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SOME PHYSICAL AND FUNCTIONAL CHARACTERISTICS
OF CHLOROPLAST DNA

by

Alan John Jaworski

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1969
I hereby recommend that this dissertation prepared under my direction by Alan John Jaworski entitled Some Physical and Functional Characteristics of Chloroplast DNA be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

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ABSTRACT

Chloroplast and nuclear DNA have been isolated from tobacco, pumpkin, and tomato. The buoyant density and renaturation properties of each DNA were investigated in an effort to establish criteria sufficient to distinguish the chloroplast and nuclear DNA's from each other. The chloroplast DNA's from all three plants were found to consist of two components designated major and minor. In agreement with other workers, pumpkin and tomato nuclear DNA's consisted of main band and satellite components while tobacco nuclear DNA does not contain a satellite component.

Buoyant densities in CsCl of the chloroplast major and minor components respectively were 1.700 and 1.707 for tobacco, 1.699 and 1.706 for pumpkin, and 1.700 and 1.707 for tomato. Both components of all three chloroplast DNA's renatured readily after denaturation. Buoyant densities in CsCl for the nuclear main band and satellite component, respectively, were 1.697 and 1.707 for pumpkin, and 1.695 and 1.706 for tomato. Tobacco nuclear DNA had a buoyant density of 1.698 in CsCl. Only the satellite components of pumpkin and tomato nuclear DNA's were found to renature readily while a readily renaturable component was not found in tobacco nuclear DNA.
Criteria were established which could detect nuclear DNA contamination of chloroplast DNA. Tobacco chloroplast DNA was considered pure if it did not contain unrenaturable DNA. Pumpkin chloroplast DNA was considered pure if the ratio of major and minor components did not change after denaturation-renaturation. The tomato nuclear main band and chloroplast component were resolvable on CsCl gradient and thus, tomato chloroplast DNA was considered pure when no DNA was detectable which had the same buoyant density as nuclear main band.

The RNA's from both 70s and 80s ribosomes hybridized with pumpkin and tobacco chloroplast DNA indicating that cistrons for both 70s and 80s rRNA are present in the chloroplast DNA of these two species. Fractionation of tobacco and pumpkin chloroplast DNA on preparative CsCl gradient followed by hybridization of each fraction to rRNA resulted in evidence that both 70s and 80s rRNA cistrons are located in the minor component of the chloroplast DNA.
INTRODUCTION

This dissertation reports the isolation of chloroplast deoxyribonucleic acid (DNA) from higher plants and information as to the nature and function of this material. The purpose of this work was twofold: (1) to ascertain whether or not chloroplast DNA contains cistrons for any of the plant ribosomal ribonucleic acids (rRNA's) and (2) to determine some of the physical and chemical properties of chloroplast DNA which might distinguish it from other DNA also present in the cell.

In eukaryotic organisms the major portion of DNA (approximately 90%) is found in nuclei. However, within the past several years it has been demonstrated that chloroplasts and mitochondria also contain DNA. Genetic analysis has served to identify many of the functional aspects of the nuclear DNA; however, very little is known about the function of the DNA found in organelles outside of the nucleus. As a start in defining the functional role of extranuclear DNA, the possible presence of ribosomal RNA genes in the chloroplast DNA was investigated.

It has been observed that chloroplasts contain only 70s ribosomes while the cytoplasm contains only 80s ribosomes. Both classes of ribosomes provide a readily available source for RNA and enable one to compare gene products.
(RNA) present in cytoplasm with those present in the chloroplast. A general working hypothesis has been that cytoplasmic RNA is derived from nuclear DNA while chloroplast RNA is derived from chloroplast DNA. It was reported, however, that nuclear DNA contains cistrons for both 70s and 80s rRNA (Tewari and Wildman, 1968) indicating that gene products found in the chloroplast could be derived from the nucleus. It was of interest, therefore, to determine whether or not chloroplast DNA contains cistrons for either or both cytoplasmic (80s) rRNA and chloroplast (70s) rRNA.

The determination of the physical properties of chloroplast DNA was considered necessary before the functional properties were investigated because previous reports on these properties were confusing and not in agreement with each other. It was therefore essential that sufficient criteria be established to not only define the chloroplast DNA, but also to serve as a measure of the purity of the DNA especially with regard to contamination by DNA from other sources. The results of these investigations establish the criteria necessary to define pure chloroplast DNA and also indicate that chloroplast DNA contains both 70s and 80s rRNA cistrons.
LITERATURE REVIEW

It is now fairly well recognized that chloroplasts contain DNA which can be extracted and shown to have unique properties. This conclusion is of fairly recent vintage and was only firmly established within the past few years, preceding slightly, and mostly concurrent with, the work done for this dissertation. Prior to the actual isolation of DNA from chloroplasts, evidence had been accumulating for several decades which strongly indicated that chloroplasts possessed genetic determinants and, as a consequence, must contain some form of nucleic acid. This early evidence is reviewed by Granick and Gibor (1967) and Rabinowitz (1968).

Geneticists detected both morphological and biochemical mutants of chloroplasts which exhibited a non-Mendelian pattern of inheritance indicating the presence of non-chromosomal or extranuclear genes. In cytological studies, chloroplasts were shown to be self-duplicating structures that result from division of pre-existing plastids or plastid precursors (Green, 1964). Since the progeny of these divisions were observed to be structurally and functionally identical to the parent, it was inferred that some transfer of genetic information occurred. Further cytological investigation demonstrated that
multiple clones of plastids co-existed within a single cell which were maintained through subsequent cell divisions thus demonstrating that at least some structural determinants did not originate in the nucleus. These observations formed the basis for investigations into the nature and function of chloroplast DNA.

**Evidence for the Existence of Chloroplast DNA**

Evidence for the existence of chloroplast DNA has been accumulated utilizing a wide variety of approaches and a broad spectrum of plant systems (Kirk, 1967). It was not until the last decade, however, that the technology was available to demonstrate directly the existence of chloroplast DNA as a species distinct from that found in the nucleus. Perhaps the simplest technique utilized was light microscopy of tissues specifically stained for DNA. Chiba (1951) reported the presence of Feulgen positive material in the chloroplasts of _Selaginella savatici_ , _Tradescantia fluminensis_ , and _Rhoeo discolor_ which disappeared when nucleic acids were removed with hot trichloroacetic acid. Feulgen staining was also used by Flaumenhaft, Conrad, and Katz (1960) to detect DNA in the chloroplasts of _Chlorella_. In this study, as well as others (Ris and Plaut, 1962; Kislev, Swift, and Bogorad, 1965), the Feulgen positive material was demonstrated to be susceptible to DNase digestion which specifically identified the stained material.
as DNA. However, studies of this type are not sensitive enough to consistently detect small amounts of DNA as evidenced by the fact that Littau (1958) was unable to detect chloroplast DNA by Feulgen staining of the same tissues utilized by Chiba.

The problems of detection encountered by the light microscopists were somewhat obviated by the use of electron microscopy and autoradiography. Ris and Plaut (1962) using electron microscopy were able to detect fibrils 25-30 Å thick in chloroplasts of *Chlamydomonas*. These fibrils could no longer be detected in chloroplasts of cells pretreated with DNase, which, together with the similarity of the fibrils to DNA fibers observed in bacteria, was offered as evidence for the existence of DNA in the chloroplasts of these cells. Kislev et al. (1965) have studied the chloroplasts of swiss chard in much the same manner as Ris and Plaut and concluded that this plant also contains chloroplast DNA. More recently, Gibbs (1968) has published electronmicrographs which show two DNA areas in chloroplasts of *Ochromonas*. She offered as additional evidence autoradiographs of cells labeled for eighteen hours with $^3$H-thymidine which showed a preferential association of grains with these areas. Autoradiography has been used by a number of other workers (Brachet, 1958; Stocking and Gifford, 1959; Wollgiehn and Mothes, 1964; Sagan, et al., 1964) to demonstrate the existence of chloroplast DNA in
such diverse species as Acetabularia, Spirogyra, Nicotiana rustica, and Euglena gracilis.

Perhaps the most direct method utilized to demonstrate the existence and, in turn, to investigate the function of chloroplast DNA has been the isolation of the DNA from purified preparations of chloroplasts. The major difficulty with this technique has been the purification of chloroplasts free from other DNA-containing material such as nuclear fragments and mitochondria. Early attempts to isolate chloroplast DNA depended on differential centrifugation to purify the chloroplasts (Kirk and Tilney-Bassett, 1967, pp. 307-310). Iwamura (1960) used this technique to purify Chlorella chloroplasts and isolated a fraction of DNA which was biochemically distinct from nuclear DNA. His method of isolation involved disruption of the cells followed by high speed centrifugation to yield a pellet rich in chloroplasts and, after additional centrifugation, a supernatant fraction containing most of the nuclear DNA. He reported that the DNA obtained from the chloroplast pellet had a different base composition from that of the nuclear DNA and he later (Iwamura, 1962) presented evidence that the chloroplast DNA was metabolically more active than the nuclear DNA. Shortly thereafter, Chun, Vaughn, and Rich (1963) isolated DNA from chloroplast enriched fractions of spinach, beet, Chlamydomonas, and Chlorella and used cesium chloride density gradient analysis to detect
differences among the DNA's isolated. This technique separates DNA species according to their guanine plus cytosine content (Schildkraut, Marmur, and Doty, 1962) and, therefore, not only provides information on the base composition of each DNA but also can detect multiple species of DNA in a single preparation. The techniques employed by Chun et al. were not sufficient to isolate chloroplasts free from nuclear contamination, however, the authors considered chloroplast DNA as a component distinct in buoyant density from that of nuclear DNA.

Due to the difficulties encountered in extracting chloroplast DNA free from nuclear contamination, much of the early information concerning chloroplast DNA was obtained by indirect approaches. Gibor and Izawa (1963) circumvented the problem of nuclear contamination by isolating chloroplasts from enucleated Acetabularia. Although this technique has limited applicability, it was significant in that it provided a system for the study of chloroplast DNA with relatively simple methods for the purification of the plastids. Leff et al. (1963) demonstrated the presence of DNA in chloroplasts of Euglena and Chlamydomonas indirectly by a comparison of CsCl density gradient profiles of DNA extracted from normal and aplastidic algae. In this study, loss of a DNA component in aplastidic cells was taken as evidence for a DNA component in chloroplasts. The work on Euglena was expanded
by a number of workers (Brawerman and Eisenstadt, 1964a; Ray and Hanawalt, 1964, 1965; Edelman, Shiff, and Epstein, 1965; Edelman et al., 1964) to include experiments in which attempts were made to purify the chloroplasts. In no case were any of the chloroplast preparations reported free from nuclear DNA. However, these workers did assign a buoyant density of 1.685 $\text{gm cm}^{-3}$ for chloroplast DNA as opposed to 1.708 $\text{gm cm}^{-3}$ for nuclear DNA on the basis of an increase in the percentage of the 1.685 component in chloroplast enriched preparations. Brawerman and Eisenstadt were able to remove most of the 1.708 component by gentle lysis of chloroplasts with MgCl$_2$ and deoxycholate which further supported the argument that the 1.685 component was chloroplastic. Base analysis of each component after purification on preparative CsCl gradients revealed that not only were the base ratios different between the two DNA's but 5-methylcytosine was found in the 1.708 or nuclear component and could not be detected in the 1.685 (chloroplast) component. Kirk (1963a, b) isolated chloroplasts from broad bean leaves by sucrose density gradient centrifugation and was able to extract a DNA which differed from nuclear DNA in base composition. However, the total chloroplast DNA in this study was analyzed for base composition by direct chemical analysis (rather than CsCl density gradients) which was not sufficient to detect the presence of small amounts of nuclear DNA.
Chloroplast DNA has been isolated from tobacco leaves to various degrees of purity and with somewhat conflicting results in regard to buoyant density values. Lyttleton and Peterson (1964) were able to detect only a single DNA component on CsCl gradients of total leaf DNA and concluded that if chloroplast DNA was present in their preparations, it must have a base composition quite similar to that of the nuclear DNA. In contrast to Lyttleton and Peterson, tobacco chloroplast DNA was reported by Shipp, Kieras, and Haselkorn (1965) to have a buoyant density of 1.703 g/cm$^3$ while that of the nucleus was 1.690 g/cm$^3$. In addition, it was reported that the rate of incorporation of radioactive phosphorus into chloroplast DNA was greater than that observed for nuclear DNA. Green and Gordon (1966) reported buoyant densities of 1.696 and 1.706 g/cm$^3$ for nuclear and chloroplast DNA respectively. Although the reported values for buoyant densities were conflicting in these two studies, the results of studies on the incorporation of DNA precursors into chloroplast DNA were, in principle, the same. In each case it was shown that chloroplast DNA incorporated label at a greater rate in developing seedlings than did the corresponding nuclear DNA. Tewari and Wildman (1966) have reported buoyant density values of 1.697 and 1.702 g/cm$^3$ for tobacco nuclear and chloroplast DNA's respectively. However, in this study the DNA was isolated from chloroplasts purified on discontinuous
sucrose density gradients whereas Shipp et al. used differential centrifugation and Green and Gordon extracted total DNA from the seedlings. Tewari and Wildman reported the isolation of chloroplast DNA free from nuclear DNA contamination and, more importantly, established that purified chloroplast DNA readily renatured after denaturation which was not true for nuclear DNA. Base analysis showed that chloroplast DNA lacked 5-methylcytosine whereas the base was present in the case of nuclear DNA in agreement with other plant systems previously studied.

Whitfeld and Spencer (1968) reported the isolation of tobacco and spinach chloroplast DNA from chloroplasts purified on discontinuous sucrose gradients according to the method of Tewari and Wildman (1966) but their preparations of chloroplast DNA had buoyant densities that were identical with those found for nuclear DNA. This agreed with the conclusion of Lyttleton and Peterson (1964) for the buoyant density of tobacco chloroplast DNA. They could offer no explanation for the difference in buoyant density values between their preparations and those of others but ruled out the possibility of nuclear contamination by the following criteria: Their chloroplast DNA renatured readily after denaturation which was not true for nuclear DNA and in neither tobacco nor spinach could they detect 5-methylcytosine in the chloroplast DNA but could detect this base in the nuclear DNA.
With increasing studies on the physical properties of chloroplast DNA, evidence began to accumulate which indicated that chloroplast DNA had more than one component; one of the components had a buoyant density similar to that of the nuclear DNA. Thus it became essential that one be able to isolate homogeneous preparations of chloroplast DNA and establish rigid criteria for the purity of the preparations. This was emphasized in a report by Bard and Gordon (1969) in which DNA extracted from purified spinach chloroplasts resolved into two components on CsCl density gradients. One component differed from nuclear DNA in buoyant density, base composition with respect to 5-methylcytosine, and renaturation properties while the other component had the same buoyant density as nuclear DNA but differed from it in base composition and renaturation properties. The fact that this latter component differed in both base composition and renaturation properties prompted the authors to conclude that the chloroplast DNA was not contaminated with nuclear DNA.

Iwamura and Kuwashima (1969), working with Chlorella have reported the isolation of two distinct DNA components from purified chloroplasts, one of which had a buoyant density similar to that of nuclear DNA. The authors concluded that both components were intrinsically associated with the chloroplasts and neither was a result of nuclear contamination. They noted that previous workers usually
assumed that DNA obtained from chloroplast preparations in which one component had a buoyant density similar to nuclear DNA was a result of contamination by the nuclear species. They concluded that this was not necessarily true and that a careful investigation is necessary before any component can be regarded as contamination.

In summary, it was not until the last few years that techniques have been established which have permitted a careful physical-chemical examination of the DNA in chloroplasts. In this context it became necessary to establish criteria sufficient to define chloroplast DNA uniquely in an effort to resolve discrepancies that have been reported by various authors in regard to the characterization of this DNA.

**Function of the Chloroplast DNA**

Much of the present information on chloroplast function has been derived from studies on isolated chloroplasts. Although, in most cases, this type of study does not yield unequivocal results concerning the role of chloroplast DNA, this approach has been of value in establishing the potential autonomy of chloroplasts. According to modern concepts of molecular biology, for complete autonomy a living system would at least require a self-duplicating genetic system (usually DNA but possibly RNA) and a mechanism to express the genes present in that
Experiments were therefore designed to establish whether or not chloroplasts contained any or all of the necessary systems required for genetic autonomy. This involved a search for DNA polymerase, RNA polymerase, and components of a protein synthesizing system which could be defined as chloroplast specific and functionally independent of the rest of the cell.

Reports from a number of different workers established that chloroplasts contain functionally independent DNA polymerase and RNA polymerase systems. A number of investigators (Spencer and Whitfeld, 1967a; Scott, Shah, and Smillie, 1968; Tewari and Wildman, 1967) were able to demonstrate the presence of DNA polymerase in chloroplasts and in all cases the product synthesized was identified as chloroplast DNA by CsCl density gradient analysis.

Chloroplast DNA replication has been studied in *Chlamydomonas* (Chiang and Sueoka, 1967) but the authors did not conclude anything concerning the origin or specificity of the polymerase involved. They did demonstrate, however, that the chloroplast DNA replicated in a semiconservative manner as is the case for bacterial DNA (Meselson and Stahl, 1958) and also that the replication occurred prior to nuclear DNA replication. Green and Gordon (1966), working with tobacco, concluded that chloroplast DNA synthesis occurred at a greater rate than nuclear DNA synthesis but did not present data concerning the mode of
replication or the origin and specificity of the enzyme involved. These studies did not rule out the possibility that the polymerase responsible for the DNA replication was coded for by a nuclear gene.

Studies by Spencer and Whitfeld, 1967b) and Kirk (1964) have demonstrated the presence of a DNA dependent RNA polymerase in chloroplasts of higher plants but it is not as yet known whether the polymerase is coded for by chloroplast DNA. However, from these studies it was concluded that chloroplasts do have the capacity to synthesize RNA from chloroplast DNA templates because in studies of this nature presumably the only available template for the RNA polymerase was the chloroplast DNA. In addition, it has been tentatively concluded by some investigators (Spencer and Whitfeld, 1967b; Brawerman and Eisenstadt, 1964b) that at least a portion of the RNA product from the DNA-dependent RNA polymerase reaction in chloroplasts was messenger RNA. Brawerman and Eisenstadt reported that fractions of chloroplast RNA isolated on a sucrose gradient were capable of stimulating the incorporation of amino acids into protein while the conclusion Spencer and Whitfeld offered was based on the rapid acquisition of a pulse label and the sedimentation behavior of the RNA.

Together, the DNA polymerase and RNA polymerase studies have shown that at least a portion of both chloroplast DNA and RNA can be synthesized within a plastid and,
if it is assumed that the respective polymerases have their origin in chloroplast DNA, then it can be concluded that chloroplasts possess an autonomous self-replicating genetic system.

The biochemical evidence for the expression of the chloroplast DNA is not as well established as is its existence, although it has been demonstrated that chloroplasts possess protein synthetic capabilities. In this regard, perhaps the most thoroughly investigated structure has been the chloroplast ribosome. Lyttleton (1962) first reported the isolation of ribosomes from spinach chloroplasts and noted that they differed from cytoplasmic ribosomes in sedimentation behavior. From this and the work of others (Sager and Hamilton, 1967; Rawson and Stutz, 1968; Chen and Wildman, 1967; Spencer and Wildman, 1964; Loening, 1968; Stutz and Noll, 1967; Brawerman, 1963; Sissakian et al., 1965) it has been established that there are two classes of ribosomes in plant cells—an 80s particle found in the cytoplasm and a 70s particle found in chloroplasts. The 70s ribosomes dissociate into 50s and 30s subunits containing 23s and 16s ribosomal RNA, respectively, while the 80s particles dissociate into 60s and 40s subunits containing 25s and 18s ribosomal RNA, respectively. The chloroplast ribosomes are capable of stimulating protein synthesis in cell free extracts as well as in isolated chloroplasts and it was shown, in at least one study
(Chen and Wildman, 1967), that protein synthesis in chloroplasts occurs on polyribosomes. This latter study is additional evidence to that of Spencer and Whitfield (1967b) and Brawerman and Eisenstadt (1964b) that messenger RNA is present in chloroplasts. Tewari and Wildman (1968) along with Scott and Smillie (1967) have concluded from DNA-RNA hybridization studies that the chloroplast DNA contains coding sites for the chloroplast ribosomal RNA. In the study by Tewari and Wildman it was also concluded that tobacco nuclear DNA contains coding sites for both 70s and 80s ribosomal RNA and they calculated that there are more 70s sites in a nucleus than in a chloroplast. The significance of the latter finding is as yet unclear.

With the exception of ribosomal RNA cistrons, very little is known about structural genes in chloroplast DNA. Smillie et al. (1967) have reported that, in intact Euglena cells, some proteins associated with the Calvin cycle and photosynthetic electron transport system of chloroplasts are synthesized on chloroplast ribosomes. His conclusion was based on the cessation of synthesis of these proteins when the chloroplast ribosomes were selectively inactivated by chloramphenicol. This approach however was only sufficient to conclude that the proteins were synthesized in the chloroplast and did not establish that the genes for those proteins were on the chloroplast DNA. With this background in mind, work was initiated on the physical and
functional properties of chloroplast DNA with the hope of at least a partial elucidation of the structural genes present in chloroplast DNA.
MATERIALS AND METHODS

Isolation of Chloroplasts

Leaves 5-9 cm long of *Nicotiana tabacum* L. var. Samsun (tobacco) were deribbed and chopped with razor blades in 2 volumes Honda medium (Honda, Hongladarom, and Wildman, 1962) (0.25 M sucrose, 2.5% Ficoll, 5% Dextran T 40, 0.025 M Tris pH 7.8, 0.001 M Mg acetate, 0.004 M mercaptoethanol) according to the method of Tewari and Wildman (1966). The resulting brei was filtered through two layers of Miracloth (Chicopee Mills, Inc.) and the filtrate was then centrifuged at 1000 x g at 4°C for fifteen minutes. Pellets consisting of chloroplasts and nuclei from 15 gm tissue were resuspended in 4 ml Honda medium, layered on discontinuous sucrose gradients, and centrifuged at 4°C for 2 hours at 23,000 r.p.m. in the Beckman SW 25.1 rotor. The discontinuous gradients were prepared by layering successively 10 ml of 60% (w/w) sucrose, 10 ml 45% sucrose, and 5 ml 20% sucrose each containing 0.01 M Tris pH 8.2 and 0.001 M MgCl₂, in 1-in x 3-in cellulose nitrate tubes. The chloroplasts, most of which banded at the 20-45% sucrose interface following centrifugation, were collected and the chloroplast suspension was diluted with 2 volumes of buffer (0.4 M sucrose, 0.05 M Tris pH 7.8, and 0.003 M CaCl₂). The chloroplasts were then pelleted by centrifugation at 10,000
x g for 20 minutes and resuspended in a minimal volume of SSC (0.15 M NaCl and 0.015 M Na citrate). Pumpkin (Cucurbita pepo L. var. Small Sugar) chloroplasts were isolated in the same manner except that leaves were 2-5 cm long and cell homogenates were centrifuged at 2,500 x g to pellet the chloroplasts and nuclei.

**Purification of Chloroplasts by Glycerol Density Gradients**

In some cases, chloroplasts, first isolated by chopping and sucrose gradient purification as described above, were treated according to the method of Jagendorf (1955). Jagendorf's glycerol medium was prepared by mixing 5 ml glycerol with 3 ml phosphate buffer (0.067 M) containing 1.3 gm sucrose. Chloroplast pellets corresponding to 45-50 gm tissue were resuspended in buffer medium (0.2 M sucrose, 0.01 M Tris pH 7.8, and 0.001 M MgCl₂) to a final volume of 5 ml and mixed with 20 ml Jagendorf's glycerol medium. The homogeneous suspension was then centrifuged for three hours at 22,500 r.p.m. and 4°C in the Spinco SW 25.1 rotor. Following centrifugation the chloroplasts which floated were collected, diluted with an equal volume of buffer medium and pelleted by centrifugation at 10,000 x g for 20 minutes.
Extraction and Purification of DNA

Chloroplast or nuclear pellets were resuspended in a minimal volume of SSC and made to 0.02 M in EDTA (disodium ethylenediaminetetraacetate) and 1% in SLS (sodium lauryl sulfate). The resulting lysate was frozen and then incubated at 50°C for 30 minutes after which Pronase (Calbiochemicals B grade) was added to a final concentration of 1 mg/ml and the suspension was further incubated at 50°C for four hours followed by an eight-ten hour incubation at 37°C. Following incubation an equal volume of Tris (0.05 M, pH 7.8) saturated phenol was added to the suspension and it was gently shaken for 30 minutes at room temperature. Phenol and aqueous phases were separated by centrifugation at 3,000 x g for five minutes. The upper aqueous phase was collected and the phenol wash repeated. Chloroplast DNA and RNA were precipitated by adding 2.5 volumes cold 95% ethanol to the aqueous phase and letting the solution stand in ice for one hour or longer. Both DNA and RNA were pelleted by centrifugation at 7,000 x g for 20 minutes and then dissolved in 1 ml 0.1 x SSC. RNA was removed by incubation of the DNA-RNA (made to SSC by the addition of 10 x SSC) with heat treated (90°C for 10 minutes) pancreatic RNase (Worthington Ribonuclease A) at a concentration of 50 μg/ml for 30 minutes at 37°C. RNase was then hydrolyzed by incubation with Pronase (100 μg/ml) at 37°C for one hour. Ribonucleotides, remaining Pronase, and other contaminants
were then separated from the DNA by Sepharose chromato-
graphy. Sepharose 4B (Pharmacia) was packed in a column 1 cm x 16 cm and washed with 1 x SSC until all U.V. absorbing material was removed. A 1-2 ml DNA sample was then applied and eluted with 1 x SSC. Fractions of approximately 1 ml were collected and absorbance at 260 μl was measured. DNA was usually found in fractions 4-6 with the remaining fractions containing the ribonucleotides and other low molecular weight material. The fractions containing DNA were pooled and either used directly or concentrated by covering a dialysis bag with Ficoll. Nuclear DNA was treated in the same manner except that following the addition of cold 95% ethanol, the solution was gently shaken back and forth in a centrifuge tube until the DNA was a fibrous precipitate which normally adhered to the glass surface of the tube. The ethanol solution was decanted and the DNA dissolved in an appropriate volume of 0.1 x SSC.

Analytical Cesium Chloride Density Gradient Analysis

Cesium chloride was dissolved in 0.05 M Tris buffer pH 8.5 containing 3 μg DNA and 1 μg Micrococcus lyso-
deikticus DNA as marker. Routinely, 0.75 gm CsCl was dissolved in a 0.6 ml DNA solution and the final density adjusted to 1.700 (+ .003) gm cm⁻³ with the aid of a Zeiss refractometer (Ifft, Voet, and Vinograd, 1961). DNA
solutions were loaded in a cell containing a plastic Kel-F centerpiece and centrifuged at 44,000 r.p.m. at 20°C in the Spinco model E analytical ultracentrifuge equipped with ultraviolet optics and the four cell mask and timer accessories. After 18-20 hours centrifugation, U.V. absorption photographs were taken on Kodak commercial film and the negatives were traced on a Beckman model RB Analytrol densitometer equipped with a film densitometer accessory. Buoyant densities were calculated according to the method of Sueoka (1961) using the following relationship:

\[ \rho = \rho_0 + 0.00892 (r^2 - r_0^2) \text{ gm cm}^{-3} \]

where

\[ \rho_0 = \text{density of the marker DNA (the DNA of } \text{M. lysodeikticus} \text{ has a buoyant density of } 1.731 \text{ in CsCl)} \]

\[ r_0 = \text{distance of the marker DNA from the axis of rotation} \]

\[ r = \text{distance of the sample DNA from the axis of rotation} \]

**Preparative Cesium Chloride Density Gradient Centrifugation**

The method of Flamm, Bond, and Burr (1966) was employed using the Spinco model L-2 ultracentrifuge with the no. 50 angle type rotor. DNA (20-40 \( \mu \)g) in 3.25 ml
0.05 M Tris buffer pH 8.5 was added to 4.25 gm CsCl and the final density adjusted with the aid of a refractometer to 1.704 (± 0.003) gm cm⁻³. The solutions were placed in 5/8 in x 2-1/2 in polyallomer tubes and the remaining volume filled with mineral oil. Samples were centrifuged at 35,000 r.p.m. at 20°C for 64-68 hours after which the rotor was allowed to come to rest without braking. After centrifugation, fractions were collected by piercing the bottom of the centrifuge tube with a 20 gauge needle and collecting an appropriate number of drops for each fraction (usually 15 or 18). Each fraction was diluted with 0.95 ml 2 x SSC and the O.D. 260 mך measured.

Denaturation-Renaturation of the DNA

DNA (2-3 µg) in 0.35 ml 2 x SSC was denatured by the addition of 0.1 ml M NaOH to raise the pH to 12-12.5. This solution was allowed to stand at room temperature for fifteen minutes after which 0.15 ml M KH₂PO₄ was added to lower the pH to 6.6-6.8. Samples were either placed in ice to keep the DNA denatured or renatured by incubation at 68°C for 2 hours followed by slow cooling for an additional three hours. Fractions from preparative CsCl gradients which had been diluted with 0.95 ml 2 x SSC were also denatured by the addition of 0.1 ml M NaOH. These samples were allowed to stand at room temperature for fifteen minutes after which 0.15 ml M KH₂PO₄ was added.
Extraction of $^3$H Labeled RNA

Labeled ribosomal RNA was prepared from roots and leaf tissue of tobacco and pumpkin. In the case of pumpkin, leaves approximately 2-3 cm long were excised from 3 week old plants grown under ordinary greenhouse conditions. Transverse slices were made approximately 2 mm apart beginning at the midrib and extending to the edge with care taken not to slice through the leaf edge (Zaitlin, Spencer, and Whitfeld, 1968, p. 92). Incubation was carried out for 48 hours at 28°C in a large petri dish containing 1 mc 5 $^3$H uridine. (ICN Spec. Act. 2.4 C/mM) in 10 ml 0.01 M $\text{KH}_2\text{PO}_4$. Washed roots were incubated in the dark for 48 hours at room temperature in a beaker containing 0.5 mc 5-$^3$H-uridine in 5 ml 0.01 M $\text{KH}_2\text{PO}_4$. During the incubation tap water was added as needed. Following incubation, the tissues were homogenized in a chilled mortar containing 2 volumes grinding medium (0.05 M Tris, pH 7.5, 0.01 M KCl, 0.01 M $\text{MgCl}_2$, 0.004 M mercaptoethanol) and then filtered through two layers of Miracloth (Chicopee Mills, Inc.). Triton X-100 (Rohm and Haas) was added to 4% in the case of leaf tissue and the homogenates centrifuged at 12,000 x g for 20 minutes. The resulting supernatants were centrifuged at 160,000 x g for 60 minutes to yield a crude ribosomal pellet which was resuspended in 2 ml 0.067 M phosphate buffer containing 1% SLS. An equal volume of phenol was added and the mixture shaken for 20 minutes after which
phases were separated by centrifugation at 3,000 x g for 5 minutes. The aqueous phase was repeatedly extracted with phenol until no visible denatured protein remained at the phenol-aqueous interface. RNA in the aqueous phase was then precipitated with 2.5 volumes of 95% ethanol and centrifuged at 7,000 x g for 15 minutes. RNA pellets were washed once with cold 95% ethanol and then dissolved in 0.067 M phosphate buffer. 4 M LiCl in distilled water was added to a final concentration of 2 M and the solution was allowed to stand at 0°C for 10 hours. This procedure is known to precipitate rRNA and leave tRNA and other low molecular weight RNA in solution (Baltimore, 1966). In addition, any remaining phenol will remain in solution. RNA thus precipitated was collected by centrifugation at 7,000 x g for 15 minutes and dissolved in 1 ml 0.067 M buffer. In order to extract tobacco rRNA, leaves 5-9 cm long were excised from plants 5 inches high grown under ordinary greenhouse conditions. Roots, also from 5 inch plants, were washed and incubated in 3H uridine while still attached to the plant as was done in the case of pumpkin. Conditions for labeling and RNA extraction were the same as described for pumpkin except that the leaf incubation was carried out in a solution which contained approximately 20 μc/ml 5-3H-uridine.
**Polyacrylamide Gel Electrophoresis of RNA**

Gel electrophoresis was carried out according to the method of Bishop, Claybrook, and Spiegelman (1967). Polyacrylamide 2.4% gels were prepared by mixing 4 ml of an aqueous stock solution of 15% recrystallized (from chloroform) acrylamide (Eastman Organic Chemicals), 0.75% recrystallized (from acetone) bis-acrylamide with 12.45 ml water, and 8.33 ml 3E buffer (0.12 M Tris, 0.06 M sodium acetate, and 0.003 M NaEDTA made pH 7.2 with 6 ml glacial acetic acid), 0.02 ml of N,N,N',N' tetramethylethylene-diamine and 0.2 ml of fresh aqueous 10% ammonium persulfate solution. The gel solution was transferred to plexiglass tubes (1/4 in x 4 in) held upright by rubber cups. Polymerization was allowed to proceed for 30 minutes after which the gels were pre-run for 30 minutes at room temperature in E buffer (one third 3E concentration), containing 0.2% SLS, at 5 mA per tube on a Canalco Model 6 gel electrophoresis apparatus equipped with a Beckman Duostat power supply. Samples of RNA (20-40 µg) in 10% sucrose were layered on top of the gels and electrophoresis was carried out for 2-5 hours at 5 mA per tube. After electrophoresis the gels were transferred to a quartz cuvette (5 mm x 100 mm x 8.5 mm) and scanned at 260 mJ on a Gilford Model 240 spectrophotometer equipped with a Gilford Model 2410 linear transport device and a Heathkit Servo-Recorder model EUW-20A.
Hybridization of RNA to DNA

Hybridization of DNA to ribosomal RNA was carried out according to the method of Gillespie and Spiegelman (1965). An appropriate amount of denatured DNA was diluted to 2 ml in 2 x SSC and passed through a Bac-T-Flex B6 nitrocellulose membrane (Schleicher and Schuell Co.) which had been pre-soaked in 2 x SSC and washed with 10 ml of the same buffer. Each membrane was then washed under gentle suction with 50 ml 2 x SSC and dried for 1-2 hours at room temperature and at 80°C in a vacuum oven for an additional 2 hours. These membranes were used almost immediately for hybridization because membranes lose their hybridization capacity with age presumably due to self annealing of the DNA in the membrane. Hybridization was carried out for 12 hours in 2 x SSC at 68°C without shaking. When hybridization was carried out on fractions collected from preparative CsCl density gradients, membranes were incubated in a common vial at an RNA concentration of 0.6 μg/ml solution. Each vial contained 10 ml labeled RNA solution which was found adequate to completely immerse as many as twenty membranes. Unhybridized RNA ("noise") was then eliminated by washing the membranes in a common beaker according to the procedure of Birnstiel et al. (1968). Membranes (15-20) were first washed for fifteen minutes in 300 ml 6 x SSC followed by three fifteen-minute washes in 300 ml 2 x SSC and a final wash in 300 ml 2 x SSC containing 10 μg/ml heat
treated RNase. Immediately after washing, membranes were dried for one hour at 80°C in a vacuum oven. For hybridization of DNA other than fractions from preparative CsCl gradients, the method of Gillespie and Spiegelman was more closely followed. This involved embedding a known quantity of DNA (ranging from 5-20 μg) on membranes as described above but hybridization was carried out in separate vials containing 1 ml labeled RNA in 2 x SSC at 68°C for 12 hours. Unlabeled RNA was eliminated by placing the membranes on Millipore platforms and washing each side with 50 ml 2 x SSC after which membranes were incubated for one hour at room temperature in 5 ml 2 x SSC containing RNase 20 μg/ml. After incubation, the membranes were again washed on each side with 50 ml 2 x SSC and dried for one hour at 80°C in a vacuum oven.

**Scintillation Counting**

Samples were counted at optimum gain on a Packard model 3320 Tri-Carb scintillation spectrometer using 10 ml 0.5% PPO (2,5 diphenyloxazole) in toluene as a scintillation cocktail. Background was usually 25-30 counts per minute.
RESULTS

Methods which were usually adequate for nuclear DNA extraction were not useful for chloroplast DNA extraction. Chloroplast DNA could not be obtained by phenol extraction of SLS solubilized chloroplasts, a method which was successful for isolation of nuclear DNA. Likewise, the method of Marmur (1961) rarely resulted in the isolation of chloroplast DNA and even then only low yields were obtained. The most consistent method utilized was the Pronase digestion method detailed in the MATERIALS AND METHODS section. Tobacco yields were usually 1 µg chloroplast DNA per gram fresh weight tissue while pumpkin and tomato yielded only 0.5 µg per gram fresh weight tissue.

In order to isolate chloroplasts free from nuclei, the tissues were hand chopped with razor blades in Honda medium. It was found that chopping the tissue was the most consistent method for disrupting cells so that the chloroplasts would not be contaminated with nuclei or nuclear fragments. Grinding the tissue in Honda medium sometimes resulted in uncontaminated chloroplasts however this method was not very consistent and often the chloroplasts would be contaminated. It should be emphasized that chopping did not always result in uncontaminated chloroplasts but on the average it was superior to grinding. In
general, gentle methods for breaking open cells will result in uncontaminated chloroplasts but as yet no single method has been found which will always result in pure plastid preparations.

**Physical Properties of Tobacco Chloroplast DNA**

Prerequisite to any study of chloroplast DNA is the isolation of purified preparations of the DNA free from contamination by DNA from other sources. It is therefore necessary to establish properties of this DNA which not only uniquely define the DNA but also serve as criteria for detection of contaminating DNA. Once the physical properties of the DNA are established and the criteria for purity defined, then the DNA can be studied with regard to function and the results unequivocally attributed to the chloroplast DNA. It is desirable that the criteria for purity be relatively simple in order that the DNA can be easily monitored for contamination. Two properties which can easily be determined are buoyant density and renaturability. The buoyant density is related to the base composition (Schildkraut et al., 1962) of the DNA while the renaturability is related to the size of the DNA and the presence of redundant sequences (Britten and Kohne, 1968). These properties therefore provide measurements of independent properties of the DNA and therefore were hoped to be
sufficient to distinguish two DNA species and at the same time define the purity of a particular DNA preparation.

The buoyant density and renaturability of tobacco chloroplast DNA were determined and compared to the corresponding properties of tobacco nuclear DNA. When tobacco leaf tissue was chopped and the chloroplasts purified on discontinuous sucrose gradients as described in MATERIALS AND METHODS, approximately 80% of the chloroplasts banded at the 20-45% sucrose interface. The nuclei formed a pellet at the bottom of the gradient tube and were used without further purification. Only the chloroplasts which banded at the 20-45% sucrose interface were used for chloroplast DNA extraction in order to prevent possible contamination of the plastids with nuclei. The same procedure was also adopted for the investigations of pumpkin and tomato chloroplast DNA.

Densitometer tracings from analytical CsCl density gradient analyses of tobacco chloroplast DNA are shown in Figure 1. Native chloroplast DNA banded in CsCl as a single peak with a very slight shoulder. This indicated the presence of two DNA components which were designated major and minor for peak and shoulder, respectively. This shoulder comprised approximately 10% of the total chloroplast DNA but this varied from preparation to preparation. Since the shoulder was only slight, it was difficult to determine the percentage of minor component. The reason
Figure 1. Microdensitometer tracings of tobacco chloroplast DNA banded in CsCl gradients.

(a) Native

(b) Denatured

(c) Denatured-Renatured

1.731
1.719
Marker DNA

1.700
1.704
1.706
for the variability in percentages of the two components could possibly be due to a variability in the percentages of intact versus damaged plastids. Although there is no available evidence, it is conceivable that the major component is membrane bound and the minor component is present as a soluble DNA which is lost if the plastids are damaged during the purification procedure. In support of this possibility, phase microscopy of the plastid preparations showed that most plastids were stripped of their outer membrane while a small percentage remained intact. Whether or not this is of any significance is not known because no direct comparison was made between DNA extracted from stripped versus intact plastids.

Buoyant densities of the major and minor components were calculated to be $1.700 \text{ gm cm}^{-3}$ and $1.707 \text{ gm cm}^{-3}$, respectively. The value of 1.700 agrees fairly well with that of Whitfeld and Spencer (1968) for tobacco chloroplast DNA while 1.707 is in agreement with that reported by Green and Gordon (1967). However, Tewari and Wildman (1966) reported that tobacco chloroplast DNA has a buoyant density of 1.703 which disagrees with values obtained in this study. Green and Gordon presented data in which DNA from plastid preparations had two components with buoyant densities of 1.696 and 1.706 but they concluded that the 1.696 component was the result of nuclear contamination and that the tobacco chloroplast DNA was the 1.706 species.
This suggested that perhaps the DNA used in this study was also contaminated. Data will be presented which indicates that the latter was not the case despite the presence of two DNA components in the preparations.

The disparity in values reported for buoyant density in different studies combined with the possibility of nuclear DNA contamination suggested the investigation of another property of the chloroplast DNA to resolve the question of whether or not both components are indeed chloroplastic or the result of contamination. Since Tewari and Wildman reported that chloroplast DNA renatures to a double stranded structure after denaturation and Whitfeld also observed the renaturation of chloroplast DNA in his preparations, it was decided to denature and renature the chloroplast DNA to determine if both components would renature. As shown in Figure 1b, both components denatured to a buoyant density of 1.719 gm cm\(^{-3}\). Renaturation of this DNA resulted in a decrease in buoyant density to 1.704 (Figure 1c) with no DNA detectable at 1.719 indicating that both components had renatured. The renaturation was carried out by subjecting denatured DNA in 2 x SSC to 68°C for two hours followed by slow cooling. The DNA strands renatured under these conditions, but did not return to native buoyant density indicating that the DNA strands did not reanneal to completely double stranded structures. The
rapidity of the renaturability did indicate, however, that there are repetitious sequences in the chloroplast DNA.

The renaturation studies did not rule out the possibility that tobacco nuclear DNA contained a readily renaturable fraction which contaminated the chloroplast DNA. To test this possibility, tobacco nuclear DNA was denatured and renatured and analyzed on CsCl gradients for the presence or absence of a renaturable fraction. Native tobacco nuclear DNA resolved into a single component of buoyant density 1.698 which denatured to 1.715 (Figure 2a, b). When denatured nuclear DNA was renatured under the same conditions used for chloroplast DNA, no renaturable fraction was detected (Figure 2c) which ruled out the possibility that one of the renaturable components of the chloroplast DNA was a nuclear contaminant. The fact that nuclear DNA did not renature also suggested that the presence of unrenaturable DNA in chloroplast DNA preparations could be used as an indication of nuclear contamination.

Results reported thus far were obtained from the chloroplasts which collected at the 20-45% sucrose interface of the discontinuous sucrose gradients. Material was also collected from the 45-60% sucrose interface which was suspected to contain both chloroplasts and nuclei. DNA extracted from this material did show an unrenaturable fraction as shown in Figure 3. In this preparation a large
Figure 2. Microdensitometer tracings of tobacco nuclear DNA banded in analytical CsCl gradients.
Figure 3. Analytical CsCl gradients of tobacco chloroplast DNA contaminated with nuclear DNA.
portion (approximately 70%) was nuclear as evidenced by the amount of unrenaturable DNA. However, if the DNA was contaminated with only 10% nuclear DNA this would be detectable as a band at 1.715 gm cm\(^{-3}\) if the preparation was subjected to denaturation-renaturation. It was concluded that tobacco chloroplast DNA preparations do not contain an unrenaturable DNA component and that tobacco nuclear DNA does not contain a detectable DNA component which is renaturable. From these results it was concluded that the tobacco chloroplast DNA used in this study was free from contamination by DNA from other sources, especially nuclei.

**Physical Properties of Pumpkin Chloroplast DNA**

Comparison of pumpkin chloroplast and nuclear DNA with respect to buoyant density and renaturability resulted in a more complex situation than in the case of tobacco DNA. Densitometer tracings of native, denatured, and denatured-renatured pumpkin chloroplast DNA are shown in Figure 4a, b, c. The native pumpkin chloroplast DNA resolved into two components with buoyant densities of 1.699 and 1.706 gm cm\(^{-3}\) for the major and minor components respectively. In contrast to the tobacco chloroplast DNA, the pumpkin chloroplast DNA minor component resolved into a prominent shoulder on the major peak. Visual inspection of the film clearly showed two bands of DNA corresponding to
Figure 4. Microdensitometer tracings of pumpkin chloroplast DNA banded in analytical CsCl gradients.
the two components observed on the densitometer tracings. Denaturation of the DNA also resulted in two components with buoyant densities of 1.714 and 1.721 gm cm\(^{-3}\) which presumably corresponded to the respective native major and minor components. After renaturation under the conditions described above for tobacco DNA, the two components decreased in buoyant density to 1.704 and 1.713 gm cm\(^{-3}\) which are slightly higher than observed for the native DNA. This result agreed with the behavior of the tobacco chloroplast DNA in that these renaturation conditions are not sufficient to reanneal the denatured DNA to completely double helical structures. It was also observed that the percentage of minor component varied among the different preparations of pumpkin chloroplast DNA but was usually close to thirty per cent of the total. There are at least two possibilities which could explain this variability. Either variable amounts of nuclear DNA contaminated the preparations or the variability was the result of differences in the per cent of intact versus damaged plastids among the chloroplast preparations. Presumably the damaged plastids could lose a portion of their DNA and thus result in decreases of one DNA component.

In order to test for the possibility of contamination by nuclear DNA, a separate study was made of the renaturation properties of the latter. Native pumpkin nuclear DNA resolved into two components with buoyant
densities of 1.697 and 1.707 gm cm\(^{-3}\) which increased to 1.713 and 1.721 gm cm\(^{-3}\) after denaturation (Figure 5a, b). The 1.707 and 1.697 components will be referred to as nuclear satellite and main band respectively. Both nuclear components renatured to a single peak with a buoyant density of 1.712 gm cm\(^{-3}\) (Figure 5c). This peak was composed of renatured satellite and unrenatured main band DNA. This conclusion was based on the following information: Matsuda and Siegel (1967) reported that pumpkin nuclear DNA contains a satellite which readily renatures. Whitfeld (1968) has isolated this nuclear satellite and found that this DNA denatured to 1.723 gm cm\(^{-3}\) and renatured to 1.710 gm cm\(^{-3}\) under the conditions used for renaturation in this study. He also determined that the purified nuclear main band denatured to 1.712 and renatured to 1.711 gm cm\(^{-3}\). Thus, the results of independent studies on nuclear satellite and main band are consistent with the renaturation of total nuclear DNA as a single peak.

Since the nuclear satellite renatured to a buoyant density similar to the minor chloroplast DNA component, it was possible that one of the chloroplast DNA components was the result of contamination by the nuclear DNA. If contamination was indeed the case, then denaturation followed by renaturation of the contaminated chloroplast DNA would result in an increase in the 1.712 fraction of the renatured DNA. On the other hand, pure chloroplast DNA should
Figure 5. Analytical CsCl gradients of pumpkin nuclear DNA.
renature with approximately the same percentage of the 1.712 component as there was 1.706 component in the native DNA. As a control, to be certain that contaminated chloroplast DNA preparations would behave in a predictable manner, DNA was extracted from chloroplasts known to be contaminated with nuclei and analyzed on CsCl density gradients before and after denaturation-renaturation. Chloroplasts were isolated by suspending a mixture of chloroplasts and nuclei in 10 ml of a glycerol-sucrose medium which was prepared by adding 4 ml sucrose buffer (0.3 M sucrose, 0.067 M phosphate pH 7.0, 0.002 M EDTA, and 0.018 M MgCl₂) to 6 ml glycerol. The suspension of chloroplasts and nuclei was centrifuged at 1,000 x g and 4°C for 12 minutes. The pellet contained mostly nuclei and was discarded. The suspension of chloroplasts and nuclei were diluted with sucrose buffer and pelleted by centrifugation at 12,000 x g for 15 minutes. Figure 6 presents the results of CsCl gradients of the DNA obtained from the mixture of nuclei and plastids. As predicted, renaturation of this DNA resulted in an increase in the percentage of the 1.712 component when compared with the percentage of 1.706 component in the native DNA. This provided a relatively simple assay for nuclear contamination of chloroplast DNA preparations assuming that the contamination was due to the presence of both nuclear satellite and main band.
Figure 6. DNA obtained from a mixture of pumpkin chloroplasts and nuclei.
Kung and Williams (1968) reported that chloroplasts can be isolated free from nuclear contamination if the chloroplasts were first purified on discontinuous glycerol gradients according to the method of James and Das (1957) and further purified according to the method of Jagendorf (1955). It was decided to compare DNA obtained by their purification method with the DNA obtained by the methods used for this study. Their procedure was modified by substituting discontinuous sucrose gradients for the glycerol gradients and using a swinging bucket rotor instead of a fixed angle rotor for the Jagendorf purification as described in Materials and Methods. DNA which was extracted from a portion of the sucrose gradient purified plastids served as a control. As shown in Figure 7, the sucrose gradient purified plastids yielded DNA that was contaminated with nuclear DNA according to the criterion established above. The profiles obtained from native and denatured DNA are similar to those obtained from DNA considered free of nuclear contamination; however, the denatured-renatured DNA showed an increase in the percentage of 1.712 component when compared with the amount of 1.700 component in the native DNA. The remainder of the plastids from the sucrose gradients were further purified according to the Jagendorf procedure. Two fractions were obtained from the Jagendorf glycerol density gradients: chloroplasts which floated to the top of the
Figure 7. Pumpkin chloroplast DNA obtained from plastids purified on a discontinuous sucrose gradient.
tube and contaminating material which pelleted. The chloroplasts yielded DNA which resolved into two components but did not show an enrichment of the 1.712 component after denaturation-renaturation (Figure 8) in agreement with the behavior observed for pure chloroplast DNA. However, the DNA from the pellet (Figure 8c) contained two components of buoyant density 1.697 and 1.707 gm cm⁻³ in agreement with values obtained from native nuclear DNA. A comparison of the yields obtained from the chloroplasts and the pellet showed that the contaminating DNA comprised 20% of the total. Thus it can be shown that both components of nuclear DNA are present in preparations which are considered contaminated by the criteria used in this study. It is therefore unlikely that variations in the percentage of (1.706) component in the native chloroplast DNA are due to a variable amount of nuclear satellite (1.707). The fact that both nuclear components are present in contaminated DNA also indicates that the renaturation behavior of the chloroplast DNA is a meaningful criterion for purity of the chloroplast DNA. Therefore, only chloroplast DNA preparations which did not demonstrate an increase in the percentage of 1.712 component after renaturation when compared with the percentage of 1.706 in native DNA were considered free from nuclear DNA. The criterion for purity of tobacco chloroplast DNA was, by comparison, more straightforward. As discussed above, tobacco chloroplast DNA was considered
Figure 8. Comparison of DNA's obtained from chloroplast and pellet fractions of Jagendorf glycerol density gradient.
pure when, after denaturation-renaturation, no DNA was detectable with a buoyant density corresponding to that of the denatured DNA.

Physical Properties of Tomato Chloroplast DNA

Tomato (*Lycopersicum esculentum*) chloroplast DNA was isolated in the same manner as described for tobacco using plants approximately four inches high. Similar to pumpkin chloroplast DNA, two components were observed in the native chloroplast DNA (Figure 9a). Tomato nuclear DNA also contained two components (Figure 9b) but the main band had a buoyant density of 1.695 gm cm$^{-3}$ as compared to 1.700 gm cm$^{-3}$ for the major component of chloroplast DNA. The tomato nuclear satellite had a buoyant density of 1.706 gm cm$^{-3}$ as compared to 1.707 gm cm$^{-3}$ for the minor component of the chloroplast DNA. The density difference between the nuclear main band and chloroplast major components suggested that a mixture of chloroplast and nuclear DNA would resolve into three components corresponding to the nuclear main band, the major chloroplast component, and a mixture of nuclear satellite and chloroplast minor components. As shown in Figure 9c a mixture of tomato nuclear and chloroplast DNA resulted in the predicted three components on CsCl density gradients. This demonstrated that in the tomato system the major chloroplast DNA component and nuclear main band DNA's have different buoyant densities.
Figure 9. Resolution of tomato chloroplast and nuclear DNA's on analytical CsCl gradients.
and can be distinguished on the basis of this criterion as well as by difference in renaturability. Tomato nuclear DNA was analogous to pumpkin nuclear DNA with regard to renaturation in that only the nuclear satellite was renaturable.

When compared with tobacco and pumpkin chloroplast DNA, tomato chloroplast DNA offered a much simpler criterion for purity. Due to the very slight density difference between the native chloroplast and nuclear DNA in tobacco it was not possible to resolve these species on analytical CsCl gradients. This necessitated the use of criteria other than buoyant density to determine the purity of a particular tobacco chloroplast DNA preparation. This was also true for pumpkin DNA. Since the tomato nuclear and chloroplast DNA's did resolve on analytical gradients, any contamination present in the chloroplast preparation would have been observed in the native DNA preparation. The fact that there is little difference between the buoyant densities of the nuclear and chloroplast DNA in both tobacco and pumpkin is not unreasonable. There is no a priori reason to assume that the buoyant densities of the DNA's from the two organelles are different or even that the DNA of plastids is present as a single component. Certainy, systems in which the two major DNA species are resolvable on CsCl gradients are more suitable for determination of nuclear contamination of chloroplast DNA. However, in
systems where chloroplast DNA contains a component of buoyant density similar to that of nuclear DNA, it has to be established that this component is not of nuclear origin, i.e., a common buoyant density is not by itself sufficient evidence that two DNA's are derived from the same organelle.

Comparison of Physical Properties of Chloroplast and Nuclear DNA's

Table 1 summarizes the data obtained for the physical properties of the chloroplast and nuclear DNA's from tobacco, pumpkin, and tomato DNA. Each value represents the average of at least three independent DNA preparations. Buoyant densities for renatured tobacco and tomato chloroplast minor component were not calculated because the denatured-renatured DNA resolved into a single peak with no obvious shoulder which could correspond to the minor component. This was probably due to a low percentage of this component in the native DNA.

A comparison of the chloroplast DNA's shows a remarkable similarity in buoyant density and renaturation behavior. Whether or not this has any biological significance or represents an evolutionary conservation of the plastid genome in higher plants cannot be ascertained as yet due to the limited data available. Tobacco nuclear DNA differs from pumpkin and tomato nuclear DNA in that the former lacks a renaturable satellite component in detectable amounts. This has proven to be of use for discriminating
Table 1. Summary of Physical Properties of Chloroplast and Nuclear DNA Species

<table>
<thead>
<tr>
<th>Plant</th>
<th>Organelle</th>
<th>Component</th>
<th>Native</th>
<th>Denatured</th>
<th>Denatured-Renatured</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Nucleus</td>
<td>--</td>
<td>1.698</td>
<td>1.715</td>
<td>1.714</td>
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<tr>
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<td>1.719</td>
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<td></td>
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<td>1.719</td>
<td>Not determined</td>
</tr>
<tr>
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<td>Nucleus</td>
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<td>1.713</td>
<td>1.712</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Satellite</td>
<td>1.707</td>
<td>1.721</td>
<td>1.712</td>
</tr>
<tr>
<td></td>
<td>Chloroplast</td>
<td>Major</td>
<td>1.699</td>
<td>1.714</td>
<td>1.704</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minor</td>
<td>1.706</td>
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</tr>
<tr>
<td>Tomato</td>
<td>Nucleus</td>
<td>Main Band</td>
<td>1.695</td>
<td>1.711</td>
<td>1.710</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Satellite</td>
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<td>1.719</td>
<td>1.710</td>
</tr>
<tr>
<td></td>
<td>Chloroplast</td>
<td>Major</td>
<td>1.700</td>
<td>1.713</td>
<td>1.705</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minor</td>
<td>1.707</td>
<td>1.719</td>
<td>Not determined</td>
</tr>
</tbody>
</table>
between the tobacco chloroplast and nuclear DNA. Matsuda and Siegel (1967) have shown that the nuclear satellite of pumpkin is greatly enriched in rRNA cistrons but as yet no biological significance has been ascribed to this satellite DNA component. It will be shown below that the minor component of chloroplast DNA also contains rRNA cistrons.

**Hybridization of Tobacco and Pumpkin Chloroplast and Plant Ribosomal RNA**

Once the criteria for purity of chloroplast DNA were established, experiments were performed to establish the function of the DNA. Since it was already established that the chloroplast contains ribosomes which are distinct from those found in the cytoplasm, it was of interest to determine whether or not the chloroplast DNA contained cistrons for either or both chloroplast and cytoplasmic ribosomal RNA. One approach to this problem was the hybridization of the ribosomal RNA to the chloroplast DNA. In principle this technique involves annealing of a particular radioactive RNA with denatured DNA immobilized on a nitrocellulose membrane. The amount of RNA which annealed to the DNA is determined by measuring the amount of radioactivity on the membrane. This technique therefore directly establishes genetic sites on the DNA.

This technique serves to detect complementary sequences between a DNA and a particular RNA and it is therefore necessary to first prepare a pure DNA and a pure
RNA. The methods and criteria used to establish the purity of the chloroplast DNA have already been discussed. The most direct approach to the isolation of pure chloroplast or cytoplasmic ribosomal RNA is to isolate the RNA from purified preparations of either class of ribosome. Attempts to isolate purified preparations of chloroplast (70s) ribosomes were unsuccessful and so another approach was adopted. Hsiao (1961) presented evidence that roots contain only cytoplasmic (80s) ribosomes while Loening and Ingle (1967) reported that leaves contain rRNA from both 70s and 80s ribosomes. It has been established that 80s ribosomes contain 25s and 18s RNA and 70s ribosomes contain 23s and 16s RNA (Loening 1968). Thus it was predicted that leaf ribosomes would contain all four rRNA species while root ribosomes would contain only the 25s and 18s species. The gel electropherograms of root and leaf rRNA from tobacco are shown in Figure 10. The leaf rRNA contained the four rRNA species known to exist in plant tissues but, more importantly, the root rRNA contained only the 25s and 18s rRNA. This provided a convenient basis for discrimination between hybridization of 70s and 80s rRNA. Hybridization to root rRNA was considered evidence for 80s rRNA hybridization while hybridization to leaf but not root indicated hybridization to 70s rRNA. Differences between leaf and root rRNA hybridization were taken as a measure of the hybridization by 70s rRNA.
Figure 10. Gel fractionation of tobacco leaf and root rRNA.

Tobacco leaf rRNA (30 μg) and tobacco root rRNA (20 μg) were subjected to electrophoresis on polyacrylamide 2.4% gels for 2.5 hours at 5 mA per gel.
Presence of Chloroplast and Cytoplasmic rRNA Cistrons in Chloroplast DNA

Hybridization of root and leaf rRNA to pumpkin and tobacco chloroplast DNA yielded evidence that chloroplast DNA contains cistrons for both 70s and 80s rRNA. The results of these experiments are presented in Table 2. As discussed above, the values for hybridization of 70s rRNA to the DNA were obtained by subtracting the root rRNA value from the leaf rRNA value. The data presented for tobacco chloroplast DNA were obtained by incubating membranes containing 20 μg DNA with 8.4 μg leaf rRNA or 8.6 μg root rRNA as indicated. The specific activities of the rRNA's were 6,900 cpm/μg and 1,200 cpm/μg for the root and leaf preparations respectively. In all cases cpm have been corrected for background by subtracting counts obtained from blank filters incubated with radioactive RNA under the conditions of the experiment.

The value obtained for hybridization of 70s rRNA with tobacco chloroplast DNA was 0.38 per cent which agrees with those of Tewari and Wildman (1968). However, the 80s rRNA hybridized to 0.12 per cent of the tobacco chloroplast DNA. This result disagrees with those of Tewari and Wildman who could not detect significant hybridization between chloroplast DNA and 80s rRNA. Scott and Smillie (1967) reported that *Euglena gracilis* chloroplast DNA contains cistrons for chloroplast rRNA but not cytoplasmic rRNA.
Table 2. Hybridization of Ribosomal RNA to Chloroplast DNA

<table>
<thead>
<tr>
<th>DNA on Membrane</th>
<th>rRNA Source</th>
<th>Net cpm&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Per Cent Hybrid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Per Cent 70s Hybrid&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco chloroplast</td>
<td>Tobacco leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(20 µg)</td>
<td>(1,200 cpm/µg)</td>
<td>118</td>
<td>0.50</td>
<td>0.38</td>
</tr>
<tr>
<td>Tobacco root (80s)</td>
<td>(6,900 cpm/µg)</td>
<td>114</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pumpkin chloroplast</td>
<td>Bean leaf</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10 µg)</td>
<td>(800 cpm/µg)</td>
<td>60</td>
<td>0.72</td>
<td>0.41</td>
</tr>
<tr>
<td>Bean root (80s)</td>
<td>(6,000 cpm/µg)</td>
<td>210</td>
<td>0.31</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Net cpm = cpm on membrane minus background counts obtained from blank filters incubated with labeled rRNA under the conditions of the experiment. Background counts were as follows: tobacco leaf 52, tobacco root 44, bean leaf 27, bean root 30.

<sup>b</sup>Per Cent Hybrid = net cpm/specific activity of rRNA, normalized to 100 µg DNA on membrane.

<sup>c</sup>Per Cent 70s Hybrid = per cent leaf hybrid minus per cent root hybrid.
which also conflicts with the results obtained in this study.

Pumpkin chloroplast DNA was also found to contain cistrons for both 80s and 70s rRNA. In this study membranes containing 10 μg DNA were incubated with 5 μg rRNA from bean (Phaseolus vulgaris L. var. Pinto) root or leaf as indicated in Table 2. The specific activities of the rRNA's were 6,000 cpm/μg and 800 cpm/μg for the root and leaf respectively. In the case of pumpkin 0.31 per cent of the chloroplast DNA was found to be complementary to the 80s rRNA with 0.41 per cent complementary to the 70s rRNA. In these experiments bean rRNA was used instead of pumpkin rRNA because of convenience. Since the rRNA's in different species of higher plants are apparently quite similar to each other (Matsuda and Siegel 1967; Matsuda, Siegel, and Lightfoot 1969), it was predicted that the same result would be obtained if pumpkin rRNA were used instead of bean rRNA. Evidence for the similarity of rRNA's in different plant species comes from experiments in which the same hybridization values were obtained for a particular DNA when a variety of rRNA's are used but, on the other hand, quite different values are obtained when a specific rRNA is used with a variety of plant DNA's. Thus the rRNA's from different plant species are qualitatively similar but there is a quantitative variation in the amount of rRNA cistrons in the DNA obtained from different plant species.
The presence of 80s cistrons on chloroplast DNA cannot be attributed to homologous sequences on the 70s and 80s rRNA. This conclusion is based on the following evidence: Using nuclear DNA Tewari and Wildman (1968) and Matsuda et al. (1969) have shown that 70s and 80s rRNA's are indeed different and that they occupy different sites on the DNA. In their experiments, they demonstrated that the 70s and 80s rRNA do not compete for the same site on the DNA indicating that there are no homologous sequences present on the two rRNA's. Thus it can be stated that cistrons for both 70s and 80s rRNA do exist on chloroplast DNA but the experiments reported in this study do not permit an accurate prediction as to the percentage of rRNA cistrons in chloroplast DNA.

Analysis of Chloroplast DNA Major and Minor Components for the Presence of Ribosomal DNA

Since the chloroplast DNA in both tobacco and pumpkin contained two components, it was of interest to determine whether the cistrons for 80s and 70s rRNA were in the same or different components. This was accomplished by fractionating tobacco chloroplast DNA on preparative CsCl gradients and hybridizing one-half of each DNA fraction with root rRNA and the other half with leaf rRNA. DNA was centrifuged in CsCl for 66 hours after which 18 drop fractions were collected as described in Materials and Methods. Each fraction was diluted with 0.95 ml 2 x SSC.
and the optical density at 260 nm measured. The fractions were divided in half prior to denaturation and embedding on nitrocellulose membranes. The distribution of 70s and 80s cistrons was determined by hybridizing one-half of each fraction with root rRNA and the other half fraction with leaf rRNA. After incubation and removal of unhybridized RNA, the radioactivity was measured. Figure 11 shows the results obtained when 30 μg tobacco chloroplast DNA were fractionated and hybridized to pumpkin leaf and root rRNA (specific activities 62,000 cpm/μg and 28,000 cpm/μg respectively).

The optical density profile shows that the DNA banded as a single peak. The minor chloroplast component could not be resolved on preparative CsCl gradients to the same extent as on analytical gradients probably due to the limited number of fractions collected and the small percentage of minor component present in any given preparation. Hybridization profiles of both leaf and root rRNA were slightly displaced to the portion of DNA with the greater buoyant density. Although this suggests that it is the minor component which contains both 70s and 80s cistrons, it is not conclusive evidence and it is still possible that some rDNA is located in the major component. Perhaps this could be resolved if the chloroplast DNA separated into more distinct components on the preparative gradients and if smaller fractions were taken.
Figure 11. Distribution of rDNA in tobacco chloroplast DNA.
This experiment was not designed to measure the degree of hybridization for each fraction due to difficulties in accurately determining the amount of DNA on each membrane and the level of saturation of hybridization. However, it can be seen that in all fractions tested the leaf (70s and 80s) rRNA hybridized to a greater extent than did root rRNA (80s) alone which is in agreement with the results obtained for the total chloroplast DNA (see Table 2) where the conditions of hybridization are better defined.

Analysis of the distribution of 70s and 80s rDNA in pumpkin chloroplast DNA yielded a result somewhat similar to that observed for tobacco chloroplast DNA except that the displacement of the rDNA was more pronounced in the case of pumpkin. Conditions of centrifugation and hybridization were the same as described for tobacco chloroplast DNA except that 15 drop fractions were taken and the gradient contained only 22 μg DNA. As shown in Figure 12 the two DNA components resolved as a peak with a slight shoulder corresponding to the major and minor components respectively. Both the 70s and 80s rDNA was found in the fractions corresponding to the minor DNA component, i.e., fractions 11-16.

The results from pumpkin chloroplast DNA indicate that it is the minor chloroplast component that contains the rDNA. The evidence for this is far from conclusive.
Figure 12. Distribution of rDNA in pumpkin chloroplast DNA.
because the components were not well separated. However, the possibility that both components contain rDNA is not very likely because in this situation one would expect at least a shoulder on the light side of the hybridization profile. As shown in the figure, both hybridization profiles are symmetrical suggesting that the major component contains very little if any rDNA. The fact that both 70s rDNA and 80s rDNA are located in the same component can be inferred from this experiment because of the coincidence of the respective peaks for rDNA distribution. Thus, there is fairly good evidence that at least a portion of the minor DNA component in both tobacco and pumpkin is composed of rRNA cistrons.
DISCUSSION

The chloroplast DNA's from all of the plant species studied contained two DNA components which were designated major and minor. This result differs from the conclusions of other workers (Chun et al. 1963; Brawerman and Eisenstadt 1961a; Edelman et al. 1964; Green and Gordon 1967) who have reported that chloroplast DNA is a single component with a buoyant density distinct from that of nuclear DNA. However, in most cases, the chloroplast DNA isolated by these workers also contained a DNA component with a buoyant density identical with that of the nuclear DNA. They presumed that this DNA was nuclear contamination and disregarded the possibility that chloroplasts contain two components one of which has a buoyant density similar to that of the nucleus. Evidence obtained in this study however, suggests the possibility that a portion of this DNA is associated with chloroplasts and can be distinguished from its nuclear counterpart by denaturation-renaturation behavior. This was shown to be the case for the three DNA's studied. In addition it was found that in the case of tomato chloroplast DNA the major chloroplast component and nuclear main band were distinguishable on the basis of buoyant density while the minor components and nuclear satellite were not readily distinguishable.
Concomitant with the work done for this study, two papers appeared which also reported the presence of two components in chloroplast DNA. Iwamura and Kuwashima (1969) and Bard and Gordon (1969), respectively, concluded that Chlorella and spinach chloroplast DNA's are composed of two DNA components. Whether or not this is a general phenomenon for chloroplast DNA's remains to be established. The minor chloroplast DNA component need not necessarily exist as a prominent species distinct from the remainder of the chloroplast DNA and thus a minor component is not always easily detected. This was found to be the case for tobacco chloroplast DNA where the minor component appears as a shoulder on the major component as opposed to pumpkin and tomato where the minor component proved to be a more distinct component. The variability in the percentage of minor component presents an unsolved aspect of chloroplast DNA study. This variation may reflect the possibility that chloroplast DNA exists both as a soluble and membrane bound species. If this were shown to be true, then it is conceivable that one could lose a component during the extraction procedure especially if the pastids were stripped of their outer membranes or placed in an osmotically unfavorable medium.

The criteria for purity of chloroplast DNA that were used in this study are not intended as an absolute measure and, at present, there are no criteria available
which could serve as an absolute measure of purity. There is still a possibility that a minute amount of nuclear DNA is present in preparations which meet the requirements set forth for purity in this study. This is especially true in the case of pumpkin where it is estimated that contamination amounting to 4% or less would be very difficult to detect. With tobacco it is estimated that greater than 2% contamination could be detected provided a sufficient amount (ca. 20 µg) of chloroplast DNA were monitored. However, although the criteria presented are not absolute, they have the common virtue of being relatively simple and, at the same time, can serve as a means to determine whether or not a particular DNA component is associated with chloroplasts. These observations serve to emphasize that a thorough investigation is necessary before it can be established that a particular component in chloroplast DNA is the result of nuclear contamination.

The observation that chloroplast DNA is readily renaturable is not only useful as a criterion for identifying chloroplast DNA but also is of importance for considerations of the genetic potential of chloroplasts. The results of this study, together with those of others, permit a more meaningful discussion of the total number of different genes present in chloroplast DNA. In this context, comparisons are often drawn between chloroplasts and bacteria because they both appear to contain approximately the same
amount of DNA. Although the estimations are variable, chloroplasts contain approximately $2 \times 10^9$ daltons of DNA (Iwamura 1966) which is comparable to the amount of DNA in a bacterial cell. In the case of bacteria this amount of DNA corresponds to approximately 4000 genes (Woese 1967, p. 8). It is of interest to note, however, that chloroplast DNA renatures more readily than bacterial DNA. An interpretation of this phenomenon is that chloroplast DNA contains repetitious base sequences whereas bacteria do not contain repetitious sequences (Britten and Kohne 1968). Thus at least a portion of chloroplast DNA may be composed of redundant sequences which reduces the potential number of different genes present in chloroplast DNA. It is therefore unlikely that the genetic potential of the chloroplast is comparable with that of a bacterium. Although it has been established that chloroplast DNA renatures quite readily (appreciably more so than does bacterial DNA) it has not been studied extensively enough to permit meaningful estimates as to the extent of redundancy that exists and, thus, the genetic potential of chloroplasts as far as diversity of gene products remains to be established.

As stated in the previous paragraph, it has been established that there is appreciable redundancy of genetic material in chloroplast DNA. The possibility is ruled out, however, that a portion of the repeating sequences are
composed of rRNA cistrons. From the data in Table 2 and the assumption that there are $2 \times 10^9$ daltons of DNA per plastid genome, it can be roughly calculated that there are four cistrons for 70s rRNA and one cistron for 80s rRNA in tobacco chloroplast DNA. In the case of pumpkin chloroplast DNA the data indicate that there are four cistrons for 70s rRNA and three cistrons for 80s rRNA. This is in contrast to the nuclear DNA where it has been estimated that the total number of rRNA cistrons in tobacco and pumpkin nuclear DNA are 1,500 and 15,000 copies respectively. In the case of pumpkin nuclear DNA, this extensive redundancy was present in a satellite component which was shown to be readily renaturable in contrast to the major portion of the DNA (Matsuda and Siegel 1967). By comparison, with only four or so copies of a particular rRNA cistron present in chloroplast DNA, one can conclude that chloroplast genes other than rRNA cistrons are present as multiple copies.

Of particular interest is the finding that chloroplast DNA apparently contains cistrons for both 70s and 80s rRNA. This result was quite unexpected because it is generally observed that the plastids contain only 70s ribosomes and thus it is not unreasonable to assume that only cistrons for 70s rRNA would be present in chloroplast DNA. By the same argument it is also observed that the cytoplasm contains only 80s ribosomes and therefore it
would be expected that nuclear DNA contains only 80s rRNA cistrons. However, cistrons for both 70s and 80s rRNA have also been detected in nuclear DNA (Tewari and Wildman, 1967, Matsuda et al., 1969). This raises the question as to which organelle DNA is functional for the synthesis of a particular rRNA. It is possible that the 80s rRNA is synthesized only from nuclear DNA templates while the 70s rRNA is produced only from chloroplast DNA templates. This would mean, however, that the nuclear 70s rRNA cistrons and chloroplast 80s rRNA cistrons are non-functional. In view of the fact that some genes necessary for plastid development are located in the nuclear DNA (Kirk, 1967), it is tempting to suggest that the presence of both 70s and 80s rRNA cistrons in chloroplast DNA represents some form of control system. There is some evidence that messenger RNA from eukaryotic organisms (which contain 80s ribosomes in the cytoplasm) exists as a nucleoprotein (Perry and Kelley, 1968) while in prokaryotic organisms (which contain 70s ribosomes) the messenger RNA presumably lacks this protein moiety. It is possible that the former type of messenger RNA (i.e., nuclear) is translated only by the 80s ribosomes while translation of the latter type of messenger (i.e., chloroplast) involves a 70s ribosome. This would require that chloroplasts contain 80s ribosomes or the potential to synthesize them in order to translate the genes related to chloroplast function which are located on the nuclear DNA.
presuming that these genes are indeed translated in the plastids. The likelihood of this hypothesis cannot be established until the mechanisms of genetic expression of the chloroplast and nuclear DNA are better understood.

There is evidence that genes associated with chloroplast function are present in the nucleus and the reverse is probably also true. It is of interest, therefore, to consider the origin of chloroplast DNA. One hypothesis put forth for the origin of chloroplast DNA is that the plastids arose from blue-green algae that had entered into a symbiotic relationship with another cell. If this hypothesis is true then the results of this study and those of others (Tewari and Wildman, 1968; Scott and Smillie, 1967; Kirk, 1967) suggest that an exchange of genetic information must have occurred during evolution. Although the extent of this exchange is not known, the present studies suggest that at least the ribosomal genes have been exchanged. Alternatively, the plastid genome could have arisen from a portion of the nuclear genome. In this case all of the plastid genes were originally in the nuclear DNA and during evolution a portion of these genes became associated with the plastids. This would explain the presence of gene for plastid function in the nucleus and at the same time offer an explanation for nuclear genes in the plastid genome. There is unfortunately no compelling reason to assume that either hypothesis is valid and perhaps
this question will be resolved only after the entire functional characteristics of chloroplast DNA have been established. The finding that chloroplast DNA contains some complementarity to nuclear DNA (Tewari and Wildman, 1967; Richards, 1967) can partly be explained by the presence of 70s and 80s rRNA in both types of DNA. Whether or not other genes are common to both types of DNA is yet to be established. From the foregoing it is obvious that the problems concerning the origin of chloroplast DNA and the functional significance of that DNA are far from understood and a more complete identification of the chloroplast and nuclear genomes is necessary before one can arrive at meaningful answers to these problems.
SUMMARY

DNA has been isolated from the purified chloroplasts of tobacco, pumpkin, and tomato. Tissues were homogenized by chopping with razor blades in Honda medium and the plastids were purified by discontinuous sucrose gradient centrifugation. In each case it was found that the chloroplast DNA was composed of two components designated major and minor. Buoyant densities in CsCl of the major and minor components respectively were 1.700 and 1.707 for tobacco, 1.699 and 1.706 for pumpkin, and 1.700 and 1.707 for tomato. Nuclear DNA has been isolated from these three plant species and it was found that both pumpkin and tomato nuclear DNA was composed of two components designated main band and nuclear satellite. In the case of tobacco, the nuclear DNA was composed of a single component. Buoyant densities in CsCl for the main band and satellite component respectively were 1.697 and 1.707 for pumpkin, and 1.695 and 1.706 for tomato. Tobacco nuclear DNA had a buoyant density of 1.698 in CsCl.

Both components of all three chloroplast DNA's renatured readily after denaturation. Only the satellite components of pumpkin and tomato nuclear DNA were found to renature readily while tobacco nuclear DNA did not contain a readily renatured component in detectable amounts.
Criteria for the purity of all three chloroplast DNA's were developed. Tobacco chloroplast DNA was considered pure if it did not contain unrenaturable DNA. Pumpkin chloroplast DNA was considered pure if the ratio of major to minor components did not change after denaturation-renaturation. The native tomato nuclear main band and chloroplast major component were resolved on CsCl gradients and thus tomato chloroplast was considered pure when no DNA was detectable which had the same buoyant density as nuclear main band.

Hybridization of 70s rRNA and 80s rRNA to pumpkin and tobacco chloroplast DNA resulted in evidence that cistrons for both 70s and 80s rRNA are present in the chloroplast DNA of these two species. Fractionation of tobacco and pumpkin chloroplast DNA on preparative CsCl gradient followed by hybridization of each fraction to rRNA resulted in evidence that both 70s and 80s rRNA cistrons are located in the minor component of the chloroplast DNA.


