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Studies on the gas-vacuoles of blue-green algae

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STUDIES ON THE GAS-VACUOLES OF

BLUE-GREEN ALGAE

By

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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 <u>Anabaena</u> <u>flos-aquae</u> 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 <u>A</u>. <u>flos-aquae</u> 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 ± 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.

CONTENTS   Page

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

Copies of the two papers by SMITH and PEAT (1967, a and
b) are located at the end of the thesis. Some of the
figures in these papers are referred to in the text of
the thesis.

Summary of Figures

Plates are located at the end of each Part of
'The Results', and are indicated as "PL" in
the list below.

Page

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.

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, PETTER (1931) showed that certain Halobacterium strains, which caused the red discolouration of fish, contained bodies of low refractic 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 'pseudovacuole'. 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 PETTER (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 1. In the present studies the Durham culture numbers of these organisms were employed to identify each strain. For example, the strain of <u>Anabaena flos-aquae</u> isolated from Windermere, was referred to as <u>A</u>. <u>flos-aquae</u> D124. All cultures were unialgal, but they were not axenic.

B. Field samples

<u>Oscillatoria agardhii</u> and <u>O</u>. <u>redekei</u> 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.

<u>Microcystis aeruginosa</u> was collected from Cole Mere, one of the Shropshire Meres, on 7.VIII.1967. On the same day, <u>Anabaena flos-aquae</u> and <u>Aphanizomenon flos-aquae</u> 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
Anabaena flos-aquae (LYNGB.) BREB.	Windermere, England.	Westfield College		124
Anabaena flos-aquae (LYNGB.) BREB.	An oxidation pond, Mississipi, U.S.A. (TISCHER, 1965)	"	60A	125
Gloeotrichia echinulata (SMITH) RICHT.		Cambridge	1432/1	126
Microcystis aeruginosa KUTZ.		"	1450/1	127
Nostoc linckia BORN. et FLAH.		C.S.S.R. culture collection	125	130
Oscillatoria agardhii 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) Benzyldimethylamine) 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

Compound	Salt used	Concentration micro-moles	mg/l
Sodium nitrate	$NaNO_3$	2,000	170.0
Magnesium sulphate	$MgSO_4.7H_2O$	200	49.0
Magnesium chloride	$MgCl_2.6H_2O$	200	41.0
Calcium chloride	$CaCl_2.2H_2O$	200	29.0
di-Potassium hydrogen orthophosphate	K_2HPO4	100	9.0
di-Sodium hydrogen orthophosphate	Na_2HPO_4	100	7.0
Ferric chloride	$FeCl_3$	4	0.65
Boric acid	H_3BO_3	40	2.0
Manganese chloride	$MnCl_2.4H_2O$	7	1.0
Zinc chloride	$ZnCl_2$	3.2	0.3
Cobaltous chloride	$CoCl_2.6H_2O$	0.08	0.02
Cupric chloride	$CuCl_2.2H_2O$	0.0008	0.001
Diaminoethanetetra-acetic acid, disodium salt	$Na_2EDTA.2H_2O$	20.0	7.0

Table 3. The composition of AC, AD and CHU 10 (mod.) media employed in the preliminary growth experiments ("Results", Section 1) 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.)
$MgSO_4 \cdot 7H_2O$	250.0	250.0	200.0	200.0	25.0	25.0
$CaCl_2$			100.0			
$CaCl_2 \cdot 2H_2O$		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
$Ca(NO_3)_2 \cdot 4H_2O$	25.0					
Na_2CO_3					20.0	20.0
Na_2SiO_3					25.0 (+2.0)	43.5
K_2HPO_4	1,000.0	1,000.0	200.0	250.0	10.0 or 5.0	10.0
$Na_2EDTA \cdot 2H_2O$		12.7		25.4		6.35
$FeCl_3$			0.40		0.80	4.85
$FeCl_3 \cdot 6H_2O$		9.7		19.4		
$Fe_2(SO_4)_3 \cdot 6H_2O$	4.0					
$NaCitrate \cdot 2H_2O$	165.0					
H_3BO_3	2.86	2.86	0.10	0.50	2.0	1.43
$MnCl_2 \cdot 4H_2O$	1.81	0.181	0.10	0.50		0.09
$MnSO_4 \cdot 4H_2O$					2.0	
$ZnCl_2$						
$ZnSO_4 \cdot 7H_2O$	0.222	0.222	0.01	0.05	2.0	0.111
$CoCl_2 \cdot 6H_2O$		0.040			1.0	0.02
$CoSO_4 \cdot 7H_2O$				0.01		
$CuSO_4 \cdot 5H_2O$	0.079	0.079	0.01	0.02		0.039
$Na_2MoO_4 \cdot 2H_2O$		0.027	0.10			0.0135
MoO_3	0.0177			0.10		
$Na_2WO_4 \cdot 2H_2O$				0.01		
$TiO(C_2O_4)_x \cdot YH_2O$				0.01		
$K_2SO_4 \cdot Cr_2(SO_4)_3 \cdot 24H_2O$				0.01		
$NiSO_4 \cdot 6H_2O$				0.01		
$AlCl_3$					2.0	
$LiCl$					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.   Inocu-
lations 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 ($^{\circ}$C).

G.   Standard growth conditions

Flasks were incubated in the tank at 20°C, 2,000 lx
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 $\log_{10}$ day
units (FOGG, 1965).

$$K = \frac{\log_{10}N - \log_{10}No}{t} \quad ,$$

where,   t = days after inoculation,

N = yield after t days,

No = yield when t = o.

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 μm 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 ηm 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 B_{14} sockets.   Samples were attached to system and evacuated for 5 minutes at the temperature of "cardice" (-60 $^{\circ}$C).   Two methods were employed for releasing the vacuole gases.
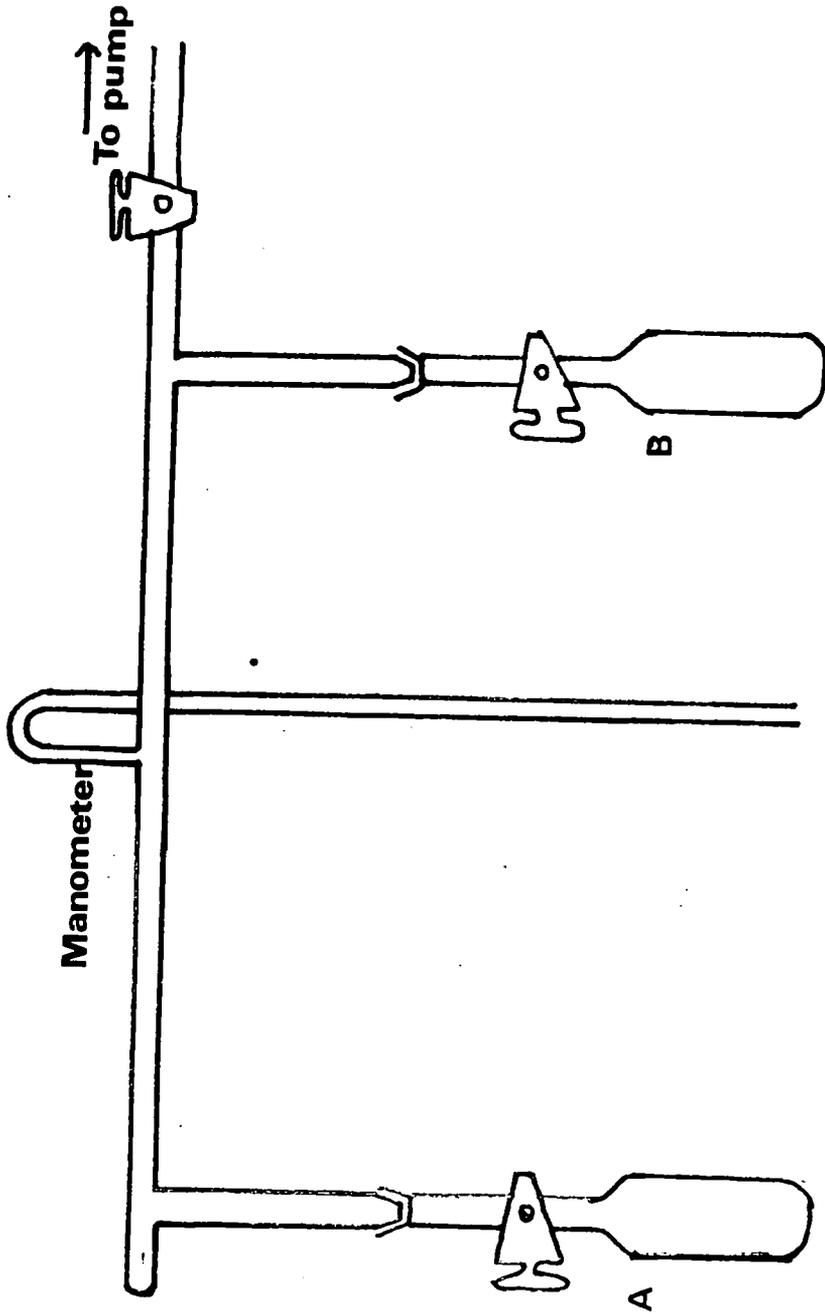
Fig. 1.   The apparatus employed for manipulating
           the gases present in gas-vacuoles.

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 N^{15}. 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 N^{15}, 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 Toepler 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 $2\frac{1}{2}$ 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 μl of 2M
ammonium carbonate buffer pH 8.6 and 5.0 μl mercaptoethanol,
and left at room temperature for 3 hours. The reduced
mixtures were alkylated with 20 mg of iodoacetamide and
50 μl 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 μg) 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 b y 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 μm 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. <u>Preliminary studies on the culturing of gas-vacuolate blue-greens and the characterisation of gas-vacuoles.</u>

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. <u>The growth rates of reported gas-vacuolate blue-greens</u>

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 <u>Gloeotrichia echinulata</u> 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 (<u>Anabaena flos-aquae</u> D124, <u>Gloeotrichia echinulata</u> D126, <u>Microcystis aeruginosa</u> D127 and <u>Oscillatoria agardhii</u> D132). However, these media supported good growth of the sessile species, <u>Nostoc linckia</u> D130, and the sewage oxidation pond isolate, <u>Anabaena flos-aquae</u> 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.)
Anabaena flos-aquae D124	0.128	No growth	Poor repli-cation	0.124
Anabaena flos-aquae D125	0.151	Good growth	Good growth	Good growth
Gloeotrichia echinulata D126	0.115	No growth	No growth	0.135
Microcystis aeruginosa D127	0.177	No growth	No growth	Some growth
Nostoc linckia D130	0.145	Good growth	Good growth	Some growth
Oscillatoria agardhii D132	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 OsO_4. 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 $KMnO_4$ 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 OsO_4 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.
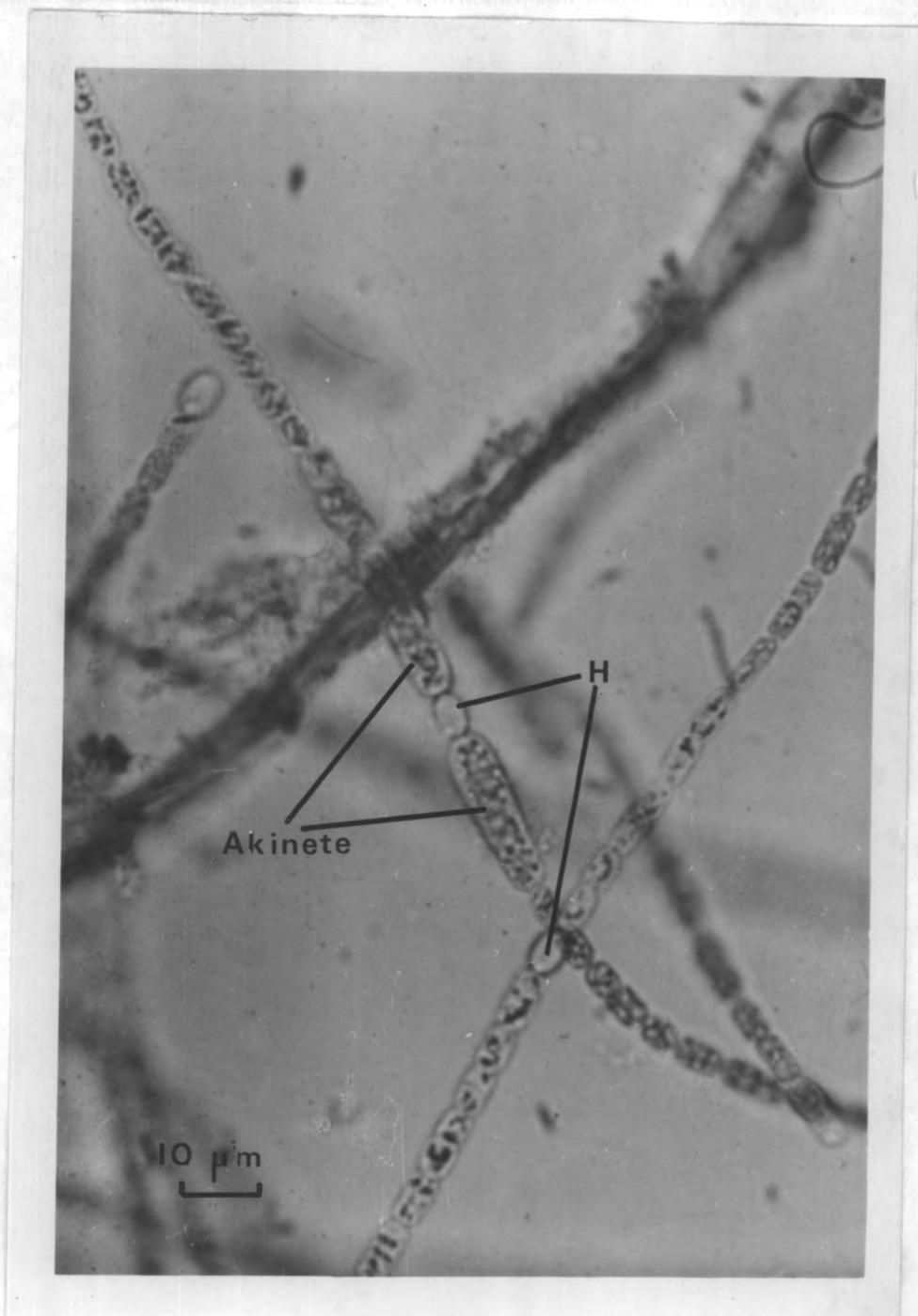
Fig. 2.   A light micrograph of day +8 Anabaena
flos-aquae D124 showing the presence of
gas-vacuoles distributed between the inner
and peripheral regions of cells and examples
of differentiation into akinetes and
heterocysts.   Mag. 1,000X.
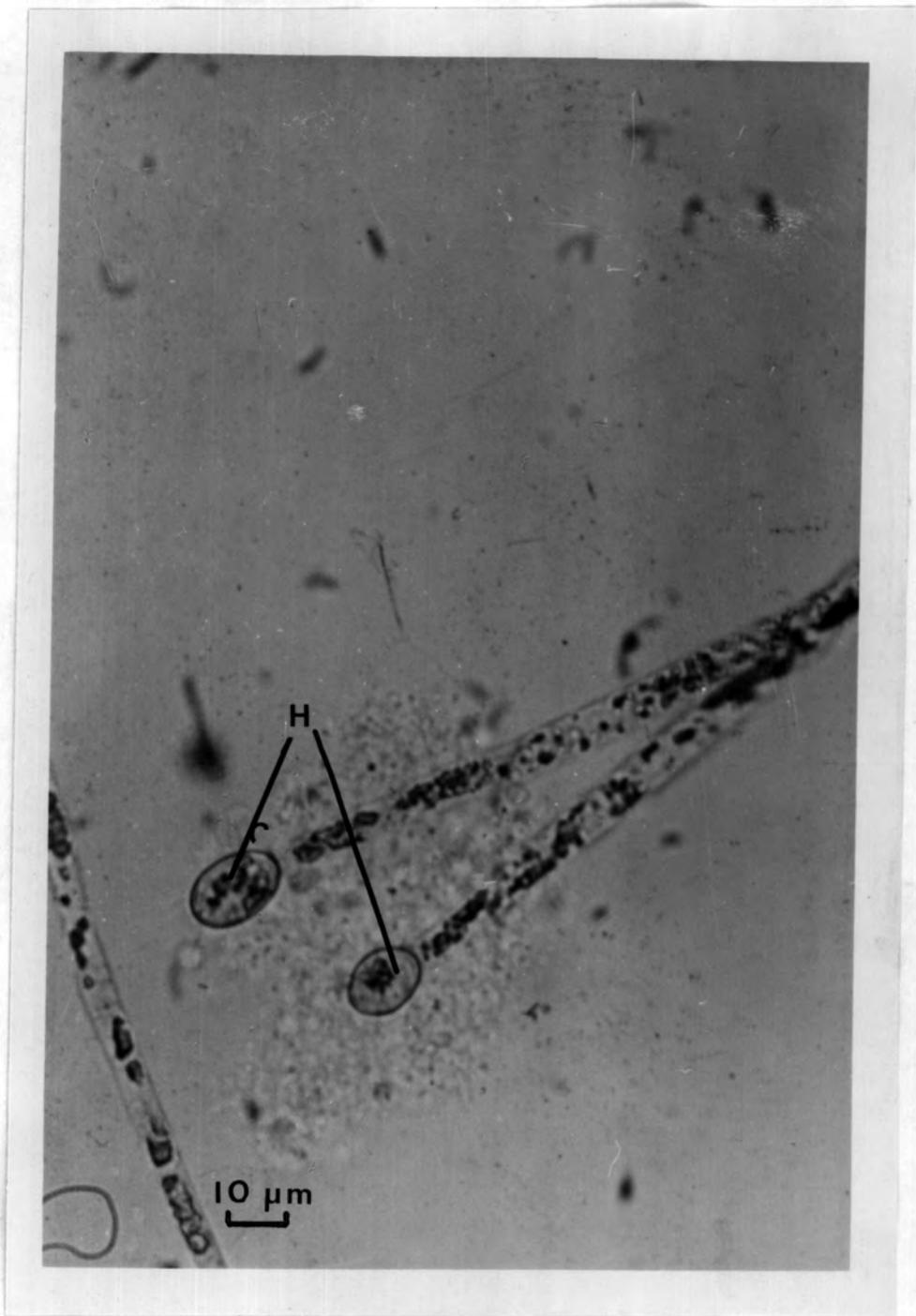
Fig. 3.   A light micrograph of day +8 Gloeotrichia
echinulata D126 showing the presence of
large gas-vacuoles and gas-vacuolate
heterocysts.   Mag. 800X.

Fig. 4. A direct preparation of a day +8 <u>Anabaena</u>
<u>flos-aquae</u> D124 cell showing the <u>distribution</u>
<u>of gas-cylinders</u>. Also present are spherical
structures (arrows) which may represent an
early stage in gas-cylinder development.
Mag. 30,000X.
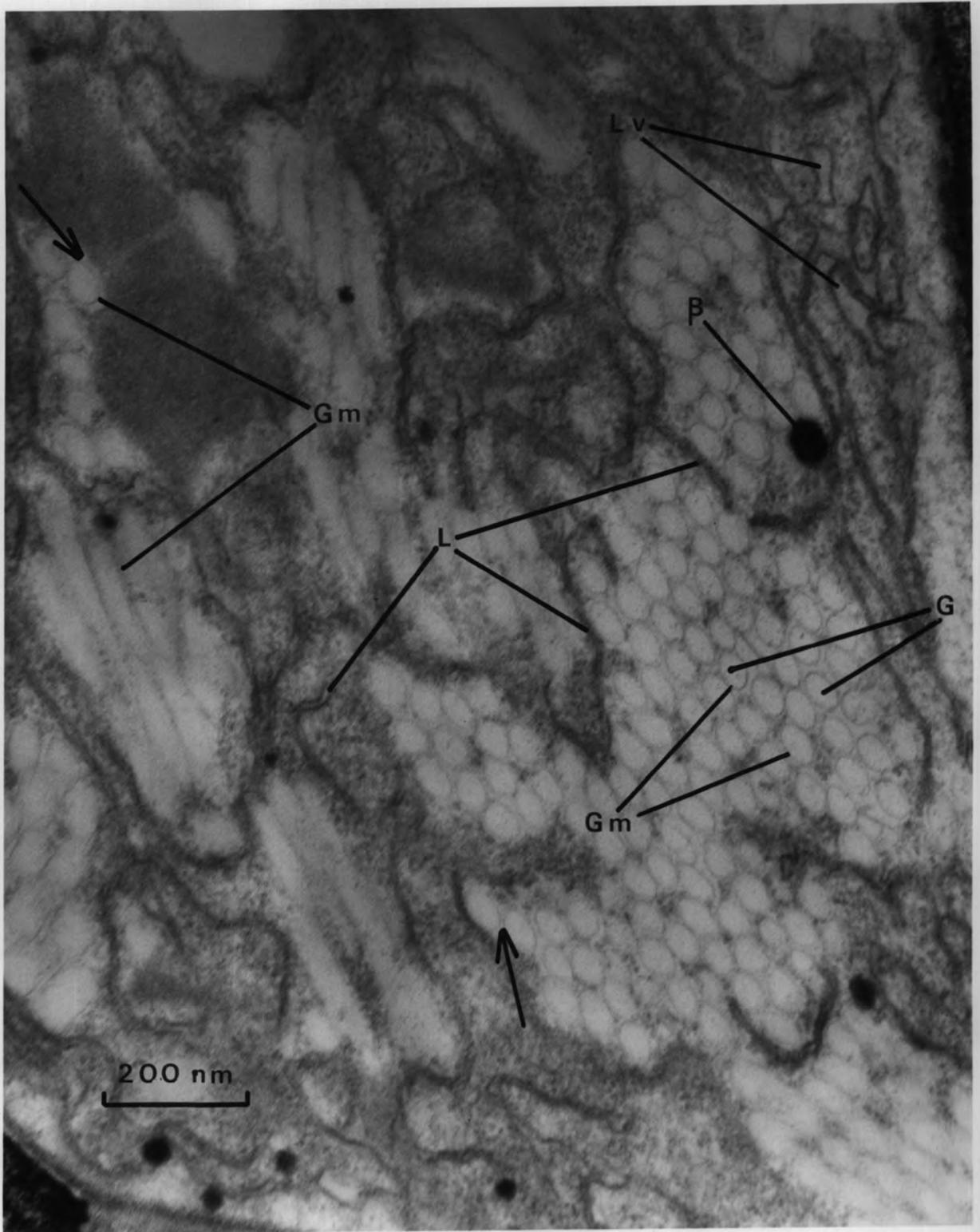
Fig. 5.   A day +8 Anabaena flos-aquae D124 cell
showing the presence of gas-cylinders
bounded by distinct membranes.   Note the
presence of electron-transparent spaces
(arrows) between gas-cylinder membranes.
OsO4 fixation.   Mag. 120,000X.

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.
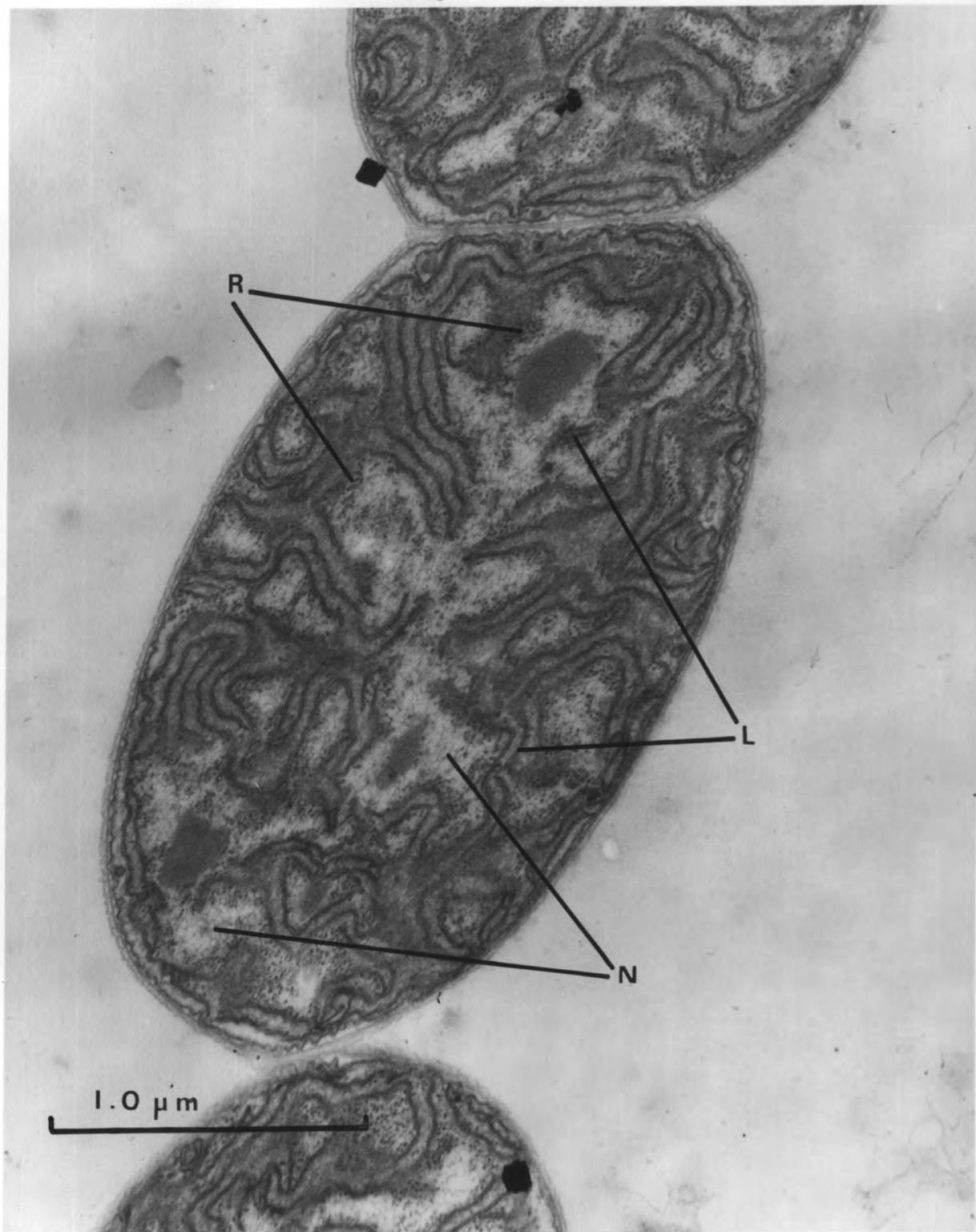
Fig. 7.   A day +8 _Anabaena flos-aquae_ D125 cell showing the absence of gas-cylinder membranes. OsO_4 fixation.   Mag. 52,000X.
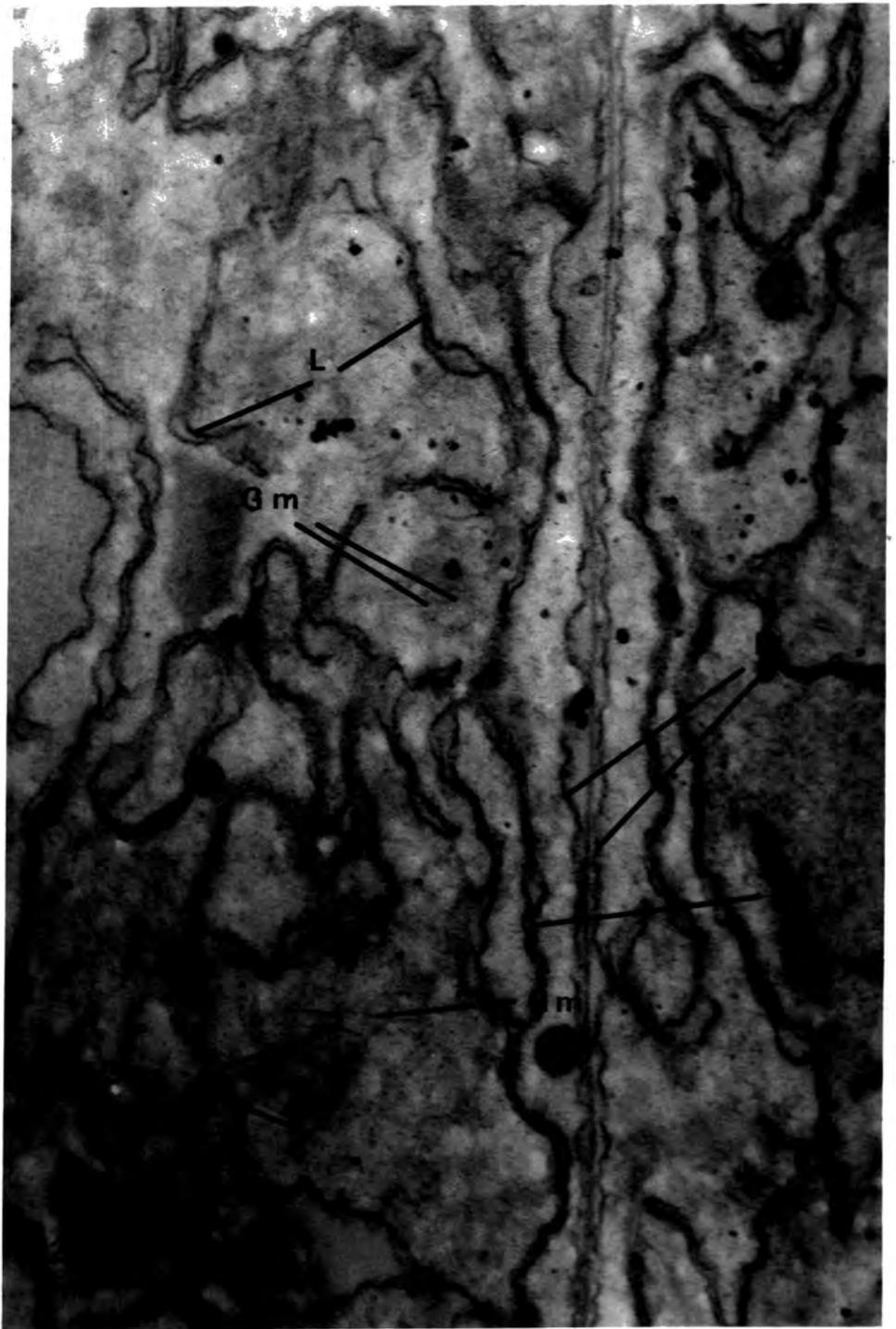
Fig. 8.   A day +8 Anabaena flos-aquae D124 cell
which has been pressure-treated prior to
fixation.   Although gas-cylinders are
absent, gas-cylinder membrane fragments
are present in the inter-lamellar areas.
OsO_4 fixation.   Mag. 68,000X.

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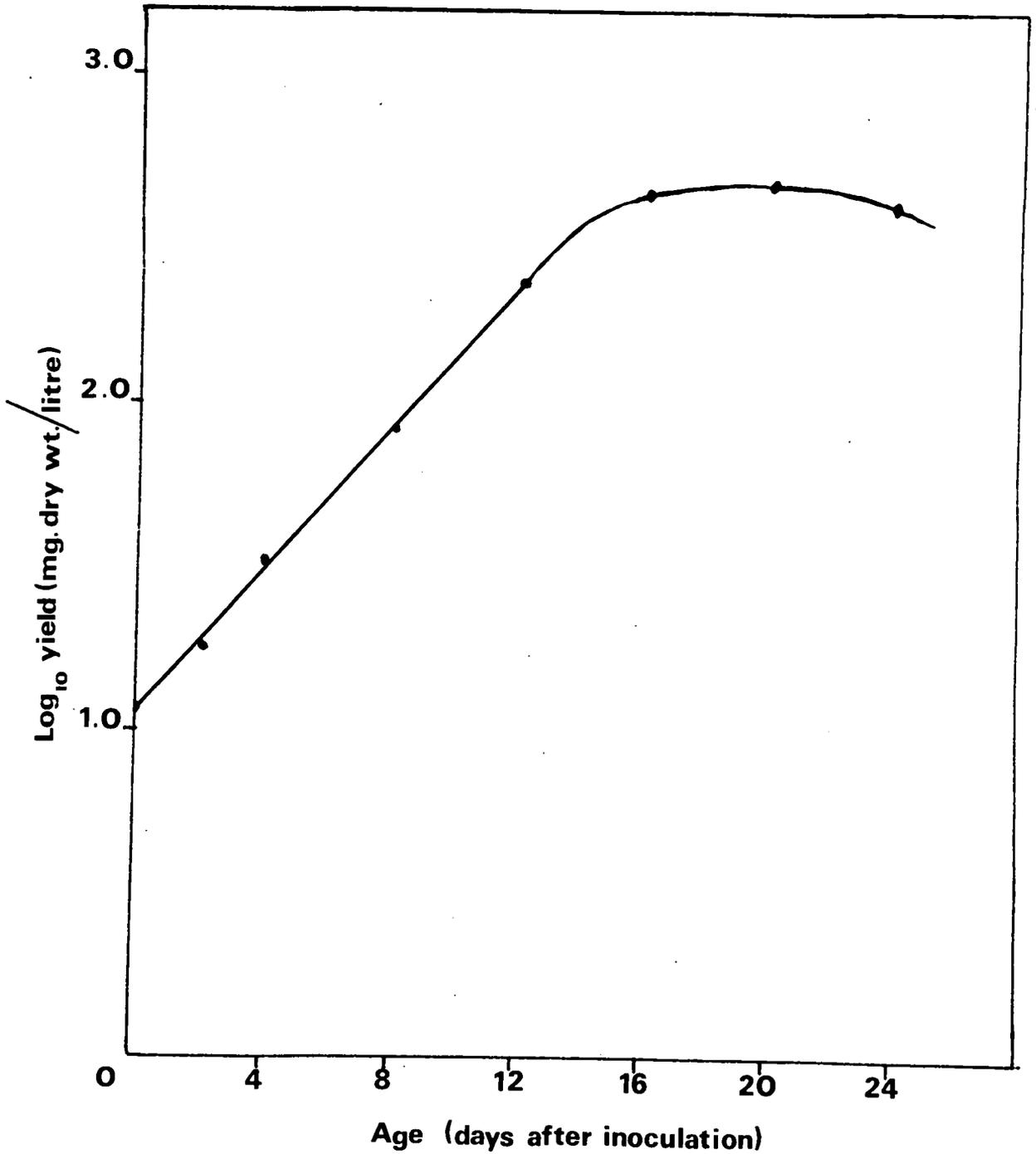
Fig. 9.   The growth characteristics of Anabaena flos-aquae D124 grown under the Standard growth conditions ('Materials and Methods', Section 3G).

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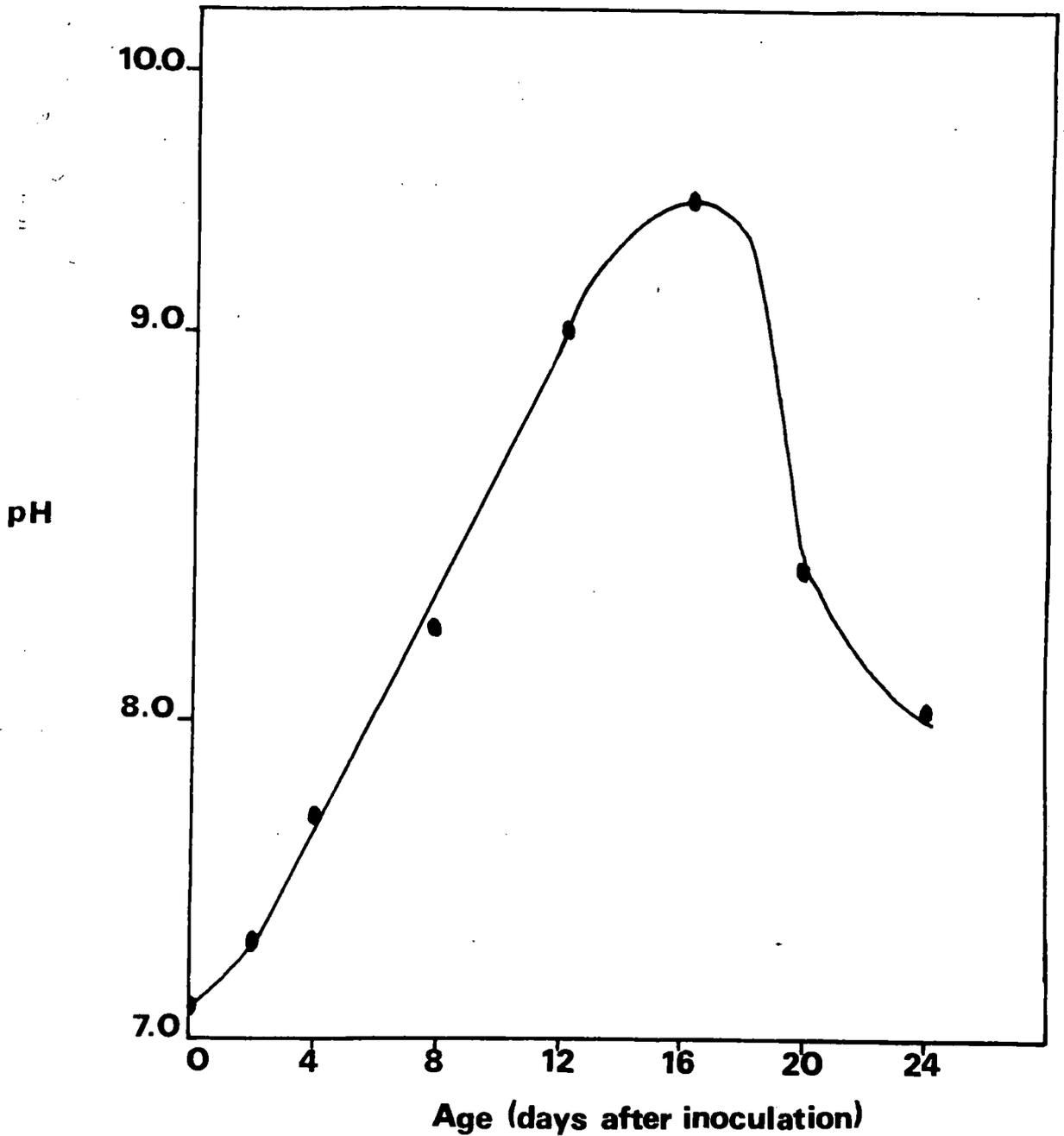
Fig. 10.   The pH changes resulting from the growth of <u>Anabaena flos-aquae</u> D124 in ASM-1 medium under the Standard growth conditions ('Materials and Methods', Section 3G).

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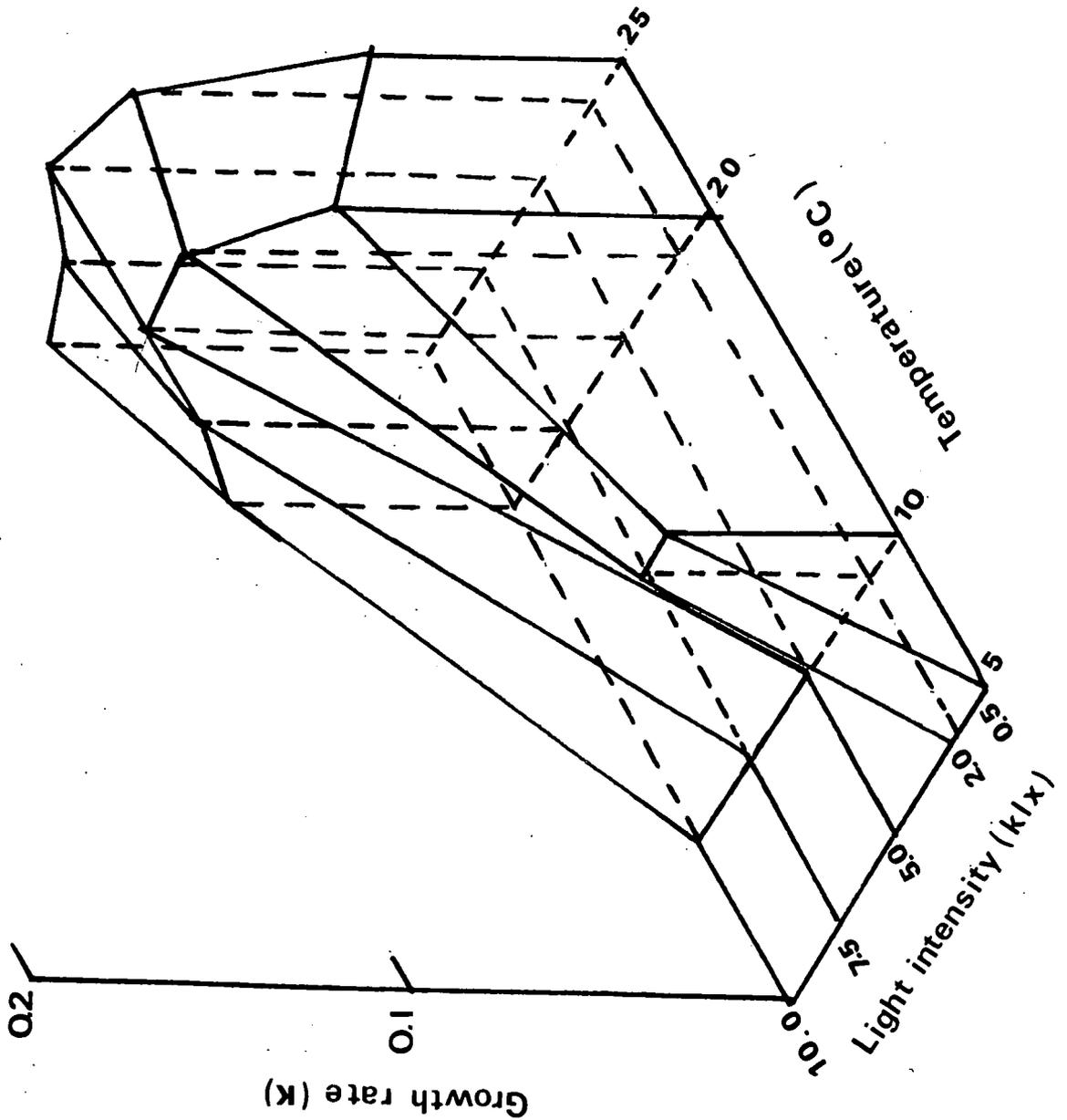
Fig. 11.   The effects of light and temperature
           on the growth rate of _Anabaena flos-aquae_
           D124 expressed in the form of a three-
           dimensional model.

Early exponential growth.
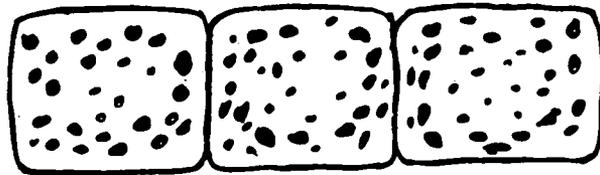
LL
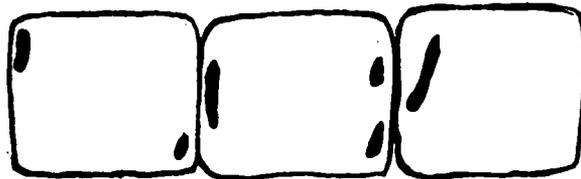
HL

Late exponential growth.
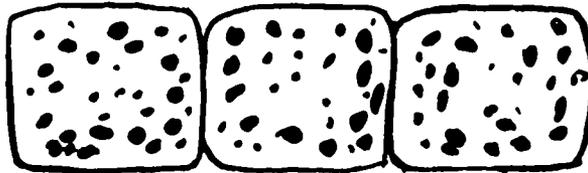
LL
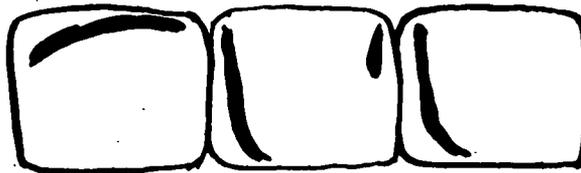
HL

Stationary phase of growth.
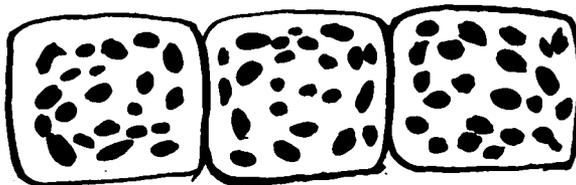
LL
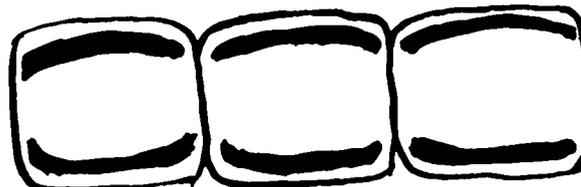
HL

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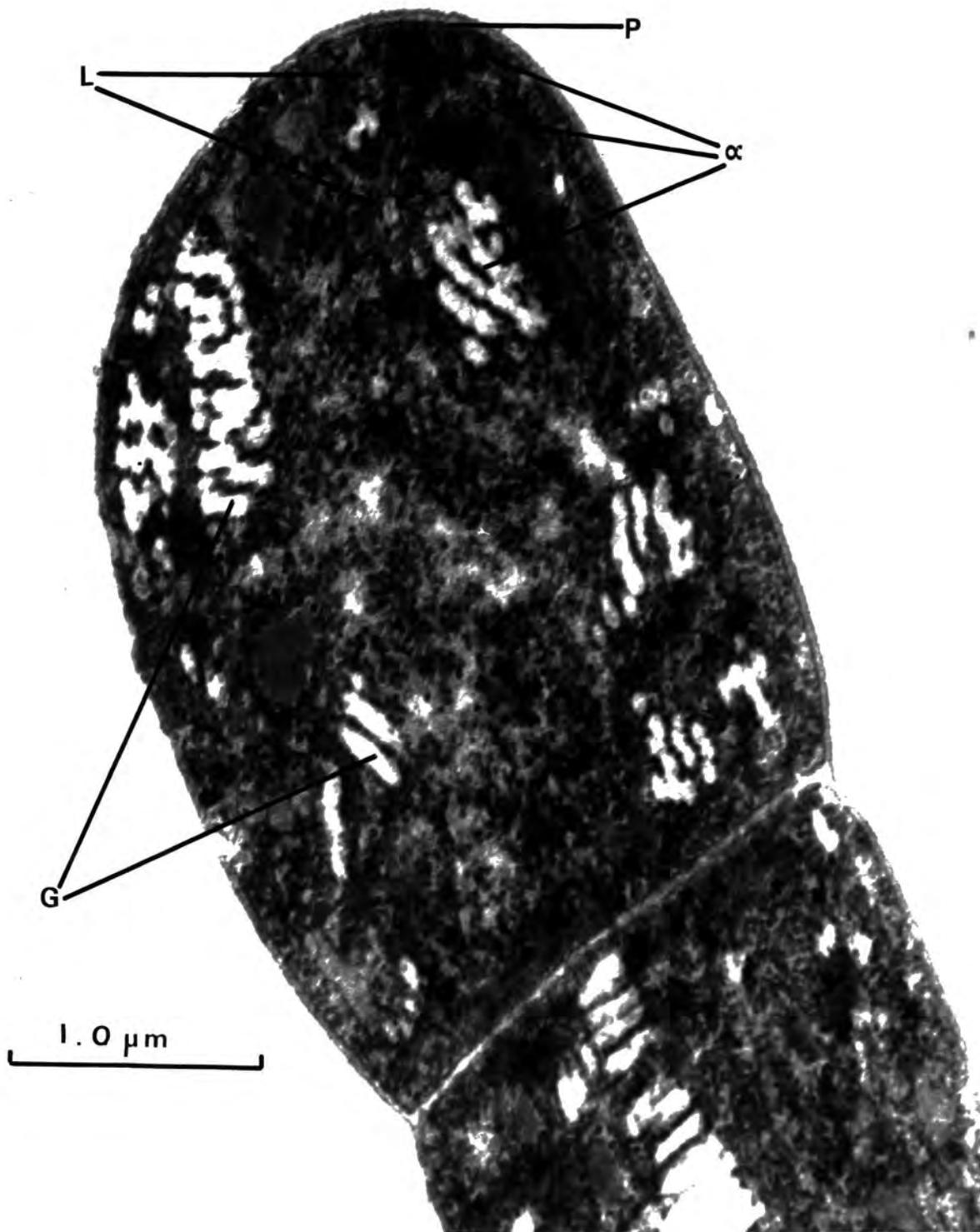
Fig. 13. A day +24 cell of <u>Anabaena flos-aquae</u> D124 showing the presence of large numbers of α-granules. Note the distribution of paired α-granules between the plasma membrane and the lamellae. $KMnO_4$ fixation. Mag. 40,000X.

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 2.0μm 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, 4.5μm 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 colume 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 longtitudinal 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 7.0μm 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 5.0μm and 4.5μm , 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
injected
time after being ~~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.
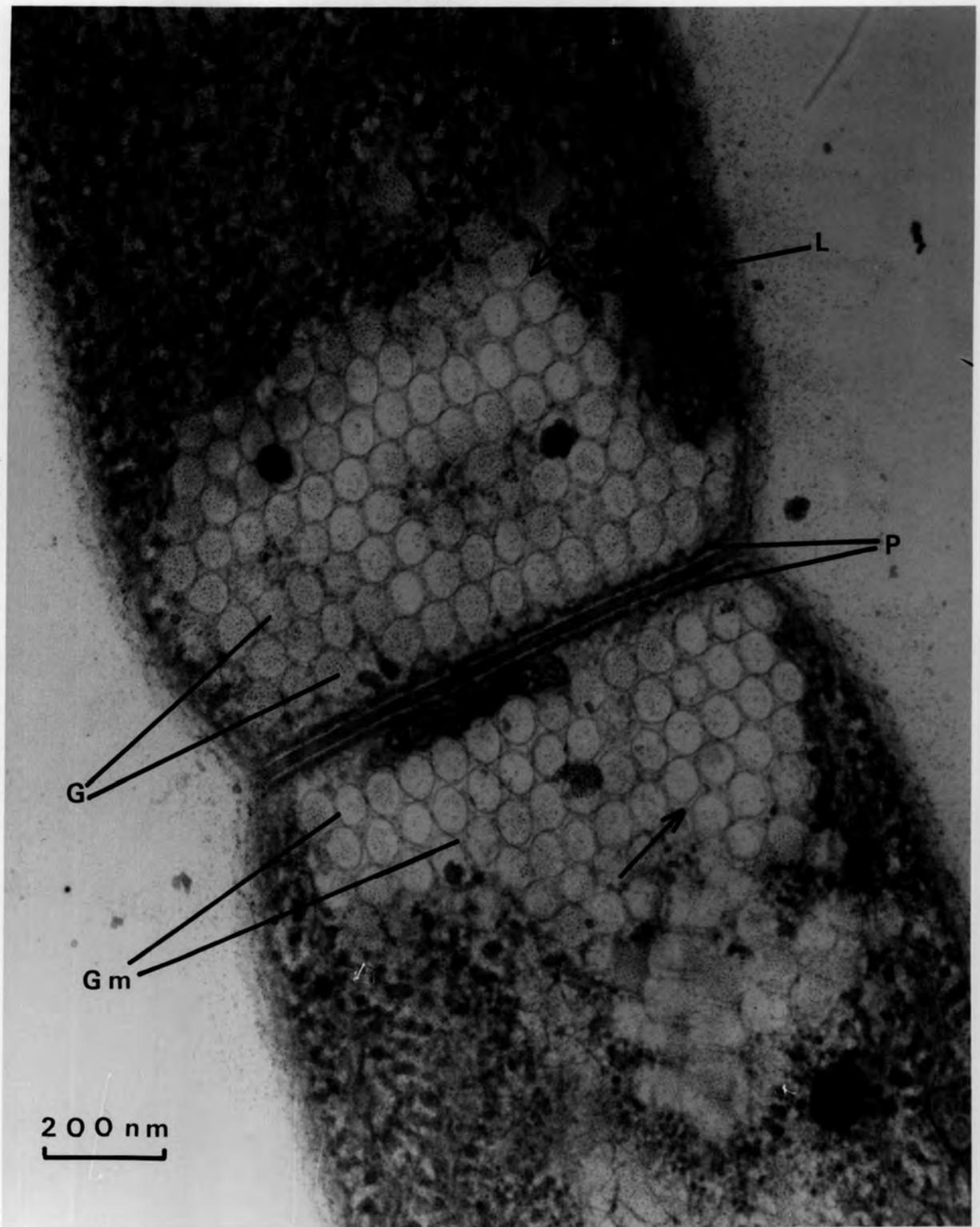
Fig. 14. Fine structure of the gas-vacuoles adjacent to
the cross-walls of Oscillatoria redekei, collected
from St. James's Park, London. Note the presence
of electron-transparent spaces (arrows) between
gas-cylinder membranes and the proliferation of
the plasma membrane which may be associated with
gas-cylinder membrane synthesis. OsO_4 fixation.
Mag. 100,000X.
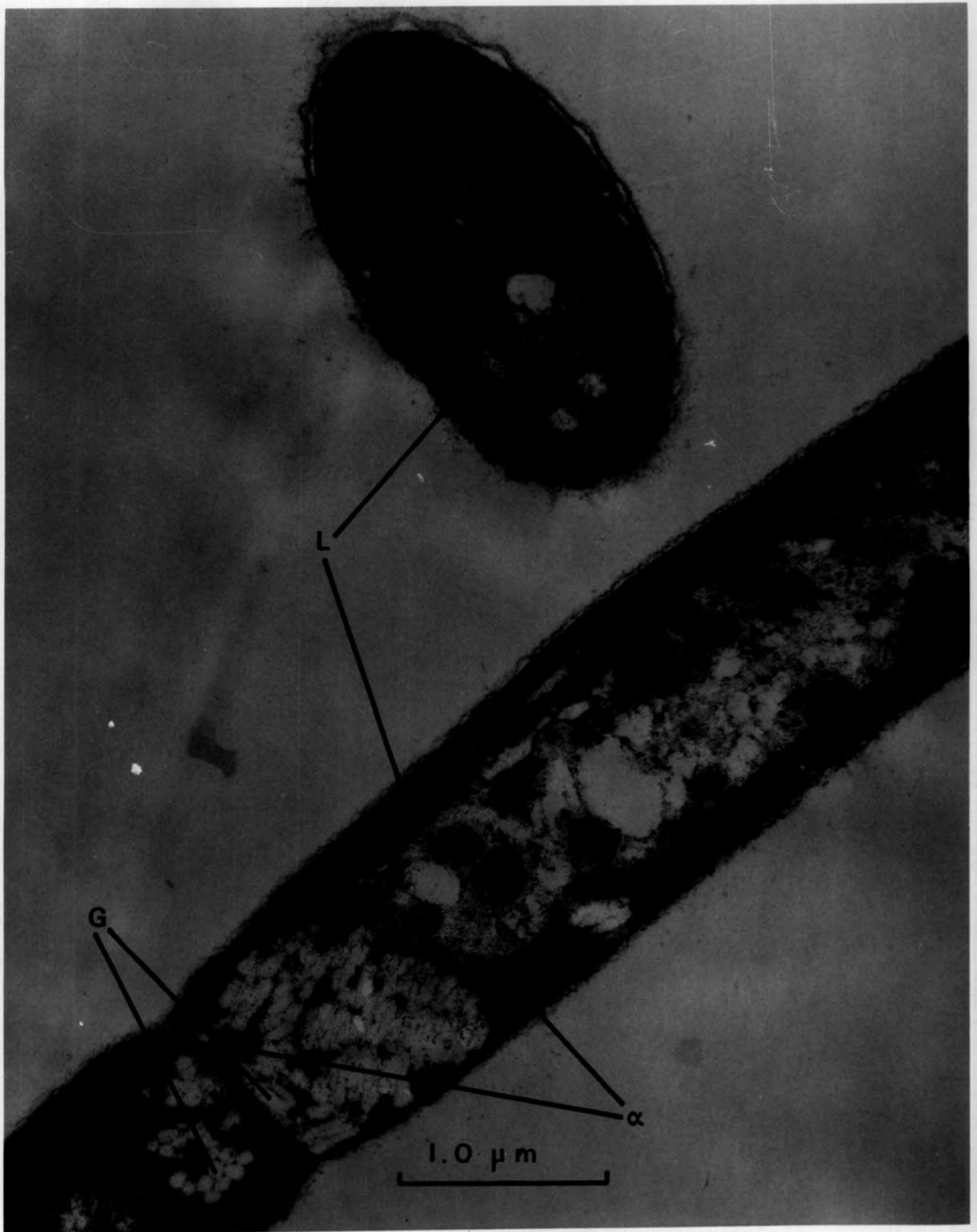
Fig. 15.   General cell views of Oscillatoria
redekei in transverse and longitudinal
section.   Note the distribution of
lamellae which run parallel to the cell
wall. KMnO$_4$ fixation.   Mag. 35,000X.
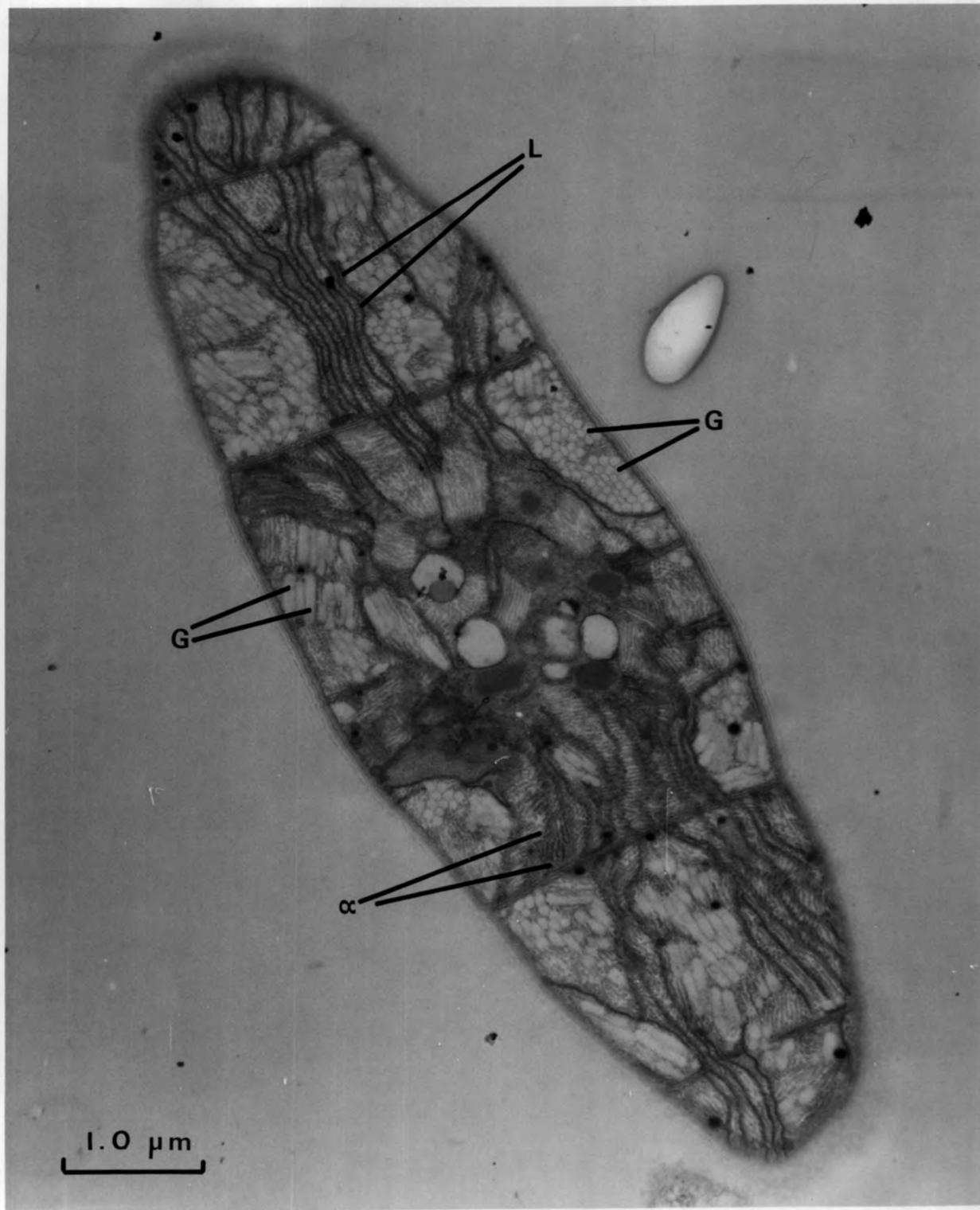
Fig. 16. An oblique section of an Oscillatoria agardhii trichome which was collected from St. James's Park, London. OsO$_4$ fixation. Mag. 22,000X.
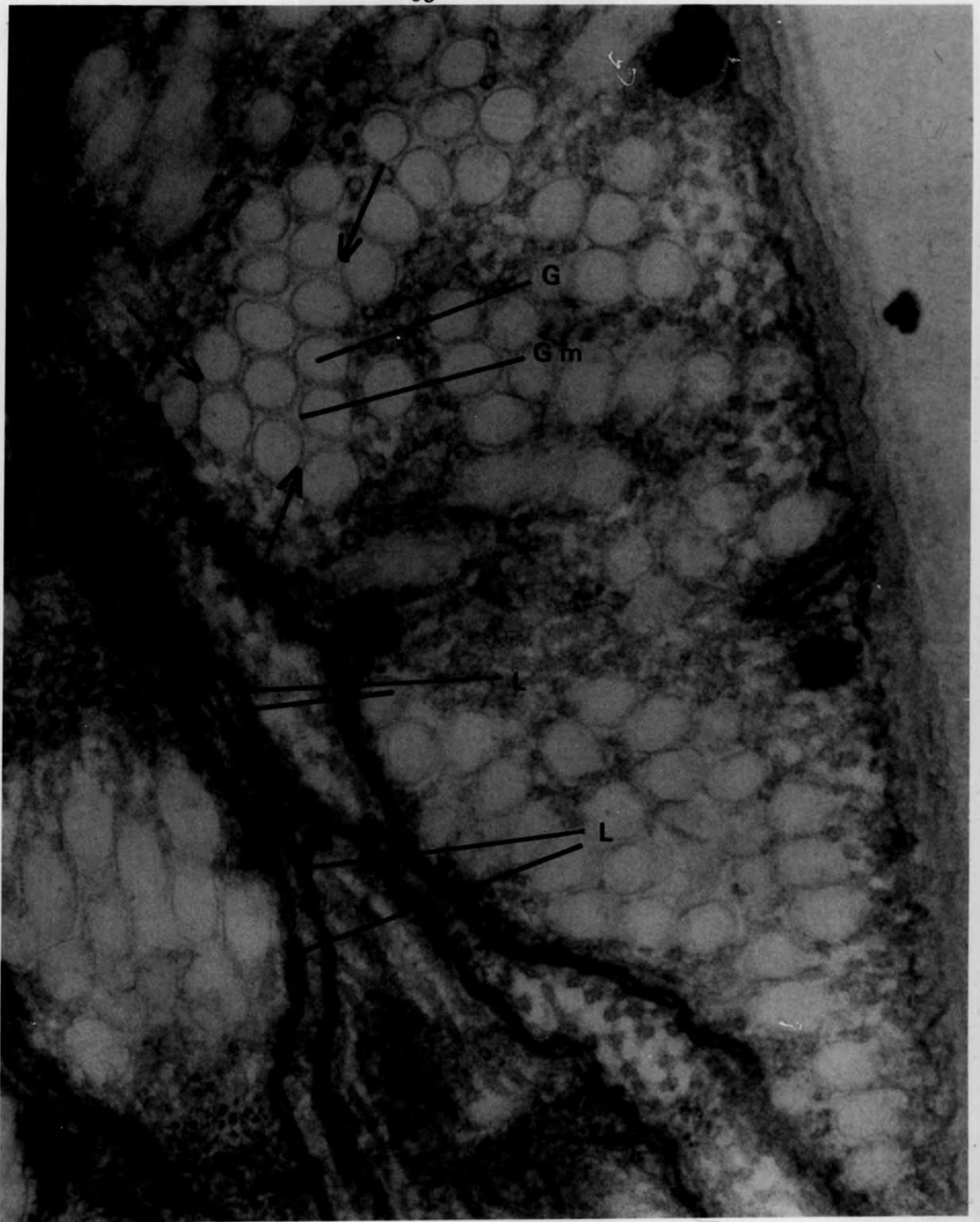
Fig. 17.   An Oscillatoria agardhii cell showing gas-
            cylinders in transverse section with electron-
            transparent spaces (arrows) between gas-
            cylinders.   Note the lamellae showing their
            typical unit membrane structure.   OsO$_4$ fixation.
            Mag. 118,000X.
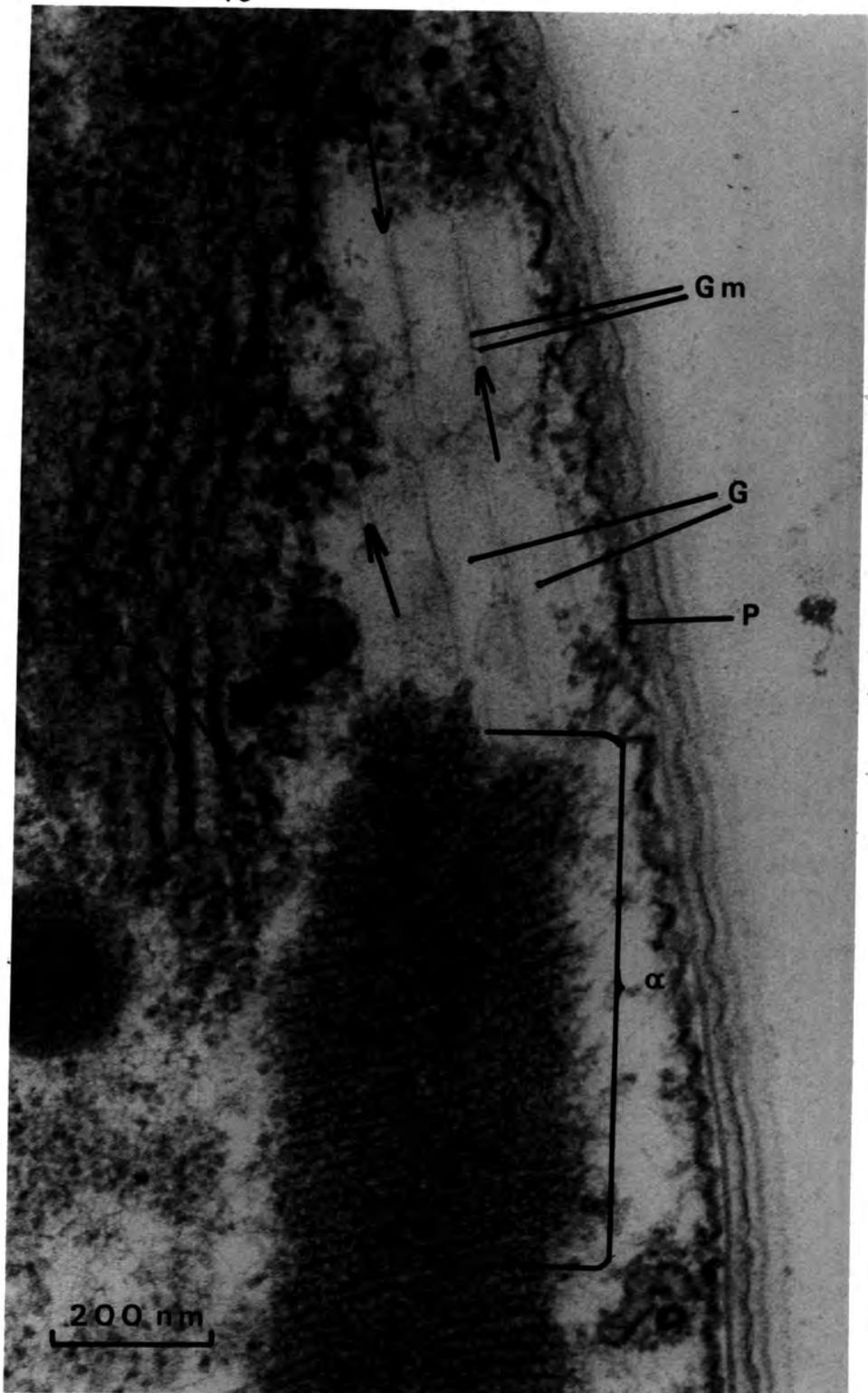
Fig. 18.   An Oscillatoria agardhii cell showing gas-
            cylinders in longitudinal section with
            electron-transparent spaces (arrows) between
            gas-cylinders.   Note the appearance of α-
            granules in this section. OsO_4 fixation.
            Mag. 118,000X.
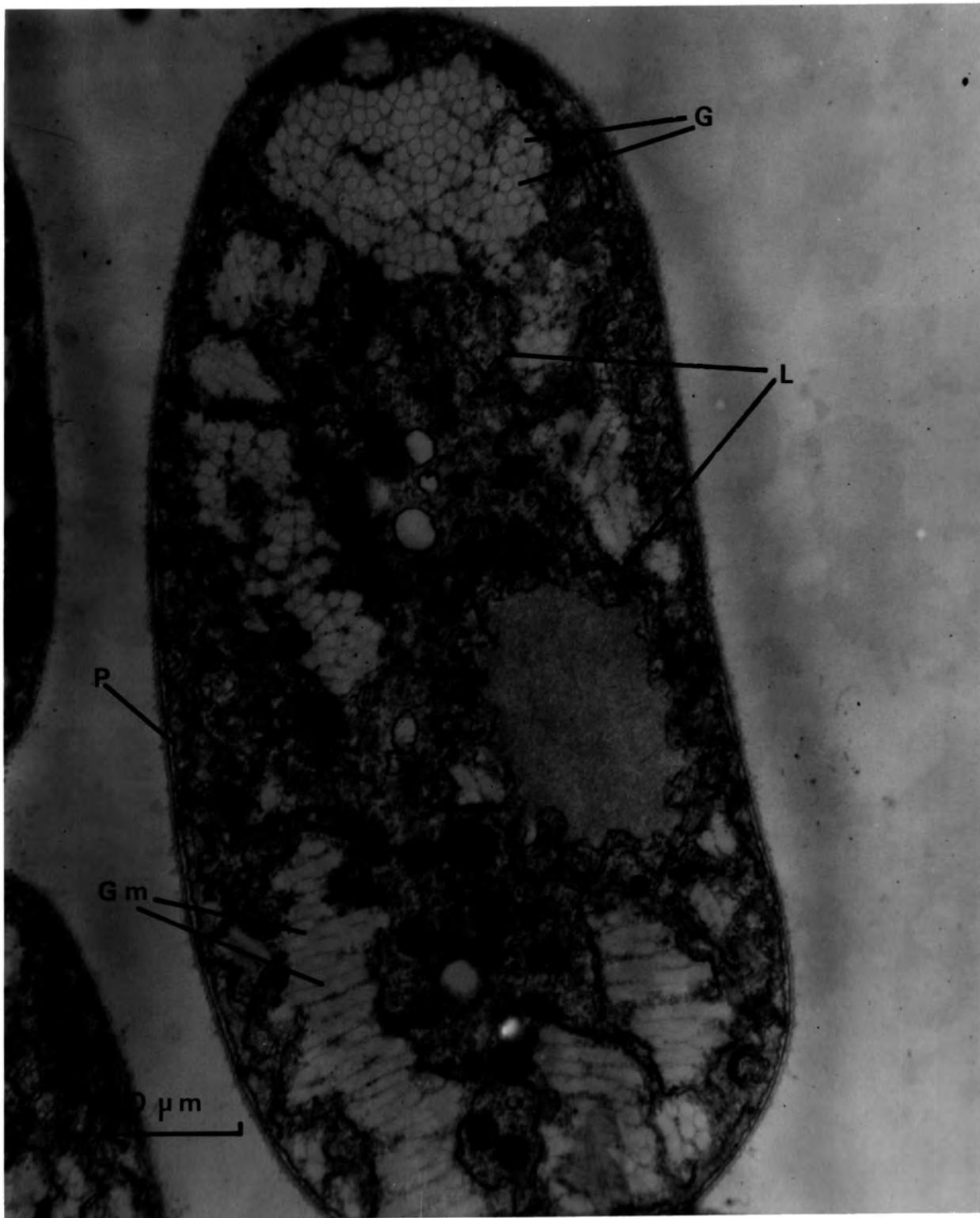
Fig. 19. An Anabaena flos-aquae cell which was
collected from White Mere, Shropshire.
OsO$_4$ fixation.   Mag. 29,000X.
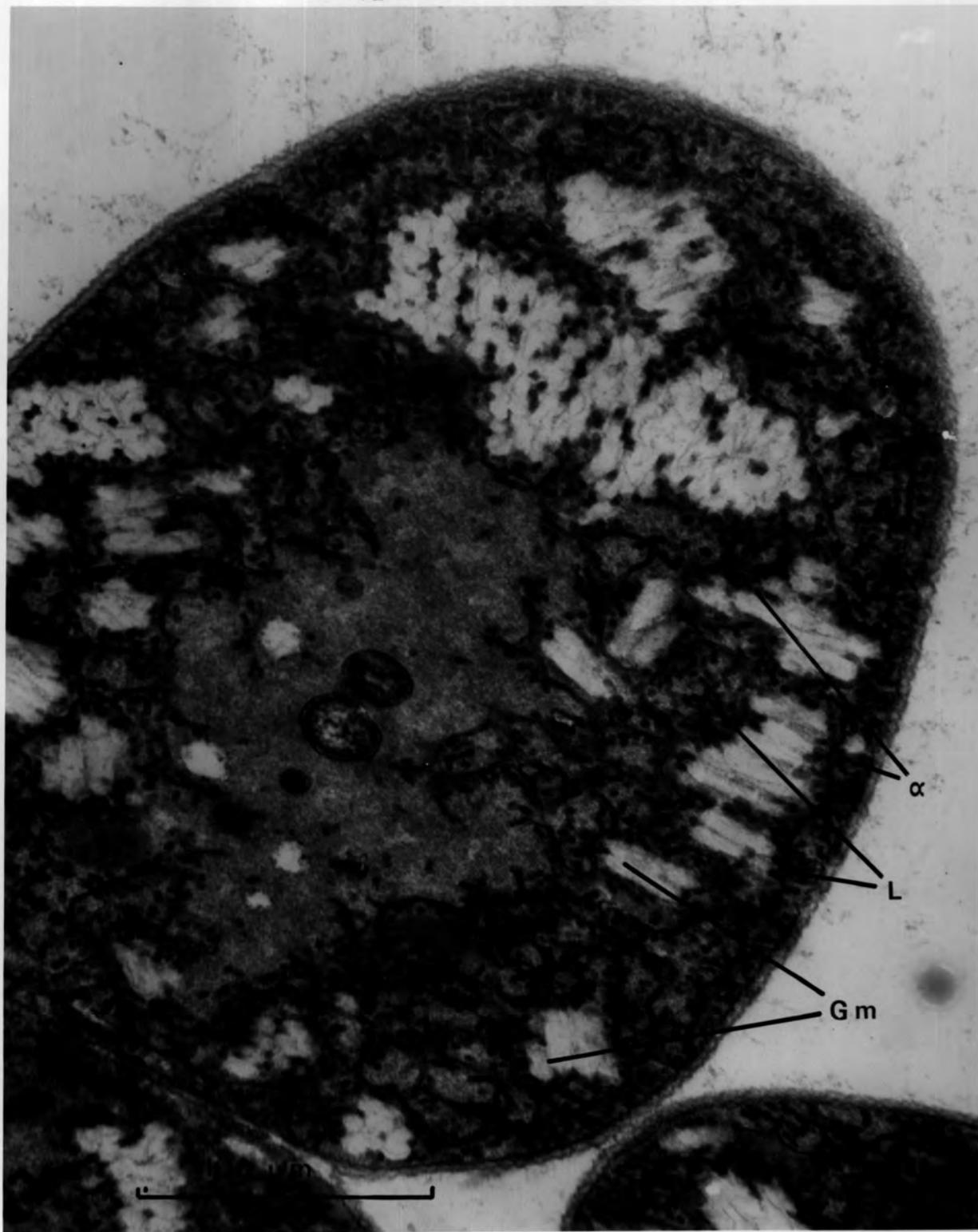
Fig. 20.   A White Mere <u>Anabaena flos-aquae</u> cell showing
a general similarity in fine structure to a
typical <u>Anabaena flos-aquae</u> D124 harvested from
the late exponential phase of growth (Fig. 6,
page 42).   KMnO$_4$ fixation.   Mag. 48,000X.
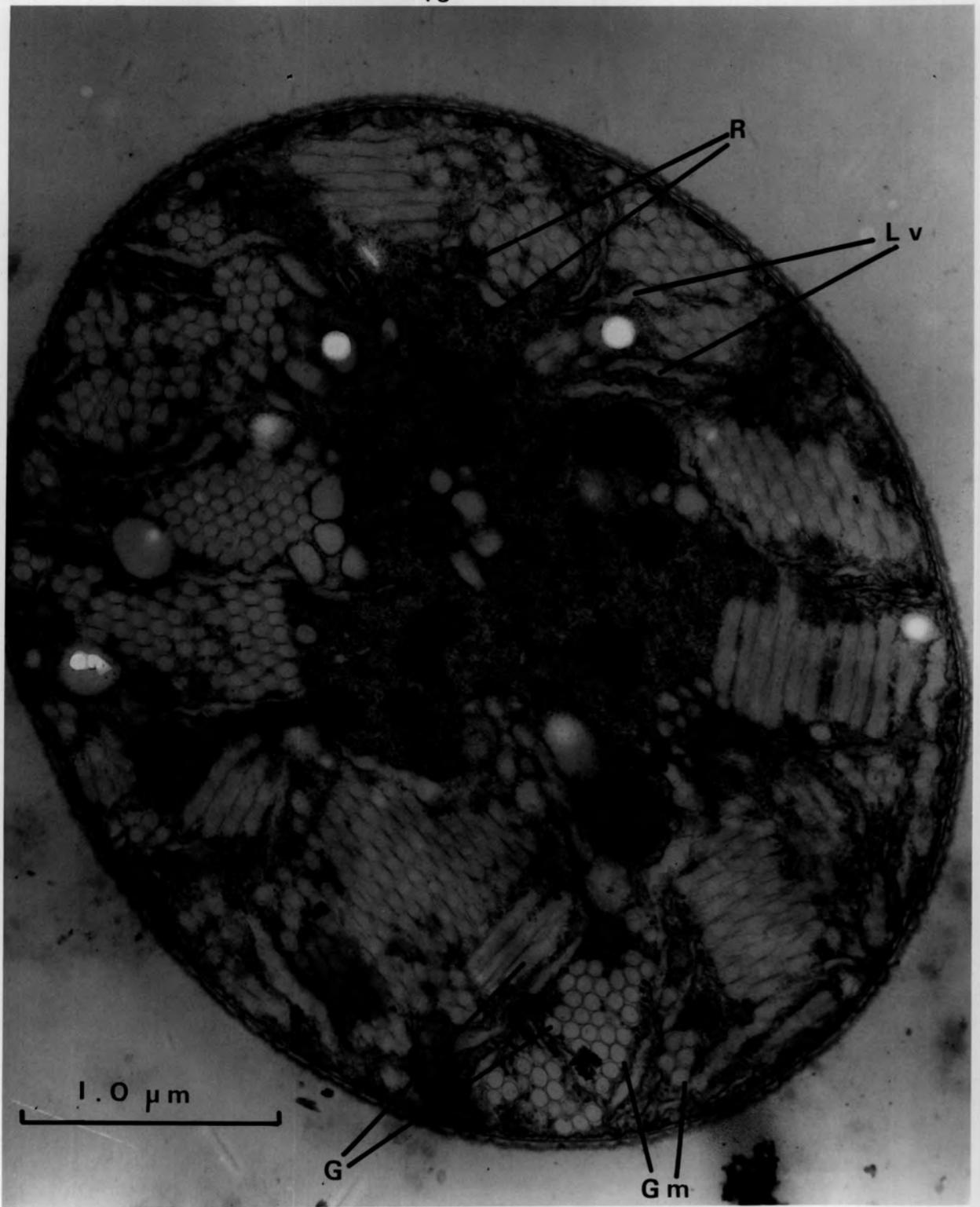
Fig. 21.   A _Microcystis_ _aeruginosa_ cell collected from
Cole Mere, Shropshire. OsO_4 fixation.
Mag. 43,000X.
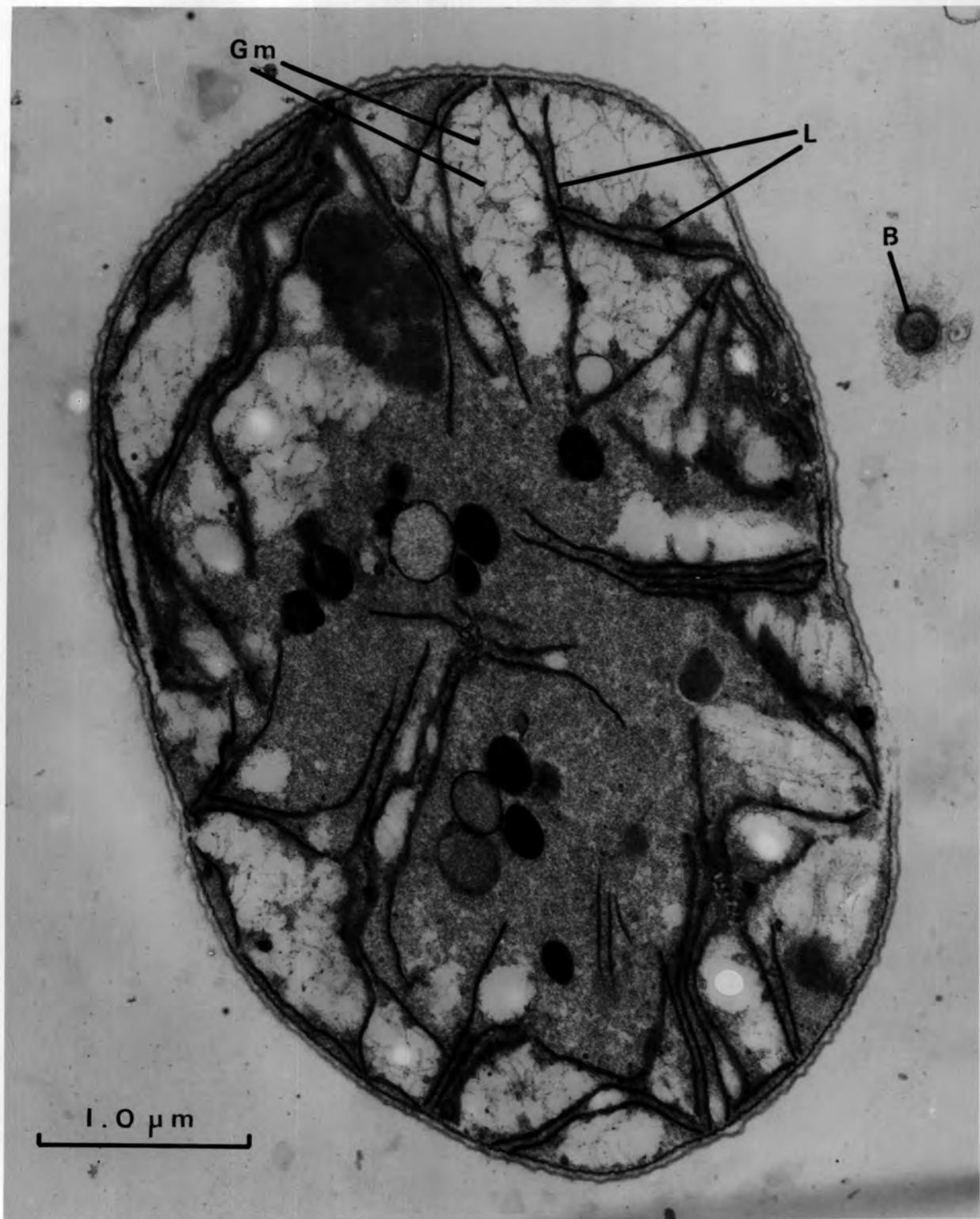
Fig. 22. A Microcystis aeruginosa cell collected from
Cole Mere, Shropshire. Note that the cell
is from a colony and that the presence of a
bacterium in this section indicates that these
organisms are present within colonies.
KMnO$_4$ fixation.   Mag. 35,000X.

Fig. 23.   A planktonic rhizopod which has ~~imbibed~~ *injested* some
gas-vacuolate <u>Microcystis aeruginosa</u> cells.
$KMnO_4$ fixation.   Mag. 15,000X.

-76-
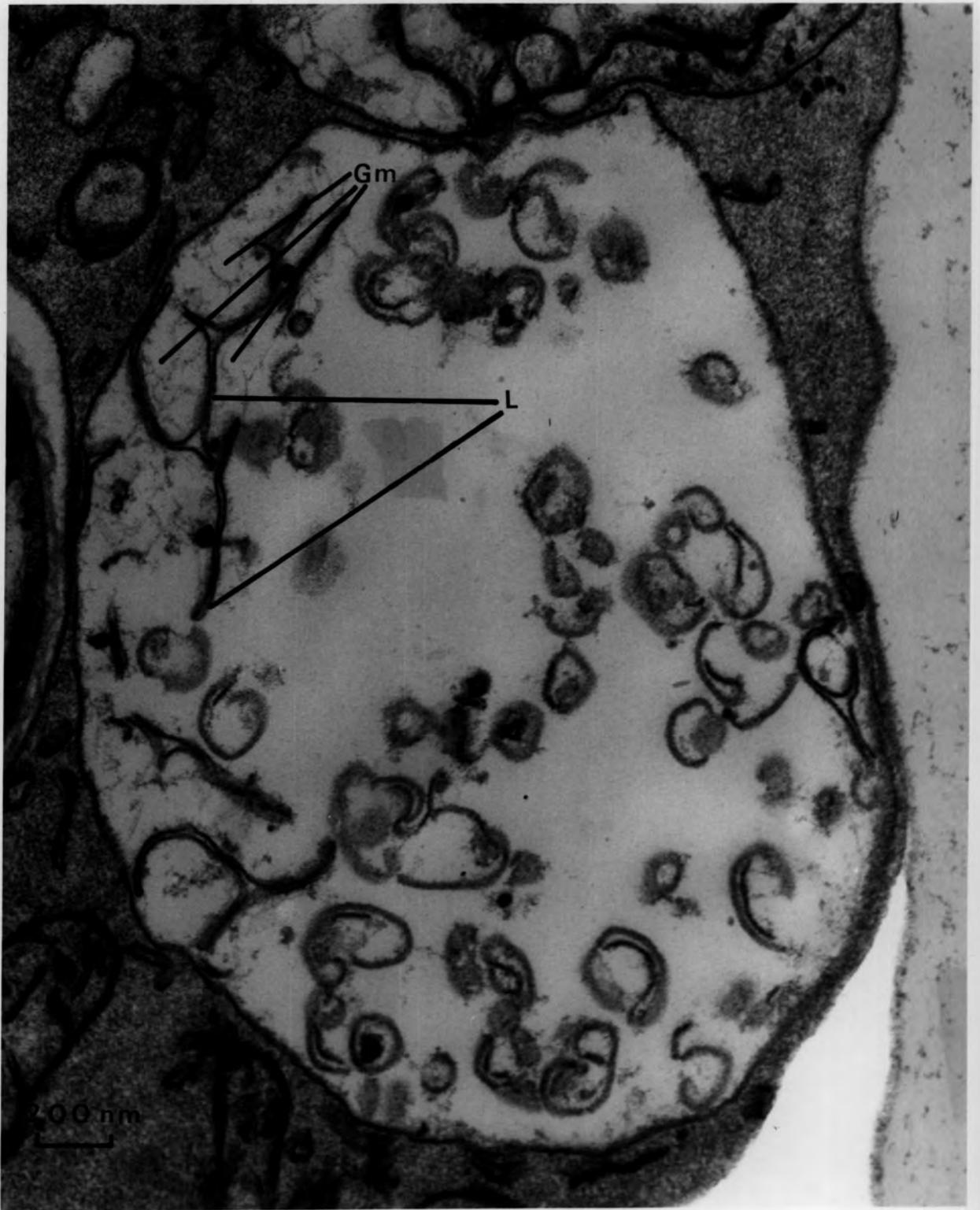
Fig. 24.   A partly digested cell of Microcystis aeruginosa
within a planktonic rhizopod.   Lamellae and
gas-cylinder membranes are still evident.
$KMnO_4$ fixation.   Mag. 59,000X.
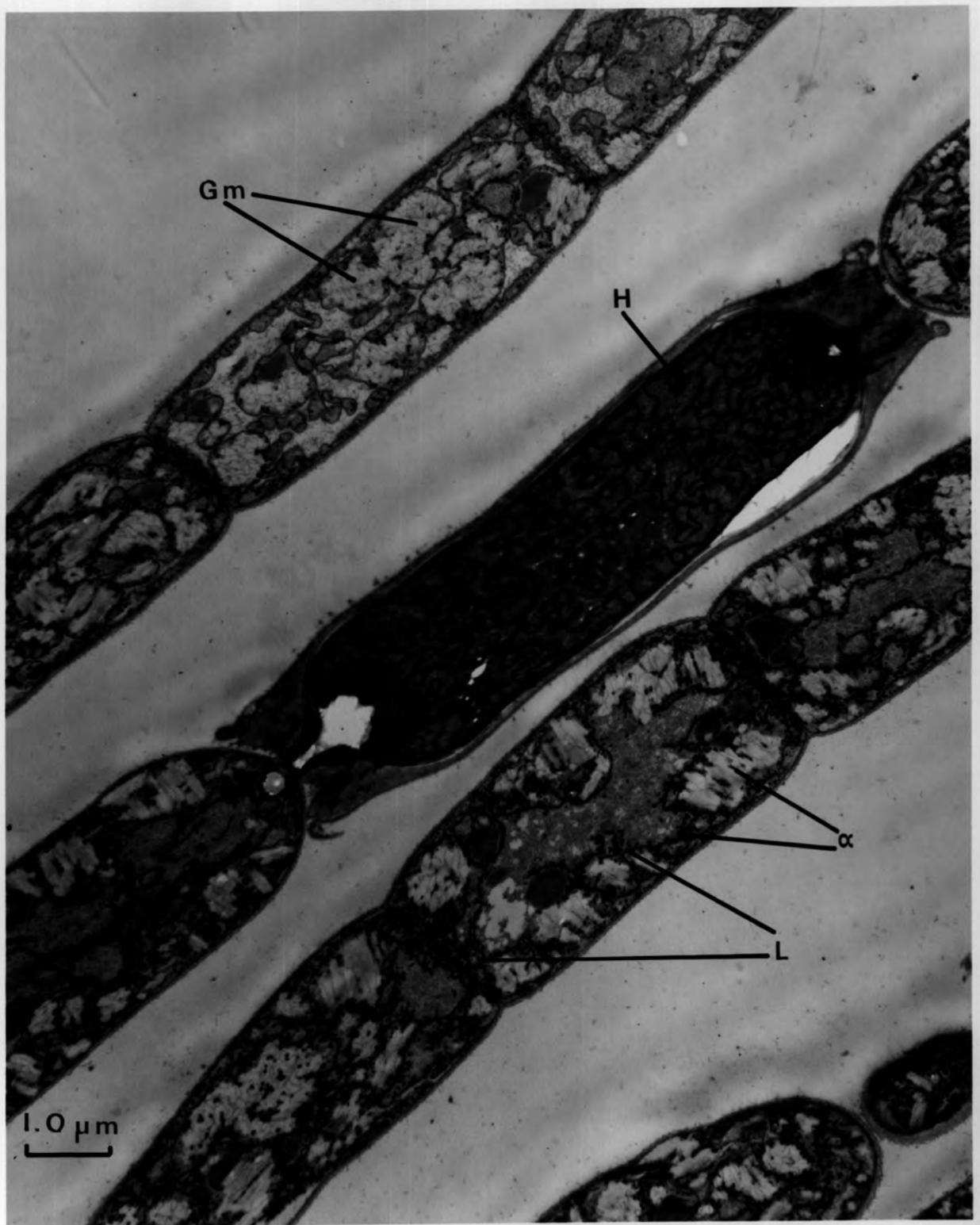
Fig. 25. A low magnification view of an <u>Aphanizomenon</u> <u>flos-aquae</u> colony collected from White Mere, Shropshire. $KMnO_4$ fixation. Mag. 35,000X.

Fig. 26.   An Aphanizomenon flos-aquae cell showing
abundance of intra-lamellar vesicles.
OsO_4 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, N^{15} 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 <u>Oscillatoria</u>
<u>agardhii</u> 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 adjoined 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.
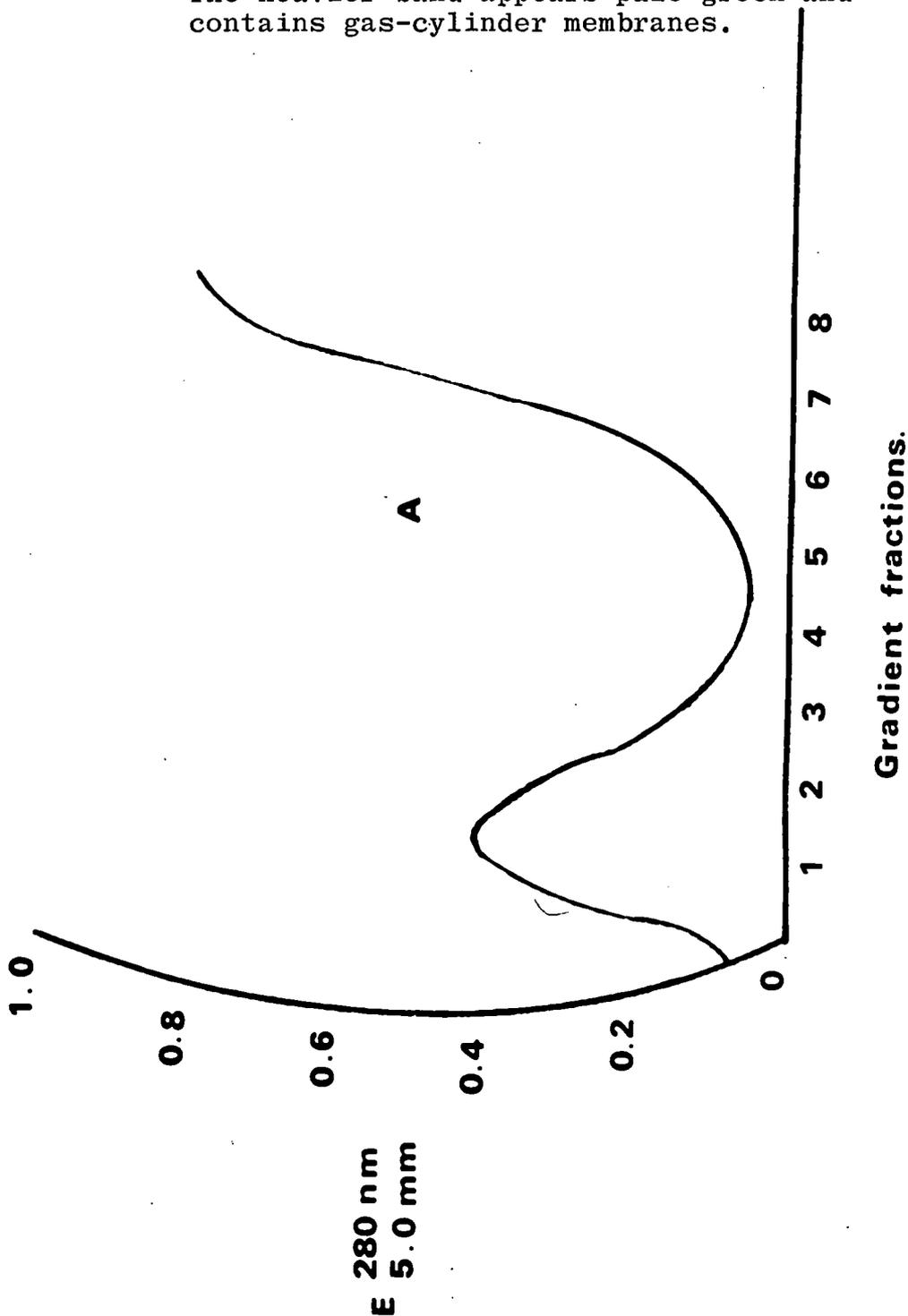
Fig. 28.   Density gradient fractionation of
membrane preparations from Anabaena
flos-aquae D125.   Compared with A.
flos-aquae D124 (Fig. 27), the heavy
band is absent and there is a blue
central peak resulting from the
presence of a large concentration
of phycocyanin.
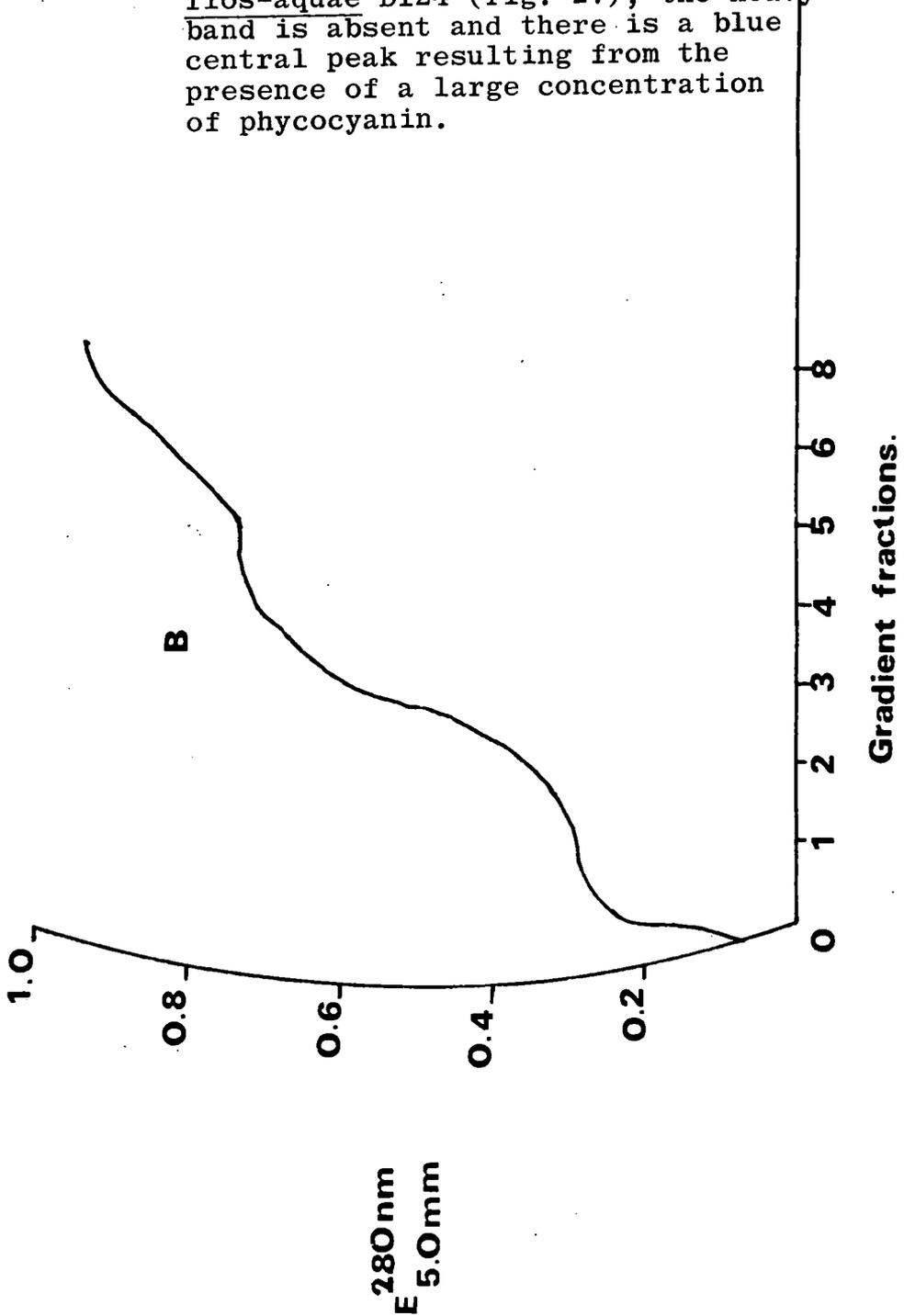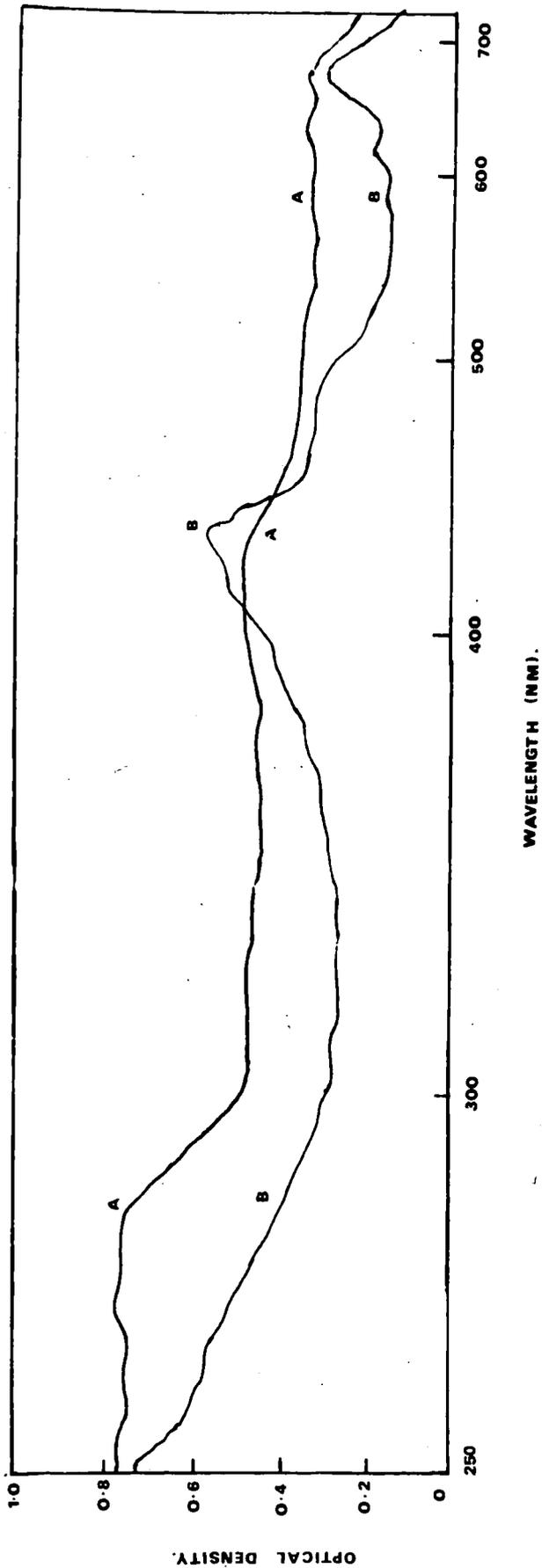
Fig. 29. The absorption spectra of the sediment fractions after density gradient centrifugation of membrane preparations from Anabaena flos-aquae D124 (A) and A. flos-aquae D125 (B).

Fractions 1 and 2 from <u>A</u>. <u>flos-aquae</u> 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 <u>A</u>. <u>flos-aquae</u> D125. Fractions 3, 4, 5 and 6, contained no examples of lamellae, occasional α-granules and, in the case of <u>A</u>. <u>flos-aquae</u> 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 <u>A</u>. <u>flos-aquae</u> 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. <u>Protein molecular weight determination of gas-cylinder membrane protein</u>

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 $22,000\pm2,000$ 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
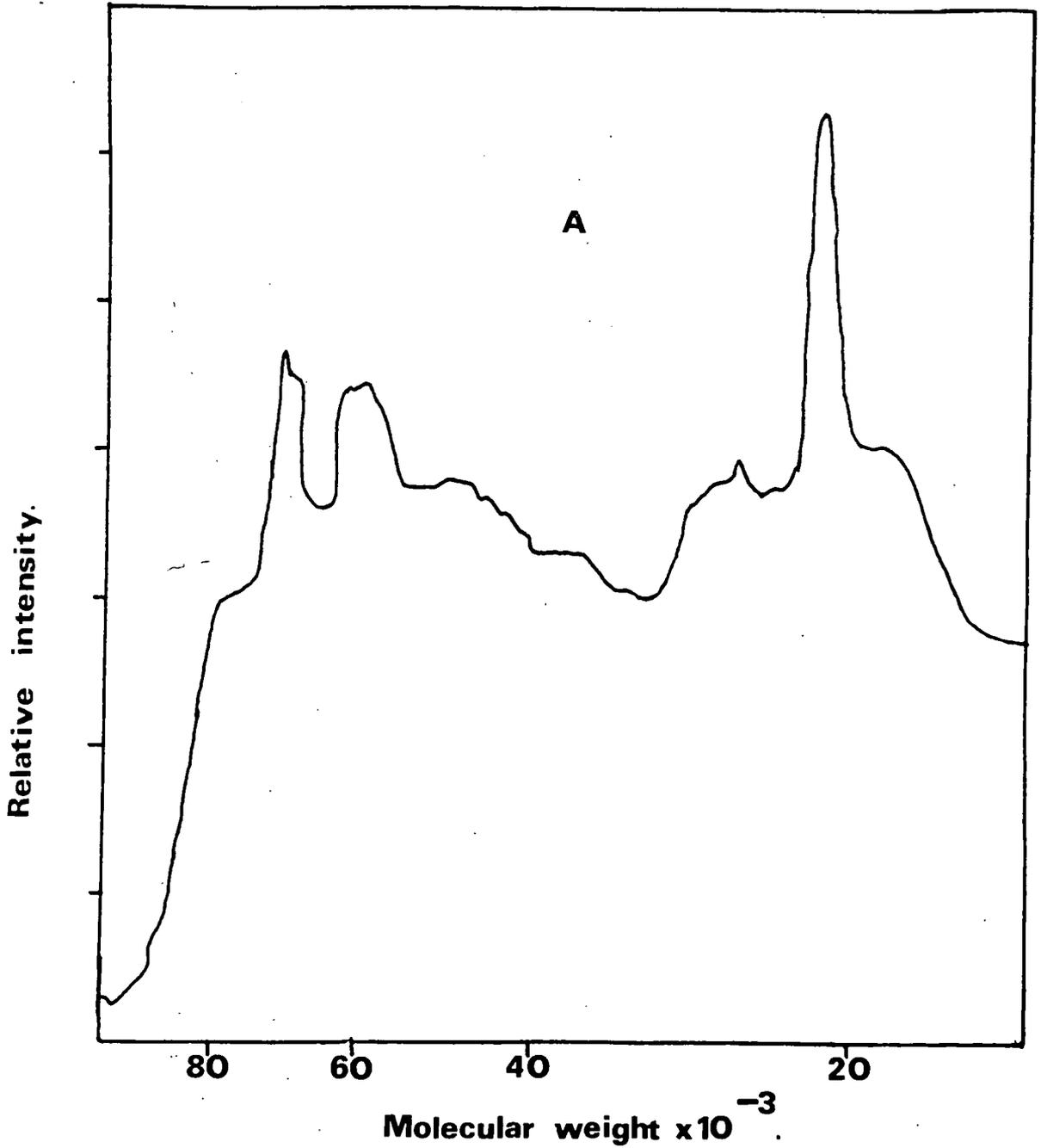
Fig. 30. The molecular weight distribution of the protein chains from the membrane fraction of Anabaena flos-aquae D124.
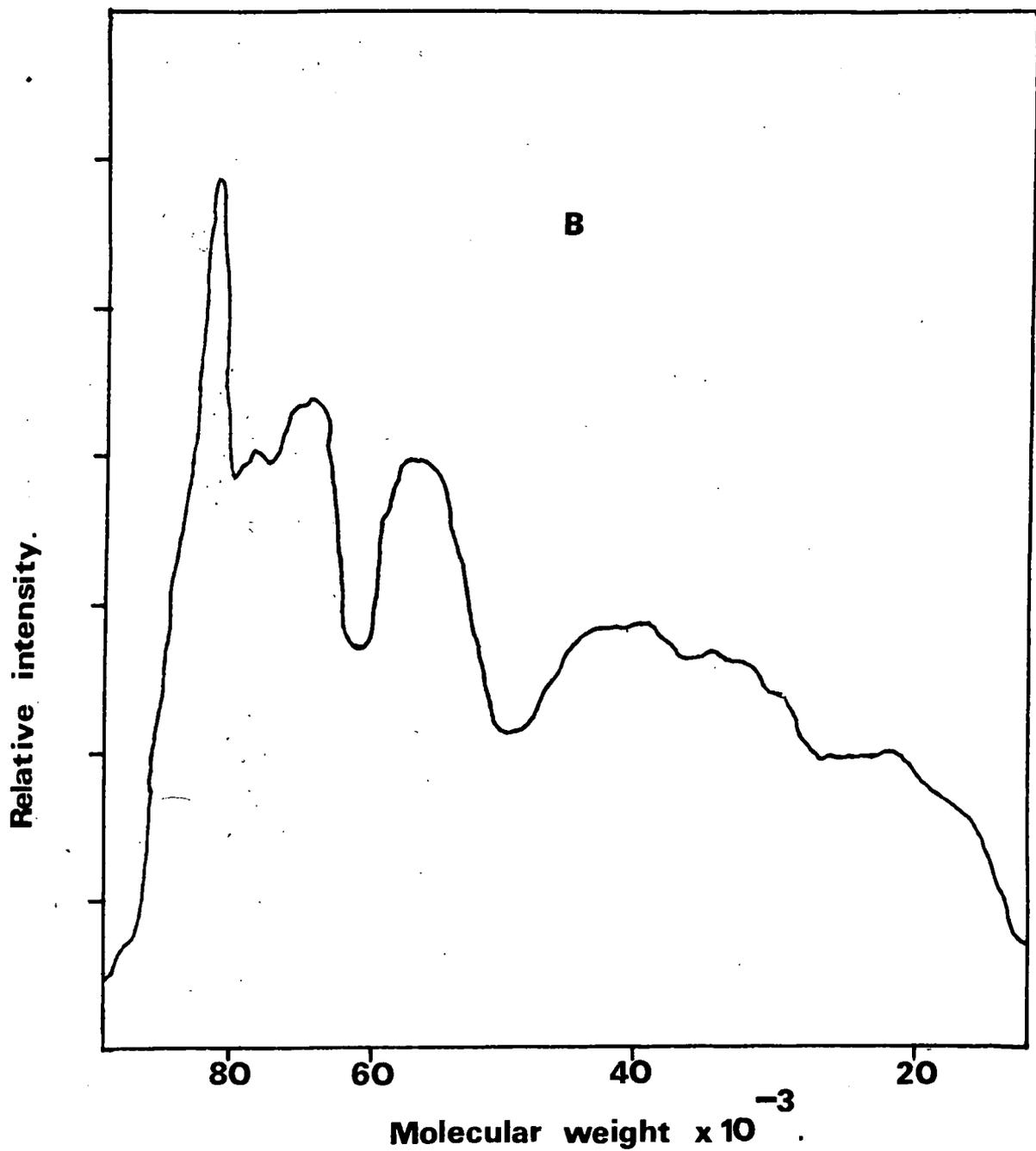
Fig. 31. The molecular weight distribution of the protein chains from the membrane fraction of Anabaena flos-aquae D125.

by poor penetration of sections by the digestate, or by some difference in the molecular branching pattern of the α-granules in <u>A</u>. <u>flos-aquae</u> D124 compared to those of <u>Oscillatoria chalybia</u> employed by GIESY (1964). The above data strongly indicate α-granules are polysaccharide. <u>Anabaena</u> <u>flos-aquae</u> 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 <u>A</u>. <u>flos-aquae</u> D124 cells.
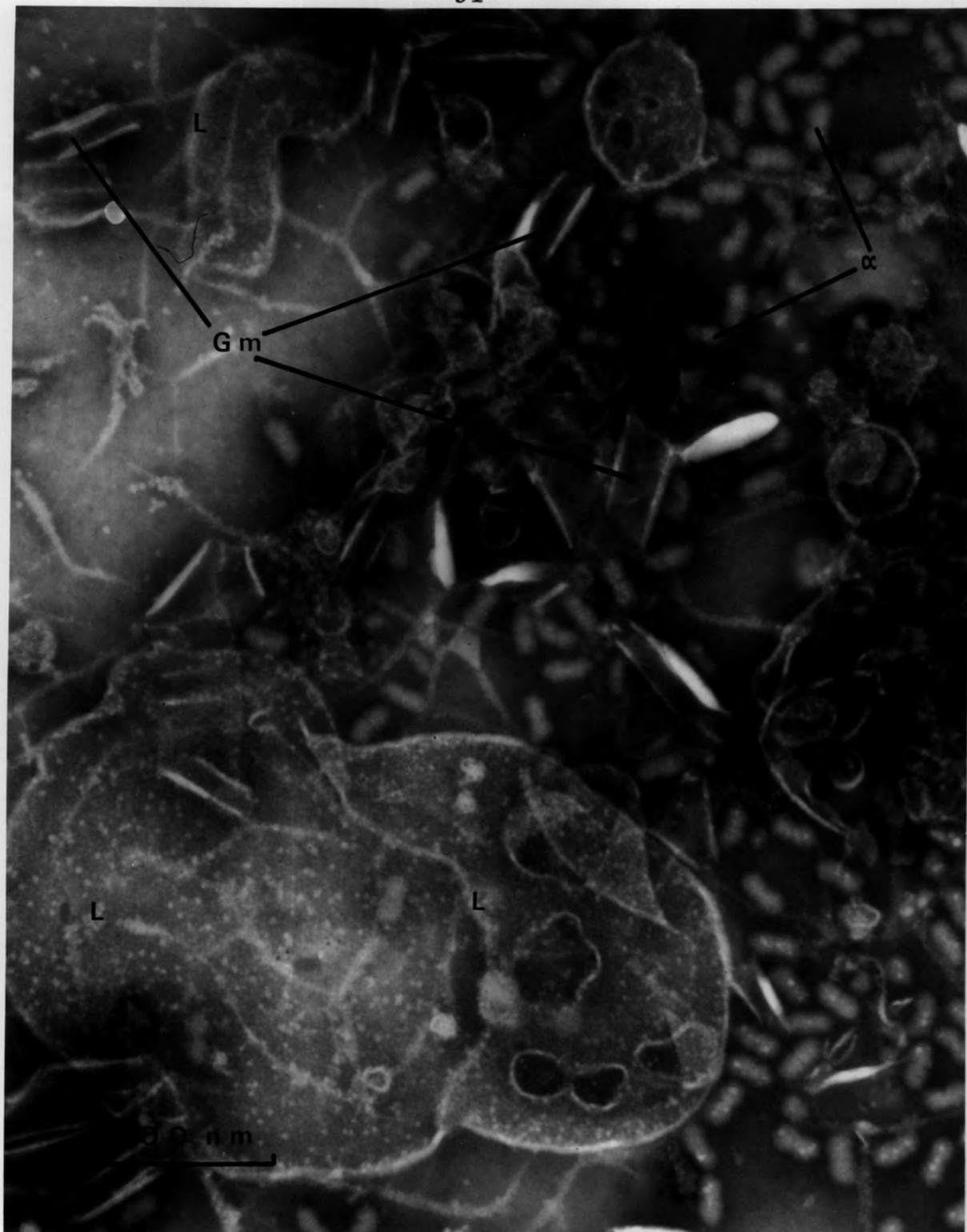
Fig. 32.   General view of a negatively-stained
preparation of the sediment from the density
gradient fractionation of Anabaena flos-aquae
D124 (Fig. 26, page 78).   Mag. 140,000X.
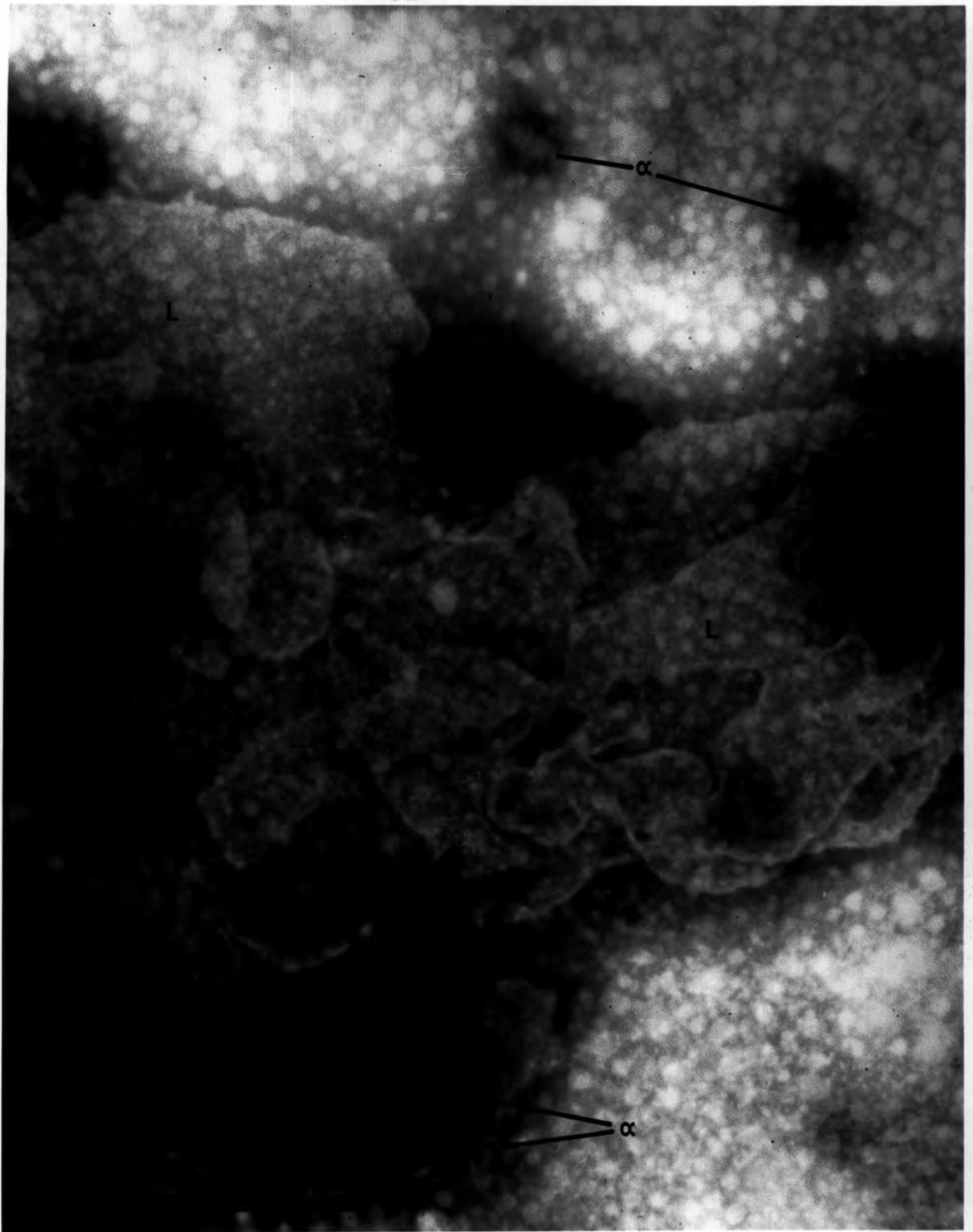
Fig. 33.   General view of a negatively-stained
preparation of the sediment from the density
gradient fractionation of Anabaena flos-aquae
D125 (Fig. 28, page 84).   Note the absence of
gas-cylinder membranes.   Mag. 135,000X.
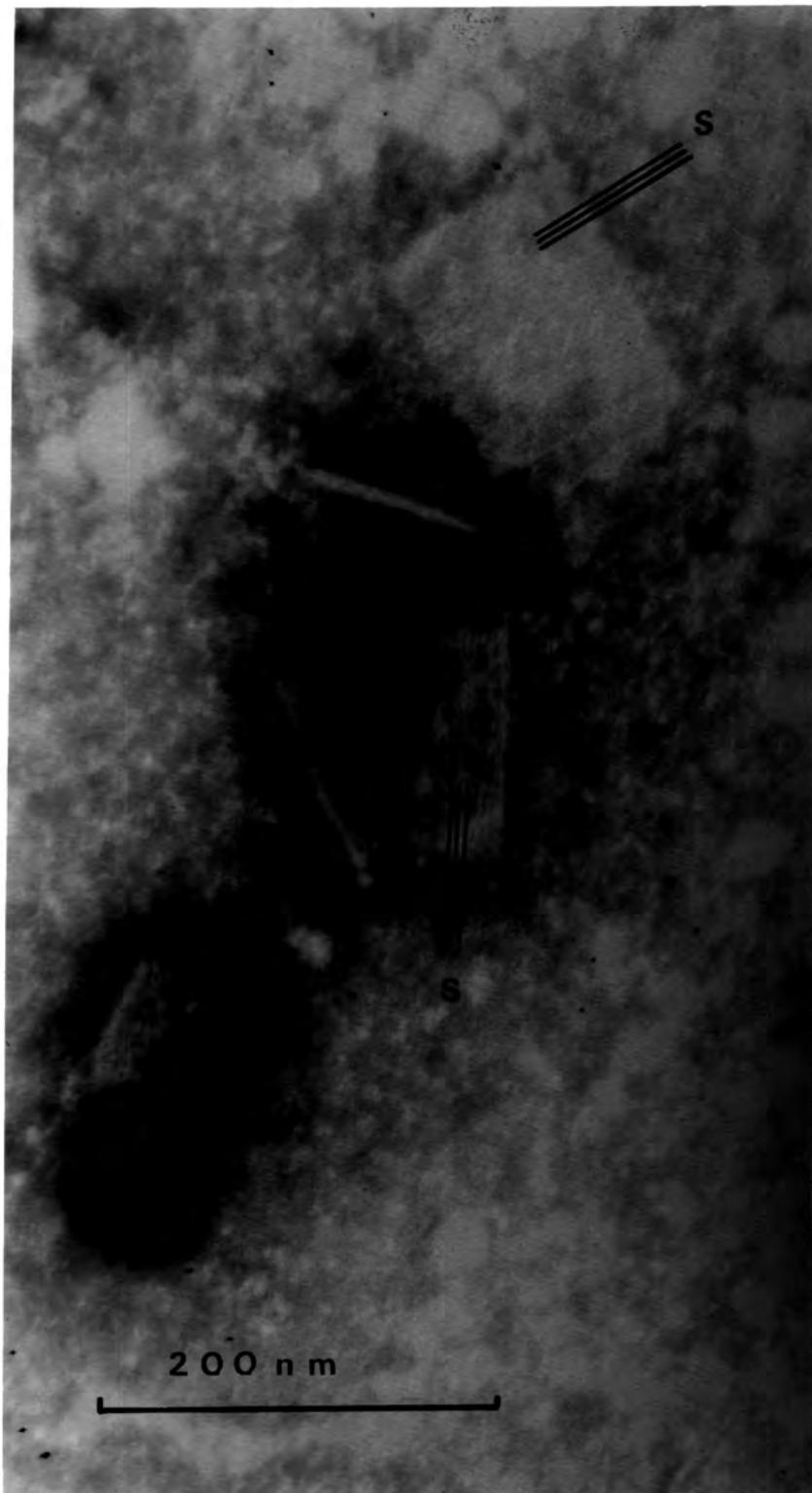
Fig. 34.   Small gas-cylinder membrane fragments
present in Fraction 2 from the density
gradient fractionation of <u>Anabaena flos-aquae</u>
D124 (Fig. 26, page 78).   Mag. 270,000X.
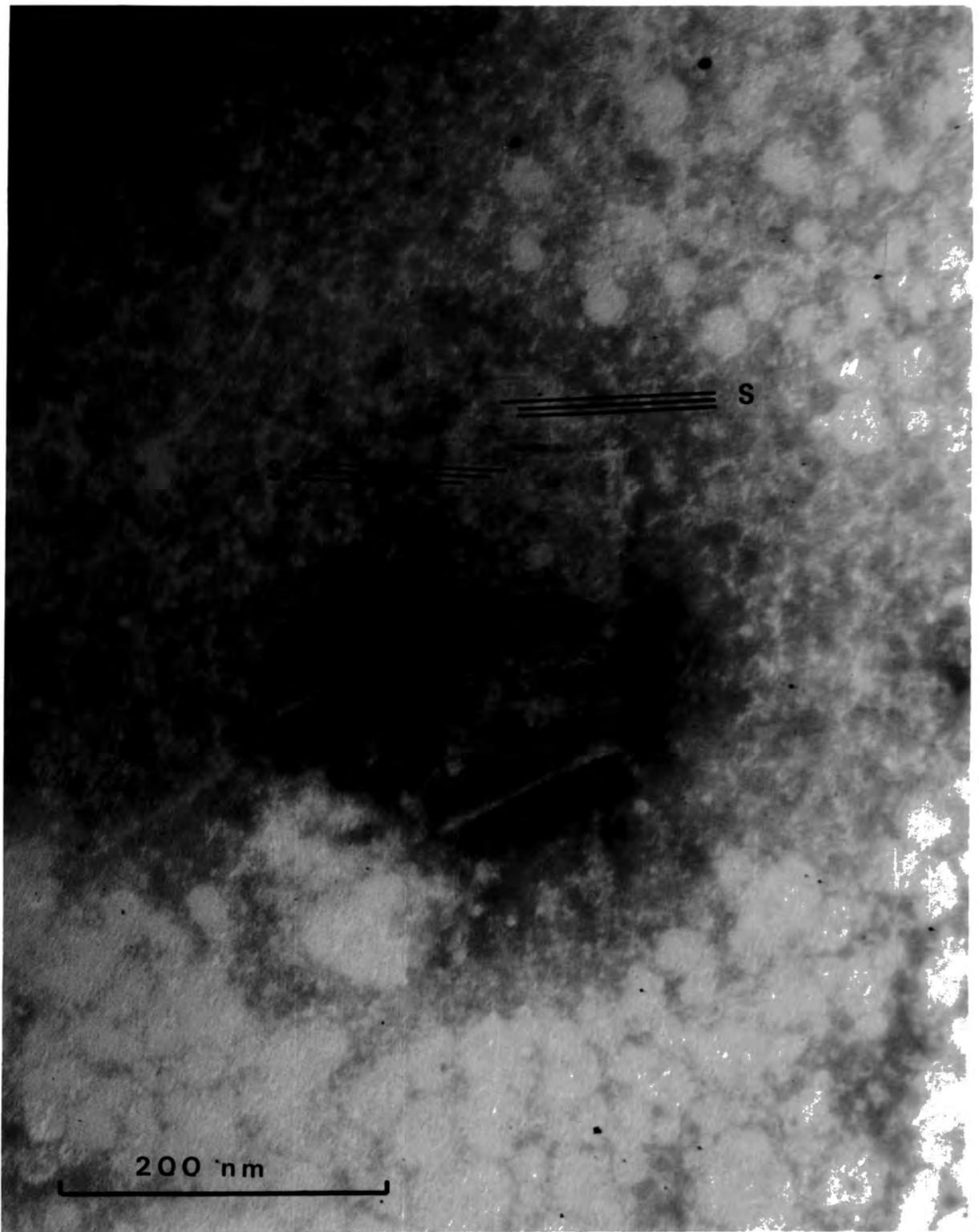
Fig. 35.   A negatively stained gas-cylinder membrane
            from the sediment of the density gradient
            fractionation of Anabaena flos-aquae D124.
            Mag. 270,000X.
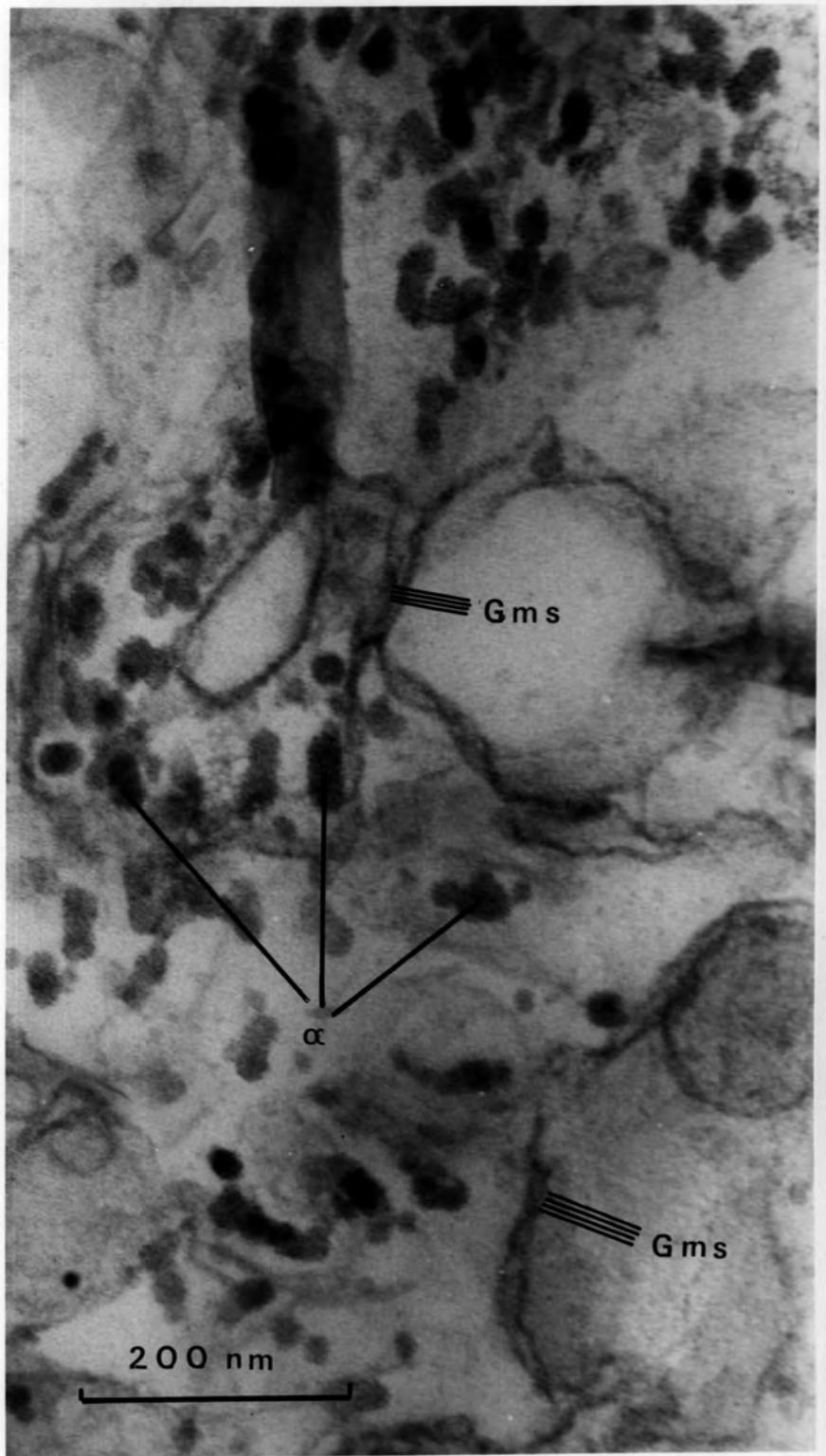
Fig. 36.   Fixed and embedded sediment from the density
gradient fractionation of Anabaena flos-aquae
D124.   Glutaraldehyde fixation.   Mag. 180,000X.

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 à 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
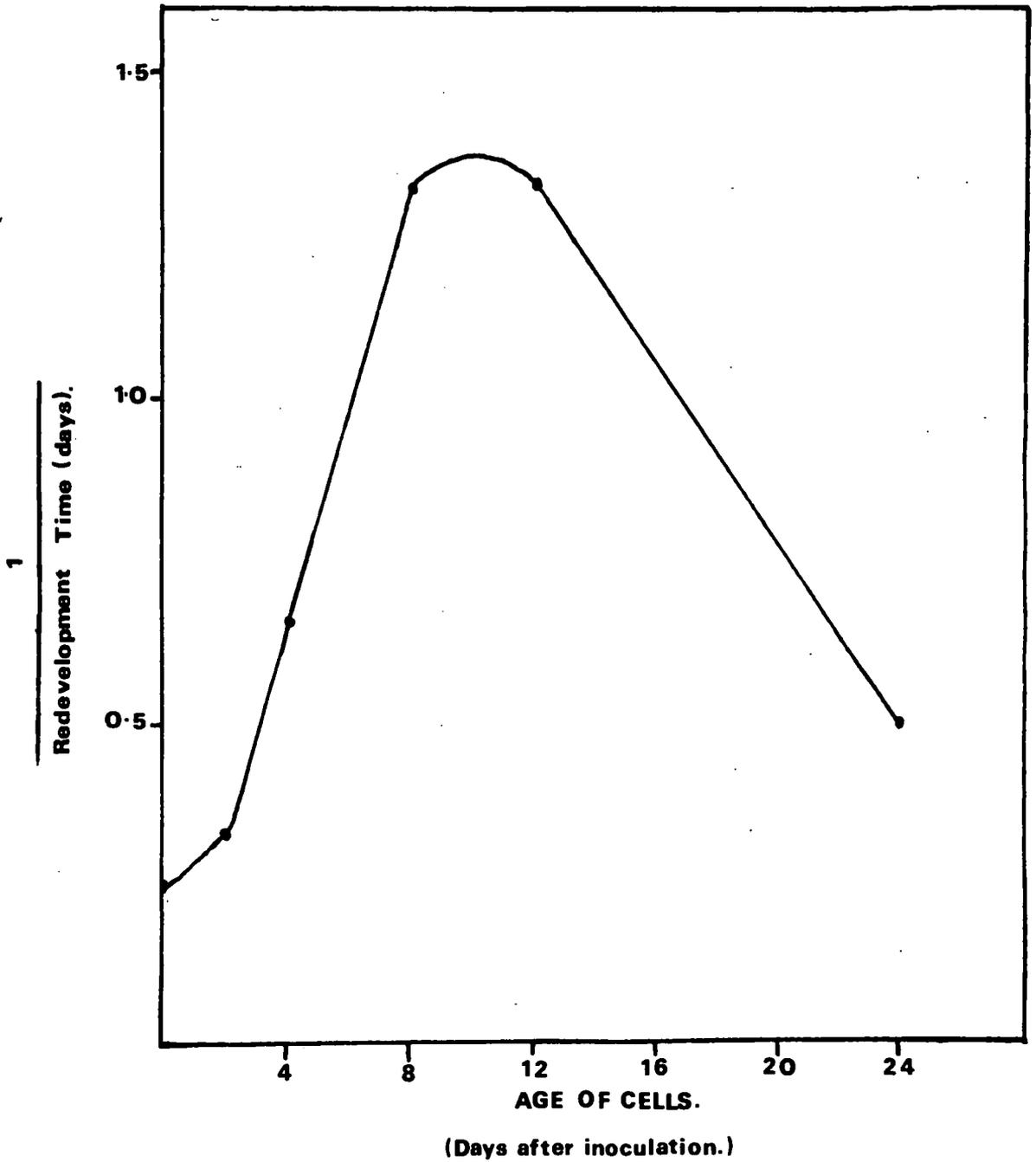
Fig. 37. The relationship between the age of cells
and the reciprocal of the time taken for
gas-vacuoles to redevelop in Anabaena
flos-aquae D124.

flos-aquae D124 immediately following gas-vacuole destruction

was $4.18 \mu m \times Sec^{-1}$ at 20 °C.   This filament size was chosen

for sedimentationary 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 sedimen-

tation rate of A. flos-aquae D125 filaments was $3.4 \mu m \times Sec^{-1}$.

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 $1.1 \mu m \times Sec^{-1}$.   At time +30 and 36 hrs. there

was/positive accumulation of filaments at the base of the
    no

sedimentation tube.   The sedimentation values plotted on

Fig. 38 for these times are shown as values of $0.0 \mu m \times Sec^{-1}$.

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 micro-

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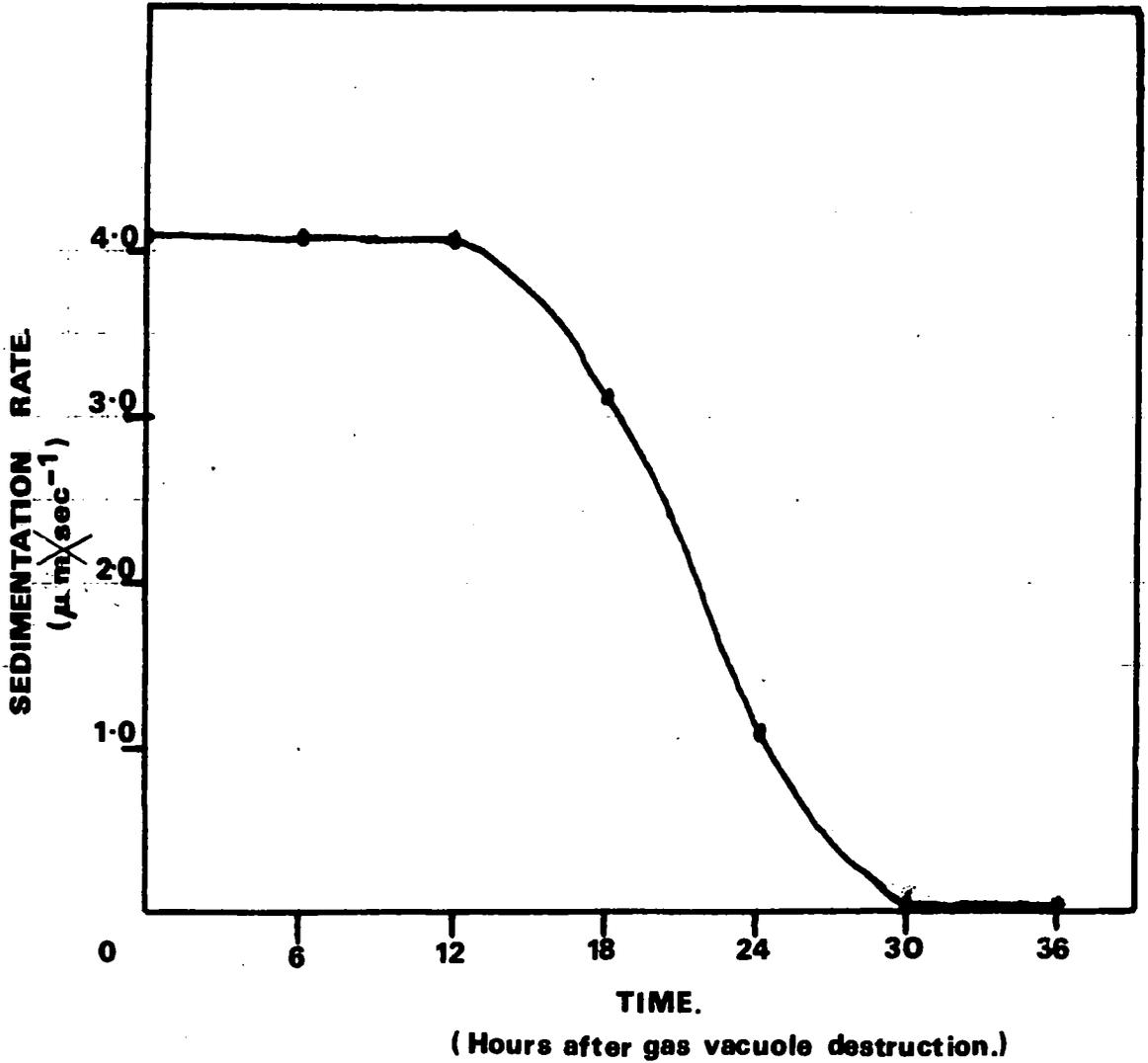
Fig. 38.   The changes in sedimentation
characteristics of 4-celled filaments of
Anabaena flos-aquae D124 during the
period of gas-vacuole redevelopment.

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. <u>The characterisation of blue-green algal gas-vacuoles</u>

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 <u>Anabaena</u> <u>flos-aquae</u> 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 <u>Trichodesmium erythaeum</u>,
which was reported by DUGDALE <u>et al</u>. (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 lipoid 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 ± 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 sub-units 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
lipoid 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 $\underline{P}$. $\underline{clathratiforme}$ 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 <u>Bacillus licheniformis</u>. 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 <u>Anabaena flos-aquae</u> 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 <u>Halobacterium</u> 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 redistri-
bution 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 m) long rods (40-60 m
length).   EBERLEY (1965) isolated Synechocystis parvala,
which is very small (0.7-0.9 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 $10\text{-}40\mu m \times sec^{-1}$. 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.

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BIBLIOGRAPHY

The abbreviations used for the titles of periodicals are those of the "World List of Scientific Periodicals", 4th ed.   Butterworths, London 1965.

ALLEN, M. B., and D. I. ARNON (1955).   Studies on nitrogen-fixing blue-green algae.   I. Growth and nitrogen fixation by Anabaena cylindrica Lemm.   Pl. Physiol., Lancaster 30, 366-372.

BAILEY-WATTS, A. E., M. E. BINDLOSS and J. H. BELCHER (1968). Freshwater primary production by a blue-green alga of bacterial size.   Nature, Lond. 220, 1344-1345.

BARSDATE, R. J., and R. C. DUGDALE (1965).   Rapid conversion of organic nitrogen to N_2 for mass spectrometry by an automated Dumas procedure.   Analyt. Biochem. 13, 1-5.

BOWEN, C. C., and T. JENSEN (1965).   Blue-green algae: fine structure of the gas-vacuoles.   Science, N.Y. 147, 1460-1462.

BRAND, F. (1901).   Bemerkungen über Grenzellen und über spontan rothe Inhaltskörper der Cyanophyceen.   Ber. dt. bot. Ges. 19, 152-159.

CANABAEUS, L. (1929).   Über die Heterocysten und Gasvacuolen der Blaualgen und ihre Beziehung zueinander. Pflanzenforschung 13.

CASPAR, D. L. D. (1963).   Assembly and stability of the tobacco mosaic virus particle.   Adv. Protein Chem. 18, 37-121.

CASPAR, D. L. D., and A. KLUG (1962).   Physical principles in the construction of regular viruses.   Cold Spring Harb. Symp. quant. Biol. 27, 1-24.

CHAPMAN, J., and M. SALTON (1962).   A study of several blue-green algae with the electron microscope.   Arch. Mikrobiol. 44, 311-322.

CHU, S. P. (1942).   The influence of the mineral composition of the medium on the growth of planktonic algae.   I. Methods and culture media.   J. Ecol. 30, 284-325.

CICAK, A., J. J. A. McLAUGHLIN and J. B. WITTENBERG (1963). Oxygen in the gas vacuole of the rhizopod protozoan Arcella.   Nature, Lond. 199, 983-985.

COHN, F. (1870).   Beitr. Biol. Pfl. 1, 141-207.

CRICK, F. H. C., and J. D. WATSON (1956).   The structure of small viruses.   Nature, Lond. 177, 473-475.

DAVSON, H. A., and J. F. DANIELLI (1935).   A contribution to the theory of permeability of thin films.   J. cell. comp. Physiol. 5, 495-508.

DUGDALE, R. C., J. J. GOERING and J. H. RYTHER (1964).   High nitrogen fixation rates in the Sargasso sea and the Arabian Sea.   Limnol. Oceanogr. 9, 507-510.

EBERLEY, W. R. (1965).   Preliminary results in the laboratory culture of planktonic blue-green algae.   Proc. Indiana Acad. Sci. 74, 165-168.

EBERLEY, W. R. (1966).   Problems in the laboratory culture of planktonic blue-green algae.   Environmental requirements of blue-green algae, Seattle.

ECHLIN, P. (1964).   Intra-cytoplasmic membranous inclusions in the blue-green alga, Anacystis nidulans.   Arch. Mikrobiol. 49, 267-274.

FISCHER, A. (1905).   Die Zelle der Cyanophyceen.   Bot. Ztg. 63, 51-130.

FITZ-JAMES, P. C. (1960). Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. J. biophys. biochem. Cytol. 8, 507-528.

FOGG, G. E. (1941). The gas-vacuoles of the Myxophyceae. Biol. Rev. 16, 205-217.

FOGG, G. E. (1949). Growth and heterocyst production in Anabaena cylindrica Lemm. II. In relation to carbon and nitrogen fixation. Ann. Bot. 13, 241-259.

FOGG, G. E. (1965). Algal cultures and phytoplankton ecology. The University of Wisconsin Press.

FRITSCH, F. E. (1945). Structure and reproduction of the algae, vol. 2. Cambridge.

GEITLER, L. (1932). Cyanophyceae. RABENHORST: Kryptogamenflora, Bd. 14. Leipzig.

GIESY, R. M. (1962). Observations on the cell structure of Oscillatoria limosa Agardh. Ohio J. Sci. 62, 119-124.

GIESY, R. M. (1964). A light and electron microscope study of interlamellar polyglucoside bodies in Oscillatoria chalybia. Am. J. Bot. 51, 388-396.

GOLDSTEIN, D. A., I. J. BENDET, M. A. LAUFFER and K. M. SMITH (1967). Some biological and physiochemical properties of blue-green algal virus LPP-1. Virology 32, 601-613.

GORHAM, P. R., J. McLACHLAN, U. T. HAMMER and W. K. KIM (1964). Isolation and culture of toxic strains of Anabaena flos-aquae (Lyngb.). Verh. int. Verein. theor. angew. Limnol. 15, 796-804.

GRIFFITHS, B. M. (1939). Early references to water-blooms in the British Isles. Proc. Linn. Soc. Lond. 151, 12-19.

HAMMER, U. T. (1964).   The succession of "bloom" species
      of blue-green algae and some causal factors.   Verh.
      int. Verein. theor. angew. Limnol. 15, 829-836.

HASELKORN, R. (1966).   Physical and chemical properties of
      plant viruses.   A. Rev. Pl. Physiol. 17, 137-154.

HOCHT, H., H. H. MARTIN and O. KANDLER (1965).   Zur Kenntnis
      der chemischen Zusammensetzung der Zellwand der
      Blaualgen.   Z. Pflanzenphysiol. 53, 39-57.

HOPWOOD, D. A., and A. M. GLAUERT (1960).   The fine structure
      of the nuclear material of a blue-green alga Anabaena
      cylindrica.   J. biophys. biochem. Cytol. 8, 813-823.

HOUWINK, A. L. (1956).   Flagella, gas vacuoles and cell-
      wall structure in Halobacterium halobium;   an electron
      microscope study.   J. gen. Microbiol. 15, 146-150.

HUTCHINSON, G. E. (1941).   Limnological studies in
      Connecticut.   IV. Mechanism of intermediary metabolism
      in stratified lakes.   Ecol. Monogr. 11, 21-60.

HUTCHINSON, G. E. (1967).   A treatise on limnology.   Volume
      2.   John Wiley, New York.

JOST, M. (1965).   Die Ultrastruktur von Oscillatoria rubescens.
      Arch. Mikrobiol. 50, 211-245.

JOST, M., and P. MATILE (1966).   Zur Charakterisierung der
      Gasvacuolen der Blaualge Oscillatoria rubescens.
      Arch. Mikrobiol. 53, 50-58.

KLEBAHN, H. (1895).   Gasvakuolen, ein Bestandteil der Zellen
      der wasserblütebildenden Phycochromaceen.   Flora, Jena
      80, 241-282.

KLEBAHN, H. (1922).   Neue Untersuchungen über die Gasvakuolen.
      Jb. wiss. Bot. 61, 535-589.

KLEBAHN, H. (1925).   Weitere Untersuchungen über die
     Gasvakuolen.   Ber. dt. bot. Ges. 43, 143-159.

KLEBAHN, H. (1929).   Ueber die Gasvakuolen der Cyanophyceen.
     Verh. int. Verein. theor. angew. Limnol. 4, 408-414.

KOLKWITZ, R. (1928).   Ueber Gasvakuolen bei Bakterien.
     Ber. dt. bot. Ges. 46, 29-34.

KRATZ, W. A., and J. MYERS (1955).   Nutrition and growth
     of several blue-green algae.   Amer. J. Bot. 42, 282-287.

KROGH, A. (1939).   Osmotic regulation in aquatic animals.
     Cambridge.

LANG, N. J. (1968).   The fine structure of blue-green algae.   A.
     Rev. Microbiol. 22, 15-46.

LARSEN, H., S. OMANG and H. STEENSLAND (1967).   On the
     gas-vacuoles of the Halobacteria.   Arch. Mikrobiol. 59,
     197-203.

LAUTERBORN, R. (1915).   Die sapropelische Lebeudt.   Verh.
     naturh.-med. Ver. Heidelb. 13, 395-481.

LEMMERMAN, E. (1910).   Algen, 1.   Kryptogamenflora der
     Mark Brandenburg 3.

LUND, J. W. G. (1965).   The ecology of the freshwater
     phytoplankton.   Biol. Rev. 40, 231-293.

MARGALEF, R. (1957).   Nueros aspectos del problema da la
     suspension en los organismos planctonicos.
     Investigacion pesq. 7, 105-116.

MARKHAM, R. (1968).   Deviations in nitrogen metabolism
     associated with viruses.   Recent aspects of Nitrogen
     Metabolism in Plants, Ed. E. J. HEWITT and C. V. CUTTING.
     Academic Press, London.

McLACHLAN, J., and P. R. GORHAM (1961).   Growth of _Microcystis_ _aeruginosa_ Kutz. in a precipitate-free medium buffered with tris.   Can. J. Microbiol. _7_, 869-882.

McLACHLAN, J., U. T. HAMMER and P. R. GORHAM (1963).   Observations on the growth and colony habits of ten strains of _Aphanizomenon_ _flos-aquae_.   Phycologia _2_, 157-168.

MOLISCH, H. (1903).   Die sogenannten gas-vacuolen und das Schweben gewisser Phycochromaceen.   Bot. Ztg. _61_, 47-58.

MOLISCH, H. (1906).   Zwei neue Purpurbakterien mit Schwebekörperchen.   Bot. Ztg. 64, 223-232.

MOLLENHAUER, H. H. (1959).   Permanganate fixation of plant cells.   J. biophys. biochem. Cytol. _6_, 431-436.

MUNK, W. H., and G. A. RILEY (1952).   Absorption of nutrients by aquatic plants.   J. mar. Res. _11_, 215-240.

van NIEL, C. B. (1957).   Bergey's Manual of Determinative Bacteriology.   7th ed.   Ed.: R. S. BREED, E. G. D. MURRAY and N. R. SMITH.   Williams and Wilkins, Baltimore.

NORTHCOTE, D. H. (1968).   Structure and function of plant-cell membranes.   Br. med. Bull. _24_, 101-112.

ORRELL, S. A., and E. BUEDING (1958).   Sedimentation characteristics of glycogen.   J. Am. chem. Soc. _80_, 3800.

OSTWALD, W. (1902).   Zur Theorie des Planktons.   Biol. Zbl. _22_, 596-605.

OUMI, T., and S. OSAWA (1966).   Int. Exchange Group Memo. 235.

PANKRATZ, H. S., and C. C. BOWEN (1963).   Cytology of blue-green algae.   I. The cells of _Symploca_ _muscorum_. Amer. J. Bot. _50_, 387-399.

PEARSON, J. E., and J. M. KINGSBURY (1966).   Culturally induced variation in four morphologically diverse blue-green algae.   Am. J. Bot. 53, 192-200.

PEAT, A., and B. A. WHITTON (1967).   Environmental effects on the structure of the blue-green alga, Chlorogloea fritschii.   Arch. Mikrobiol. 57, 155-180.

PETTER, H. F. M. (1931).   On bacteria of salted fish. Proc. Sect. Sci. K. ned. Akad. Wet. 34, 1417.

PFENNIG, N., and G. COHEN-BAZIRE (1967).   Some properties of the green bacterium Pelodictyon clathratiforme. Arch. Mikrobiol. 59, 226-236.

PFENNIG, N., M. C. MARKHAM and S. LIAAEN-JENSEN (1968). Carotenoids of Thiorbodaceae.   8. Isolation and characterization of a Thiothece, Lamprocystis and Thiodictyon strain and their carotenoid pigments. Arch. Mikrobiol. 62, 178-191.

PRINGSHEIM, E. (1966).   The nature of pseudovacuoles in Cyanophyceae.   Nature, Lond. 210, 549-550.

REYNOLDS, C. (1967).   The breaking of the Shropshire Meres - some recent investigations.   Shropshire Conservation Trust Bulletin 10, 9-14.

REYNOLDS, C. (1969).   The "Breaking of the Meres" - some recent investigations.   Br. phycol. Bull.   (In press).

REYNOLDS, E. S. (1963).   The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17, 208-212.

RICHTER, P. (1894).   Gloeotrichia echinulata, eine Wasserblüte des Gr. und Kl. Plöner Sees.   ForschBer. biol. Sta. Plön. 2, 31.

ROBERTSON, J. D. (1959). The ultrastructures of cell membranes and their derivatives. Biochem. Soc. Symp. 16, 3-43.

RODHE, W. (1948). Environmental requirements of fresh-water plankton algae. Symb. bot. Upsal. 10, 1-149.

ROTHFIELD, L., and A. FINKELSTEIN (1968). Membrane biochemistry. A. Rev. Biochem. 37, 463-496.

SHAPIRO, A. L., E. VINUELA and J. V. MAIZEL (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. biophys. Res. Commun. 28, 815-820.

SMITH, K. M. (1965). Plant virus-vector relationships. Adv. Virus Res. 11, 61-95.

SMITH, R. V., and A. PEAT (1967a). Comparative structure of the gas-vacuoles of blue-green algae. Arch. Mikrobiol. 57, 11-122.

SMITH, R. V., and A. PEAT (1967b). Growth and gas-vacuole development in vegetative cells of Anabaena flos-aquae. Arch. Mikrobiol. 58, 117-126.

STAUB, R. (1961). Erhahrungsphysiologisch-autokologische Untersuchungen an der planktischen Blaualge Oscillatoria rubescens DC. Schweiz. Z. Hydrol. 23, 82-198.

STOECKENIUS, W., and W. H. KUNAU (1968). Further characterisation of particulate fractions from lysed cell envelopes of Halobacterium halobium and isolation of gas-vacuole membranes. J. Cell Biol. 34, 365-393.

STOECKENIUS, W., and R. ROWEN (1967). A morphological study of Halobacterium halobium and its lysis in media of low salt concentration. J. Cell Biol. 34, 365-393.

STRODTMANN, S. (1895). Die Ursache des Schwebevermögens bei den Cyanophyceen. Biol. Zbl. _15_, 113-115.

SUN, C. H. (1961). Electron microscope observations on _Gloeocapsa_ sp. Bull. Torrey bot. Club _88_, 106-110.

TISCHER, R. G. (1965). Pure culture of _Anabaena flos-aquae_ A-37. Nature, Lond. _205_, 419-420.

TUOMINEN, F. W., and R. W. BERNLOHR (1967). Energetics and motility in _Bacillus lichenformis_. J. Bact. _93_, 1725-1726.

UEDA, K. (1966). Virus-like structures in the cells of the blue-green alga _Oscillatoria princeps_. Exptl Cell Res. _40_, 671-673.

WALSBY, A. E. (1969). Permeability of blue-green algal gas-vacuole membranes to gas. Proc. R. Soc. B (In Press).

WALSBY, A. E., and H. H. EICHELBERGER (1968). The fine structure of gas-vacuoles released from cells of the blue-green alga _Anabaena flos-aquae_. Arch. Mikrobiol. _60_, 76-83.

WHITTAKER, V. P. (1968). Structure and function of animal-cell membranes. Br. med. Bull. _24_, 101-106.

WHITTON, B. A. (1969). Algae in St. James's Park Lake, London. Lond. Nat. (In Press).

WILLIAMS, W. T., and D. A. BARBER (1961). Mechanisms in Biological Competition. Symp. Soc. exp. Biol. _15_, 132-144.

WINOGRADSKY, S. (1888). Zur Morphologie und Physiologie der Schwefelbakterien. A. Felix, Leipzig.

ZEHNDER, A. (1963). Kulturversuche mit _Gloeotrichia echinulata_ (J. E. Smith) P. Richter. Schweiz. Z. Hydrol. _25_, 65-83.

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 gutullata, 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.
Sapropelic 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
                        with
bacteria associated/sapropelic blue-greens.

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 <u>Microcystis</u> <u>aeruginosa</u> 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. werneri.

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 pseudovacuolata*.

(TOTAL: 68 species).

REFERENCES

BÖCHER, T. W. (1949).   Studies on the sapropelic flora of the lake Flyndersø with special reference to the Oscillatoriaceae.   Biol. Skr. 21, 1-46.

BORNET, E., and G. THURET (1880).   Notes algologiques II. Paris.

GEITLER, L. (1932).   Cyanophyceae.   RABENHORST: Kryptogamenflora, Bd. 14.   Leipzig.

GORBUNOVA, N. P. (1960).   Gazovye vakuoli v kletkakh gormogoniev Amorphonostoc punctiforme (Kütz.). Elenk. Bot. Zhur. 45, 1546-1548.

HORTOBAGYI, T. (1954).   Quelques precisions sur la formation et la signification systematique des pseudovacuoles gazeuses des Cyanophycees.   Rapp. et communs. huitieme congr. intern. bot. Paris 17, 12-14.

JOST, M., and A. ZEHNDER (1966).   Die Gasvakuolen der Blaualgen Microcystis aeruginosa.   Schweiz. Z. Hydrol. 28, 1-3.

KOLKWITZ, R. (1928).   Uber Gasvacuolen bei Bakterien. Ber. dt. bot. Ges. 43, 29-34.

LANG, N. J. (1968).   The fine structure of blue-green algae. A. Rev. Microbiol. 22, 15-46.

LAUTERBORN, R. (1915).   Die sapropelische Lebewelt.   Verh. naturh.-med. Ver. Heidelb. 13, 395-481.

LEMMERMAN, E. (1898).   Der grosse Waterneverstorfer Binnensee. Forschungsberichte Biologische Station Plön. 4.

LEMMERMAN, E. (1901).   Zur kenntnis der algen flora des Saaler Boddens.   Forschungsberichte Biologische Station Plön. 8.

PFENNIG, N. (1967).   Photosynthetic bacteria.   A. Rev.
     Microbiol. 21, 285-324.

SMITH, R. V., and A. PEAT (1967a).   Comparative structure
     of the gas-vacuoles of blue-green algae.   Arch.
     Mikrobiol. 57, 111-122.

VAN GOOR, A. C. J. (1918).   Zur Kenntnis der Oscillatoriaceen.
     Rec. Trav. bot. neerl. 15, 255-262.

VAN GOOR, A. C. J. (1925).   Contribution a la physiologie
     des Cyanophycées.   Sur les pseudovacuoles rouges et
     leur signification.   Revue algol. 2, 19-38.

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

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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, Gloeotrichia 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 mμ 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 mμ to 500 mμ and in *A. flos-aquae* it is from 100 mμ to 1300 mμ. 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 mμ and 2 mμ 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.

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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
Anabaena flos-aquae	Westfield College, London		124
Gloeotrichia echinulata	Cambridge	1432/1	126
Oscillatoria agardhii	F.B.A., Windermere		132
Nostoc linckia	C.S.S.R. culture collection	125	130
Microcystis aeruginosa	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 OsO_4 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% $KMnO_4$ 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). Fixed material was dehydrated through an ethanol series and embedded in Araldite. Sections were cut on an LKB ultrotome 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
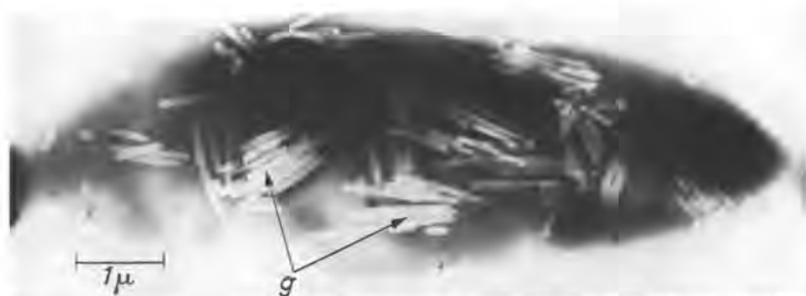

Fig. 1. Direct preparation of *A. flos-aquae* showing distribution of gas-cylinders. OsO_4 fixation. Mag. 12,000 ×. The symbols used for all photographs are as follows: *ed* electron-dense body; *med* membrane surrounding electron-dense body; *f* nucleoplasmic fibril; *g* gas-cylinder; *gm* gas-cylinder 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
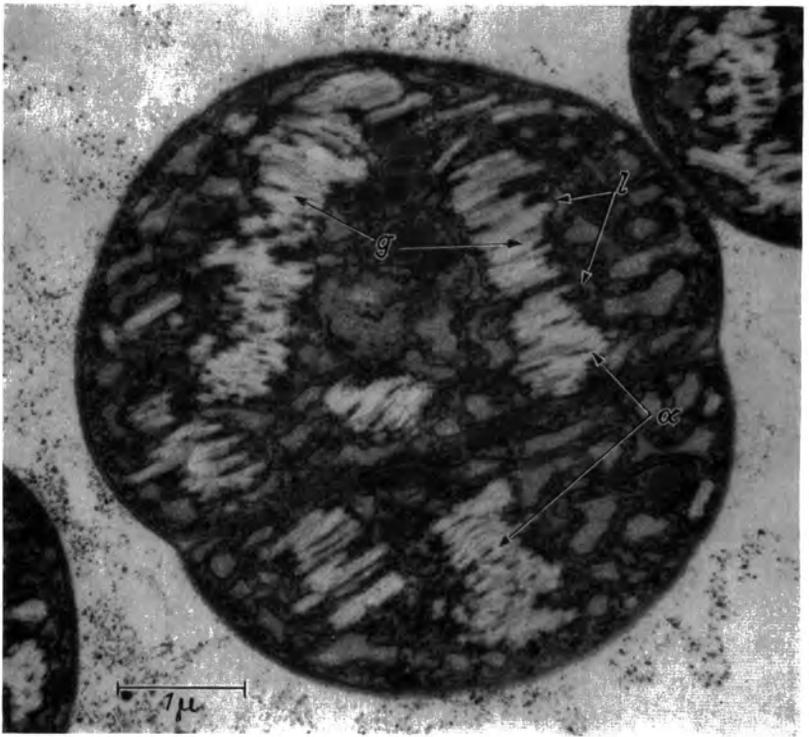
Fig. 2. General cell view of *A. flos-aquae* showing distribution of gas-cylinders and their association with lamellae and α-granules. KMnO$_4$ fixation. Mag. 17,500×
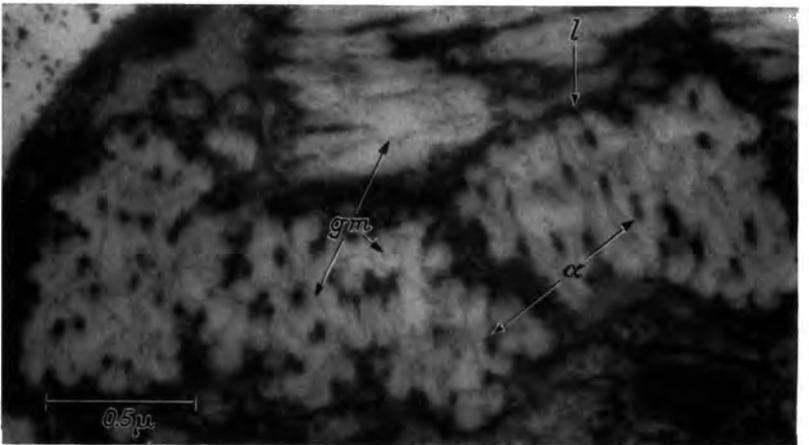

Fig. 3. Appearance of gas-cylinders in transverse sectional view in *A. flos-aquae*. KMnO$_4$ fixation. Mag. 41,000×

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.
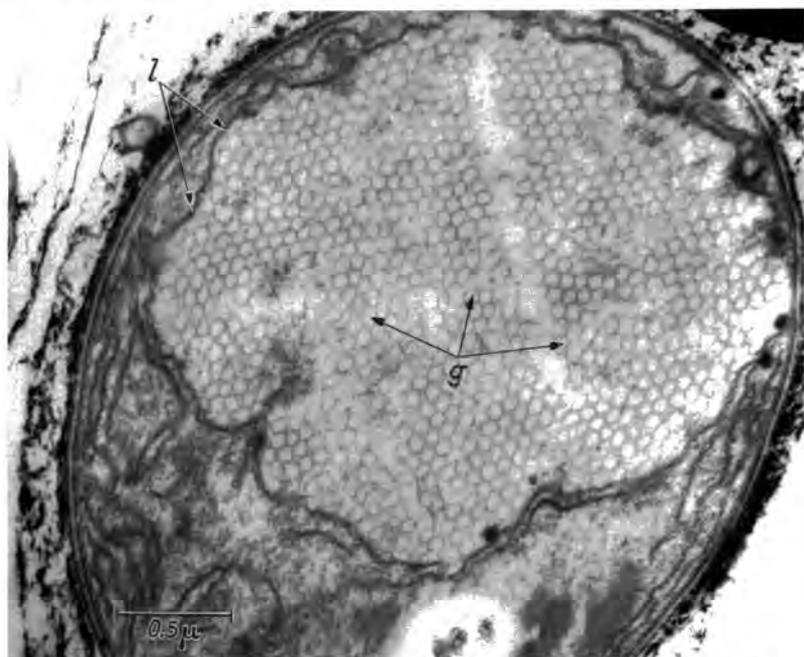
Fig. 4. Transverse section of *G. echinulata* trichome showing a collection of gas-cylinders and the associated lamellae. OsO_4 fixation. Mag. 31,000 ×

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 $KMnO_4$ 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. OsO₄ fixation. Mag. 22,000×

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.
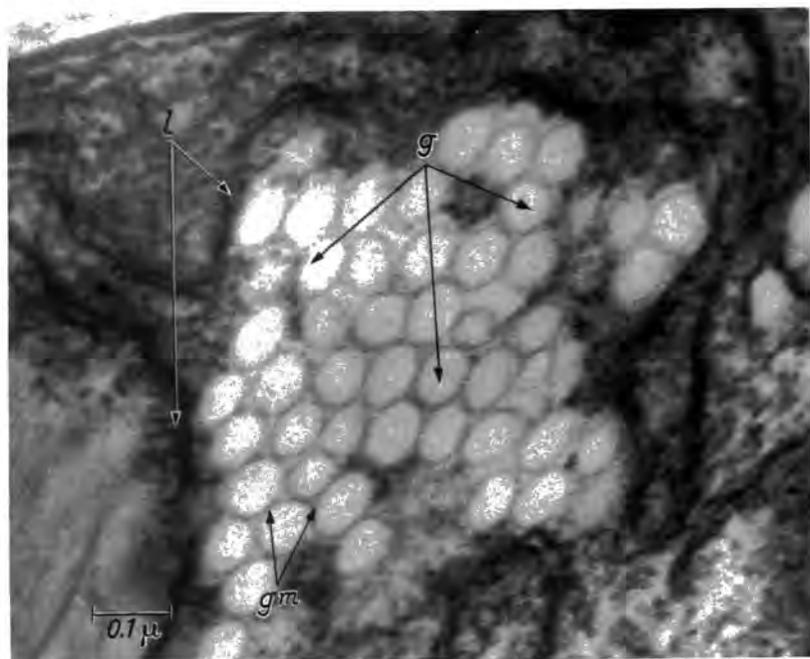
Fig.6. Appearance of gas-cylinders in transverse section in *A. flos-aquae*. OsO₄ fixation. Mag. 103,000 ×

d) Fine structure of gas-cylinders

The gas-cylinders in all organisms studied have a standard width of 70 mμ. The range of length is 100 mμ to 500 mμ in *G. echinulata* and *O. agardhii*. *A. flos-aquae* has a wider range in lengths of gas-cylinders (100 mμ—1300 mμ) than other organisms examined (see Table 2 and Fig. 1). The width of the gas-cylinder membrane is 2 mμ after OsO₄ 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 KMnO₄ fixation. Figs. 2, 3, 7 and 8 show the appearance of gas-cylinder membranes after KMnO₄ 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 KMnO₄ fixation with that obtained by OsO₄ can be made by studying Figs. 3 and 6. Good preservation of gas-cylinder membranes in *G. echinulata* is shown
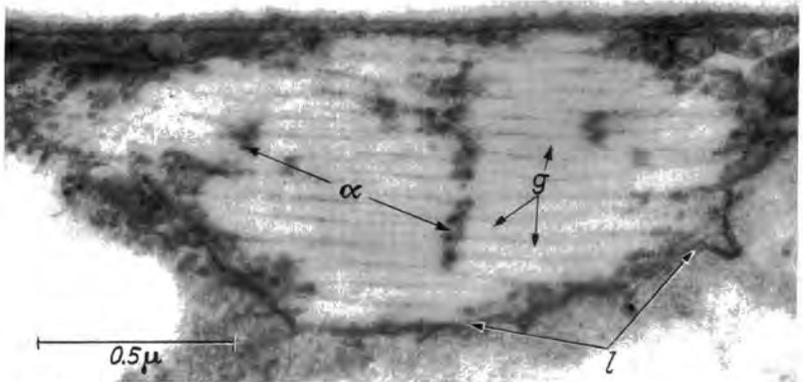
Fig. 7. Appearance of gas-cylinders in longitudinal section in trichome of *G. echinulata* and their association with lamellae and α-granules. KMnO₄ fixation. Mag. 47,500 ×
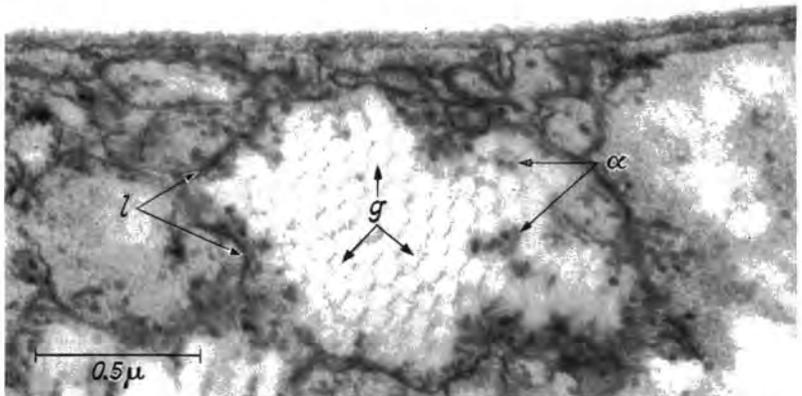

Fig. 8. Appearance of gas-cylinders in cross-section in trichome of *G. echinulata*. KMnO₄ fixation. Mag. 53,500 ×

Table 2. *Summary of electron microscope observations*

Organism	Width of gas-cylinders in mμ	Range of lengths of gas-cylinders in mμ	Mean % volume of cells occupied by gas-vacuoles
A. flos-aquae	70	100 — 1300	22
G. echinulata	70	100 — 500	22
O. agardhii	70	100 — 500	39
Aphanizomenon flos-aquae (Bowen and Jensen, 1965)	75	100 — 1000	—
Oscillatoria rubescens (Jost and Matile, 1966)	65	210 — 430	—
Microcystis aeruginosa (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 3 mμ.
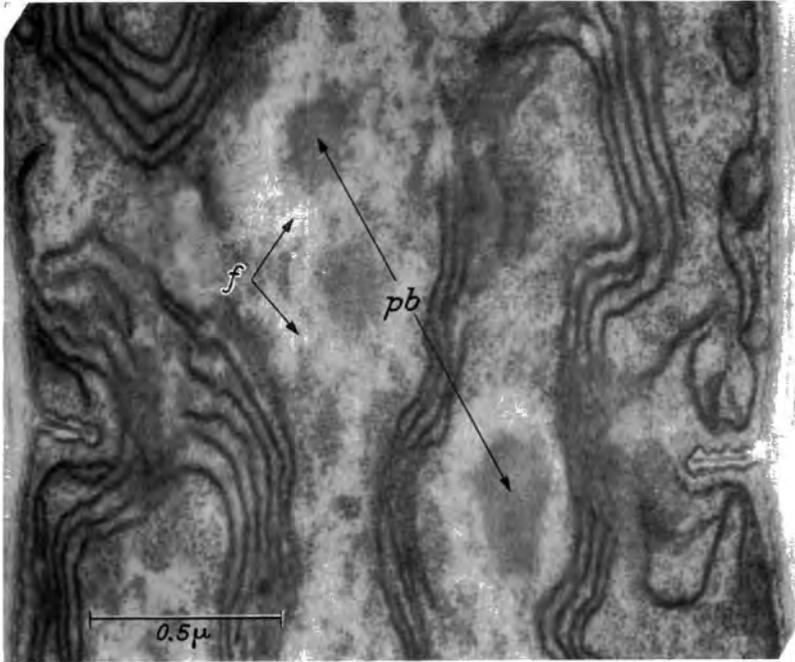

Fig. 9. Appearance of central area of a *N. linckia* cell showing nucleoplasmic fibrils associated with polyhedral bodies. OsO$_4$ fixation. Mag. 52,000×
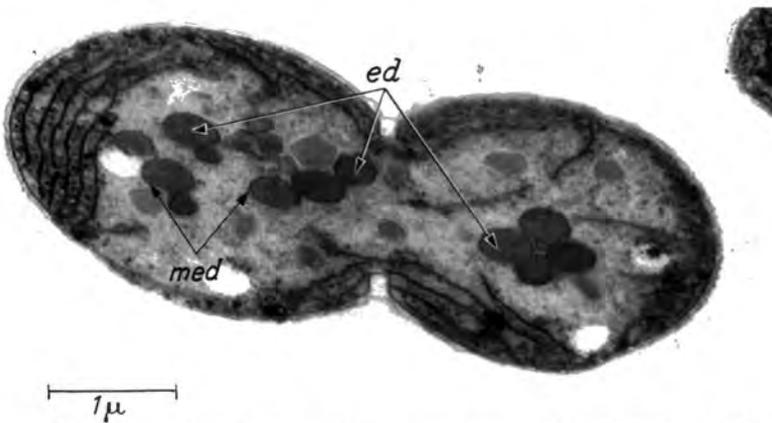

Fig. 10. Appearance of central area of a *M. aeruginosa* cell showing electron-dense bodies partially bounded by membranes. KMnO$_4$ fixation. Mag. 17,500×

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 mμ 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 GEITLER (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 GORBU-NOVA (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 mμ 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 mμ) 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 mμ), plasma membranes (9—10 mμ) and Golgi membranes (6 mμ). The width of the gas-cylinder membrane is 2 mμ 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.

References

BENEDETTI, E. L., and B. BERTOLINI: The use of the phosphotungstic acid (P.T.A.) as a stain for the plasma membrane. J. roy. micr. Soc. 81, 219—222 (1963).

BOWEN, C. C., and T. JENSEN: Blue-green algae: fine structure of the gas-vacuoles. Science 147, 1460—1462 (1965).

CANABAEUS, L.: Über die Heterocysten und Gasvakuolen der Blaualgen und ihre Beziehungen zueinander. Pflanzenforsch. Bd. 13. Jena 1929.

CHAPMAN, J., and M. SALTON: A study of several blue-green algae with the electron microscope. Arch. Mikrobiol. 44, 311—322 (1962).

FOGG, G. E.: The gas-vacuoles of *Myxophyceae.* Biol. Rev. 16, 205—217 (1941).

FRITSCH, F. E.: Structure and reproduction of the algae, Vol. 2. Cambridge 1945.

GEITLER, L.: *Cyanophyceae.* In RABENHORST: Kryptogamenflora, Bd. 14. Leipzig 1932.

Giesy, R. M.: Observations on the cell structure of *Oscillatoria limosa* Agardh. Ohio J. Sci. **62**, 119—124 (1962).

— A light and electron microscope study of interlamellar polyglucoside bodies in *Oscillatoria chalybia*. Amer. J. Bot. **51**, 388—396 (1964).

Gorham, P. R., J. McLachlan, U. T. Hammer, and W. K. Kim: Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) Bréb. Verh. int. Ver. Limnol. **15**, 796—804 (1964).

Gorbunova, N. P.: Gazovye vakuoli v kletkakh gormogoniev *Amorphonostoc punctiforme* (Kütz.) Elenk. Bot. Zhur. **45**, (10), 1546—1548 (1960).

Hall, W. T., and G. Claus: The fine structure of the coccoid blue-green alga, nom. prov. *Synechococcus oceanica*. Rev. Biol. **5**, 63—74 (1964/65).

Hopwood, D. A., and A. M. Glauert: The fine structure of the nuclear material of a blue-green alga *Anabaena cylindrica*. J. biophys. biochem. Cytol. **8**, 813—823 (1960).

Jost, M.: Die Ultrastruktur von *Oscillatoria rubescens*. Arch. Mikrobiol. **50**, 211—245 (1965).

—, and P. Matile: Zur Charakterisierung der Gasvacuolen der Blaualge *Oscillatoria rubescens*. Arch. Mikrobiol. **53**, 50—58 (1966).

—, and A. Zehnder: Die Gasvakuolen der Blaualgen *Microcystis aeruginosa*. Schweiz. Z. Hydrol. **28**, 1—3 (1966).

Klebahn, H.: Neue Untersuchungen über die Gasvacuolen. Jb. wiss. Bot. **61**, 535—589 (1922).

Pankratz, H. S., and C. C. Bowen: Cytology of blue-green algae. I. The cells of *Symploca muscorum*. Amer. J. Bot. **50**, 387—399 (1963).

Peat, A., and B. A. Whitton: Environmental effects on the structure of the blue-green alga, *Chlorogloea fritschii*. (In Press.)

Pringsheim, E.: The nature of pseudovacuoles in *Cyanophyceae*. Nature (Lond.) **210**, 549—550 (1966).

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**, 208—212 (1963).

Ris, H., and R. N. Singh: Electron microscope studies on blue-green algae. J. biophys. biochem. Cytol. **9**, 639—680 (1961).

Robertson, J. D.: The ultrastructures of cell membranes and their derivatives. Biochem. Soc. Symp. **16**, 3—43 (1959).

Sjöstrand, F. S.: A comparison of plasma membranes, cytomembranes, and mitochondrial membrane elements with respect to ultrastructural features. J. Ultrastruct. Res. **9**, 561—580 (1963).

Sun, C. N.: Electron microscope observations on *Gleocapsa* sp. Bull. Torrey Bot. Club **88**, 106—110 (1961).

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

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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 $20^0/_0$ during exponential growth when the doubling time is 56.5 hours, increasing to $34^0/_0$ 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.
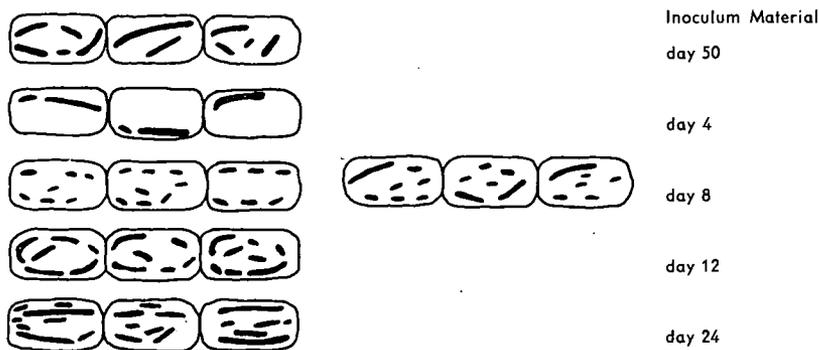

Fig. 1. Summary of light microscope observations of gas-vacuoles in cells from different growth stages

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 OsO₄ fixation (Figs. 2, 3, 5 and
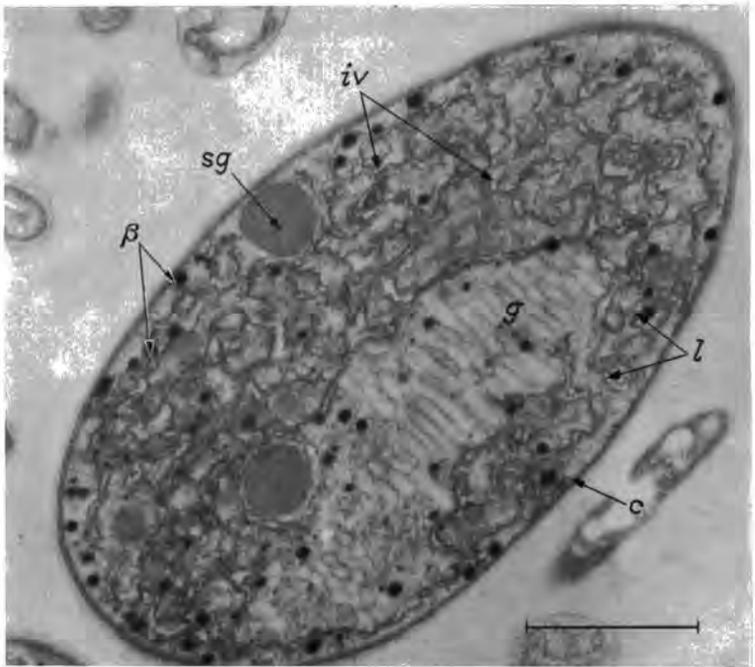8). Figs. 4 and 6 show their appearance after KMnO₄ fixation, when the

Fig. 2. A day + 2 cell showing lamellae distributed throughout the cell and structur-
ed granules. OsO₄ fixation. Mag. 23,000 ×. The symbols used for all photographs
are as follows: α α-granule; β β-granule; c connection between the plasma mem-
brane and lamellae; g gas-cylinder; *hed* hollow electron-dense body; *in* invagi-
nation 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
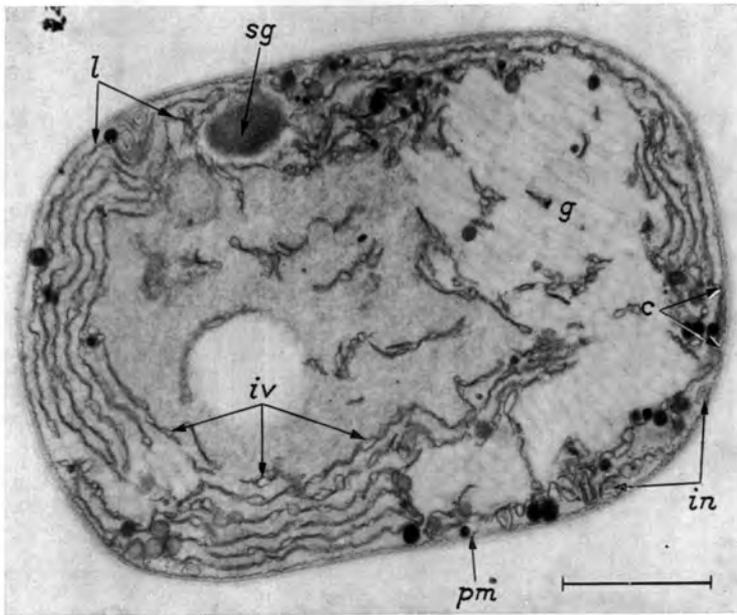gas-cylinders, were also present. In day + 4 cells the gas-cylinders were

Fig. 3. A day + 4 cell showing invaginations of the plasma membrane and lamellae lying parallel to the cell wall. OsO₄ fixation. Mag. 20,000 ×
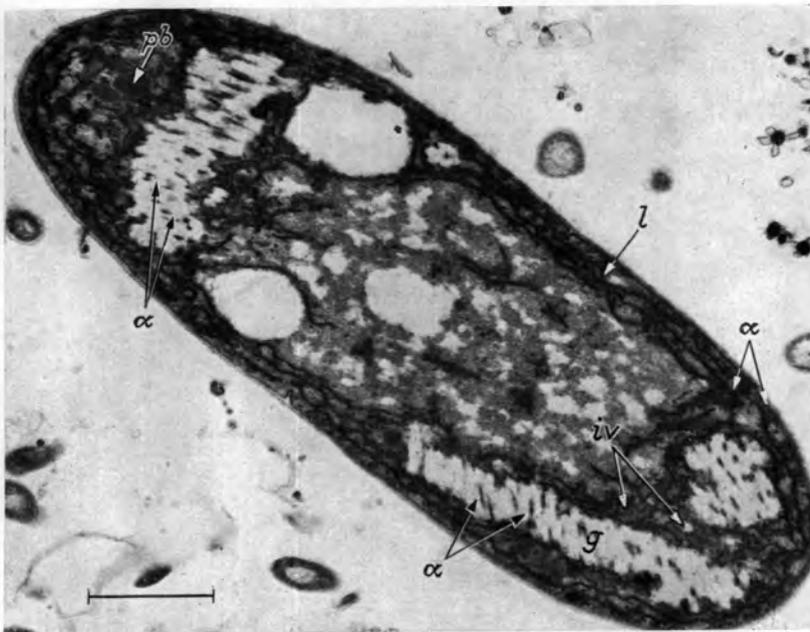

Fig. 4. A day + 4 cell showing lamellae lying parallel to the cell wall and a cylindrical grouping of gas-cylinders. KMnO₄ fixation. Mag. 17,000 ×
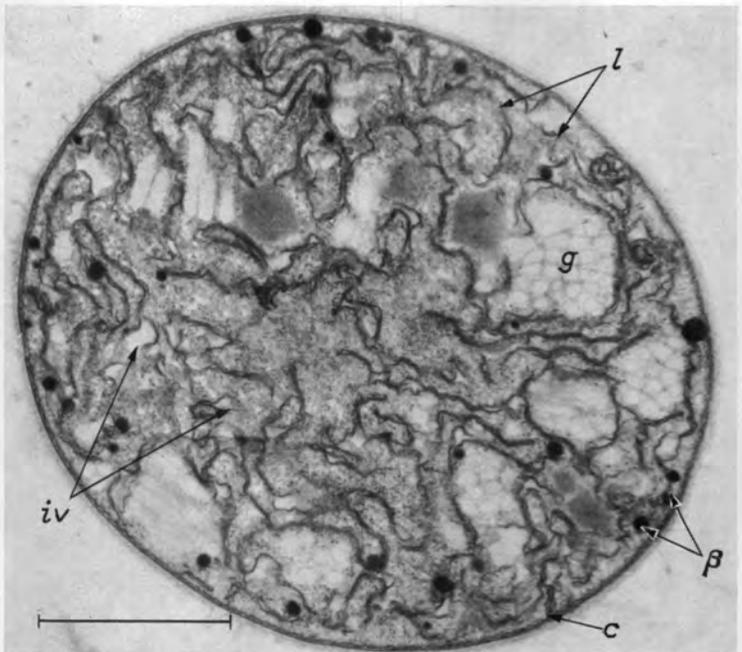
Fig. 5. A day + 8 cell showing lamellae distributed throughout the cell and small groups of gas-cylinders bounded by lamellae. OsO₄ fixation. Mag. 26,500 ×
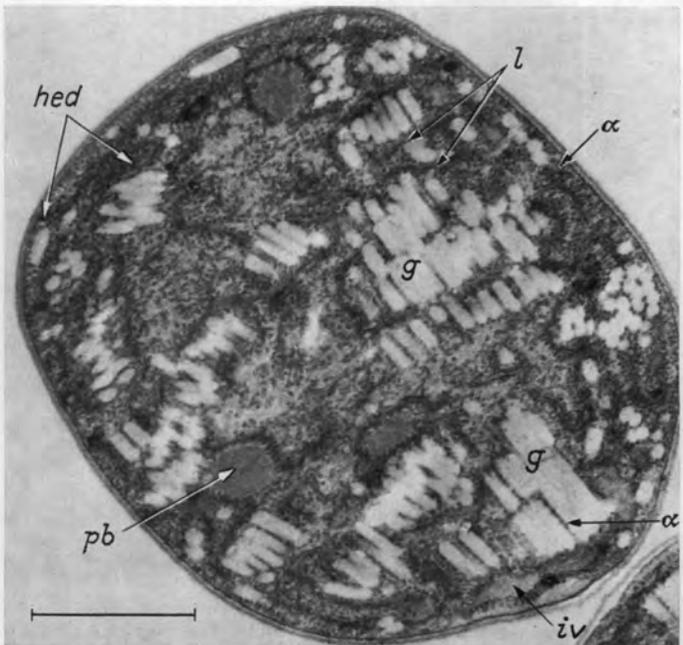

Fig. 6. A day + 12 cell showing α-granules distributed in rows between gas-cylinders. KMnO₄ fixation. Mag. 22,000 ×

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
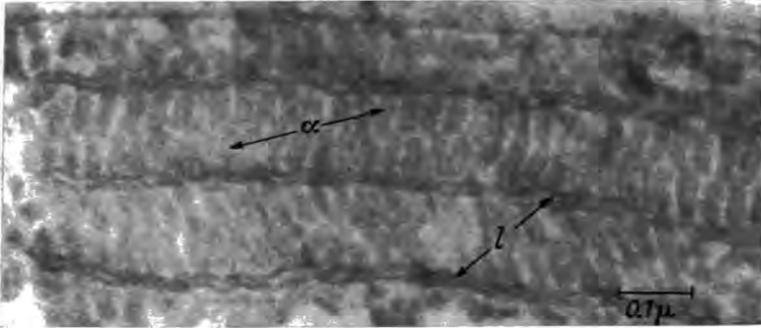numbers of gas-cylinders and they were distributed throughout the cells.

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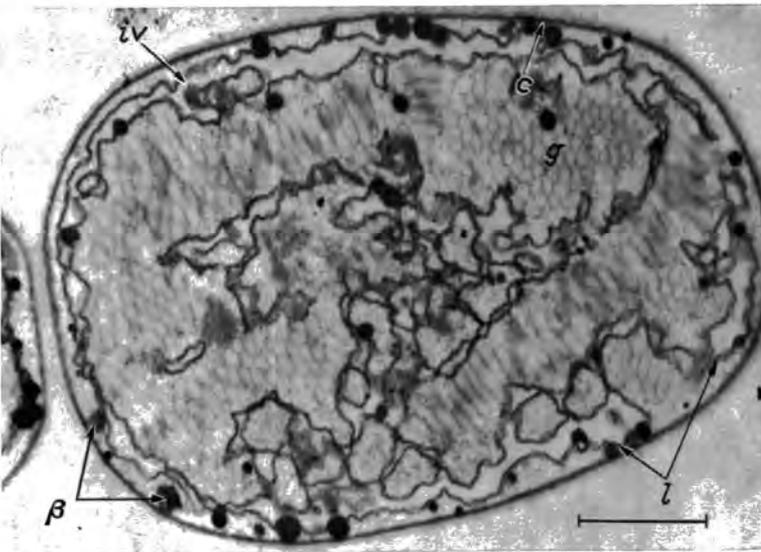(from a day + 12 cell). $KMnO_4$ fixation. Mag. 100,000 ×

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

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 intra-lamellar 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. FRITSCH (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.

References

ECHLIN, P., and I. MORRIS: The relationship between blue-green algae and bacteria. Biol. Rev. **40**, 143—187 (1965).

FRITSCH, F. E.: Structure and reproduction of the algae, Vol. 2. Cambridge: University press 1945.

FUHS, G. W.: Spherical subunits in photosynthetic membranes of two *Cyanophyceae* and the bacterium *Rhodospirillum rubrum*. Arch. Mikrobiol. **54**, 253—265 (1966).

GIESY, R. M.: A light and electron microscope study of interlamellar polyglucoside bodies in *Oscillatoria chalybia*. Amer. J. Bot. **51**, 388—396 (1964).

GORHAM, P. R., J. McLACHLAN, U. T. HAMMER, and W. K. KIM: Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.). Breb. Verh. int. Ver. Limnol. **15**, 796—804 (1964).

HORTOBAGYI, T.: Quelques précisions sur la formation et la signification systématique des pseudovacuoles gazeuses des *Cyanophycées*. Rapp. et communs. huitième congr. intern. bot. Paris, sect. **17**, 12—14 (1954).

JOST, M.: Die Ultrastruktur von *Oscillatoria rubescens*. Arch. Mikrobiol. **50**, (3), 211—245 (1965).

—, and A. ZEHNDER: Die Gasvakuolen der Blaualgen *Microcystis aeruginosa*. Schweiz. Z. Hydrol. **28**, 1—3 (1966).

LUND, J. W. G.: Buoyancy in relation to the ecology of the freshwater phytoplankton. Brit. Phycol. Bull. **1**, (7), 1—17 (1959).

— The ecology of the freshwater phytoplankton. Biol. Rev. **40**, 231—293 (1965).

PANKRATZ, H. S., and C. C. BOWEN: Cytology of the blue-green algae. I. The cells of *Symploca muscorum*. Amer. J. Bot. **50**, 387—399 (1963).

PEARSON, J. E., and J. M. KINGSBURY: Culturally induced variation in four morphologically diverse blue-green algae. Amer. J. Bot. **53**, 192—200 (1966).

PEAT, A., and B. A. WHITTON: Environmental effects on the structure of the blue-green alga, *Chlorogloea fritschii*. Arch. Mikrobiol. **57**, 155—180 (1967).

PRINGSHEIM, E.: *Oscillatoria agardhii* var. *suspensa* nov. var. Kleine Mitteilungen über Flagellaten und Algen. X. Arch. Mikrobiol. **50**, 401—413 (1965).

— The nature of pseudovacuoles in *Cyanophyceae*. Nature (Lond.) **210**, 549—550 (1966).

SERBANESCU, M.: Sur quelques aspects du cycle de développement de l'algue bleue *Gloeotrichia natans*. Rev. Algologique, N. S. 8, 189—195 (1966).

SMITH, R. V., and A. PEAT: Comparative structure of the gas-vacuoles of blue-green algae. Arch. Mikrobiol. **57**, 111—122 (1967).

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