Studies on Spinach Chloroplast and Nuclear DNA Using Large-scale Tissue Preparations[1]

Stephen A. Bard and Milton P. Gordon

Department of Biochemistry, University of Washington, Seattle, Washington 98105

Abstract. A method for the large-scale preparation of spinach chloroplasts using the Spinco Model L-4 zonal ultracentrifuge and for the extraction of DNA from the chloroplasts is described. Thirty-five percent of the chloroplast DNA (ρ = 1.706 g/cc) differs from nuclear DNA (ρ = 1.695 g/cc) in buoyant density, T_m, base composition, and renaturation properties. Sixty-five percent of the chloroplast DNA (ρ = 1.696 g/cc) has the same buoyant density and T_m as nuclear DNA, but it differs in base composition and renaturation properties.

The presence of DNA in subcellular organelles has been convincingly demonstrated during the last several years (10). The major method used to characterize these satellite DNAs has been the determination of buoyant density in CsCl. Generally, these buoyant density determinations have shown that in DNA isolated from chloroplasts there is a minor DNA species which differs in buoyant density from nuclear DNA. In almost every case, however, the major DNA species isolated from chloroplasts has had the same buoyant density as nuclear DNA, and this major chloroplast DNA has, therefore, been ascribed to contamination by nuclear DNA (13). For example, Chun et al. (8), working with spinach, found buoyant densities of 1.695 g/cc for nuclear DNA, 1.695 g/cc for the major chloroplast DNA, and 1.705 g/cc and 1.719 g/cc for 2 minor chloroplast DNA species.

There have been 3 reports describing the isolation of only a single species of DNA from chloroplasts. Tewari and Wildman (17) use a razor blade homogenization procedure which provides a single chloroplast DNA from tobacco; this chloroplast DNA does differ in buoyant density from nuclear DNA. Whitfeld and Spencer (19) isolated a single chloroplast DNA from both tobacco and spinach, but these DNAs had the same buoyant densities as the respective nuclear DNAs. A single chloroplast DNA intermediate in buoyant density between nuclear DNA and a "heavy" satellite DNA was isolated by Wells and Birnstiel (18) from several higher plant species. All of these studies indicate the presence of DNA in chloroplasts, but there is much conflicting data as to its nature and buoyant density. The data to be

presented in this paper suggest that some of this conflict may be due to the presence of 2 distinct types of DNA in chloroplasts.

Sufficient quantities of chloroplast DNA differing in buoyant density from nuclear DNA were accumulated to determine the actual base composition for tobacco (17) and *Euglena* (6). In addition, the chloroplast DNAs of tobacco and spinach which had the same buoyant densities as the respective nuclear DNAs were analyzed for base composition (19). In all of these cases the chloroplast DNA contains no 5-methylcytosine, whereas the nuclear DNA does contain a significant amount of 5-methylcytosine in the range of 2 to 6 mole percent.

The work to be described was directed towards the development of a procedure for the large-scale preparation of spinach chloroplasts as free of nuclear contamination as possible. This provided a source of much larger amounts of chloroplast DNA than heretofore available.

Materials and Methods

Preparation of Spinach Nuclei. Spinach (*Spinacia oleracea*) was purchased on the day of use from local supermarkets and stored at 4°. The method used was essentially that of Green and Gordon (11). The buffered sucrose used in this method and those to be described later consisted of a certain percentage of sucrose (w/w) and 0.005 M EDTA, 0.01 M NaCl, 0.004 M mercaptoethanol, and 0.02 M tris, pH 8.0. About 100 g of leaves are homogenized in 60 % buffered sucrose with a mortar and pestle. After squeezing through cheesecloth, the liquid part of the homogenate is centrifuged at 23,000 rpm in the Spinco SW 25.2 rotor for 45 min. The nuclear pellet is washed in 0.25 M sucrose-NET (NET = 0.1 M NaCl, 0.01 M EDTA, and 0.01 M tris, pH 8.0) and pelleted at 4000g.

Preparation of Spinach Chloroplasts. For the preparation of chloroplasts from about 500 g of leaf tissue, the following procedure was developed. The leaves are washed thoroughly in cold water, and as much of the water as possible is shaken off. Only the stems, not the midribs, are removed in order to conserve time. The remainder of the procedure is performed at 4°. The leaves in bunches of 4 to 6 at a time are placed in the inlet of a motor-driven meat grinder assembled with an outlet plate that has 0.5 cm holes. They are forced down into the grinder with a wooden implement designed for that purpose. The green homogenate is caught in 2 layers of cheesecloth held over a large beaker. During the course of the grinding, about 80 ml of 50 % buffered sucrose is added for each 500 g of leaves. This will normally give a final volume of homogenate of about 450 ml. The liquid part of the homogenate is then filtered through 6 layers of cheesecloth and is now ready for centrifugation in the Spinco Model L-4. The B-IV batch zonal rotor is filled with a 1200 ml linear sucrose gradient from 20 % to 50 % buffered sucrose, and a 525 ml 50 % buffered sucrose cushion is placed under this gradient. The homogenate and 50 ml of overlay is pumped into the rotor, thus displacing about 500 ml of the 50 % sucrose cushion (3). Centrifugation is carried out at 37,000 rpm for 45 min at 5°. After reducing to 5000 rpm, the rotor contents are pumped out using 50 % sucrose. The chloroplasts band in about 200 ml between 40 % and 44 % sucrose. The nuclei accumulate on the outer rotor wall and are not recovered by this procedure. The chloroplasts are diluted by twice their volume with NET and are collected by centrifugation at 10,000*g* for 20 min. This is followed by 3 resuspensions of the chloroplast pellet in 0.25 M sucrose-NET with 13,000*g* centrifugation after each resuspension.

Extraction of DNA. The method described by Marmur (14) was used to extract DNA from nuclei. Originally, the Marmur procedure with the omission of the ethanol precipitation step and the addition of exhaustive dialysis was also used to extract DNA from small amounts of chloroplasts. With larger amounts of chloroplasts, an improved method of DNA extraction was used. The chloroplasts are suspended in an equal volume of 4 % Triton X-100 in 0.25 M sucrose-NET, and the suspension is incubated at 65° for 10 min with gentle stirring. The suspension is centrifuged at 10,000*g* for 10 min. The supernatant is then incubated at 37° for 4 hr with pronase at a final concentration of 1 mg/ml. An equal volume of water-saturated redistilled phenol is added, and gentle hand shaking in a large screwcap tube with a teflon-lined cap is carried out for 10 min. Following low-speed centrifugation, the aqueous layer is removed with an inverted pipette. Two volumes of cold ethanol are added, and the solution is stirred. At this stage, the precipitated material will not wind around a rod, but it may be

collected by centrifugation at 10,000*g* for 10 min. It is dissolved in 0.1 SSC (SSC = 0.15 M NaCl, 0.015 M Na₃citrate), and the concentration is increased to 1 × SSC with 10 × SSC. The solution is then treated with 50 μg/ml of pancreatic RNase at 37° for 1 hr. The RNase had previously been heated to 100° for 10 min to destroy any DNases. A phenol deproteinization is carried out followed by ethanol precipitation; fibers can now be wound around a rod and are redissolved in 0.1 SSC. The concentration is increased to SSC as before, and another phenol deproteinization is done. The DNA is ethanol-precipitated again, wound around a rod, and redissolved in 0.1 SSC. This is followed by dialysis for 48 hr *versus* 1 change of SSC (500 volumes).

Preparative CsCl Density Gradient Centrifugation. These centrifugations were performed as described by Flamm *et al.* (9) except that a Spinco No. 50 rotor was used; this allowed an increase in speed to 39,000 rpm and a decrease in time to 48 hr.

Analytical CsCl Density Gradient Centrifugation. These centrifugations were performed as described by Green and Gordon (11), but 2 cells were run simultaneously with the use of the 2-cell mask and timer assembly for the Model E. *Mixococcus xanthus* DNA having a buoyant density of 1.729 g/cc or *Micrococcus lysodeikticus* DNA having a buoyant density of 1.731 g/cc were used as the marker. All
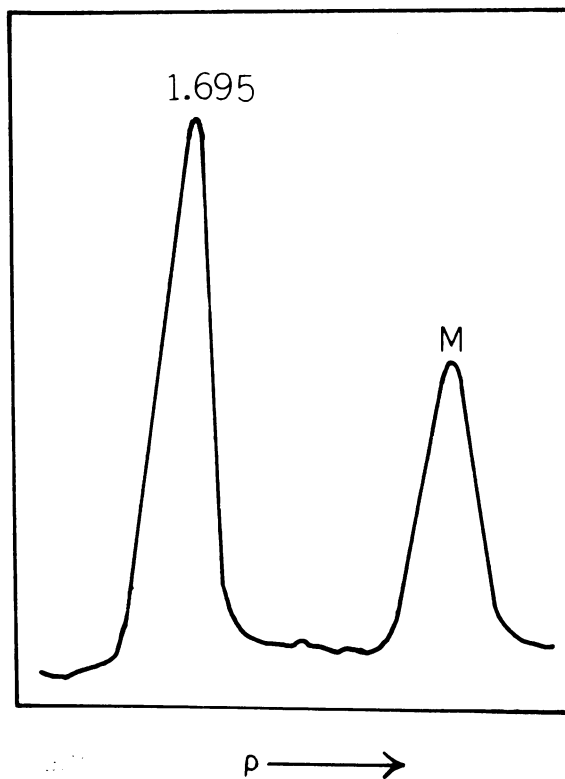

Fig. 1. Buoyant density (g/cc) of nuclear DNA. M = *M. xanthus* DNA, ρ = 1.729 g/cc.

buoyant densities are based on a value of 1.710 g/cc for *E. coli* DNA.

Base Composition. The method for determining DNA base composition described by Bendich (4) was adapted for use with smaller amounts of DNA. Duplicate analyses with 150 to 200 μg of DNA were made. Hydrolyses were carried out in 125 μl of 88 % formic acid in combustion tubes made from small diameter Pyrex tubing. The tubes were sealed and heated at 175° for 30 min. After cooling, 10 μl was removed for phosphate determination (2), and
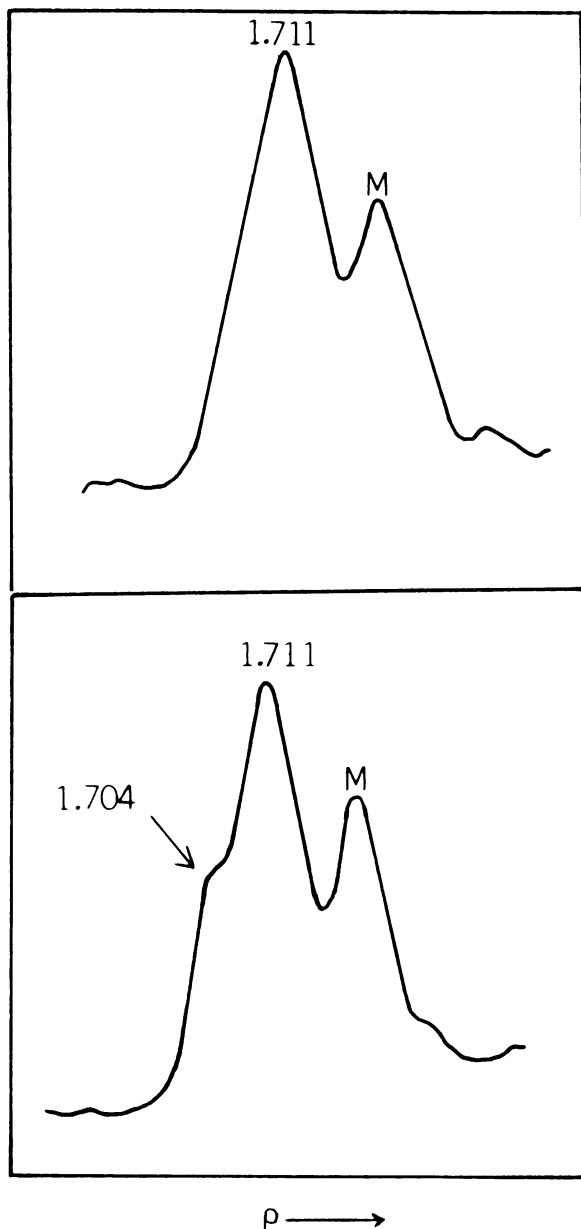

FIG. 2a (top). Buoyant density (g/cc) of fast cooled nuclear DNA. FIG. 2b (bottom). Buoyant density (g/cc) of slow cooled nuclear DNA. M = *M. xanthus* DNA, ρ = 1.729 g/cc.
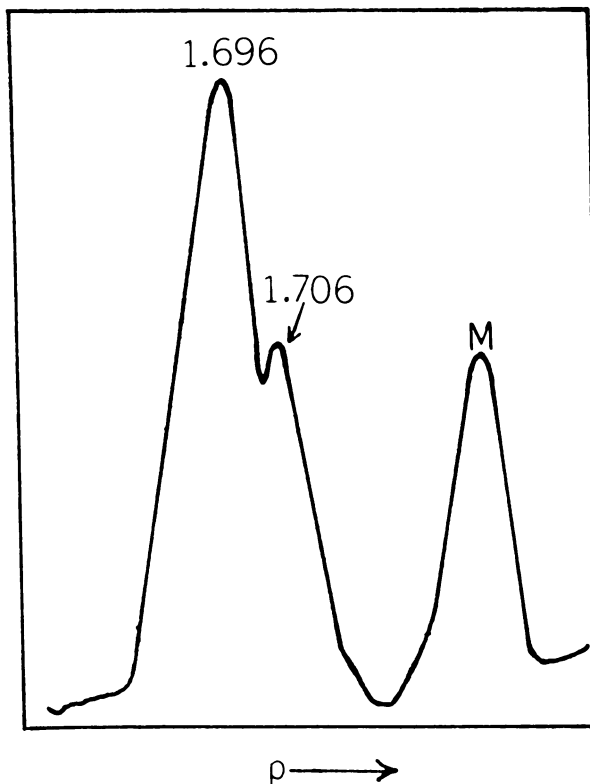

FIG. 3. Buoyant density (g/cc) of chloroplast DNA. M = *M. xanthus* DNA, ρ = 1.729 g/cc.

100 μl was used for paper chromatography. Whatman No. 1 paper and the 2 N HCl-isopropanol solvent system were used for paper chromatography of the hydrolyzed DNA. 5-Methylcytosine content was determined by the isotope dilution method, and the amounts of the 4 normal bases were determined by the differential extinction technique (4).

Heat Denaturation-Renaturation. DNA at concentrations of 10 to 20 μg/ml in SSC was denatured for 10 min at 100°. Fast-cooled samples were placed immediately in ice. Slow-cooled samples were allowed to incubate at 60° for 6 hr, then slowly cooled to room temperature over a 4-hr period.

T_m *Measurements.* These were made on a Gilford Model 2000 capable of automatically measuring the T_m of 4 DNA samples. DNA concentrations were 10 to 20 μg/ml in SSC.

Results

The yield of purified spinach nuclear DNA was about 500 μg per 100 g of leaf tissue. The buoyant density of nuclear DNA was 1.695 g/cc (Fig. 1), and the T_m was 84° in SSC. This DNA was completely sensitive to DNase. Upon heat denaturation and fast cooling, the increase in buoyant density was 0.016 g/cc (Fig. 2a). Under slow cooling renaturation conditions, the nuclear DNA showed little re-

Table I. *Base Composition of Spinach Nuclear and Chloroplast DNAs (mole %)*

	Nuclear DNA ρ = 1.695 g/cc	Major chloroplast DNA, ρ = 1.696 g/cc	Minor chloroplast DNA, ρ = 1.706 g/cc
Guanine	19.8 ± 0.5 %	20.2 ± 0.3 %	23.7 ± 0.5 %
Cytosine	14.0 ± 0.2 %	17.0 ± 0.5 %	23.3 ± 0.1 %
5-Methylcytosine	3.6 ± 0.3 %	1.6 ± 0.3 %	<0.6 %
Adenine	32.1 ± 0.5 %	31.3 ± 0.4 %	27.2 ± 0.6 %
Thymine	30.5 ± 0.2 %	30.1 ± 0.3 %	26.8 ± 0.4 %
Recovery[1]	91.8 ± 0.9 %	93.3 ± 1.7 %	94.7 ± 1.5 %
$\dfrac{A + T}{G+C+5\text{-MeC}}$	1.67	1.58	1.18
$\dfrac{G}{C+5\text{-MeC}}$	1.12	1.08	1.06
$\dfrac{A}{T}$	1.05	1.03	1.01
G+C+5-MeC (from base composition)	37.4 %	38.8 %	45.9 %
G+C (from buoyant density)	35.7 %	36.7 %	46.9 %
G+C (from T_m)	35.8 %	35.8 %	44.4 %

[1] Based on phosphate as a 100 % value.

naturation (Fig. 2b). Since there is a shoulder on the low-density side of the peak, however, it is possible that some renaturation of nuclear DNA has occurred. This could be due to the phenomenon of repetitious sequences as described by Britten and Kohne (7), and which is apparently common to a variety of plant DNAs. The base composition of nuclear DNA is listed in table I. The relatively high amount (3.6 %) of 5-methylcytosine is of particular interest.

The yield of spinach chloroplast DNA was about 500 μg per 500 g of leaf tissue. This DNA consists of 2 different components as seen in Fig. 3. The major component comprising about 65 % of the DNA had a buoyant density of 1.696 g/cc, and the minor component comprising about 35 % of the DNA had a buoyant density of 1.706 g/cc. The relative amounts of the 2 chloroplast DNA components were practically constant in all of 8 DNA preparations. In order to characterize the DNA obtained from chloroplasts, it was, therefore, necessary to separate the 2 DNA species actually present and to study them individually. This separation was accomplished in 2 cycles of preparative CsCl density gradient centrifugation in a fixed angle rotor. The effectiveness of this separation can be seen in Fig. 4a and 4b. There is no indication that more than one DNA species is present in the separated light and heavy chloroplast DNAs.

The T_m of light chloroplast DNA was 84° while that of heavy chloroplast DNA was 87.5° in SSC. Upon heat denaturation and fast cooling, the light chloroplast DNA increased in buoyant density by

0.015 g/cc (Fig. 5a) while heavy chloroplast DNA increased by 0.014 g/cc (Fig. 6a). Under slow cooling renaturation conditions, the heavy chloroplast DNA renatured almost completely (Fig. 6b), but the light chloroplast DNA renatured to 1.703 g/cc, a value intermediate between its native and denatured buoyant densities (Fig. 5b). The base composition of the 2 chloroplast DNA species is listed in table I. Again, the relative amounts of 5-methylcytosine are of interest. The heavy chloroplast DNA has no detectable amount of this base, within the limits of the lower level of sensitivity of the isotope dilution, which is 0.6 % 5-methylcytosine. The light chloroplast DNA has 1.6 % 5-methylcytosine. For all of the base analyses, the results of the duplicate analyses are in good agreement, and the recovery of bases using the amount of phosphate as a 100 % value is excellent.

In several experiments, chloroplasts were obtained from a 1-step gradient in the Spinco Model L-4 rather than from a linear gradient. This 1-step gradient consisted of about 500 ml of 20 % buffered sucrose layered over about 1200 ml of 50 % buffered sucrose. After centrifugation, the chloroplasts band at the interface of the 2 layers. Mitochondria would also band at this interface and would, therefore, be recovered along with the chloroplasts (1). The DNA extracted from the "chloroplasts" obtained from this step gradient showed an additional minor band when run in the Spinco Model E. This was at a buoyant density of 1.719 g/cc and was approximately equal in amount to the 1.706 g/cc minor band (Fig. 7).

Discussion

The use of the B-IV rotor in the L-4 zonal ultracentrifuge for chloroplast isolation provides about a 5-fold increase per run in the amount of chloroplasts obtained than can be obtained from the SW 25.2 rotor. It is possible to process 2 kg of leaves per day if several L-4 runs are made. Due to the large physical separation between chloroplasts and nuclei in the B-IV rotor, the chloroplasts are probably more nearly free of any nuclear contamination than chloroplasts isolated by any other centrifugal method. This expectation was borne out by both light microscopy and fluorescence microscopy using acridine orange staining; in nuclear preparations intact nuclei
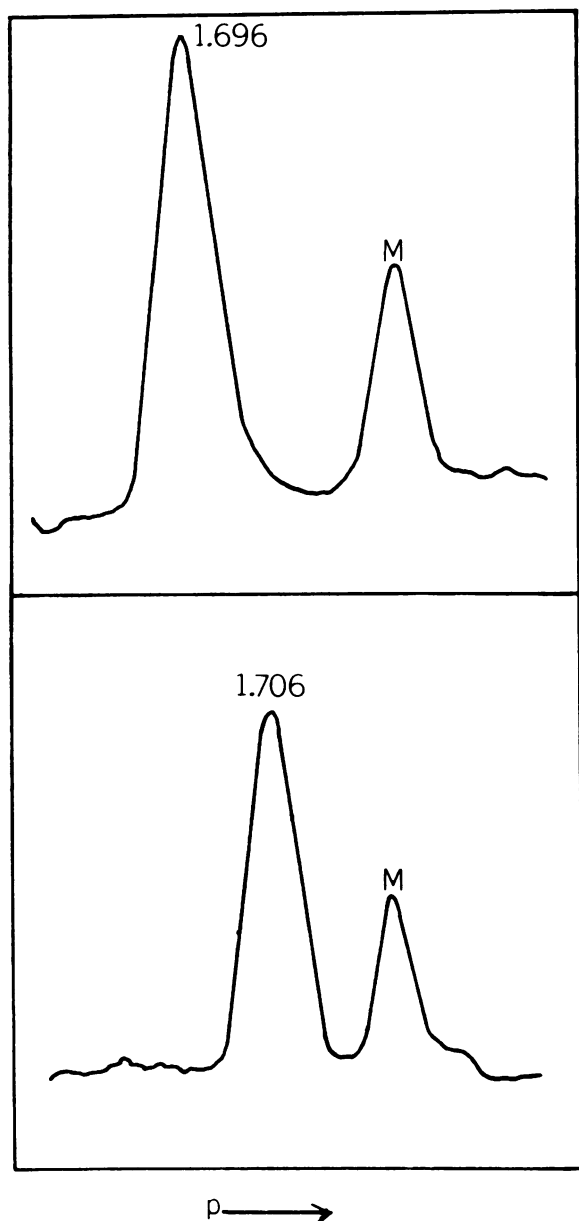
Fig. 4a (top). Buoyant density (g/cc) of purified light chloroplast DNA. Fig. 4b (bottom). Buoyant density (g/cc) of purified heavy chloroplast DNA. M = M. xanthus DNA, ρ = 1.729 g/cc.
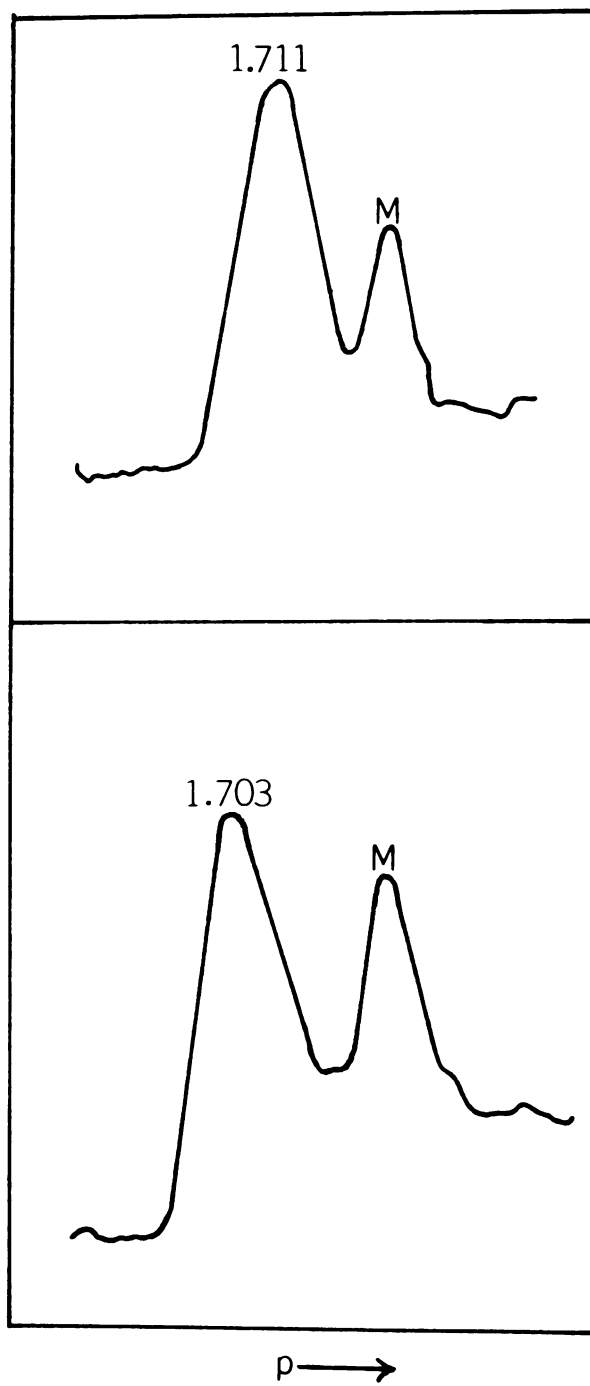
Fig. 5a (top). Buoyant density (g/cc) of fast cooled light chloroplast DNA. Fig. 5b (bottom)). Buoyant density (g/cc) of slow cooled light chloroplast DNA. M = M. xanthus DNA, ρ = 1.729 g/cc.

could be identified by both methods. In the chloroplast preparations, no contamination by intact nuclei could be detected. As a further precaution against contamination by nuclear DNA, the chloroplasts were lysed with Triton X-100 which does not lyse nuclei or bacteria (5, 15, 19). It is, therefore, doubtful

that the chloroplast DNA contains nuclear DNA arising from the contamination of the chloroplasts by intact nuclei. Previous work in this laboratory has shown that bacterial DNA contamination is negligible using the techniques described (11).

It is still possible, however, that there is nuclear DNA contamination which arises from nuclear fragments or nuclear DNA which adhere to the chloroplast membrane following the homogenization step during which there is probably some breakage of nuclei. These nuclear fragments or DNA would have to adhere quite firmly to the chloroplasts in order to survive during L-4 centrifugation and the 4 subsequent washings in isotonic buffered sucrose which the chloroplasts undergo during the isolation procedure. Pollard (16) found that spinach chloroplasts had little tendency to adsorb nuclear material following deliberate attempts to contaminate purified chloroplasts with intact and broken nuclei. Experiments in this laboratory with ^{14}C-labeled _E. coli_ DNA added during the homogenization of spinach leaves showed that there is also little tendency for free DNA to adsorb to intact chloroplasts. Only 0.41 % of the final chloroplast DNA was labeled.

The major component of chloroplast DNA does have the same buoyant density and T_m as nuclear DNA, but it seems unlikely that it is due entirely to nuclear DNA contamination for the reasons just given. In addition, this major chloroplast DNA does
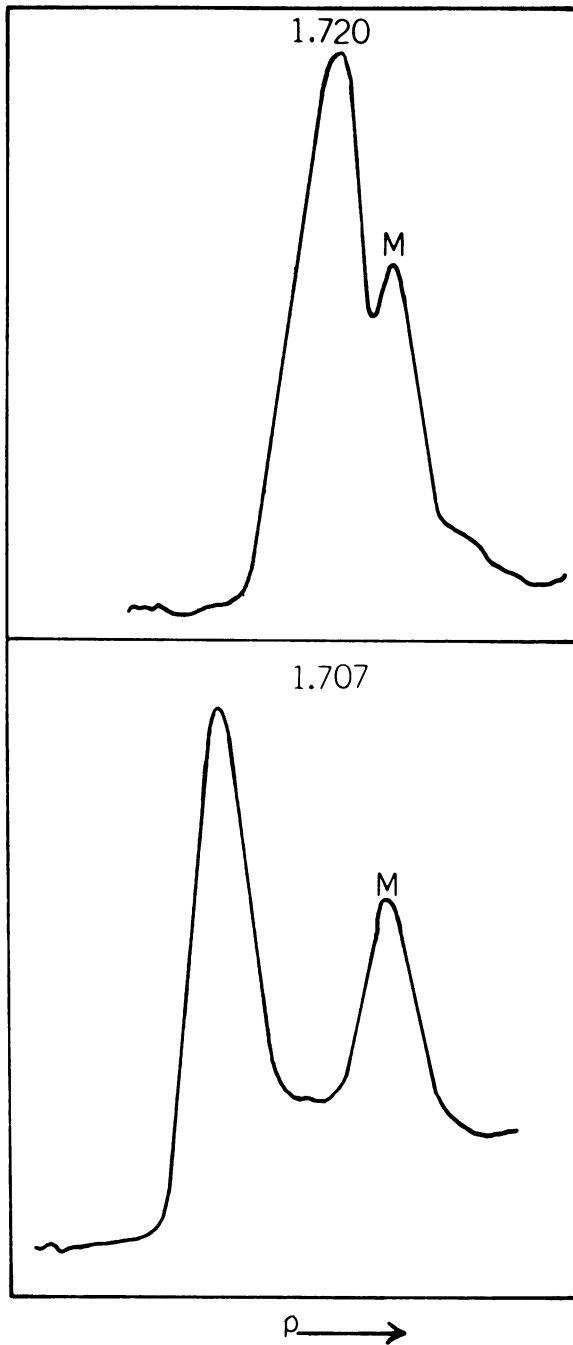

FIG. 6a. (top) Buoyant density (g/cc) of fast cooled heavy chloroplast DNA. FIG. 6b. (bottom) Buoyant density (g/cc) of slow cooled heavy chloroplast DNA. M = _M. lysodeikticus_ DNA, ρ = 1.731 g/cc.
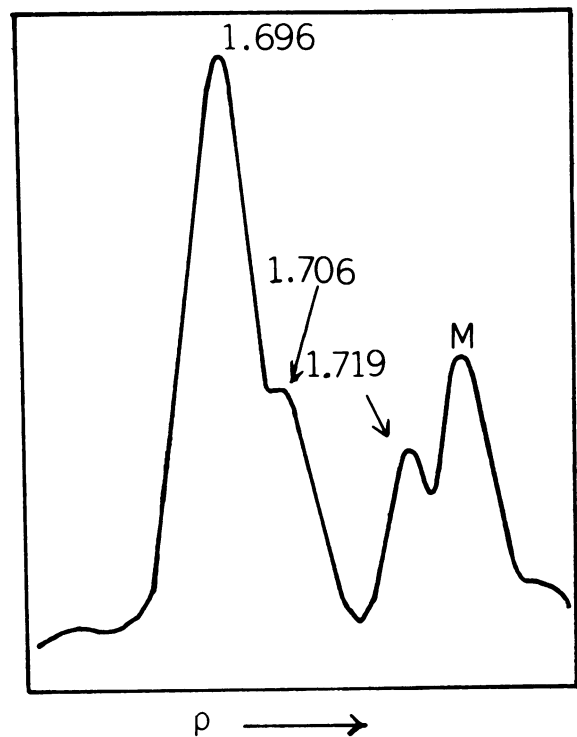

FIG. 7. Buoyant density (g/cc) of "chloroplast-mitochondrial" DNA. M = _M. xanthus_ DNA, ρ = 1.729 g/cc.

differ clearly from nuclear DNA in its actual base composition and renaturation properties. With mitochondrial DNA, it has been possible to selectively eliminate most contaminating nuclear DNA by treatment of intact mitochondria with DNase. This treatment has not been successful with chloroplasts, however, because the chloroplast membrane is apparently not resistant to DNase penetration (19). This fact has been confirmed in the present studies using low concentrations of DNase in the range of 0.5 to 5.0 μg/ml and short 30-min incubation times. With less than 2.0 μg/ml of DNase, there was no decrease in the amount of major and minor DNA obtained from the chloroplasts: with 2.0 μg/ml or higher DNase concentrations, no DNA could be obtained from the chloroplasts. This indicates the susceptibility of chloroplasts to low DNase concentrations, and it also suggests that the 2 DNA species isolated from the chloroplasts are both contained within the chloroplast.

The minor component of spinach chloroplast DNA differs from nuclear DNA in buoyant density, T_m, base composition and renaturation properties. Its buoyant density of 1.706 g/cc is almost the same as one of the chloroplast DNA components isolated by Chun et al. (8). As a result, there is little doubt that this minor chloroplast DNA component is a true chloroplast DNA.

If there are indeed 2 distinct chloroplast DNAs, then there are still 2 unresolvable alternatives at this time. Either both the major and the minor chloroplast DNAs are contained within the same chloroplast, or there are 2 types of chloroplasts present each containing its own unique DNA. The first alternative is supported by observations that in wheat chloroplasts, DNA fibrils are found in several discrete locations within the organelle (21). Recently the electron microscope work of Woodcock and Fernandez-Moran (20) has demonstrated that the chloroplast DNA from individual spinach chloroplasts apparently exists in 2 different conformations which conceivably could be 2 types of DNA differing in buoyant density within the same chloroplast. The second alternative finds support in the observations that cells apparently can have "mixed" populations of chloroplasts or more than 1 type of chloroplast in a cell (10, 12, 13).

Chun et al. (8) found a second chloroplast DNA with a buoyant density of 1.719 g/cc: using the normal procedures developed during the course of this work, however, such a second satellite DNA was never found in this laboratory. A 1.719 g/cc second satellite DNA was found under conditions where mitochondrial contamination of the chloroplasts was to be expected, but further work is required before the source of this satellite can be attributed to mitochondria or chance bacterial contamination.

In conclusion, a procedure has been developed for the large-scale preparation of spinach chloroplasts. This has, in turn, provided larger quantities of chloroplast DNA than possible by other procedures. Thirty-five percent of the chloroplast DNA has a different buoyant density, T_m, base composition, and renaturation properties than nuclear DNA. Sixty-five percent of the chloroplast DNA has the same buoyant density and T_m as nuclear DNA, but differs in base composition and renaturation properties. A second satellite DNA with a buoyant density of 1.719 g/cc was found.

Literature Cited

1. ANDERSON, W. G., W. W. HARRIS, S. A. BARBER, C. T. RANKIN, AND E. D. CANDLER. 1966. Separation of subcellular components and viruses by combined rate- and isopycnic-zonal centrifugation. The development of zonal centrifuges. NCI Monograph No. 21, United States Government Printing Office, Washington, D. C. p 253–69.

2. AMES, B. N. AND D. T. DUBIN. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. 235: 769–75.

3. BECKMAN INSTRUCTION MANUAL L4-1M-1. 1966. Model L-4 Preparative Ultracentrifuge. Spinco Division Technical Publications Department, Beckman Instruments, Incorporated, Palo Alto, California.

4. BENDICH, A. 1957. Methods for the characterization of nucleic acids by base composition. Methods In Enzymology 3: 715–23.

5. BOARDMAN, N. K., R. I. B. FRANCKI, AND S. G. WILDMAN. 1966. Protein synthesis by cell-free extracts of tobacco leaves. J. Mol. Biol. 17: 470–89.

6. BRAWERMAN, G. AND J. M. EISENSTADT. 1964. Deoxyribonucleic acid from the chloroplasts of Euglena gracilis. Biochim. Biophys. Acta 91: 477–85.

7. BRITTEN, R. J. AND D. E. KOHNE. 1968. Repeated sequences in DNA. Science 161: 529–40.

8. CHUN, E. H. L., M. H. VAUGHAN, AND A. RICH. 1963. The isolation and characterization of DNA associated with chloroplast preparations. J. Mol. Biol. 7: 130–41.

9. FLAMM, W. G., H. E. BOND, AND H. E. BURR. 1966. Density-gradient centrifugation of DNA in a fixed-angle rotor. Biochim. Biophys. Acta 129: 310–19.

10. GRANICK, S. AND A. GIBOR. 1967. The DNA of chloroplasts, mitochondria, and centrioles. Progr. Nucl. Acid Res. Mol. Biol. 6: 143–86.

11. GREEN, B. R. AND M. P. GORDON. 1967. The satellite DNA's of some higher plants. Biochim. Biophys. Acta 145: 378–90.

12. HAGEMANN, R. 1963. Advances in the field of plastid inheritance in higher plants. Genetics Today, Vol. 3. Pergamon Press, New York. p 613–25.

13. KIRK, J. T. O. AND R. A. E. TILNEY-BASSETT. 1967. The Plastids. W. H. Freeman and Company, New York.

14. MARMUR, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3: 208–18,

15. PARENTI, F. AND M. M. MARGULIES. 1967. *In vitro* protein synthesis by plastids of *Phaseolus vulgaris.* Plant Physiol. 42: 1179–86.

16. POLLARD, C. J. 1964. The deoxyribonucleic acid content of purified spinach chloroplasts. Arch. Biochem. Biophys. 105: 114–19.

17. TEWARI, K. K. AND S. G. WILDMAN. 1966. Chloroplast DNA from tobacco leaves. Science 153: 1269–71.

18. WELLS, R. AND M. L. BIRNSTIEL. 1967. A rapidly renaturing deoxyribonucleic acid component associated with chloroplast preparations. Biochem. J. 105: p 53–54.

19. WHITFELD, P. R. AND D. SPENCER. 1968. Buoyant density of tobacco and spinach chloroplast DNA. Biochim. Biophys. Acta 157: 333–43.

20. WOODCOCK, C. L. F. AND H. FERNANDEZ-MORAN. 1968. Electron microscopy of DNA conformations in spinach chloroplasts. J. Mol. Biol. 31: 627–31.

21. YOTSUYANAGI, Y. AND C. GUERRIER. 1965. Mise en evidence par des techniques cytochimiques et al microscopie electronique d'acide desoxyribonucleique dans les mitochondres et les proplastes d'allium cepa. Compt. Rend. 260: 2344–49.