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STUDIES ON THE GAS-VACUOLES OF
BLUE-GREEN ALGAE

By

Roger Vincent Smith (B.Sc. Dunelm)

A thesis submitted for the degree
of Doctor of Philosophy in the
University of Durham.

April, 1969.



A B S T R A C T

Gas-vacuoles were examined with optical and electron microscopes in species of blue-green algae cultured in the laboratory and collected from the field. Gas-vacuoles were characterised with the normal transmission optical microscope as reddish, pressure-sensitive structures, which possess a lower refractive index than the surrounding cellular contents. They were resolved with the electron microscope into closely packed bundles of gas-cylinders (70.0 nm wide) with α -granules lying in ordered rows between the individual gas-cylinders. The groups of gas-cylinders were found in association with lamellae at all stages of growth of Anabaena flos-aquae D124. Changes in the arrangement of lamellae were reflected in the appearance of the groups of gas-cylinders.

Analyses of vacuole gases with the mass-spectrometer indicated the presence of nitrogen. However, the finding that gas-cylinder membranes are freely permeable to gases indicates that the composition of vacuole gases will reflect that of the surroundings.

Gas-cylinder membranes and α -granules were isolated from A. flos-aquae D124. The membranes were striated with a periodicity of 5.0 nm and appeared globular in section. The 4.0 nm globules were proteinaceous with molecular weights

of 22,000 \pm 2,000. Possible homologies of these membranes with viral coat protein are discussed. α -granules were shown by a variety of techniques to be polysaccharide.

A. flos-aquae D124 was grown under a variety of different environmental conditions. Two generalisations were apparent from these studies. Gas-vacuoles occupy a greater proportion of cell volume in the stationary phase of growth compared with exponential growth. Gas-vacuoles in cells grown at inhibitory light intensities are restricted in their development to the cell periphery adjacent to cell walls.

Some physiological studies were conducted on the effects of the absence of gas-vacuoles on growth of A. flos-aquae D124, and the changes in sedimentation rate of cells associated with gas-vacuole redevelopment. After consideration of a variety of different evidence it was concluded that the main function of gas-vacuoles is to lower the specific gravity of blue-green algal cells.

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The abbreviations employed for the labelling of the plates are shown below. The terminology of PANKRATZ and BOWEN (1963) has been employed. For a review of the various descriptive terms used by other authors, see LANG (1968).

B	=	Bacterium.
G	=	Gas-cylinder.
Gm	=	Gas-cylinder membrane.
Gms	=	Gas-cylinder membrane sub-unit.
H	=	Heterocyst.
L	=	Lamella.
Lv	=	Intralamellar vesicle.
N	=	Nucleoplasmic fibril.
P	=	Plasma membrane.
R	=	Ribosome.
S	=	Striation.
α	=	α -granule.
β	=	β -granule.

(x)

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INTRODUCTION

An effect of the increase in human population in the last few decades has been the nutrient enrichment of natural waters by waste disposal and the leaching of agricultural fertilizers from surface soils. In lakes and reservoirs this excessive nutrient richness has led to the development of blooms of planktonic blue-green algae. These blooms are a great nuisance to water supply undertakings, to fish farmers and anglers, and, under certain circumstances, may cause severe poisoning of cattle. However, a blue-green algal bloom is not only a contemporary phenomenon. Early references to water blooms in the British Isles were collected by GRIFFITHS (1939), who reported that several early chroniclers recorded the occurrence of a red bloom at Finchampstead, Berkshire, in 1100 A.D. Similarly, the turning of the River Nile blood-red reported in Exodus 7: 19-21, and the colouration of the Red Sea may possibly be ascribed to blue-green algal blooms.

Several species of blue-green algae from a number of distinct genera produce blooms*. Three of the most important species from different genera (Anabaena, Aphanizomenon, and Microcystis) all bear the specific name of 'flos-aquae'. It is possible for a bloom to be unialgal, or to be a mixed bloom consisting of several species (HAMMER, 1964). These

*See the Appendix (p.142) for a summary of the species reported to contain gas-vacuoles by GEITLER (1932).

species may be divided into epilimnetic and hypolimnetic forms (EBERLEY, 1966). In general, epilimnetic forms are species from the genera described above and are surface blooms, hypolimnetic forms are species from the genus Oscillatoria and are deep-water blooms.

The cells of these planktonic species of blue-green algae possess distinctive inclusions of low refractive index which appear reddish when observed with the optical microscope. These structures were reported by RICHTER (1894) to be amorphous sulphur. However, subsequent studies revealed the absence of extractable sulphur from cells containing these structures. KLEBAHN (1895) termed these red inclusions 'gas-vacuoles'. Klebahn based his characterisation on the evidence of their low refractive index and their destruction by pressure treatment. An elegant experiment devised by STRODTMANN (1895), which demonstrated the significant contribution made by these structures in lowering the specific gravity of cells, supported Klebahn's hypothesis that they were gas-filled. In the present study the term 'gas-vacuole' will be used to describe these structures. Its use will be restricted to observations made with the optical microscope.

Gas-vacuoles were described by COHN (1870), WINOGRADSKY (1888), MOLISCH (1906) and LAUTERBORN (1915) in cells of

certain strains of purple and green sulphur bacteria collected from natural habitats. WINOGRADSKY (1888) termed the gas-vacuoles, 'Höhlungen'. Gas-vacuoles were also observed by LAUTERBORN (1915) in certain aquatic, colourless filamentous bacteria which he called Pelonema and Peloploca and by KOLKWITZ (1928) in Thiothrix and Sarcina ventriculi. Unfortunately, most of these observations must be accepted with caution because these workers did not invariably use pressure treatment to characterise the reported gas-vacuoles. However, PETER (1931) showed that certain Halobacterium strains, which caused the red discolouration of fish, contained bodies of low refractive index which could be induced to disappear after pressure treatment. She therefore provided convincing evidence of presence of gas-vacuoles in a group of organisms not closely related to blue-green algae.

Klebahn's gas-vacuole hypothesis was vigorously opposed by BRAND (1901), MOLISCH (1903), FISCHER (1905) and LEMMERMAN (1910). Two properties of the blue-green algal gas-vacuoles, which were incompatible with the known characteristics of small gas bubbles were the main grounds for this criticism. In the first instance, gas-vacuoles did not disappear under vacuum and, secondly, they could be isolated from the blue-green algal cell by gentle

homogenisation or by chemical digestion of the cell wall (MOLISCH, 1903). However, KLEBAHN (1922, 1925, 1929) attempted to explain these anomalies by suggesting that the gas was enclosed in a rigid, impermeable membrane. He carried out a series of ingenious experiments to demonstrate that gas could be extracted from these structures and also attempted an identification of the gas present.

Unfortunately, Klebahn was limited by the inadequacies of the technology of his period. He did not use fresh material and most of the gas he extracted from the gas-vacuolate material was derived from air in solution. However, although the experimental evidence for Klebahn's conclusion that the gas was nitrogen was unsatisfactory, he was able to show that gas was released from cells on destruction of their gas-vacuoles.

FOGG (1941) reviewed the early studies on blue-green gas-vacuoles (by German workers) and thus provided an English introduction into the subject of gas-vacuoles. Following Klebahn's investigations in the 1920's, the unique problems presented by gas-vacuoles were neglected. The cause of this neglect may be partly due to the need for new instrumentation which had to await technological advances in other disciplines. However, the main reason for the absence of any significant published work on the

subject for nearly forty years may be ascribed to the state of disrepute of the subject following critical attacks on the gas-vacuole hypothesis by BRAND (1901), MOLISCH (1903), FISCHER (1905), LEMMERMAN (1910) and LAUTERBORN (1915).

These workers did very little meaningful experimentation on the gas-vacuoles and their contribution to the subject must be said to have been a negative one. LEMMERMAN (1910) proposed that gas-vacuoles should be described by the misleading term 'pseudovacuoles'. Unfortunately, this term has been employed by recent workers in the field (PRINGSHEIM, 1966; PEARSON and KINGSBURY, 1966) and is used in the text of FRITSCH (1945) and by VAN NIEL (1957) in 'Bergey's Manual of Determinative Bacteriology'.

In the past two decades the advent of modern culture methods has led to a great increase in our understanding of the biology of the blue-green algae. However, blue-green algal research has centred on organisms which have been selected for their fast growth rates and capacity to produce high yields in laboratory culture. Despite their economic importance, the planktonic blue-green algae containing gas-vacuoles have been relatively neglected because of difficulties experienced in their culturing. Consequently, little is known about the physiology of these gas-vacuolate organisms, and our knowledge of blue-green algal physiology

and biochemistry rests on information obtained from a few species without gas-vacuoles which grow well in laboratory culture.

However, since 1960 some progress has been made in the culturing of planktonic species by workers at the National Research Council at Ottawa. The following epilimnetic species have been grown successfully in laboratory culture: Microcystis aeruginosa (McLACHLAN and GORHAM, 1961), Aphanizomenon flos-aquae (McLACHLAN, HAMMER and GORHAM, 1963), Gloeotrichia echinulata (ZEHNDER, 1963) and Anabaena flos-aquae (GORHAM et al., 1964). STAUB (1961) cultured a hypolimnetic species, Oscillatoria rubescens, and published an extensive account of its physiology and ecology. The first blue-green algae examined with the electron microscope were not planktonic organisms. However, several workers (HOPWOOD and GLAUERT, 1960; SUN, 1961; CHAPMAN and SALTON, 1962, and GIESY, 1962) reported the presence of irregular lacunae in blue-green algal cells which they mis-identified as gas-vacuoles. BOWEN and JENSEN (1965) collected field material of the gas-vacuolate organism, Aphanizomenon flos-aquae and correlated their observations of fine structure with a light microscope characterisation of gas-vacuoles. JOST (1965) cultured the planktonic organism Oscillatoria rubescens in the laboratory, employing the

medium developed by STAUB (1961) and studied its fine structure. Both these studies revealed that gas-vacuoles consisted of cylindrical sub-units with conical ends closely packed in parallel arrays. Individual cylindrical sub-units were about 70 nm wide and were bounded by an unusual membrane about 2.0 nm wide. These structures were termed 'Hohlspindeln' by JOST (1965) and 'gas vesicles' by BOWEN and JENSEN (1965). In the present study the term 'gas-cylinder' is employed.

Since 1966, when the present thesis studies were started, there has been a great revival of interest in the subject of gas-vacuoles in blue-green algae and bacteria. This work is intimately related to the present studies and will be briefly summarised and discussed below in more detail. JOST and MATILE (1966) attempted to isolate gas-cylinders from Oscillatoria rubescens by employing a sucrose gradient and high speed centrifugation. They reported that gas-cylinder membranes had a high lipoid and carotenoid content. WALSBY and EICHELBERGER (1968) showed that gas-cylinders could be released from Anabaena flos-aquae D124 and that on isolation they retained their shape and structure. These authors also reported some preliminary studies on the gas-composition of the gas-vacuoles. Employing the mass-spectrometer for their analysis they confirmed the result

of KLEBAHN (1922) that the gas present was mainly nitrogen.

The studies on the gas-vacuoles of bacteria have been of great comparative interest. HOUWINK (1956) was the first to demonstrate with the electron microscope the presence of small vesicles in a Habolacterium strain which corresponded with the gas-vacuoles observed with the light microscope by PETER (1931). LARSEN et al. (1967) and STOEKENIUS and ROWEN (1967) showed that these structures varied in size between 100 and 300 nm and had a striated surface structure similar to the appearance of gas-cylinders described by WALSBY and EICHELBERGER (1968). STOEKENIUS and KUNAU (1968) reported that the membranes of these structures were highly proteinaceous. PFENNIG and COHEN-BAZIRE (1967) have demonstrated the presence of 70 nm tubular vesicles in the green bacterium Pelodictyon clathratiforme. These appear homologous to the gas-cylinders present in blue-green algae, but they are not closely packed into bundles. PFENNIG, MARKHAM and LIAAEN-JENSEN (1968) made observations with the light microscope on gas-vacuoles present in strains of Lamprocystis roseopersicina and Thiodictyon elegans.

In the present study the nature and functional significance of gas-vacuoles in blue-green algae has been investigated. The problem assumes some economic importance

because of the nuisance caused in certain reservoirs by these organisms. The ability of these organisms to float, a property which may be attributed to the presence of gas-vacuoles, causes great difficulties in coagulation and filtration when blue-green algae are present in raw water. A subsidiary aim of the present study was, therefore, to identify the environmental conditions which stimulate gas-vacuole development.

A consequence of these aims was a need to provide structural, physiological and biochemical information relating to gas-vacuoles and gas-vacuolate organisms. The "Results" section of the present thesis is organised into six Parts, each dealing with one line of enquiry. The culturing, harvesting and preparation of gas-vacuolate organisms for electron microscope examination presented problems not normally associated with studies on blue-green algae. Specialised techniques for studying these organisms were developed in Part I of the present study. As a result of these preliminary studies Anabaena flos-aquae D124 was chosen as a suitable experimental organism for subsequent investigations. Parts, I, II, III and V each have brief introductory sections which outline the aims of the particular study. As the results of Parts I and II were important to the development of a suitable approach to

subsequent studies they are discussed in "Results",
Sections 5 and 12, respectively.

MATERIALS AND METHODS

1. Algal materials

A. Unialgal cultures

Details of the origins of the cultures are given in Table I. In the present studies the Durham culture numbers of these organisms were employed to identify each strain. For example, the strain of Anabaena flos-aquae isolated from Windermere, was referred to as A. flos-aquae D124. All cultures were unialgal, but they were not axenic.

B. Field samples

Oscillatoria agardhii and O. redekei were collected from St. James's Park Lake, London, on 31.VII.1967. The organisms were transported to Durham in a polythene 250 ml collecting bottle and allowed to stand overnight at room temperature. On 1.VIII.1967 the material that had collected at the surface of the suspension was examined with the optical microscope, and fixed and embedded for subsequent electron microscope studies.

Microcystis aeruginosa was collected from Cole Mere, one of the Shropshire Meres, on 7.VIII.1967. On the same day, Anabaena flos-aquae and Aphanizomenon flos-aquae were collected from White Mere. Within six hours of collection these organisms were examined with the optical microscope and

Table 1. Details of the origins of the organisms studied

Organisms	Site of isolation	Source of culture	Culture no. at source	Culture no. at Durham
<u>Anabaena flos-aquae</u> (LYNGB.) BREB.	Windermere, England.	Westfield College		124
<u>Anabaena flos-aquae</u> (LYNGB.) BREB.	An oxidation pond, Mississippi, U.S.A. (TISCHER, 1965)	"	60A	125
<u>Gloeotrichia echinulata</u> (SMITH) RICHT.		Cambridge	1432/1	126
<u>Microcystis aeruginosa</u> KUTZ.		"	1450/1	127
<u>Nostoc linekia</u> BORN. et FLAH.		C.S.S.R. culture collection	125	130
<u>Oscillatoria agardhii</u> GOM.	Windermere, England.	F.B.A., Windermere		132

fixed and embedded for subsequent electron microscope studies at Durham.

2. Chemicals

Except for those listed below, chemicals were obtained from the British Drug Houses Ltd., and were of Analar grade.

Solid carbon dioxide ("Cardice").	The Distillers Co. Ltd., Gateshead, Co. Durham.
Araldite) Benzylidimethylamine) Glutaraldehyde) Propylene oxide)	G. T. Gurr Ltd., London.
Osmium tetroxide) Potassium permanganate)	Johnson Matthey Co. Ltd., London.
Formvar	Shawinigin Ltd., London.
Bovine serum albumin) Catalase)	Sigma Chemical Co. Ltd., London.

3. Culture methods

A. Cleaning of glass-ware

Conical, 250 ml Pyrex flasks were employed for growing all organisms. These were washed by soaking overnight in a hot 2% (w/v) solution of Quadralene Laboratory detergent. After soaking they were scrubbed to remove any stubborn algal deposits, and rinsed thoroughly in tap water. Flasks were given a final rinse in distilled water and dried in an oven

at 105 °C.

Pipettes that had come into contact with organic materials were cleaned by soaking overnight in a mixture of 1 volume of saturated sodium nitrate and six volumes of concentrated sulphuric acid. They were rinsed in the same way as for flasks.

B. Apparatus for growth of cultures

Experiments were carried out in flasks incubated in tanks illuminated from below by a bank of fluorescent tubes. Light intensities were adjusted by reducing the number of tubes and by wrapping flasks in black, fine-meshed cloth gauze. The design of apparatus allowed one to make easy and rapid modifications of the desired shaking rate and incubation temperature.

C. Media

Media were prepared with distilled water and chemicals of Analar grade. Apart from the preliminary growth experiments described in "Results", Part I, only one medium was employed. This medium (termed ASM-1) was developed by GORHAM et al., (1964). Its composition is shown in Table 2. Details of the other media used in Part I are summarised in Table 3.

D. Sterilisation

Flasks were stoppered with non-absorbent cotton wool

Table 2. Composition of ASM-1 medium

Compôund	Salt used	Concentration	
		micro- moles	mg/l
Sodium nitrate	NaNO ₃	2,000	170.0
Magnesium sulphate	MgSO ₄ .7H ₂ O	200	49.0
Magnesium chloride	MgCl ₂ .6H ₂ O	200	41.0
Calcium chloride	CaCl ₂ .2H ₂ O	200	29.0
di-Potassium hydrogen orthophosphate	K ₂ HPO ₄	100	9.0
di-Sodium hydrogen orthophosphate	Na ₂ HPO ₄	100	7.0
Ferric chloride	FeCl ₃	4	0.65
Boric acid	H ₃ BO ₃	40	2.0
Manganese chloride	MnCl ₂ .4H ₂ O	7	1.0
Zinc chloride	ZnCl ₂	3.2	0.3
Cobaltous chloride	CoCl ₂ .6H ₂ O	0.08	0.02
Cupric chloride	CuCl ₂ .2H ₂ O	0.0008	0.001
Diaminoethanetetra- acetic acid, disodium salt	Na ₂ EDTA.2H ₂ O	20.0	7.0

Table 3. The composition of AC, AD and CHU 10 (mod.) media employed in the preliminary growth experiments ("Results", Section I) with the original descriptions of these media

Salt used	"C" medium of KRATZ and MYERS (1955)	AC medium	Medium of FOGG (1949)	AD medium (Microelements of ALLEN and ARNON, 1955)	CHU 10 medium (CHU, 1942)	CHU 10 (mod.)
MgSO ₄ ·7H ₂ O	250.0	250.0	200.0	200.0	25.0	25.0
CaCl ₂			100.0			
CaCl ₂ ·2H ₂ O		15.0		50.0		
NaCl		23.0		23.0		
KNO ₃	1,000.0	1,000.0				
Ca(NO ₃) ₂					40.0	40.0
Ca(NO ₃) ₂ ·4H ₂ O	25.0					
Na ₂ CO ₃					20.0	20.0
Na ₂ SiO ₃					25.0 (+2.0)	43.5
K ₂ HPO ₄	1,000.0	1,000.0	200.0	250.0	10.0 or 5.0	10.0
Na ₂ EDTA·2H ₂ O		12.7		25.4		6.35
FeCl ₃			0.40		0.80	
FeCl ₃ ·6H ₂ O		9.7		19.4		4.85
Fe ₂ (SO ₄) ₃ ·6H ₂ O	4.0					
NaCitrate·2H ₂ O	165.0					
H ₃ BO ₃	2.86	2.86	0.10	0.50	2.0	1.43
MnCl ₂ ·4H ₂ O	1.81	0.181	0.10	0.50		0.09
MnSO ₄ ·4H ₂ O					2.0	
ZnCl ₂						
ZnSO ₄ ·7H ₂ O	0.222	0.222	0.01	0.05	2.0	0.111
CoCl ₂ ·6H ₂ O		0.040			1.0	0.02
CoSO ₄ ·7H ₂ O						
CuSO ₄ ·5H ₂ O	0.079	0.079	0.01	0.02		0.039
Na ₂ MoO ₄ ·2H ₂ O		0.027	0.10			0.0135
MoO ₃	0.0177			0.10		
Na ₂ WO ₄ ·2H ₂ O				0.01		
TiO(C ₂ O ₄) _x ·YH ₂ O				0.01		
K ₂ SO ₄ ·Cr ₂ (SO ₄) ₃ ·24H ₂ O				0.01		
NiSO ₄ ·6H ₂ O				0.01		
AlCl ₃					2.0	2.0
LiCl					1.0	1.0

Concentrations are expressed in mg/l.

plugs and sterilised by autoclaving at 15 lb per sq. in. for 15 min. Pipettes were similarly sterilised. Inoculations were carried out in a room sterilised by ultra-violet irradiation. Before inoculation the room was sprayed with absolute ethanol to remove suspended material from the atmosphere, and the inoculation bench was swabbed with absolute ethanol.

E. Inoculum material

Inoculum material consisted of 5.0 ml of a culture between 28 and 38 days old, which had been grown on ASM-1 medium under the standard conditions described in "Materials and Methods", Section 3G.

F. Measurement of physical features

Light intensities were measured with an "EEL" Lightmaster photometer at the plane of the middle of the fluid in the growth flasks, and orientated at right angles to the source of light. Light intensity will be expressed in lux (lx). pH values were measured with a "PYE" Dynacap pH meter sensitive to 0.01 differences. Temperatures will be expressed in degrees Celsius ($^{\circ}\text{C}$).

G. Standard growth conditions

Flasks were incubated in the tank at 20°C , 2,000lx and shaken 64 times per min. through a horizontal movement

of 30.0 mm. Cells were harvested from the late log phase of growth when they were in a healthy, actively growing state.

4. Growth measurements

Material was harvested by centrifugation at 5,000 x g at 2 day intervals, for a period of 12 days. Cells were washed with distilled water, transferred to vitreosil crucibles and dried for 24 hours at 105°C. Growth rates were expressed in terms of the relative growth constant, K, in log₁₀ day units (FOGG, 1965).

$$K = \frac{\log_{10}N - \log_{10}N_0}{t},$$

where, t = days after inoculation,

N = yield after t days,

N₀ = yield when t = 0.

The mean doubling time, G, can be derived from K:

$$G = \frac{0.301}{K}$$

For the study of the growth characteristics of Anabaena flos-aquae D124 in "Results", Part II, material was harvested on days +2, 4, 8, 12, 16, 20, 24 and 28.

5. Electron microscopy

A. Direct preparation technique

Cells were fixed with osmic acid vapour for 30 seconds

on 200 mesh formvar coated grids. The preparation was then dried in an oven for 10 minutes at 40 °C. After washing in distilled water and subsequent drying, the cells were examined with an optical microscope prior to examination with the electron microscope.

B. Negative staining

Some difficulty was encountered in obtaining good negatively stained preparations of membrane fractions. The following procedure was found the most satisfactory:

(i) Drop of fraction placed on formvar-carbon coated grids and allowed to stand for 30 seconds.

(ii) Grid held vertically and several drops of distilled water run over the surface to remove excess material and sucrose.

(iii) Grid held upside down for 1 minute in contact with a drop of 2% (w/v) phosphotungstic acid neutralised with potassium hydroxide.

(iv) Excess stain removed by touching the edge of the grid with filter paper.

(v) Grid allowed to dry at room temperature.

The use of 1% (w/v) uranyl acetate at pH 4.1 as a negative stain, and the addition of 1% (w/v) bovine serum albumin, did not improve the quality of the preparations.

C. Preparation of sectioned material

Since gas-vacuoles are destroyed by centrifugation, cells were concentrated on Millipore filters (3.0 μ m pore width).

I. Fixatives. Two fixatives were employed in all studies:

- (i) 2% (w/v) potassium permanganate, unbuffered, at room temperature for 1 hour (MOLLENHAUER, 1959).
- (ii) 1% (w/v) osmic acid, buffered at pH 6.1, at room temperature for 3 hours (PANKRATZ and BOWEN, 1963).

For examination of membrane fractions the following fixation procedure was found to be the most satisfactory:

- (i) Membrane fractions collected on Millipore filters (10 μ m pore width).
- (ii) Filters washed with 0.2 M phosphate buffer (pH 7.0).
- (iii) Material fixed for 3 hours at 4 °C with 5% (w/v) glutaraldehyde in buffer.
- (iv) Re-washed in buffer and post fixed with 1% (w/v) osmic acid in buffer for 3 hours at 4 °C.
- (v) Washed in distilled water.

II. Embedment. In "Results", Parts I and II, fixed material was treated in the following way:

- (i) Dehydrated in an ethanol series.
- (ii) Propylene oxide (P.O.) for 20 minutes.
- (iii) 10% (v/v) Araldite (prepared by mixing 26 ml of araldite (CY212) with 24 ml of hardener (HY964) plus one drop of benzyldimethylamine to each ml of mixture in P.O. for 30 minutes at 60 °C.
- (iv) 20% (v/v) araldite in P.O. for 30 minutes at 60 °C.
- (v) 40% (v/v) araldite in P.O. for 30 minutes at 60 °C.
- (vi) 50% (v/v) araldite in P.O. overnight at room temperature with the cap off the container.
- (vii) 100% araldite for 6 hours at room temperature.
- (viii) Material transferred to capsules containing fresh araldite and cured for 48 hours at 60 °C.

In "Results", Parts III, V and VI, improved penetration of araldite into material was accomplished by modifying the above procedure. After treatment of dehydrated material with P.O. for 20 minutes, it was soaked in 50% (v/v) araldite in P.O. overnight. The material was then transferred to capsules containing fresh araldite at 40 °C for 48 hours and finally cured at 60 °C for a further 48 hours. The ratio of accelerator to araldite was lowered to one drop of benzyldimethylamine to 5 ml of araldite mixture.

III. Sectioning. Sections were cut on an LKB ultratome using glass knives and collected on formvar coated grids.

D. Electron microscope examination

Sections were stained with lead citrate (REYNOLDS, 1963) before examination with an AEI EM6B electron microscope. Examination was routinely carried out at 60 Kv. Ilford N50 plates were used throughout the present study.

6. Gas experiments

A. The composition of vacuole gas

Gas-vacuolate cells of Anabaena flos-aquae D124 were concentrated by low speed centrifugation at 1,500 x g (a pressure not high enough to rupture gas-vacuoles). The concentrate was divided into two samples. One sample was treated with 3.72 atmospheres pressure by centrifugation at 5,000 x g. Optical microscope examination revealed that gas-vacuoles had been ruptured by this treatment. Both samples were then resuspended in previously boiled water and reconcentrated. For certain experiments the samples were freeze-dried to remove water and sealed under vacuum. A vacuum system was constructed for the manipulation of the vacuole gases (Fig. 1). Evacuation was effected using a vacuum unit rotary pump, "Speedivac", model 12E6/1288. Access to the system was possible at two points utilising B14 sockets. Samples were attached to system and evacuated for 5 minutes at the temperature of "cardice" (-60 °C). Two methods were employed for releasing the vacuole gases.

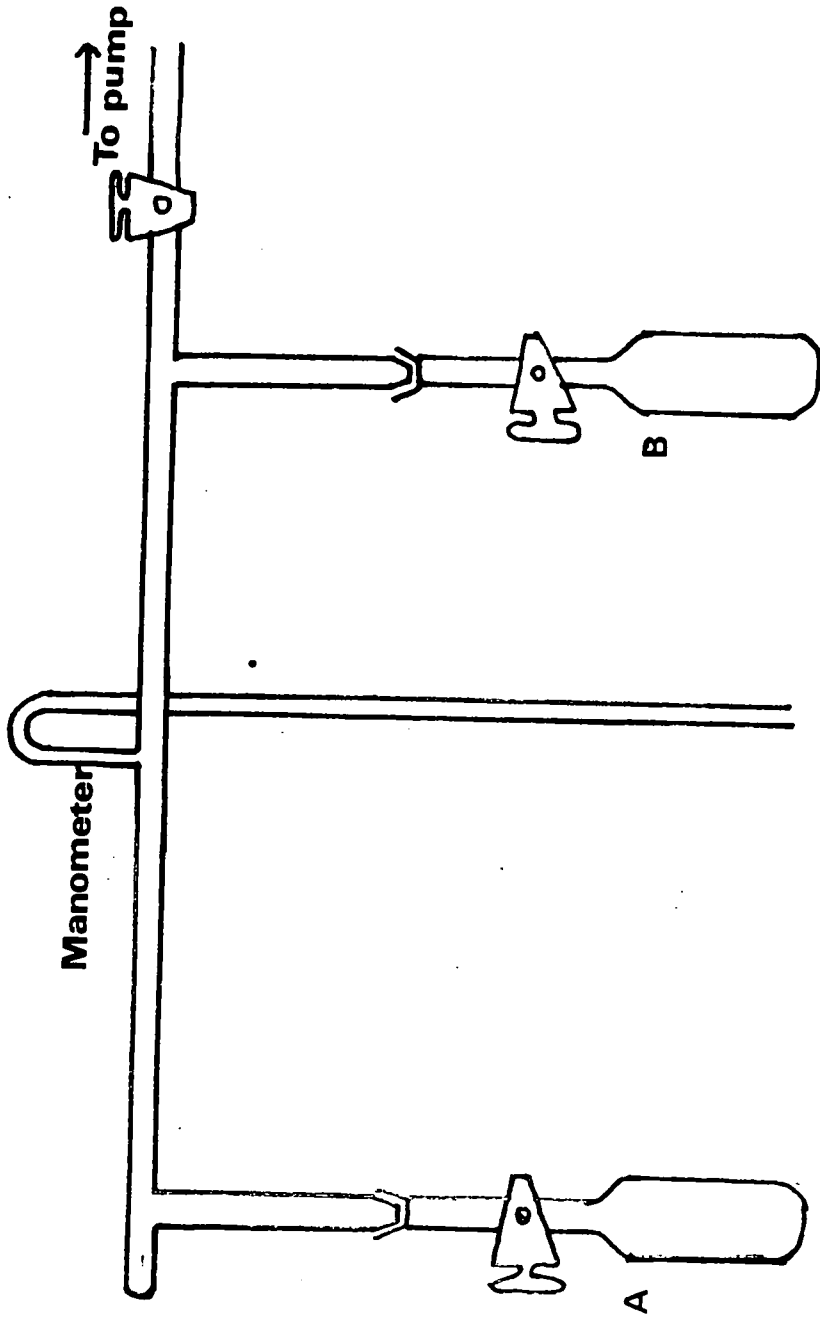


Fig. 1. The apparatus employed for manipulating the gases present in gas-vacuoles.

Method I. Chloroform was solidified with liquid nitrogen (-195°C). After prior evacuation of the system the chloroform was allowed to vaporise and was used to disrupt gas-vacuolate cells by distillation from flask A to flask B.

Method II. After prior evacuation at -60°C the samples were allowed to reach room temperature. The pressures generated during the process of thawing disrupted the gas-vacuoles. Analyses of vacuole gases were made in conjunction with Mr. P. Nutter of the Department of Chemistry, Durham, on an AEI M.S.9 mass spectrometer. The mass spectrum of the samples was measured from mass 1 to mass 130.

B. Argon and N^{15} uptake experiments

The following experiments were carried out at the Department of Botany, Westfield College, University of London, in conjunction with Mr. A. E. Walsby. Anabaena flos-aquae D124 was grown at Westfield. Oscillatoria agardhii D132 was grown at Durham under the standard growth conditions ("Materials and Methods", Section 3G), with the modification that the light intensity was lowered to 1,000 lx.

Argon uptake. Two concentrated 5.0 ml samples of Anabaena flos-aquae D124 were prepared. One sample was pressure treated to disrupt gas-vacuoles. They were placed in Warburg flasks and attached to a manifold suitable for flushing with different gas mixtures. The flasks were

evacuated for one minute and shaken for 10 minutes while 100% argon was flushed through the system.

Incubation with N¹⁵. Four 5 ml samples of Oscillatoria agardhii D132 were placed in Warburg flasks and flushed with an 80% argon, 20% oxygen, 0.04% carbon dioxide mixture.

After prior evacuation, 0.2 atmospheres of N¹⁵, plus 0.8 atmospheres of the 80% argon, 20% oxygen and 0.04% carbon dioxide mixture were added to the flasks. These flasks were incubated for 24 hours at 1,000 lx and 20 °C.

Dumas combustion. Material was prepared for Dumas combustion by concentrating it on filter paper and adding copper oxide powder. This was placed in a combustion tube, copper oxide added, and the tube topped up with fines. The combustion tubes were treated by the method of BARSDATE and DUGDALE (1965). The apparatus employed by these workers was simplified by omitting the Pirani gauge and the ^TJoeppler pump. The masses of the gases released by the combustion were estimated with a mass spectrometer (AEI M.S.3).

7. Sucrose gradient centrifugation

Washed cells were ground with acid washed sand and 8% (w/v) sucrose in 0.05M tris-HCl buffer (pH 7.5). More 8% sucrose buffer was added and the homogenate was centrifuged at 2,000 x g for 10 minutes to remove sand and whole cells. Approximately 1.0 ml suspension was carefully layered on

to the surface of a sucrose density gradient in a 23 ml centrifuge tube. The gradient was similar to that employed by JOST and MATILE (1966): 70% to 10% (w/v) sucrose. The gradient was centrifuged at 105,000 x g AVE for 2½ hours at 4 °C using an M.S.E. 3 x 23 swingout rotor, catalogue no. 59590 on an M.S.E. "superspeed 65" centrifuge.

After centrifugation, the gradient was fractionated by a modified method of OUMI and OSAWA (1966). A thin steel rod was carefully inserted down the side of the tube until it rested at the bottom. The gradient was pumped out of the tube at a flow rate of 0.5 ml per minute, using a peristaltic pump, and the optical density at 280 nm was monitored using an Isco Ultraviolet Analyser, model 222.

8. The isolation and molecular weight determination of membrane proteins

A. Preparation of membrane proteins

Cells were washed with ASM-1 medium and ground with acid-washed sand and one volume of 0.3M sucrose, 0.05M tris-HCl (pH 7.5). The homogenate was extracted with 10 volumes of buffer and centrifuged at 5,000 x g for 15 minutes to remove sand, unbroken cells and large membrane fragments. The sediment was discarded and the supernatant was centrifuged at 20,000 x g for 30 minutes to obtain a sediment containing membranes. This was drained briefly, taken up in 0.5 ml of

6M guanidine hydrochloride, containing 25 μ l of 2M ammonium carbonate buffer pH 8.6 and 5.0 μ l mercaptoethanol, and left at room temperature for 3 hours. The reduced mixtures were alkylated with 20 mg of iodoacetamide and 50 μ l of 2M ammonium carbonate buffer (pH 8.6) at room temperature for 1 $\frac{1}{2}$ hours. After overnight dialysis against tap water the protein was precipitated with two volumes of acetone and collected by a brief centrifugation at 3,000 x g. It was washed thoroughly with acetone and dried.

B. Polyacrylamide gel electrophoresis

Molecular weights of proteins were determined on 10% polyacrylamide gels by the method of SHAPIRO et al. (1967). The protein samples were dissolved in 1% (w/v) sodium dodecyl sulphate (pH 7.1). This solution was diluted to 0.1% sodium dodecyl sulphate before use. Duplicate samples (20-50 μ g) of the proteins, to one of which was added cytochrome c as a marker, were run on the gels. The gels were stained in 0.25% (w/v) coomassie blue in methanol, acetic acid, water, (5.0/5.0/1.0, (v/v/v)) for 30 minutes and destained by overnight washing in 7% (v/v) acetic acid. Results were recorded by scanning the gels on the Joyce-Loebl Chromoscan. The solubilisation of membrane protein and the determination of molecular weights were carried out in conjunction with Dr. C. J. Bailey of the Dept. of Botany, Durham.

9. The isolation of α -granules

α -granules were prepared as described for the preparation of membrane protein (see "Materials and Methods", Section 8A), with the modification that the 5,000 x g supernatant was centrifuged at 60,000 x g for 30 minutes to form a pellet. This pellet was washed with buffer and recentrifuged.

10. Buoyancy experiments

A. Preparation of the cell suspension

Cells were harvested from the late exponential phase of growth after having been grown under the standard growth conditions described in "Materials and Methods", Section 3G. Material was centrifuged at a pressure of 3.72 atmospheres to destroy gas-vacuoles. Cells were resuspended in the supernatant after it had been Millipore filtered (5.0 μ m pore width) to remove any gas-vacuolate cells. The suspension was glass-wool filtered to remove any clumps, mucilage and long filaments and returned to the growth tank. When samples were taken in order to measure the changes in sedimentation characteristics of cells, a second filtration was made.

B. Measurement of the sedimentation rates of cells

Some difficulty was encountered in making accurate determinations of small changes in the sedimentation

characteristics of cells. A spectrophotometric method was first used in an attempt to measure the sinking rate of the cell suspension in a cuvette. However, this method only provided mean results relating to a mixed population containing filaments of varying lengths. To obtain standard measurements relating to filaments of specific length an alternative method was devised. The sinking rates of filaments in a soda glass-tube were observed with an inverted microscope. The soda glass-tube was sealed at its base with a round, glass cover slip. The method employed for sealing the cover slip to the tube is summarised below:

- (i) Base of the soda glass-tube was ground flat.
- (ii) A paste was made by adding water to the soda glass powder and this was applied to the base of the tube with a camel hair brush.
- (iii) The cover slip was placed in a furnace on top of a copper sheet and the tube placed centrally on it.
- (iv) Paste was added to the join, in order to seal it, and the furnace was heated to 600°C and maintained at that temperature for ten minutes.
- (v) The oven was allowed to slowly equilibrate with room temperature before it was opened.

In order to estimate sedimentation rates one ml of cell suspension was added to the tube. Filament counts

were made at 5 minute intervals on the 4-celled filaments which had sunk to the base of the tube.

RESULTS

PART 1. Preliminary studies on the culturing of gas-vacuolate blue-greens and the characterisation of gas-vacuoles.

Five species of blue-green algae reported to contain gas-vacuoles by GEITLER (1932) were studied in order to select a suitable organism and a standard growth medium for later studies. A subsidiary aim of the study was to characterise the features common to gas-vacuoles of blue-green algae and the development of a suitable descriptive nomenclature. The terminology of PANKRATZ and BOWEN (1963) was used to describe cellular morphology.

1. The growth rates of reported gas-vacuolate blue-greens

The results of the growth studies are shown in Table 4. All species grew well on ASM-1 medium. These organisms all showed some growth on CHU 10 (mod.) and in the case of Gloeotrichia echinulata D126 the medium supported a faster growth rate ($K = 0.135$) than on ASM-1 ($K = 0.115$). The more concentrated AC and AD media inhibited growth of the organisms originally isolated from the plankton (Anabaena flos-aquae D124, Gloeotrichia echinulata D126, Microcystis aeruginosa D127 and Oscillatoria agardhii D132). However, these media supported good growth of the sessile species, Nostoc linckia D130, and the sewage oxidation pond isolate, Anabaena flos-aquae D125.

Table 4. Growth rates of organisms in different media grown under standard growth conditions ("Materials and Methods", Section 3G) expressed in terms of the growth constant 'K'.

Organism	Medium			
	ASM-1	AC	AD	CHU 10 (mod.)
<u>Anabaena flos-aquae</u> DI24	0.128	No growth	Poor replication	0.124
<u>Anabaena flos-aquae</u> DI25	0.151	Good growth	Good growth	Good growth
<u>Gloeotrichia echinulata</u> DI26	0.115	No growth	No growth	0.135
<u>Microcystis aeruginosa</u> DI27	0.177	No growth	No growth	Some growth
<u>Nostoc linckia</u> DI30	0.145	Good growth	Good growth	Some growth
<u>Oscillatoria agardhii</u> DI32	0.131	No growth	No growth	Some growth

Quantitative growth measurements were not made where poor replication was obtained between flasks, and growth is indicated in these cases by "poor replication". Where quantitative measurements were not made growth is indicated as either "no growth", "some growth", or "good growth".

EBERLEY (1965) similarly concluded that ASM medium supported the best growth of the five planktonic species which he had attempted to grow on several recognised media. The ASM-1 medium employed in the present study is a more concentrated modification of the original ASM medium.

2. Optical microscope observations

The vegetative cells of Anabaena flos-aquae D124 when viewed at low magnification contained black, irregular shaped structures. At higher magnification (400 X) these non-refractile structures appeared reddish with black margins. Under dark phase contrast illumination they appear as bright structures. Fig. 2 is a light micrograph of this organism and shows examples of heterocysts and akinetes. These cells represented about 4% and 1%, respectively, of the cell population. The reddish structures were distributed between the peripheral and central regions of the vegetative cells. Most heterocysts contained one or two of these structures which were very striking in contrast to the pale green colouration of these cells.

The reddish structures were absent from the cells of Anabaena flos-aquae D125, Microcystis aeruginosa D127, and Nostoc linckia D130. However, these cells have pink central areas which do not appear black at low magnification. Cells from trichomes of Gloeotrichia echinulata D126 contained

large examples of these structures which often occupied the total volume of both heterocysts and vegetative cells (Fig. 3). Oscillatoria agardhii D132 cells have a reddish granular appearance.

The reddish structures present in the cells of Anabaena flos-aquae D124 and Gloeotrichia echinulata D126 and the reddish appearance of Oscillatoria agardhii D132 were destroyed by centrifugation at a pressure of 3.72 atmospheres. The same result was achieved by applying pressure to a cover slip mounted above a preparation of cells on a slide. No changes were observed in the appearance of cells of Anabaena flos-aquae D125, Microcystis aeruginosa D127 and Nostoc linckia D130 after pressure treatment.

3. Examination of direct preparations with the electron microscope

Cells of the 6 species were fixed with OsO_4 vapour on grids and examined with the optical microscope. No changes in structure were observed after this treatment. Cells were then examined with the electron microscope. Fig. 4 shows an example of a cell of Anabaena flos-aquae D124. Bundles of electron transparent cylinders were observed in positions which appeared to correlate with the position of the non-refractile reddish structures observed with the optical microscope. These cylinders were also observed in Gloeotrichia echinulata D126 cells, but were absent from those

of Anabaena flos-aquae D125, Microcystis aeruginosa D127 and Nostoc linckia D130. These cylinders were not easily resolved in Oscillatoria agardhii D132 trichomes which had a highly vacuolated appearance and tended to disrupt when the electron beam was focussed on each trichome. No cylinders were observed in cells which had been pressure treated.

4. Examination of fixed and embedded material with the electron microscope

Fig. 5 shows a section of a vegetative cell of Anabaena flos-aquae D124 fixed with OsO₄. A distinctive feature of the cell was presence of membranes consisting of a single electron dense which is about 2.0-3.0 nm wide. These appeared circular in favourable sections and appeared to correspond with the electron transparent cylinders (Fig. 4). These membranes contrasted with the lamellae membranes which were about 7.5 nm wide and consisted of two electron dense lines (about 2.0 nm wide) with a space of about 3.5 nm between them. After KMnO₄ fixation (Fig. 6) these membranes were wider (about 3.0 nm wide), but the cylindrical organisation was not obvious after treatment with this fixative. The width of the cylinders after OsO₄ treatment was 70.0 nm. Similar structures were present in sectioned cells of Gloeotrichia echinulata D126 and Oscillatoria agardhii D132 (see Figs. 4 and 5, respectively, of SMITH and PEAT, 1967a). Present in association with cylinder membranes were α -granules.

They are best preserved by KMnO_4 fixation (Fig. 6). In Anabaena flos-aquae D124 and Gloeotrichia echinulata D126 the bundles of cylinders are surrounded by the lamellae. Examples of this association are best shown in Figs. 2, 3, 4 and 6 of SMITH and PEAT (1967a). Cells of Anabaena flos-aquae D124 which were fixed and embedded after pressure treatment did not contain any cylindrical structures. However, Fig. 8 shows the presence of membranes with interlamellar distribution which appear to be collapsed cylinder membrane fragments.

No cylinders were observed in sectioned cells of Anabaena flos-aquae D125 (Fig. 7) and were also absent from cells of Microcystis aeruginosa D127 and Nostoc linckia D130 (see Figs. 9 and 10, respectively, of SMITH and PEAT, 1967a).

5. The characterisation of blue-green algal gas-vacuoles and the development of standard preparative procedures for fine structural studies (Discussion)

From the results described above it was concluded that gas-vacuoles were present in the strains of Anabaena flos-aquae D124, Gloeotrichia echinulata D126 and Oscillatoria agardhii D132 examined and absent from Anabaena flos-aquae D125, Microcystis aeruginosa D127, and Nostoc linckia D130, grown under the conditions employed in the present study. Gas-vacuoles may be identified with the optical microscope as reddish, non-refractile structures which are destroyed by applying

pressure treatment. With the electron microscope the gas-vacuole is resolved into groups of closely packed cylinders (termed 'gas-cylinders') with α -granules present in the inter gas-cylinder spaces. These groupings are bounded by lamellae.

The normal preparative procedures employed during fixation and embedment of cells for electron microscope examination involve the use of centrifugation to concentrate cells. This procedure cannot be applied to the pressure sensitive gas-vacuoles of blue-green algae and Millipore filters must be used routinely to collect cells. The complementary results with OsO_4 and KMnO_4 fixation described above reflect the need to use both fixatives on identical samples as a standard procedure. Although OsO_4 fixation provides better preservation of gas-cylinders it is inadequate to fix α -granules, which lack contrast, and are not easily distinguished from ribosomes and phycobilin molecules when the latter are aggregated adjacent to lamellae (LANG, 1968).

Although bacteria were present in the gas-vacuolate cultures they did not effect the good replication of growth results which was obtained. However, as the culture of Gloeotrichia echinulata D126 was also contaminated with a small protozoan it was decided to employ Anabaena flos-aquae D124 as a standard organism for studying gas-vacuoles.

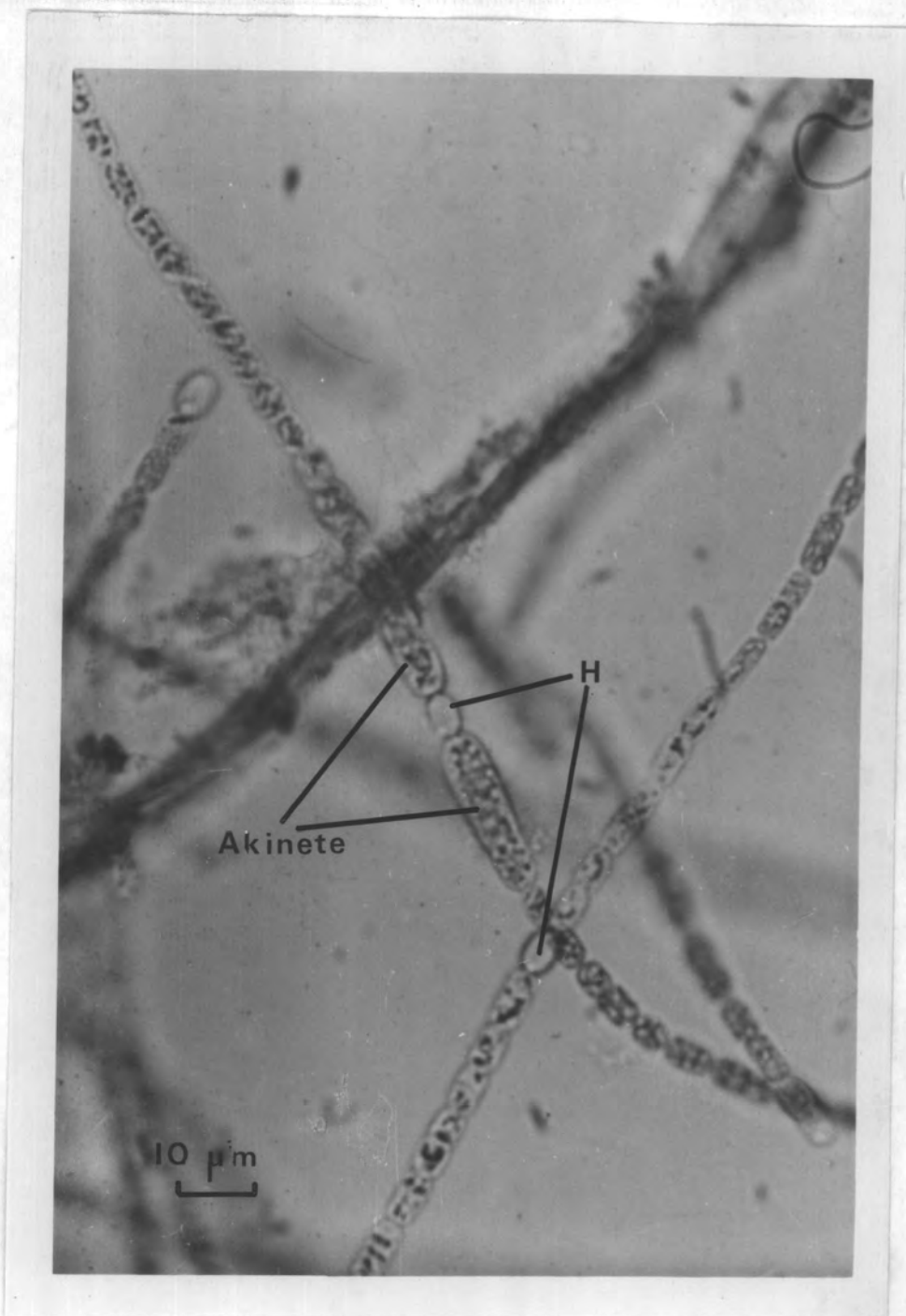


Fig. 2. A light micrograph of day +8 Anabaena flos-aquae D124 showing the presence of gas-vacuoles distributed between the inner and peripheral regions of cells and examples of differentiation into akinetes and heterocysts. Mag. 1,000X.

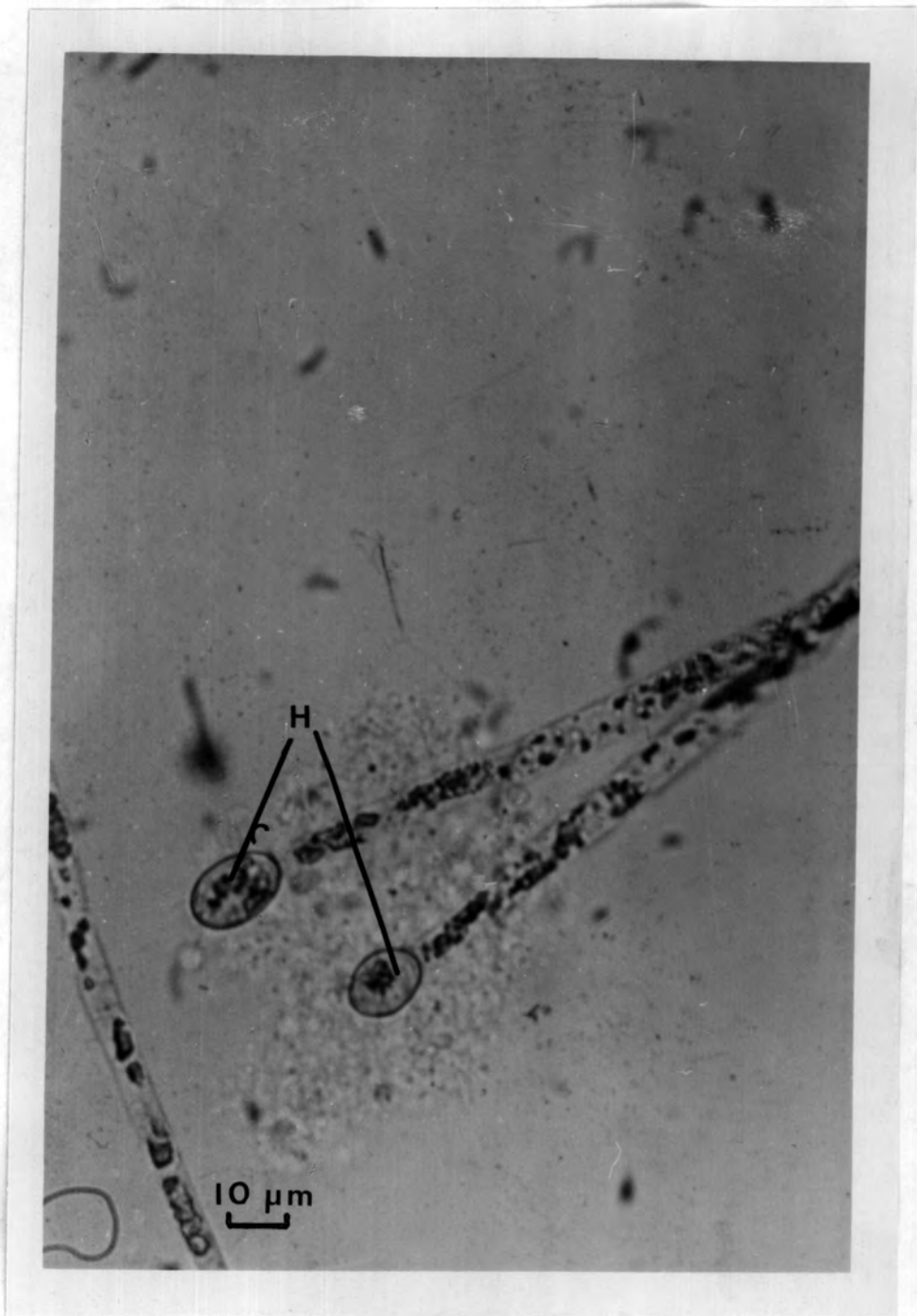


Fig. 3. A light micrograph of day +8 *Gloeotrichia echinulata* D126 showing the presence of large gas-vacuoles and gas-vacuolate heterocysts. Mag. 800X.



Fig. 4. A direct preparation of a day +8 Anabaena flos-aquae D124 cell showing the distribution of gas-cylinders. Also present are spherical structures (arrows) which may represent an early stage in gas-cylinder development. Mag. 30,000X.

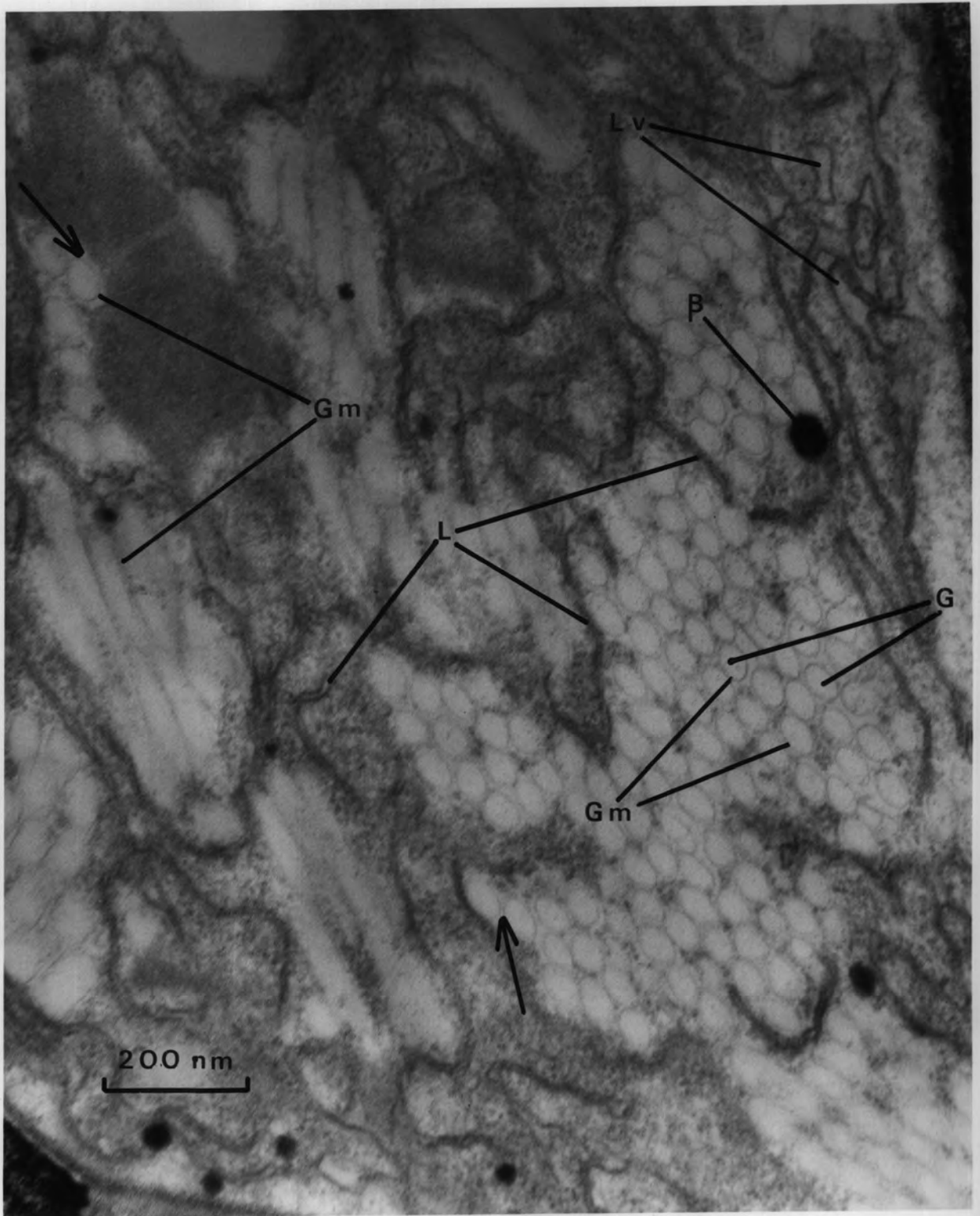


Fig. 5. A day +8 *Anabaena flos-aquae* D124 cell showing the presence of gas-cylinders bounded by distinct membranes. Note the presence of electron-transparent spaces (arrows) between gas-cylinder membranes. OsO₄ fixation. Mag. 120,000X.



Fig. 6. A day +8 *Anabaena flos-aquae* D124 cell showing the presence of α -granules associated with gas-cylinder membranes. KMnO_4 fixation. Mag. 48,000X.

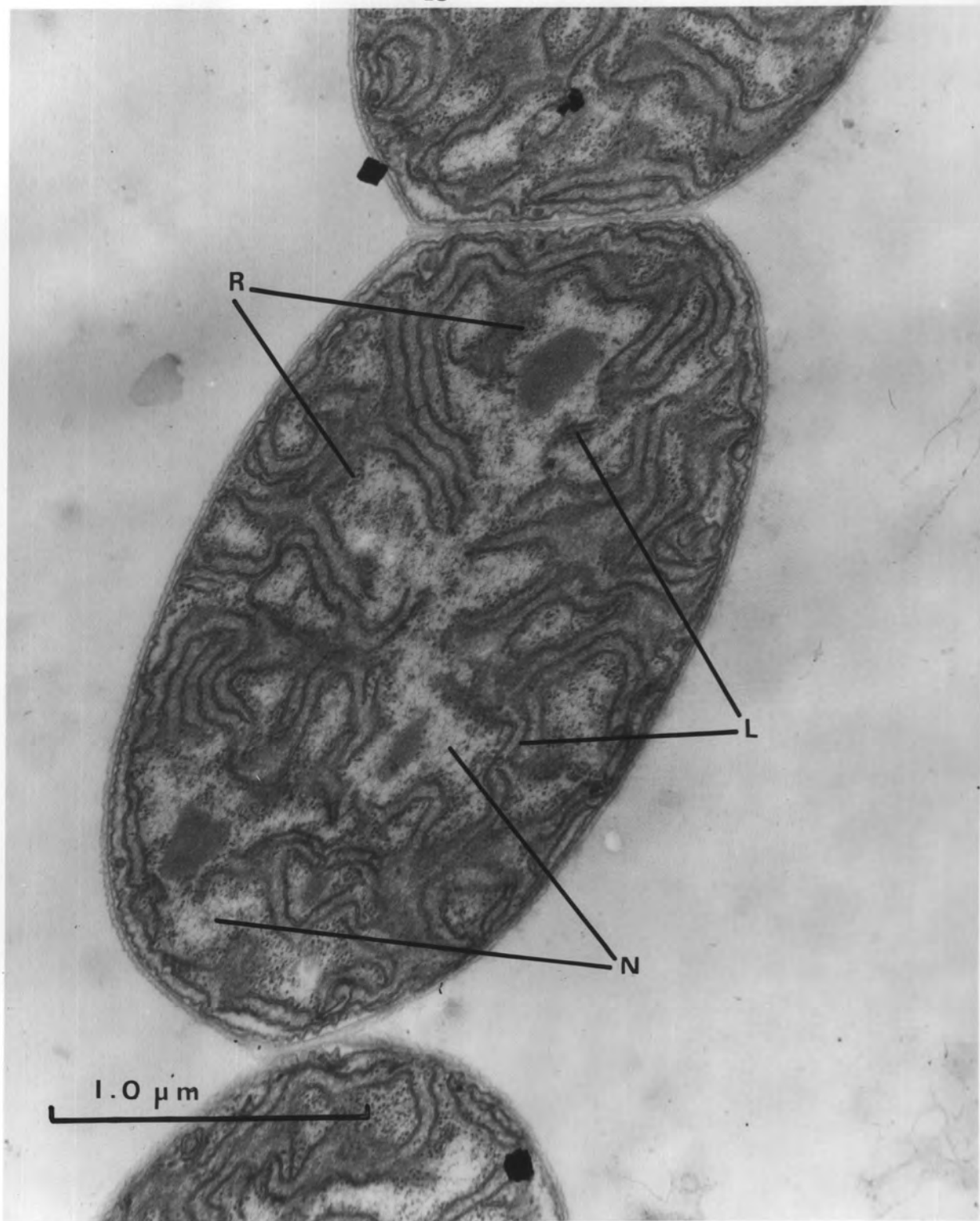


Fig. 7. A day +8 *Anabaena flos-aquae* D125 cell showing the absence of gas-cylinder membranes. OsO₄ fixation. Mag. 52,000X.

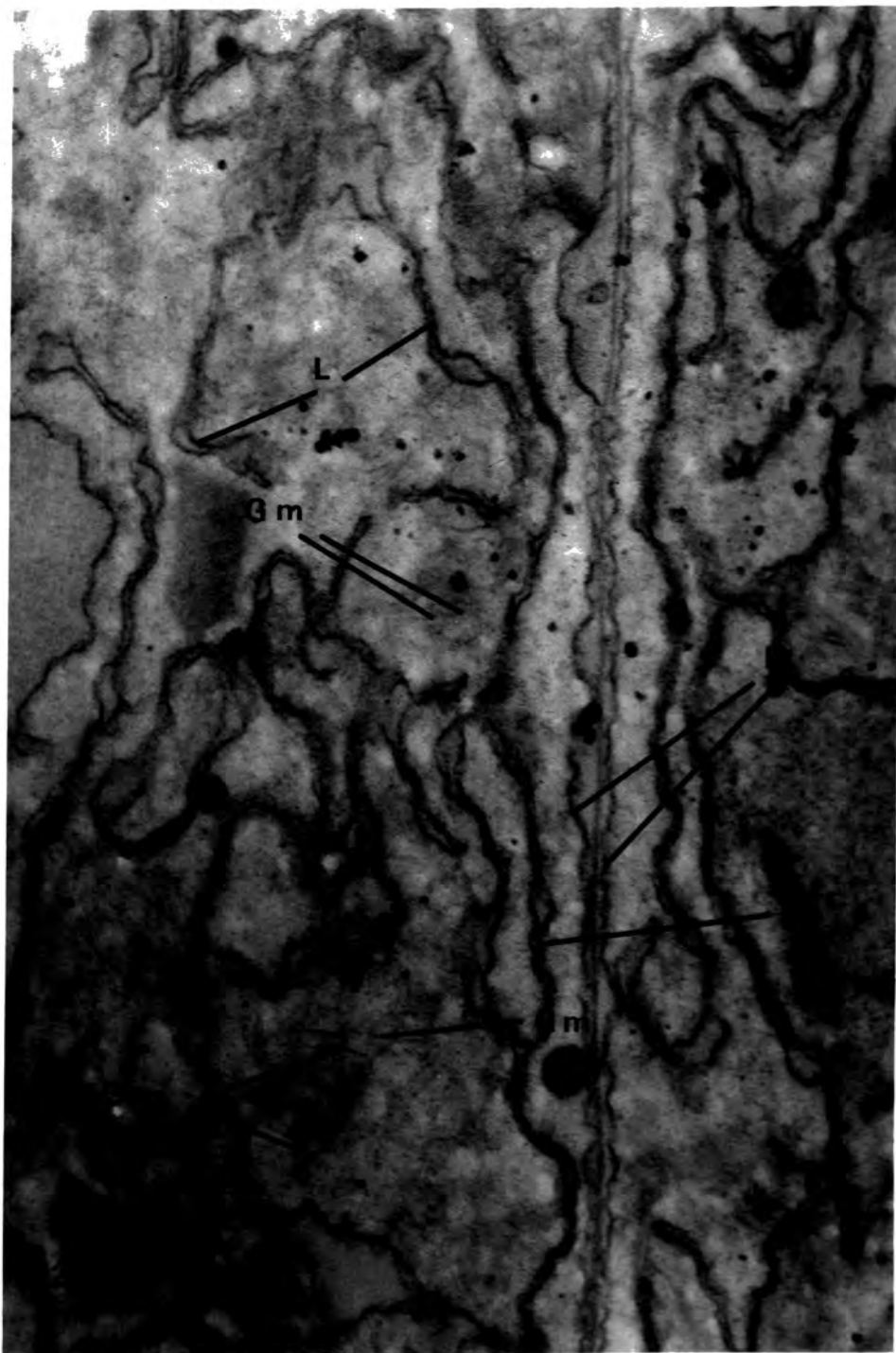


Fig. 8. A day +8 *Anabaena flos-aquae* D124 cell which has been pressure-treated prior to fixation. Although gas-cylinders are absent, gas-cylinder membrane fragments are present in the inter-lamellar areas. OsO₄ fixation. Mag. 68,000X.

PART II. The effects of environmental conditions on growth and gas-vacuole development in *Anabaena flos-aquae* D124.

The reported observations relating gas-vacuoles to stages of growth are meagre and often contradictory. According to the work of CANABAEUS (1929) cells which possess gas-vacuoles which have been induced artificially, show no further growth. These observations led FRITSCH (1945) to speculate whether gas-vacuoles were symptomatic of cells being in a senescent state. In the present study an attempt was made to relate gas-vacuolation to stages during growth of *Anabaena flos-aquae* D124 grown under the standard growth conditions ('Materials and Methods', Section 3G). Samples were taken for electron microscope examination on days +2, 4, 8, 12 and 24, and attention was focussed on the relation of gas-cylinders to lamellae and α -granules. Day +50 material was also examined. The effects of light intensity, temperature, inoculum size and shaking rate on growth rate and gas-vacuole development were also examined.

6. Growth characteristics of *Anabaena flos-aquae* D124

There was no detectable lag phase following inoculation into ASM-1 medium of 38 day old *A. flos-aquae* D124 to give a concentration of 12.0 mg/l. Exponential growth was slow (doubling time of 56.5 hrs) and lasted 12 days (Fig. 9). The growth rate declined from day +12 to day +20 and at day +24 growth was stationary. The final yield obtained was

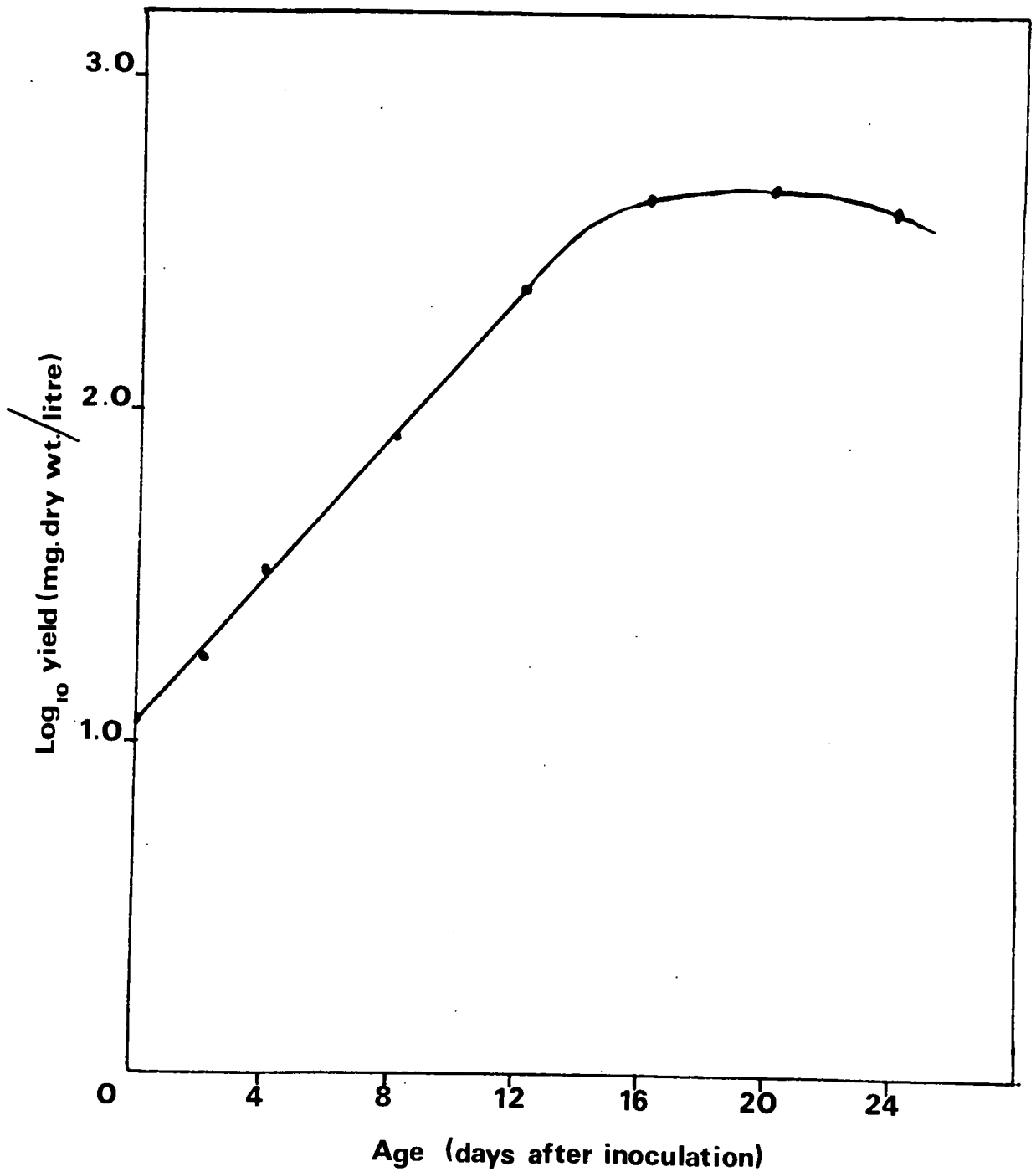


Fig. 9. The growth characteristics of Anabaena flos-aquae D124 grown under the Standard growth conditions ('Materials and Methods', Section 3G).

416.0 mg/l. After day +28 the culture showed a gradual loss in dry weight. Good replication of these results were obtained following repeat experiments. Exponential growth is accompanied by a pH change in the medium from an initial pH of 7.1 to a pH of 9.4 on day +16 (Fig. 10). The decline in growth rate is accompanied by a drop in pH to a value of 8.0 on day +24.

7. Structural changes associated with growth of Anabaena flos-aquae D124

A. Optical microscope observations

A summary of the optical microscope observations of gas-vacuoles in cells from different growth stages is shown in Fig. 1 of SMITH and PEAT (1967b). The inoculum material contained large, elongated gas-vacuoles and pressure-resistant, refractile vacuoles which had a pinkish colouration. During early exponential growth these latter vacuoles disappeared and gas-vacuoles were reduced in numbers to one or two large cylindrical gas-vacuoles adjacent to cell walls. By day +8 small, granular gas-vacuoles made their appearance and had a distribution between the peripheral and central regions of cells (Fig. 2). During late exponential growth these gradually increased in size. The declining phase in growth was characterised by an increase in the numbers of gas-vacuoles per cell, and by day +24 these had a distribution throughout the volume of each cell. The pinkish, pressure-resistant vacuoles redeveloped in the latter cells.

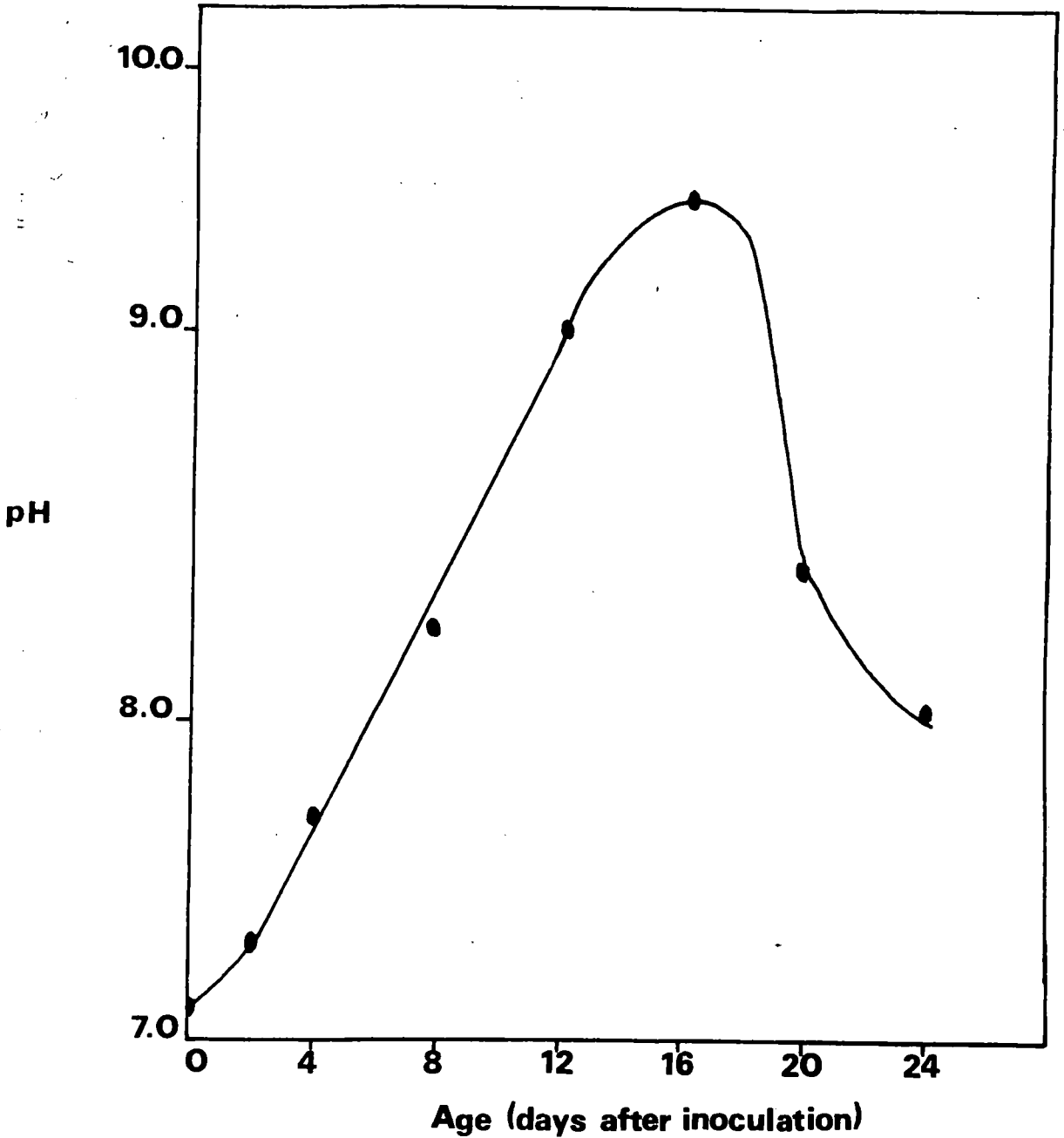


Fig. 10. The pH changes resulting from the growth of *Anabaena flos-aquae* D124 in ASM-1 medium under the Standard growth conditions ('Materials and Methods', Section 3G).

B. Electron microscope observations

At all stages of growth gas-vacuoles, as observed with the optical microscope, showed an excellent correlation with groups of gas-cylinders observed with the electron microscope. The gas-cylinders were in association with lamellae and α -granules. Changes in the arrangement of the lamellae were reflected in the appearance of the groups of gas-cylinders. In day +4 cells lamellae tended to lie parallel to the cell wall in association with cylindrical groupings of gas-cylinders (see Figs. 3 and 4, SMITH and PEAT, 1967b). By day +8, and also in days +12 and +24, lamellae were distributed throughout the cells and small groups of gas-cylinders clearly bounded by lamellae were present (see Figs. 5 and 6, SMITH and PEAT, 1967b). α -granules were observed at all stages of growth in rows between gas-cylinders (Figs. 6 and 13). They were present in low numbers in day +2 cells and increased in numbers during exponential growth. In days +12 and +24 (Fig. 13) the α -granules were present in large numbers, and they were often found in pairs at right angles to the lamellae. A direct estimate of the percentage volume of vegetative cells occupied by gas-cylinders and hence, gas-vacuoles was made by examining a series of micrographs of sections from different cells. The mean percentage volume of cells occupied by gas-vacuoles remained constant at about 20% during exponential growth and rose to 34% by day +24.

Small intralamellar vesicles (PEAT and WHITTON, 1967)

were present in cells at all stages of growth. Their contents were electron transparent after OsO_4 fixation (see Figs. 2, 3, 5 and 8 of SMITH and PEAT, 1967b). Large intralamellar vesicles were present in day +24 cells and occasionally in some day +2 cells. As large intralamellar vesicles were present in day +50 cells, these were presumed to be a feature of old cells. Their presence in sectioned cells correlated with the occurrence of the pressure-resistant refractile cells observed with the light microscope.

8. The effects of light and temperature

RODHE (1948) reported that the response of an alga to light intensity is dependent on the ambient temperature. The shaking culture tanks employed in the present study provided an excellent technical basis for the study of the relationship between light and temperature on the growth rate of A. flos-aquae D124. Because of the impossibility of employing identical inoculum material in all growth studies, comparison of growth rates at a particular temperature were always made with a control grown under the standard conditions described in 'Materials and Methods', Section 3G. In all experiments the growth rate of the control fell within the range $K = 0.128-0.132$. In one case, growth of the control was very poor and there was heavy bacterial contamination. The experiment was repeated successfully by employing different inoculum material.

A. Growth Rates

The growth rates of *A. flos-aquae* D124 at light intensities from 500-10,000 lx and from 5 °-25 °C temperature are summarised in the form of a three-dimensional model, Fig. 11. No growth occurred at 5 °C, and growth only occurred at low light intensities at 10 °C. Optimum growth occurred at 2,000 lx light intensity, 20 °C temperature, whilst some inhibition of growth occurred at higher light intensities at this temperature. Some additional experiments were made at higher light intensities and temperatures. No growth occurred at light intensities of 15,000 lx and, although some growth was shown at 27.5 °C, it was completely inhibited at a temperature of 30 °C. In general, growth of *A. flos-aquae* D124 was inhibited at comparatively low temperatures and light intensities. Light inhibition was accentuated by lowering the temperature.

B. Optical microscope observations on gas-vacuoles

At 20 °C and 25 °C, and light intensities above 2,000 lx, there was a marked reduction in gas-vacuolation following inoculation. About 40-50% of cells were non-gas-vacuolate at this stage of growth. The gas-vacuolate cells possessed small gas-vacuoles usually distributed at the cross-walls of cells (Fig. 12). At the end of the exponential phase of growth, large, cylindrical gas-vacuoles made their appearance adjacent to cell walls. Similar gas-vacuole development was

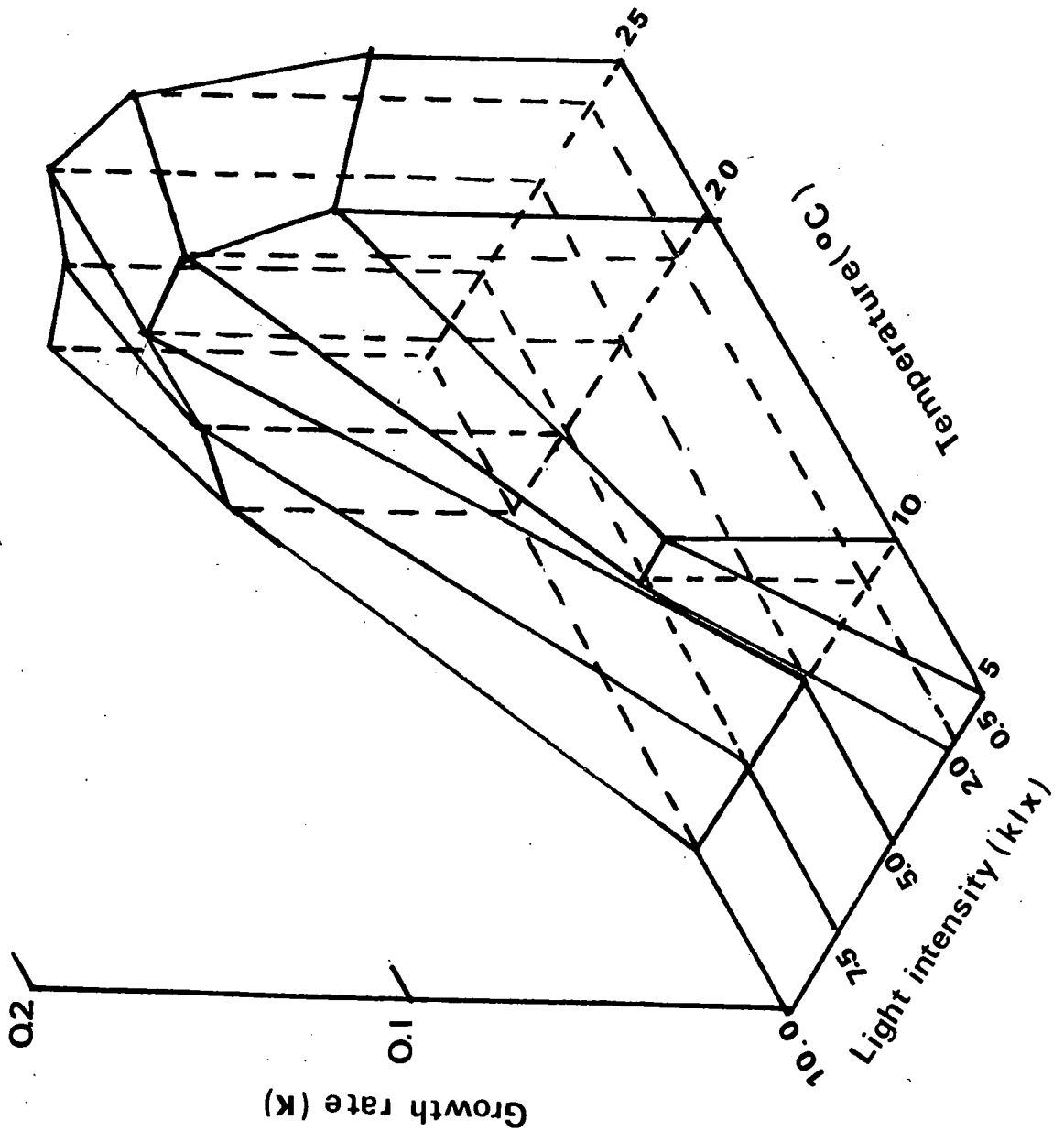
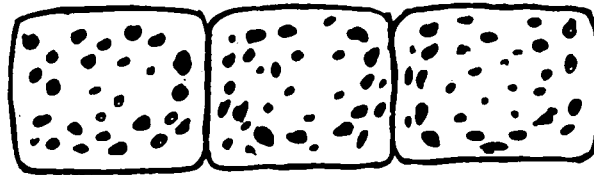
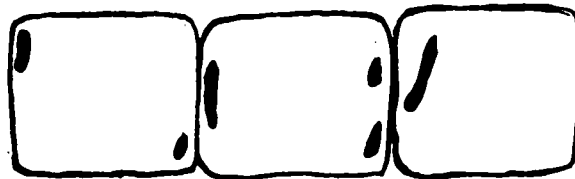


Fig. 11. The effects of light and temperature on the growth rate of Anabaena flos-aquae D124 expressed in the form of a three-dimensional model.

Early exponential growth.

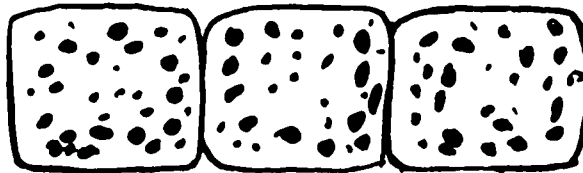


LL

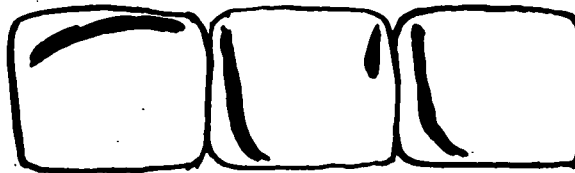


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Late exponential growth.

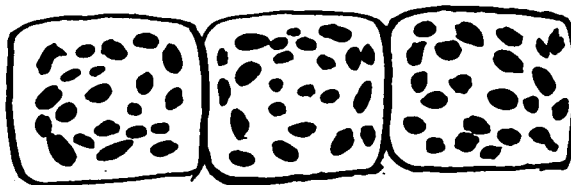


LL

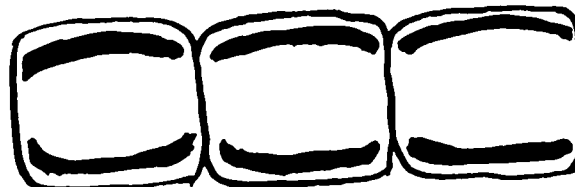


HL

Stationary phase of growth.



LL



HL

Fig. 12.

Summary of optical microscope observations on gas-vacuole development in Anabaena flos-aquae D124 at low and high light intensities (LL and HL, respectively).

shown by cells grown at 10 °C, 2,000 lx. In contrast, cells grown at 20 °C and 25 °C and light intensities below 2,000 lx showed pronounced gas-vacuolation at all stages of growth following inoculation. The gas-vacuoles were small and granular and appeared to be distributed throughout the volume of cells.

In the stationary phase of growth cells grown at all combinations of light and temperature are markedly gas-vacuolate. However, there were marked differences in the appearance and distribution of gas-vacuoles in cells grown in different light and temperature combinations. At 20 °C and 25 °C and light intensities above 2,000 lx gas-vacuolation took the form of ^acylinder beneath the cell wall which completely enclosed the central region of the cell. Cells grown at 10 °C, 2,000 lx had a similar appearance. Cells grown at 20 °C and 25 °C and lower light intensities possessed large, granular gas-vacuoles distributed throughout the cell volume.

9. The effects of change of shaking rate

The results of this experiment are shown in Table 5. As no significant differences in growth rate were observed between 64 and 90 oscillations/min this experiment was not extended further to include a wider range of speeds. No differences in gas-vacuolation were observed. As was to be expected, the higher shaking rate induced fragmentation of filaments to produce a high proportion of 4-celled units.

Table 5. Growth rate of Anabaena flos-aquae D124 in relation to shaking rate. Material was grown under the standard growth conditions ('Materials and Methods', Section 3G).

Shaking rate	Growth rate ('K')
Standing culture	0.110
64 oscillations/min.	0.132
90 oscillations/min.	0.134

10. The effects of inoculum size

The following concentrations of inoculum were employed: 1.2, 2.4, 12.0, 24.0, 48.0 mg/l. Concentrations of 12.0 mg/l and above showed no significant differences in growth rate. Attempts at measuring the growth rate of cultures with inocula of 1.2 and 2.4 mg/l proved unsatisfactory because no growth was observed in these cultures until a lag phase of 12 days had occurred. In both 1.2 and 2.4 mg/l cultures no growth was observed in some flasks 24 days after inoculation. No significant differences in gas-vacuolation were observed in cells from cultures with inocula of 12.0 mg/l and above. One interesting effect on gas-vacuolation was observed in day +2 cells from cultures of 1.2 and 2.4 mg/l. These cells were attached to the occasional cotton wool strands which were present in the culture medium and they showed no obvious

gas-vacuolation. However, day +6 cells possessed single, large gas-vacuoles which were normally distributed at cross-walls or adjacent to cell walls.

11. Effects of competition with *Anabaena flos-aquae* D125 on growth of *Anabaena flos-aquae* D124

Flasks were inoculated with equal quantities of *Anabaena flos-aquae* D124 and *A. flos-aquae* D125. There was some initial growth on *A. flos-aquae* D124, but after day +12 there was a great reduction in the number of cells present and by day +24 no cells could be detected.

In competition with *A. flos-aquae* D124, *A. flos-aquae* D125 attached itself to the glass surface at the bottom of the flask. This behaviour was not shown by *A. flos-aquae* D125 when it was grown in control flasks from which *A. flos-aquae* D124 was absent. After day +12, however, *A. flos-aquae* D125 began to resuspend itself and by day +24 a clumped suspension of pure *A. flos-aquae* D125 was obtained.

12. Gas-vacuole development in *Anabaena flos-aquae* D124 (Discussion)

The above studies show that gas-vacuoles are present in cells of *Anabaena flos-aquae* D124 grown under a variety of conditions. Two generalisations are apparent from these studies. Firstly, gas-vacuoles occupy a greater proportion of cell volume in the stationary phase of growth compared with exponential growth. This generalisation is applicable

to cells grown under all the light and temperature combinations investigated. Secondly, cells grown at light intensities which are high enough to cause some inhibition of growth rate possess gas-vacuolation which is normally restricted in its development to the periphery of the cell adjacent to the cell walls.

FRITSCH (1945) states that it is not clear whether material possessed of gas-vacuoles is actually in a healthy condition and whether cell division continues. The present studies show that gas-vacuoles are a normal feature of Anabaena flos-aquae D124 cells and they are not in any way characteristic of senescent cells. However, the refractile, pressure-resistant vacuoles (large intralamellar vesicles) observed in old Anabaena flos-aquae D124 cells are certainly a characteristic feature of senescence in this organism.

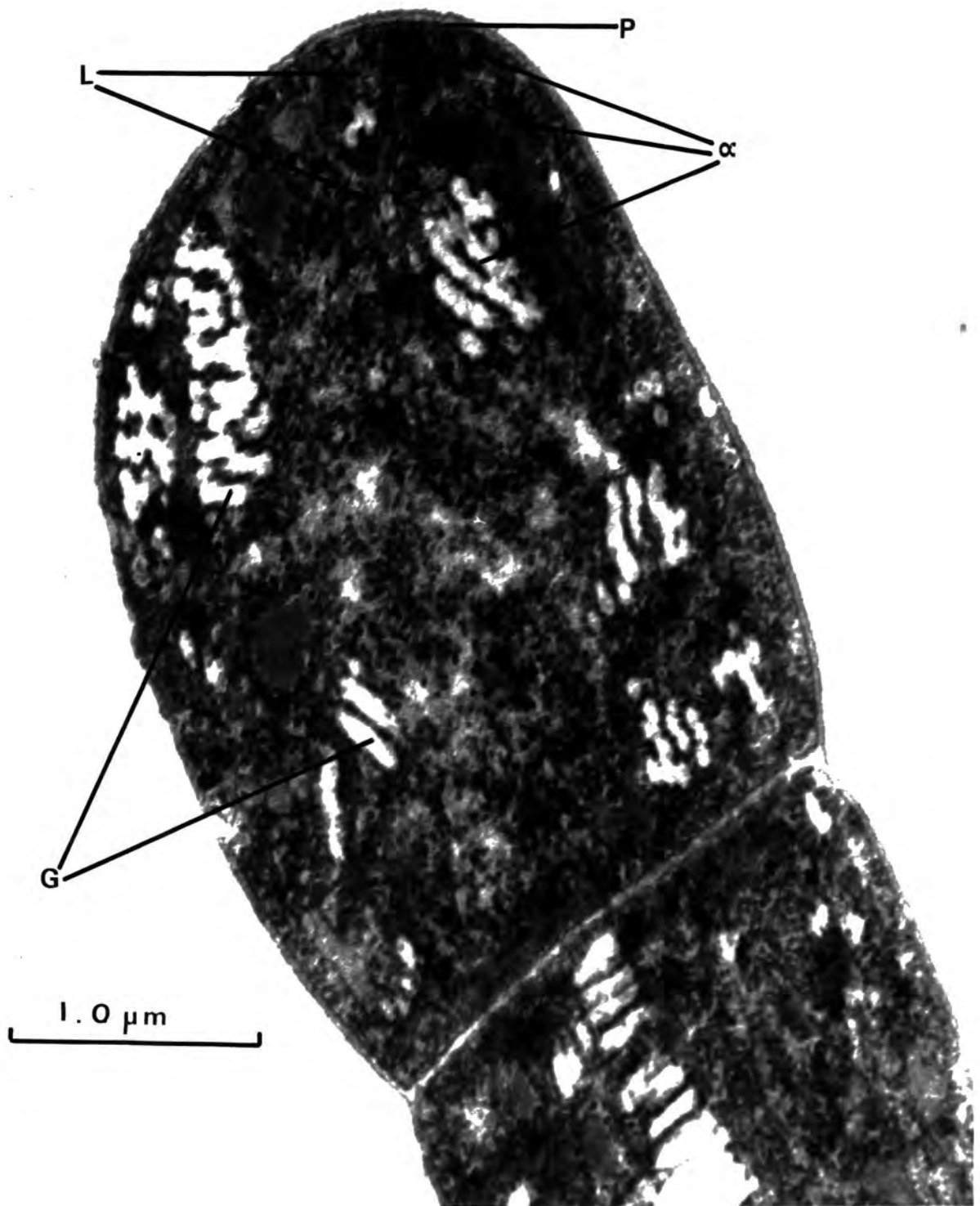


Fig. 13. A day +24 cell of *Anabaena flos-aquae* D124 showing the presence of large numbers of α -granules. Note the distribution of paired α -granules between the plasma membrane and the lamellae. KMnO_4 fixation. Mag. 40,000X.

PART III. Observations on organisms collected from the field.

The observations reported in Part I and II of the present thesis have been restricted to cultures maintained in the laboratory. The physical environment of these organisms does not correspond with that in which they grow naturally. It was, therefore, decided to collect material from natural situations in order to make comparative studies. Two sites were chosen: St. James's Park Lake, London, and the Shropshire Meres. These lakes had the advantage over other sites because at each site biological studies were in progress and the planktonic blue-green algae were regularly being sampled (REYNOLDS, 1969, and WHITTON, 1969). Electron microscope observations were restricted to the structure and distribution of gas-cylinders, lamellae and α -granules. A more detailed description of the fine structure of these organisms will be published in a future paper.

13. Organisms collected from St. James's Park Lake, London

A. Optical microscope observations and age of populations

Two gas-vacuolate blue-green algae were present:

Oscillatoria redekei and O. agardhii. The former organism was about 2.0 μ m wide and had a colourless appearance. In about 50% of the trichomes no gas-vacuoles were observed.

Where gas-vacuoles were observed they were small, rounded and

were invariably positioned adjacent to the cross-walls of trichomes. O. agardhii cells were highly gas-vacuolate, 4.5 μ m wide, and had the appearance of the O. agardhii D132 trichomes described in 'Results', Section 2, p.33. The proportion of O. redekei to O. agardhii trichomes was about 3:1.

The O. redekei population had been observed in the lake for 18 months prior to collection and its numbers had not fluctuated greatly in the 4 months prior to collection. The O. agardhii population had developed more recently and was first observed only 2 months before material was collected.

B. Electron microscope observations

Oscillatoria redekei

Fig. 14 shows a median section through the gas-vacuoles associated with the cross-walls in O. redekei. Gas-cylinders were closely packed together and they appeared to be associated with proliferations of the plasma membrane. These proliferations bore some similarities to the lamellasomes described by ECHLIN (1964) in Anacystis nidulans and the mesosomes of bacilli (FITZ-JAMES, 1960). The proliferations may also have a comparable role in cells to mesosomes and, similarly, represent sites of high biosynthetic activity, possibly associated with gas-cylinder membrane synthesis. Lamellae were orientated parallel to the cell wall and had a

peripheral distribution (Fig. 15). α -granules were present adjacent to lamellae and between gas-cylinders. In favourable sections bacteria could be observed inside the sheath of this organism. The Oscillatoria redekei trichomes examined were notable for the small percentage of their cell volume (less than 10%), which was occupied by gas-cylinders. However, the degree of gas-vacuolation in this organism shows considerable variation. The percentage of the cell volume occupied by gas-vacuoles may be greater than 10% when O. redekei is grown and harvested under different conditions (PEAT and WHITTON, in preparation).

Oscillatoria agardhii

Fig. 16 shows an O. agardhii trichome which has been sectioned obliquely. Its structure is similar to that described by SMITH and PEAT (1967a) in O. agardhii D132. It was notable for the high percentage of its cell volume occupied by gas-cylinders and the closely packed organisation of the α -granules. Depending on the orientation of section, α -granules showed considerable variation in appearance. However, favourable sections demonstrated that the large, electron-dense area with its striations about 20 nm wide (Fig. 18) was composed of α -granules, showing an unusual orientation. It seems probable that the report by UEDA (1966) of a 19 nm wide virus present in Oscillatoria

princeps was a misinterpretation of this arrangement of α -granules. Figs. 17 and 18 show good examples of gas-cylinders in transverse and longitudinal section, respectively. Many examples of the presence of electron-transparent spaces between gas-cylinders were observed in favourable sections of this organism.

14. Organisms collected from the Shropshire Meres

A. Optical microscope observations and age of populations

Microcystis aeruginosa formed small colonies composed of gas-vacuolate cells about $7.0\mu\text{m}$ wide. This organism represented about 10% of the blue-green algal population in Colemere and was first observed in the Mere only one month previous to collection. Aphanizomenon flos-aquae and Anabaena flos-aquae colonies were present in equal numbers in Whitemere at the time of collection. The widths of the cells of these species were $5.0\mu\text{m}$ and $4.5\mu\text{m}$, respectively. No data was available on the age of the Aphanizomenon flos-aquae population. Gas-vacuoles were observed in vegetative cells and absent from heterocysts in this species. Anabaena flos-aquae was first observed in Whitemere two months prior to collection. At the time of collection its doubling time was about 3.5 days. The gas-vacuoles of vegetative cells were distributed adjacent to cell walls. This distribution of gas-vacuoles was similar to that of A. flos-aquae D124 cells grown at light intensities

which cause some inhibition of growth (see 'Results', Section 8, p.50).

B. Electron microscope observations

Anabaena flos-aquae

Cells fixed with OsO_4 and KMnO_4 are shown in Figs. 19 and 20, respectively. Lamellae were distributed throughout cells and showed no tendency to lie parallel to the cell wall. Groups of gas-cylinders bounded by lamellae generally had a peripheral distribution. α -granules were present in association with lamellae and gas-cylinder membranes.

In general, the appearance of these cells was consistent with structure of day +12 cells of A. flos-aquae D124 described in 'Results', Section 7, p.49. However, larger groups of gas-cylinders, possessing a peripheral distribution, were present in the cells of A. flos-aquae collected from the field and α -granules were not as abundant in these cells compared with typical day +12 cells.

Microcystis aeruginosa

Micro-colonies of Microcystis aeruginosa were sectioned for electron microscope examination. Figs. 21 and 22 are typical examples of cells fixed in OsO_4 and KMnO_4 , respectively. Of interest was the presence of bacteria within these colonies (Fig. 22). In comparison with M. aeruginosa D127 cells described in 'Results', Section 4 (see Fig. 10 of SMITH and

PEAT, 1967a) the field material showed two differences. Firstly, gas-cylinders were present in these cells in closely-packed groupings and showed a peripheral distribution. Secondly, α -granules were almost totally absent from the field material. Fig. 23 shows a planktonic rhizopod which has ^{injected}~~imbibed~~ some cells of M. aeruginosa. These cells still contained intact gas-cylinders and lamellae systems. An example of a partly digested cell is shown in Fig. 24. Of interest was the absence of all recognisable cell structures except for the presence of lamellae showing a tendency to vesiculate, and gas-cylinder membranes. From these observations it is tempting to speculate whether these structures remain functional for any significant length of time after being ^{injected}~~imbibed~~ and whether significant changes occur in the buoyancy characteristics of the animal.

Aphanizomenon flos-aquae

A low magnification view of colony fixed by $KMnO_4$ fixation is shown in Fig. 25. A higher magnification view of material fixed with OsO_4 is shown in Fig. 26. BOWEN and JENSEN (1965) similarly examined field material of this organism. The present observations confirm the presence of gas-cylinders in this organism and also the presence of the large intralamellar vesicles which were reported by these workers. However, BOWEN and JENSEN (1965) stated that, "membranes of gas

vesicles are not preserved by fixation with KMnO_4 ". Fig. 25 shows the presence of gas-cylinder membranes after KMnO_4 fixation. Although typical gas-cylinders were not observed, abundant gas-cylinder membranes and α -granules were present in the interlamellar areas.

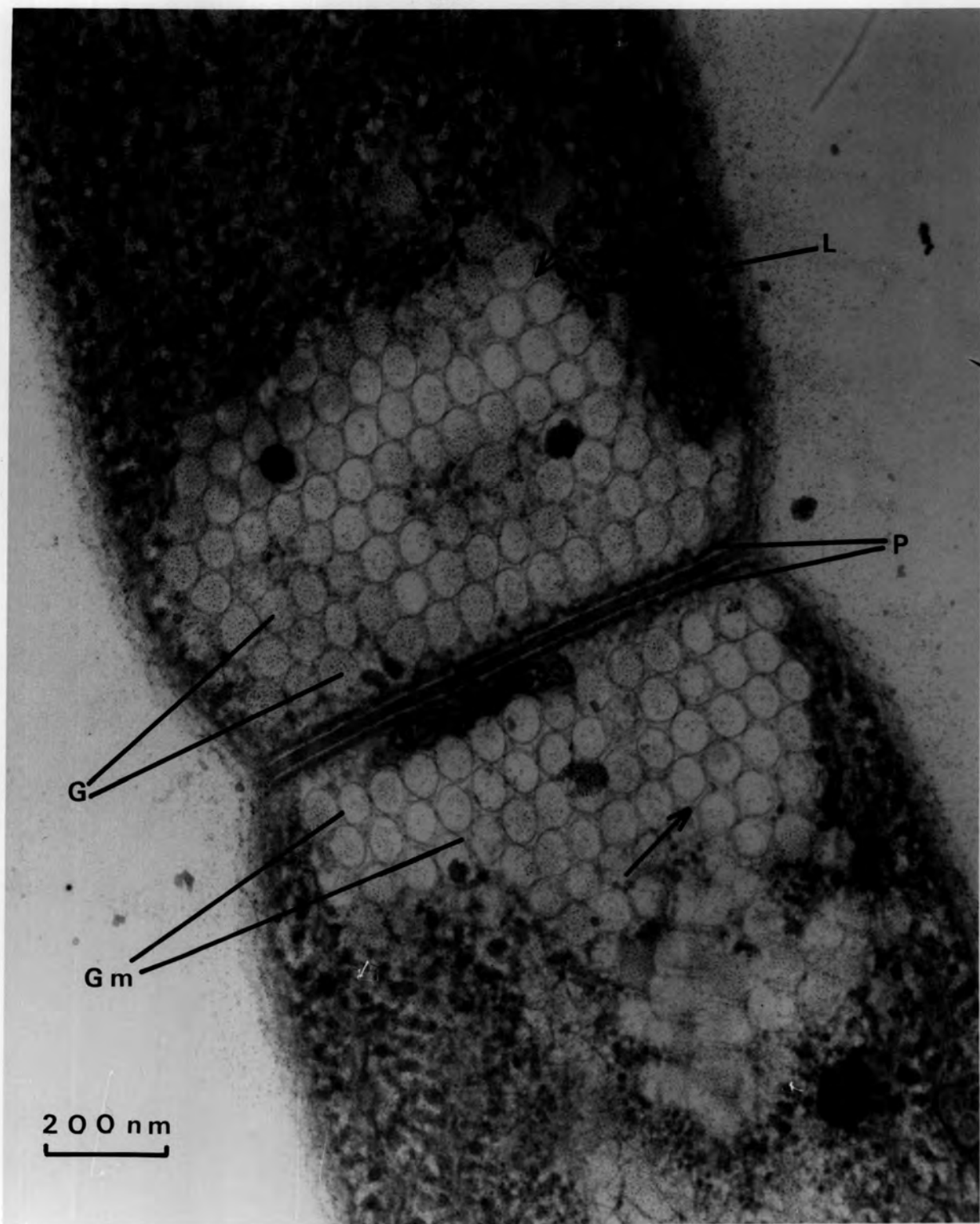


Fig. 14. Fine structure of the gas-vacuoles adjacent to the cross-walls of *Oscillatoria redekei*, collected from St. James's Park, London. Note the presence of electron-transparent spaces (arrows) between gas-cylinder membranes and the proliferation of the plasma membrane which may be associated with gas-cylinder membrane synthesis. OsO_4 fixation. Mag. 100,000X.

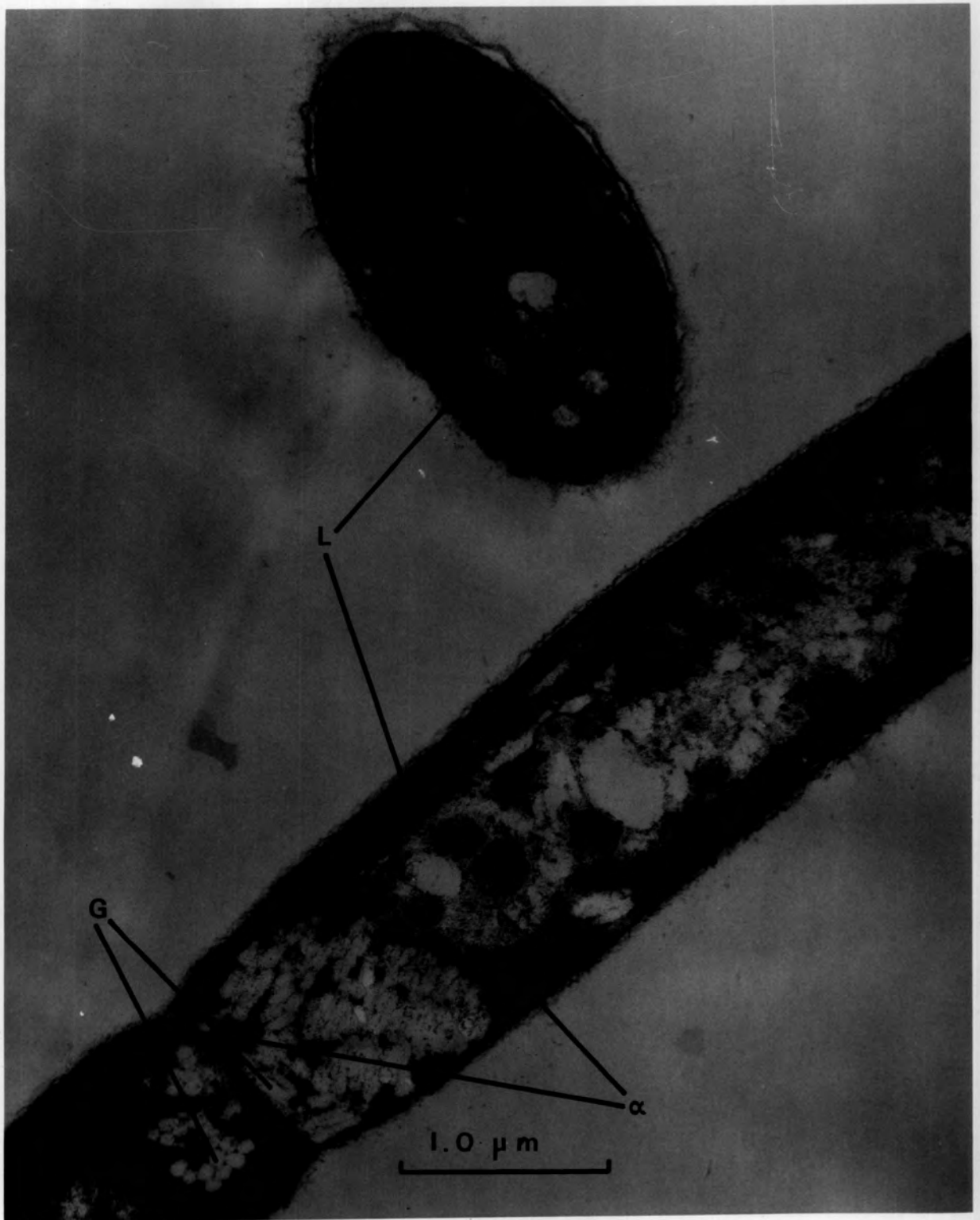


Fig. 15. General cell views of *Oscillatoria redekei* in transverse and longitudinal section. Note the distribution of lamellae which run parallel to the cell wall. KMnO_4 fixation. Mag. 35,000X.

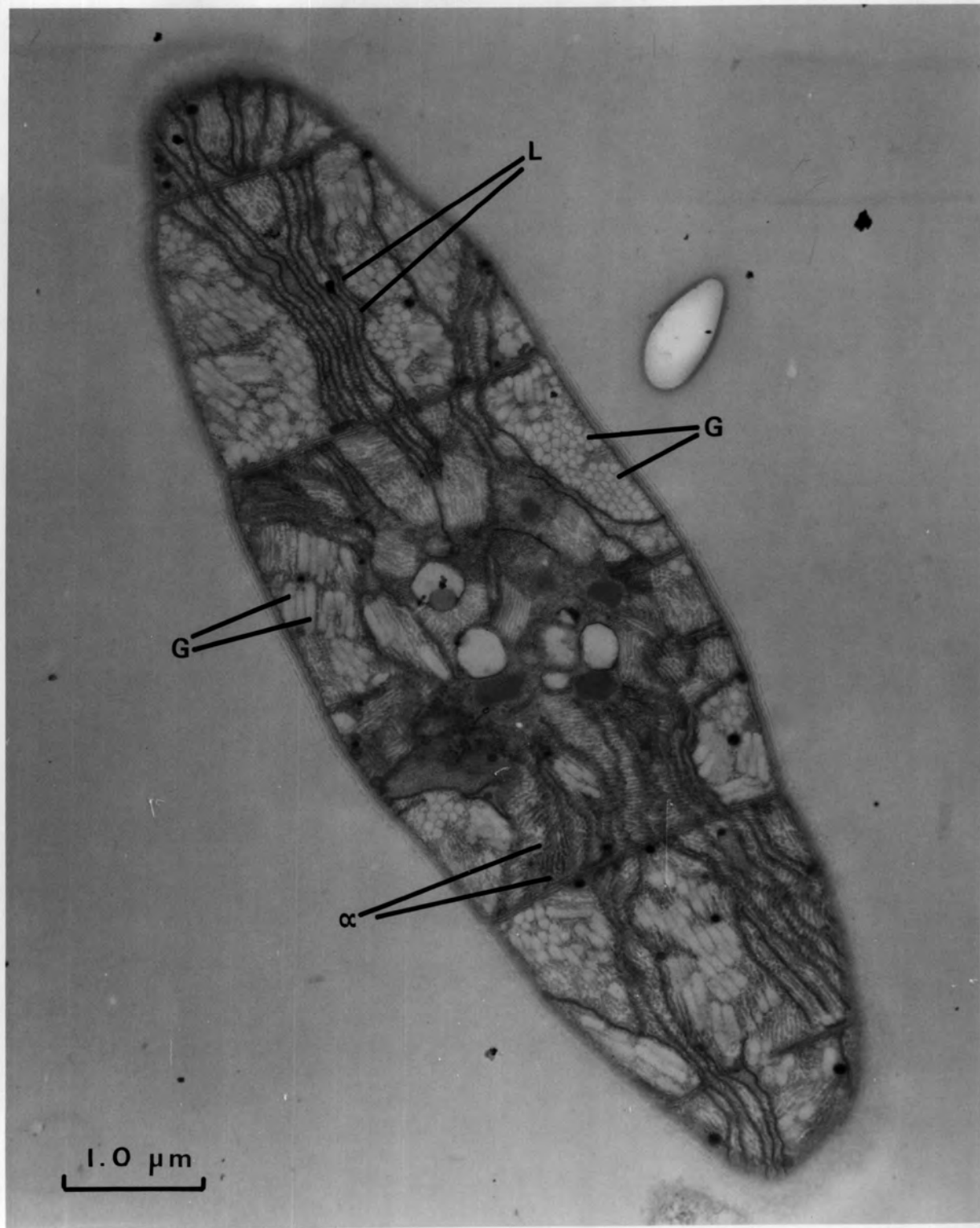


Fig. 16. An oblique section of an Oscillatoria agardhii trichome which was collected from St. James's Park, London. OsO₄ fixation. Mag. 22,000X.

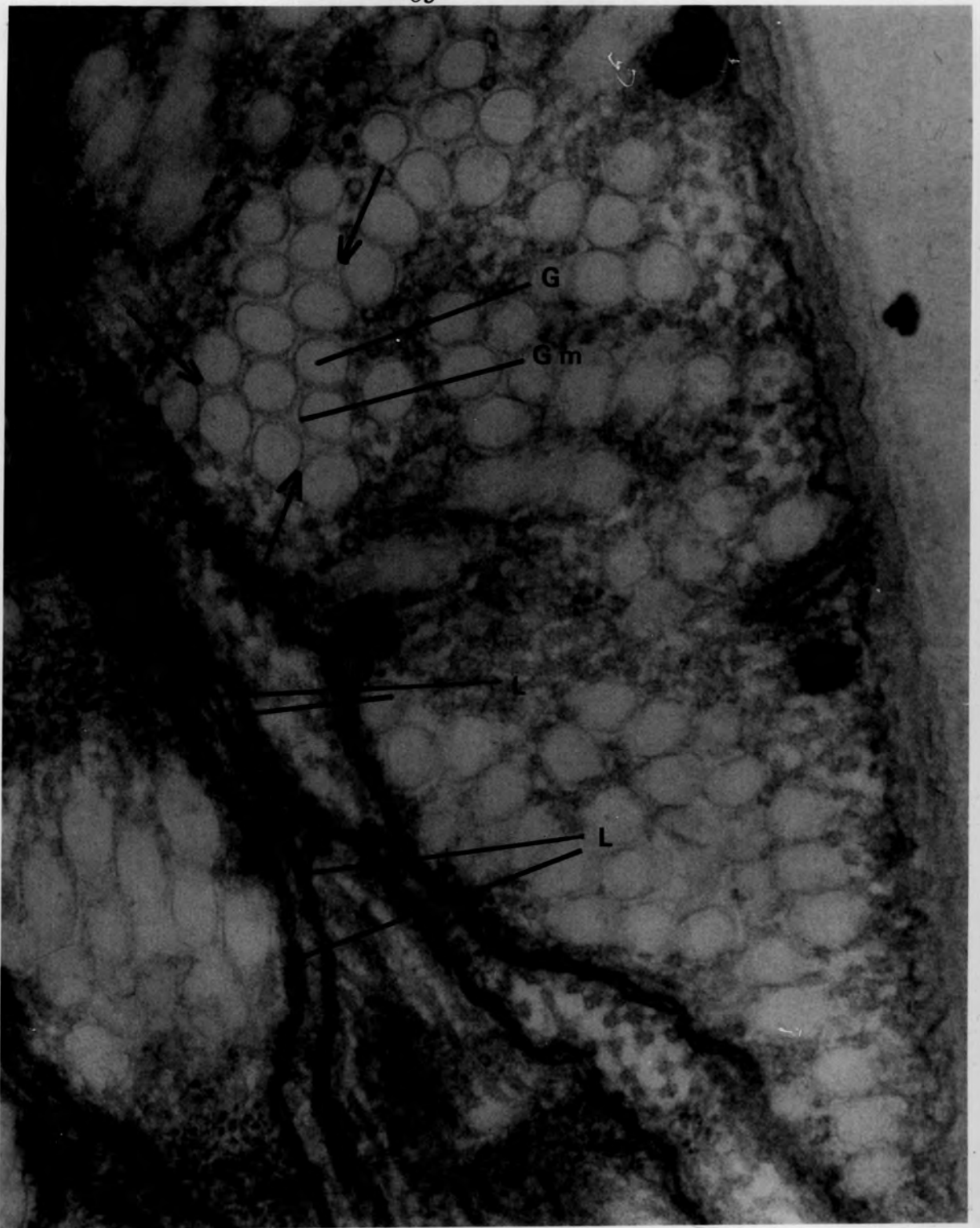


Fig. 17. An *Oscillatoria agardhii* cell showing gas-cylinders in transverse section with electron-transparent spaces (arrows) between gas-cylinders. Note the lamellae showing their typical unit membrane structure. OsO₄ fixation. Mag. 118,000X.

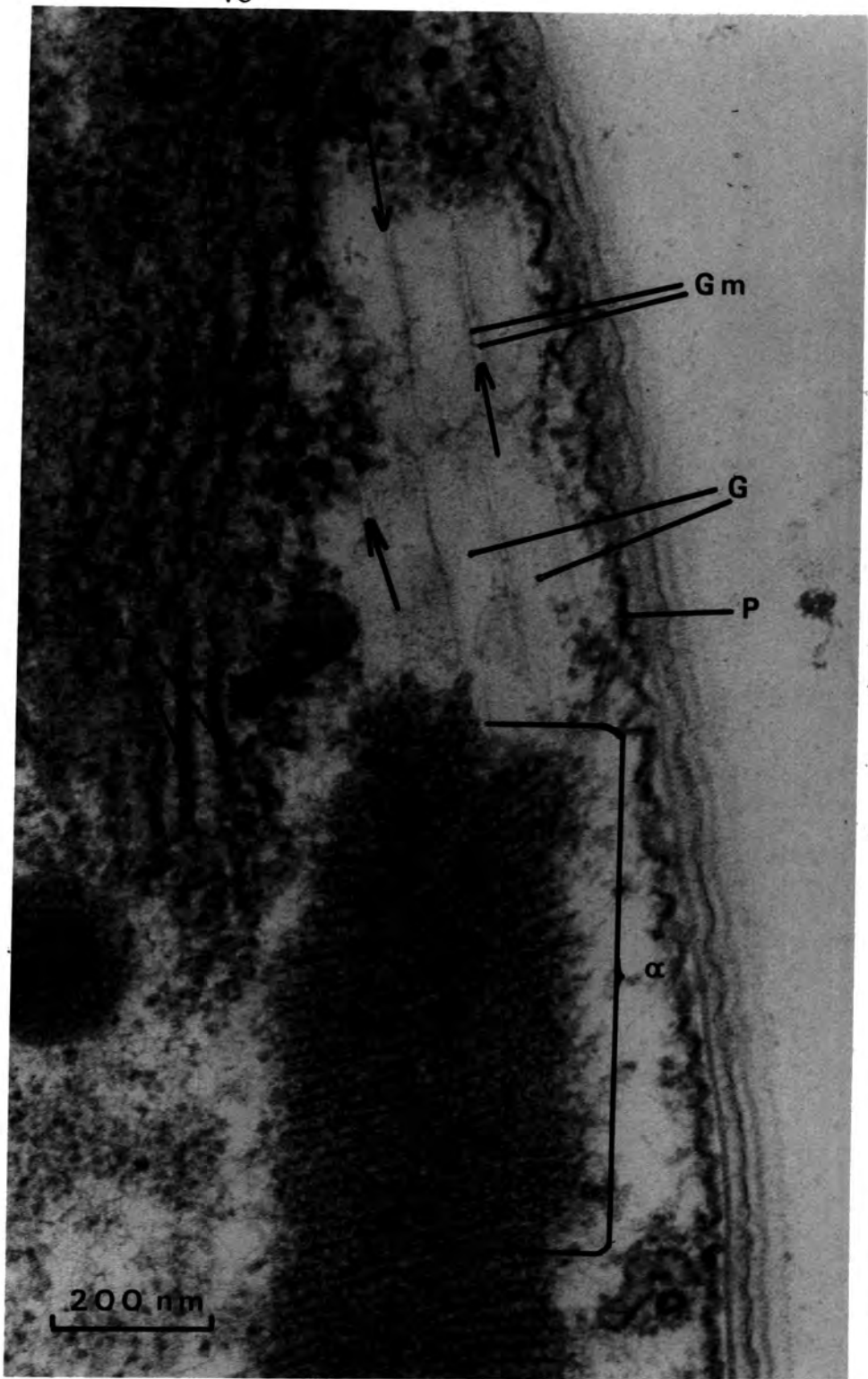


Fig. 18. An *Oscillatoria agardhii* cell showing gas-cylinders in longitudinal section with electron-transparent spaces (arrows) between gas-cylinders. Note the appearance of α -granules in this section. OsO_4 fixation. Mag. 118,000X.

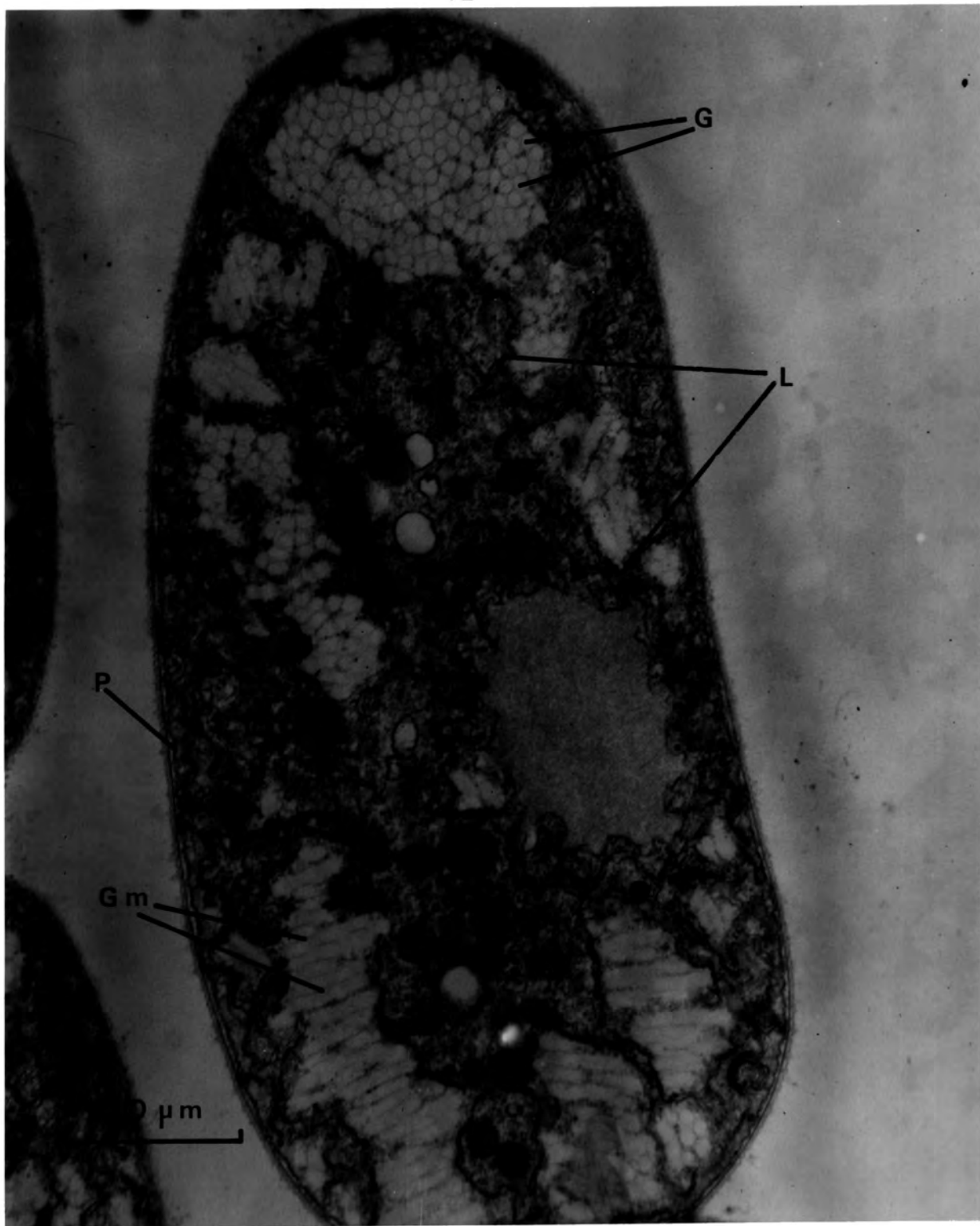


Fig. 19. An Anabaena flos-aquae cell which was collected from White Mere, Shropshire. OsO₄ fixation. Mag. 29,000X.



Fig. 20. A White Mere *Anabaena flos-aquae* cell showing a general similarity in fine structure to a typical *Anabaena flos-aquae* D124 harvested from the late exponential phase of growth (Fig. 6, page 42). KMnO_4 fixation. Mag. 48,000X.

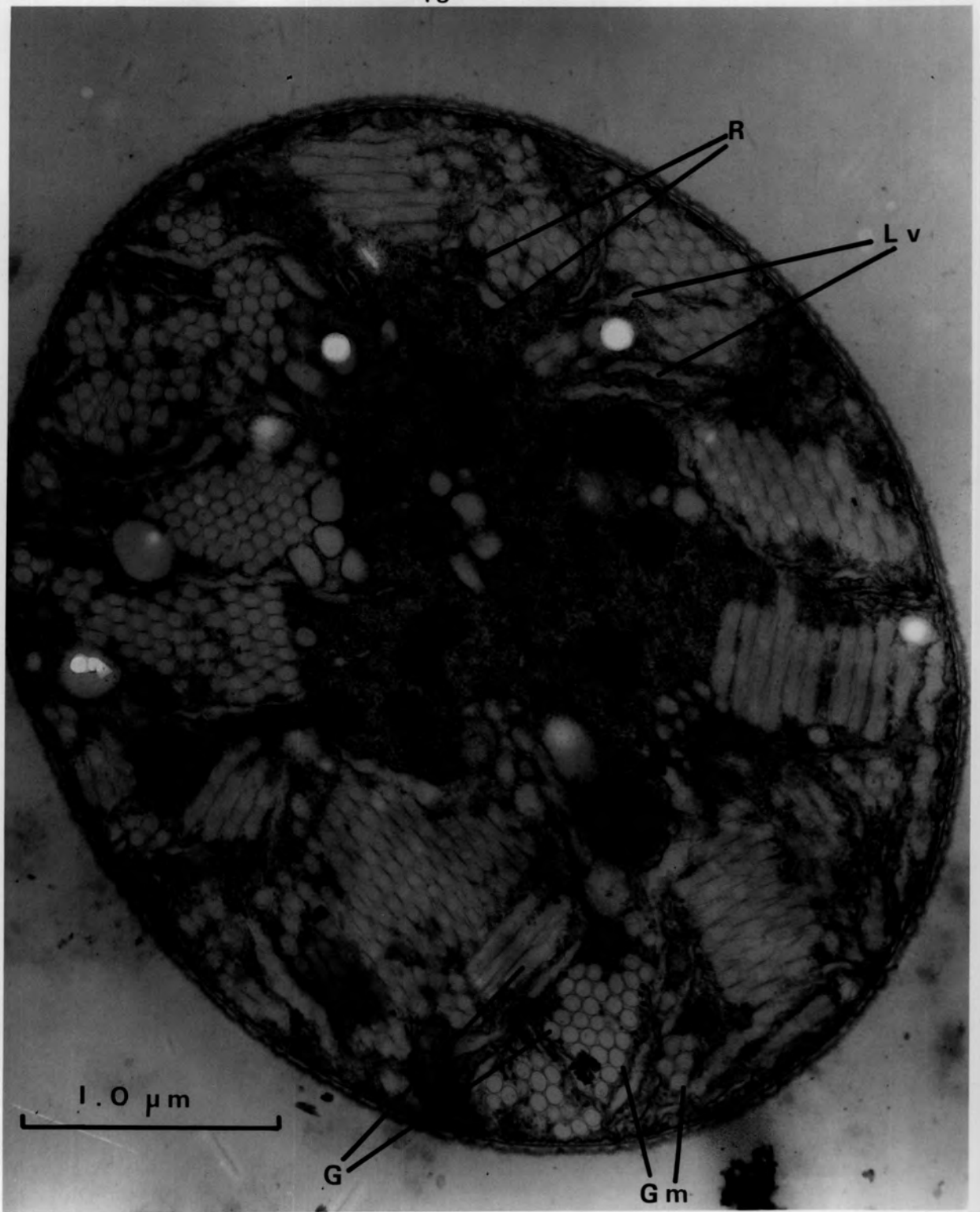


Fig. 21. A *Microcystis aeruginosa* cell collected from Cole Mere, Shropshire. OsO₄ fixation. Mag. 43,000X.

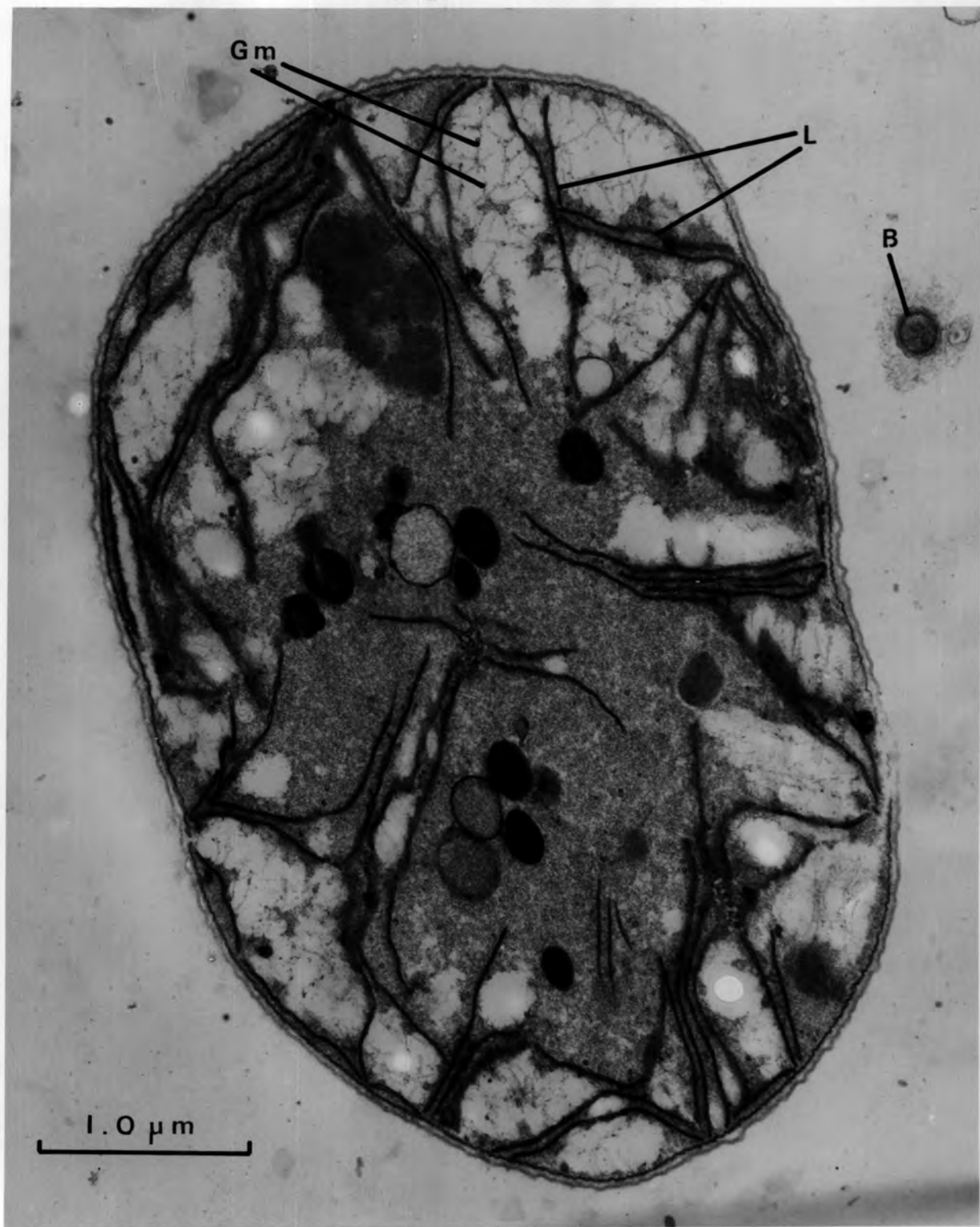


Fig. 22. A *Microcystis aeruginosa* cell collected from Cole Mere, Shropshire. Note that the cell is from a colony and that the presence of a bacterium in this section indicates that these organisms are present within colonies. KMnO_4 fixation. Mag. 35,000X.



Fig. 23. A planktonic rhizopod which has ^{injected} ~~imbibed~~ some gas-vacuolate Microcystis aeruginosa cells. KMnO₄ fixation. Mag. 15,000X.

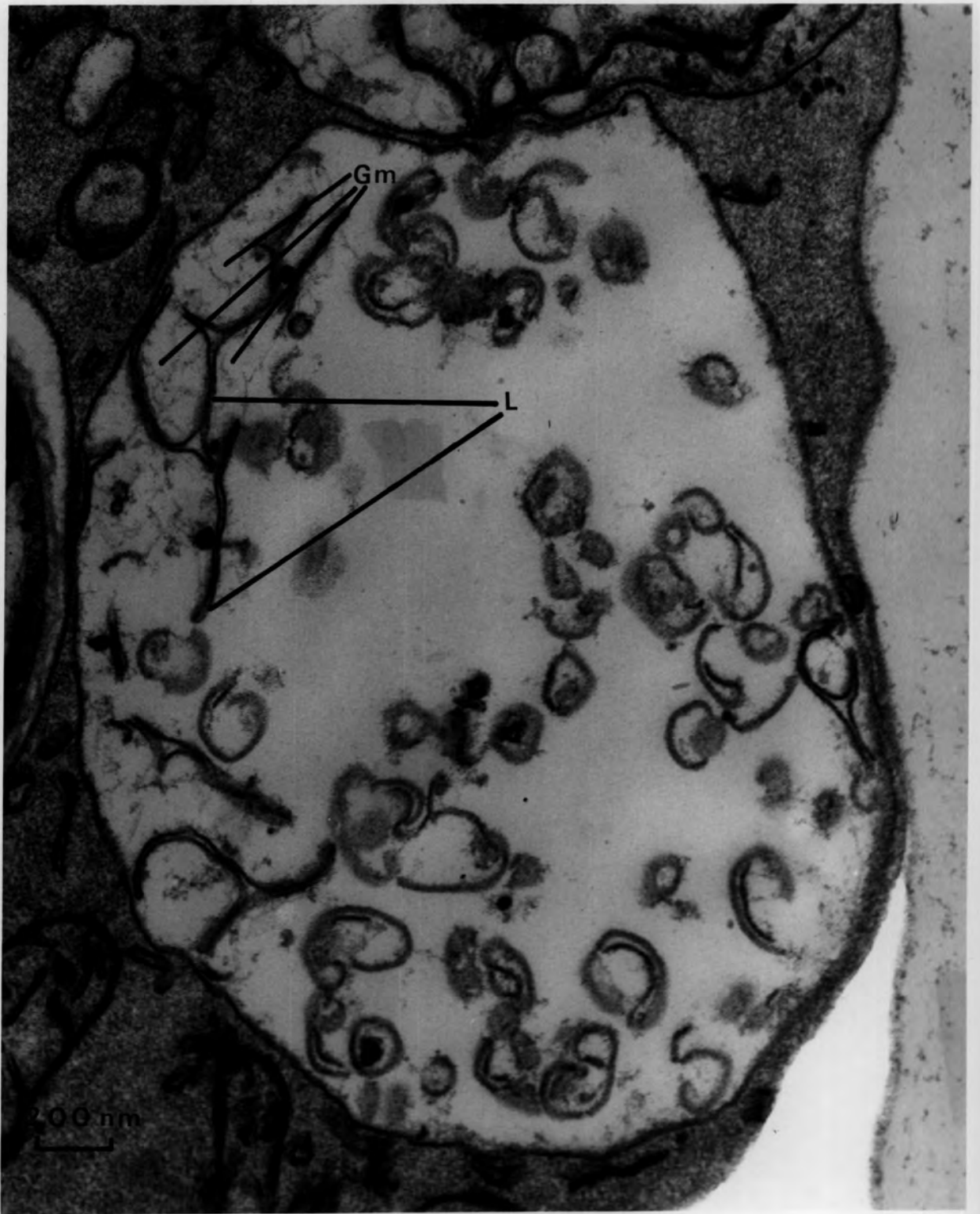


Fig. 24. A partly digested cell of *Microcystis aeruginosa* within a planktonic rhizopod. Lamellae and gas-cylinder membranes are still evident. KMnO_4 fixation. Mag. 59,000X.

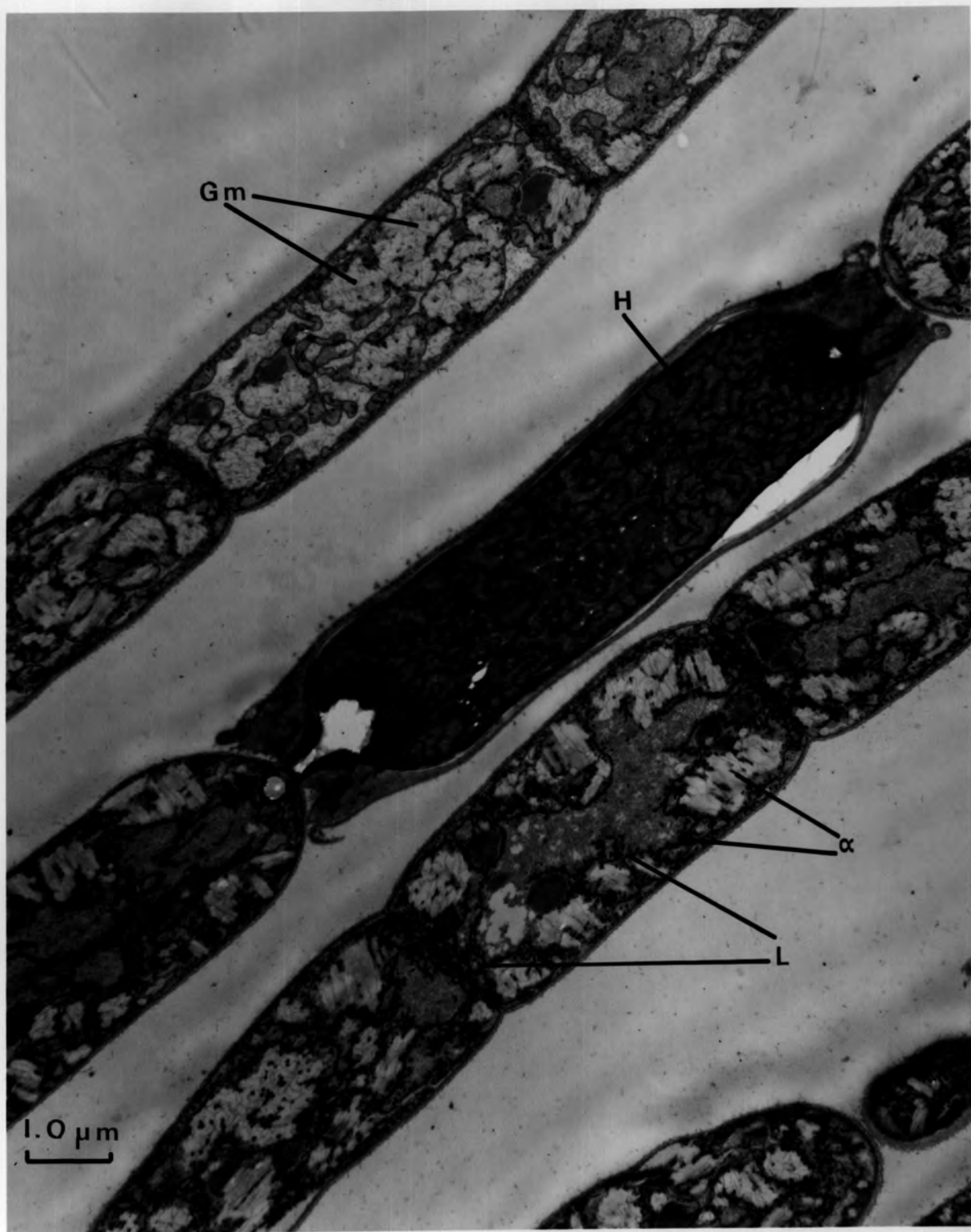


Fig. 25. A low magnification view of an Aphanizomenon flos-aquae colony collected from White Mere, Shropshire. KMnO_4 fixation. Mag. 35,000X.



Fig. 26. An *Aphanizomenon flos-aquae* cell showing abundance of intra-lamellar vesicles. OsO₄ fixation. Mag. 35,000X.

PART IV. The gases present in gas-vacuoles.

15. The composition of vacuole gas

Method I

Freeze-dried material containing gas-vacuoles and sealed under vacuum released no excess gas, compared with the control, after chloroform treatment. However, Method I proved unsatisfactory because of the large number of chloroform degradation products released during the process of mass-spectrometer analysis. Products containing chlorine were present from masses 35-38 and 47-50.

Method II

Analysis with the mass-spectrometer of the gases released after freezing and thawing of cells, revealed a significant excess of nitrogen in the gas-vacuolate sample compared with the control. The Nitrogen:oxygen ratio was 10:1 compared with 5:1 in the sample in which gas-vacuoles had been previously destroyed. No other differences could be detected.

16. Gas uptake experiments

No significant increase in argon was detected in the gas-vacuolate sample after the Dumas combustion was complete. Following incubation, N¹⁵ uptake was estimated in two samples with controls in which gas-vacuoles had been disrupted prior to the Dumas Combustion. The results obtained are shown

in Table 6.

Table 6. N^{15} uptake by cells of *Oscillatoria agardhii* D132 with and without gas-vacuoles

	Atom % excess N^{15}
Gas-vacuolate	
Sample (i)	-0.039
Control	+0.03
Gas-vacuolate	
Sample (ii)	-0.025
Control	+0.05

The results showed no tendency towards any excess N^{15} uptake by the samples compared with the controls. It was concluded that when gas uptake is estimated by this method (the method of BARSDATE and DUGDALE, 1965) gas is lost from the gas-vacuoles during the preparation procedures. The most likely stage at which this occurs is when the sample is purged with carbon dioxide prior to combustion. The gases present in the gas-vacuoles are flushed away and replaced by carbon dioxide. These results indicate the permeable nature of the gas-vacuole membrane and contradict the assumption made by KLEBAHN (1922) that the gas-vacuole membranes are impermeable to the gases they contain.

PART V. The isolation and chemical characterisation of gas-vacuole components from *Anabaena flos-aquae* D124.

The density gradient method described by JOST and MATILE (1966) was used to attempt to isolate gas-cylinder membranes from *Anabaena flos-aquae* D124. However, *A. flos-aquae* D125 (Fig. 7, p. 43) was also used in the present study as a control organism because it does not possess gas-vacuoles, thus allowing an unequivocal identification of gas-cylinder membranes to be made in various preparations.

17. Sucrose gradient centrifugation

A. Absorption spectra

The fractionation of *A. flos-aquae* D124 and *A. flos-aquae* D125 is shown in Figs. 27 and 28 respectively. Fractions were monitored at 280 nm wavelength in order to detect absorption by proteins. The heavy fractions 1 and 2 showed a peak at this wavelength in *A. flos-aquae* D124 which was absent from the corresponding functions of *A. flos-aquae* D125. However, fractions 1 and 2 from both organisms were yellow-green in colouration. Another difference between the two organisms was the high absorption at 280 nm of fractions 4 and 5 from *A. flos-aquae* D125. These fractions were dense blue due to the presence of phycocyanin. Fraction 8 at the top of the gradient had a pale brown colouration. The absorption spectra of the sediments from the two gradients were also

examined (Fig. 29). The A. flos-aquae D124 sediment showed increased absorption at 270-280 nm compared with A. flos-aquae D125.

B. Examination of fractions with the electron microscope

Figs. 32 and 33 show negatively stained preparations of the sediment from A. flos-aquae D124 and A. flos-aquae D125 respectively. The only difference observed between the two preparations was the presence in the A. flos-aquae D124 sediment of membranes 110 nm wide with lengths not exceeding 600 nm. They often had a characteristic fold about 300 nm from their conical ends. Fig. 35 shows an example of one of these membranes which has been magnified in order to examine its surface structure. Striations having a 50 nm spacing were observed. Similar striations were reported by WALSBY and EICHELBERGER (1968) on the surface of isolated gas-cylinders. As these membranes also possess a width (110 nm) which is the predicted size for a fully flattened 70 nm gas-cylinder it was concluded that they were gas-cylinder membranes. Also present in sediments from both organisms were membranes which were identified as lamellae because of their characteristic granular surface structure (NORTHCOTE, 1968). Granules which had the appearance of two closely adjoining spheres, each of which was 40 nm in diameter, were identified as α -granules.

Fig. 27. Density gradient fractionation of membrane preparations from Anabaena flos-aquae D124. The heavier band appears pale green and contains gas-cylinder membranes.

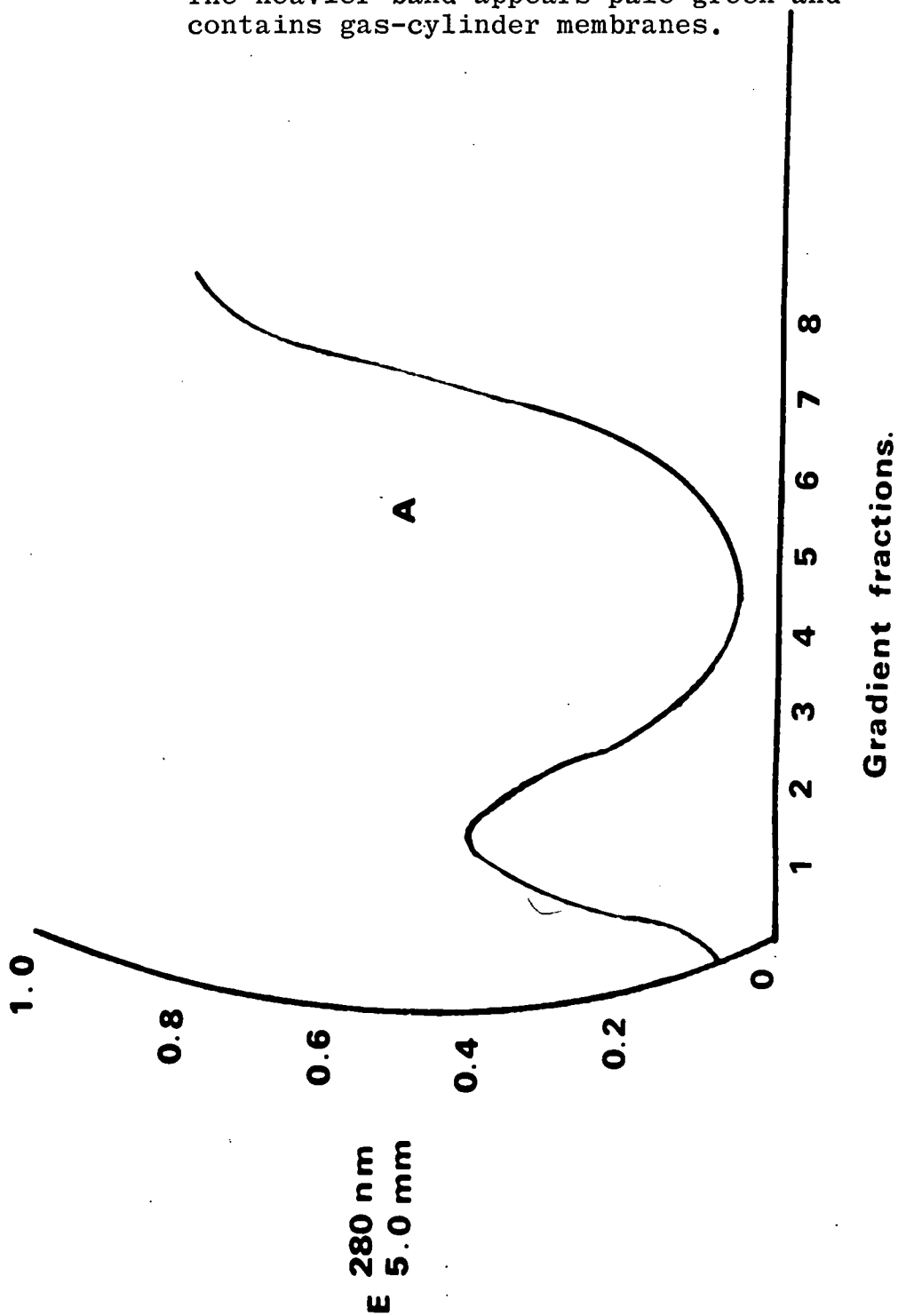
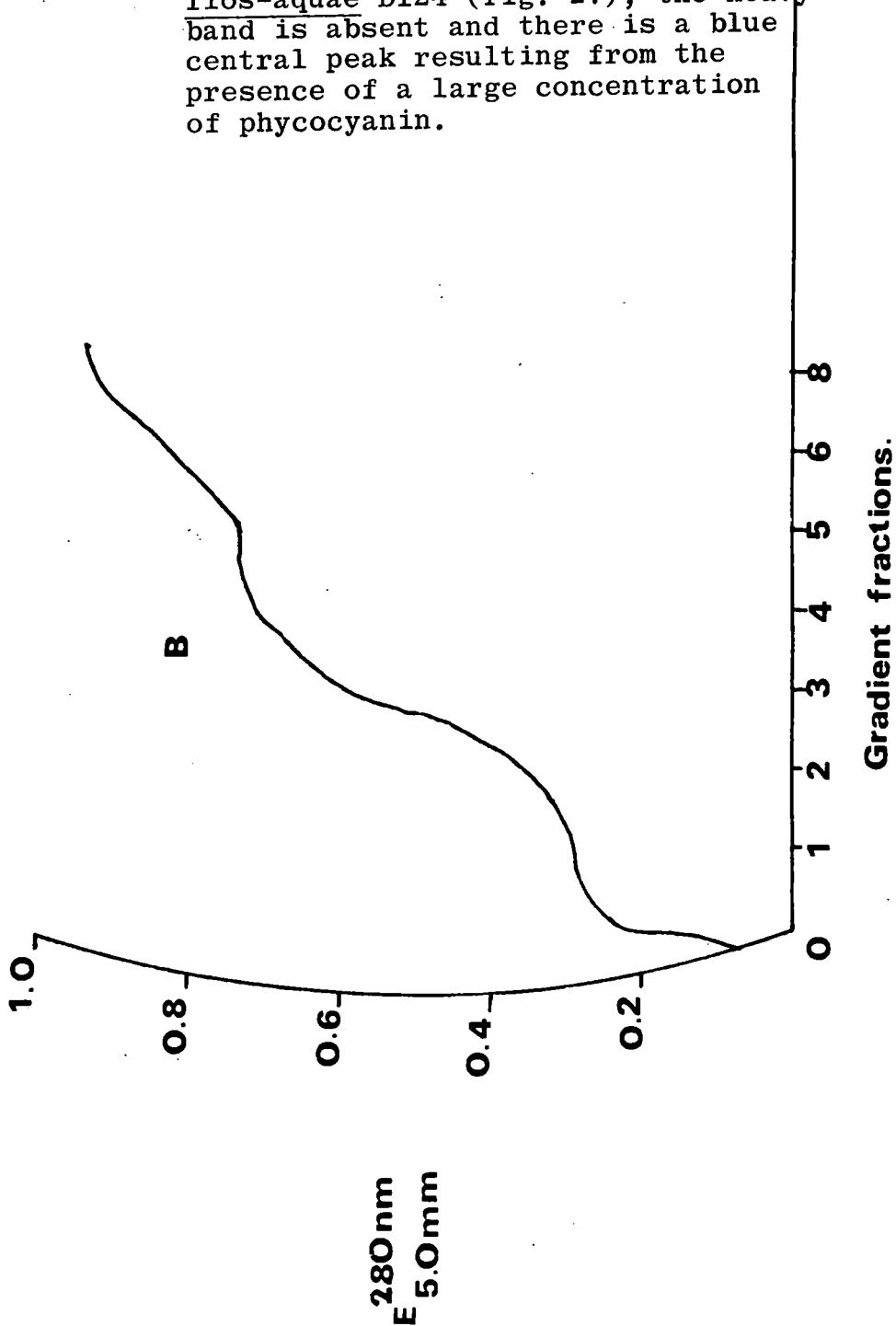


Fig. 28. Density gradient fractionation of membrane preparations from *Anabaena flos-aquae* D125. Compared with *A. flos-aquae* D124 (Fig. 27), the heavy band is absent and there is a blue central peak resulting from the presence of a large concentration of phycocyanin.



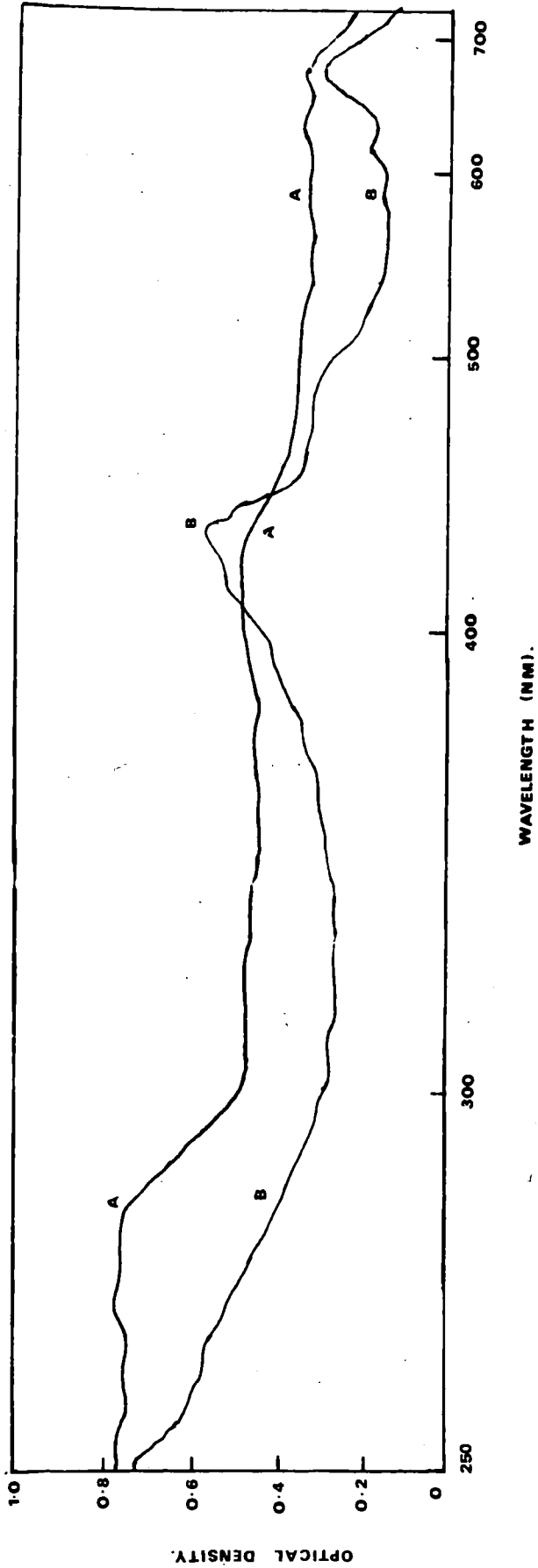


Fig. 29. The absorption spectra of the sediment fractions after density gradient centrifugation of membrane preparations from Anabaena flos-aquae D124 (A) and A. flos-aquae D125 (B).

Fractions 1 and 2 from A. flos-aquae D124 contained smaller gas-cylinder membrane fragments (Fig. 34), some lamellae fragments, and α -granules. Gas-cylinder membrane fragments were absent from the corresponding fractions from A. flos-aquae D125. Fractions 3, 4, 5 and 6, contained no examples of lamellae, occasional α -granules and, in the case of A. flos-aquae D124, one or two scattered gas-cylinder membrane fragments. Fractions 7 and 8 contained no examples of α -granules, gas-cylinder membranes and lamellae.

Fig. 36 shows a general view of the fixed and embedded sediment fractions from A. flos-aquae D124. Gas-cylinder membranes were observed in cross-section as electron dense lines with a globular appearance. The spacing of the globules was about 4.0 nm. In tangential section there were some indications of striations on the surface of these membranes. α -granules were also observed and, in favourable sections, these had the characteristic appearance of two closely adjoined spheres, each of which was about 40.0 nm diameter.

18. Protein molecular weight determination of gas-cylinder membrane protein

Because of the presence of both gas-cylinder membranes and lamellae in fractions prepared by sucrose gradient centrifugation, this method has no advantage over simple differential centrifugation. The latter method was therefore

used to prepare membrane samples for protein studies. The molecular weight distributions of the protein chains from the membrane fractions of A. flos-aquae D.24 and A. flos-aquae D125 are shown in Figs. 30 and 31 respectively. The samples were similar in that both contain complex mixtures of polypeptide chains with molecular weights in the range 55,000-85,000 and smaller amounts of a continuous spectrum of chains in the molecular weight range 20,000-50,000. The samples probably contained similar polypeptide units in different proportions. The most obvious difference in the samples was the presence of a high concentration of a pure polypeptide chain of molecular weight $22,000 \pm 2,000$ in A. flos-aquae D124 (Fig. 30).

19. α -granule characterisation

α -granules showed sedimentation characteristics similar to those of animal glycogen described by ORRELL and BUEDING (1958). Treatment of α -granules with 2% diastase and subsequent examination of negatively stained preparations demonstrated the disappearance of α -granules. A positive reaction was obtained when paraffin wax embedded material was treated with the periodic acid-Schiff technique. Sections of A. flos-aquae D124 treated with 2% diastase for 30 minutes after prior bleaching (GIESY, 1964) did not show any disappearance of α -granules. This result may be explained

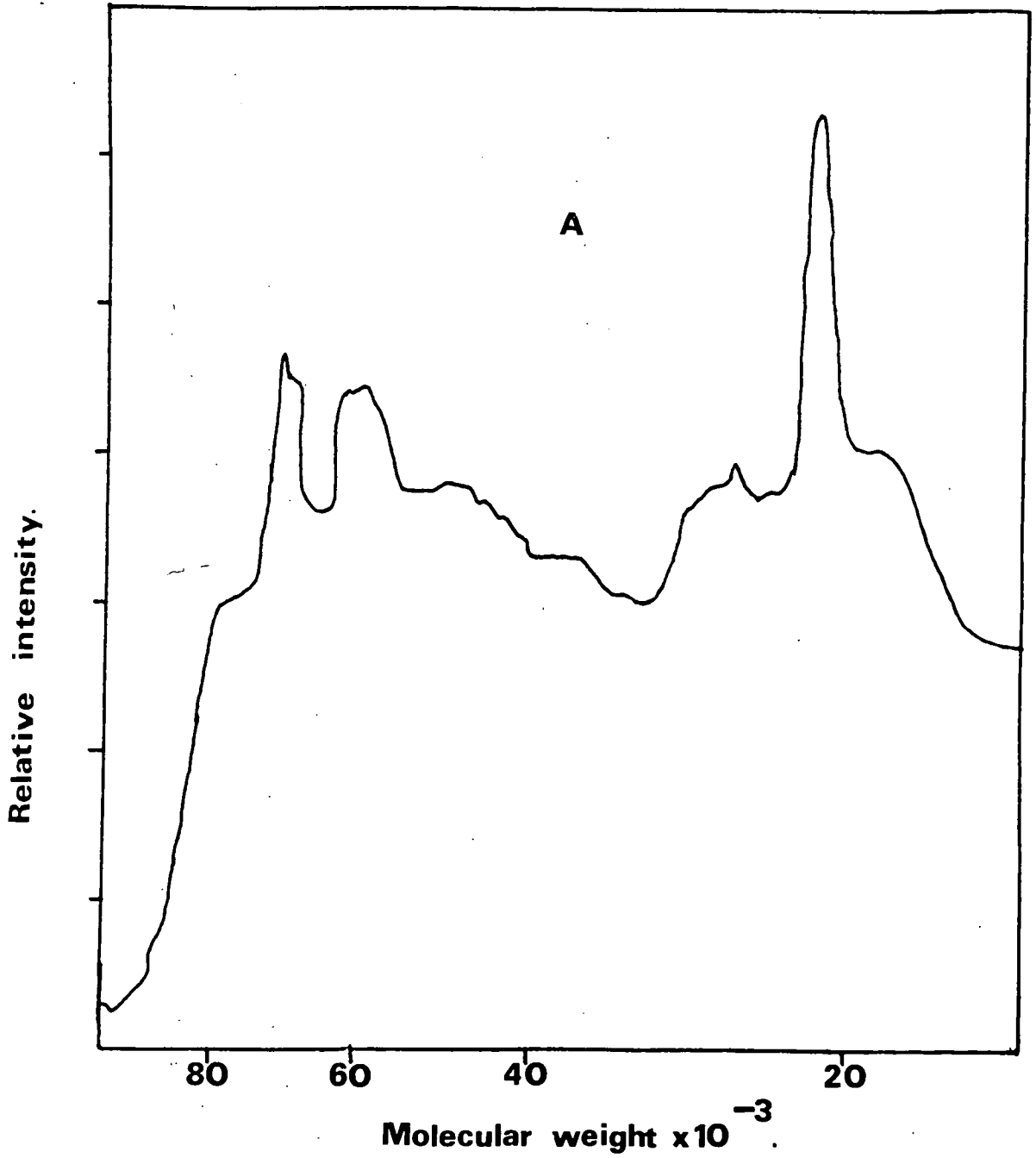


Fig. 30.

The molecular weight distribution of the protein chains from the membrane fraction of Anabaena flos-aquae D124.

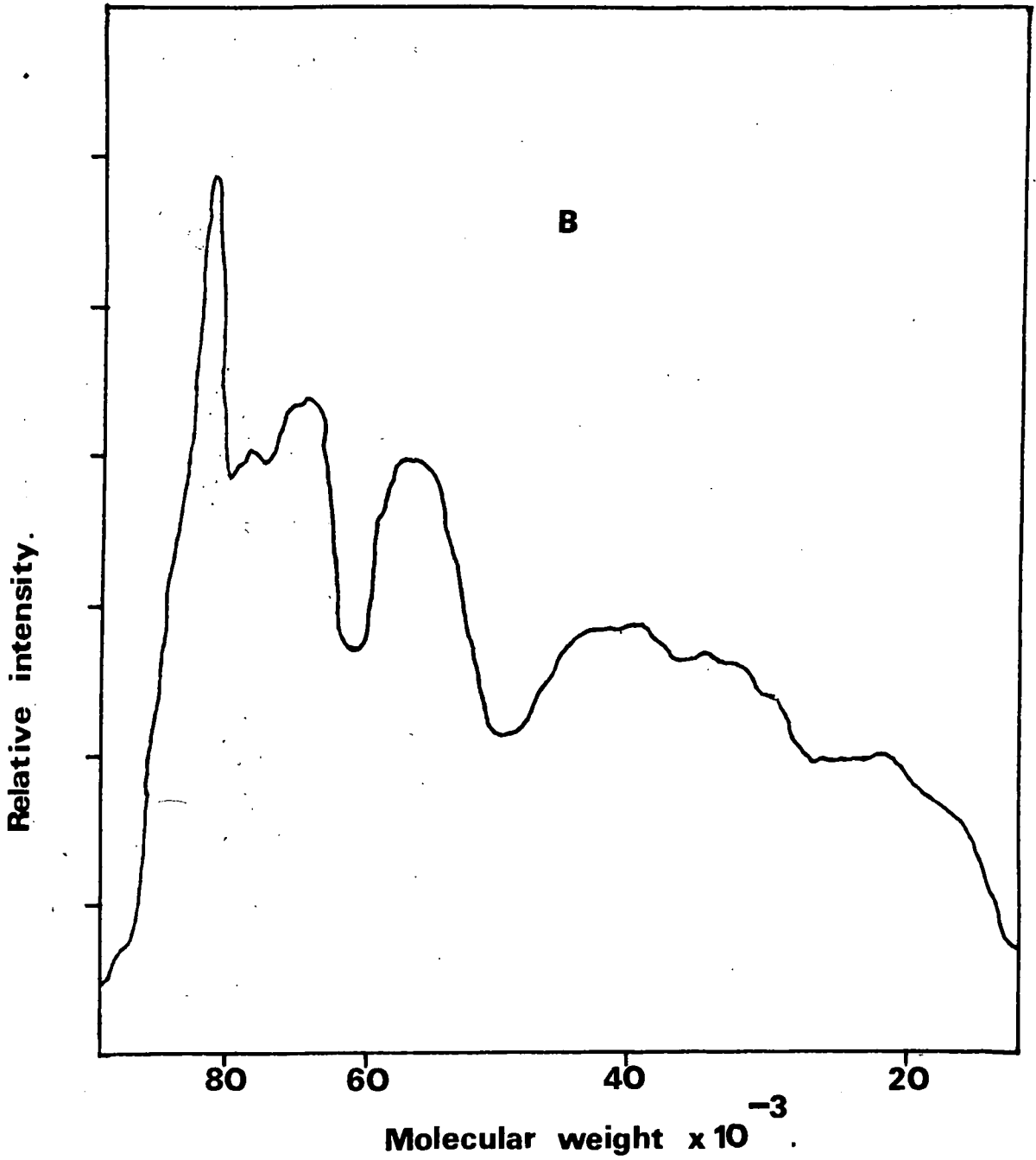


Fig. 31. The molecular weight distribution of the protein chains from the membrane fraction of Anabaena flos-aquae D125.

by poor penetration of sections by the digestate, or by some difference in the molecular branching pattern of the α -granules in A. flos-aquae D124 compared to those of Oscillatoria chalybia employed by GIESY (1964). The above data strongly indicate α -granules are polysaccharide. Anabaena flos-aquae D124 cells were incubated in the dark for 48 hours after inoculation into fresh medium. Examination of these cells with the electron microscope revealed a great reduction in numbers of α -granules in the interlamellar areas. This result indicates that α -granules polysaccharide may act as a food reserve in A. flos-aquae D124 cells.

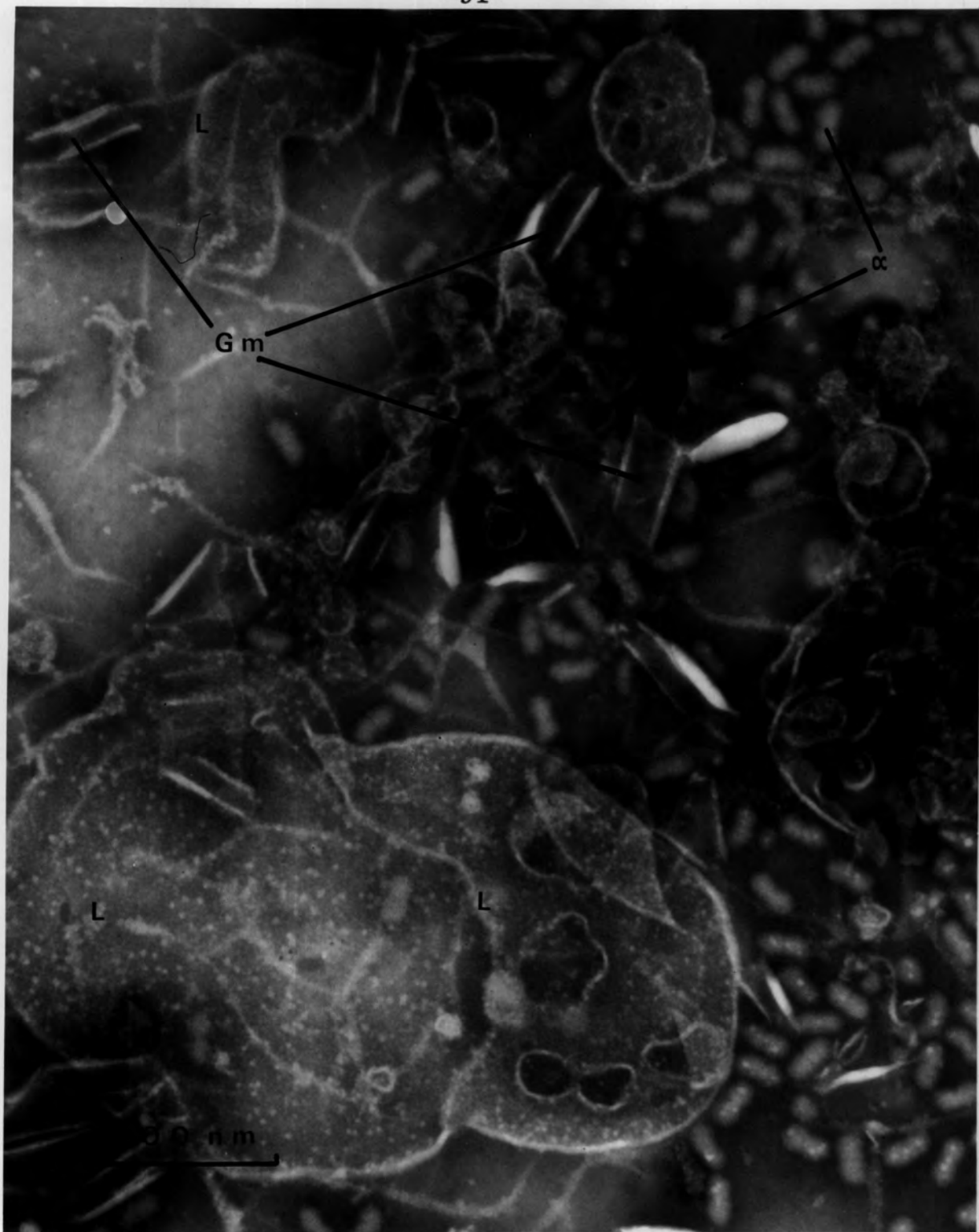


Fig. 32. General view of a negatively-stained preparation of the sediment from the density gradient fractionation of *Anabaena flos-aquae* D124 (Fig. 26, page 78). Mag. 140,000X.

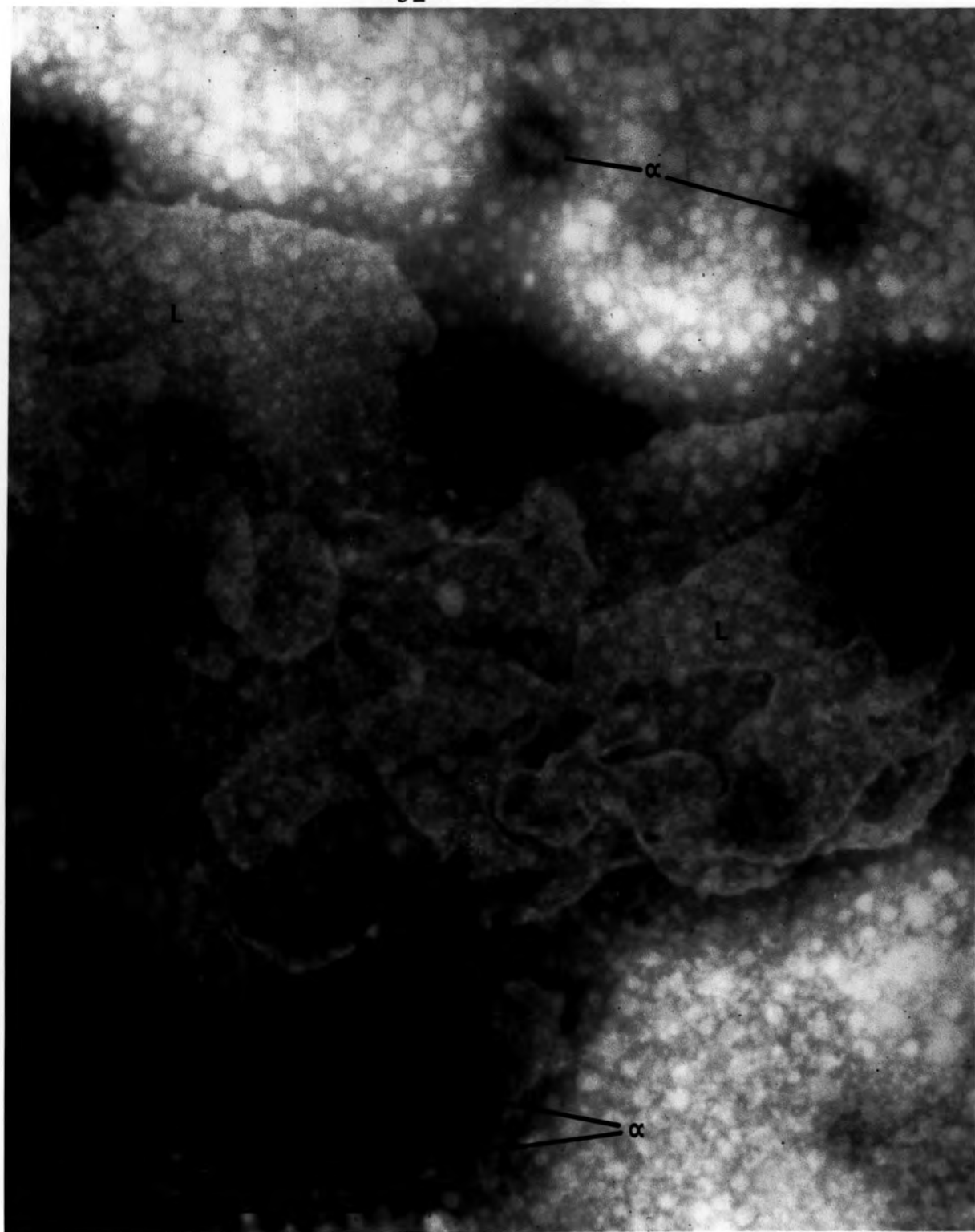


Fig. 33. General view of a negatively-stained preparation of the sediment from the density gradient fractionation of *Anabaena flos-aquae* D125 (Fig. 28, page 84). Note the absence of gas-cylinder membranes. Mag. 135,000X.

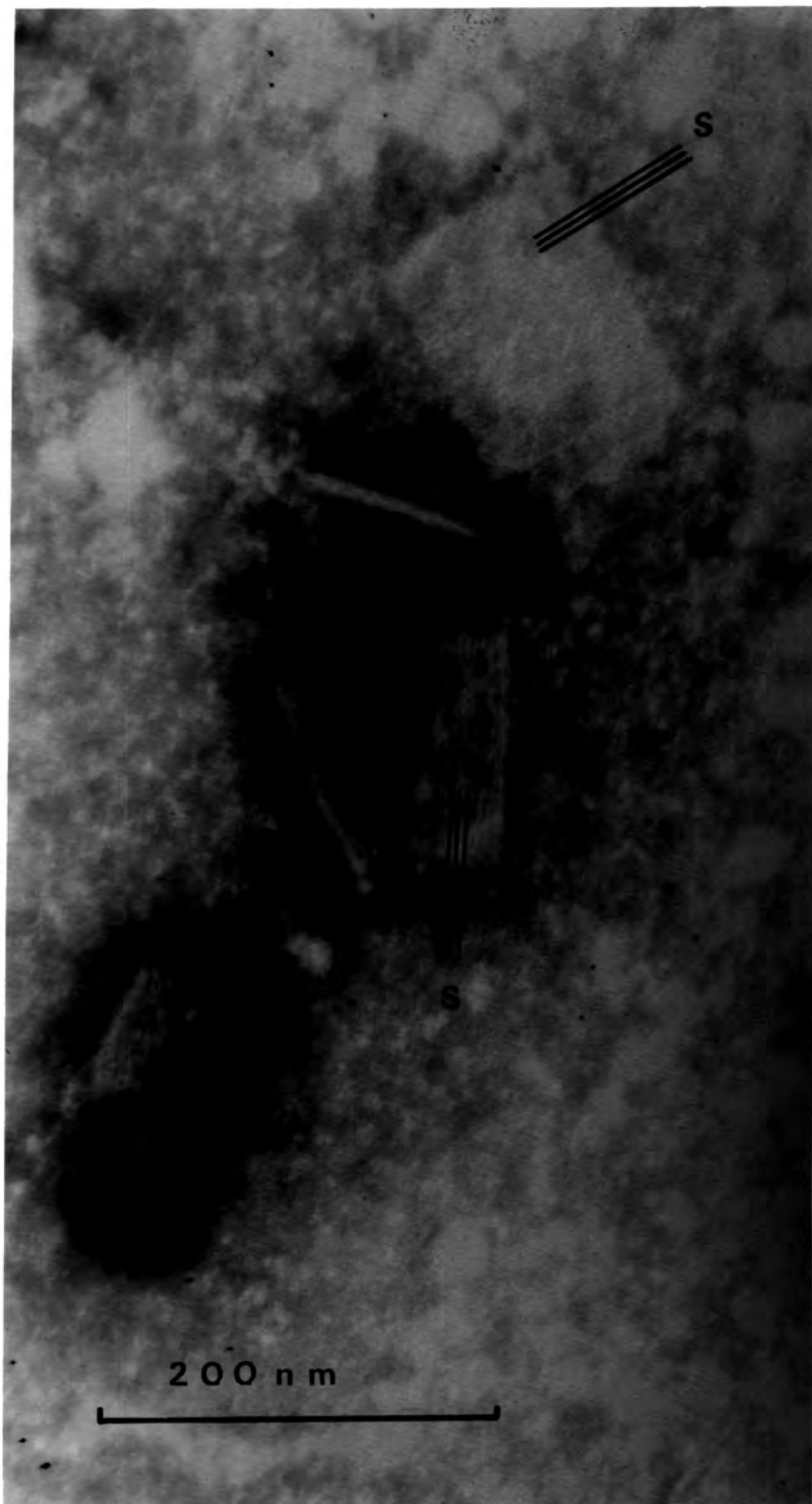


Fig. 34. Small gas-cylinder membrane fragments present in Fraction 2 from the density gradient fractionation of *Anabaena flos-aquae* D124 (Fig. 26, page 78). Mag. 270,000X.

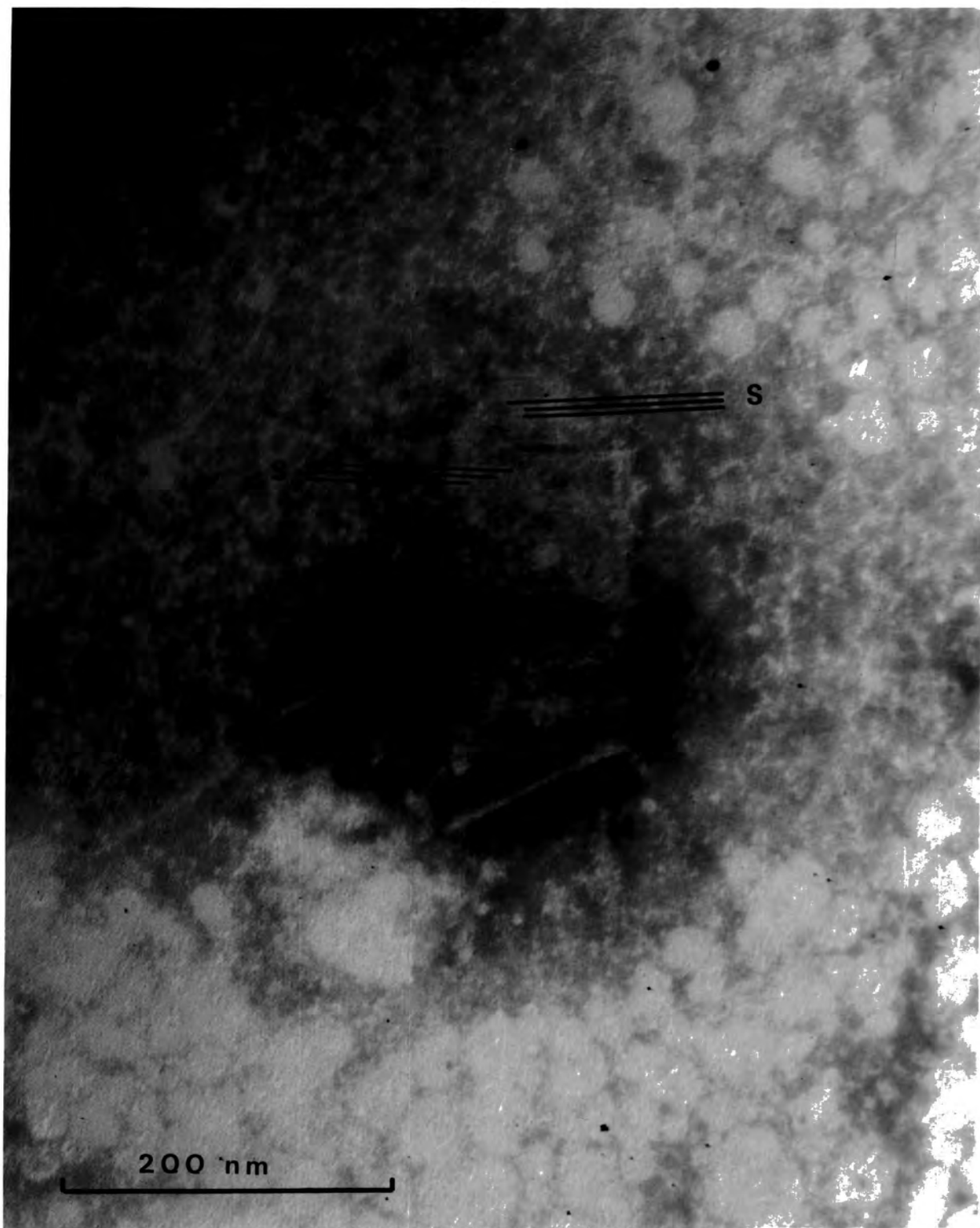


Fig. 35. A negatively stained gas-cylinder membrane from the sediment of the density gradient fractionation of Anabaena flos-aquae D124. Mag. 270,000X.

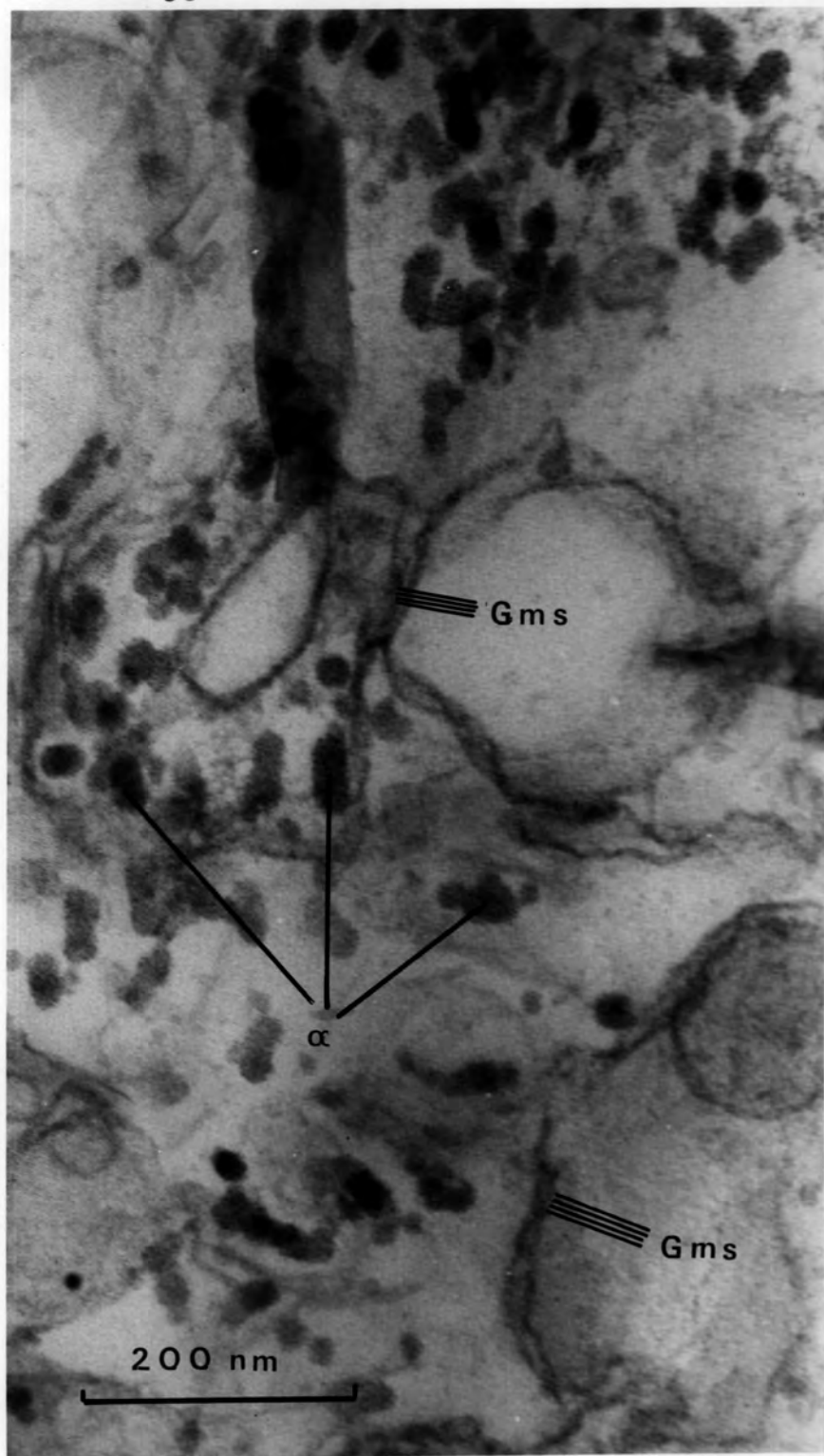


Fig. 36. Fixed and embedded sediment from the density gradient fractionation of *Anabaena flos-aquae* D124. Glutaraldehyde fixation. Mag. 180,000X.

PART VI. Physiological studies on gas-vacuoles in
Anabaena flos-aquae D124

20. Effects of the absence of gas-vacuoles on
growth rate

Cells were prepared as described in 'Materials and Methods', Section 10A, p.28, with the modification that pressure-treated cells were resuspended in freshly prepared ASM-1 medium. The growth rate of these cells during the time taken for gas-vacuoles to redevelop (4.0 days) was measured by taking optical density readings at 650 nm. The value obtained by this method was $K = 0.137$. The growth rate of gas-vacuolate cells grown under identical conditions is $K = 0.132$. It was concluded that no inhibition of growth rate occurs when gas-vacuoles are absent from Anabaena flos-aquae D124 cells grown under the above conditions. As LEMMERMAN (1910) proposed that gas-vacuoles might provide a light shielding function it was decided to examine the effect of the absence of gas-vacuoles on cells incubated at an inhibitory light intensity. Cells were grown under the standard growth conditions ('Materials and Methods', Section 3G) with the modification that the light intensity was increased to 12,000 lx. The growth rate of gas-vacuolate cells under these conditions was $K = 0.68$. Pressure-treated cells redeveloped gas-vacuoles in 4.0 days. The effect on growth rate of the absence of gas-vacuoles

was to induce a lag phase in these cells which lasted 4-6 days.

21. The redevelopment of gas-vacuoles following pressure treatment.

A. The relationship between the growth rate of cells and the time taken for gas-vacuoles to redevelop

The aim of this experiment was to investigate whether the times taken for gas-vacuoles to redevelop bear a simple relationship to the growth rate of cells at different stages of growth. From the above results ('Results', Section 19), it was concluded that the growth rate of cells grown at medium light intensities was not significantly affected by gas-vacuole destruction. However, this conclusion was only based on results with day +0 cells, and the assumption was made in the present experiment that the growth of cells of different ages was likewise unaffected by gas-vacuole destruction.

Cells grown under the Standard growth conditions ('Materials and Methods', Section 3G) were pressure treated (3.72 atmospheres) to destroy gas-vacuoles and the cells resuspended. The suspension was returned to the growth tank and observations were made at 6 hour intervals on the time taken for the first indications of gas-vacuole redevelopment to appear in 50% of cells. The redevelopment times for cells pressure treated on day 0, +2, 4, 8, 12, 24, and 50, were

3.5, 3.0, 1.5, 0.75, 0.75, 2, and 8 days, respectively. The reciprocal of the time taken for gas-vacuoles to redevelop is plotted against the age of cells in Fig. 37. The fastest redevelopment times were thus observed in the cells from the late exponential phase of growth (day +8 and 12 cells) when the growth rate of cells was $K = 0.132$. However, although cells from the early exponential phase of growth (day +2 and 4) also had a growth rate of $K = 0.132$, these cells exhibited much slower development rates than the day +8 and 12 cells. Growth is stationary in day +24 cells. However, these cells redevelop gas-vacuoles in only 2 days. Day +12 cells incubated in the dark redeveloped gas-vacuoles in 24 hrs., a time which was only reduced to 18 hrs. when these cells were incubated in the light. The redevelopment times of gas-vacuoles do not, therefore, bear any simple relationship to the growth rate of cells. Addition of 2-4-dinitrophenol to a concentration of 2.5 mM prevented gas-vacuole redevelopment. This result confirmed that of LARSEN et al. (1967) who employed a similar concentration of 2-4-dinitrophenol to prevent recovery of gas-vacuoles in Halobacterium.

B. Changes in the sedimentation rate of cells associated with gas-vacuole redevelopment

The sedimentation rate of 4-celled filaments of Anabaena

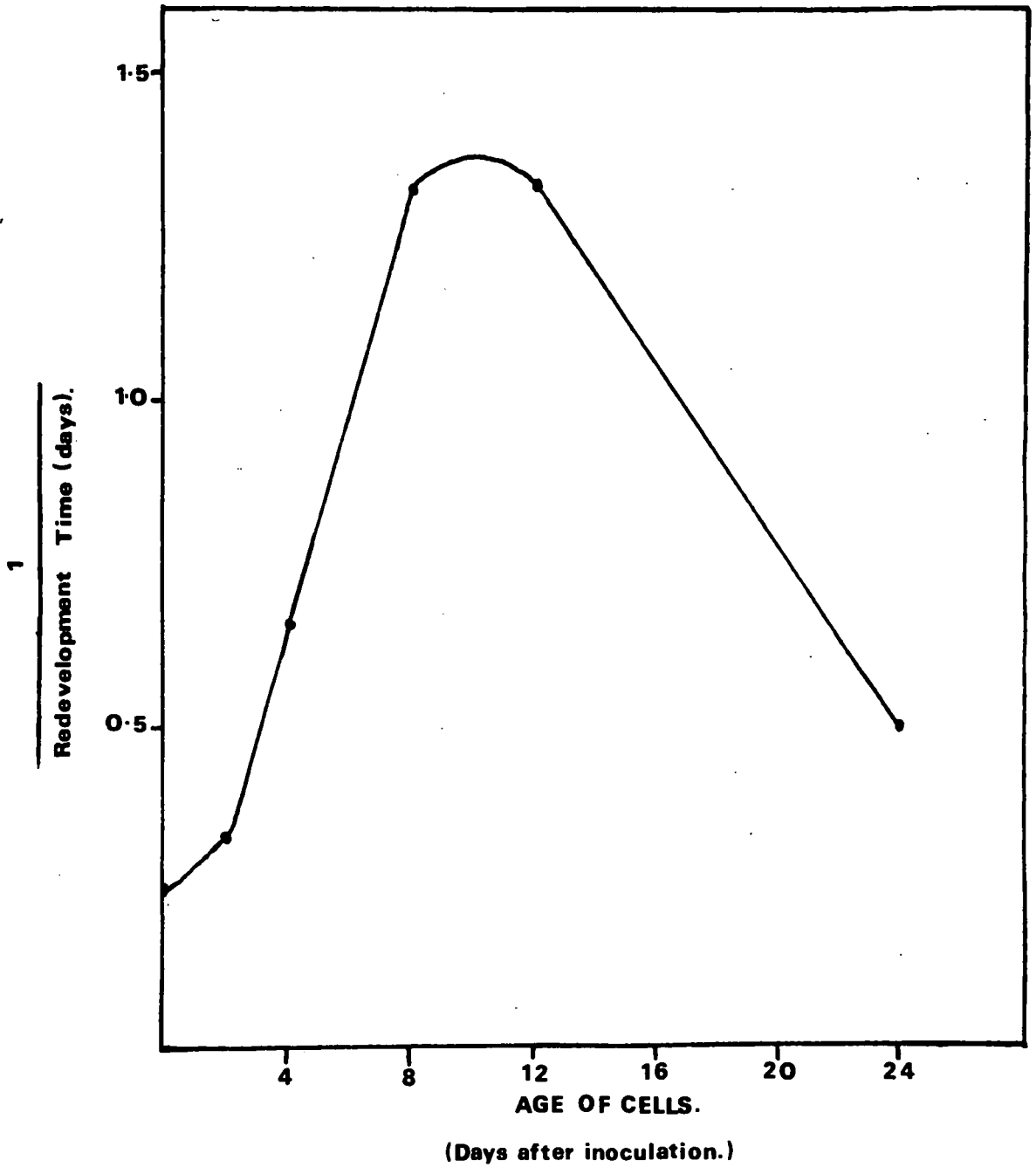


Fig. 37.

The relationship between the age of cells and the reciprocal of the time taken for gas-vacuoles to redevelop in Anabaena flos-aquae D124.

flos-aquae D124 immediately following gas-vacuole destruction was $4.18 \mu\text{m} \times \text{Sec}^{-1}$ at 20°C . This filament size was chosen for sedimentation studies because of the high proportion of 4-celled filaments present in the sample following filtration. Fortunately, no heterocysts or akinetes were observed in these filaments. These types of cell exhibit faster sinking rates than vegetative cells. The sedimentation rate of A. flos-aquae D125 filaments was $3.4 \mu\text{m} \times \text{Sec}^{-1}$.

A change in the sedimentation rate of A. flos-aquae D124 filaments was first observed at time +18 hrs. (18 hrs. after gas-vacuole destruction). When measurements were repeated at time +24 hrs. (Fig. 38) the sedimentation rate had been reduced to only $1.1 \mu\text{m} \times \text{Sec}^{-1}$. At time +30 and 36 hrs. there was ^{no} positive accumulation of filaments at the base of the sedimentation tube. The sedimentation values plotted on Fig. 38 for these times are shown as values of $0.0 \mu\text{m} \times \text{Sec}^{-1}$. However, it is possible that filaments may exhibit negative sedimentation rates at these times. It was not possible to make accurate estimates of these rates by employing the standard sedimentation technique used in the present studies.

C. Observations on gas-vacuole redevelopment with optical and electron microscopes

Gas-vacuoles were first observed with the optical microscope in 4-celled filaments of Anabaena flos-aquae D124 at time +18 hours (18 hours after gas-vacuole destruction). At this time the gas-vacuoles were very indistinct, and

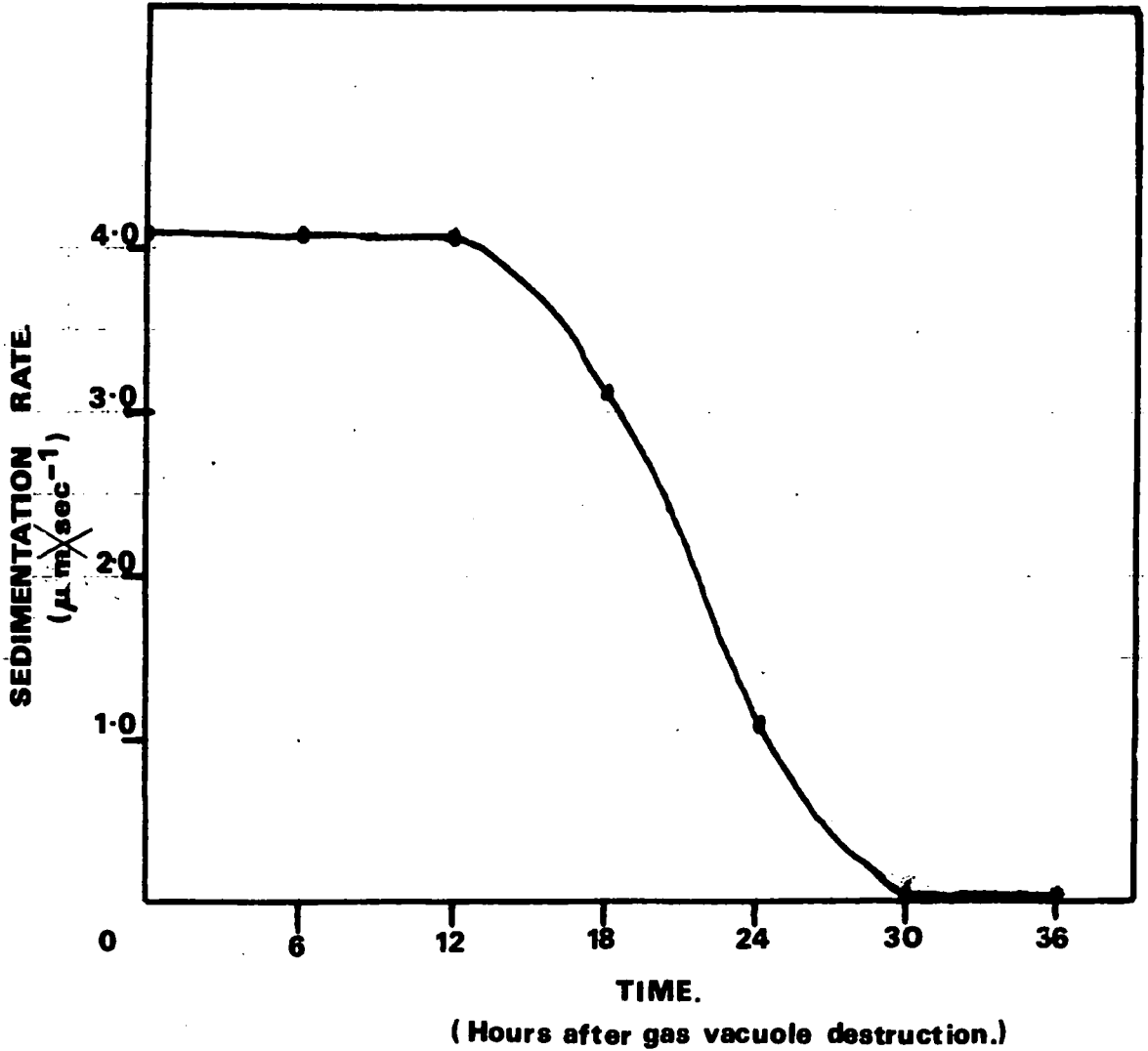


Fig. 38. The changes in sedimentation characteristics of 4-celled filaments of Anabaena flos-aquae D124 during the period of gas-vacuole redevelopment.



there were only occasional examples of definite granules in some cells. At time +24 hours gas-vacuoles were observed in all cells. They appeared as distinct, rounded granules distributed between inner and peripheral regions of the cells. The number and size of gas-vacuoles had increased considerably when observations were made at time +30 and 36 hours.

Attempts were made to examine direct preparations of cells at time +18 hours with the electron microscope. However, the cells fixed on grids proved to be very unstable when attempts were made to focus the electron microscope. There appear to be two possible explanations for their instability. Firstly, cells containing sites of gas-vacuole resynthesis may be inherently unstable. Secondly, the 4-celled filaments employed in the present studies contained large rounded cells which may not stabilise during the drying process as satisfactorily as the cells employed for previous direct preparations (see Fig. 4, p.40).

DISCUSSION

1. The characterisation of blue-green algal gas-vacuoles

The subject of the optical and electron microscope characterisation of gas-vacuoles has been briefly discussed in 'Results', Section 5, p.36. The optical microscope observations on blue-green gas-vacuoles are also reviewed in the 'Appendix' to the present thesis. In both species cultured in the laboratory ('Results', Part I), and species collected from the field ('Results', Part III), gas-vacuoles appeared under the normal transmission optical microscope as reddish, non-refractile structures which were pressure-sensitive. The shape and distribution of the gas-vacuoles in these organisms varied considerably between different species. In Anabaena flos-aquae D124 there was also variation in gas-vacuole development in relation to changes in environmental conditions ('Results', Part II). The nature of this variation has been discussed in 'Results', Section 12, p.56.

The gas-vacuoles of all the species examined in the present study ('Results', Parts I and III) were resolved with the electron microscope into groups of gas-cylinders, 70.0 nm in width. Although the length of the cylinders and the size of the groupings showed some variation between species, all cylinders were bounded by unusual membranes about 3.0 nm wide.

2. The gas present in gas-vacuoles

Recent studies have confirmed the hypothesis of KLEBAHN (1895) that the reddish, pressure-sensitive structures present in certain blue-green algae are gas-filled vacuoles. The present work ('Results', Part IV, p.79) indicated the presence of nitrogen in gas-vacuoles of Anabaena flos-aquae D124. KLEBAHN (1922), in attempting to explain the persistence of gas-vacuoles in cells examined under vacuum, postulated that the gas-vacuole wall must be a rigid, impermeable structure. KLEBAHN based his postulate on optical microscope observations of gas-vacuoles. However, the present studies showed that gas-vacuoles were resolved, with the electron microscope, into groups of cylindrical sub-units (gas-cylinders) and that there was no rigid, outer wall enclosing these structures.

Two observations indicated that the gas-cylinders were permeable to gases. Firstly, no gas was obtained from gas-vacuolate material which had been freeze-dried and sealed under vacuum ('Results', Section 15, p.79), and, secondly, vacuole gases may be easily flushed away as was demonstrated during gas-uptake experiments ('Results', Section 16, p.80). WALSBY (1969) in an extensive study on the permeability of the gas-cylinders in cells of A. flos-aquae D124, similarly concluded that they were freely permeable to gases. He

employed a manometric method to measure the quantity of gas released after ultrasonic destruction of these structures and demonstrated that the pressure of gas-cylinder gas was about one atmosphere. By modifying a Warburg apparatus he was able to demonstrate that gas-cylinders were very permeable to nitrogen, oxygen and argon.

These results indicate that the blue-green algal gas-cylinders are not homologous to the vacuole present in the rhizopod Arcella. In this organism the gas present is exclusively oxygen which is actively transported to the vacuole (CICAK, 1963). Instead, the blue-green algal groups of gas-cylinders show some similarities to the hydrostatic organ of Chaoborus, a larval diptera. Two pairs of kidney-shaped sacs are developed from the main tracheal trunks of this organism. The sacs contain gases which are in equilibrium with the mixture dissolved in the water in which the larva is living (KROGH, 1939). Similarly, the composition of gas-cylinder gas of blue-green algae will be close to the mixture present in the surrounding solution. It will be modified, however, by the processes of photosynthesis, respiration, and, in some cases, nitrogen fixation.

In regard to nitrogen fixation by gas-vacuolate blue-green algae, it was suspected that the presence of gas-cylinders might falsify some nitrogen fixation results

obtained by the method of BARSDATE and DUGDALE (1965). For example, the uptake of N^{15} by Trichodesmium erythaeum, which was reported by DUGDALE et al. (1964), might be explained by exchange of N^{15} with gas present in gas-cylinders rather than true fixation. However, the gas-uptake experiments ('Results', Section 15, p.79) clearly indicated that, because of the permeability of the gas-cylinders, all N^{15} gas was removed by the flushing process incorporated into the method of BARSDATE and DUGDALE (1965). Results obtained by this method are not, therefore, falsified by the presence of gas-cylinders.

FOGG (1941) after reviewing the early studies on gas-vacuoles concluded that, 'the unusual properties of the gas-vacuoles might depend not so much on the gas as on the membrane which enclosed it'. Recent studies have supported his view and revealed that the presence of gas in the vacuoles is a consequence of the permeability of gas-cylinders.

3. The gas-cylinder membrane

The molecular organisation of cellular membranes has been the subject of vigorous discussion for many years (for a review of the literature, see ROTHFIELD and FINKELSTEIN, 1968). Until recently the generally accepted model has been that of DAVSON and DANIELLI (1935) as modified by ROBERTSON (1959). In this view (the unit membrane concept) all

membranes consist of continuous bimolecular leaflets of lipids, with proteins mainly located in the polar surfaces of the leaflets. However, a uniform unit membrane structure is inconsistent with variations in lipid and protein composition seen in isolated membranes. The only true similarity between different cellular membranes is their general resemblance in electron micrographs, when they exhibit a characteristic trilaminar 'unit membrane' structure consisting of two electron dense lines about 2.0 nm thick with a clear space of about 3.5 nm between them. WHITTAKER (1968) interpreted this resemblance between different membranes in electron micrographs as simply representing a stable configuration of membranes which is brought about by the physical and chemical transformations of the membranes during the process of fixation.

However, the gas-cylinder membrane does not stabilise after fixation as two electron dense lines. It appears as a single electron dense line (about 3.0 nm wide) and can be resolved into globules with a spacing of about 4.0 nm. Clearly the gas-cylinder membrane is an unusual membrane with distinctive properties and is unlikely to represent 'half a unit membrane' as suggested by BOWEN and JENSEN (1965).

JOST and MATILE (1966) isolated a gas-cylinder membrane fraction from Oscillatoria rubescens and reported that it had a high carotenoid and lipid content. However, they did not

use the typical cylindrical shape, constant width (110 nm after isolation), or striated surface structures of gas-cylinder membranes to characterise this fraction. Unless the gas-cylinder membranes of O. rubescens possess a very different structure from those of Anabaena flos-aquae D124, one must conclude that this fraction was misidentified.

By employing the above morphological criteria in the present studies, gas-cylinder membranes were identified in membrane fractions from A. flos-aquae D124. A high concentration of a pure polypeptide chain of molecular weight $22,000 \pm 2,000$ was present in the samples (Fig. 30, p.88). The calculated size of a spherical protein sub-unit with this molecular weight is 3.8 nm. The polypeptide appears to correspond with the globules observed in fixed gas-cylinder membranes. A globular sub-structure has been observed in the gas-vesicle membranes of Halobacterium halobium by LARSEN, OMANG and STEENSLAND (1967). STOECKENIUS and KUNAU (1968) reported that these membranes consisted mainly of protein and that no lipid could be extracted. These results strongly indicate the gas-cylinder and gas-vesicle membranes in both bacteria and blue-green algae are composed of globular sub-units which are protein in nature.

From the above fine structural and biochemical results

it appears that the gas-cylinder membranes bear some resemblances to the structure of the protein coat of viruses. Similarly, the lack of variation in construction between gas-cylinders from different species of blue-green algae ('Results', Parts I and III) and from different growth stages ('Results', Section 7B, p.49), indicates that their formation must be the simple, straightforward process following one fixed path, akin to viral shell formation. Therefore, a useful approach to the interpretation of the molecular organisation of gas-cylinder membranes may be to refer to the structure of viral coat protein.

The present theories of virus construction stem from the suggestion of CRICK and WATSON (1956) that all viruses are built up of identical protein sub-units (about 20,000 MW) packed together in a regular manner, to provide a protein shell for the nucleic acid. It might appear, at first sight, that there is an enormous variety in the ways in which this could be done. However, CASPAR and KLUG (1963) have shown that there are only a limited number of efficient designs possible for a biological container which can be constructed from a large number of identical protein molecules. The two basic designs are helical tubes and icosahedral shells. The construction of the gas-cylinder appears closest to that of the helical tube.

CASPAR (1963) reported that no rod-shaped viruses (the helical tube design) are known to be built according

to principles other than those determined for TMV (Tobacco Mosaic Virus). The gas-cylinder, however, does not possess an obvious helical structure. However, when isolated TMV protein is reaggregated it assumes its lowest energy conformation and does not form a helix. The protein subunits crystallise in hexagonally packed sheets; the sheet is then rolled up to produce a hollow cylinder, which from the side looks like a pile of stacked discs with a periodicity of 5.0 nm (HASELKORN, 1966). This appearance is comparable to that of the gas-cylinder which is similarly striated with a periodicity about 5.0 nm (Fig. 35, p.94). The normal structure of the TMV protein coat is assumed when RNA is inserted, resulting in a periodic dislocation of discs to give a helix.

From these comparisons with viral coat protein the application of the term 'membrane' to the structure enclosing the gas of the gas-cylinder must be reconsidered. If one defines a cellular membrane as a flexible structure capable of growth, its size being determined by its contents, and a shell as a relatively rigid structure which can only assume a fixed structure (CASPAR and KLUG, 1963) the gas-cylinder membrane seems more akin to a shell than a typical membrane.

It is tempting to speculate that gas-cylinders may have a viral origin. The protein of viruses by virtue of

their inherent self-polymerising potentialities, may form structures other than virus particles (for a review of the literature see MARKHAM, 1968). An example of such structures is the 'stacked disc' form of TMV protein which was described above. It is possible that an over-production of viral coat protein in viral-infected cells of blue-green algae resulted in tubes resembling gas-cylinders being assembled. However, the gas-cylinder membrane probably possesses additional non-protein components. Electron transparent spaces are present outside the 'membrane' as observed after OsO_4 fixation (see Figs. 17 and 18, pp.69 and 70). These may represent a component that has been lost by leaching during electron microscope preparation procedures. STOEKENUS and KUNAU (1968) reported that protein accounts for only 70% of the dry weight of the Halobacterium gas-vesicle membranes.

This additional component may represent the need to stabilise the proteinaceous tube. Viral coat protein, for example, without the addition of its nucleic acid component can be disaggregated under milder conditions than either the native or reconstituted virus. The increase in width of the gas-cylinder membrane observed after KMnO_4 fixation may reflect the presence of the OsO_4 leached component. This leachable substance is unlikely to be lipid because this is normally more stable after OsO_4

fixation. A possible suggestion as to the chemical nature of this component is that it represents a carbohydrate which is covalently bonded to gas-cylinder polypeptide. Such glycoprotein associations have been demonstrated in bacterial cell envelope preparations (ROTHFIELD and FINKELSTEIN, 1968), and have been shown to partly stabilise the wall architecture of Tolypothrix (HÖCHT et al., 1965). It is also of comparative interest that GOLDSTEIN et al. (1967) report that only 93% of the weight of the intact blue-green algal virus LPP-1 can be accounted for by protein and DNA. Although these authors propose that the remaining 7% might be protein destroyed through hydrolysis, they also suggest that it might represent a carbohydrate component.

4. Gas-vacuole synthesis and development

The present studies have not examined in detail the mode of synthesis of gas-cylinders. However, groups of gas-cylinders in association with lamellae were found at all stages of growth of A. flos-aquae D124 (see 'Results', Section 7B, p.49). Changes in the arrangement of lamellae were reflected in the appearance of the groups of gas-cylinders. Because of this intimate relationship which exists between the lamellae and the gas-cylinders, it is tempting to suggest that the gas-cylinders may be synthesised in association with the lamellae.

Gas-cylinder synthesis appears often to be restricted to particular sites in blue-green algal cells. For example, gas-cylinders were restricted in location to the regions immediately adjacent to cross-walls in Oscillatoria redekei (Fig. 14, p.66). Similarly, gas-vacuoles were restricted to the regions adjacent to the cell walls of Anabaena flos-aquae D124 cells grown at high light intensity (Fig. 12, p.53). The fact that gas-cylinders are closely packed in bundles, rather than evenly distributed in blue-green algal cells, again tends to suggest that these structures are synthesised at particular cellular sites. Whether these sites of gas-cylinder synthesis are associated with specialised biosynthesis by the lamella system cannot be ascertained with any certainty at the present time.

In this regard it appears to be of some value to make comparisons with the organisation of cells of Pelodictyon clathratiforme, a green bacterium. Cylindrical gas-vesicles are present in this species which appear homologous to the gas-cylinders of blue-green algae. However, lamellae are absent from this organism and, instead, chlorobium vesicles are the sites of photosynthesis (PFENNIG and COHEN-BAZIRE, 1967). In contrast to the closely packed bundles of gas-cylinders observed in the blue-green algae, the tubular gas-vesicles of this species are distributed throughout cells.

This difference may be related to the absence of a lamella system in P. clathratiforme and the consequent change in biosynthetic organisation imposed by its substitution by individual chlorobium vesicles.

When gas-cylinders redevelop following pressure-treatment it is uncertain whether gas-cylinder membrane fragments are used to rebuild gas-cylinders. It seems more probable that individual gas-cylinder membrane sub-units are re-employed in their construction. The differences in redevelopment times observed in cells of different ages (Fig. 37, p.99) indicate that their reconstruction is in some way related to the metabolic states of these cells. The redevelopment of gas-cylinders is certainly an active energy-requiring process as evidenced by their more rapid redevelopment in cells from the late exponential phase of growth and the inhibition of this process of 2-4-dinitrophenol. The accumulation of gas within gas-cylinders is not an energy-requiring process (WALSBY, 1969). The energy must therefore be required for either the reconstruction of gas-cylinders during the re-employment of gas-cylinder membrane sub-units, or, for the synthesis of gas-cylinder membrane sub-units de novo.

The slow redevelopment times of cells from the early exponential phase of growth probably reflected the net reduction in gas-vacuolation from 34% to about 20%, which occurs in the inoculum cells during the early exponential

phase of growth. These cells probably undergo considerable reorganisation of their metabolic pathways which requires high rates of protein biosynthesis and hence energy demand. When these demands are reduced in older cells it is probable that there is an increased availability of biosynthetic sites and energy for gas-cylinder re-synthesis. A comparable example of re-organisation of metabolic pathways was reported by TUOMINEN and BERNLOHR (1967) in Bacillus licheniformis. These authors observed that motility ceased for a 45 minute period during the exponential growth of this organism. Motility resumed when cellular rates of protein biosynthesis decreased.

An increase in the percentage of the cell volume of Anabaena flos-aquae D124 occupied by gas-cylinders was observed during the stationary phase of growth in this organism ('Results', Section 7B, p.49). An increase in the number of gas-vesicles present in Halobacterium was also observed by STOECKENIUS and ROWEN (1967) at this stage of growth. Other optical microscope observations on increased gas-vacuole development at particular growth stages are summarised in the 'Appendix' to the present thesis. The functional significance of the increase in gas-vacuole development in planktonic blue-green algae during the stationary phase of growth, is discussed below ('Discussion',

Section 5).

5. The functional significance of gas-vacuoles

As recent studies have shed some new light on the problem of the nature of gas-vacuoles, it seems of value to reconsider the functional significance of these structures. In order to prove that a particular structure performs a suggested function it is necessary for the hypothesis to fulfil the following four postulates (WILLIAMS and BARBER, 1965):-

- (i) The structure is necessary for the successful growth of the plant in competition with others.
- (ii) The structural provision is adequate for the requirements of the function it is supposed to serve.
- (iii) These requirements could not have been met with markedly greater economy by some other available means.
- (iv) Provision is not markedly more than is necessary to fulfil the functional requirements.

WILLIAMS and BARBER (1965) approached the problem of the functional significance of aerenchyma in plants by testing various hypothesis against the above four postulates. As this approach proved quite successful in the above case, it will be applied below to three theories of the function of gas-vacuoles in planktonic blue-green algae: gas storage

reservoir, light protection, and buoyancy theories.

The gas storage reservoir theory does not fulfil postulate (ii). The quantity of gas that could be stored within groups of gas-cylinders at one atmosphere pressure would be very small. In any case, this gas would rapidly equilibrate with gases present in solution in the surrounding medium (WALSBY, 1969). Thus gas-cylinders are not an adequate structural provision for gas storage.

The hypothesis that gas-vacuoles provide protection against high light intensities in blue-green algae at the surface waters of lakes was first proposed by LEMMERMAN (1910). REYNOLDS (1969) has observed increased gas-vacuolation in blue-green algae after they collect at the surface of the Shropshire Meres. The presence of gas-vacuoles in a blue-green algal cell will alter the refractile properties of the cell and it will reflect more light than a cell without gas-vacuoles (WALSBY, 1969). In the present studies ('Results', Section 20, p.96) it was shown that at inhibitory light intensities a lag phase in the growth of Anabaena flos-aquae D124 was induced by destruction of its gas-vacuoles.

These observations tend to suggest that gas-vacuoles might provide some photo-protection under certain conditions. However, if we consider light protection as the main function of gas-vacuoles and test this hypothesis against the four

postulates the evidence for the theory is very unsatisfactory. Results of laboratory culturing of gas-vacuolate blue-greens indicate that, in general, they are inhibited at comparatively low light intensities. However, these results must be accepted with caution because the spectral composition of the light employed in the laboratory is different from that of the light available to these organisms growing under natural conditions. Growth of Anabaena flos-aquae D124 was completely inhibited at light intensities above 15,000 lx ('Results', Section 8A, p.51) and ~~EBERLEY~~ (1965) reported that optimum growth of the species he studied was at 500 lx. Therefore, there are no definite indications that because gas-vacuolate species possess an effective photo-protective structure that they are more successful in competition with other blue-green algae which do not possess gas-vacuoles. Thus, postulate (i) appears to be contravened.

If the light protection function is tested against postulate (ii), it is again apparent that a postulate is contravened. Firstly, there are no indications from studies on Anabaena flos-aquae D124 ('Results', Section 8B, p.51) that gas-vacuolation increased during growth of the organism at inhibitory light intensities. The peripheral distribution of the gas-vacuoles in these cells suggests a possible functional role. However, their distribution

could be adequately explained by a change in the distribution of gas-cylinder synthetic sites associated with a redistribution of the lamella system at high light intensities. Secondly, gas-vacuoles seem unlikely to provide sufficient photoprotection by virtue of their scattering properties at the surface of lakes where light intensities of 50,000 lx may be recorded. Some additional photoprotection may be provided by the presence of carotenoids in gas-cylinder membranes as suggested by JOST and MATILE (1966). However, as discussed above ('Discussion', Section 3), recent studies have indicated that pigments are absent from gas-cylinder membranes.

The main function of gas-vacuoles, therefore, is unlikely to be photoprotection. The observations of LEMMERMAN (1910) and REYNOLDS (1969) that there was increased gas-vacuolation under conditions of high light intensity at the surface of lakes may be explained by assuming that the increased gas-vacuolation in these cells was associated with a reduction in their growth rate and not a direct response to light.

The hypothesis that the main function of gas-vacuoles is to lower the specific gravity of cells and thus provide a buoyancy function as suggested by STRODTMANN (1895), KLEBAHN (1895) and MOLISCH (1903), does not appear to

contravene any of the four postulates. The possession of gas-vacuoles appears to be a necessary condition for a planktonic existence, for those strains that contain them. Non gas-vacuolate blue-green algal strains of comparable size and type to these planktonic species are not observed in the plankton of deep lakes. The possession of gas-vacuoles does, therefore, appear to be necessary for the successful growth of these blue-green algae in competition with other species (postulate (i)).

The provision of gas-vacuoles in blue-green algae certainly appears to be adequate to lower the specific gravity of these cells and allow these organisms to maintain their position in the water column of lakes (postulate (ii)). The buoyancy experiments described in 'Results', Section 21B, (p.98) demonstrate that when gas-vacuoles redevelop they effectively lower the sedimentation rate of cells.

It is of comparative interest to consider how the sedimentation rates of blue-green algae could be reduced by other means than the possession of gas-vacuoles (postulate (iii)). A useful approach to this problem is to consider the expression of OSTWALD (1902):-

$$\text{Sedimentation rate (1) = } \frac{\text{Specific gravity of organism - medium specific gravity (2)}}{\text{Viscosity of water (3) x Form resistance (4)}}$$

It is apparent that the possession of gas-vacuoles by an organism will result in lowering (2) to small or negative values and reduce the sedimentation rate (1). The only other examples of algae which possess specific gravities less than that of water are some of the green algae such as Botryococcus braunii, which possesses numerous oil droplets (KLEBAHN, 1895). However, blue-green algae do not appear to show excess development of oil inclusions.

MARGALEF (1957) points out that (3) has been considered a qualitative property of water that can be assessed easily. He points out, however, that the viscosity that should be considered is a "structural viscosity", modified by the chemical and the electrostatic properties of the cell surface which could control the arrangement of water molecules in the vicinity of the cell. Until this suggestion is experimentally investigated, it is uncertain whether it has any effect on the sedimentation rates of blue-green algae.

HUTCHINSON (1967) reviews the subject of form resistance (4) in general terms. There appear to be three ways of increasing form resistance available to planktonic blue-greens: reduction in size, possession of a gelatinous sheath and increase in length to form a cylinder. It seems apparent from the comparatively few reports of planktonic blue-greens without gas-vacuoles, that they lower their

sedimentation rates by some of these mechanisms. The Synechococcus species reported by BAILEY-WATTS et al. (1968), consists of very narrow (0.7-0.9 μ m) long rods (40-60 μ m length). EBERLEY (1965) isolated Synechocystis parvala, which is very small (0.7-0.9 μ m), and occurs in pairs of cells with a very fine mucilagenous envelope.

There may be more examples of non gas-vacuolate blue-green algae than the few reports indicate. However, there seems to be no evidence that directly contravenes postulate (iii), that the possession of gas-vacuoles is the most economical adaptation available to blue-green algae occupying the planktonic niche.

WILLIAMS and BARBER (1961) stress that postulate (iv) is of great importance and is often neglected by physiologists. The fact that gas-vacuole redevelopment increases after sedimentation rates of cells has been reduced to very low values (Fig. 38, p.101), and the observations of decaying blooms floating at the surface of lakes, indicate that gas-vacuole development is greater than would appear necessary to adequately suspend cells in the water column of lakes. Thus the provision of gas-vacuoles appears markedly greater than is necessary and, at first sight, postulate (iv) appears to be contravened. It would appear, therefore, that it is important to

explain why the quantitative provision of gas-vacuoles is greater than is required to fulfil the buoyancy function. One possibility is that gas-cylinder synthesis and hence gas-vacuole development, is not adequately controlled by blue-green algae except during early exponential growth. In a lake when optimum nutrient requirements are available, the organism would apparently remain in the exponential phase of growth until nutrients are depleted. At this point there will be an increase in gas-vacuole development associated with a reduction in growth rate. The negative sinking rate associated with increased gas-vacuole development may be advantageous in that it will provide more rapid utilisation of dissolved nutrients than if the organism is at rest.

REYNOLDS (1969) reported that the flotation rates of colonies of Anabaena flos-aquae collected from the Shropshire Meres, were within the range $10-40 \mu\text{m} \times \text{sec}^{-1}$. Although it is ordinarily assumed that the sinking of a non-motile planktonic organism is disadvantageous, this is not necessarily true in turbulent water in which the sinking rate permits more rapid nutrient uptake, and so faster division than would be possible for a stationary cell (MUNK and RILEY, 1952). Although MUNK and RILEY (1952) were concerned with the positive sinking rates of diatoms,

their generalisation seems applicable to the flotation rates of gas-vacuolate blue-green algae. Therefore, under conditions of high turbulence, the apparent "excess" gas-vacuole development exhibited by these organisms may be advantageous, because it will result in more rapid nutrient uptake. An increased nutrient uptake rate will also be a consequence of the increase in the ratio of surface area of cell to non gas-vacuolate cell volume resulting from increased gas-vacuole development.

REYNOLDS (1967) has shown that when turbulence falls to a minimum, the gas-vacuolate blue-green algae float to the surface of the Shropshire Meres. This sudden accumulation at the surface does not appear to be a result of any increase in the division rate of the organisms, and is simply a consequence of their negative sinking rates. LUND (1965) pointed out the apparent disadvantages to these organisms of surface exposure to high light intensity and temperature. The observations that they release phycocyanin and accumulate at the sides of lakes, indicate that surface accumulation has deleterious consequences to the individual organism.

However, if one examines this phenomenon in a wider context, it becomes apparent that surface bloom formation may be advantageous to the aquatic ecosystem. The conditions associated with surface bloom formation such as

high temperature and lack of turbulence, will also result in the establishment of a thermocline. Under these conditions nutrient exchange between surface waters and the nutrient rich hypolimnion will be prevented. The breakdown of blue-green algae in the littoral may provide a significant source of nutrients, phosphorus in particular. HUTCHINSON (1941) has shown that in Linsley Pond, Connecticut, ~~that~~ the phosphorus of the epilimnion is replaced from the littoral about once every three weeks during the summer. Similarly, the high concentration of blue-greens per unit volume in the surface waters will provide ideal conditions for effective predation by herbivores, resulting in rapid nutrient re-cycling.

6. The control of blue-green algal blooms by destruction of gas-vacuoles

It is apparent that the success of the planktonic blue-green algae in lakes and reservoirs is in great measure due to their ability to float. If one seeks effective methods of controlling these organisms, an obvious target is the gas-vacuoles, because these structures fulfil the buoyancy function. Two methods of destroying gas-vacuoles appear available: the selective destruction of the gas-vacuole membrane, and the disruption of gas-vacuoles by pressure treatment.

The former method does not seem very practical in the light of the present studies, which indicate the proteinaceous nature of the gas-cylinder membranes ('Discussion', Section 3). Proteolytic enzymes are not in general very specific and structural protein "in vivo" is very resistant to disruption. It is possible that some organisms may have evolved enzymes to disrupt viral coat protein as part of a cellular defence mechanism. If one accepts that this protein is homologous to gas-cylinder membrane protein, then these hypothetical enzymes should be sought. However, viral coat protein does appear to be resistant to digestion as evidenced by the stability of viruses within the gut of aphid species (SMITH, 1965). Therefore, the search for a selective agent to disrupt gas-cylinder membranes does not appear to be a very fruitful one.

Disruption of gas-vacuoles by pressure treatment appears to be a suitable approach to the controlling of blue-green algal blooms. If this method is applied, it is important that it is timed to coincide with maximum development of the blue-green algae. At this stage most of the available nutrients will have been imbibed by these organisms. Destruction of their gas-vacuoles will result in the sedimentation of the organisms into the hypolimnion and removal of available nutrients for a recurrent bloom.

If nutrients are not removed, and, in the absence of competition from blue-green algae, it is probable that other organisms would develop. There are other conditions for the successful application of this method of controlling blue-green algal blooms. Firstly, it must be applied during conditions of stratification and low wind speeds, otherwise the organisms might be resuspended by turbulence. Secondly, the treatment must be reapplied on successive days to prevent effective gas-vacuole redevelopment ('Results', Section 21).

In conclusion to the present studies, it is hoped that the hypothesis that gas-vacuoles have a viral origin will prove a fruitful basis for future studies on these unique structures. The wealth of our knowledge on viruses and the numerous techniques available to investigate them suggests that this hypothesis could be adequately tested. If the viral origin of gas-cylinders was proved, then the problems of gas-cylinder construction, synthesis and development would become special examples of viral development and justly belong within the field of virology.

ACKNOWLEDGEMENTS

As the thesis covered a broad spectrum of biological topics it was necessary to seek advice and cooperate with several people. I wish to thank above all my supervisor, Dr. B. A. Whitton, for his encouragement and helpful advice during my postgraduate training, and for stimulating my interest in the problems presented by the blue-green algae when I was an undergraduate. I cooperated with Mr. Alan Peat in the employment of the electron microscope, in most of the fine structural studies. I am indebted to him for the interest he showed in my research problem and the training he has given me. I wish to thank Dr. M. Weston of the Department of Chemistry, for permission to use the massspectrometer. Miss Margaret Berryman typed the thesis, and during my three years at Durham has provided me with excellent secretarial assistance. Mrs. K. Hill assisted in the labelling of the plates, and, in conjunction with Mr. G. Bainbridge, helped in numerous small ways in the culturing of the research organisms. Finally, I wish to thank Professor D. Boulter for permission to carry out my project in his Department, and Professor D. H. Valentine for assisting me to obtain a Science Research Council Studentship.

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APPENDIX

A review of the optical microscope observations on
gas-vacuoles in blue-green algae

The present thesis has been concerned with the nature and functional significance of gas-vacuoles in planktonic blue-green algae and has reviewed some of the optical microscope observations of gas-vacuoles in these organisms. However, there are a considerable number of early references to gas-vacuoles being present in non-planktonic species of blue-green algae. As these observations are of some comparative interest they are briefly summarised below. A complete reference list of all the gas-vacuolate species (planktonic and non-planktonic) reported by GEITLER (1932), is given at the end of this review.

A problem arises in the interpretation of some of the references because authors do not invariably use pressure-treatment to characterise gas-vacuoles. The reported gas-vacuoles may represent pink, refractile nucleoplasm (SMITH and PEAT, 1967a), or pink, refractile vacuoles (intralamellar vesicles) which occur in the older cells of Anabaena flos-aquae D124 ('Results', Section 12, p.56). The term "refractile" employed in these above descriptions is used to mean that the structures have a refractile index greater than that of their surrounding medium.

LANG (1968) and PFENNIG (1967) describe individual gas-vacuoles as highly refractile. This use of the term "refractile" merely implies that gas-vacuoles possess a different refractive index from that of the surrounding medium. However, to avoid confusion with other cell inclusions it is more exact to describe gas-vacuoles as structures of low refractive index within a medium of higher refractive index. When observed with the normal transmission optical microscope, gas-vacuoles appear as reddish structures with black margins. This appearance is different from all other cell inclusions and, on its own, is probably an adequate diagnostic feature with which to characterise gas-vacuoles. Thus, although some of the following references must be accepted with caution, because gas-vacuoles were not characterised by pressure treatment, most references are probably valid, if the authors were familiar with the distinctive optical properties of gas-vacuoles.

Gas-vacuoles were reported to be present in sapropelic mud-inhabiting forms of blue-green algae by BORNET and THURET (1880), VAN GOOR (1918) and VAN GOOR (1925). Oscillatoria guttullata, O. limosa, and O. chalybea, were observed in the mud examined by VAN GOOR (1918). Gas-vacuoles occupied positions adjacent to cross-walls in the latter two species and O. guttullata was reported to possess

single, perfectly spherical gas-vacuoles in some cells. Saproelic Oscillatoriaceae containing gas-vacuoles, were also reported by LAUTERBORN (1915) and BÖCHER (1949).

BÖCHER (1949) conducted a thorough survey of the mud at the bottom of a small brackish-water lagoon in N. W. Zealand. He reported that of the 44 species of blue-green algae which were present, seven species were gas-vacuolate. The gas-vacuolate species were, Oscillatoria putrida, O. fulgens, O. mirabilis, Pseudanabaena biceps, P. galeata, Synechococcus cedrorum and Synechocystis miniscula. The Oscillatoria species showed gas-vacuole development which was restricted to the regions of cells adjacent to cross-walls. Some of the associated bacteria showed similar gas-vacuole development to the blue-green algae. KOLKWITZ (1928) also observed gas-vacuoles in bacteria associated/sapropelic blue-greens with

Other references to gas-vacuoles being present in blue-green algae which are not truly planktonic are restricted to the reports of gas-vacuolate hormogonia. Calothrix anomala (Cambridge Culture Collection number 1410/4) was seen to detach its terminal cells to form gas-vacuolate hormogonia (R. V. SMITH, personal observations). BORNET and THURET (1880) were the first to report this type of development in Nostoc linckia and observed gas-vacuoles

("gros granules rougeâtres tres refringent") in hormogonia which developed from germinating spores.

LEMMERMAN (1898) observed gas-vacuoles in hormogonia of Phormidium ambiguum, and suggested that they played an important role in the dispersal of this species.

LEMMERMAN (1901) reported a similar development of hormogonia in Lyngbya aestuarii. GORBUNOVA (1960) cultivated Amorphonostoc punctiforme on agar plates and observed hormogonia development.

Initially, hormogonia cells were densely packed with gas-vacuoles. However, gas-vacuoles started to disappear after 12 hours and had completely disappeared by the fifth day.

It is apparent from the many reports of gas-vacuole development in non-planktonic species that gas-vacuole development may only last a short time. In contrast, gas-vacuole development in planktonic species appears to be a reasonably constant feature of these organisms. Apparent exceptions to this generalisation are the observations of non-gas-vacuolate cells in colonies of Microcystis flos-aquae (HORTOBAGYI, 1954) and Microcystis aeruginosa (JOST and ZEHNDER, 1966). It also is apparent from examining the list of species reported to contain gas-vacuoles by GEITLER (1932), shown below, that only in planktonic species are gas-vacuoles employed as a diagnostic character in the identification of blue-green algae. The presence of gas-

vacuoles in Microcystis aeruginosa was used as a diagnostic feature in the identification of this species by GEITLER (1932). However, the observation of the absence of gas-vacuoles in the strain of the organism examined in Part I of the present thesis shows the danger of relying on one diagnostic character to differentiate species.

The following species are reported by GEITLER (1932) to contain gas-vacuoles. Those species which are definitely not plankton forms are indicated as "+". Species where gas-vacuoles are a diagnostic character in their identification are indicated as "*".

Anabaena aphanizomenoides, *A. baltica*, *A. bergii*, *A. bolochonzewii*, *A. circinalis*, *A. elliptica*, *A. flos-aquae*, *A. halbfassi*, *A. levanderi*, *A. macrospora*, *A. planctonica*, *A. scheremetievi*, *A. spiroides*, *A. utermohli*, *A. variabilis*, *A. wernerii*.

Anabaenopsis arnoldii, *A. elenkini*, *A. milleri*, *A. nadsonii*.

Aphanizomenon flos-aquae, *A. ovalisporum*.

*Aphanocapsa sideroderma**.

*Aphanothece pulverulenta**.

*Aulosira planctonica**.

*Chroococcus cumulatus**.

*Coelosphaerium dubium**, *C. naegelianum**; *C. natans**.

*Gloeotrichia echinulata**, *G. natans*.

Haliarachne lenticularis.

Lyngbya aestuarii, *L. borgerti*, *L. hieronymusii*, *L. pseudospirulina*.

*Merismopedia marssonii**, *M. trolleri**.

*Microcystis aeruginosa**, *M. aphanothecioides*⁺, *M. elabens*,
M. elabentioides⁺, *M. firma**, *M. flos-aquae**, *M. fusca*⁺,
*M. marginata**, *M. protocystis**, *M. pseudofilamentosa**,
*M. scripta**, *M. viridis**.

*Nostoc kihlmani**, *N. planctonicum**.

*Oscillatoria agardhii**, *O. amphigranulata*⁺, *O. guttulata*⁺,
O. lacustris, *O. lauterbornii*⁺, *O. mougeotii*⁺, *O. planctonica*,
O. prolifica, *O. raciborskii**, *O. redekei*, *O. rosea*,
O. rubescens, *O. trichoides*.

Pelagothrix clevei.

Phormidium ambiguum⁺.

*Spirulina pseudovacuoolata**.

(TOTAL: 68 species).

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Comparative Structure of the Gas-vacuoles of Blue-green Algae

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Received January 27, 1967

Summary. An investigation was made of 5 species of blue-green algae reported to contain gas-vacuoles. All organisms were grown and harvested under standard conditions. Gas-vacuoles were characterised as reddish structures which are destroyed by applying pressure. Using a simple direct preparation technique gas-cylinders were observed with the transmission electron microscope in gas-vacuolate cells. Gas-vacuoles were present in the strains of *Anabaena flos-aquae*, *Gloeoctrichia echinulata* and *Oscillatoria agardhii* studied and absent from *Microcystis aeruginosa* and *Nostoc linckia*. The reddish, refractile central area of *N. linckia* and *M. aeruginosa* cells was tentatively identified as nucleoplasm. Gas-vacuoles are collections of gas-cylinders 70 μ wide, which in *A. flos-aquae* and *G. echinulata* are clearly bounded by photosynthetic lamellae and associated with α -granules. The presence of bounding photosynthetic lamellae in these species is suggested as a causal factor of the unusual optical properties of their gas-vacuoles. The range of lengths of gas-cylinders in *G. echinulata* and *O. agardhii* is from 100 μ to 500 μ and in *A. flos-aquae* it is from 100 μ to 1300 μ . The percentage of cell volume occupied by gas-vacuoles was estimated by direct measurement. In *A. flos-aquae* and *G. echinulata* it was 22%. In *O. agardhii* gas-cylinders were not clearly associated with photosynthetic lamellae and α -granules and occupied 39% of cell volume. Gas-cylinder membranes showed reasonable preservation in KMnO_4 and excellent preservation in OsO_4 . The widths of membranes after treatment with these two fixatives was 3 μ and 2 μ respectively.

Gas-vacuoles of blue-green algae are characterised with the light microscope as reddish structures of low refractive index which are destroyed by applying pressure. The early literature is reviewed by FOGG (1941).

HOPWOOD and GLAUERT (1960), SUN (1961), CHAPMAN and SALTON (1962), and GIESY (1962) undertook fine structure studies of species of blue-green algae and reported irregular lacunae which were identified as gas-vacuoles. These authors did not attempt to correlate their observations of fine structure with light microscope characterisation of gas-vacuoles. BOWEN and JENSEN (1965), JOST (1965), JOST and MATILE (1966), and JOST and ZEHNDER (1966) made light and electron microscope studies of gas-vacuolate species and characterised the gas-vacuoles as three-dimensional packed arrays of electron transparent cylinders. A disadvantage of the published accounts is the absence of any attempt to relate the gas-vacuoles to other features of cell structure.

The present paper reports the results of an investigation of 5 species reported to contain gas-vacuoles by GEITLER (1932). Observations were made on normal vegetative cells of these species. The presence of gas-vacuoles in heterocysts, akinetes and other cell types will be reported in a later paper. The terminology of PANKRATZ and BOWEN (1963) was used throughout the paper to describe the cellular morphology.

Material and Methods

The details of the methods of cultivation of the organisms and the preparation procedures for electron microscopy are in general the same as those used by PEAT and WHITTON (in press).

Species Studied. Details of the origins of the cultures used are given in Table 1.

Table 1. *Details of cultures studied*

Organism	Source	Culture no. at Source	Culture no. at Durham
<i>Anabaena flos-aquae</i>	Westfield College, London		124
<i>Gloetrichia echinulata</i>	Cambridge	1432/1	126
<i>Oscillatoria agardhii</i>	F.B.A., Windermere		132
<i>Nostoc linckia</i>	C.S.S.R. culture collection	125	130
<i>Microcystis aeruginosa</i>	Cambridge	1450/1	127

Cultivation. The organisms were grown in shake culture in modified ASM-1 medium (GORHAM *et al.*, 1964) with twice the level of phosphate, at a light intensity of 2000 metre candles and a temperature of 20°C. Cells of each organism were harvested from the late log phase of growth when they are in a healthy, actively-growing state.

Direct Preparation Technique. Cells were fixed on a grid with OsO₄ vapour for 30 seconds. The preparation was then dried in an oven for 10 minutes at 40°C. After washing in distilled water and subsequent drying, the cells were first examined with a light microscope and then with an EM 6 B electron microscope.

Preparation of Sectioned Material. Since gas-vacuoles are destroyed by centrifugation, cells were concentrated on Millipore filters. The filters were placed directly in the fixing fluid and dissolved in the subsequent preparation procedures. Two methods of fixation were employed: 2% KMnO₄ for 1 hour at room temperature and 1% OsO₄ buffered at pH 6.1 for 3 hours at room temperature (PANKRATZ and BOWEN, 1963). Fixed material was dehydrated through an ethanol series and embedded in Araldite. Sections were cut on an LKB ultratome and stained with lead citrate (REYNOLDS, 1963) before examination with an EM 6 B electron microscope.

Results

1. *Light microscope observations*

In the log phase of growth all normal vegetative cells of *A. flos-aquae*, when viewed at low magnification, contain black structures. At high magnification these non-refractile structures appear reddish with black

margins. They are distributed between the peripheral and central parts of the cell. Cells from trichomes of *G. echinulata* contain large structures which sometimes occupy almost the total volume of the cells. These have optical properties similar to those found in *A. flos-aquae*. Areas of the cell which appear uniformly green and occasionally containing small irregularly shaped reddish bodies are interspersed with these structures. Cells of *O. agardhii* trichomes have a reddish, granular appearance. No discrete reddish structures of the type described for *A. flos-aquae* and *G. echinulata* can be observed. Cells of *M. aeruginosa* and *N. linckia* have red, refractile central areas which do not appear black at low magnification.

The reddish structures present in the cells of *A. flos-aquae* and *G. echinulata* and the reddish appearance of *O. agardhii* cells are destroyed by centrifugation at a pressure of 3.72 atmospheres. The cells have a translucent green appearance after pressure treatment. No changes were observed in the appearance of *M. aeruginosa* and *N. linckia* cells after such treatment.

2. Electron microscope observations

a) Distribution of gas-cylinders

Fig.1 shows a direct preparation of *A. flos-aquae*. The reddish, non-refractile areas of cells remain visible after fixation with OsO_4 vapour. The position of these cells on the grid was determined with the light



Fig.1. Direct preparation of *A. flos-aquae* showing distribution of gas-cylinders. OsO_4 fixation. Mag. 12,000 \times . The symbols used for all photographs are as follows: *ed* electron-dense body; *med* membrane surrounding electron-dense body; *f* nucleoplasmic fibril; *g* gas-cylinder; *gm* gas-cylinder membrane; *l* lamellae; *pb* polyhedral body; α α -granules

microscope and they were then examined with the electron microscope. The bundles of gas-cylinders correlated with the presence of reddish, non-refractile areas and this technique provides a simple test for the presence of gas-cylinders. Gas-cylinders were observed in preparations

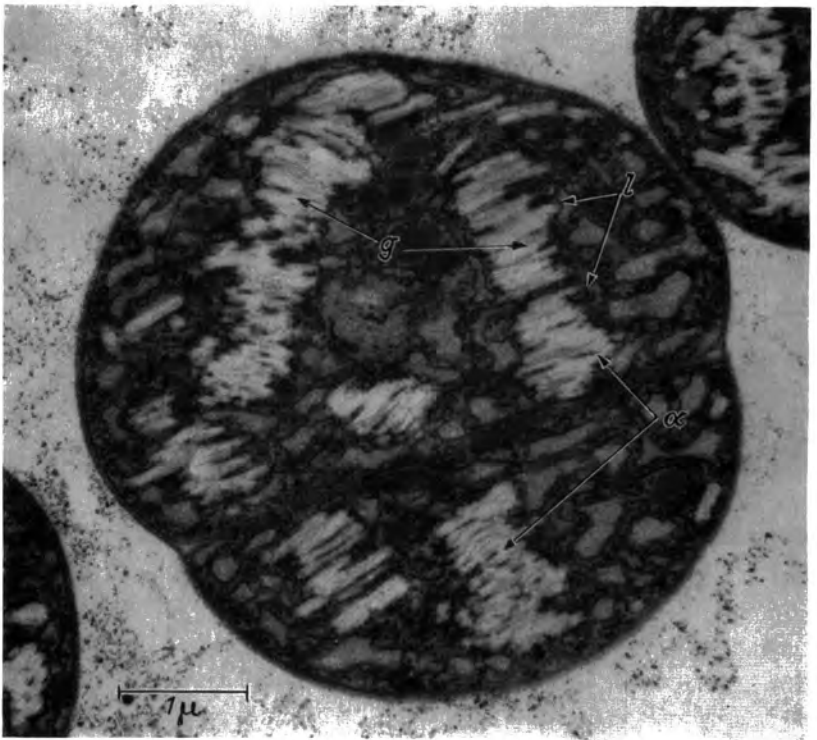


Fig. 2. General cell view of *A. flos-aquae* showing distribution of gas-cylinders and their association with lamellae and α -granules. KMnO_4 fixation. Mag. 17,500 \times

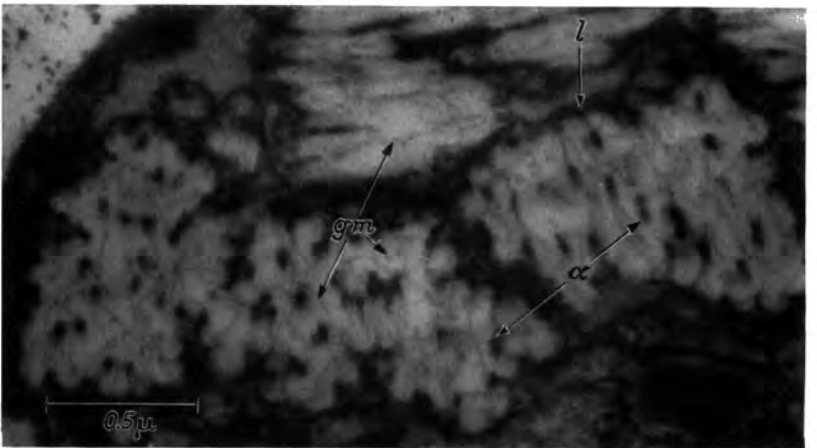


Fig. 3. Appearance of gas-cylinders in transverse sectional view in *A. flos-aquae*. KMnO_4 fixation. Mag. 41,000 \times

of *A. flos-aquae*, *G. echinulata* and *O. agardhii* but were absent from those of *M. aeruginosa* and *N. linckia*.

Figs. 2, 4 and 5 show sections of the vegetative cells of *A. flos-aquae*, *G. echinulata* and *O. agardhii* respectively. The distribution of gas-cylinders in *A. flos-aquae* and *G. echinulata* is identical to the distribution of the reddish, non-refractile areas observed under the light microscope.

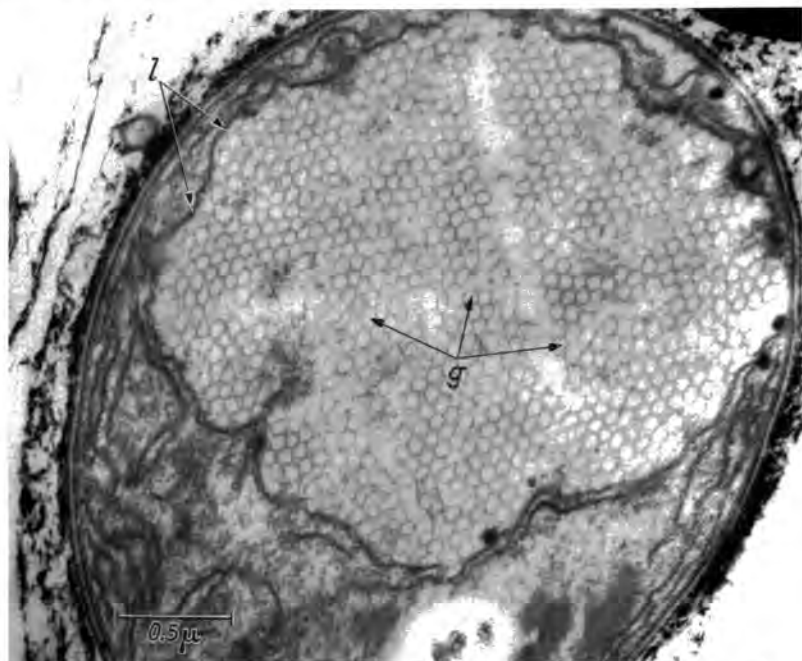


Fig. 4. Transverse section of *G. echinulata* trichome showing a collection of gas-cylinders and the associated lamellae. OsO₄ fixation. Mag. 31,000 \times

b) Relationship of gas-cylinders to general cell structure

In *A. flos-aquae* and *G. echinulata* the bundles of gas-cylinders are surrounded by photosynthetic lamellae. This is shown in Figs. 2, 3, 4, 6, 7 and 8. This relationship between gas-cylinders and photosynthetic lamellae is not clearly shown by *O. agardhii*. Also present in association with the gas-cylinders are granules which on the basis of their size and shape are identified as α -granules. These granules have been characterised as polyglucoside by GIESY (1964). They are best preserved by KMnO₄ fixation and are shown in Figs. 2, 3, 7 and 8.

c) Percentage volume occupied by gas-cylinders

KLEBAHN (1922) estimated that the volume of cell occupied by gas-vacuoles in *G. echinulata* was 0.8%. He used a suspension of *G. echinulata*

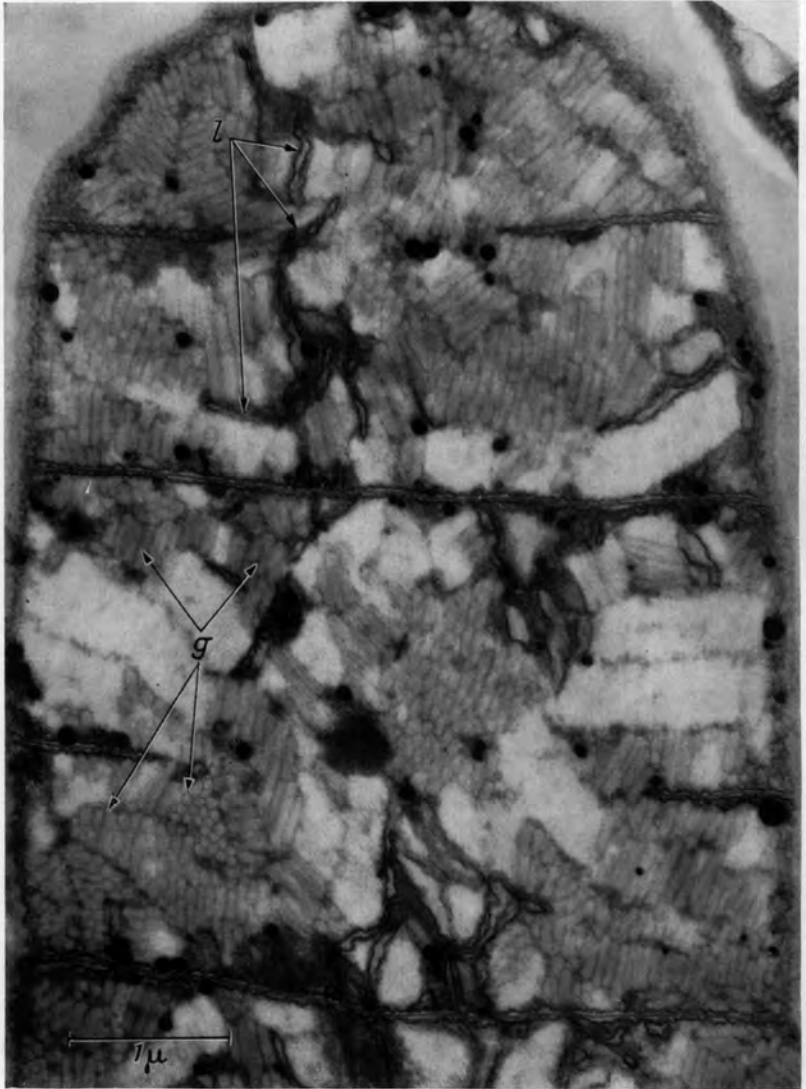


Fig. 5. Longitudinal section of a dividing *O. agardhii* trichome showing distribution of gas-cylinders. OsO_4 fixation. Mag. 22,000 \times

in water and observed a decrease in volume of the cells when sufficient pressure was applied to destroy the gas-vacuoles. A direct estimate of the percentage volume of vegetative cells occupied by gas-vacuoles was made by examining a series of micrographs of sections from different vegetative cells. The value obtained by this method for *A. flos-aquae* and *G. echinulata* was 22%. The average percentage volume occupied by

gas-vacuoles in *O. agardhii* was 39%. It must be noted that KLEBAHN (1922) used as his material a mixed population of cells which would include heterocysts, spores and possibly bacteria.

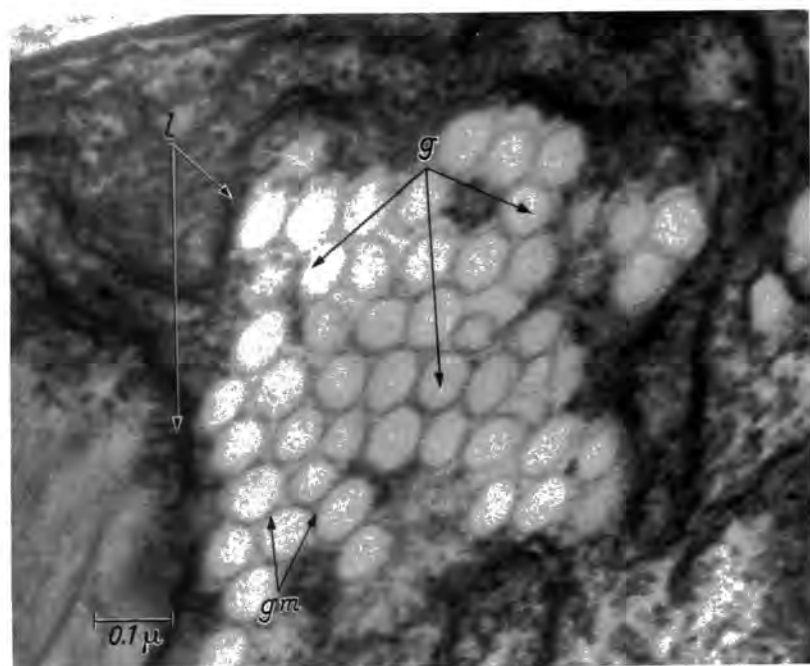


Fig.6. Appearance of gas-cylinders in transverse section in *A. flos-aquae*. OsO_4 fixation. Mag. 103,000 \times

d) Fine structure of gas-cylinders

The gas-cylinders in all organisms studied have a standard width of 70 $\text{m}\mu$. The range of length is 100 $\text{m}\mu$ to 500 $\text{m}\mu$ in *G. echinulata* and *O. agardhii*. *A. flos-aquae* has a wider range in lengths of gas-cylinders (100 $\text{m}\mu$ —1300 $\text{m}\mu$) than other organisms examined (see Table 2 and Fig. 1). The width of the gas-cylinder membrane is 2 $\text{m}\mu$ after OsO_4 fixation. This confirms the result of BOWEN and JENSEN (1965). These authors together with JOST (1965), and JOST and MATILE (1966) report that the gas-cylinder membrane is destroyed by KMnO_4 fixation. Figs. 2, 3, 7 and 8 show the appearance of gas-cylinder membranes after KMnO_4 fixation. Partial destruction of the membrane is shown in *A. flos-aquae* (Fig. 3). Disorganisation of the gas-cylinders is particularly evident in cross-sectional view. A comparison of their appearance after KMnO_4 fixation with that obtained by OsO_4 can be made by studying Figs. 3 and 6. Good preservation of gas-cylinder membranes in *G. echinulata* is shown

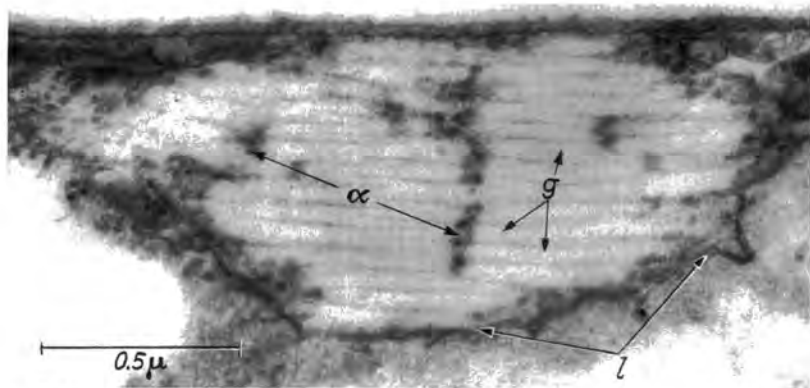


Fig. 7. Appearance of gas-cylinders in longitudinal section in trichome of *G. echinulata* and their association with lamellae and α -granules. KMnO_4 fixation. Mag. 47,500 \times

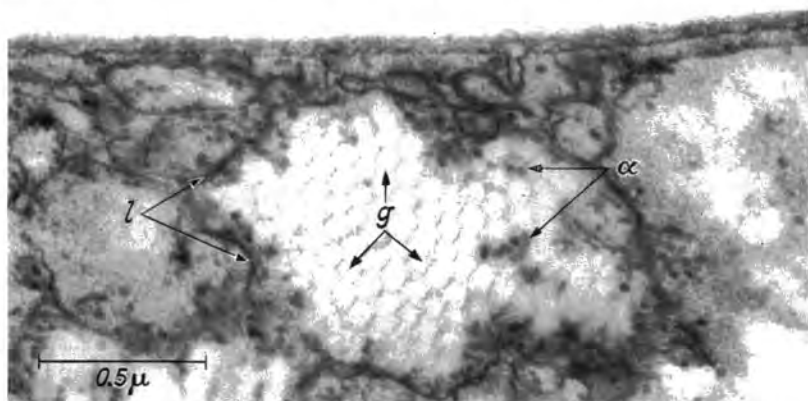


Fig. 8. Appearance of gas-cylinders in cross-section in trichome of *G. echinulata*. KMnO_4 fixation. Mag. 53,500 \times

Table 2. Summary of electron microscope observations

Organism	Width of gas-cylinders in $m\mu$	Range of lengths of gas-cylinders in $m\mu$	Mean % volume of cells occupied by gas-vacuoles
<i>A. flos-aquae</i>	70	100–1300	22
<i>G. echinulata</i>	70	100–500	22
<i>O. agardhii</i>	70	100–500	39
<i>Aphanizomenon flos-aquae</i> (BOWEN and JENSEN, 1965)	75	100–1000	—
<i>Oscillatoria rubescens</i> (JOST and MATILE, 1966)	65	210–430	—
<i>Microcystis aeruginosa</i> (JOST and ZEHNDER, 1966)	c. 75*	c. 100–500*	—

* Estimated from micrograph in this publication.

after KMnO_4 treatment. Fig. 7 shows gas-cylinders in longitudinal section; Fig. 8 shows them in transverse section. The width of the membrane after KMnO_4 fixation is 3μ .

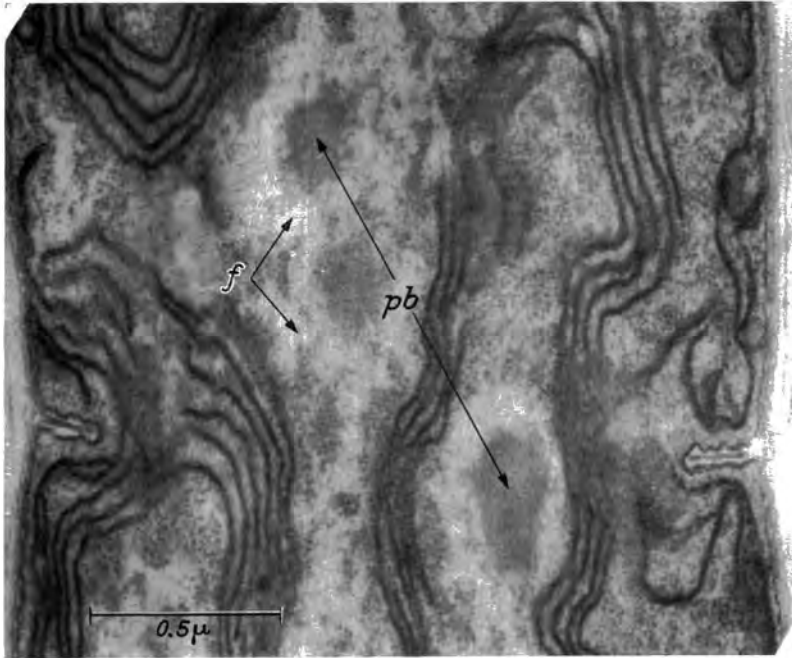


Fig. 9. Appearance of central area of a *N. linckia* cell showing nucleoplasmic fibrils associated with polyhedral bodies. OsO_4 fixation. Mag. $52,000\times$

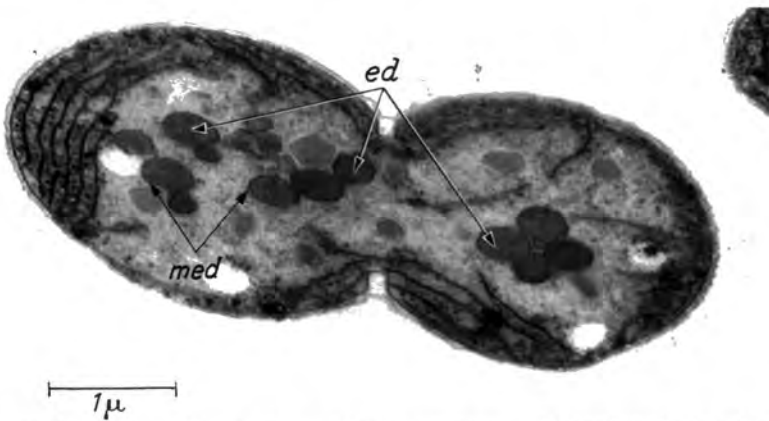


Fig. 10. Appearance of central area of a *M. aeruginosa* cell showing electron-dense bodies partially bounded by membranes. KMnO_4 fixation. Mag. $17,500\times$

e) Fine structure of the reddish central areas in
N. linckia and *M. aeruginosa*

Fig. 9 shows the appearance of the central area of *N. linckia*. This consists of an anastomosing network of fibrils 3 m μ in width which surround the polyhedral bodies. Similar organisations of fibrils were characterised as the Feulgen-positive nucleoplasm in *Symploca muscorum* by PANKRATZ and BOWEN (1963), and in *Nostoc muscorum* by RIS and SINGH (1961). The central region of *M. aeruginosa* is occupied by bodies of varying electron density with no evidence of fibrillar nucleoplasm. The denser bodies are partially enclosed by membranes (see Fig. 10). These bodies bear some resemblance to the electron dense bodies described by HALL and CLAUS (1965) in *Synechococcus oceanica*. However, they were not membrane bounded and were of uniform electron density.

Discussion and Conclusions

Cells of the strains of *M. aeruginosa* and *N. linckia* examined contain reddish refractile central areas which are not destroyed by applying pressure. The fine structure of this area differs in the two species, but the central area in both was tentatively characterised as nucleoplasm. It was concluded that gas-vacuoles are absent from these strains. The presence of gas-vacuoles in *M. aeruginosa* is used by GETTLER (1932) as a diagnostic character in the identification of this species. The absence of gas-vacuoles from the cells of *M. aeruginosa* examined throws doubt upon the use of the character "presence or absence of gas-vacuoles" as a diagnostic feature in the identification of blue-green algal species. Gas-vacuoles are reported to be present in *N. linckia* by CANABAEUS (1929) and by GORBUNOVA (1960). It seems possible that the gas-vacuoles reported to be present in this species are in fact simply the reddish, refractile nucleoplasmic regions described in this paper.

It was concluded that gas-vacuoles are present in the strains of *A. flos-aquae*, *G. echinulata* and *O. agardhii* examined. The feature common to the fine structure of all the gas-vacuoles of species studied is the presence of gas-cylinders 70 m μ in width, organised in bundles. In *O. agardhii* the gas-vacuoles do not appear as the discrete non-refractile reddish structures which are characteristic of *A. flos-aquae* and *G. echinulata*. As the gas-vacuoles in these latter species are surrounded by photosynthetic lamellae, it is tentatively suggested that their peculiar optical properties may be due to the presence of bounding photosynthetic lamellae containing photosynthetic pigments.

BOWEN and JENSEN (1965) characterise the gas-cylinder membrane as a half-unit membrane. In common with JOST (1965) they state that the membrane is destroyed by KMnO_4 . This result contradicts the interpretation of gas-cylinder membranes as half-unit membranes because

typical unit membranes are preserved by KMnO_4 fixation. The gas-cylinder membranes of the organisms studied in this paper showed reasonable preservation in KMnO_4 . Recently considerable doubt has been cast on the unitary concept of membrane structure. The unit membrane concept of ROBERTSON (1959) was based on the argument that all membranes would be essentially of similar size (7.5 $\text{m}\mu$) and structure, *i. e.*, a bimolecular leaflet of lipid molecules sandwiched between two protein layers. The studies of BENEDETTI and BERTOLINI (1963) and SJÖSTRAND (1963) show that even in the same cell type the chemical composition and the ultrastructure of membranes vary significantly. SJÖSTRAND (1963) reports in mouse kidney tissue the following widths of membranes after OsO_4 fixation: mitochondrial membranes (5 $\text{m}\mu$), plasma membranes (9—10 $\text{m}\mu$) and Golgi membranes (6 $\text{m}\mu$). The width of the gas-cylinder membrane is 2 $\text{m}\mu$ after OsO_4 fixation. Clearly the gas-cylinder membrane is best regarded as an unusual membrane having distinctive properties and to attempt to classify it as a half-unit membrane is of little predictive value.

FRITSCH (1945) states that it is not clear whether material possessed of gas-vacuoles is actually in a healthy condition and whether cell division continues. Our studies confirm those of PRINGSHEIM (1966) who found that gas-vacuoles are characteristic of young actively growing cells. Further functional studies on these unique organelles will give us increased insight into the physiology of the procaryotic cell.

Acknowledgements. The authors are very grateful to Mr. A. E. WALSBY of Westfield College for kindly supplying a culture of *A. flos-aquae* and to Dr. J. W. G. LUND and Mr. G. JAWORSKI of the F. B. A., Windermere, for supplying a culture of *O. agardhii*. They are indebted to Professor D. BOULTEB, Mr. G. H. BANBURY and Dr. B. A. WHITTON for helpful criticism and Miss M. BERRYMAN for secretarial help. This work was carried out while one of the authors (R. V. S.) was in receipt of a grant from S. R. C.

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Growth and Gas-Vacuole Development in Vegetative Cells of *Anabaena flos-aquae*

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Received May 6, 1967

Summary. Gas-vacuoles consisting of collections of gas-cylinders, and continuity of the plasma membrane with lamellae, are observed at all stages of growth of *Anabaena flos-aquae*. In day + 4 cells, increased numbers of invaginations of the plasma membrane are observed and the lamellae tend to lie parallel to the cell wall. Some evidence is presented which suggests an associated synthesis of α -granules and gas-cylinders by lamellae. Features of older cells are increased numbers of gas-cylinders, α -granules, structured granules, and large intralamellar vesicles which appear to correlate with the presence of pinkish vacuoles as observed with the light microscope. The mean percentage of the volume of cells occupied by gas-vacuoles is about 20% during exponential growth when the doubling time is 56.5 hours, increasing to 34% in day + 24 cells when growth is stationary.

The gas-vacuoles of blue-green algae are characterised as reddish structures, consisting of collections of gas-cylinders, which are destroyed by applying pressure (SMITH and PEAT, 1967). Such structures were described in cells taken from the late exponential phase of growth of *Anabaena flos-aquae*, *Gleotrichia echinulata* and *Oscillatoria agardhii*. In the present study attention is focussed on the arrangement of lamellae and on the distribution of interlamellar structures in vegetative cells of *A. flos-aquae* sampled at all stages during growth.

Observations which relate gas-vacuoles to stages in growth are meagre and often contradictory. FRITSCH (1945) states that it is not clear whether material possessed of pseudo-vacuoles (gas-vacuoles) is actually in a healthy state and if cell division continues. HORTOBAGYI (1954) reported the development of gas-vacuoles in the older, peripheral cells of *Microcystis flos-aquae* colonies, whilst JOST and ZEHNDER (1966) demonstrated the presence of both gas-vacuolate and non gas-vacuolate cells in *Microcystis aeruginosa*. SERBANESCU (1966) and PRINGSHEIM (1966) observed that gas-vacuoles characterise young, actively dividing cells of *Gleotrichia natans* and *Oscillatoria agardhii* respectively.

Material and Methods

Anabaena flos-aquae was kindly supplied by the Botany Department, Westfield College, London, in 1966. The organism was isolated originally from Windermere and subsequently maintained at the Freshwater Biological Association Laboratories.

The Durham University culture number is 124. The culture is unialgal but is contaminated with bacteria.

Cultivation. The organism was grown in shake culture in 250 ml flasks containing 100 ml of ASM-1 medium (GORHAM *et al.*, 1964). Inoculum material consisted of 5 ml of a 38 day old culture (day + 38) which had been grown under conditions identical to those used for the growth experiments. All experiments were carried out at 20°C, 2000 lux, in tanks illuminated from below by a bank of fluorescent tubes. The flasks were shaken 64 times per minute through a horizontal movement of 3 cm. For repeat experiments the inoculum consisted of day + 38 material which had been stored in standing culture for a few days at 20°C, 750 lux.

Growth Measurements. Material was harvested on days + 2, 4, 8, 12, 16, 20, 24 and 28. Cells were removed by centrifugation, washed with distilled water, transferred to vitreosil crucibles and dried for 24 hours at 105°C.

Light Microscope Observations. These were made at daily intervals throughout the experiment on material taken from replicate flasks. The observations were confirmed by running 2 repeat experiments. Cultures older than 28 days were also examined.

Preparation of material for electron microscopy. The details of the preparation procedures are reported by SMITH and PEAT (1967). Samples were taken from replicate flasks on days + 2, 4, 8, 12 and 24. In order to characterise the features of older cells which form the inoculum day + 50 material was also embedded. The presence of relatively large numbers of bacteria in days + 2, 4 and 50, no doubt contributed to the difficulties experienced in embedding these samples. Further samples were therefore taken on days + 2 and 4, during the repeat growth experiments. Two methods of fixation were employed: 2% KMnO_4 for 1 hour at room temperature and 1% OsO_4 buffered at pH 6.1 for 3 hours at room temperature (PANKRATZ and BOWEN, 1963).

Results

1. Growth Measurements

Growth was characterised by the absence of a lag phase and a period of slow exponential growth lasting 12 days, during which the mean doubling time was 56.5 hours. The growth rate declined from day + 12 to day + 20 and at day + 24 it was stationary. After day + 28 the culture showed a gradual loss in dry weight.

2. Light Microscope Observations

These are summarised diagrammatically in Fig. 1. The day + 38 culture, from which the inoculum was taken, was orange in colour. The individual cells appeared yellowish and they contained large, non-refractile, reddish structures which were destroyed by applying a pressure of 3.72 atmospheres. They therefore correspond to the definition of a gas-vacuole given earlier. During early exponential growth when the cells were green in colour, the gas-vacuoles were fewer in number and by day + 4 the cells contained one or two large, cylindrical gas-vacuoles adjacent to the cell wall. By day + 8 small granular gas-vacuoles were distributed between the peripheral and central regions of

the cells. During late exponential growth, when the culture had a characteristic pale green appearance, these gradually increased in size. The declining phase in growth was characterised by an increase in the number of gas-vacuoles and by day +24 they were distributed throughout the cells.

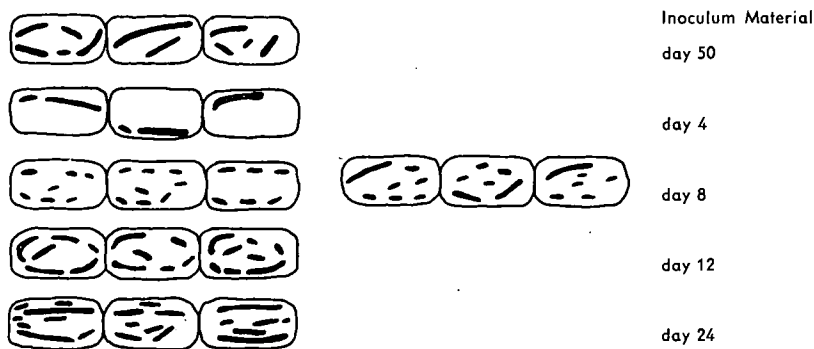


Fig.1. Summary of light microscope observations of gas-vacuoles in cells from different growth stages

Pinkish structures, which were pressure resistant, were present in the inoculum material. They were absent from days +4 to +20, but by day +24 they could be seen in some cells. These structures often appeared as refractile vacuoles at one end of the cell adjacent to the cell wall.

3. Electron Microscope Observations

a) Arrangement of Lamellae

1. *Distribution.* Day +2 cells showed considerable variation in the arrangement of lamellae. Presumably the old cells which formed the initial inoculum were undergoing reorganisation. Examination of day +50 material, which had many features in common with that from day +2, has led to this conclusion. The lamellae were usually distributed throughout the cell (Fig.2). In day +4 cells the lamellae tended to lie parallel to the cell wall (Figs.3 and 4). By day +8 and also in days +12 and +24 the lamellae were again distributed throughout the cell (Figs.5, 6 and 8 respectively).

2. *Continuity of Lamellae and Plasma Membrane.* In *A. flos-aquae* the plasma membrane and the lamellae were connected at all stages of growth (Figs.2, 3, 5 and 8). Examination of these figures, which were representative of the samples, indicated that the connections were more numerous on days +4 and +8, when the culture was in the exponential phase. The presence of large numbers of invaginations of the plasma membrane, from which lamellae would presumably develop, was a characteristic feature of the day +4 cells.

3. *Intralamellar Vesicles*. Small intralamellar vesicles (PEAT and WHITTON, 1967) were present in cells at all growth stages. Their contents were electron transparent after OsO_4 fixation (Figs. 2, 3, 5 and 8). Figs. 4 and 6 show their appearance after KMnO_4 fixation, when the

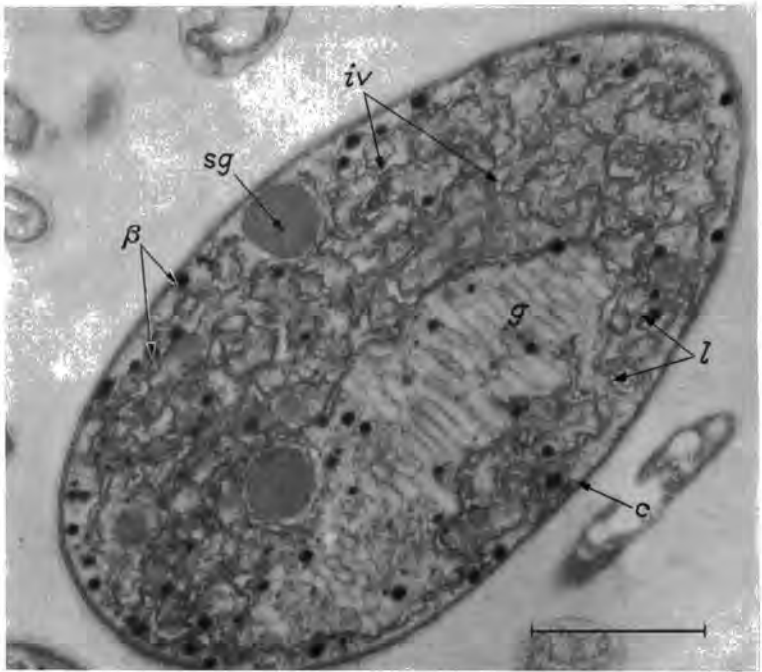


Fig. 2. A day + 2 cell showing lamellae distributed throughout the cell and structured granules. OsO_4 fixation. Mag. 23,000 \times . The symbols used for all photographs are as follows: α α -granule; β β -granule; *c* connection between the plasma membrane and lamellae; *g* gas-cylinder; *hed* hollow electron-dense body; *in* invagination of plasma membrane; *iv* intralamellar vesicle; *l* lamella; *pb* polyhedral body; *sg* structured granule. The scale shown on all photographs is 1.0 μ unless otherwise stated

vesicular contents were of medium electron density. Large intralamellar vesicles were found in some day + 24 cells (Fig. 8) and occasionally in some day + 2 cells. As large intralamellar vesicles were present in day + 50 cells, these were presumed to be a feature of old cells.

b) Distribution of Interlamellar Structures

1. *Gas-cylinders*. These were present at all stages of growth. In day + 2 cells groups were found to be partially bounded by lamellae (Fig. 2), and in some cells structures, which could be interpreted as remnants of gas-cylinders, were also present. In day + 4 cells the gas-cylinders were

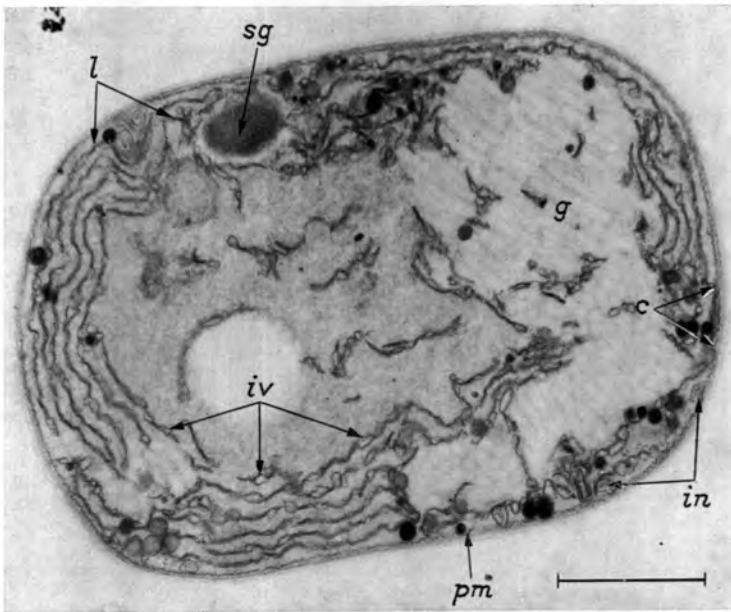


Fig. 3. A day + 4 cell showing invaginations of the plasma membrane and lamellae lying parallel to the cell wall. OsO_4 fixation. Mag. 20,000 \times

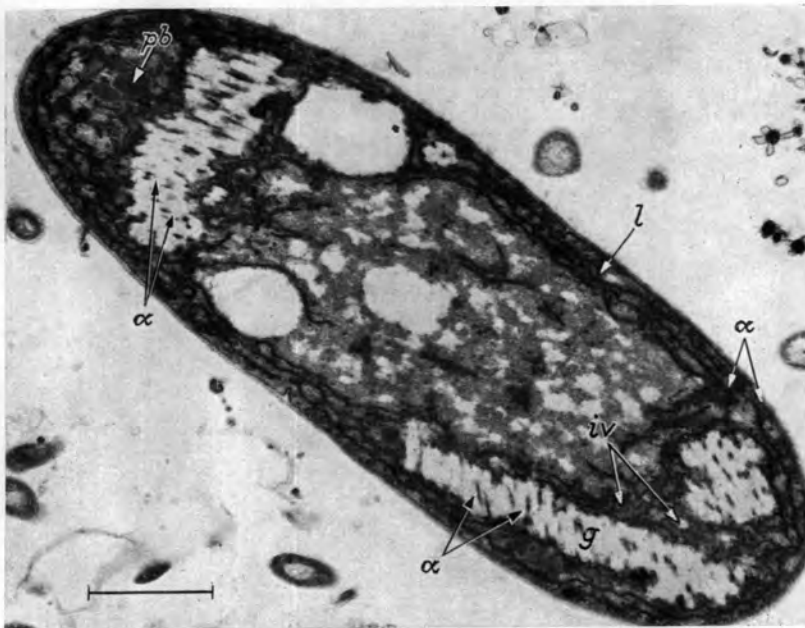


Fig. 4. A day + 4 cell showing lamellae lying parallel to the cell wall and a cylindrical grouping of gas-cylinders. KMnO_4 fixation. Mag. 17,000 \times

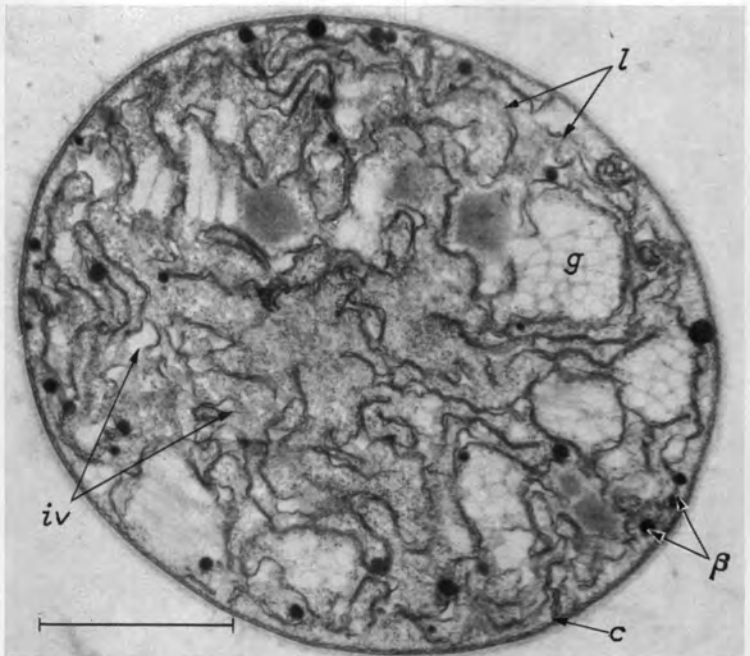


Fig. 5. A day + 8 cell showing lamellae distributed throughout the cell and small groups of gas-cylinders bounded by lamellae. OsO_4 fixation. Mag. 26,500 \times

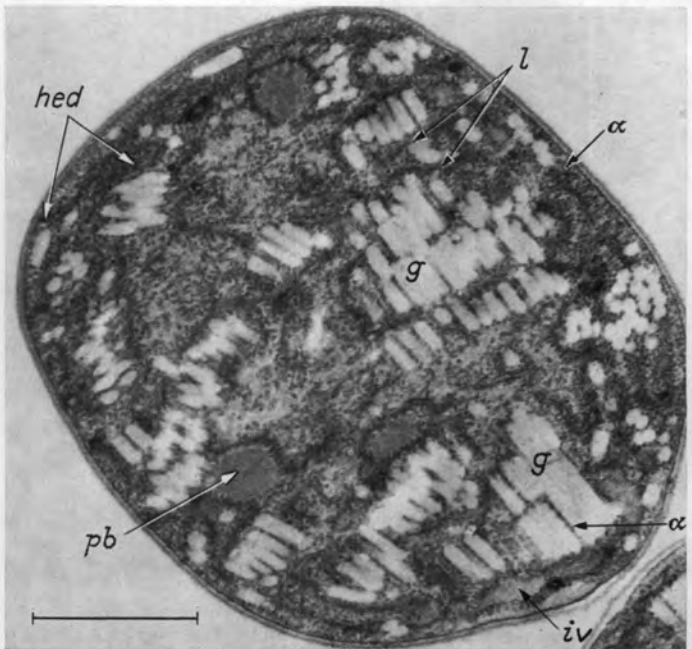


Fig. 6. A day + 12 cell showing α -granules distributed in rows between gas-cylinders. KMnO_4 fixation. Mag. 22,000 \times

often arranged in cylindrical groupings next to the cell wall. By days +8 and +12 small groups of gas-cylinders clearly bounded by lamellae were present (Figs.5 and 6). By day +24 there was an increase in numbers of gas-cylinders and they were distributed throughout the cells.

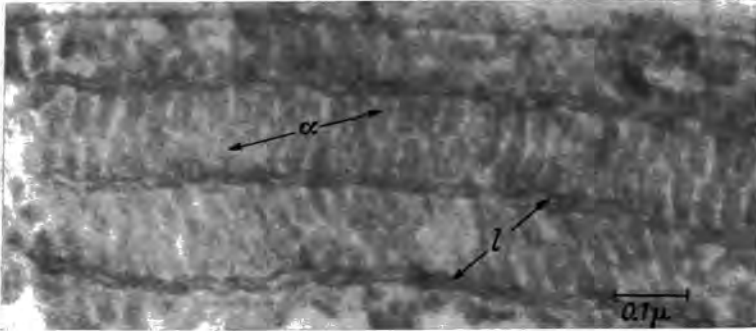


Fig.7. α -granules in pairs at right angles to the lamellae in the interlamellar spaces (from a day +12 cell). KMnO_4 fixation. Mag. 100,000 \times

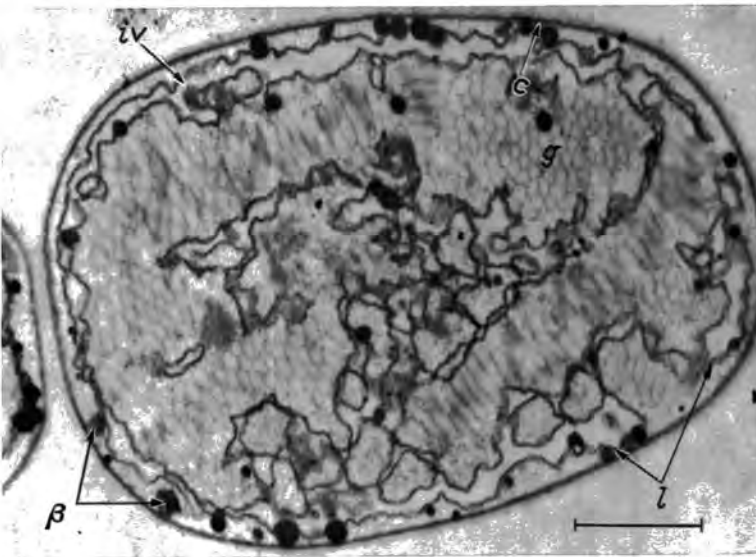


Fig.8. A day +24 cell showing increased numbers of gas-cylinders and intralamellar vesicles. OsO_4 fixation. Mag. 17,000 \times

The mean percentage of the volume of cells occupied by gas-cylinders and hence, gas-vacuoles remained constant at 20% during exponential growth. By day +24 the percentage volume had increased to 34%.

2. α -Granules. Granules which on the basis of their size and shape were identified as α -granules, were present at all stages of growth.

Similar granules were characterised as polyglucoside by GIESY (1964) in *Oscillatoria chalybia*. They were present in low numbers in day + 2 cells and increased in number during exponential growth. They were most obvious after KMnO_4 fixation. At all stages of growth they were distributed in rows between gas-cylinders (Figs. 4 and 6). In days + 12 and + 24 the α -granules were present in large numbers, and they were often found in pairs at right angles to the lamellae. In the interlamellar areas, pairs in association with one lamella alternate with those in association with the other (Fig. 7). Similarly, pairs of α -granules were found in association with the plasma membrane.

3. *Other Structures.* Polyhedral bodies, β -granules and hollow electron-dense bodies did not show any variation with growth. Structured granules (PANKRATZ and BOWEN, 1963) were found in day + 2 cells (Fig. 2) and occasionally in day + 4 cells (Fig. 3). They were present in large numbers in day + 50 cells.

Discussion

The gas-vacuoles observed with the light microscope show an excellent correlation with the groups of gas-cylinders seen with the electron microscope. In day + 4 cells, for example, the cylindrical gas-vacuoles correspond with the presence of cylindrical groupings of gas-cylinders bounded by lamellae. The presence of pinkish, pressure-resistant, refractile vacuoles in old cells appears to correspond with the large intralamellar vesicles seen with the electron microscope.

PEARSON and KINGSBURY (1966) observed the appearance with age of similar refractile vacuoles in a number of blue-green algae and used the term "pseudo-vacuole" to describe these structures. FRITTSCH (1945) employed the term "pseudo-vacuole" to describe structures which would be characterised as gas-vacuoles by the present authors. To avoid confusion it is suggested that the term "pseudo-vacuole" should no longer be used as an alternative to "gas-vacuole".

Groups of gas-cylinders and α -granules, in association with lamellae, are found in *A. flos-aquae* at all stages of growth. Changes in the arrangement of lamellae will therefore be reflected in the appearance of the groups of gas-cylinders. The ordered appearance of α -granules, at right angles to the lamellae, would suggest that they may be synthesized by the lamellae. This would be in accord with their characterisation as polyglucoside by GIESY (1964). Although α -granules are present at all stages during growth, they are more obvious as the culture ages. A similar observation was reported by GIESY (1964) and PEAT and WHITTON (1967). It has been stated that α -granules are often distributed in rows between gas-cylinders. Because of the intimate relationship existing between lamellae, gas-cylinders and α -granules, it is tempting to suggest

that the gas-cylinders may also arise as a result of active synthesis by the lamellae.

The formation of lamellae in blue-green algae by invagination of the plasma membrane has been reported by ECHLIN and MORRIS (1965), JOST (1965) and FUHS (1966). FUHS however states that it is an exceptional event during normal growth of *Oscillatoria amoena*. In *A. flos-aquae* invaginations of the plasma membrane and continuity between the plasma membrane and the lamellae are not confined to any particular growth stage. These invaginations of the plasma membrane are most obvious in day +4 cells and probably give rise to the lamellae which lie parallel to the cell wall in these cells.

There are many reports of blue-green algae which contain gas-vacuoles accumulating at the surface of lakes in late summer (LUND, 1959 and LUND, 1965). PRINGSHEIM (1965) reported that *Oscillatoria agardhii* tends to accumulate near the surface in old cultures. It would seem plausible to suggest that the increased gas-vacuolation observed in day +24 cells of *A. flos-aquae* may be a factor contributing to the increased buoyancy of these cells.

Acknowledgements: The authors are indebted to Dr. B. A. WHITTON for helpful criticism. Our thanks are due to Miss S. TARREN and Mr. J. RICHARDSON for technical assistance and to Miss M. BERRYMAN for patient secretarial help. This work was carried out while one of the authors (R. V. S.) was in receipt of a grant from S.R.C.

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