. The DNA samples were adjusted to a density of 1.710 g.cm.⁻³ by the addition of caesium chloride and centrifuged at 44000 rev./min. at 25° for 20 hr. (a) Whole-cell DNA shows the main nuclear band at 1.694 g.cm.⁻³ and a small satellite band at 1.706 g.cm.⁻³. (b) DNA from the chloroplast fraction (sp.gr. 1.30–1.35) gives a unimodal band at 1.697 g.cm.⁻³ that is distinct from both the nuclear and the satellite bands of the whole-cell DNA. (c) DNA from the fraction of sp.gr. 1.00–1.25 gives a broad band with a peak at about 1.706 g.cm.⁻³; as this fraction is low in chloroplast content it suggests that the 1.706 g.cm.⁻³ satellite is not of chloroplastal origin.

Table 1. Buoyant density of DNA from non-aqueously prepared plant fractions in a caesium chloride gradient

	ρ , Density of the organic phase.		
	Buoyant density (g.cm. ⁻³)		
	Nuclear DNA ($\rho \geq 1.35$ g.cm. ⁻³)	Chloroplastal DNA ($\rho = 1.30$ – 1.35 g.cm. ⁻³) ($\rho = 1.00$ – 1.25 g.cm. ⁻³)	
Sweet pea	1.695	1.697	—
Bean	1.695	1.697	1.705
Spinach	1.694	1.697	1.706
Lettuce	1.694	1.697	1.706

Fig. 2. Microdensitometer tracing of the photographic record obtained after isopycnic CsCl -density-gradient centrifugation of DNA from aqueously prepared lettuce fractions. Marker *Micrococcus lysodeikticus* DNA is at 1.731 g.cm.^{-3} . (a) Chloroplast fraction without deoxyribonuclease treatment; the DNA is bimodal with peaks at 1.694 and 1.697 g.cm.^{-3} . (b) Mitochondrial fraction without deoxyribonuclease treatment; this again shows two bands, at 1.694 and 1.706 g.cm.^{-3} . (c) DNA prepared from nuclei shows a unimodal band at 1.694 g.cm.^{-3} . (d) DNA from the chloroplast fraction after deoxyribonuclease treatment; this now shows a unimodal band at 1.697 g.cm.^{-3} , the 1.694 g.cm.^{-3} material having been removed by the deoxyribonuclease action. (e) DNA from the mitochondrial fraction after inclusion of the deoxyribonuclease step; this shows a similar result to that given by the chloroplast fraction, the 1.694 g.cm.^{-3} band having been removed by the deoxyribonuclease action, leaving a unimodal band at 1.706 g.cm.^{-3} .

with that of the non-aqueous fraction of specific gravity 1.00 – 1.25 (Tables 1 and 2).

Base composition of lettuce nuclear and DNA chloroplastal DNA. Table 3 shows the base com-

Table 2. Buoyant density of DNA from aqueously prepared plant fractions in a caesium chloride gradient

	Buoyant density (g.cm.^{-3})		
	Nuclear DNA	Chloroplastal DNA	Mitochondrial DNA
Bean	1.695	1.697	1.705
Lettuce	1.694	1.697	1.706

Table 3. Base compositions of DNA from aqueously prepared lettuce fractions

The base compositions were determined by the method of Wyatt (1955). B.D., below detection, estimated as 0.3 mole/100 moles; the s.d. was about 0.8% . The discrepancy of adenine and thymine in chloroplast DNA was also observed by Whitfield & Spencer (1968).

	Base composition (moles/100 moles)	
	Nuclear DNA	Chloroplastal DNA
Adenine	28.5	31.1
Thymine	28.3	25.8
Guanine	21.0	22.5
Cytosine	17.2	21.4
5-Methylcytosine	5.0	B.D.

position of lettuce nuclear DNA and chloroplastal DNA. This confirms findings by other workers that chloroplastal DNA can be differentiated from nuclear DNA by its lack, within detection limits, of 5-methylcytosine (Brawerman & Eisenstadt, 1964; Ray & Hanawalt, 1964; Ruppel & van Wyck, 1965; Whitfield & Spencer, 1968).

Renaturation of DNA fractions from lettuce. Heat-denatured nuclear DNA, chloroplastal DNA and mitochondrial DNA from lettuce band in caesium chloride at densities of 1.710 , 1.713 and 1.721 g.cm.^{-3} respectively (Fig. 3). When the denatured DNA components are allowed to renature in SSC at a DNA concentration of 30 – $40 \mu\text{g./ml.}$ for 200 min. , the buoyant densities of 1.699 and 1.707 g.cm.^{-3} for the chloroplastal DNA and mitochondrial DNA were close to those of the native molecules (Figs. 3b and 3c). By contrast, the nuclear DNA showed only a slight return towards banding at the position of native DNA (Fig. 3a).

Chloroplastal DNA and mitochondrial DNA obviously renature rapidly and extensively, whereas the bulk of the nuclear DNA does not. To study the progress of renaturation more quantitatively, we measured the loss of E_{260} of the DNA components with time, and such curves for lettuce chloroplastal DNA, mitochondrial DNA and nuclear DNA are shown in Fig. 4. Renaturation of chloroplastal DNA and mitochondrial DNA resulted

Fig. 3. Microdensitometer tracing of the photographic record obtained after isopycnic CsCl-density-gradient centrifugation of lettuce DNA fractions. Marker *Micrococcus lysodeikticus* DNA is at 1.731 g.cm.⁻³. (a) Nuclear DNA, in native, denatured and renatured states (top, middle and bottom respectively); the nuclear DNA partly renatures with a return to buoyant density 1.705 g.cm.⁻³ from that of denatured DNA, 1.710 g.cm.⁻³; the narrowing of the band suggests some network formation. (b) Chloroplastal DNA in native, denatured and renatured states; it appears to renature as a homogeneous fraction (1.699 g.cm.⁻³) with a return almost to the native buoyant density (1.697 g.cm.⁻³). (c) Mitochondrial DNA in native, denatured and renatured states; this shows a similar result to that for the chloroplastal DNA, with a value for renatured DNA of 1.707 g.cm.⁻³ compared with that for native DNA of 1.706 g.cm.⁻³. In all cases the renatured DNA fractions were obtained from the experiment similar to that in Fig. 4, after the DNA components had re-annealed for some 200 min.

in a recovery of hypochromicity of 70–80%, whereas in the same time the recovery of hypochromicity was only 25–30% in nuclear DNA. The half-life, t_1 , values for chloroplastal DNA, mitochondrial DNA, bacteriophage T₄ DNA and *Escherichia coli* DNA are given in Table 4.

The kinetics of renaturation for chloroplastal DNA are truly second-order. This is deduced from the finding (Table 5) that t_1 is inversely proportional to DNA concentration (Britten & Kohne, 1967; Wetmur & Davidson, 1968). As expected, the relative speed of the reaction was governed by the single-stranded molecular weight of the DNA and exhibited the square-root relationship (cf. Table 6) between the second-order rate constant and the molecular weight (Wetmur & Davidson, 1968).

For evaluation of second-order constants, the reciprocal of the remaining hypochromicity, $E_{260(t)} - E_{260(\infty)}$, where $E_{260(\infty)}$ and $E_{260(t)}$ are the E_{260} values of native DNA and partly renatured DNA respectively, is usually plotted against time

(Wetmur & Davidson, 1968). The second-order rate constant, k'_2 , is then the slope. Homogeneous DNA fractions are expected to give a straight line and this was observed for bacteriophage T₄ DNA and *E. coli* DNA (Fig. 5). Chloroplastal DNA and mitochondrial DNA behaved heterogeneously. The curve for chloroplastal DNA (see the Discussion section) can be subdivided into a section with an apparent k'_2 of $11.5 \pm 1.3 \text{ mole}^{-1} \text{ sec}^{-1}$, and another of $4.6 \pm 0.3 \text{ mole}^{-1} \text{ sec}^{-1}$. From the intercepts of the regression curves it is deduced that the slower component represents about 76% of the DNA. The k'_2 values and mass ratios of the two components are given in Table 6.

Alternatively, the renaturation data may be analysed by recording the fraction of renatured DNA $(E_{260(t)} - E_{260(\infty)})/E_{260(\infty)}$ against $\log(ct)$. This presentation affords an alternative method for the determination of the kinetic complexity.

A k'_2 value may be derived from the time point at half renaturation, since then $ct_1 k'_2 = 1$ (Britten &

Fig. 4. Renaturation curves for lettuce DNA fractions. Denatured DNA ($30\text{--}40\text{ }\mu\text{g./ml.}$) was incubated at $(T_m - 25)^\circ$ in $1\times\text{SSC}$. After initial renaturation of reiterated nucleotide sequences the annealing reaction in nuclear DNA (Δ) becomes very slow. Chloroplastal DNA ($\circ$) renatures rapidly with a 70% recovery of hypochromicity. Mitochondrial DNA ($\square$) shows a somewhat lower rate of renaturation than does chloroplastal DNA, with a similar recovery of hyperchromicity.

Table 4. Renaturation half-lives of DNA fractions

The DNA was renatured at $(T_m - 25)^\circ$ in $1\times\text{SSC}$ at concentrations of $15\text{--}50\text{ }\mu\text{g./ml.}$ The DNA preparations were treated ultrasonically, to yield material with a single-stranded molecular weight of approx. 0.3×10^6 daltons. The half-life values given were adjusted for a DNA concentration of $100\text{ }\mu\text{g./ml.}$ (Britten & Kohne, 1967). The renaturation half-life is the time required to obtain a $E_{260(t)}$ equal to $1.18\times E_{260(\infty)}$.

	$t_{1/2}$ (min.)
Bacteriophage T ₄ DNA	10.4
<i>E. coli</i> DNA	138
Lettuce chloroplastal DNA	6.9
Lettuce mitochondrial DNA	14.0

Kohne, 1967). Owing to the disparate definition of the second-order rate constant, k'_2 is always $2k_2$. We have used the cot plot in our work mainly because it shows at a glance whether the system under investigation is homogeneous or not.

Table 5. Renaturation half-lives of lettuce chloroplastal DNA at different concentrations

Conditions were as given in Table 4.

Concn. (c) of DNA ($\mu\text{g./ml.}$)	Renaturation half-life ($t_{1/2}$) (min.)	$ct_{1/2}$
12	53	636
25	21	525
32	17	544
45	14	630

Bacteriophage T₄ DNA, when evaluated by this method, exhibits a slope at 50% renaturation characteristic of a homogeneous DNA (Britten & Kohne, 1967). Apart from an initial section, the slope of the curve generated by chloroplastal DNA approximates to that of bacteriophage T₄ DNA for most of the annealing reaction (Fig. 6). By contrast, mitochondrial DNA, and especially nuclear DNA, diverge from the ideal behaviour of a homogeneous DNA throughout.

DISCUSSION

Identification of chloroplastal DNA. We have shown for a variety of higher plants that, in addition to the nuclear DNA component(s), there are two distinct DNA satellites. The 1.706 g.cm.^{-3} satellite is always well resolved, but we find that in unfractionated leaf material the 1.697 g.cm.^{-3} DNA normally bands within the Gaussian distribution of the 1.695 g.cm.^{-3} nuclear DNA. In addition to their unique banding properties in caesium chloride gradients the two satellite DNA species are distinguished from nuclear DNA in that they renature rapidly and completely. The great ease with which they renature excludes the possibility that they arise from bacterial contaminants. The 1.697 g.cm.^{-3} DNA component is differentiated from nuclear DNA by its lack of 5-methylcytosine. In contrast with nuclear DNA both satellite DNA species are found in a form not easily accessible to deoxyribonuclease.

On the basis of the above consideration and the results obtained from both aqueous and non-aqueous cell-fractionation techniques we attribute the DNA component banding in the caesium chloride gradient at 1.706 g.cm.^{-3} to mitochondria and that banding at 1.697 g.cm.^{-3} to chloroplasts. The assignment of the latter is reinforced by the considerable degree of purity of the chloroplastal preparations when examined by electron microscopy.

We note the difficulty that others (Whitfield & Spencer, 1968; Wolstenholme & Gross, 1968) have experienced in the use of deoxyribonuclease in the purification of the chloroplasts for the preparation

Table 6. Renaturation data for lettuce chloroplastal DNA when treated as a population system

Conditions were as given in Table 4. The single-stranded molecular weights of the DNA molecules were determined at alkaline pH by the method of Studier (1965).

Prep. no.	Mol.wt. (daltons)	k'_2 (slow fraction) (mol ⁻¹ sec. ⁻¹)	Ratio $\frac{k'_2 \text{ (fast fraction)}}{k'_2 \text{ (slow fraction)}}$	Slow fraction (% of total)
29	3×10^5	4.6	2.4	76
30	3×10^5	4.7	2.5	77
38	3×10^5	4.4	2.9	77
7	5×10^5	6.1	2.7	81
37	5×10^5	6.2	2.4	79
36	6×10^5	6.0	2.4	70
14	2×10^6	11.4	2.8	85
17	9×10^6	25.2	3.2	83
Mean			2.5	76

Fig. 5. Reciprocal k'_2 plot for partly renatured DNA from various sources. In this presentation the reciprocal absolute value of the remaining hypochromicity of the partly renatured DNA is measured against time. Conditions were those given in Fig. 4. $E_{260(\infty)}$ and $E_{260(t)}$ are the E_{260} values of the solution for the native DNA and for the partly renatured DNA at a given time t during the annealing reaction respectively (Wetmur & Davidson, 1968). Bacteriophage T₄ DNA (●) and *E. coli* DNA (○) approximate to the expected linear relationship. Chloroplastal DNA (○) and mitochondrial DNA (□) behave heterogeneously.

of chloroplastal DNA. The iso-osmoticity of the extraction medium, the absence of amyloplasts and a fair proportion of only little-differentiated chloroplasts in our preparation may all contribute to the resistance of the chloroplastal DNA to attack by deoxyribonuclease. The relatively high molecular weight of the chloroplastal DNA ($> 5 \times 10^6$ daltons) is an indication of a good preservation of the DNA molecules.

Treatment of the mitochondrial preparations with deoxyribonuclease eliminated any DNA that was present in the contaminating chloroplastal frag-

Fig. 6. c_0t plot for various DNA components. The amount of renatured DNA is expressed as a fraction of the total DNA mass and recorded against the logarithm of concentration, c_0 , of the DNA (expressed in moles of nucleotides) multiplied by the time interval (in sec.) (Britten & Kohne, 1967). The DNA fractions have not been standardized to an identical molecular weight. The plot for bacteriophage T₄ DNA (●) is identical with that reported by Britten & Kohne (1967). Chloroplastal DNA (○) is homogeneous in the slowly renaturing sequences, whereas mitochondrial DNA (□) and nuclear DNA (△) are polydisperse.

ments. DNA prepared from deoxyribonuclease-treated or non-aqueously prepared chloroplasts exhibited a unimodal distribution on caesium chloride density gradients, a feature not previously reported. That nuclear DNA, in particular its non-reiterated DNA portion, was quantitatively removed by the deoxyribonuclease treatment may be deduced from the observation that on renaturation the chloroplastal DNA banded homogeneously

and did not include DNA that had remained denatured and hence of high buoyant density.

The identification of the 1.697 g.cm.⁻³ DNA component as chloroplastal by Wells & Birnstiel (1967), Whitfield & Spencer (1968) and Wolstenholme & Gross (1968) is in contradiction to the work of Chun *et al.* (1963), Kislev, Swift & Bogorad (1965), Shipp *et al.* (1965) and Green & Gordon (1967), who identified the 1.706 (1.700–1.707) g.cm.⁻³ satellite as chloroplastal. When the DNA of the isolated cell fraction was investigated by analytical or preparative caesium chloride density-gradient centrifugation and compared with unfractionated cellular DNA an enrichment of the 1.706 g.cm.⁻³ component was observed by the latter groups of workers. But, contrary to our experience, the DNA was seen to band heterogeneously with at least two density modes. The DNA component banding at lower buoyant density, which was usually present in large amount, was assumed, on the basis of its density, to represent contaminating nuclear DNA and was disregarded. It is possible that this DNA was made up, at least in part, of chloroplastal DNA. That contamination of isopycnically prepared chloroplasts by mitochondria may be an important factor has been pointed out by Green, Heilborn, Limbosch, Boloukhere & Brachet (1967).

Base-sequence complexity of chloroplastal DNA. The speed with which DNA renatures is critically dependent on the diversity of the basic non-repeating nucleotide sequences it contains. From the cot_1 value (Britten & Kohne, 1967) or second-order rate constant, k'_2 , of the reaction (Wetmur & Davidson, 1968) it is possible to derive a value for the kinetic complexity of any given DNA. Such calculations are simple only if the DNA in question behaves homogeneously, like bacteriophage T₄ DNA, which served as a standard in our experiments. Calf thymus DNA (Britten & Kohne, 1967) and ascites-tumour DNA (Wetmur & Davidson, 1968) are only two examples of DNA containing differing degrees of reiteration of the DNA sequences. In these cases estimation of the kinetic complexity from half-life determinations is inadmissible since the half-life is strongly influenced by early renaturation of DNA, at which time the 'fast' fractions are most active.

From the overall half-life for chloroplastal DNA from lettuce we derived a kinetic complexity of about 8×10^7 daltons (Wells & Birnstiel, 1967). From the k'_2 plot of the renaturation data it has since become evident that chloroplastal DNA is kinetically heterogeneous. A k'_2 value is easily obtained from the initial slope of the k'_2 plot (Fig. 5) for the fast-renaturing DNA sequences. From the cot plot (Fig. 6) it is evident that the late-renaturing chloroplastal DNA may be treated as a single unique base sequence (see the Results section). We there-

fore consider that we are justified in also fitting a straight line to the late time points of the k'_2 plot.

From these considerations it follows that chloroplastal DNA is made up of two classes of DNA sequences, a 'fast' and a 'slow' fraction. The two apparent k'_2 rate constants are 11.5 and 4.6 mole⁻¹ sec.⁻¹ respectively.

The assumption is made that the contribution of each fraction can be calculated from extrapolation of the amount of slow fraction to its zero-time value. A mean value of 76% is obtained for the slow fraction and a value of 24% for the fast fraction (Table 6). To arrive at an approximation of the true k'_2 values we have adopted a procedure which was suggested to us by Dr J. O. Bishop and Dr N. Davidson. At late times in the experiment when most of the fast fraction has already reacted the slope of the k'_2 plot represents primarily the more slowly renaturing DNA fraction. The apparent k'_2 for the second, final, slope approximates closely to the true second-order rate constant for the slow DNA component. The hyperchromicity contribution of the fast fraction may be calculated by determining the hyperchromicity of the slow fraction from the Wetmur & Davidson plot and subtracting it from the total hyperchromicity value at each time point. The reciprocal of the hyperchromicity values for the fast fraction alone can then be replotted against time and a corrected k'_2 value of 220 mole⁻¹ sec.⁻¹ is obtained. Since these calculated hyperchromicity values are more sensitive to experimental error and the correction is necessarily large, the corrected k'_2 constant for the fast fraction must represent, at best, a crude approximation.

We now consider factors other than differences in base-sequence complexity that might produce the heterogeneous pattern observed for chloroplastal DNA. These are heterogeneity in guanine + cytosine content or in molecular weight of the DNA fragments, cross-linkage of DNA strands and contamination by mitochondrial DNA.

(1) Heterogeneity can be caused by variation in guanine + cytosine content of the DNA sequences (Thrower & Peacocke, 1968). The work of Wetmur & Davidson (1968) suggests that the kinetic complexities of homogeneous DNA species with guanine + cytosine contents 71 and 34% respectively are respectively 0.7 and 1.4 times the analytical complexity. The factor required to explain the corrected k'_2 ratios for the fast and slow reaction is about 48. It seems therefore unlikely that heterogeneity in guanine + cytosine content could by itself account for the biphasic reciprocal plot.

(2) Chloroplastal DNA at all extents of ultrasonic treatment sediments at alkaline pH in a unimodal manner. There is no indication that the heterogeneity on renaturation is caused by the presence

of DNA fractions of widely different rates of sedimentation. Since the rate of renaturation is proportional to the square root of the single-stranded molecular weight (Wetmur & Davidson, 1968), the factor for the DNA fragment would have to be approx. 900 to obtain the observed heterogeneity.

(3) The fast fraction does not represent cross-linked DNA. This is deduced from the observation that the rate of reaction for the first phase exhibits the usual reciprocal relationship to concentration. The initial slope remains unchanged in the k'_2 plot, although shifted in parallel, when DNA is renatured at different concentrations.

(4) Finally, we can exclude the possibility that the heterogeneous pattern is elicited through contamination by mitochondrial DNA. Mitochondrial DNA is always well resolved from chloroplastal DNA in the analytical ultracentrifuge. A contamination of about 24% would become apparent on caesium chloride density gradients even if this DNA is of low molecular weight. In addition, mitochondrial DNA renatures slowly and cannot constitute the fast fraction.

By comparing the second-order rate constant with that of bacteriophage T₄ DNA, which is of low guanine + cytosine content, as is chloroplastal DNA, it is evident that the chloroplastal DNA components possess kinetic complexities of about 1.2×10^8 and 3×10^6 daltons. Because the faster-renaturing base sequences are of very low kinetic complexity it follows that they occur in the chloroplastal DNA in greater multiplicity than do the more slowly renaturing DNA stretches.

We have not been able to amass sufficient quantities of mitochondrial DNA from lettuce for exhaustive analysis of this DNA. However, as in chloroplastal DNA, the k'_2 pattern is complex (Fig. 5). A k'_2 value of $4.4 \text{ mole}^{-1} \text{ sec}^{-1}$, corresponding to a kinetic complexity of $\sim 1.4 \times 10^8$ daltons, is available from the initial slope. The slope of the k'_2 plot decreases gradually and no straight line can be fitted to the later parts of the

curve. A curved appearance of the k'_2 plot would be expected for a DNA in which the base sequences diverge gradually, rather than discontinuously. The c_0t plot reinforces this view, since there is a marked deviation from the slope representative of a homogeneous DNA. Such a deviation could result from a considerable base-sequence heterogeneity (Britten & Kohne, 1967). The above results would be most satisfactorily explained by the hypothesis that in mitochondrial DNA the bulk of the DNA segments (1.4×10^8 daltons) are very similar and anneal indiscriminately with one another, but are accompanied by an ever-decreasing amount of base sequences that have been altered by random mutational events. Since our experiments were carried out at relatively low ionic strength, the annealing conditions may have been taxing enough to reveal even small differences in the nucleotide sequences. Whatever the exact interpretation of these results, it is certain that mitochondrial DNA from lettuce possesses greater kinetic complexity ($> 10^8$ daltons) than that expected from the circular structure of mitochondrial DNA from animal sources ($\sim 10^7$ daltons). The possibility has already been raised by Wolstenholme & Gross (1968) that mitochondrial DNA from plants may well have a greater degree of complexity than that obtained from animals.

The analytical complexity (Wetmur & Davidson, 1968) of chloroplastal DNA, i.e. the amount of DNA per chloroplast, in algae and higher plants has been reported to be about 10^{-16} (Gibor & Izawa, 1963) to 10^{-14} g. (Chiba & Sugahara, 1957; Brawerman & Eisenstadt, 1964; Edelman, Cowan, Epstein & Schiff, 1964; Chun *et al.* 1963; Ruppel & van Wyck, 1965) about 6×10^8 – 6×10^{10} daltons of DNA. Our own estimations on lettuce chloroplasts are consistent with a content of about 2×10^9 daltons of DNA. From the observed discrepancy between analytical and kinetic complexities (Table 7) we deduce that chloroplastal DNA is extensively reiterated. The kinetic studies indicate that the

Table 7. *Comparison of kinetic and analytical complexities of DNA from different sources*

The definitions for kinetic and analytical complexity are those given by Wetmur & Davidson (1968). The k'_2 values have been adjusted to a single-stranded molecular weight of 0.3×10^6 daltons according to the formula of Wetmur & Davidson (1968).

	Apparent k'_2 ($\text{mole}^{-1} \text{ sec}^{-1}$)	Corrected k'_2 ($\text{mole}^{-1} \text{ sec}^{-1}$)	Kinetic complexity (daltons)	Analytical complexity (daltons)
Bacteriophage T ₄ DNA	4.8	4.8	Used as standard	1.3×10^8
<i>E. coli</i> DNA	0.37	0.37	2.5×10^9	2.5×10^9
Lettuce chloroplastal DNA				2×10^9
Fast fraction	11.5	220	3×10^6	
Slow fraction	4.6	4.6	1.2×10^8	
Lettuce mitochondrial DNA	4.4		1.4×10^8	

base-sequence diversity in chloroplastal DNA is some 20-fold less complex than that of bacteria, but is equivalent to that of, say, bacteriophage T₄ DNA.

The renaturation results do not show how these redundant base sequences are arranged in the chloroplastal DNA. However, we note, with some satisfaction, that DNA filaments have been isolated and made visible in the electron microscope from spinach chloroplasts (Woodcock & Fernandez-Moran, 1968) which extend to about 75 μ m. and 150 μ m. A 75 μ m. DNA strand represents about 1.4×10^8 daltons of DNA, a value comparable with the kinetic complexity of lettuce chloroplastal DNA. The possibility therefore exists that these DNA molecules represent the base sequences detected in our annealing experiments.

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