Purification and Properties of Unicellular Blue-Green Algae (Order *Chroococcales*)

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To the memory of E. G. Pringsheim (1881–1970).

INTRODUCTION

Blue-green algae occupy an anomalous position in the biological world. They are treated by botanists as a division (or class) of algae because they are photoautotrophs that use water as an electron donor and contain the two photopigments (chlorophyll *a* and *β*-carotene) that are the chemical hallmarks of plant photosynthesis. However, it has long been recognized that in cellular and organismal respects they resemble the bacteria. This was first noted by Cohn (14), who subsequently proposed (15) to place the two groups in a common division, the *Schizophytae*, even though their shared properties then appeared to be negative ones. The problem of defining in positive terms the properties that are shared by bacteria and blue-green algae remained intractable

for many decades (16, 58, 69, 74) and was solved only when the structure of the cell in these two groups could be studied at the level of resolution afforded by the electron microscope. Electron microscopic investigations, together with analytical data on cell wall composition and ribosomal structure, have now revealed the common denominators, which are fundamental: bacteria and blue-green algae are the only organisms with cells of the procaryotic type (6, 10, 21, 47, 70).

The designation blue-green algae is therefore misleading, although this common name is now so firmly established that its use can probably never be eradicated. These organisms are not algae; their taxonomic association with eucaryotic algal groups is an anachronism, formally equivalent to classifying the bacteria as a constituent group of the fungi or the protozoa. In view of their cellular structure, *blue-green algae can now*

be recognized as a major group of bacteria, distinguished from other photosynthetic bacteria by the nature of their pigment system and by their performance of aerobic photosynthesis. Their properties are still very poorly known. Much of the available information about them lies in the domain of natural history, not biology.

The structural diversity of blue-green algae is considerable, and for this reason algologists have been able to develop elaborate taxonomic treatments of the group based solely on structural properties, determined through microscopic examination of field collections (19, 28). Most algal taxonomists recognize several different orders. The unicellular blue-green algae are assigned to a single order, *Chroococcales*, containing two families, *Chroococcaceae* and *Entophysalidaceae* (28). Members of the *Chroococcaceae* are coccoid or rod-shaped organisms which multiply by binary fission, and they may form loose colonies in which the constituent cells are held together by a common slime layer or by sheaths. In structural terms, they appear to be counterparts of unicellular true bacteria. Members of the *Entophysalidaceae* are coccoid organisms, distinguished primarily by their colonial growth habit. They grow as dense, parenchymatous masses of cells, typically found on the surface of moist rocks.

In the study of any microbial group, the transition from natural history to biology is largely dependent on the solution of a technical problem: the isolation and maintenance of its members in pure culture. The present paucity of information about blue-green algae reflects the difficulty that has been encountered in the purification of these microorganisms. The total number of strains purified so far is small, and the pure strains now available are far from representative of the evident biological diversity of the group. Nevertheless, the nutrient requirements of these organisms are simple, and it is easy to obtain growth on solid media. The primary obstacle to obtaining pure cultures seems to lie in the synthesis by many blue-green algae of a copious extracellular slime layer, which harbors bacteria.

Some filamentous blue-green algae have been purified by taking advantage of their vigorous gliding movement and inducing phototactic migration of trichomes or hormogonia over (or through) agar plates (41). This does not always eliminate bacteria, however, and purification of some filamentous strains has been achieved only by treatment of trichomes or resting structures with ultraviolet light (9), toxic chemicals (27), or heat (75).

In principle, the purification of unicellular blue-green algae should be simpler than that of filamentous forms, since most of the unicellular members of the group are immotile and, therefore, will form small, compact colonies on solid media. The most successful pioneer in the purification of blue-green algae, M. B. Allen (1), isolated one unicellular strain, the organism known as *Anacystis nidulans*, by standard plating methods. Her strain remained for many years the sole representative of the *Chroococcales* available in pure culture, and for this reason it has been widely used in physiological and biochemical investigations (*see* 33). A few additional unicellular strains have been purified from marine sources by Van Baalen (73), and Castenholz (11) has purified several thermophilic unicellular strains from hot springs.

Our work on this problem began about 8 years ago when M. M. Allen (2) succeeded in purifying by plating methods some unicellular strains that had been received as unialgal cultures from other laboratories. This convinced us that purification is not difficult, provided that suitable material is available in the form of unialgal cultures or crude cultures that contain a preponderance of unicellular blue-green algae. However, these organisms typically occur in nature as minor components of mixed algal populations; since their nutritional requirements do not differ significantly from those of many other algae, they cannot be specifically enriched from such mixed populations by nutritional selection. Natural populations that consist largely of unicellular blue-green algae may arise sporadically as water blooms in lakes or ponds (28) but are of relatively constant occurrence only in hot springs, where they are composed of a few physiologically highly specialized thermophilic forms (11).

Allen and Stanier (5) discovered that many mesophilic blue-green algae, both filamentous and unicellular, can be enriched successfully from mixed algal populations by temperature selection, since their temperature maxima are significantly higher than those of nearly all eucaryotic algae from the same environments. When samples of soil or fresh water are placed in a suitable mineral medium and incubated in the light at 35 C, the population that develops consists almost exclusively of blue-green algae. This enrichment technique is particularly valuable for the isolation of unicellular blue-green algae because, if present in the inoculum, they eventually far outnumber filamentous members of the group as the enrichment culture develops. Experience with its use for several years has shown that one or more kinds of unicellular blue-green algae are present in almost every natural sample of fresh water, even if no algae can be detected in them by direct microscopic examination. M. M. Allen (2) purified several unicellular strains from fresh-

water enrichments, and we have subsequently isolated many more strains by the same method.

Our collection of pure unicellular blue-green algae now consists of more than 40 strains. In addition to our own isolates, it includes pure cultures previously isolated by M. B. Allen (1), Van Baalen (73), and Castenholz and his collaborators (57, 65), as well as a number of strains received from other investigators in the unialgal state and purified by us. Although this collection is certainly not yet representative of the entire order *Chroococcales*, its size and diversity are now sufficient to permit a preliminary comparative survey of the properties of unicellular blue-green algae.

The dependence on structural characters alone for the classification of blue-green algae has caused particular difficulties in the *Chroococcales*, the simplest members of the group in structural respects. Our initial goal was, accordingly, to examine other phenotypic traits, determinable only with pure cultures, which might prove to be of taxonomic value. At the same time, we determined the deoxyribonucleic acid (DNA) base composition of every strain because the one previous study of this genetic character in blue-green algae (22) indicated that it might be particularly valuable for the subdivision of the *Chroococcales*.

Edelman et al. (22) determined DNA base compositions for 29 strains of blue-green algae— probably a large fraction of all strains then available in pure culture. The material was biased in favor of filamentous representatives, since 22 strains belonged to the *Nostocaceae*, *Oscillatoriaceae*, *Scytonemataceae*, and *Rivulariaceae*. Among these strains, the range of DNA base composition was small and showed no clear-cut taxonomic correlations; the extreme limits were 39 and 51 moles % guanine and cytosine (GC), and for 20 strains the values lay between 42 and 48 moles % GC. Only six strains of the order *Chroococcales* were analyzed, but their range of base composition extended from 35 to 71 moles % GC, a span little narrower than that found in the entire group of procaryotes, about 25 to 75 moles % GC (30).

DNA base composition is now recognized to be a character of major taxonomic significance among bacteria; it has been found that the taxonomic subgroups long recognized on purely phenotypic grounds are, in most cases, also characterized by a relatively narrow and distinctive span of DNA base composition (30). The situation which presented itself in *Chroococcales* was somewhat different, since the phenotaxonomy of these organisms has remained at the level which had been attained, with respect to the bacteria, in the era of Ferdinand Cohn (15). It seemed

possible, therefore, that the major internal divergences of DNA base composition in *Chroococcales* revealed by the work of Edelman et al. (22) might provide primary clues for the development of a more satisfactory system of classification and actually direct the search for taxonomically useful phenotypic characters.

MATERIALS AND METHODS

Sources of Strains Examined

Strain histories are summarized in Tables 1 and 2. In these tables, the strains are arranged according to typological groups, of which the distinguishing properties are described in a later section (*see* Table 4). We have noted the names under which strains from other laboratories were received, accompanied by a parenthetic G (Geitler, reference 28) or D (Drouet and Daily, reference 20) to designate the nomenclatural system reflected by each name. The state of each of these strains when it was received in the Berkeley culture collection is indicated by the symbols P (pure culture) or U (unialgal culture). Strains found on receipt to be unialgal were subsequently purified by us.

Media and Conditions of Cultivation

With the exception of two marine strains (7002 and 7003) previously shown by Van Baalen (73) to require vitamin B_{12}, all strains examined can grow in strictly mineral media. The medium which we have adopted [devised by M. M. Allen (3)] for general use (BG-11) is a slight modification of medium G-11 of Hughes, Gorham, and Zehnder (36). Its composition is shown in Table 3. For the preparation of solid media, it is supplemented with 1% (w/v) Difco agar, sterilized separately from the mineral base (both at double strength).

Medium BG-11 is neutral after sterilization. It will support growth either in air or in an atmosphere slightly enriched with CO_2. For routine maintenance, cultures are incubated in air, but more rapid growth and higher yields are obtainable if cultures are continuously aerated with N_2 containing 0.5 to 1.0% (v/v) CO_2. All our unicellular strains can grow well in BG-11. A supplement of vitamin B_{12} (final concentration 1 μg/liter) is added for the cultivation of strains 7002 and 7003, and the latter strain also requires the addition of NaCl (1%, w/v) for satisfactory growth.

Medium BG-11 has a low phosphate content and is poorly buffered. Several other media which have been used to cultivate blue-green algae are better buffered and provide a larger phosphate supply—e.g., medium C of Kratz and Myers (42)

TABLE 1. *Pure strains of unicellular blue-green algae: typological group I*

Typo-logical group	Ber-keley strain no.	Name (if any) and other strain designations	Isolator	Subsequent strain history[a]	Refer-ences
IA	6301	*Anacystis nidulans* (D)	M. B. Allen	$\xrightarrow{P}$ BCC,[a] 1963	1, 52, 60
	6311	Berkeley isolate	M. M. Allen		2
	6312	Berkeley isolate	M. M. Allen		2
	6715	*Synechococcus lividus* (G)	D. L. Dyer	→ D. S. Berns $\xrightarrow{U}$ BCC, 1967	23
	7002	*Agmenellum quadruplicatum* (D) PR-6	C. Van Baalen	$\xrightarrow{P}$ BCC, 1970	73
	7003	*Coccochloris elabens* (D) 17-A	C. Van Baalen	$\xrightarrow{P}$ BCC, 1970	73
	6716	*Synechococcus* sp. (G) Cl 53	R. Castenholz	$\xrightarrow{P}$ BCC, 1967	57
	6717	*Synechococcus* sp. (G) Y 52	R. Castenholz	$\xrightarrow{P}$ BCC, 1967	65
	6908	*Synechococcus cedrorum* (G), IUCC 1911	Gassner	→ IUCC[b] $\xrightarrow{U}$ BCC, 1969	
	6605	Berkeley isolate	M. M. Allen		
	6910	*Gloeocapsa alpicola* (D) 1051	G. P. Fitzgerald	$\longrightarrow$ BCC, 1969	
	6307	*Coccochloris peniocystis* (D)	G. P. Fitzgerald	$\xrightarrow{U}$ BCC, 1963	2
	6603	Berkeley isolate	M. M. Allen		
	6706	Berkeley isolate	R. Kunisawa		
	6707	Berkeley isolate	R. Kunisawa		
	6708	Berkeley isolate	R. Kunisawa		
	6709	Berkeley isolate	R. Kunisawa		
	6710	Berkeley isolate	R. Kunisawa		
	6713	Berkeley isolate	R. Kunisawa		
	6907	*Synechococcus elongatus* (G) IUCC 563	E. G. Pringsheim	→ IUCC $\xrightarrow{U}$ BCC, 1969	
	6911	*Microcystis aeruginosa* (G) NRC-1 (?)	P. R. Gorham	→ R. Safferman → R. Haselkorn $\xrightarrow{U}$ BCC, 1969	
	7001	*Anacystis marina* (D), 6	C. Van Baalen	$\xrightarrow{P}$ BCC, 1970	73
IB	6802	Berkeley isolate	A. Neilson		
	6901	Berkeley isolate	A. Neilson		
	6903	Berkeley isolate	A. Neilson		

[a] Berkeley Culture Collection.
[b] Indiana University Algal Culture Collection.

and the medium of Van Baalen (73). Whereas many of our unicellular strains grow excellently in these particular media, some do not; and it is for this reason that we have adopted medium BG-11 as a general culture medium for unicellular strains.

We routinely use Warm White DeLuxe fluorescent tubes (General Electric) as light sources, although other fluorescent tubes or incandescent bulbs are also satisfactory. The light intensity is usually adjusted to lie in the range of 2,000 to 3,000 lux at the surface of culture vessels, as measured with a Weston illumination meter, model 756. A few strains (6507 and 6909) require lower light intensities for normal growth (500 lux or less).

With the exception of thermophilic strains (6715, 6716, and 6717), all strains now in culture grow satisfactorily at 25 C, although this is considerably below the temperature maximum for most mesophilic strains. Largely as a matter of convenience, we have incubated plate cultures and stock cultures at room temperature. However, growth is more rapid at higher temperatures; for experimental purposes, mesophilic strains are usually grown in water baths maintained at temperatures in the range of 30 to 35 C. The thermophils must be incubated at a temperature of 40 C (or higher) to obtain good growth.

Liquid cultures of small volume (including stock cultures) are grown without aeration in test tubes or Erlenmayer flasks of 125-ml capacity, either in water baths provided with lateral illumination from banks of fluorescent tubes or

TABLE 2. *Axenic strains of unicellular blue-green algae: typological groups II and III*

Typo-logical group	Berk-eley strain no.	Name (if any) and other strain designations	Isolator	Subsequent strain history	Refer-ences
IIA	6308	*Gloeocapsa alpicola* (D) 1051	G. P. Fitzgerald	$\xrightarrow{U}$ BCC,[a] 1963	
	6701	Berkeley isolate	J. Hauxhurst		
	6711	Berkeley isolate	R. Kunisawa		
	6804	Berkeley isolate	R. Kunisawa		
	6807	Berkeley isolate	R. Kunisawa		
	6808	Berkeley isolate	R. Kunisawa		
	6714	Berkeley isolate	R. Kunisawa		
	6702	Berkeley isolate	J. Hauxhurst		
	6803	Berkeley isolate	R. Kunisawa		
	6805	Berkeley isolate	R. Kunisawa		
	6806	Berkeley isolate	R. Kunisawa		
IIB	6501	Berkeley isolate	M. M. Allen		4, 62
	6909	*Gloeocapsa* sp. (G) IUCC 795	Markle	→ IUCC[b] $\xrightarrow{U}$ BCC, 1969	62, 76
IIC	7005	*Microcystis aeruginosa* (G)	G. C. Gerloff	→ SAUG[c] $\xrightarrow{U}$ BCC, 1969	
III	6712	Berkeley isolate	R. Kunisawa		
		Chlorogloea fritschii Mitra	A. K. Mitra	→ Botany Dept., Univ. of Calif. (Berkeley) → BCC, 1967	24, 25, 26, 46

[a] Berkeley Culture Collection.
[b] Indian University Algal Culture Collection.
[c] Sammlung von Algenkulturen der Universität Göttingen.

in a light cabinet. Cultures of larger volume (1.5 liters) are grown in Fernbach flasks equipped with magnetic stirrers and are incubated in water baths. The most convenient light source for such cultures is a circular fluorescent tube suspended around the culture vessel; it can be raised or lowered to provide the desired light intensity. These flask cultures are gassed with N_2 containing 0.5% (v/v) CO_2, introduced through a Pasteur pipette positioned in the cotton plug so that the tip of the pipette is slightly above the surface of the culture medium. In aerating cultures, it is not desirable to bubble gas through the medium. This may cause moistening of the cotton plug, which favors contamination, particularly if the culture is incubated for several weeks, often necessary with slowly growing strains. Cultures with a volume of 10 liters are grown in carboys equipped with magnetic stirrers and gassed above the level of the medium with N_2 containing 1% (v/v) CO_2. Illumination is provided by two banks of fluorescent tubes on either side of the carboy.

In large flasks and carboys, the growth rate becomes limited by light at relatively low population densities, as a result of self shadowing by

TABLE 3. *Medium BG-11[a]*

Compound	Amt (g/liter)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4 \cdot 7 \ H_2O$	0.075
$CaCl_2 \cdot 2 \ H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium magnesium salt)	0.001
Na_2CO_3	0.02
Trace-metal mix A5[b]	1 ml/liter

[a] After autoclaving and cooling, pH of medium 7.1.
[b] H_3BO_3, 2.86 g/liter; $MnCl_2 \cdot 4H_2O$, 1.81 g/liter; $ZnSO_4 \cdot 7H_2O$, 0.222 g/liter; $Na_2MoO_4 \cdot 2 \ H_2O$, 0.39 g/liter; $CuSO_2 \cdot 5H_2O$, 0.079 g/liter; $Co(NO_3)_2 \cdot 6H_2O$, 0.0494 g/liter.

the growing culture. For this reason, the growth yields obtainable in such cultures are relatively low; growth is virtually arrested when the cell mass reaches a density of 1 to 2 g (wet weight) per liter.

We have found it advisable to incubate plate cultures in clear plastic boxes. (Commercial

vegetable crispers are excellent for this purpose.) It is often necessary to keep plate cultures for several weeks, and their enclosure in boxes greatly reduces external contamination and water loss by evaporation. Plate cultures of thermophilic strains are incubated in shallow, gasketed plastic boxes immersed in an illuminated water bath.

Two factors of critical importance for the cultivation of blue-green algae are the cleanliness of glassware and the purity of the water used to prepare media. Experience has taught us that if both factors are not carefully controlled, sporadic failures to obtain satisfactory growth are almost certain to occur. We now employ a special system (Millipore Super-Q water purifier) to prepare water for use in media and for the final rinsing of glassware. All glassware is washed by hand and then rinsed successively with tapwater, concentrated HNO_3, and purified water.

The effects of organic compounds on growth were determined on plates of medium BG-11 supplemented singly with various organic compounds. The basal medium was modified by using NH_4NO_3 in place of $NaNO_3$ as a nitrogen source. Penicillin sensitivity was determined on plates of medium BG-11 containing gradients of penicillin G (0 to 1, 0 to 10, and 0 to 50 units/ml). Temperature maxima were determined by visual estimation of growth in tubes of medium BG-11 incubated in water baths at a series of different temperatures.

Enrichment, Isolation, and Purification of Unicellular Blue-Green Algae

Of the 40 strains listed in Tables 1 and 2, 25 were isolated at Berkeley from local samples of fresh water (ponds, streams, or reservoirs). In nearly every case, the inoculum was subjected to primary enrichment in a tube of medium BG-11 incubated in an illuminated water bath at 35 C. When microscopic examination revealed the presence of unicellular blue-green algae in an enrichment tube, streaked plates were prepared. Immotile unicellular blue-green algae form compact, deeply pigmented colonies on plates. Even when they are too small to be seen with the naked eye, such colonies are therefore easy to detect if plates are examined with a dissecting microscope. Early transfer of colonies, performed under a dissecting microscope, greatly facilitates purification. Numerous restreakings may be required to eliminate contaminating bacteria, but given sufficient time and patience this simple technique almost never fails. The purification of immotile unicellular blue-green algae is tedious but not difficult.

A few strains (notably 6901 and 6903) are very actively motile, and these were purified by the technique which has proved effective with some motile filamentous blue-green algae. A small patch of material was placed on the agar surface at one side of a petri dish which was illuminated from the opposite side. The blue-green algae responded phototactically by gliding across the plate towards the region of higher light intensity; as soon as some organisms had reached the opposite side, they were picked and subjected to a repetition of the same procedure.

When the macroscopic and microscopic appearance of the material on plates suggested that a pure culture had been obtained, a transfer was made to a tube of liquid medium BG-11 used for the maintenance of stock cultures. The purity of liquid cultures can be controlled in several ways. In our experience, the most reliable control is a careful microscopic examination of ageing cultures in which the blue-green algae have become senescent and have begun to die. The presence of contaminating bacteria is very evident under these conditions. We have also routinely tested liquid cultures for bacterial contamination by making transfers to a complex liquid medium (yeast extract-glucose broth), subsequently incubated in the dark at 30 C. The absence of growth under these conditions is a necessary criterion of purity, but far from a sufficient one. This test will, of course, reveal gross bacterial contamination; but if only one or a few kinds of bacteria still contaminate a culture, they will not necessarily be able to grow in an arbitrarily selected complex medium, incubated under a single set of environmental conditions. To devise cultural tests that would reveal all possible nutritional categories of contaminating bacteria is scarcely feasible; it is for this reason that we have relied primarily on the microscopic control of purity.

Measurement of Absorption Spectra

Absorption spectra of cell suspensions were determined in a Cary spectrophotometer, using a diffusing plate of opal glass to minimize the effects of scattering (66).

Determination of Fatty-Acid Composition

Techniques for determining fatty-acid composition have been described by Kenyon and Stanier (39).

Extraction of DNA

Cells were harvested by centrifugation, washed in buffer [0.03 M tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0), containing 0.15 M NaCl and 0.001 M ethylenediaminetetraacetic acid (EDTA)], resuspended in a minimal

volume of 0.15 M NaCl-0.1 M EDTA (pH 8.0), and stored in the frozen state at -40 C until extraction.

The majority of the unicellular blue-green algae are refractory to lysis by osmotic shock with or without prior lysozyme treatment (18, 22). Strains 7002, 7003, 7005, and 6707 are sensitive to lysozyme. These were treated with 100 µg of lysozyme per ml at 37 C in the saline-EDTA buffer for 2 hr; addition of sodium dodecyl sulfate (SDS) to a final concentration of 2% (v/v) at 60 C resulted in prompt lysis.

Numerous other methods were employed to disrupt the remaining strains. The cells were incubated in lysozyme as above, followed by an excess of cell wall lytic enzymes of *Streptomyces albidoflavus* as previously described (64). A larger measure of success with this method was achieved when the washed cells were incubated in 1 M β-mercaptoethanol (29) for 2 hr at 37 C, centrifuged once, and resuspended in lysozyme and then *S. albidoflavus* enzymes; lysis was then completed with SDS as described above.

Cells of strains 6716, 6801, 6501, and 6701 lyse poorly or not at all after enzymatic treatment. These cells were then disrupted in a Hughes press at -18 C. The frozen paste was thawed in the presence of SDS in saline-EDTA buffer. For comparative purposes, one lot of cells of strain 6501 was disrupted in a French pressure cell (22).

Nucleic acids were separated from cellular debris by shaking with an equal volume of saline/EDTA-saturated phenol at pH 8.0 and a drop of chloroform. The phases were separated by centrifugation at 8,000 × g at 5 C. Whole cells appear at the bottom of the centrifuge tube below the phenol layer; denatured proteins and much of the cell wall and sheath components concentrate at the interface. The aqueous layer was removed with a wide-mouth pipette and the nucleic acids were concentrated by layering thereon two volumes of 95% ethyl alcohol and "spooling" fibers on a thin glass rod. The fibers were washed three times in small volumes of 70% ethylalcohol and then were dissolved in a small volume of SSC (0.15 M NaCl, 0.015 M Na₃ citrate, pH 7.0). Such partially purified preparations were stored at 5 C over $CHCl_3$ and were analyzed within 2 weeks.

When large amounts of material were available (strain 7002), the DNA was purified by the method of Marmur (45).

Determination of Mean DNA Base Composition

Buoyant densities in CsCl were determined for each sample in two different Spinco model E ultracentrifuges, using four-cell rotors and optical masks. One centrifuge was equipped with a photomultiplier scanner in addition to the usual camera assembly. Ultraviolet absorbance in the cells was determined at 265.4 nm after centrifugation of 1 to 3 µg of the DNA for 23 hr at 42,040 rev/min at 25 C. Centers of the peaks of the ultraviolet-absorbing bands were measured from scanner tracings or from densitometer tracings of the ultraviolet photographs prepared with the aid of a Joyce-Loebl mark III recording densitometer. The densities were calculated with respect to an internal standard of *Bacillus subtilis* bacteriophage 2C DNA of a density of 1.7420 ± 0.0004 g/cm^3, which was measured with respect to DNA of *Escherichia coli* K-12 DNA assumed to be 1.7100 g/cm^3 (63).

The identification of ultraviolet-absorbing bands as nucleic acid or as polysaccharide cannot be done by examination by schlieren optics (22) when multicell operation is employed, as in this report. This identification was made by examination at 280 nm and at 320 nm. The light absorption by nucleic acids at 280 nm is about half that at 265.4 nm, and they do not absorb light at 320 nm. Polysaccharides appear as ultraviolet-absorbing bands by virtue of light scattering, and their apparent absorption at 320 nm is half that at 265.4 nm. Only one strain (7002) yielded DNA appreciably contaminated with polysaccharide material. Removal of this material by a phase separation (7) did not alter the recorded density of the DNA.

ASSIGNMENT OF STRAINS TO TYPOLOGICAL GROUPS

Classification of *Chroococcales* is controversial, and even generic identifications require a choice between two very different taxonomic treatments of the order (20, 28). Since we were not disposed to make this choice on a priori grounds, we provisionally assigned all our strains to a series of unnamed typological groups, each defined by a few salient structural properties (Table 4).

We have used the plane or planes of successive cell divisions as a primary determinative character. This property is, to some degree, correlated with cell shape. Many strains that reproduce by repeated binary fission in a single plane (group I) have cylindrical or ellipsoidal cells; however, the cells in some strains of this group are spherical (or nearly so) immediately after division. Strains that reproduce by successive binary fissions in two or three planes at right angles to one another (groups II and III) have spherical cells.

It should be emphasized that the planes of successive cell divisions cannot always be determined with certainty by simple microcscopic examination because in many strains the separation of

TABLE 4. *Key to the typological groups of unicellular blue-green algae*

A. Reproduction by repeated binary fission in a single plane, frequently resulting in the formation of short chains of cells.   Group I
  1. Cells cylindrical, ellipsoidal, or spherical immediately after division; cells do not contain refractile polar granules.   Group IA
  2. Cells cylindrical or ellipsoidal after division; cells always contain refractile polar granules.
                                                            Group IB
B. Reproduction by binary fission in two or three successive planes at right angles to one another; cells spherical.
  1. Cell divisions occur at regular intervals; never form parenchymatous masses of cells.
                                                            Group II
    a. Cells not ensheathed; do not contain gas vacuoles.   Group IIA
    b. Cells ensheathed; do not contain gas vacuoles.   Group IIB
    c. Cells not ensheathed; contain gas vacuoles.
                                                            Group IIC
  2. Cell division irregular; growth leads to the formation of parenchymatous, tightly packed masses of cells.   Group III

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daughter cells occurs shortly after the completion of cell division. When necessary, we determined this character by observing the growth of strains in slide cultures (4).

Most strains that divide successively in a single plane form short chains of cells. The extent of chain formation is frequently variable within a single strain, being dependent both on the conditions of cultivation and the stage of growth. This raises a fundamental determinative question: what is the distinction between a unicellular blue-green alga which can form short chains of cells and a filamentous blue-green alga in which a trichome is the vegetative unit of structure? There is no clear-cut answer to this question, as shown by the controversy which has already surrounded the correct taxonomic assignment of strain 6301. A typical representative of our typological group I, this strain was purified in 1952 by M. B. Allen (1) who submitted it to Francis Drouet for identification (Allen, *personal communication*). He initially identified it as a member of the *Chroococcaceae*, under the name *Anacystis nidulans*. However, according to Silva (67), Drouet subsequently reidentified this strain as *Phormidium mucicola* (viz., a member of the *Oscillatoriaceae*). Pringsheim (60) also interpreted the chains of cells produced by strain 6301

as trichomes, though of an unusual kind. He disagreed with its assignment to *Phormidium* and proposed the new genus *Lauterbornia* for it. On the other hand, Allen and Stanier (4), Padmaja and Desikachary (52), and Kunisawa and Cohen-Bazire (43) argued that chain formation by 6301 and similar strains is insufficient ground for excluding them from the *Chroococcales*. A similar phenomenon is characteristic of many eubacteria (e.g., *Streptococcus* and *Lactobacillus*) which are conventionally regarded as unicellular organisms. We shall adopt this view here and consider all members of group I to be representatives of the *Chroococcales*, even though the great majority of them can form short chains of cells. The strains for which this interpretation may be most questionable are 6901 and 6903 (group IB) which typically occur as chains of at least two to four cells. We might have been inclined to interpret these two strains as filamentous blue-green algae which produce short trichomes were it not for their numerous similarities to the third strain of group IB, which is unicellular under the same growth conditions.

The microscopic appearance of each strain is shown in Fig. 1–5 and 7–9. These photomicrographs were selected to illustrate the typical size, shape, and arrangement of cells from young cultures in the course of active growth, as seen with phase-contrast illumination. With the exception of Fig. 7 and 9, all were printed at the same magnification, indicated by the scale marker accompanying each group of prints. The strains were arranged in these figures according to their assignments to typological groups and subgroups; in the two largest typological subgroups (IA and IIA), strains of markedly different DNA base composition were placed in separate figures (Fig. 1 and 2 for strains of group IA; Fig. 4 and 5 for strains of group IIA).

Within group I, we have distinguished two typological subgroups. The larger of these, IA (Fig. 1 and 2), comprises strains without any distinguishing intracellular structures visible in the light microscope. The cells may be cylindrical, ellipsoidal, or spherical, and the size range is considerable. Group IB (Fig. 3) comprises three strains with rod-shaped cells which have conspicuous refractile granules at each pole. This is a constant and regular structural property, which permits a ready differentiation from all strains of group IA. The polar granules arise in the course of septum formation, as can be seen from the
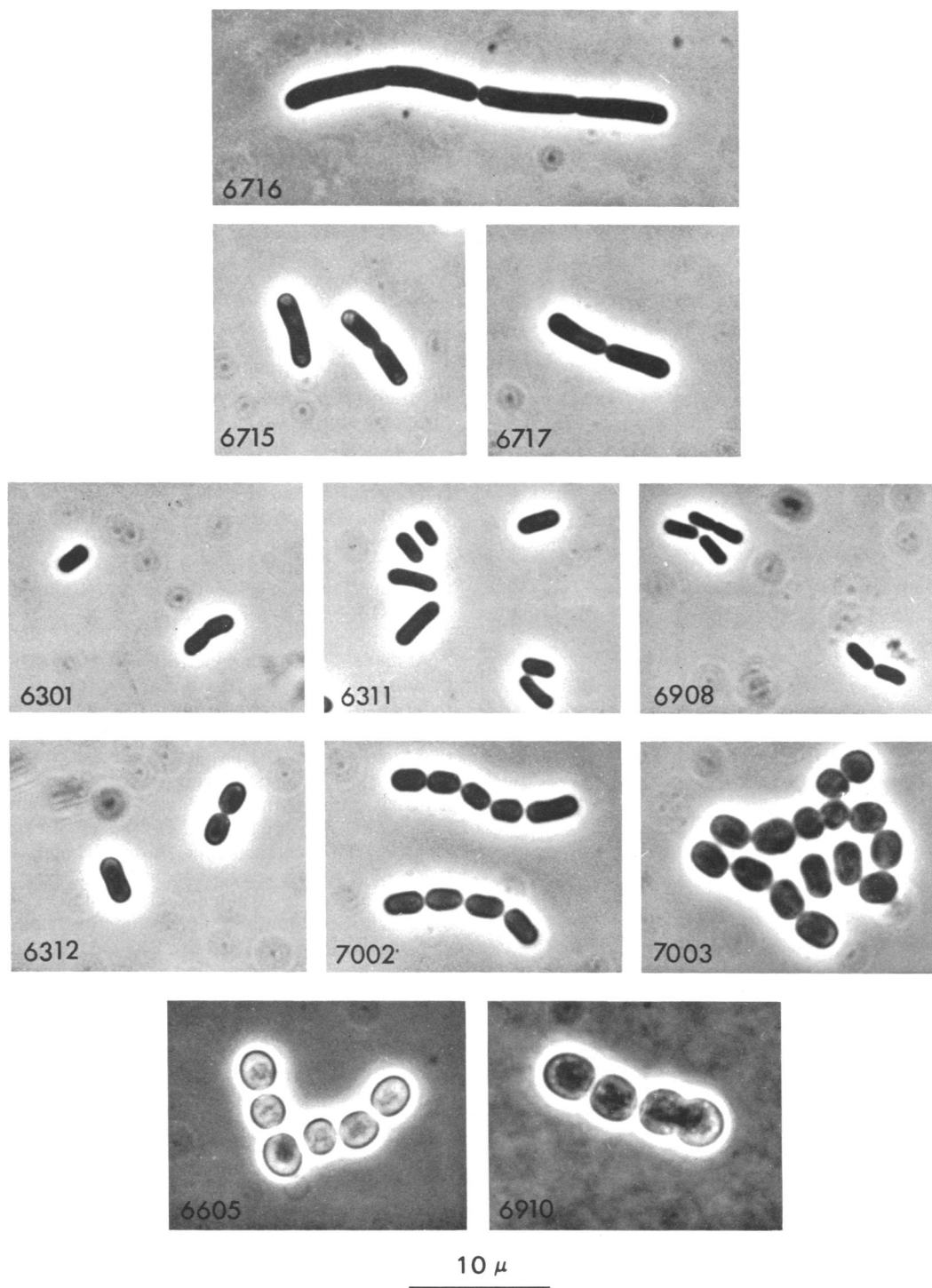
FIG. 1. *Photomicrographs of strains belonging to typological group IA. All strains illustrated have DNA of low GC content (48 to 56 moles %).*
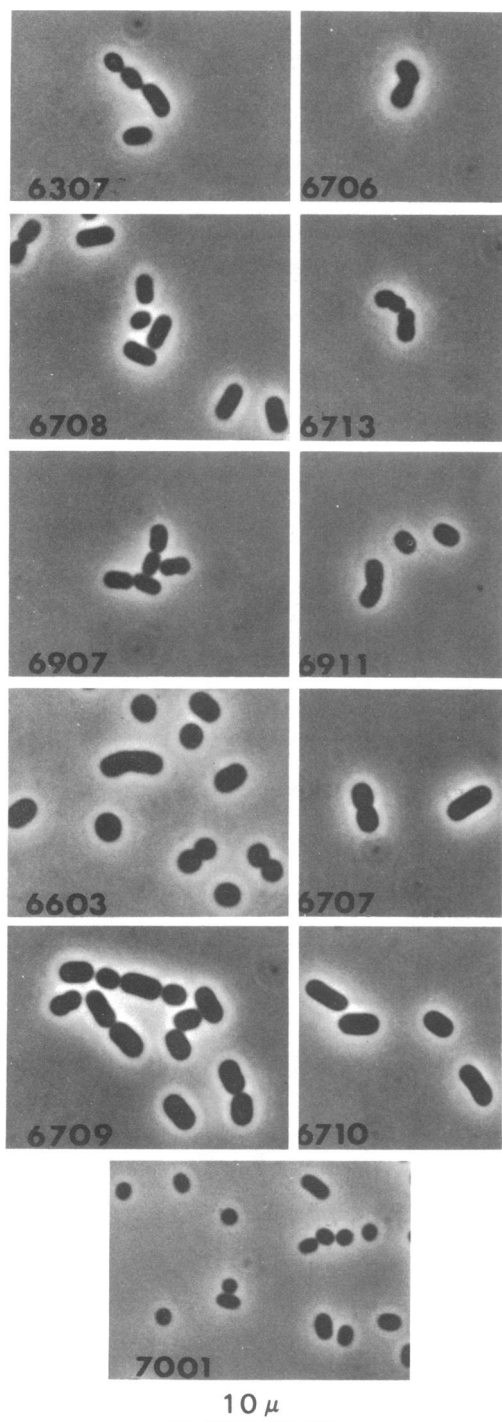
10 μ

FIG. 2. *Photomicrographs of strains belonging to typological group IA. All strains illustrated have DNA of high GC content (66 to 71 moles %).*

appearance of dividing cells of strain 6903 (arrows) in Fig. 3.

Within group II, we have distinguished three subgroups. The strains of group IIA (Fig. 4 and 5) do not produce sheaths or gas vacuoles, although the cells may be surrounded by a diffuse slime layer, demonstrable in India-ink preparations (Fig. 6). The two strains of group IIB (Fig. 7) form conspicuous, sharply defined, multilaminate sheaths. Geitler (28) expressed some doubts about the distinctness of sheaths and slime layers in *Chroococcaceae*. However, we have never found any difficulty in making a clear distinction between these two kinds of extracellular structures. Furthermore, the property of sheath formation has remained constant in strain 6501 through the period of more than five years, during which this strain has been kept in pure culture. We believe, therefore, that this structural character is a significant and taxonomically useful one. The single strain assigned to group IIC (7005) forms gas vacuoles, visible in the light microscope as small refractile bodies (Fig. 8). It is not ensheathed, and it can be distinguished typologically from the strains of group IIA only by the property of gas-vacuole formation.

A single strain with spherical cells that divide in more than one successive plane (strain 6712) differs markedly in growth habit from the strains of group II, and has been assigned to a separate typological group III. The size of the cells in this strain is highly variable, and it grows as dense, irregular, parenchymatous masses in which many cells have a polygonal shape as a result of close packing. The characteristic growth habit of strain 6712 is best revealed by successive photomicrographs of a developing slide culture (Fig. 9), which show how the parenchymatous aggregates develop from single cells. The colony structure of this strain is also unusual: colonies on plates are hard, dry, and coherent, in contrast to the butyrous or slimy colonies formed by other unicellular strains.

One other blue-green alga of uncertain taxonomic position which has been isolated in pure culture has a somewhat similar mode of growth. This is the organism originally described by Mitra (46) under the name *Chlorogloea fritschii*, a strain of which we have compared with 6712. As shown by its development in a slide culture (Fig. 10), *C. fritschii* closely resembles 6712 in vegetative growth habit, except for the occasional formation of short chains of cells, a character that we have not observed in 6712. However, as will be discussed, the two strains differ in other
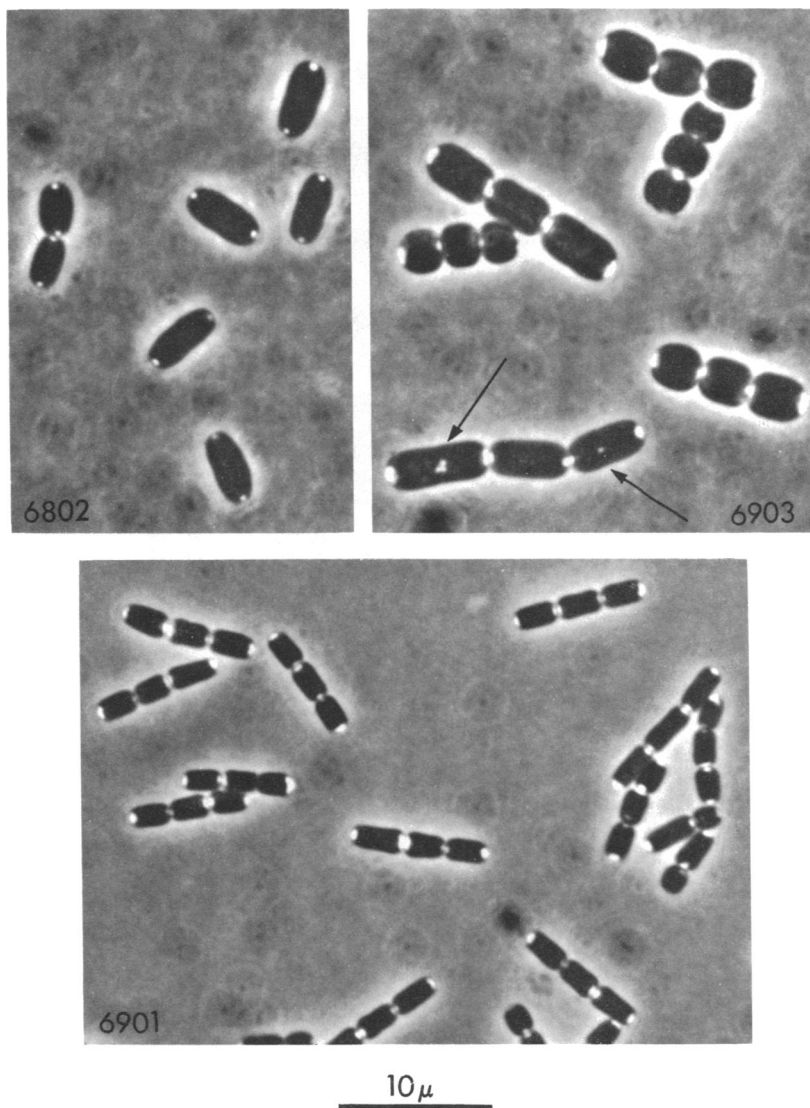
FIG. 3. *Photomicrographs of strains belonging to typological group IB. Note the refractile, bipolar granules which distinguish strains of group IB from those of group IA. Arrows on the photomicrograph of strain 6903 point to newly formed polar granules which are developing at the site of septum formation in two dividing cells.*

phenotypic respects and probably are not closely related taxonomically.

DNA BASE COMPOSITION

The buoyant densities and calculated mean DNA base compositions of the strains are listed in Table 5, which also includes the values previously published (22) for six of these strains. The agreements between the independent determinations are as close as can be expected. It should be noted that the value previously published for strain 6604 was in fact determined on strain 6603, an error that we have corrected in Table 5.

The present, much more extensive analyses have not extended the over-all range of base composition for the *Chroococcales*; the limits remain at approximately 35 and 70 moles % GC. However, they do reveal significant facts about the distribution of DNA base composition within the order (Fig. 11). The strains of typological groups
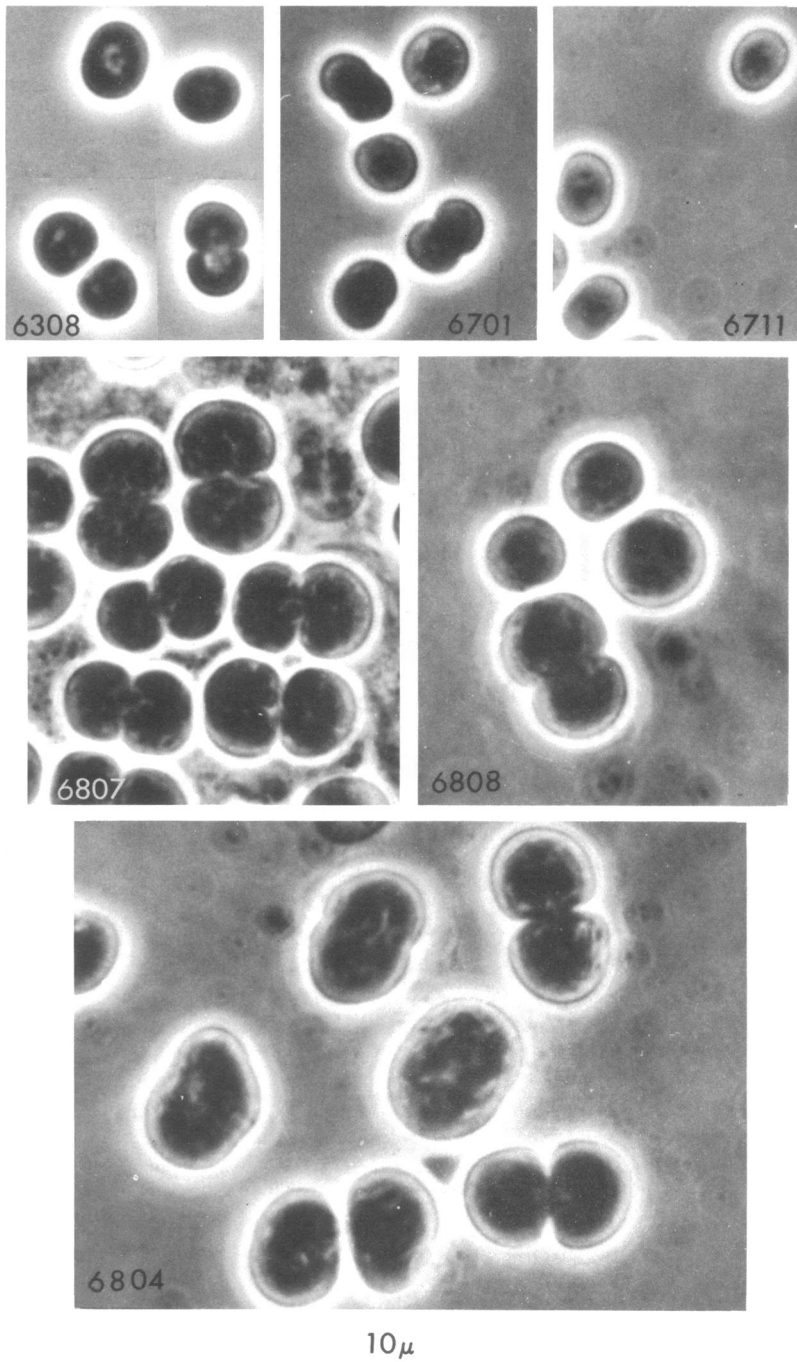
FIG. 4. *Photomicrographs of strains belonging to typological group IIA. All strains illustrated have DNA of low GC content (35 to 37 moles %).*

I and II show ranges that are almost entirely distinct. The range for group I extends from 45 to 72 moles % GC; for group II, from 35 to 47 moles % GC. The value for the one strain of group III falls within the range characteristic for group II. In Fig. 11, the ranges established by Edelman et al. (22) for the families *Nostocaceae*, *Oscillatoriaceae*, and *Scytonemataceae* have also
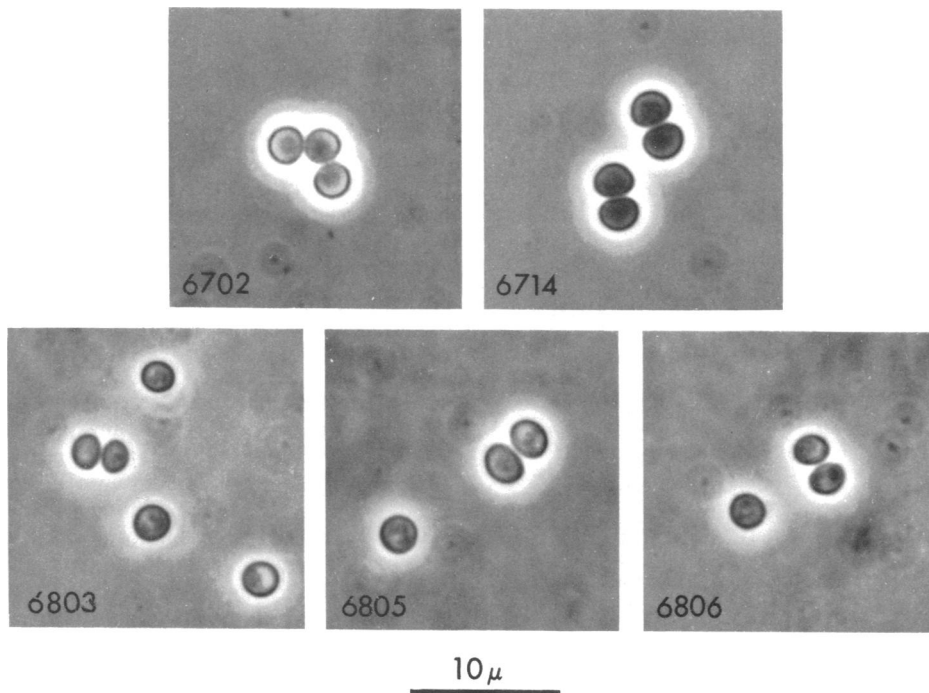
FIG. 5. *Photomicrographs of strains belonging to typological group IIA. All strains illustrated have DNA of high GC content (46 to 47 moles %).*
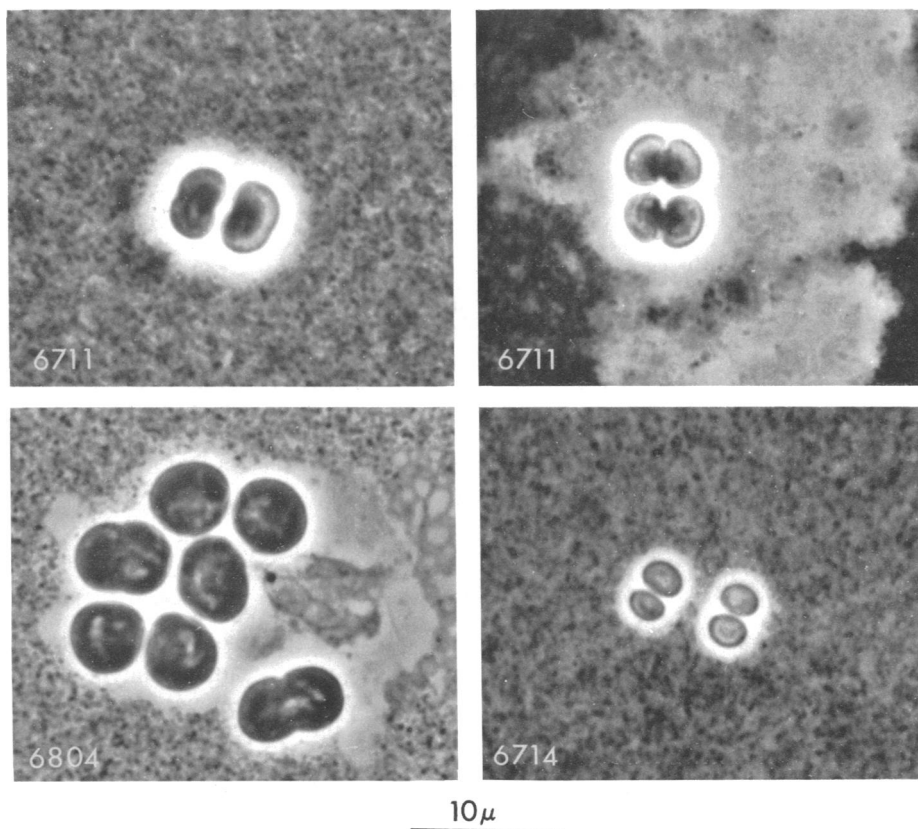

FIG. 6. *Photomicrographs (wet mounts in India ink) of three strains belonging to typological group IIA, to show the slime layers formed by some representatives of this typological group.*
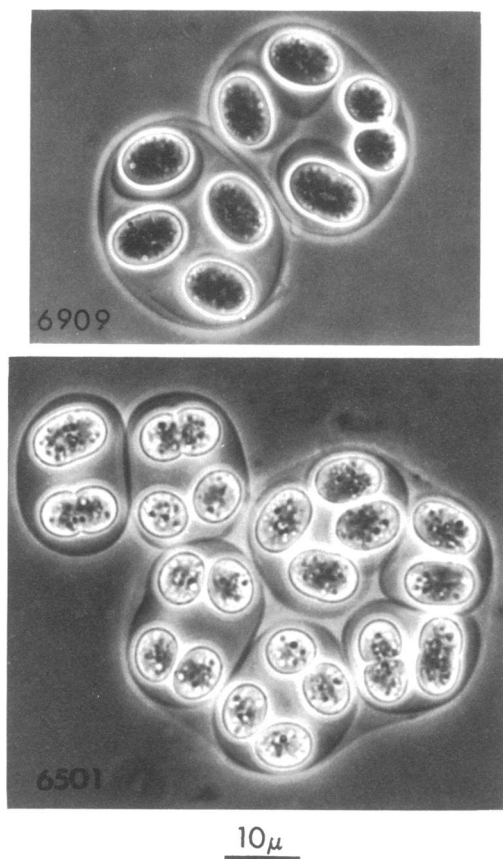
FIG. 7. *Photomicrographs of strains belonging to typological group IIB. Note the thin, multilaminate sheaths which unite the cells into microcolonies, entirely different in structure from the slime layers (Fig. 6) produced by some strains of group IIA.*

been included. They are comparatively narrow and cover the region of overlap between the ranges for groups I and II of the *Chroococcales*.

The strains of group I fall into two widely separated base compositional clusters, each of which has a comparatively narrow range. These clusters are not well correlated with typological subdivisions. The 14 strains with values in the range of 45 to 56 moles % GC include 11 strains of group IA and all 3 strains of group IB; the 11 strains with values in the range of 66 to 73 moles % GC are all of group IA. A comparison of the photomicrographs of individual strains of group IA in Fig. 1 (low-GC cluster) and 2 (high-GC cluster) shows, furthermore, that many strains differing by over 10 moles % GC in base composition cannot be distinguished by cell size or shape. The base compositional data therefore provide prima facie evidence that the existing

classification of unicellular blue-green algae on the basis of structural characters has very poor taxonomic resolving power.

Group II comprises three separate base compositional clusters, again poorly correlated with typological subdivisions. The strains of group IIA fall into two clusters at the extreme ends of the base compositional span (35 to 37 and 46 to 47 moles % GC). A comparison of the photomicrographs of the individual strains of these two clusters (Fig. 4 and 5, respectively) does, however, reveal that they are distinguishable from one another by cell size: strains of high GC content have smaller cells than strains of low GC content. The strains of group IIB, characterized typologically by multilaminate sheaths, are intermediate in base composition (40 to 42 moles % GC) between the strain clusters of group IIA. The single gas-vacuolate strain of group IIC is indistinguishable in base composition from the high-GC cluster of group IIA. Strain 6712 (typological group III) is similar in DNA base composition (40.8 moles % GC) to *Chlorogloea fritschii* (42.9 moles % GC).

PHENOTYPIC PROPERTIES

Motility

Gliding movement is widespread, perhaps universal, among filamentous blue-green algae. In some of these organisms the trichomes are motile; in others motility is confined to hormogonia (37). There have been a number of reports, summarized by Pringsheim (61), that certain members of the *Chroococcales* are motile, but the extent to which gliding movement occurs in this order is still unclear.

A majority of the unicellular strains that we have examined appear to be permanently immotile. However, we have observed gliding movement in some representatives of both typological groups I and II. All strains of group IB are vigorous gliders. Movement is particularly rapid in strains 6901 and 6903, which regularly grow as short chains of cells. Rapid gliding movement is also characteristic of one strain in group IA, 6910. Motile strains of group I show a positive phototactic response on plates exposed to unilateral illumination (Fig. 12).

More restricted motility occurs in several strains of group IIA. The movement of these strains is always very slow, and we have not been able to recognize it with certainty by microscopic examination of wet mounts. Motility was first discovered on plate cultures of strain 6711 (Fig. 13a). As can be seen, the phototactic response is negative. During maintenance in liquid culture, strain 6711 gives rise to nonmotile variants
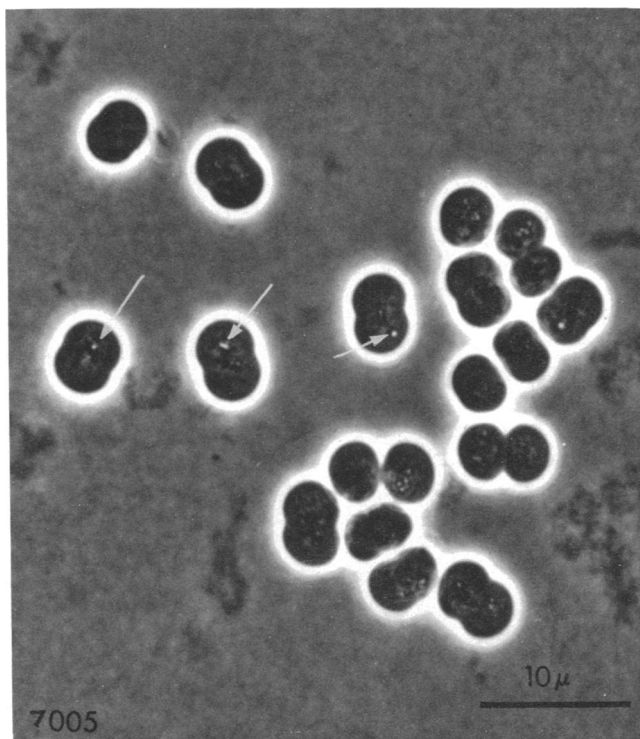
FIG. 8. *Photomicrograph of strain 7005, the sole representative of typological group IIC. Arrows point to small gas vacuoles.*

(Fig. 13b). We have been able to preserve a motile stock of this strain by repeated selection of motile clones on plates.

Other strains of group IIA in which colony movements have been occasionally observed are 6701, 6803, 6804, and 6808. Movement of these strains on plates is both sporadic and of limited extent, and we do not understand the factors that determine its occurrence.

Accordingly, our experience shows that gliding movement is rare in typological group I, but when it does occur in strains of this group it is rapid and easily recognized. In typological group IIA, the potential capacity to perform slow gliding movement may be fairly common, but it is difficult to detect for the reasons given above. Since motility seems to be an unstable character in strain 6711, it could have escaped detection in other strains of group IIA as a result of its loss during their maintenance in liquid culture. We have not detected movement in strains of groups IIB, IIC, and III.

The marked differences between motile strains of groups I and II with respect to the regularity and the rate of movement may perhaps indicate that the mechanisms of movement are dissimilar. Pringsheim (61) suggested, on the basis of micros-

copic observations, that two types of movement occur in blue-green algae. Gliding movement (Gleitbewegung) is seen characteristically in members of the *Oscillatoriaceae*; it is relatively smooth and rapid, and accompanied by axial rotation of the filament. Jerking movement (Ruckbewegung) is slow, irregular, and frequently interrupted. The movement of strains of group I can clearly be described as gliding movement. It is possible that the comparatively slow migration of colonies on plates we have observed in strains of group IIA reflects the occurrence of jerking movement, although, as already mentioned, we have not been able to detect it microscopically.

Growth in Darkness and Utilization of Organic Compounds

Pure cultures are essential for a study of the utilization of organic compounds by blue-green algae; consequently, information about this aspect of their physiology is still very limited. Reviewing the information then available, Holm-Hansen (33) concluded that most blue-green algae are obligate phototrophs, although there are well-authenticated reports that a few strains can grow in the dark at the expense of organic compounds.
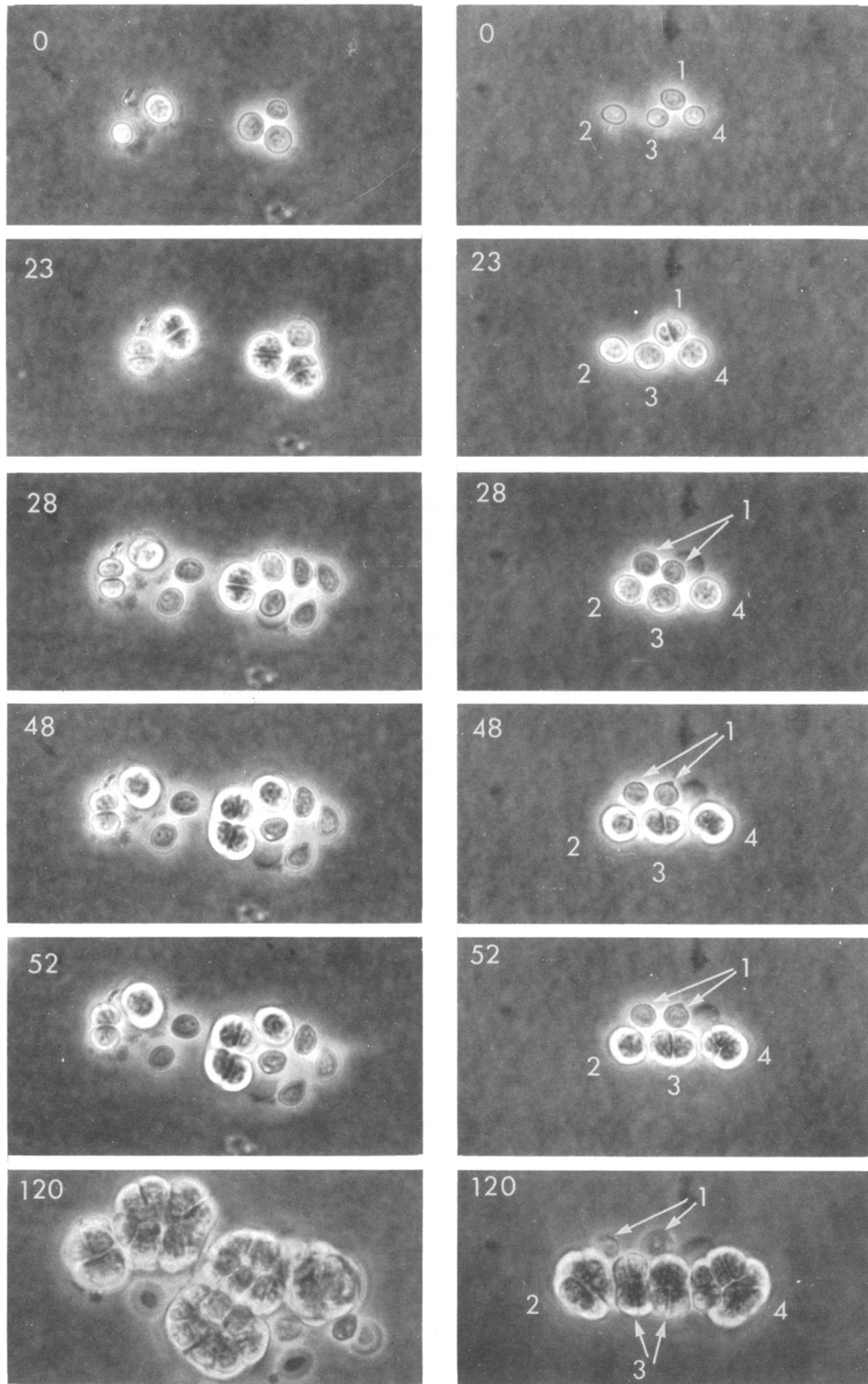
FIG. 9. *Two series of photomicrographs to illustrate the growth of strain 6712 (typological group III) in a slide culture. The number in the top left corner of each print indicates the time (in hours) after the preparation of the slide culture at which the photograph was taken. In the series on the right, the four cells initially present and their progeny have been numbered. A comparison of the development of each cell reveals the irregular timing of cell division in this strain. The parenchymatous aggregates of compressed cells produced after 120 hr are typical for strain 6712.*

186

10μ

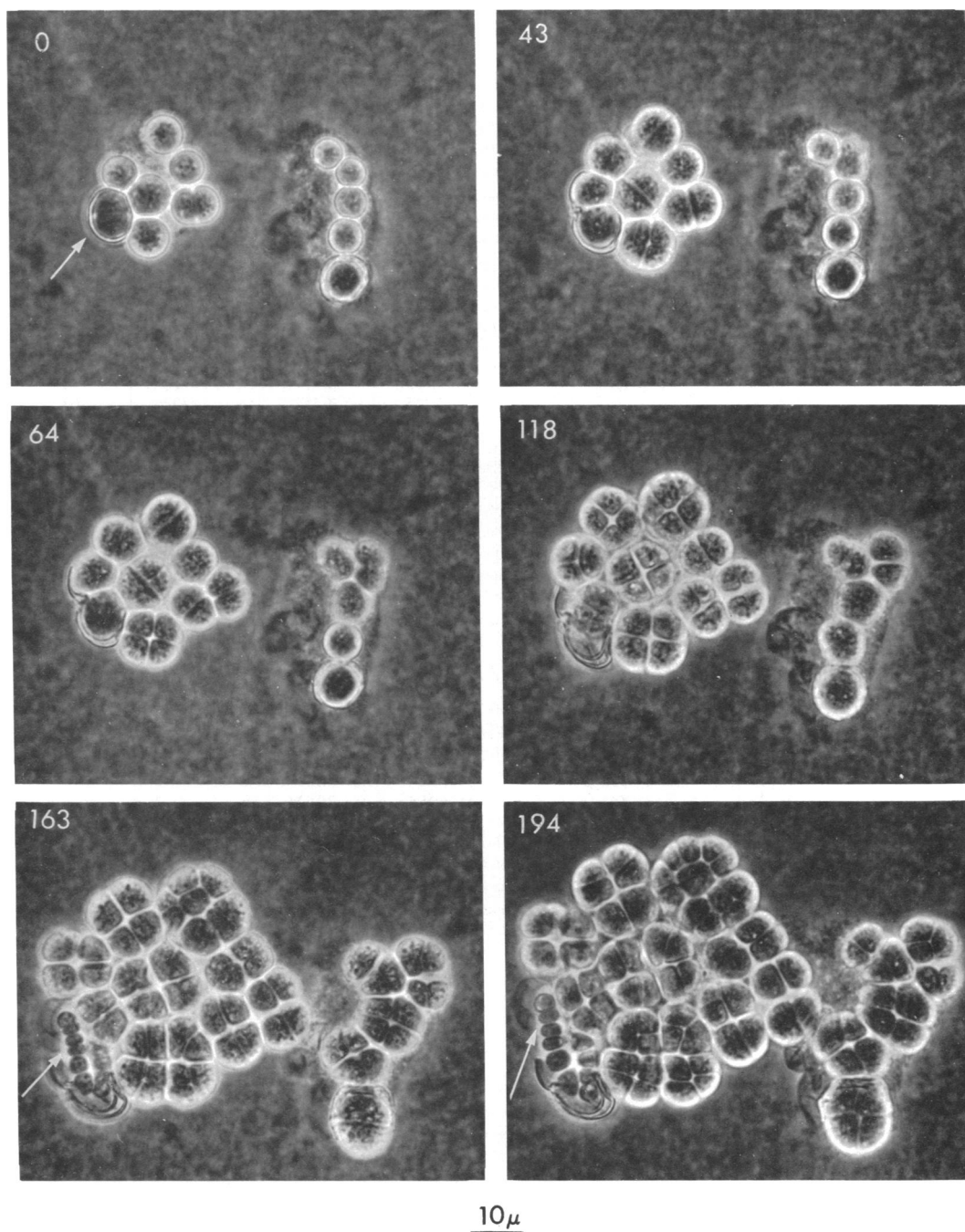FIG. 10. *Series of photomicrographs to illustrate the growth of Chlorogloea fritschii in a slide culture. The number in the top left corner of each print indicates the time (in hours) after the preparation of the slide culture at which the photograph was taken. One cell in the initial group (arrow), distinguishable by its relatively thick wall, eventually gave rise (arrows at 163 and 194 hr) to a chain of 6 small cells.*

TABLE 5. *Buoyant densities and guanine-plus-cytosine (GC) contents of deoxyribonucleic acid from strains of unicellular blue-green algae*

Typological group	Strain	Cell disruption method[a]	Mean density (g/cm³)	GC (moles %)	Mean density (g/cm³)[b]	GC (moles %)[b]
IA	6715	B	1.7125	53.6		
	6716	D	1.7125	53.6		
	6717	B	1.711	52		
	6301	B	1.714	55.1	1.715	56
	6311	B	1.714	55.1	1.715	56
	6908	C	1.7145	55.6		
	6312	A	1.709	50.2		
	7002	A	1.708	49.0		
	7003	A	1.7085	49.5		
	6605	B	1.706	46.9		
	6910	C	1.707	48.0		
	6307	B	1.7285	69.7	1.730	71
	6603	B	1.7245	65.7	1.722	63
	6706	B	1.729	70.2		
	6707	A, C	1.7263	67.7		
	6708	B	1.728	69.4		
	6709	B	1.7265	67.9		
	6710	B	1.727	68.4		
	6713	C	1.728	69.4		
	6907	C	1.730	71.4		
	6911	C	1.725	66.3		
	7001	B	1.728	69.4	1.728	69
IB	6802	D	1.705	45.9		
	6901	C	1.705	45.9		
	6903	C	1.704	44.9		
IIA	6308	B	1.694	34.7	1.694	35
	6701	B	1.695	35.7		
	6711	C, D	1.696	36.7		
	6804	C	1.6955	36.2		
	6807	C	1.695	35.7		
	6808	C	1.695	35.7		
	6714	B	1.7065	47.4		
	6702	B	1.7065	47.4		
	6803	B	1.7065	47.4		
	6805	C	1.707	48.0		
	6806	C	1.7055	46.4		
IIB	6501	C, D, E	1.700	40.8		
	6909	C	1.7005	41.3		
IIC	7005	A	1.7045	45.4		
III	6712	B	1.700	40.8		
	Chlorogloea fritschii	D	1.702	42.9		

[a] A, lysozyme-sodium dodecyl sulfate (SDS); B, lysozyme, *Streptomyces albidoflavus* enzyme, SDS; C, B, after β-mercaptoethanol; D, Hughes press; E, French pressure cell.
[b] From data of Edelman et al. (22).

In analyzing this question, a clear distinction must be made between the utilization of organic compounds in the light and in the dark. Provided that it is permeable to organic compounds, even an obligate phototroph may well be able to assimilate them in the light, using photochemical reactions for the generation of adenosine triphosphate (ATP) and reducing power. The photosynthetic growth rate may be increased by the provision of organic substrates, if they can serve
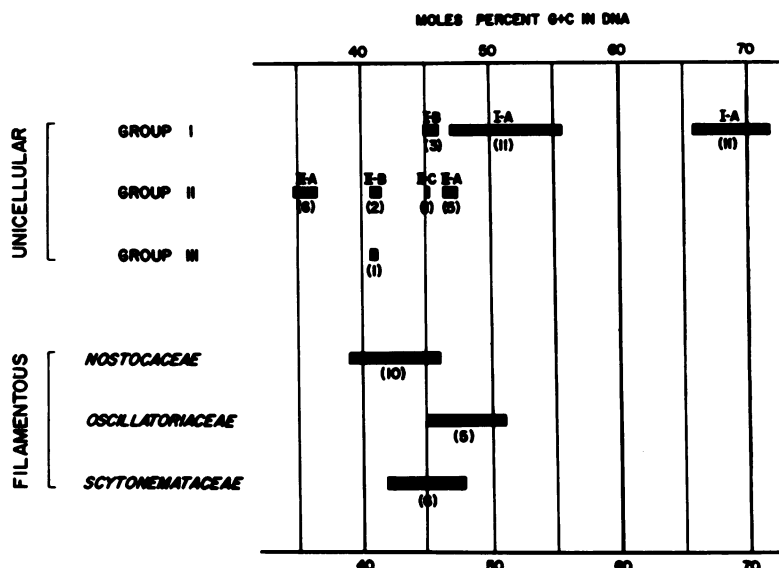
FIG. 11. *Ranges of mean DNA base composition among the blue-green algae. Data for the Chroococales, taken from Table 5, show the compositional ranges for the subgroups defined in Table 4. Data for filamentous families are taken from Edelman et al. (1967). Numbers in parentheses under each bar denote the number of strains analyzed.*

as general sources of cellular carbon and if the rate of photosynthetic growth is normally limited by the rate of CO_2 assimilation. However, to grow with organic compounds in the dark, a photosynthetic organism must be able to use them as sources of ATP and reducing power, generated through respiration or fermentation, and not merely as sources of assimilable carbon.

Smith, London, and Stanier (68) studied the utilization of organic compounds in the light by three of the unicellular strains in our collection: 6301 and 6307 (typological group IA) and 6308 (typological group IIA). All three strains could assimilate organic acids, amino acids, and glucose, although none of the compounds tested increased the rate of photosynthetic growth. The uptake of these compounds by cells was light dependent. Even acetate, the most rapidly assimilated compound that was examined, contributed only some 10% of the carbon incorporated into newly synthesized cell material. An investigation of the patterns of incorporation of ^{14}C-labeled acetate and other substrates into the cellular amino acids suggested that the tricarboxylic acid cycle was not operative, and this inference was supported by the failure to detect a key enzyme of the cycle, α-ketoglutarate dehydrogenase, in cell-free extracts.

With respect to the photoassimilation of acetate, essentially similar results were reported by Pearce and Carr (55) and Hoare et al. (31) for

several unicellular and filamentous blue-green algae. Hoare and his associates (32) subsequently extended such studies to many other strains, including several in which growth in the dark had previously been reported. The absence of a functional tricarboxylic acid cycle has been confirmed for every blue-green alga so far examined.

Smith et al. (68) were unable to demonstrate reduced nicotinamide adenine dinucleotide oxidase activity in extracts of the unicellular strains that they studied; however, low levels of this activity were subsequently reported in blue-green algae by some workers (35), but not by others (8, 44). The mechanisms and physiological significance of respiratory electron transport in these organisms still remain to be elucidated.

Since none of the blue-green algae appears to have a functional tricarboxylic acid cycle, it is evident that a normal respiratory metabolism of organic compounds in the dark cannot occur; growth in the dark, therefore, probably takes place at the expense either of glycolysis or of the pentose phosphate cycle (56). It should be noted that carbohydrates are the only organic substrates that have been reported to support the growth of blue-green algae in the dark (24, 40). This has been confirmed (R. Rippka, *unpublished observations*) for several of the filamentous blue-green algae in our collection: only glucose, fructose, and sucrose permit growth in the dark. It would be surprising if blue-green algae that
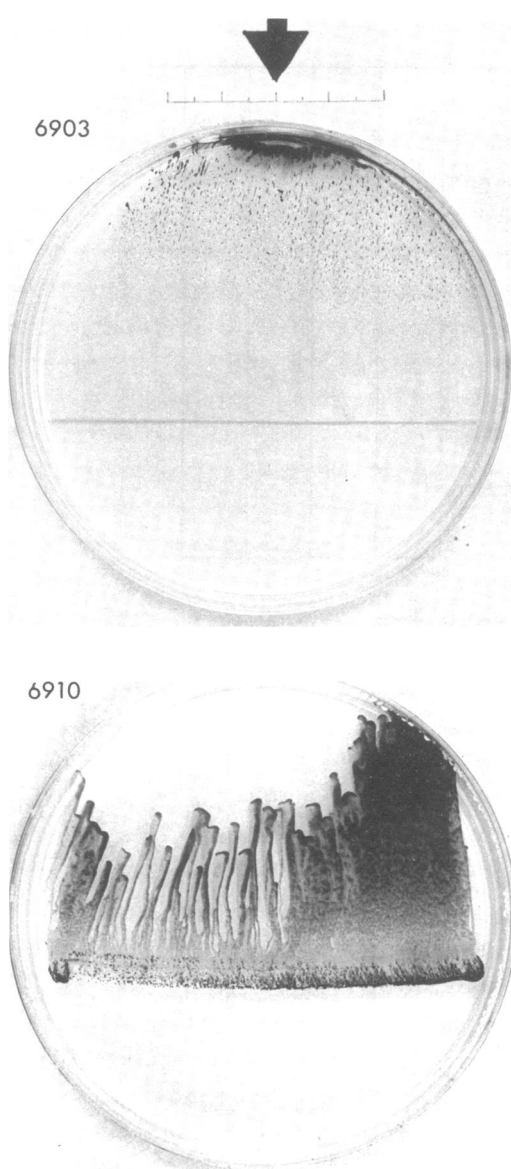
FIG. 12. *Phototactic migration on plates of strains 6903 (typological group IB) and 6910 (typological group IA). Each plate was inoculated with a single horizontal streak slightly below the center. Photographs were taken after incubation at 30 C for 6 days. Illumination was unidirectional (arrow). Both strains are positively phototactic.*

FIG. 13. *Phototactic migration on plates of strain 6711 (typological group IIA). Figure 13a shows a small group of colonies grown for about 2 weeks on a plate with unidirectional illumination (arrow); all have migrated from the points of initial growth (at top) away from the light source. Figure 13b shows a plate streaked from a liquid stock culture of the same strain after incubation for 10 days with unidirectional illumination (arrow). The heterogeneity with respect to motility is evident; many colonies show no evidence of movement. Phototaxis is, again, invariably negative.*

possessed an effective machinery of respiration were unable to grow at the expense of such compounds as succinate and pyruvate, known to be assimilable in the light (68). The strict dependence on carbohydrates as energy-yielding organic substrates suggests a very limited respiratory capacity.
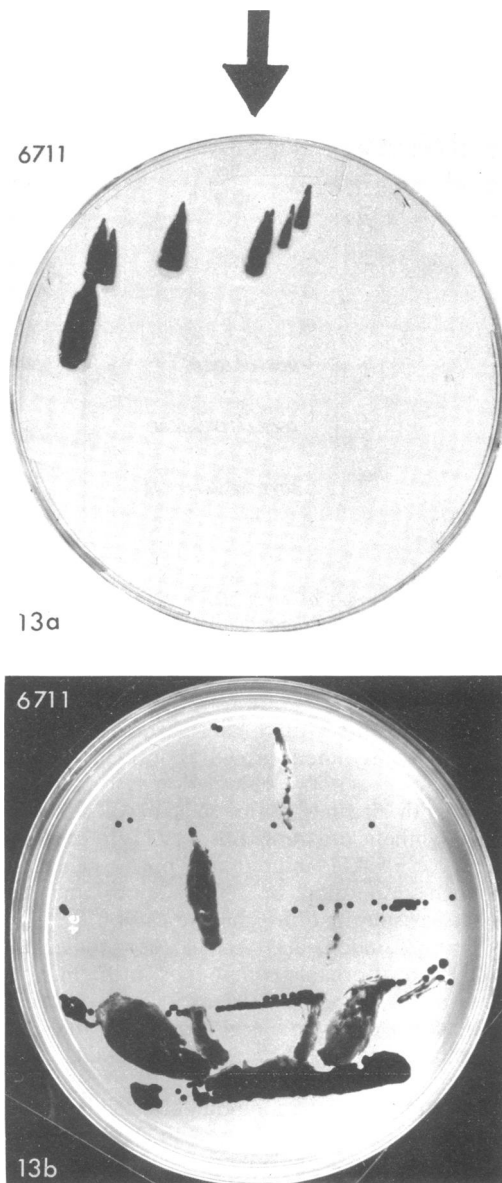
We have screened all strains of groups I, II, and III qualitatively for growth in the dark at the expense of a series of organic compounds. The compounds tested were glucose (0.25%, w/v);

acetate, succinate, pyruvate, and glutamate (furnished as sodium salts at a concentration of 0.1%, w/v). The experiment was conducted by using plates of mineral agar to which the organic compounds were added singly. $NH_4 NO_3$ was used in place of $NaNO_3$ as a nitrogen source to preclude the possibility that nitrate is not a utilizable nitrogen source in the dark, as might be the case if its assimilatory reduction were a light-dependent process. The plates were inoculated by placing small drops of liquid cultures on the agar surface. One set of plates was incubated at 30 C in the dark and one set was incubated at the same temperature in the light. Growth of most strains in the set of plates incubated in the light was clearly evident after 6 days, and the more slowly developing strains showed growth within 2 weeks. However, after a month no strains showed detectable growth on the set of plates incubated in the dark.

The growth of nostocaceous blue-green algae in the dark at the expense of sugars is sometimes extraordinarily slow and may become clearly evident only after incubation periods of several months. Hence, the results of this experiment with unicellular blue-green algae do not rigorously exclude the possibility that some strains may be capable of comparably slow growth in the dark. Even if this phenomenon could be demonstrated, it would obviously be of little physiological or ecological significance. For all practical purposes, the unicellular blue-green algae that we have examined can be considered obligate phototrophs.

Even among filamentous blue-green algae, we have not yet encountered a strain able to grow in the dark with sugars at a rate remotely comparable to its growth rate in a mineral medium in the light. Hence, although it is formally correct to describe some filamentous blue-green algae as facultative chemoheterotrophs, the potential for chemoheterotrophy appears to be very limited in all blue-green algae tested so far.

Nitrogen Fixation

Until recently, the capacity for nitrogen fixation among blue-green algae appeared to be invariably associated with the ability to form heterocysts. Heterocysts are not produced by unicellular blue-green algae, with the exception of *Chlorogloea fritschii*, an organism whose assignment to the *Chroococcales* is controversial (26). In 1969, however, Wyatt and Silvey (76) reported that a *Gloeocapsa* species can grow well with N_2 as a nitrogen source under aerobic conditions, and they demonstrated by appropriate assay methods the presence of nitrogenase in this strain.

We have screened most strains of groups I, II,

and III for the ability to grow well through repeated transfers in liquid medium BG-11 devoid of nitrate, and we have found only two that possess this property. One is 6909, the strain studied by Wyatt and Silvey (76); the other is 6501, a closely similar strain isolated at Berkeley. The formation of nitrogenase by strain 6501 has been confirmed by the technique of acetylene reduction (62). Both nitrogen-fixing strains belong to group IIB, characterized typologically by enclosure of the cells in multilaminate sheaths. Since these are the only representatives of group IIB so far isolated in pure culture, it is not yet possible to know whether nitrogen fixation is a universal character in this typological group. It should be noted that strain 6712 (typological group III), which resembles *Chlorogloea fritschii* in some structural respects, does not share its ability to grow at the expense of N_2.

Maximal Temperature for Growth

We have determined approximate temperature maxima for all strains by visual estimation of growth in medium BG-11, incubated in illuminated water baths at various temperatures (Fig. 14). Excluding 3 thermophilic isolates from hot springs, for which the temperature maximum is 53 C or higher, 35 of the 37 strains examined had temperature maxima between 35 and 43 C. Most strains isolated at Berkeley had been preselected in the course of their isolation for the ability to grow at 35 C. However, 11 of 12 nonthermophilic strains received from other laboratories had temperature maxima in the range of 35 to 43 C, even though, to the best of our knowledge, they had been isolated at temperatures below 30 C. A relatively high temperature maximum
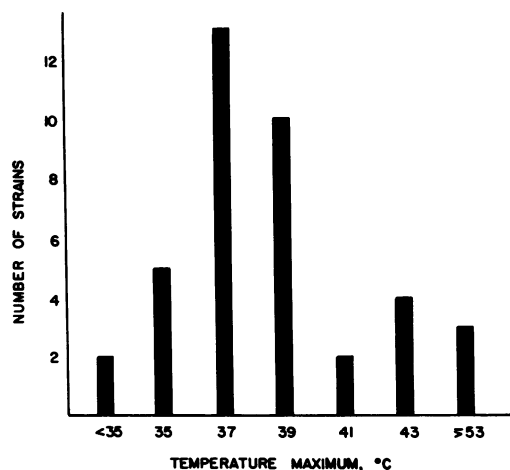

FIG. 14. *Frequency distribution of the temperature maximum for growth of 40 unicellular strains of blue-green algae.*

TABLE 6. *Maximal temperatures of growth for unicellular blue-green algae*

Maximal temperature	Strains belonging to typological groups							
	IA, low GC[a] cluster	IA, high GC cluster	IB	IIA, low GC cluster	IIA, high GC cluster	IIB	IIC	III
<35 C	6605						7005	
35 C		6307, 6603, 6710, 6713				6909		
37 C	6312	6707, 6708, 6709, 6706, 6907, 6911	6901, 6903	6308, 6711, 6804	6806			
39 C	7003, 6910		6802	6701, 6808	6702, 6803, 6805, 6714	6501		6712
41 C	7001			6807				
43 C	6908, 6301, 6311, 7002							
53 C (or higher	6715, 6716, 6717							

[a] Guanine plus cytosine.

therefore appears to be characteristic of many unicellular blue-green algae isolated from non-thermal habitats. It should be noted that the temperature maxima of three strains isolated by Van Baalen from marine sources (73) lay between 39 and 43 C, even though organisms from this habitat might have been expected to possess lower temperature ranges than fresh-water isolates.

Table 6 shows the distribution of temperature maxima among strains belonging to different typological groups. Within typological group IA, there is a fair correlation between DNA base composition and temperature maximum. Of the 11 strains of high GC content (66 to 72 moles %), 10 have relatively low temperature maxima (35 to 37 C). Of the 11 strains of low GC content (48 to 56 moles %), 7 have temperature maxima in excess of 40 C. In typological group II, only 1 of 14 strains can grow at temperatures in excess of 40 C.

Sensitivity to Light

Fluorescent light intensities of 2,000 to 3,000 lux are not deleterious to most unicellular strains. However, the representatives of group IIB (6501 and 6909) are unusually light sensitive, and they become partly bleached through destruction of phycobiliproteins if exposed to light intensities in this range. The highest fluorescent light intensity at which these two strains show normal growth and pigmentation is approximately 500 lux. Several strains of group IIA (6702, 6308, 6714, 6711, 6804, 6805, and 6808) bleach when exposed to 5,000 lux.

Penicillin Sensitivity

Semiquantitative experiments with gradient plates show that most unicellular blue-green algae are extremely sensitive to penicillin G. The only comparatively resistant strain is 6712 (typological group III), which can grow in the presence of at least 50 units/ml. Five strains (6301, 6311, 6803, 6805, and 6806) can grow in the presence of 1 unit/ml, but not of 10 units/ml. The remaining 34 strains are inhibited by penicillin concentrations of 1 unit/ml or less. Therefore, the inhibitory concentrations for the majority of unicellular blue-green algae are similar to those for gram-positive eubacteria and far below the levels required to inhibit growth of most gram-negative eubacteria.

Cellular Absorption Spectra

The photopigments characteristic of blue-green algae are chlorophyll *a*, phycobiliproteins (C-phycocyanin, *allo*-phycocyanin, and in some strains phycoerythrin), and a variable array of carotenoids (51, 72). In intact cells, the main visible absorption maxima are attributable to chlorophyll *a*, C-phycocyanin (which always predominates over *allo*-phycocyanin), and phycoerythrin, if this phycobiliprotein is present.

Table 7 shows the positions of the in vivo absorption maxima for the unicellular strains in our collection. The position of the chlorophyll *a* peak is essentially constant, at 680 to 683 nm. The position of the C-phycocyanin peak shows a greater variation, extending from 627 to 638 nm. This probably reflects strain differences with respect to the intracellular state of molecular aggregation of C-phycocyanin. The absorption maximum of the isolated pigment from several different strains lies at 622 to 625 nm, and breakage of the cells typically causes an immediate shift of the peak position to a shorter wavelength. A few strains also contain phycoerythrin, whose

TABLE 7. *Positions and relative heights of chlorophyll a and phycobiliprotein peaks in intact cells of unicellular strains[4]*

Typo-logical group	Strain	Phyco-eryth-rin	Phyco-cyanin	Chloro-phyll a	Relative peak heights, phycocy-anin/chloro-phyll a
IA	Low GC cluster				
	6715	—[b]	634	682	0.83
	6716	—	628	680	0.71
	6717	—	635	680	0.76
	6301	—	630	683	0.80
	6311	—	630	682	1.08
	6908	—	629	680	0.92
	6312	—	636	682	0.88
	7002	—	634	682	0.96
	7003	—	635	680	1.17
	6605	577	629	683	0.87
	6910	—	625	680	0.78
	High GC cluster				
	6307	—	637	683	1.20
	6603	—	636	683	1.25
	6706	—	636	681	1.26
	6707	—	637	680	1.32
	6708	—	637	683	1.19
	6709	—	638	683	1.28
	6710	—	637	682	1.21
	6713	—	638	683	1.22
	6907	—	635	680	1.57
	6911	—	630	681	1.39
	7001	—	632	681	1.03
IB	6802	580	632	682	0.79
	6901	—	633	682	0.98
	6903	—	630	681	0.98
IIA	Low GC cluster				
	6308	—	629	683	0.79
	6711	570	627	682	0.84
	6701	570	627	682	0.68
	6804	—	625	680	0.87
	6807	573	628	683	0.74
	6808	577	629	680	0.72
	High GC cluster				
	6714	—	628	682	0.92
	6702	—	628	680	0.94
	6803	—	627	681	0.98
	6805	—	628	681	0.98
	6806	-	630	682	1.02
IIB	6501	574	627	681	0.80
	6909				
IIC	7005	—	628	680	0.73

[a] Spectra measured behind opal glass.
[b] Relatively low concentration, so that its presence is not necessarily evident from the color of the cells.

presence is revealed by a peak or shoulder in the in vivo spectrum at approximately 570 nm. Some strains contain a relatively low concentration of phycoerythrin, so that its presence is not necessarily evident from the color of the cells as judged by visual inspection.

The last column of Table 7 shows the ratio of the peak heights of C-phycocyanin (627 to 638 nm) and chlorophyll a (680 to 683 nm) as measured from the in vivo absorption spectra. In most strains, this ratio does not exceed 1.0. However, an exceptionally large ratio (1.19 to 1.36), indicative of a very high phycocyanin content, is characteristic of many strains of group IA. Almost all the strains which show this property have DNA of high GC content (65 to to 72 moles % GC).

Cellular Fatty-Acid Composition

Nearly all bacteria, including the photosynthetic purple and green bacteria, contain only saturated and monounsaturated fatty acids (38). Eucaryotes also contain polyunsaturated fatty acids. With respect to cellular fatty-acid composition, the blue-green algae are heterogeneous (34, 49, 50, 54). The most extensive comparative analysis so far published (39) showed that the majority of filamentous blue-green algae (15 of 17 strains examined) contain large amounts of polyunsaturated fatty acids, whereas the majority

TABLE 8. *Fatty-acid composition of unicellular blue-green algae of typological group I*

Group	Mean deoxyribo-nucleic acid base compo-sition (moles % GC)	Strain	Percentage (by weight) of polyunsat-urated fatty acids[a]	Presence of[b] Linoleic acid	α-Lino-lenic acid	γ-Lino-lenic acid
IA	48–56	6605	58	+	+	—
		6910	12	+	+	±
		6301	2	±	—	—
		6311	0	—	—	—
		6312	0	—	—	—
		6715	1	±	—	—
		6716	1	±	—	—
		6717	0	—	—	—
		7002	24	+	+	—
		7003	31	+	+	—
	66–71	6307	1	±	—	—
		6603	0	—	—	—
		6706	0	—	—	—
		6707	0	—	—	—
		6708	0	—	—	—
		6709	0	—	—	—
		7001	23	+	+	—
IB	45–46	6802	43	+	±	+
		6901	45	+	+	—
		6903	36	+	+	—

[a] Content of linoleic (18:2), α-linolenic (18:3α), and γ-linolenic (18:3γ) acids, as percentage (by weight) of the total fatty acids of the cell.
[b] Key: + indicates major fatty acid; ± indicates minor fatty acid.

TABLE 9. *Fatty-acid composition of unicellular blue-green algae of typological groups II and III*

Group	Mean deoxyribonucleic acid base composition moles % GC	Strain	Percentage (by weight) of polyunsaturated fatty acids[a]	Presence of[b]		
				Linoleic acid	α-Linolenic acid	γ-Linolenic acid
IIA	46–48	6702	47	+	−	+
		6714	55	+	−	+
		6803	45	+	±	+
		6805	43	+	±	+
		6806	42	+	±	+
	35–37	6308	4	±	−	−
		6701	1	±	−	−
		6711	1	±	−	−
		6804	4	±	−	−
		6807	2	±	−	−
		6808	3	±	−	−
IIB	41	6501	3	±	−	−
IIC	45	7005	45	+	−	+
III	41	6712	3	±	−	−
	43	Chlorogloea fritschii	28	+	+	−

[a] Content of linoleic (18:2), α-linolenic (18:3α), and γ-linolenic (18:3γ) acids, as percentage (by weight) of the total fatty acids of the cell.

[b] Key: + indicates major fatty acid; ± indicates minor fatty acid.

of unicellular ones (14 of 20 strains examined) do not. Nearly all the unicellular strains in our collection have now been analyzed for fatty-acid composition. The detailed data will be published elsewhere by C. Kenyon, but we have abstracted from them the information which appears to be of particular taxonomic significance (Tables 8 and 9).

It is evident that unicellular blue-green algae fall into two general groups in terms of fatty-acid composition. Some have a high content of polyunsaturated fatty acids, which represent between 12 and 50% by weight of the total fatty acids of the cells. Such strains always contain significant amounts of triply unsaturated fatty acids: either the α- or the γ-isomer of linolenic acid. Other strains either do not contain detectable polyunsaturated fatty acids or have a very low content, ranging from 1 to 4% of the total fatty-acid content of the cells. Furthermore, such strains never contain either isomer of linolenic acid; the only polyunsaturated fatty acid present is the doubly unsaturated linolenic acid.

Strains of high polyunsaturated fatty-acid content occur in both typological groups I and II. In strains of group II, γ-linolenic acid is always the predominant triply unsaturated fatty acid; α-linolenic acid, if present at all, is a minor

constituent. In most strains of group I, α-linolenic acid is the predominant triply unsaturated fatty acid. Fatty-acid composition is of particular taxonomic significance in group IIA, where it is perfectly correlated with the internal subdivision of the group in terms of DNA base composition. Strains of high GC content (46 to 48 moles %) have a high polyunsaturated fatty-acid content, whereas strains of low GC content (35 to 37 moles %) have a low polyunsaturated fatty-acid content. Strain 7005 (group IIC) resembles the high GC cluster of group IIA with respect both to DNA base composition and fatty-acid composition. It should also be noted (Table 9) that fatty-acid composition distinguishes strain 6712 (typological group III) from *Chlorogloea fritschii*, which it resembles in both DNA base composition and growth habit.

PROBLEM OF GENERIC NOMENCLATURE IN THE ORDER CHROOCOCCALES

Taxa in the *Chroococcales* are defined by structural properties, determined in the first instance on field collections; type materials have usually been conserved as dried herbarium specimens. Over the course of time, many genera and species have been proposed. The traditional taxonomy of the *Chroococcales* has been excellently epitomized by Geitler (28). His treatment is a critical one that points out the numerous uncertainties: differences between named species are often small, and in some instances generic boundaries are unclear. Despite its limitations, this taxonomic treatment of the group remains the one most widely employed.

Drouet and Daily (20) proposed a major taxonomic revision of the *Chroococcales* which involved a drastic reduction in the number of genera and species. However, this so-called revision did not embody any extension of knowledge about the properties of the group. It was based in large part on a systematic comparative study of published descriptions and surviving herbarium specimens, some dating from the 18th century. The generic and specific descriptions of Drouet and Daily convey a minimum of factual information and consist largely of lists of synonyms. Furthermore, it is very difficult for other taxonomists to evaluate their allegations concerning synonymy, since the evidence presented consists of a series of photomicrographs of herbarium specimens, for the most part of poor technical quality.

The danger of attempting to base the classification of organisms as structurally simple as the members of the *Chroococcales* on the examination of herbarium specimens scarcely needs to be emphasized. These specimens almost always

TABLE 10. *Generic and specific names previously assigned to 10 strains of group IA*[a]

Strain No.	Proposed name
System of Drouet and Daily (20)	
6301	*Anacystis nidulans*
7002	*Agmenellum quadruplicatum*
7003	*Coccochloris elabens*
6307	*Coccochloris peniocystis*
7001	*Anacystis marina*
System of Geitler (28)	
6715	*Synechococcus lividus*
6716	*Synechococcus* sp.
6717	*Synechococcus* sp.
6908	*Synechococcus cedrorum*
6907	*Synechococcus elongatus*

[a] Short rods or cocci, 0.8 to 2.0 μm wide, reproducing by binary fission in a single plane.

contain a diversity of microbial corpses, and they vary greatly in their state of dilapidation. Any assertions about synonymy on such grounds are debatable, and there is no obvious way to resolve a difference of opinion about the identity or non-identity of two herbarium specimens. Therefore, archeological explorations through the herbaria of the world cannot be expected to contribute usefully to the classification of the *Chroococcales*. Unfortunately, however, nomenclatural priority rests with these herbarium specimens, no matter how uninformative the original published descriptions may have been or how unrecognizable the preserved material may have become through the vicissitudes of time. By their assertions of synonymy, Drouet and Daily succeeded in bringing back from limbo many ancient generic and specific names which had been mercifully forgotten by other taxonomists. For this reason, some of the genera that they recognize are not even mentioned in Geitler's treatment of the *Chroococcales*.

Fortunately, we can now evaluate pragmatically the relative determinative merits of the treatments of Geitler (28) and Drouet and Daily (20). Of the strains assigned to typological group IA, 10 were named strains received from other laboratories. Five had been named in accordance with the system of Drouet and Daily; these had all been identified by F. Drouet, on the basis of the examination of cultures submitted to him (M. B. Allen, C. Van Baalen, and G. P. Fitzgerald, *personal communications*). Five had been named by other investigators who used the system of Geitler. The proposed generic and specific assignments of these 10 strains are shown in Table 10.

Detailed comment is unnecessary. Even applied by one of its creators, the system of Drouet and

Dailey (20) leads to three different generic assignments for strains that are very similar in gross structural respects; therefore, this taxonomic system is a determinatively worthless one. Identification through the system of Geitler (28) resulted in a consistent generic assignment for five other strains belonging to the same typological group. Despite its shortcomings, this system provides the only reasonable point of departure for future taxonomic work.

POSSIBLE CORRESPONDENCES BETWEEN TYPOLOGICAL GROUPS AND TRADITIONAL FORM GENERA IN THE CHROOCOCCACEAE

In Table 11, we have summarized what appear to be the most important determinative characters of some of the principal form genera in the *Chroococcaceae*, as described by Geitler (28). Apart from *Microcystis*, which is very loosely defined, a primary differentiation is made on the basis of cell shape and successive planes of division. *Aphanocapsa*, *Gloeocapsa*, and *Chroococcus* possess spherical cells which divide in two or three successive planes; *Aphanothece*, *Gloeothece*, and *Synechococcus* possess ellipsoidal or cylindrical cells which divide transversely in one plane. Broadly speaking, the three former genera correspond to our typological group II, and the three latter to our typological group I.

One character emphasized in most of Geitler's generic descriptions is colony structure. In unicellular blue-green algae that produce multilaminate sheaths, the formation of colonies is determined by the continued enclosure of cells, after division, in a common sheath layer. This type of colony structure is readily recognizable in the sheath-forming strains of typological group IIB (*see* Fig. 7). However, various more-or-less distinctive colony structures are also described by Geitler for genera and species which do not form sheaths (e.g., *Microcystis*). In such cases, the colony is produced by the enclosure of many cells in a common and abundant slime layer. A similar phenomenon occurs in the bacterial genus *Zoogloea*, in which it seems to be taxonomically significant, since the characteristic zoogloeal colonies are formed in pure culture as well as in natural populations (17). However, none of the unicellular blue-green algae without sheaths that we have studied regularly produces distinctive colonial aggregates in pure culture. This is true even of our strain of *Microcystis aeruginosa*, a species for which Geitler (28) gives a precise description of colony form: "Young colonies spherical or elongated, compact; older colonies with net-like interruptions, and an

TABLE 11. *Comparison of the properties of certain genera of the Chroococcaceae, as defined by Geitler (28)*

Genus	Cell shape	Planes of division	Sheaths	Slime layers	Gas vacuoles	Colony structure *and* other characteristics
Aphanocapsa	Spherical	2 or 3	−	+	−	Many cells in a slime layer of irregular form
Gloeocapsa	Spherical	3	+	V[a]	−	2–8 cells enclosed by multilaminate sheaths; cells spherical after division
Chroococcus	Spherical	2 or 3	+	V	−	2–16 or more cells enclosed by multilaminate sheaths; cells hemispherical after division
Microcystis	Spherical or cylindrical	1 or many	−	+	V	Many cells in slime layer; colony can assume a number of different forms
Aphanothece	Ellipsoidal or cylindrical	1	−	+	−	Many cells in a slime layer, usually of irregular form
Gleothece	Ellipsoidal or cylindrical	1	+	V	−	As in *Gloeocapsa*
Synechococcus	Ellipsoidal or cylindrical	1	−	−	−	Never forms colonies

[a] Character that is variable within the genus.

indistinctly delineated slime layer." This character is, accordingly, undeterminable for most of the strains in our collection. Unless it could be shown (as has been done for *Zoogloea*) that some unicellular blue-green algae regularly produce colonies of distinctive form held together by a slime layer even in pure culture, this type of colony structure must be considered a character of dubious taxonomic value.

Among the form genera with spherical cells that divide in two or three planes, *Aphanocapsa* can be distinguished from *Gloeocapsa* and *Chroococcus* by its inability to form sheaths. This distinction probably appeared less significant to Geitler than it does to us, since he doubted whether slime layers and sheaths were really different kinds of cellular envelopes. Our experience suggests that the distinction is both significant and readily determinable. If one excludes from the generic definition of *Aphanocapsa* Geitler's qualifications concerning colony form (in any case very indefinite), the strains of typological group IIA fall into this form genus.

The distinction between *Gloeocapsa* and *Chroococcus*, both of which produce sheaths, is less clear cut. Geitler evidently felt that his verbal definitions were unsatisfactory, since he commented (28): "the comparison of illustrations (of *Chroococcus*) with those of *Gloeocapsa* reveals the generic differences better than many words. The distinction is often difficult for small-celled species, whose generic assignment is frequently purely conventional." If one follows Geitler's advice, the most striking difference revealed by his illustrations concerns the shape of cells after division. In most species of *Gloeocapsa*, a gradual

constriction occurs in the plane of division, so that daughter cells are spherical after separation. In *Chroococcus*, on the other hand, there is little or no constriction in the plane of division, which appears to be occupied by a cell plate: the daughter cells are hemispherical after division. This form is maintained through the following division, with the result that in ensheathed packets of four cells each cell has the shape of a quarter sphere. If this is indeed the basic distinction between the two genera, our ensheathed strains of group IIB are assignable to *Gloeocapsa*.

The form genus *Microcystis* is clearly an unsatisfactory one, as Geitler admitted. It includes both rods and cocci, dividing in a single plane or in more than one plane. The strain of *Microcystis aeruginosa* in our collection (typological group IIC) can be distinguished from the strains of typological group IIA (*Aphanocapsa*) by only one major character, the formation of gas vacuoles. However, Geitler records this as a variable property of *Microcystis*. Evidently, this genus needs to be defined in more restrictive terms, if it is to be maintained at all. The majority of the described species possess spherical cells that divide in two or three planes. The genus should probably be restricted to gas-vacuolate unicellular blue-green algae of this type.

Of the genera defined by the possession of ellipsoidal or cylindrical cells that divide in one plane, *Gloeothece* is distinguished from *Aphanothece* and *Synechococcus* by the presence of sheaths. The validity of the genus *Gloethece* seems highly questionable to us. In ensheathed coccoid strains of group IIB (= *Gloecapsa*), there is a

marked tendency to synchronous division of the cells within a single colony. Consequently, at a certain stage of development, all the cells in a colony may be ellipsoidal. Observed in a field collection, such a colony would almost certainly be identified as *Gloeothece*. Hence, this genus may be synonymous with *Gloeocapsa*.

The distinction between *Aphanothece* and *Synechococcus* seems to rest entirely on the property of colony formation, determined by the presence of an extensive slime layer. This is characteristic of *Aphanothece* and absent from *Synechococcus*. In terms of this distinction, all strains of typological group I appear to be assignable to *Synechococcus*.

Table 12 shows the probable correspondences between our typological groups I and II and form genera of the family *Chrococcaceae* recognized by Geitler. Even these proposed correspondences can be maintained only by making certain revisions of the Geitlerian definitions—notably, the omission of colony structure as a generic character for organisms that do not form sheaths and the restriction of *Microcystis* to spherical organisms that divide in two or three planes and produce gas vacuoles. Although these revisions lead to genera that appear satisfactory for determinative purposes, it must be emphasized that both *Synechococcus* and *Aphanocapsa* as now defined encompass organisms that differ widely in DNA base composition and in certain phenotypic respects. Eventually, a subdivision of each of these genera into at least two genera should probably be undertaken. However, before any new taxonomic proposals with respect to the *Chrococcales* are put forward, one essential and long-overdue action must be taken, the establishment of a reasonable nomenclatural date of departure for the order.

We formally propose that the starting date should be C. Nägeli's monograph *Gattungen Einzelliger Algen* (48), published in 1849. It contains good descriptions of most of the major genera of unicellular blue-green algae now recognized, accompanied by excellent line drawings to illustrate their salient structural properties. International agreement to accept Nägeli's monograph as the oldest source of valid generic and specific names in the *Chroococcales* would largely prevent nomenclatural instability of the kind introduced by the monograph of Drouet and Daily (20), which has been a source of great confusion during the past 15 years.

MERISMOPEDIA: A GROWTH FORM OF APHANOCAPSA?

One of the oldest form genera in the Chroococcaceae is *Merismopedia*. It comprises organisms with spherical cells not enclosed by sheaths, which divide synchronously in two planes to form flat, rectangular colonies that contain 16, 32, 64, or even more cells in regular two-dimensional array (28). Unicellular blue-green algae of this distinctive type have been observed frequently in field collections, and many species have been named.

Pringsheim (61) was the first to describe cultured material of *Merismopedia*. Two of the three strains that he examined were unquestionably authentic, since he had established them by direct isolation of organisms with the characteristic colony form. In culture, however, the cells occurred mostly as pairs or irregular aggregates; Pringsheim's sketch shows cell arrangements that are not significantly different from those characteristic of young, healthy cultures of our strains of group IIA, which we believe to be assignable to the genus *Aphanocapsa*.

By chance, we recently found that under special circumstances some strains of group IIA can produce *Merismopedia*-like colonies. Liquid cultures of a number of group IIA strains had been grown with continuous illumination for a week at 37 C and subsequently left undisturbed for over a month at room temperature on a table, where they were exposed to a diurnal light-dark cycle. Wet mounts showed that two of these strains, 6807 and 6808, had produced aggregates with the typical form and perfectly synchronized division characteristic of *Merismopedia*. These aggregates were interspersed with single cells, pairs of cells, and irregular clumps, many obviously dead or senescent. The *Merismopedia*-like colonies were particularly well developed in the culture of strain 6808 (Fig. 15). Both strains had been in pure culture for over 2 years, and repeated microscopic examinations of material grown under more favorable conditions had not previously revealed such aggregates.

This experience suggests that the ability of *Aphanocapsa* strains to produce *Merismopedia*-like growth forms may be relatively common. Binary fission in two planes at right angles to one another is clearly a necessary condition, but not a sufficient one. The history of the cultures in which these colonies developed points to two other factors that may be of importance: imposed synchrony of division (by a diurnal light-dark cycle) and minimal disturbance of the medium, so that the slowly growing aggregates are not physically disrupted.

Coupled with the report of Pringsheim (61), our observations cast some doubt on the validity of the genus *Merismopedia*. Most (if not all) of the described species may well be growth forms of *Aphanocapsa* species, which have developed in

TABLE 12. *Probable correspondences between form genera and typological groups*

Typo-logical group	Cell shape	Planes of divi-sion	Sheaths	Gas vac-uoles	Genus
IIA	Spherical	2 or 3	−	−	*Aphano-capsa*
IIB	Spherical	2 or 3	+	−	*Gloeocapsa*
IIC	Spherical	2 or 3	−	+	*Microcys-tis*
I	Cylindrical, el-lipsoidal, or spherical	1	−	−	*Synecho-coccus*

nature under conditions that favor the formation of *Merismopedia*-like colonies. The mainten-ance of *Merismopedia* as a separate genus would be justified only if it could be shown that the for-mation of such colonies by some coccoid blue-green algae is a constant property, of regular oc-currence in cultured material. The possible exist-ence of such blue-green algae certainly cannot be discounted, since the same mode of growth is char-acteristic of the bacterium *Lampropedia hyalina*, and is maintained in pure cultures of this organism (59). *L. hyalina* has a very complex wall structure, and the two outer layers of the wall enclose the rectangular tablets of cells (13, 53). Its distinctive
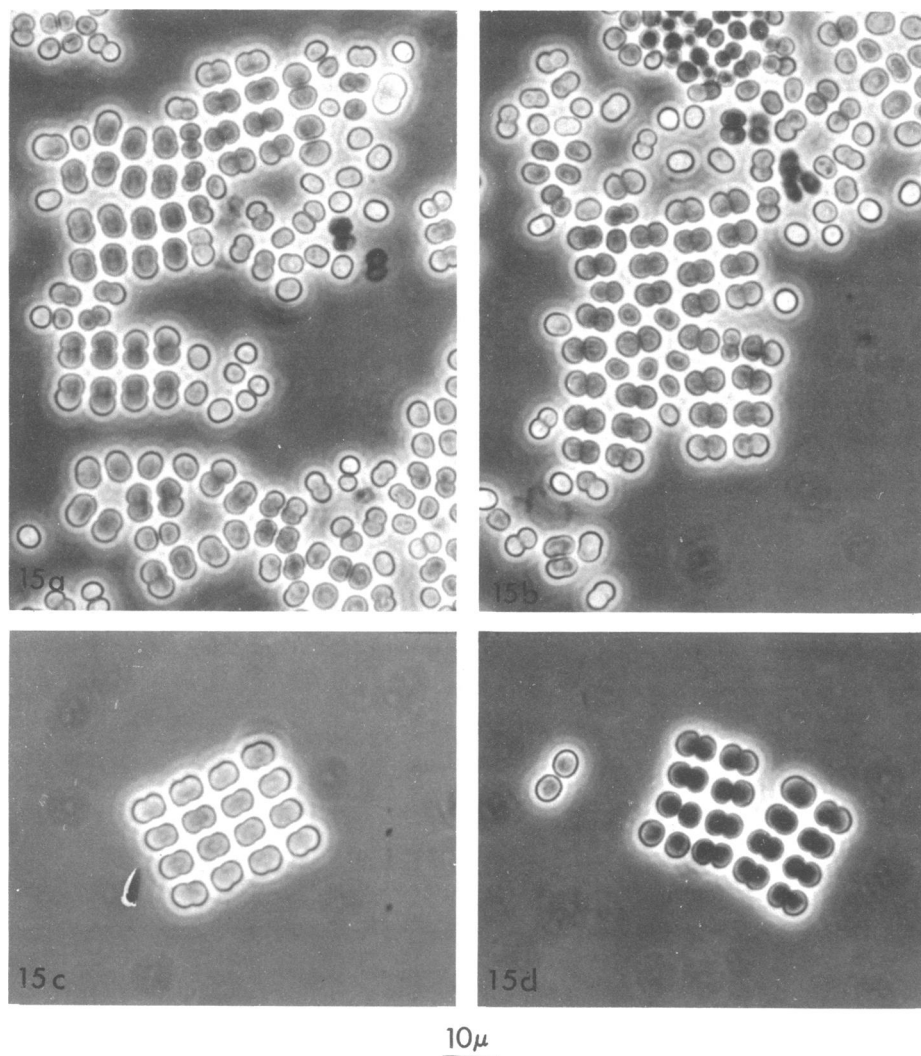

10μ

FIG. 15. *Merismopedia-like aggregates in an old culture of strain 6808. (See text for growth conditions.) Representative fields, also containing many single cells and cell pairs, are shown in Fig. 15a and 15b. Figures 15c and 15d show two particularly regular, 16-celled colonies of which the cells are undergoing division in almost perfect synchrony.*

growth habit is therefore probably determined by the special properties of the cell wall.

GENERA CHLOROGLOEA AND CHLOROGLOEOPSIS

One of our unicellular isolates, strain 6712 (typological group III) does not appear to be assignable to the family *Chroococcaceae*. Its structure and development (Fig. 9) correspond well to the descriptions and illustrations by Geitler (28) of the genus *Chlorogloea* (family *Entophysalidaceae*). However, as shown in Table 13, strain 6712 differs in a number of respects from *Chlorogloea fritschii* Mitra (46), the only other blue-green alga available in culture that has been placed in this genus.

The formation of heterocysts by *C. fritschii*, which was discovered (25, 26) subsequent to its original description (46), excludes this strain by definition from the *Chroococcales*. Fay, Kumar, and Fogg (26) proposed its transfer to the genus *Nostoc* (order *Nostocales*), whereas Mitra and Pandey (46a) suggested the creation for it of a new genus *Chlorogloeopsis*, which they assign to the order *Stigonematales*. The vegetative growth of this organism (Fig. 10) does not resemble that of *Nostoc* species, and for this reason the taxonomic solution suggested by Mitra and Pandey (46a) appears to be the more appropriate one.

Strain 6712 fully conforms to the description of the form genus *Chlorogloea*. It does not fix nitrogen or develop heterocysts, and it has not been observed to form chains of cells by successive divisions in a single plane. This strain is apparently the first true representative of the genus *Chlorogloea* to have been obtained in pure culture.

SPECIATION IN THE CHROOCOCCACEAE

Structural characters, DNA base composition, and physiological and chemical properties permit the subdivision of typological groups I and II into series of strain clusters, most of which

TABLE 13. *Comparison of the properties of strain 6712 and Chlorogloea fritschii*

Determination	Strain 6712	*C. fritschii*
Deoxyribonucleic acid base composition[a]	40.8	42.9
Formation of short filaments	−	+
Formation of heterocysts	−	+
Nitrogen fixation	−	+
Content of polyunsaturated acids	Low	High

[a] Expressed as moles % guanine plus cytosine.

probably deserve recognition as separate species. However, the problem of assigning specific names is difficult. In the genera to which these strains appear to belong, many nomenspecies have already been proposed, but the information content associated with existing specific names is so small that a single specific epithet could frequently be applied to several different strains or strain-clusters, readily distinguishable from one another if properties that have hitherto not been considered in the taxonomy of the *Chroococcaceae* are taken into account. Eventually, a solution to this problem must be found. We can see no alternative to deciding arbitrarily that an existing specific name be attached to a particular strain, which would thereafter constitute the neotype for the species in question. Other strains of similar structure which differ markedly from the neotype in DNA base composition or in physiological and chemical respects could then be assigned new specific names. This is the solution which was adopted in bacteriological nomenclature (71) to fix the type of *B. subtilis*, of which the original description, based solely on structural criteria, was later found to apply to more than one specific entity.

At the present time, we are unwilling to make formal proposals to this effect. We shall indicate, therefore, for groups I and II the strains and strain clusters which in our judgement merit specific recognition, but without attaching specific names to them except in the rare cases in which identification with an existing nomenspecies can be made without ambiguity.

Subdivisions in Typological Group I (Synechococcus)

Most of the strains of typological group I are immotile short rods or cocci, 2.0 μm or less in width, without polar granules. Structurally speaking, they present few distinguishing characters and might well be placed in a single species by a taxonomist who considered only structural properties. Nevertheless, these 20 strains can be subdivided into a series of distinct clusters when additional characters are taken into consideration (Table 14). Clusters 1 and 2 are distinguished by the GC content (66 to 71 moles %) of their DNA, at least 10 moles % higher than the GC content of the DNA of all other strains in group I. The 10 strains of cluster 1 are all very small rods or cocci, ranging in width from 0.8 to 1.5 μm. Although they show slight variations in cell size and in DNA base composition, they appear very similar in all other respects. One strain of high GC content, 7001, has been placed in a separate cluster 2, since it differs from the strains of cluster 1 with respect to fatty-acid composition, phycocyanin:

TABLE 14. *Strain clusters of typological group I* (*Synechococcus*)

Cluster	Cell width (μm)	Polar gran-ules	Mo-tility	Temp maxi-mum (C)	Content of poly-unsat-urated fatty acids	Requirement for		Phyco-erythrin	Peak ratio of phycocyanin: chlorophyll *a*	Deoxyri-bonucleic acid base compo-sition[a]	Strains included
						Vit-amin B12	1% NaCl				
1	0.8–1.5	—	—	35–37	Low	—	—	—	1.19–1.57	66–71	6307, 6603, 6706, 6707, 6708, 6709, 6710, 6713, 6907, 6911
2	0.8	—	—	41	High	—	—	—	1.03	69	7001
3	1.5	—	—	>53	Low	—	—	—	0.76–0.83	52–54	6715, 6716, 6717
4	1.0	—	—	43	Low	—	—	—	0.80–1.08	55–56	6301, 6311, 6908
5	1.5	—	—	37	Low	—	—	—	0.88	50	6312
6a	1.5	—	—	43	High	+	—	—	0.96	49	7002
6b	2.0	—	—	39	High	+	+	—	1.17	49.5	7003
7	3	—	—	<35	High	—	—	+	0.87	47	6605
8	3	—	+	41	High	—	—	—	0.78	48	6910
9a	2.5–3.0	+	+	37	High	—	—	+ or —	0.79–0.98	46	6802, 6903
9b	1.5	+	+	37	High	—	—	—	0.98	45	6901

[a] Expressed as moles % guanine plus cytosine.

chlorophyll *a* ratio, and temperature maximum for growth. It should also be noted that this strain is of marine origin (although it grows well in me-um BG-11 without added NaCl), whereas the strains of cluster 1 are all isolates from fresh water.

Strain 6907 (cluster 1) was received under the name *Synechococcus elongatus*. According to Geitler (28), *S. elongatus* has the smallest cells of any named *Synechococcus* species, 1.4 to 2 μm in width. These dimensions fit moderately well for 6907 and other strains of cluster 1. However, the same name could be applied with equal cogency to clusters 2, 4, or 5. Strain 7001 (cluster 2) was identified by Drouet (Van Baalen, *personal communication*) as *Anacystis marina*. For reasons already discussed, we do not accept this proposal.

Among the strains that are similar in cell structure to those of clusters 1 and 2 but contain DNA of lower GC content, clusters 3, 4, and 5 resemble one another in most phenotypic respects but can be distinguished by DNA base composition, as well as by one phenotypic trait—the temperature maximum for growth. The strains of cluster 3 (DNA base composition: 52 to 54 moles % GC) were isolated from thermal habitats, and they have a temperature maximum of 53 C or higher. The cells are relatively long rods, and this structural property also serves to distinguish them from strains of clusters 1, 2, 4, and 5 (*See* Fig. 1 and 2). One of these strains was received under the name

Synechococcus lividus. The remaining two strains were received from R. W. Castenholz as *Synechococcus* species. However, Castenholz (12) has also applied the specific name *lividus* to thermophilic *Synechococcus* strains of this type, and we believe that it can be considered the correct specific epithet.

The three strains of cluster 4 and the single strain of cluster 5 are all isolates from fresh water; they can be construed as nonthermophilic counterparts of *S. lividus*. The temperature maximum for the strains of cluster 4 is unusually high (43 C), a character that distinguishes them from other strains of similar structure from nonthermal habitats. The single strain of cluster 5 has a much lower temperature maximum (37 C) and differs in GC content by 5 moles % from the strains of cluster 4.

One strain of cluster 4 (6908) was received under the name *S. cedrorum*. However, the width of the cells in cluster 4 is considerably less than the range of 3 to 4.3 μm given by Geitler (28) for *S. cedrorum*, and this name is therefore clearly incorrect. Cluster 4 also includes 6301, the classic isolate by M. B. Allen (1952), to which Drouet applied the name *Anacystis nidulans*. Padmaja and Desikachary (52) carefully compared the dimensions of *A. nidulans* and of a number of *Synechococcus* strains and concluded that Allen's strain corresponded in size to *Synechococcus elongatus*, an opinion with which we concur. Unfortunately, however, this name could be ap-

plied with equal justification to strain clusters 1 and 5, which cannot be clearly distinguished from cluster 4 either by structural properties or by habitat. The only solution to this problem is to designate a neotype strain of *S. elongatus*. On historical grounds we believe that M. B. Allen's strain (6301) would be the most appropriate neotype. If this suggestion is accepted by other taxonomists, the name *S. elongatus* will become fixed to strain cluster 4. Other specific epithets can then be proposed for strain clusters that are distinguishable from cluster 4 only by nonstructural characters.

Cluster 6 contains two strains of marine origin distinguished by their requirement for vitamin B_{12} (73). Both show a pronounced tendency to chain formation (*see* Fig. 1). Although virtually identical in DNA base composition (49 to 49.5 moles % GC), they differ from one another by several phenotypic characters and have been placed in separate subclusters, 6a and 6b. Strain 7002 (subcluster 6a) has cylindrical cells 1.5 μm wide, whereas the cells of strain 7003 (subcluster 6b) are 2.0 μm wide and spherical immediately after division. The temperature maxima differ markedly (Table 14). Strain 7003 shows a salt requirement and will not grow satisfactorily unless the culture medium is supplemented with 1% (w/v) NaCl. The other two strains of marine origin in the collection (7001 and 7002) appear to grow equally well with or without the addition of NaCl.

Drouet assigned the two strains of cluster 6 to different genera, identifying 7002 as *Agmenellum quadruplicatum* and 7003 as *Coccochloris elabens* (Van Baalen, *personal communication*). In Geitlerian terms, both should be assigned to *Synechococcus*, but we have not been able to identify them with named species of this genus.

Two strains of group I (6605 and 6910) can be distinguished from the strain clusters so far discussed by their relatively large, ellipsoidal cells, 3 μm in width. They differ from one another with respect to motility, temperature maximum, and the presence of phycoerythrin, and they have been assigned to separate clusters, 7 and 8. In terms of cell size, they fit the specifications given by Geitler (28) for *Synechococcus cedrorum*; here again, however, a single specific epithet could be applied with equal justification to two organisms that are clearly representatives of different species. This problem can be solved only by the designation of a neotype strain for *S. cedrorum*.

The three strains of group IB (6802, 6901, and 6903) are collectively distinguishable from other members of group I by a structural character, the regular presence of refractile polar granules. They also differ from all other strains of group I, except

6910, by their rapid gliding movement. To the best of our knowledge, unicellular blue-green algae of this type have not been previously described. Although their assignment to a new genus might be appropriate, we shall regard them provisionally as members of the genus *Synechococcus* and assign them to a special cluster 9. Strains 6802 and 6903 (subcluster 9a) can be distinguished from strain 6901 (subcluster 9b) by their considerably larger cells, 2.5 to 3.0 μm in width; the cells of strain 6901 are 1.5 μm in width. The two strains assigned to cluster 9a differ with respect to chain formation (absent from strain 6802) and the presence of phycoerythrin (absent from strain 6903).

Subdivisions in Typological Group II
(Aphanocapsa, Gloeocapsa, Microcystis)

Table 15 summarizes the distinguishing properties of strain clusters in typological group II. Eleven strains are assignable to *Aphanocapsa*, and they can be subdivided into five clusters. Cluster 1 comprises five strains with DNA of high GC content (46 to 48 moles %) and of high polyunsaturated fatty-acid content, and this cluster is internally homogeneous in all phenotypic respects.

The remaining six strains assigned to *Aphanocapsa*, all with DNA of low GC content (35 to 37 moles %) and of low polyunsaturated fatty-acid content, are subdivisible into four clusters by differences in cell size and temperature maximum and by the presence or absence of phycoerythrin. None of these clusters can be identified satisfactorily with existing nomenspecies.

The one strain (7005, group IIC) assigned to the genus *Microcystis* can be identified by cell size and gas-vacuole formation as *Microcystis aeruginosa*, but in our hands it has never formed the distinctive colonial aggregates described as characteristic of this species in nature. Zehnder and Gorham (77), who studied a crude culture of another strain of *M. aeruginosa*, reported that its ability to form colonies was rapidly lost after isolation. Hence, this character is evidently an unstable one.

The somewhat tenuous nature of the generic distinction between *Microcystis* and *Aphanocapsa* must be emphasized; it can be maintained only by taking into account the property of gas-vacuole formation. With respect to both DNA base composition and polyunsaturated fatty-acid content, *M. aeruginosa* resembles the strains assigned to cluster 1 of *Aphanocapsa*, although it can be distinguished from them at the specific level by its larger cell size and lower temperature maximum, as well as by the property of gas-vacuole formation. It is conceivable that a more

TABLE 15. *Differential properties of strains of group II (Aphanocapsa, Gloeocapsa, Microcystis)*

Genus	Cluster	Sheaths	Gas vacuoles	Diameter of cells (μm)	Temp maximum (C)	N_2 fixation	Phycoerythrin	Polyunsaturated fatty acids	Deoxyribonucleic acid base composition[a]	Strains included
Aphanocapsa	1	−	−	2–3	37–39	−	−	High	46–48	6702, 6714, 6803, 6805, 6806
	2	−	−	4–5	37	−	−	Low	35	6308
	3	−	−	3–4	37–39	−	+	Low	36–37	6701, 6711
	4	−	−	6–7	41	−	+	Low	36	6807, 6808
	5	−	−	7	37	−	−	Low	36	6804
Microcystis	1	−	+	4	<35	−	−	High	45	7005
Gloeocapsa	1	+	−	6	35–39	+	+	Low	41	6501, 6909

[a] Expressed as moles % guanine plus cytosine.

satisfactory generic arrangement of nonensheathed strains with spherical cells that divide in two or three planes could be achieved by the recognition of two genera, distinguished primarily by DNA base composition and fatty-acid content. In this event, *M. aeruginosa* and cluster 1 of *Aphanocapsa* would comprise two species in one of these genera, and the remaining strain clusters of *Aphanocapsa* a series of species in the other genus.

The two strains assignable to *Gloeocapsa* are readily distinguishable from all other strains of group II by their distinctive, multilaminate sheaths, as well as by one physiological property, the capacity for nitrogen fixation. Apart from a difference in temperature maximum (35 C for 6909, 39 C for 6501) they appear phenotypically identical and also have nearly identical DNA base compositions. We consider them to be representatives of a single species, but we have been unable to identify it with certainty with any of the numerous nomenspecies of *Gloeocapsa* described by Geitler (28).

CONCLUDING COMMENTS

Most of the taxonomic conclusions which have been presented here are tentative ones. This is ascribable primarily to the difficulties encountered in assigning strains characterized on the basis of pure-culture studies to taxa originally proposed on the basis of an entirely different methodology. In fact, the principal conclusion to be drawn from our work is a methodological one. We have shown beyond any question that the members of the *Chroococcales*, like most other unicellular bacteria, cannot be adequately characterized solely by gross structural properties; many good species remain unrecognizable in terms of these criteria. We believe accordingly that *all future taxonomic work on the Chroococ-*

cales should be based on the isolation and comparison of pure strains.

The isolation of unicellular blue-green algae in pure culture is admittedly time consuming and it demands a certain degree of microbiological competence. It might be argued, therefore, that future taxonomic work would be conducted more expeditiously and conveniently on impure (so-called unialgal) cultures. This hope is probably illusory, since properties now recognizable as being of taxonomic importance (the entire range of physiological properties, fatty-acid composition, DNA base composition) cannot be determined reliably on such material. Furthermore, when one deals with microorganisms as small and structurally unspecialized as are many members of the *Chroococcaceae,* the unialgal state of an impure culture can never be taken for granted, unless its blue-green algal component has been cloned by single-cell isolation or by plating. The danger of assuming that a culture is unialgal on the basis of inadequate evidence can be specifically documented from our experience with two impure cultures received from other laboratories.

The first case history concerns a supposedly unialgal culture, labeled *Gloeocapsa alpicola* 1051, which we received in 1963 and from which a pure culture (strain 6308, typological group IIA) was then isolated. Some years later, fearing that strain 6308 had been lost, we again obtained a culture of *G. alpicola* 1051 from the original donor, and again proceeded to isolate a pure culture from it. On this occasion, a totally different organism (strain 6910, typological group IA) was isolated. The properties of strains 6308 and 6910 are compared in Table 16. The two pure strains do not differ very markedly in size and microscopic appearance (*see* Fig. 1 and 4), although the cells of 6910 are definitely ellipsoidal, whereas those of 6308 are spherical. The differ-

TABLE 16. *Properties of the two strains isolated from a supposedly unialgal culture, Gloeocapsa alpicola 1051*

Strain	Cell width (μm)	Cell shape	Planes of successive division	Motility	Deoxyribonucleic acid base composition[a]	Polyunsaturated fatty acid content
6308	4–5	Spherical	2	–	35	Low
6910	3	Ellipsoidal	1	+	48	High

[a] Expressed as moles % guanine plus cytosine.

ence between them with respect to planes of successive division is also not easy to recognize by simple microscopic examination, since in both strains daughter cells usually separate soon after the completion of division. If one did not suspect that *G. alpicola* 1051 contained two different species of unicellular blue-green algae, its microscopic appearance might well be interpreted as reflecting clonal variation of a single strain. However, the many differences between the two pure strains isolated from *G. alpicola* 1051 show that it was bialgal, not unialgal.

The second case history concerns a culture received with the label *Microcystis aeruginosa* NRC-1. This culture was unquestionably unialgal when we received it; however, the blue-green alga that it contained, strain 6911, was a small-celled *Synechococcus* of high GC content (Fig. 2), completely different in microscopic appearance from *M. aeruginosa* (Fig. 8). Although the previous strain history of 6911 is complex (*see* Table 1), its strain designation (NRC-1) and history indicate that it is derived from a culture that once contained *M. aeruginosa*, and was then believed to be unialgal (36, 77). The methods used to isolate this strain of *M. aeruginosa* did not include cloning on plates. As a result of the small cell size of 6911, its pigmentation is not detectable microscopically. Hence, its presence in a crude culture of *M. aeruginosa* that also contained bacteria could easily have escaped notice. Furthermore, if *M. aeruginosa* had died out during later transfers, this fact would not have been evident macroscopically, since the culture would still have appeared blue-green. We suspect that strain 6911 is derived from a culture that was once bialgal and also contained *M. aeruginosa*, although this inference cannot be definitely proven.

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