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Deoxyribonucleic Acid of the Blue-Green Algae (*Cyanophyta*)

MARVIN EDELMAN,[1] DAVID SWINTON,[2] JEROME A. SCHIFF, H. T. EPSTEIN, AND BERNICE ZELDIN

Department of Biology, Brandeis University, Waltham, Massachusetts 02154

INTRODUCTION

The *Cyanophyta* or blue-green algae share the kingdom *Monera* with the *Schizomycophyta* or bacteria, because both are distinguished by a procaryotic type of intracellular organization, where the protoplast of the cell does not contain organelles conspicuously delimited from the surrounding cytoplasm. The blue-green algae, however, share the property of an oxygen-evolving type of photosynthesis with the eucaryotic algae and higher plants and produce phycoerythrin and phycocyanin, two photosynthetic pigments characteristic of the *Rhodophyta*, or red algae, and one or two other smaller groups. It has been suggested that the organelles of eucaryotic cells, such as the chloroplast, arose from a blue-green algal-like endosymbiont within an otherwise nonphotosynthetic cell. The phylogenetic affinities of the blue-green algae, therefore, are of considerable interest.

With the finding (25) that deoxyribonucleic acid (DNA) base compositions from related organisms are similar and can be employed as an index of phylogenetic relatedness (as judged from more conventional taxonomic evidence), and with the accumulation of DNA base composition data for certain bacteria and for chloroplasts of several of the eucaryotic algae and higher plants, it became of interest to compare the DNA base compositions of various blue-green algal species with each other, with those of other groups of organisms, and with various chloroplast DNA's.

This paper presents a characterization of the DNA of a number of blue-green algae with respect to several physical and chemical properties including their base-pair compositions. The DNA characteristics are reported and discussed with regard to their taxonomic and phylogenetic significance and their relation to hypotheses of chloroplast origin. Several points of interest concerning certain polysaccharides, which were consistently encountered in the DNA fraction of the *Cyanophyta*, are presented. A brief account of this work has already appeared (M. Edelman, D. Swinton, J. A. Schiff, and H. T. Epstein, Plant Physiol. (Suppl.) p. x, 1966).

MATERIALS AND METHODS

Culture Conditions

Axenic cultures of blue-green algae, obtained from several sources (acknowledged in Table 3), were usually grown at 25 to 30 C in 500-ml Erlenmyer flasks containing 100 ml of liquid

[1] Present address: Department of Biochemistry, Weizmann Institute of Science, Rehovoth, Israel.
[2] Present address: Department of Biology, Stanford University, Stanford, Calif.

media on a rotary shaker under an atmosphere of 1 to 5% CO_2. Occasionally, when larger amounts were required, several liters of media were used. Intensity of illumination was from 50 to 200 ft-c. Algae were harvested when growth appeared sufficient to yield a 2- to 5-ml packed volume of cells. Stock cultures were maintained on medium solidified with 1% agar without added CO_2.

All cultures were adapted to, and grown on Soil Extract Medium (58) except the following: *Nostoc* species were maintained in a modified Bristol's Nitrate Broth (24), marine species (*Anacystis marina, Dermocarpa violacea, Lyngbya* sp. 77, and *Plectonema* sp. 52) were grown in Artificial Seawater Medium (40) and *Cyanophora paradoxa* was grown in *Cyanophora* Conservation Medium, II (Provasoli, *personal communication*).

Before a culture was used experimentally, it was routinely checked for contamination. In addition to microscopic examination, a sample of culture was added to 5 ml of sterile Brain Heart Infusion Medium (Difco) and aerated for 24 hr at 30 C. When turbidity appeared at the end of this period, a large amount of the contaminating cells was grown up, and the DNA was extracted and subjected to analytical ultracentrifugal analysis. The density pattern thus obtained was compared with a similar DNA density pattern from the parent blue-green algal culture. In each instance of cell contamination encountered by us (e.g., in cultures of *Nostoc muscorum* and *Nostoc punctiforme*, 384), a minor band in the blue-green algal DNA pattern could be matched with the DNA profile of the freshly grown contaminant and thereby identified and eliminated from consideration. The identity of each of the various blue-green algal species used in this study was accepted as indicated on the label of the culture tube upon arrival. Other than microscopic examination to verify gross morphological features, no attempt was made to confirm the various assignments.

Extraction of Blue-Green Algal DNA

Cultures were harvested at 7,000 to 10,000 × *g* for 5 min and suspended in 5 to 10 ml of saline ethylenediaminetetraacetic acid (EDTA) [0.15 M NaCl, 0.1 M EDTA, *p*H 8 (29)]. Filamentous blue-green algae were subjected, at this point, to homogenization at 4 C for 30 sec with a Teflon homogenizer to reduce the length of their filaments. Cell breakage was accomplished with a French pressure cell (4 C) applying from 12,000 to 18,000 psi. Occasionally, breakage by lysozyme treatment (8) was also employed. The French press effluent (or lysozyme extract) was

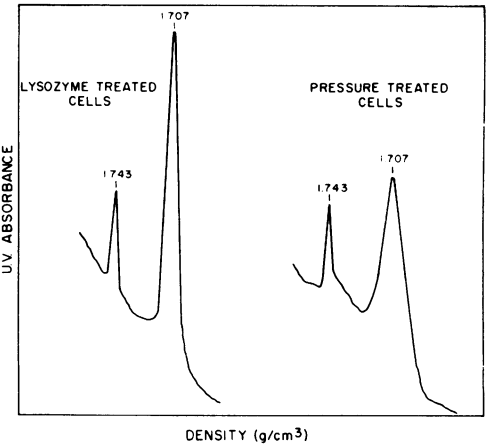

FIG. 1. *DNA density profiles from Plectonema boryanum cells treated with lysozyme or pressure. Microdensitometer tracings from ultraviolet-absorption photographs of Plectonema DNA centrifuged to equilibrium at 44,770 rev/min and 25 C in CsCl density gradients. Photographs taken after 20 hr in the analytical ultracentrifuge. The peaks at 1.743 g/cm³ are due to SP8 DNA, which was added as a density standard to calibrate the gradients. Peaks at 1.707 g/cm³ are due to Plectonema DNA.*

directed into a chilled test tube containing 1 ml of a 10% sodium dodecyl sulfate solution (2 × recrystallized) and was gently mixed with this soap solution for 5 min to complete lysis.

DNA was isolated by the method of Marmur (29), with minor modifications (12, 26). The isopropanol precipitation step was not omitted in this study.

Figure 1 compares ultracentrifugal DNA patterns from *Plectonema boryanum* cells broken by pressure and lysozyme treatment. As anticipated, pressure-treated samples yielded a larger variance in the DNA banding pattern than did lysozyme-treated samples, thus indicating the shearing effects of the former treatment on the DNA molecules. However, both techniques produced identical buoyant density medians at 1.707 g/cm³. The band of density 1.743 g/cm³ is a density marker (SP8 phage DNA) introduced to calibrate the CsCl gradient. Since the average buoyant density was the datum sought in this study, and cell breakage by lysozyme treatment is not feasible for many blue-green algal species (8), pressure was employed routinely.

Fractionation of Cyanophora paradoxa

Cells were harvested at 500 × *g* for 10 min at 25 C and were resuspended in two times their packed volume of saline-EDTA with the aid of a machine-driven Teflon homogenizer for 15

sec. From 60 to 80% of the host protozoan cells were ruptured by this procedure, but few cyanelles (blue-green algal inclusions) were broken. The homogenate was divided into two parts. One was treated directly with 0.05% lysozyme in 0.03 M sodium phosphate buffer, *p*H 6.8, for 4 hr at 37 C, and then with 1.0% (final concentration) sodium dodecyl sulfate (SDS) for 5 min at 20 C. DNA isolated from this fraction is designated as the whole cell extract. Incubation of a sample of this extract with deoxyribonuclease produced the deoxyribonuclease-treated whole cell extract.

The second part of the homogenate was subjected to 0.01% (final concentration) SDS for 1 min at 20 C. This resulted in lysis of the remaining whole protozoan cells, but not of the cyanelles. These were sedimented at 5,000 × *g* for 5 min at 4 C and the supernatant fluid was decanted and extracted, yielding supernatant fraction DNA.

The 5,000 × *g* pellet, containing cellular debris and cyanelle particles, was further divided into two parts. The first part was resuspended directly in 2 to 3 volumes of saline-EDTA, treated with lysozyme, and with 1.0% (final concentration) SDS as described above; then the lysate was extracted to give the pellet fraction DNA. A second sample was taken up in 1 ml of 0.25 M sucrose and 0.005 M $MgCl_2$ and was treated with deoxyribonuclease. Just before the start of incubation, 50 μg of SP8 phage DNA (density 1.743 g/cm³) was added to the reaction mixture as a check on the effectiveness of the enzymatic treatment. At the termination of the incubation period (45 min), the mixture was sedimented once more at 5,000 × *g* for 5 min at 4 C, the supernatant fluid was saved for optical density measurements at 260 mμ, and the pellet was resuspended in 2 volumes of saline-EDTA. Lysozyme treatment, followed by the addition of 1.0% SDS (final concentration), brought about lysis of the isolated cyanelle particles, as confirmed by the release of the photosynthetic pigments into the medium and by direct microscopic observation. DNA was extracted from this lysate to yield the cyanelle fraction. An outline of the fractionation procedure is shown in Fig.2.

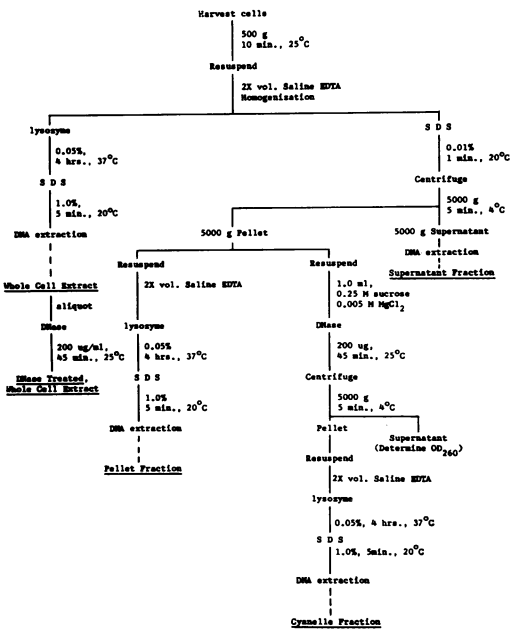

FIG. 2. *Fractionation scheme for Cyanophora paradoxa. Designations are explained in the text.*

Density-gradient Centrifugation

CsCl (Harshaw, optical grade) density-gradient centrifugation (35, 51) was carried out in a Spinco model E analytical ultracentrifuge, as previously described (12). A 0.5-μg amount of SP8 or SP01 phage DNA was introduced into the centrifuge cells in all experiments, to serve as a density standard. Experimental samples were investigated at two different concentrations: one, at 1 to 2 μg of DNA per centrifuge cell, to determine mean buoyant densities, and the other, at 25 to 50 μg of DNA per centrifuge cell, to enable detection of minor DNA components, either endogenous or exogenous, down to the 1% level (M. Edelman, Ph.D. Thesis, Brandeis Univ., Waltham, Mass., 1965). Densitometer tracings were made with a Joyce-Loebl double-beam recording microdensitometer.

Preparative gradient ultracentrifugation was carried out in cellulose nitrate tubes in the SW 39 rotor of a Spinco model L ultracentrifuge. A 4-ml sample in a CsCl solution was centrifuged to equilibrium at 39,000 rev/min for 48 hr at a chamber temperature of 4 C. Fractions were collected dropwise through a puncture in the bottom of the centrifuge tube and diluted to 5 ml with SSC [0.15 M NaCl, 0.015 M trisodium citrate, *p*H 7.0 (29)] for optical density measurements (reported per milliliter) at 260 mμ.

Chemical and Enzymatic Analyses

DNA was determined by a modification of the Dische diphenylamine procedure (4). RNA was estimated as pentose-reacting material by the Mejbaum modification of the Bial orcinol reaction (34) and the dichromatic procedure used by Dische (11). Total protein was assayed with Folin reagent according to the method of

Lowry et al. (28). Carbohydrates were qualitatively assayed by a modification of the orcinol test described by Brown (3) and quantitatively determined by using Dreywood's anthrone reagent as reported by Morris (36).

Deoxyribonuclease treatment was accomplished with 200 μg of pancreatic deoxyribonuclease (Worthington) in 1 ml of 0.25 M sucrose and 0.005 M $MgCl_2$. Incubation was carried out at 25 C for 45 min, and the reaction was terminated by the addition of 2 volumes of saline-EDTA. Ribonuclease treatment was carried out as described by Marmur (29). Trypsin (100 μg/ml) digestion was performed at *p*H 7.5, 25 C, for 30 min.

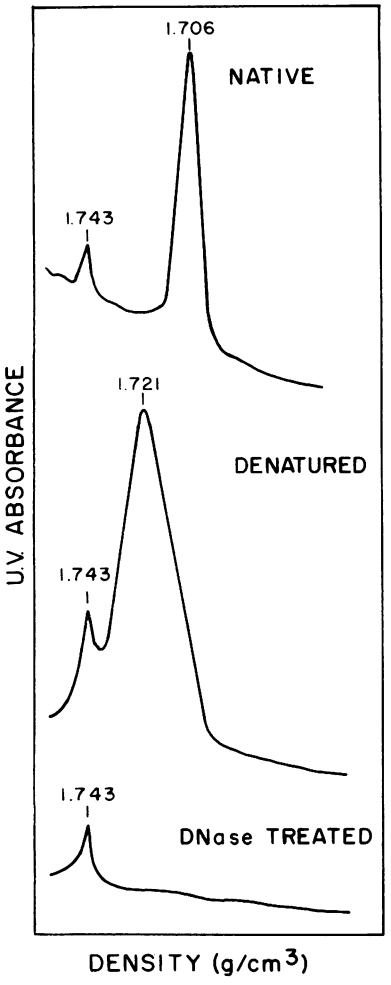

FIG. 3. *DNA density profiles of Phormidium luridum, a representative blue-green alga. Conditions of centrifugation are as in Fig. 1. Peaks at 1.743 g/cm³ are of density marker, SP8 DNA. Upper tracing shows the native Phormidium DNA profile peaking at 1.706 g/cm³. Lower tracing reveals the sensitivity of the native DNA preparation (1.706 g/cm³) to deoxyribonuclease treatment.*

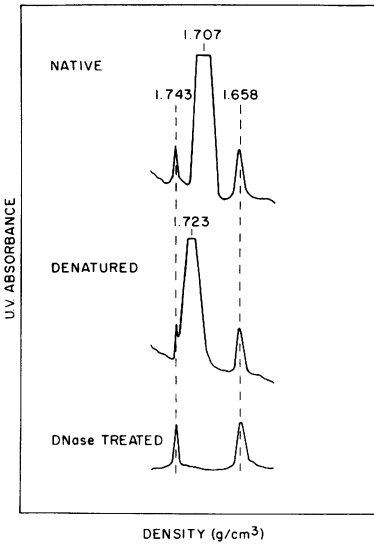

FIG. 4. *Satellite components in the DNA fraction of Plectonema boryanum. Conditions of centrifugation are as in Fig. 1. Peaks of density 1.743 g/cm³ show SP8 standard DNA. Upper tracing shows native DNA (35 μg) with a main band at 1.707 g/cm³ and a satellite component at 1.658 g/cm³. Middle tracing shows denatured DNA (25 μg) with the main band shifted to 1.723 g/cm³ and the satellite component remaining at 1.658 g/cm³. Lower tracing shows the deoxyribonuclease-treated, native DNA profile. A 35-μg amount of DNA was subjected to enzymatic treatment; a single peak, at 1.658 g/cm³, remains after treatment.*

RESULTS AND DISCUSSION

Blue-green Algal DNA Patterns

When DNA was extracted from a typical, axenically grown blue-green alga (in this case, *Phormidium luridum*, a representative member of the *Oscillatoriales*) and subjected to analytical ultracentrifugation, the CsCl buoyant density profiles shown in Fig. 3 were obtained. The band of density 1.743 g/cm³ is due to SP8 phage DNA, which was introduced as a density marker in all profiles shown. Native DNA (upper tracing), after centrifugation to equilibrium, showed a unimodal density distribution at 1.706 g/cm³. Treatment to heat-denature the DNA (middle tracing) resulted in an upward shift in buoyant density of 0.015 g/cm³ characteristic of double-stranded molecules (30), and produced an increase in band width in accordance with a reduction in molecular weight (35). Treatment of the DNA sample with deoxyribonuclease (lower

tracing), followed by dialysis against SSC, resulted in the failure of the treated sample to produce a band in the CsCl gradient, due to enzymatic degradation of the DNA polymer. These characteristics were shared by all of the blue-green algal DNA samples tested by us.

Question of Satellite Components

In an attempt to determine whether minor or "satellite" components were present in any of the DNA samples investigated, large amounts of DNA (from 25 to 50 μg) were introduced into analytical ultracentrifuge cells and were centrifuged to equilibrium. As is evident from Fig. 4, certain of the purified DNA samples did, indeed, show a satellite band. *Plectonema boryanum* extracts are shown in which a main band (1.707 g/cm³) and satellite band (1.658 g/cm³) are evident (upper tracing). However, this satellite material neither increased in density upon heat treatment (middle tracing) nor exhibited a sensitivity to incubation with deoxyribonuclease (lower tracing), while the main band DNA did exhibit such changes in each instance. Moreover, this satellite band exhibited an unusually low CsCl buoyant density for a DNA species. [Crab testes poly-AT DNA and synthetic poly-AT band at 1.680 g/cm³ and are the least dense DNA species reported (51, 55).]

Nature of the Satellite Component

The satellite band material of Fig. 4 was subjected to several preparative density gradient centrifugations and collections to further purify it for analysis. The final gradient pattern is shown in Fig. 5A, where a single peak at an average density of 1.658 g/cm³ accounts for essentially all of the material measurable at 260 mμ.

Several tests were made on this purified material (Table 1). The enzymatic and chemical tests for DNA, RNA, and protein clearly indicated that the satellite band was not composed of

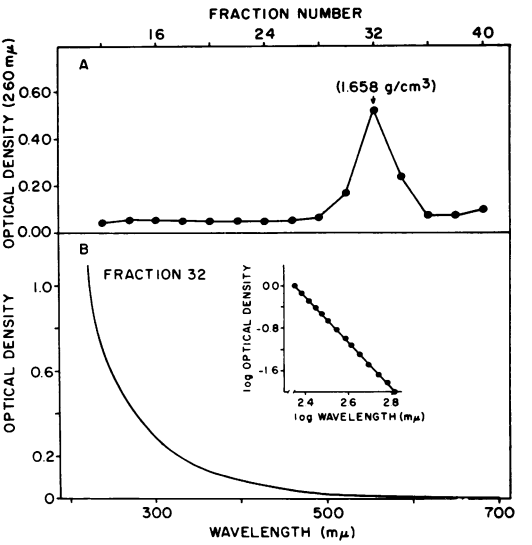

FIG. 5. *Purification and spectrophotometric profile of the Plectonema satellite band. (A) Preparative gradient ultracentrifugal pattern of the deoxyribonuclease-treated DNA fraction from Fig. 3. Conditions of centrifugation as in Materials and Methods. Buoyant density was calculated from refractive index measurements (51). (B) Spectrophotometric profile of purified satellite band material. Fraction 32 of (A), dialyzed against distilled water, was scanned in the visible and ultraviolet regions of the spectrum in a Cary model 14 recording spectrophotometer. Insert shows a plot of the log of the optical density against the log of the wavelength for this data. Slope of the resulting straight line is −4.2.*

TABLE 1. *Analysis of blue-green algal satellite band material*[a]

Substance sought	Treatment[b]	Result
Deoxyribonucleic acid	Deoxyribonuclease	Resistant[c]
	Diphenyl amine	<1 μg
Ribonucleic acid	Ribonuclease	Resistant[c]
	Orcinol	<5 μg[d]
Protein	Trypsin	Resistant[c]
	Folin phenol	<1 μg
Carbohydrate	Orcinol	Positive for hexose[d]
	Anthrone	1,500 μg (equivalents of glucose)[e]

[a] From fraction 32, Fig. 5A.
[b] See Materials and Methods.
[c] Material having an optical density at 260 mμ of 0.10 was treated. "Resistant" refers to the unaltered density, concentration, and band width of the satellite component in CsCl equilibrium gradients after treatment.
[d] See Fig. 6 for relevant spectra.
[e] Material having an optical density at 260 mμ of 0.50 was treated.

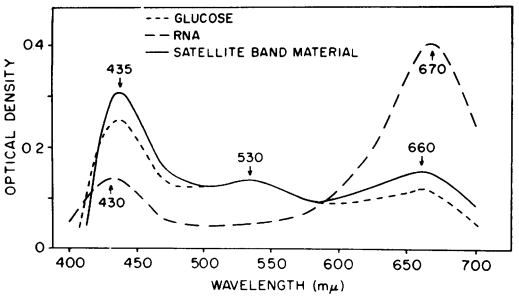

FIG. 6. *Absorption spectra of Plectonema satellite band material after treatment with modified orcinol reagent (3). Spectrochemical characteristics of similar concentrations (approximately 100 μg) of RNA, glucose, and satellite material were determined with a Cary model 14 recording spectrophotometer. Satellite-band material and glucose peak at 660, 530, and 435 mμ, and RNA pentose peaks at 670 and 430 mμ.*

these molecular species. On the other hand, positive tests (qualitative and quantitative) for carbohydrates were obtained and a value of 1,500-μg equivalents of glucose per 0.5 optical density units at 260 mμ of satellite band material was determined.

Figure 6 shows comparative data for the absorption spectra of RNA (Worthington, commercial), glucose, and the purified satellite band material after treatment with orcinol reagent (34). The spectrum for glucose exhibited maxima at 660, 530, and 435 mμ (3), as did the satellite band material. The spectrum produced by RNA pentose was qualitatively different, showing only two maxima, at 670 and 430 mμ.

The nondialysable nature of the satellite material, together with the banding it exhibited in equilibrium density gradients (Fig. 5A), required it to be of a fairly high molecular weight (35). Coupled with the results of the analytical tests in Table 1 and Fig. 6, a polysaccharide was indicated. Further evidence for this identification (Fig. 5B) was in the spectrophotometric profile of the purified satellite material. It was apparent that the carbohydrate-positive satellite material was scattering, rather than absorbing, UV light. The insert in Fig. 5B shows a plot of the log of optical density against the log of the wavelength, in which the experimentally obtained linear slope is −4.2, closely approximating the expectation of −4 from Rayleigh's law for light scattering by ideal particles. The milky-white appearance of the concentrated, purified satellite band in preparative CsCl gradient tubes and its formation of a colloidal suspension in water substantiated this finding. Such light scattering behavior is characteristic of large polysaccharides, such as starch or glycogen.

Distinguishing Between Light-absorbing and Light-scattering Macromolecules in the Analytical Ultracentrifuge

From a consideration of the spectrophotometric data of Fig. 5B and the quantitative determination of carbohydrate (in glucose equivalent units) by the anthrone test (Table 1), the specific extinction coefficient (E_{260}) of the satellite band polysaccharide material from *Plectonema boryanum* was estimated to be 60-fold lower than the E_{260} for DNA. This difference between DNA and the polysaccharide material can be exploited to provide a simple and rapid method for distinguishing between these two macromolecular species in a single analytical ultracentrifuge sample.

Figure 7 shows photographs of such a sample taken with both the UV and Schlieren optical systems. In the first case, optical density, and in the second, the rate of change in refractive index, was measured across the gradient. In the upper photograph (UV optics), the band at 1.710 g/cm³ represents 1 μg of *Escherichia coli* DNA, and that of density 1.658 g/cm³ represents 60 μg of the purified *Plectonema* polysaccharide material based on the anthrone test shown in Table 1. These amounts were chosen to produce approximately equal blackening of the UV-sensitive film by both macromolecular species. A comparison of the areas under the curves obtained in microdensitometer tracings (not shown) of this film showed that this condition was approximately met. In the lower photograph, taken with the Schlieren optical system, the difference between the optically similar bands in the UV-sensitive photograph is revealed. The polysaccharide material at 1.658 g/cm³, due to its greater concentration, produced a much larger disturbance in the refractive index gradient than did the lesser amount of DNA at 1.710 g/cm³. Thus, the degree of disturbance of refractive index measured across the gradient, and the different apparent absorbancies of the two substances in UV light, permitted a clear distinction to be made between light-absorbing and light-scattering macromolecules present at a given density in a sample.

Based on this method of detection, a number of polysaccharide species have been found in the DNA fractions from several blue-green algae. Some were undetectable using UV-absorption photographs but were clearly visible in Schlieren photographs of the same sample. Some samples

contained two such bands. Table 2 gives the sources and buoyant densities in CsCl of these polysaccharides. All the blue-green algal species found by us to contain a polysaccharide in their isolated DNA fractions belong to the order *Oscillatoriales*, whose members possess extensive mucopolysaccharide sheaths surrounding their filaments. Possibly, therefore, these DNA-fraction contaminants were derived from this material. However, no attempt was made by us to localize or further characterize these polysaccharides.

The buoyant densities of the polysaccharide

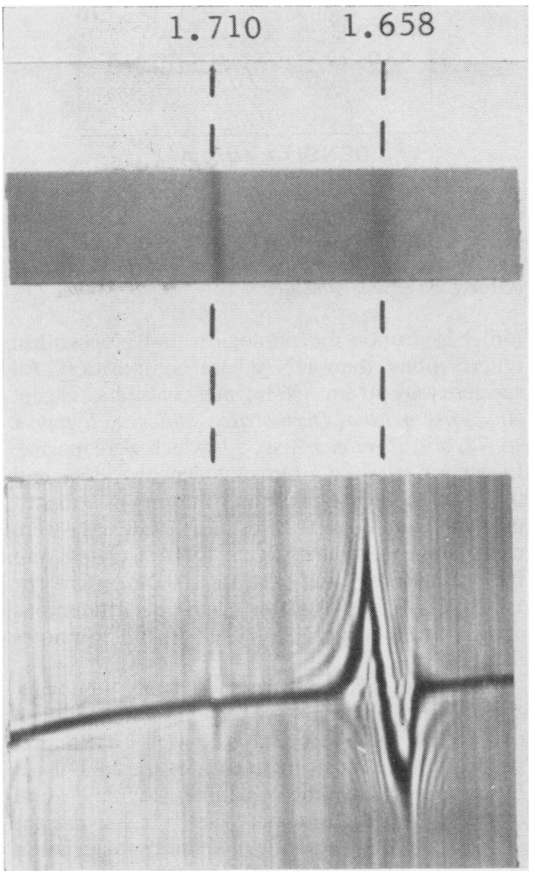

FIG. 7. *Distinguishing between light-absorbing and light-scattering macromolecules in the analytical ultracentrifuge. Conditions of centrifugation are as in Fig. 1. Upper photograph made with the ultraviolet-absorbing optical system; lower photograph made with the Schlieren-refractive index optical system. The band at 1.710 g/cm³ is from 1 μg of Escherichia coli DNA, and that at 1.658 g/cm³ from 60 μg of Plectonema polysaccharide material.*

TABLE 2. *Buoyant densities of blue-green algal polysaccharides in CsCl*

Species	Density (g/cm³)
Anabaena spiroides	1.654
Tolypothrix sp	1.655
Lyngbya sp., 77	1.656
Plectonema boryanum, 581	1.658; 1.672
Anabaenopsis sp.	1.659
Anabaena variabilis	1.664
Plectonema sp., 52	1.665
Nostoc muscorum 12·3·2	1.665; 1.672
Nostoc punctiforme, 384	1.665; 1.675
Nostoc muscorum	1.666
Nostoc sp., 586	1.668
Lyngbya sp., 487	1.684
Nodularia sphaerocarpa, 11·1·1	1.704; 1.729
Calothrix parietina	1.753

polymers shown in Table 2 varied to a slightly greater degree from one determination to another than did the usual DNA density values; they were reproducible to ± 0.003 g/cm³. Although many of these polymers have densities lower than those of known DNA species, the major portion of the DNA density spectrum (51) is encompassed by these polysaccharide densities as well.

Observations concerning polysaccharide contaminants in DNA fractions have recently been made by other investigators. The presence of these polymers in chloroplast (41), Drosophilia (47), and frog egg (10) DNA fractions has been noted. Substances in *E. coli* lysates, which band in the DNA buoyant density region in CsCl and behave similarly to the polysaccharides described here with respect to their Schlieren optical properties, have been described by Schumaker and Wagnild (52). In mammalian systems, polysaccharides have been identified in HeLa cell DNA preparations (54) and characterized in mouse liver DNA fractions (7, 54).

Thus, in a wide spectrum of biological systems, the DNA fraction of the cell, extracted and purified according to current methods (20, 29), appears to be contaminated with carbohydrate polymers. As it is now evident that these polysaccharides can be responsible for the appearance of minor components or "satellite bands" in UV-absorption photographs of purified DNA solutions, it is suggested that a comparison of UV-absorption and refractive-index patterns of ultracentrifuge samples be routinely made (as in Fig. 7) whenever multicomponent patterns are encountered and are to be interpreted. This method should supplement, rather than replace,

identification of DNA by deoxyribonuclease sensitivity and other conventional assays which are desirable.

DNA Density Shifts Due to Presence of Polysaccharides

Two or more macromolecular components, present in small amounts and sufficiently dissimilar in buoyant density, will not measurably distort each other's position in a gradient at equilibrium. The use of DNA species of dissimilar but previously determined densities as standards in CsCl gradients is based upon this fact (51). However, when the conditions mentioned above do not obtain, distorting effects may appear (M. Edelman, Ph.D. Thesis, Brandeis University, 1965).

Figure 8 shows DNA extracts from *Nodularia sphaerocarpa*, which produced bimodal distributions in the analytical ultracentrifuge. The upper tracing indicates the native, low concentration (6 μg) pattern. Based upon heat denaturation treatment, observation of the Schlieren refractive index patterns, and deoxyribonuclease treatment (middle tracing), the component of density 1.698 g/cm^3 was DNA and that of 1.703 g/cm^3 a carbohydrate polymer. Upon centrifugation of larger amounts of the native material (30 μg), the pattern shown in the lower tracing was produced. The density differential between the medians of the two components had now increased considerably, giving the DNA an apparent density of 1.691 g/cm^3 and the polysaccharide a value of 1.712 g/cm^3. The maintenance of separate modalities, and the shifts in density produced under these conditions of centrifugation, raised the possibility that such carbohydrate polymers may be useful in physically separating closely banding DNA species in a gradient.

The materials examined in the present investigation were studied under conditions which did not introduce these distortions, and the DNA density values reported throughout represent undistorted values.

Taxonomic and Phylogenetic Significance of the DNA Base Compositions

Lee et al. (25) were the first to recognize the possibilities of DNA base analysis as a taxonomic aid. Extensive analyses by several workers examining bacteria showed that organisms which are very closely related genetically have similar DNA base compositions (31, 33). Table 3 summarizes the DNA base-pair compositions of the various species of the *Cyanophyta* investigated by us. The organisms are grouped according to a commonly accepted classification for this phylum

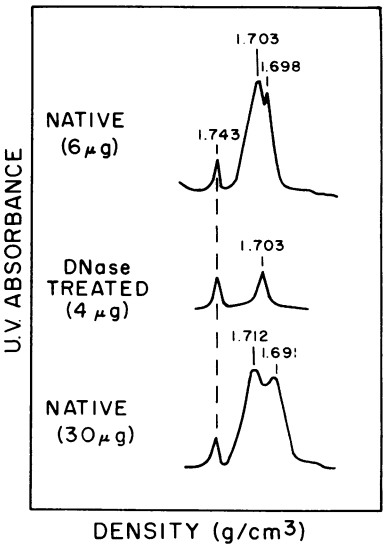

FIG. 8. *DNA fraction of Nodularia sphaerocarpa. Conditions of centrifugation are as in Fig. 1. Peaks at 1.743 g/cm^3 represent SP8-phage, standard DNA. The amount of material introduced into the centrifuge cell in each case is indicated.*

(56) based upon morphological and biochemical criteria other than DNA base composition. All species were from fresh-water habitats, except *Anacystis marina*, *Dermocarpa violacea*, *Lyngbya* sp. 77, and *Plectonema* sp. 52, which were marine. In general, the CsCl buoyant density values and the calculated base-pair compositions in terms of per cent guanine plus cytosine (% GC; 51) were based upon several separate DNA isolates, and the values are significant to $\pm$0.001 g/cm^3 or 1% GC. Single isolates, and determinations made on cell lysates, are noted in the footnotes to Table 3.

The linear relationship between DNA buoyant density and base-pair composition, upon which the % GC values of Table 3 were based, presupposes the exclusive presence of the four usual DNA bases: adenine, guanine, thymine, and cytosine. Anomalous or "rare" bases present in a sample might lead to a significant deviation from this correlation (51). In this regard, the thermal transition temperature (t_m) of *Anacystis nidulans* DNA was determined to be 92 C in SSC (% GC = 56; 30), in agreement with the value calculated from buoyant density measurements for this strain. Chemical base analysis data are also available for this species [B. B. Biswas, Plant Physiol. (Suppl.) **35:** xxx, 1960] and are in good agreement with the base-pair percentages determined by the two indirect physical methods mentioned above.

The most conspicuous feature of Table 3 is the homogeneity of base-pair compositions among the members of the *Oscillatoriales*. The mean % GC for the 20 different species of this group examined by us is 45 ± 6. The exhibited homogeneity is noteworthy, because it is clear from other studies that a wide diversity in base composition is generally characteristic of groups of unicellular organisms (33). This has been shown to be the case for bacteria (31), protozoa (M. Mandel, Chem. Zool., *in press*), and algae and fungi (50). The results obtained for the

TABLE 3. *Densities and base compositions of blue-green algal DNA*

Biological classification[a]	Strain	Source	Density (g/cm³)	% GC[b]
Order 1. *Chroococcales*				
Family 1. *Chroococcaceae*				
Anacystis marina		R. Lewin	1.728	69
A. nidulans		M. M. Allen (from M. B. Allen)	1.715	56
Anacystis sp.	6311	M. M. Allen	1.715	56
Coccochloris peniocystis	6307	M. M. Allen	1.730	71[c]
Coccochloris sp.	6604	M. M. Allen	1.722	63[d]
Gleocapsa alpicola	6308	M. M. Allen	1.694	35[c]
Order 2. *Chamaesiphonales*				
Family 2. *Dermocarpaceae*				
Dermocarpa violacea		R. Lewin	1.703	44[c]
Order 3. *Oscillatoriales*				
Suborder 1. *Oscillatorineae*				
Family 1. *Oscillatoriaceae*				
Lyngbya sp.	478	IUCC[e]	1.706	47
Lyngbya sp.	77	R. Lewin	1.710	51
Microcoleus sp.	6401	M. M. Allen	1.704	45[d]
M. vaginatus	6304	M. M. Allen	1.707	48[d]
Phormidium luridum var. olivacea	426	IUCC	1.706	47
Suborder 2. *Nostochineae*				
Family 1. *Nostocaceae*				
Anabaena spiroides		M. M. Allen (from M. Shilo)	1.705	46
A. variabilis		J. Meyers	1.703	44
Anabaenopsis sp.		M. Gibbs	1.701	42
Nodularia sphaerocarpa	11·1·1	KFRI[f]	1.698	39
Nostoc punctiforme	384	IUCC	1.703	44
N. muscorum		N. Lazaroff	1.702	43
N. muscorum	12·3·2	KFRI	1.702	43
Nostoc sp.	12·4·1	KFRI	1.703	44
Nostoc sp.	586	IUCC	1.703	44
Nostoc sp.	6305	M. M. Allen	1.703	44
Family 2. *Scytonemataceae*				
Fremyella diplosiphon	481	IUCC	1.701	42
Plectonema boryanum	581	IUCC	1.707	48
P. boryanum	9·2·5	KFRI	1.707	48
P. calothricoides	598	IUCC	1.707	48
Plectonema sp.	52	R. Lewin	1.702	43
Tolypothrix sp.		M. Gibbs	1.702	43
Family 4. *Rivulariaceae*				
Calothrix parietina		M. M. Allen (from M. B. Allen)	1.702	43

[a] After Smith (56).
[b] Per cent guanine plus cytosine (% GC); determined from bouyant density (51).
[c] Determined from single sample.
[d] Determined from cell lysate (single sample); not examined for possible contamination.
[e] IUCC, Indiana University Culture Collection (58).
[f] KFRI, Kaiser Foundation Research Institute Collection, Richmond, Calif.

TABLE 4. *DNA of bacterial species thought to be related to the blue-green algae*

Group	Species	Density	% GC[a]	Reference
Filamentous gliding bacteria	*Flexibacter rubrum*	1.696	37	Mandel (*personal communication*)
	Saprospira thermalis	1.696	37	Mandel (*personal communication*)
	Flexothrix sp.	1.697	38	Mandel (*personal communication*)
	Vitreoscilla sp.	1.703	44	Mandel (*personal communication*)
	Vitreoscilla sp.	1.704	45	Mandel (*personal communication*)
	Saprospira grandis	1.706	47	Mandel (*personal communication*)
	Flexibacteria elegans	1.707	48	Mandel (*personal communication*)
	Leucothrix mucor (11 strains)	1.708	49	(2)
Nonfilamentous gliding bacteria	*Cytophaga johnsonii*		33	(31)
	Sporocytophaga myxococcoides		35	(31)
	Cytophaga fermentans		39	(31)
	C. aurantiaca		39	(31)
	Myxococcus fulvus		67	(31)
	M. virescens		67	(31)
	M. xanthus		67	(31)
	Polyangium cellulosum		69	(31)

[a] Per cent guanine plus cytosine; determined from buoyant density (51).

Oscillatoriales suggested that this group, as postulated from other evidence (14), may indeed be a conservative one which has undergone limited genetic diversification over the course of evolution.

Morphological heterogeneity permits the division of this group into two suborders and several families. The sampling of DNA base-pair compositions permitted by the availability of axenic cultures may appear to reflect these divisions, but the differences are not statistically significant.

Only one member of the *Chaemosiphonales* was available, and the base-pair composition of this species, *Dermocarpa violacea*, falls within the range of the base compositions of the *Oscillatoriales*.

The *Chroococcales* exhibit a much larger range of buoyant densities in CsCl than do the *Oscillatoriales*, their base compositions varying from 35 to 71% GC for the six samples examined by us. In this respect, they resemble most of the other unicellular groups investigated. Although the evidence presented for the *Oscillatoriales* is in agreement with a common evolutionary origin for the various species, the members of the *Chroococcales* exhibit sufficiently diverse base-pair compositions (from this limited sample) to suggest polyphyletic origins for this group. Indeed, the genera placed in this order are characterized by negative rather than positive characteristics, and form the residuum left after exclusion of the strictly filamentous and regularly endospore-forming *Cyanophyta* (56).

In view of the data in Table 3, it is of interest to examine the DNA base-pair compositions of certain other members of the *Monera*, e.g., the "gliding bacteria," which have been thought to be related to the blue-green algae (17, 39, 57).

The DNA base compositions of several members of the filamentous gliding bacteria, generally acknowledged to be closely related to the *Oscillatoriales* (39), and the nonfilamentous gliding bacteria, more distant, but possibly also related to the *Cyanophyta* (39), have been characterized by Mandel and co-workers and are listed in Table 4. Figure 9 compares these values with those of the blue-green algae. Note that the DNA base compositions of the filamentous organisms overlap with those of the *Oscillatoriales*. Thus, strains of *Vitreoscilla*, *Saprospira*, *Leucothrix*, and representatives of the *Flexibacteriaceae* (Soriano, *Bergey's Manual of Determinative Bacteriology*, 6th ed., 1948) all fall within, or quite close to, the limits of base composition exhibited by the *Oscillatoriales* in the present study.

The nonfilamentous unicellular gliding bacteria generally fall outside the range exhibited by the *Oscillatoriales* and appear similar to the nonfilamentous, unicellular blue-green algae (*Chroococcales*) in that both show large differences in base compositions among the various species investigated. The unicellular gliding bacteria also

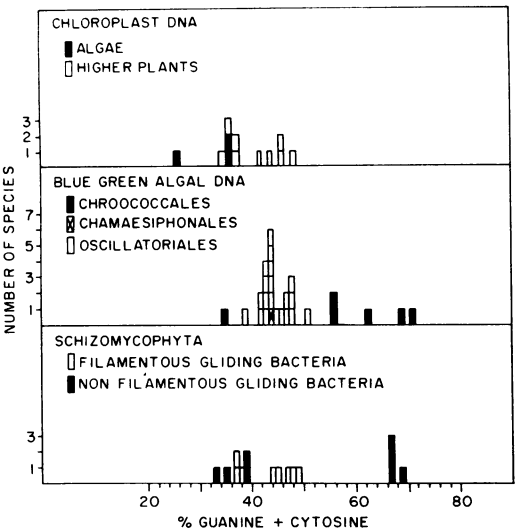

FIG. 9. *DNA base composition relationships of the blue-green algae, the gliding bacteria, and eucaryotic chloroplasts. Blue-green algal, Schizomycophyta, and chloroplast DNA values are from Tables 3, 4, and 5, respectively.*

appear to be a heterogeneous group based upon DNA homology studies (J. L. Johnson and E. J. Ordal, Bacteriol. Proc., p. 304, 1966).

It appears that, in several of the filamentous gliding bacteria, there are affinities indicated, on the basis of DNA base composition, which substantiate current phylogenetic notions based on other criteria.

Relationships Between Blue-green Algal and Chloroplast DNA

Basically, the same two theories which have been suggested for the origins of viruses have been suggested to account for the origin of chloroplasts in eucaryotic cells (50). In the first, a piece of the central DNA of the cell is presumed to have become detached from the main genome and established itself as a separate entity in the cytoplasm. In time, this episomal DNA would organize about itself a structure implicit in its own code and might continue to evolve, dividing along with the rest of the cell. This would predict that the DNA of this episomally derived structure (which might evolve as far as a chloroplast or other organelle) should be related to the host's DNA. It is possible, however, that, in terms of base composition, the host DNA could be heterogeneous along its length, and the detached piece might not be representative of the host DNA as a whole (e.g., see 43). It is also possible that, after detachment, the cytoplasmic fragment and the major DNA of the cell diverged in base composition as a result of subsequent evolution.

A second theory supposes that a primitive, procaryotic, photosynthetic cell, such as a primitive blue-green alga, invaded a nonphotosynthetic procaryote and established itself as an endosymbiont. With time, interrelationships would develop which would further ensure that the endosymbiont divided along with the host cell, and evolutionary pressure might lead to certain losses in the invader which would no longer, normally, permit its existence outside the host. This theory does not require the invader and host necessarily to have DNA's of related base composition.

Although there is little direct evidence to support these two theories (or several others which might be proposed), bits of relevant information exist from studies of the DNA of contemporary organisms.

Table 5 shows the DNA base composition data for several algal and higher plant species. A close relationship between nuclear and chloroplast DNA base compositions in these organisms would be viewed as supporting an episomal theory of chloroplast origin. However, even from the limited sampling available at present from the literature, it is obvious that this condition does not obtain in the algae where chloroplast DNA is from 21 to 28% richer in adenosine plus thymidine content than the corresponding nuclear DNA. In the case of higher plants, the situation is less obvious, with the chloroplast-nuclear DNA differential ranging from 0 to 13% for the species investigated.

If chloroplasts originated by blue-green algal invasion and endosymbiosis, a relationship at the level of DNA base-pair composition might exist between the present-day representatives. A comparison of the blue-green algal (Table 3) and chloroplast (Table 5) DNA base compositions is shown in Fig. 9. Significant correlation at this level between organism and organelle would be in agreement with the postulated relationship between the two. However, too few algal species (which are of greater interest for this purpose) have been investigated to permit a meaningful comparison of their chloroplast DNA data with the data from the *Cyanophyta*. For the higher plants, as well as the green algae (*Chlorella* and *Chlamydomonas*), existing data indicate a partial matching of chloroplast DNA with the DNA of *Oscillatoriales*.

For the present, we will assess possible relationships between blue-green algal DNA and chloroplast DNA by comparing certain macromolecular characteristics shared by procaryotic

TABLE 5. *DNA of chloroplast-containing species*

Group	Species	Chloroplasts		Nucleus		References
		Density (g/cm³)	%GC[a]	Density (g/cm³)	%GC[a]	
Algae	*Euglena gracilis* Z	1.685	25[b]	1.708	51[b]	(1)
	Euglena gracillis v. bacillaris	1.686	26[b]	1.707	52[b]	(12,42)
	Chlamydomonas reinhardi, Y_1	1.694	35	1.721	62	(27,49)
	Chlamydomonas reinhardi	1.695	36	1.723	64	(6)
	Chlorella ellipsoidea	1.695	36	1.716	57	(6,18)
Higher plants	*Phaseolus vulgaris*, v. Saxa (bean)	1.694[c]	35	1.694	35	(Beridze et al. Mol. Biologiya, *in press*, 1967)
	Brassica rapa (turnip)	1.695	36	1.692	33	(59)
	Antirrhinum majus (snapdragon)		37[b]		41[b]	(48)
	Vicia faba (broad bean)		37[d]		40[d]	(21)
	Beta vulgaris v. cicla (swiss chard)	1.700	42	1.689	30	(22,61)
	Nicotiana tabacum (tobacco)	1.703	44	1.690	31	(53)
	Beta vulgaris (beet)	1.705[e]	46	1.695	36	(6)
	Spinacia oleracea (spinach)	1.705[e]	46	1.695	36	(6)
	Nicotiana rustica (turkish tobacco)	1.707[c]	48			(Green and Gordon, Federation Proc. **24**:539, 1965)

[a] Per cent guanine plus cytosine determined from bouyant density (51).
[b] Determined from chemical analyses.
[c] Unidentified minor components are also present in the chloroplast fraction as isolated.
[d] Chloroplast fraction, A:G = 1.67; nuclear fraction, A:G = 1.54.
[e] The authors also report a satellite band of density 1.719 g/cm³ in the chloroplast fraction.

organisms and eucaryotic organelles. Chloroplasts and mitochondria, viewed under the electron microscope, contain clumped filamentous components closely resembling structures in the bacterial nucleus (37, 44, 61). These clumps, produced during specimen dehydration, are not characteristic of nuclear chromatin of higher organisms, presumably due to the presence of DNA-histone complexes in the latter (61). The observation that exactly similar clumps occur in the nuclear regions of blue-green algae (46, 62; L. V. Leek, J. Cell Biol. **31**:67A, 1966) is of specific interest. In addition, after fixation with Kellenberger's procedure, the nucleoplasm of eucaryotic chloroplasts and mitochondria, along with that of the blue-green algae and bacteria, exhibit masses of ribonuclease-resistant, deoxyribonuclease-sensitive fibrils 25 A thick (37, 45, 46).

Another similarity between blue-green algal and chloroplast DNA, and, in general, between DNA of procaryotes and eucaryotic organelles, is the apparent unimodality of both in CsCl equilibrium density gradients. The numerous bacterial DNA samples investigated have consistently been found to be unimodal, unless an episomal or similar type of particle is present (31, 50). The organism investigated by Joshi, Guild, and Handler (19) is the only exception. DNA satellite bands are not found in our blue-green algal preparations (Fig. 3). This characteristic, unique at the organismal level, to the DNA of procaryotic cells which lack organized intracellular compartmentalization, appears to find a parallel in organellular DNA from eucaryotes. Occasionally, in isolated chloroplast and mitochondrial fractions, several DNA species are encountered. On further purification or enzymatic treatment, however, a single DNA species, giving a unimodal distribution in CsCl, often emerges free from cellular DNA contaminants (12, 23). To establish whether such a pattern is characteristic of all organelle samples will require further elucidation.

Other DNA characteristics in common between cells of the *Monera* and organelle samples, but not reported present in eucaryotic chromatin, are the circular state of the bacterial (5) and mitochondrial (23) DNA molecule and the similarity in the frequency of occurrence of the dinucleotide CpG (9). Equivalent studies with blue-green algal and chloroplast DNA have not yet been reported.

These similarities in DNA behavior at the

electron microscopic and ultracentrifugal level between the blue-green algae and bacteria on the one hand and chloroplasts and mitochondria of eucaryotes on the other, are consistent with the suggestion that a genetic relationship may exist between certain procaryotic cells and eucaryotic organelles. Evidence supporting this hypothesis is presented in the next section.

DNA of Cyanophora paradoxa

The hypothesis of chloroplast origin by invasion and endosymbiosis is attractive, because some instances of extensive symbiosis can be found in contemporary organisms. Examples of blue-green algae, associated to various degrees with a variety of other organisms, are to be found in the literature (cf., 14, 56). In several of these cases, cyanophytes coexist in a symbiotic relationship with various fungi, protozoa, higher animals, and plants. Often, the two partners can be separated and grown independently of each other, indicating that the relationship is not an extremely close or reciprocal one. However, in at least two separate cases (15, 16, 27), a blue-green alga appears to have established a more intimate relationship with a protistan cell. In the case of *Cyanophora paradoxa*, a cryptomonad, a blue-green alga has become habituated in an animal cell, has lost its cell wall, and divides along with its host (15). It might be but a small series of steps from here to becoming a chloroplast. Our attempts to grow the "cyanelle," so designated by Pascher (38), outside of the cryptomonad have not been successful.

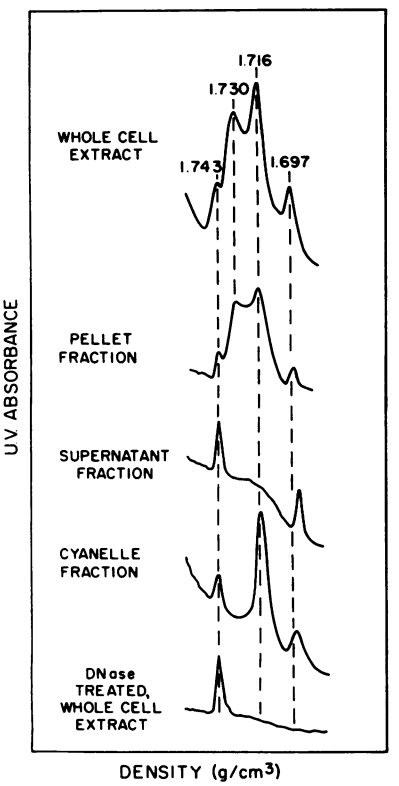

FIG. 10. *DNA density profiles of Cyanophora paradoxa. Conditions of centrifugation are as in Fig. 1. SP8 phage DNA (1.743 g/cm³) was added as a density marker in each case. Fractionation procedures and designations are explained in the text.*

The DNA of this organism was extracted and examined in detail. Figure 10 shows the CsCl equilibrium density profiles for the various fractions of the cell prepared according to Fig. 2. The band at 1.743 g/cm³ in each case is a density standard introduced into the ultracentrifuge cell to calibrate the gradient. The whole cell extract, in which lysis of all cellular components occurs, exhibits a trimodal DNA distribution with two components of about equal concentrations at 1.730 g/cm³ and 1.716 g/cm³ and a minor component at 1.697 g/cm³.

The DNA banding profile of the pellet fraction, containing broken nuclei, large cellular debris, and unlysed cyanelles, is shown below (Fig. 10). A pattern similar to the whole cell extract is obtained, but the minor component is slightly diminished in concentration. The supernatant fraction (Fig. 10), which by microscopic examination is completely free of cyanelle particles as well as any large debris, contains the remainder of the minor DNA band free from other components. The cellular localization of this DNA species is discussed below.

Since the protozoan nuclei do not remain intact during preparation of the pellet fraction, treatment of this fraction with deoxyribonuclease before DNA extraction was performed in an attempt to degrade selectively any susceptible DNA fragments which might be adhering to the membranes of the intact cyanelle particles. A 50-μg amount of DNA of known density (SP8 phage DNA) was added to the pellet before incubation, as a check on the effectiveness of the enzyme (13). At the end of the incubation period, the membrane-enclosed cyanelles were recentrifuged, and an amount of low molecular weight, ultraviolet-absorbing material (optical density maximal at 263 mμ) in excess of the added SP8 DNA was recovered from the supernatant fraction. When the recentrifuged material was lysed and subjected to DNA extraction, the profile designated "Cyanelle fraction" was obtained. Here, the DNA species of 1.730 g/cm³, present in the whole cell extract and in the pellet

fraction in a partially degraded form (observe band widths), is totally absent. Bands of density 1.716 g/cm³ and 1.697 g/cm³ are present, the former enriched about 50% over the latter. In other cyanelle fraction preparations, the DNA of density 1.716 g/cm³ constituted over 99% of the total. This DNA species, then, is identified with the blue-green endosymbiont of *Cyanophora paradoxa*.

We also tested the possibility that the deoxyribonuclease resistance exhibited by the bands present in the cyanelle fraction might be due to polysaccharide material or to some unusual property of the DNA. A comparison of refractive-index and UV-absorbance patterns in the ultracentrifuge, however, revealed no evidence for the presence of polysaccharides. Treatment of the whole cell extract DNA with enzyme, as above, and dialyzing overnight against SSC, produced the deoxyribonuclease-treated, whole cell extract (bottom profile, Fig. 10) in which all bands are shown to be sensitive to enzymatic degradation under these non-membrane-protected conditions.

The DNA profile of the protozoan partner can now be assigned. Main band DNA of density 1.730 g/cm³ is probably nuclear in origin, since it constitutes approximately 75% of the DNA of the whole cell extract minus the cyanelle-band DNA, is absent from the 5,000 × *g* supernatant fraction, and is present in a degraded form in the pellet fraction in which ruptured nuclei are observed.

Satellite band DNA of density 1.697 g/cm³ is present in all fractions of the cell investigated. It is not associated with the cyanelles, since it is prominently present in the 5,000 × *g* supernatant fraction, which is observed to be free of the blue-green particles. By its appearance in the cyanelle fraction preincubated with deoxyribonuclease, it seems to be contained within a membrane-protected structure. These criteria suggest a localization of this DNA species within an organelle of the protozoan such as the mitochondrion or nucleolus. Mitochondria are known to protect their DNA from enzymatic digestion, under the conditions employed in this study (13, 41), and both organelles would be, to some extent, sedimented at 5,000 × *g*, the force necessary to concentrate the cyanelles in the pellet fraction. The percentage of satellite DNA (about 25% of the DNA of the whole cell extract minus the cyanelle-band DNA) is appreciably greater than has been shown to be present in protozoan (60) and other (23, 59) mitochondrial fractions. [The case of frog egg DNA, where at least two-thirds of the total egg DNA has been shown to be mitochondrial, is clearly different from the above. Dawid (10) has shown the phenomenon of excess cytoplasmic DNA in amphibian eggs to be caused by the large size of these cells and by the great number of mitochondria they contain.] The high percentage of satellite DNA in *Cyanophora* may be evidence in favor of a nucleolar location for this DNA species, since nuclear-localized minor components constitute a greater percentage of the total DNA than do mitochondrial components and range as high as 30% of the total DNA (T. Beridze, M. Odintsova, and N. Sissakian, Mol. Biologiya, *in press*). Alternatively, the high percentages of both satellite and cyanelle DNA encountered in the whole cell extract could result from selective degradation of the DNA of the ruptured protozoan nuclei during the 4-hr period of lysozyme treatment at 37 C, which occurs before inactivation of endogenous nucleases with sodium dodecyl sulfate. From its phylogenetic position in the evolutionary scale, one might expect the protozoan nucleus to contain appreciably more DNA than the two to eight blue-green endosymbionts within it (33).

Based upon the CsCl buoyant density values (51), the mole per cent of GC for the nuclear, cyanelle, and satellite DNA of *Cyanophora paradoxa* is 71, 57, and 38, respectively. The GC content of the cyanelle DNA (57%) places it outside the DNA base-composition range of the *Oscillatoriales* and very close to several species of *Chroococcales* (see Table 3). On morphological grounds, Hall and Claus (15) placed the cyanelle of *Cyanophora paradoxa* precisely in this same taxonomic group, calling it *Cyanocyta Korschikoffiana* in a new family *Cyanocytaceae* of the *Chroococcales*. *Cyanocyta* DNA is 14% richer in adenosine plus thymidine content than is the nuclear DNA of its protozoan host. This is consistent with the previous postulation that it is not necessary for invader and host to have DNA of similar base composition. In this respect, *Cyanocyta* DNA resembles the algal chloroplast DNA's shown in Table 3.

While the presence of organisms, such as *Cyanophora paradoxa*, speaks in favor of the hypothesis of chloroplast origin through invasion, the heterogeneity in base composition of chloroplast DNA among the algal genera studied, as well as among the higher plants (Table 5), suggests that, if its origin were through invasion and habituation, there may have been several different cases of invasion rendering the origins of chloroplasts in different species polyphyletic (50). In a similar case to that of *Cyanophora paradoxa*, a blue-green alga lacking the typical cyanophycean wall has been found inside an apochlorotic green alga, *Glaucocystis nostochinearum* (16, 27). This cyanophyte, on mor-

phological grounds, is also classified in the *Chroococcales*, but in a different genus than *Cyanocyta*. If a propensity had existed for different members of the early *Chroococcales* to become associated in such a manner with early protistan cells, then the present heterogeneity of this group in DNA base composition (Table 3) would account for the concomitant heterogeneity observed in chloroplast DNA's (Table 5).

Although data for comparison are scanty, the blue-green algal DNA parameters described and discussed in the preceding sections appear to be in agreement with an hypothesis of chloroplast origin based on invasion and endosymbiosis. Further characterization of chloroplast DNA, in terms of base composition and other physiochemical parameters, is necessary if greater significance is to be given to the present comparisons. Molecular hybridization studies, as a more direct test of base sequence homology between related genomes (32), should be undertaken to augment the existing data.

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