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PHYSIOLOGICAL AND MORPHOLOGICAL STUDIES
ON TAPERING BLUE-GREEN ALGAE

by

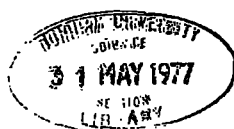
Christine Sinclair (B.Sc. Dunelm)

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

Department of Botany

January, 1977



This thesis is entirely the result of my own work.
It has not been accepted for any other degree, and
is not being submitted for any other degree.

C. Sinclair

ABSTRACT

A study was made of factors influencing hair development in 34 heterocystous and 2 non-heterocystous strains of Rivulariaceae.

When grown with a high level of $\text{NO}_3\text{-N}$, 33 of the heterocystous strains lost their heterocysts; 19 became untapered, but 14 produced tapered trichomes which resembled those of Homoeothrix or Ammatoidea. One strain was unchanged in morphology. Hair development was reduced but not eliminated in the 3 strains with long hairs in the heterocystous state, and was unaffected in the 2 strains with short hairs. When one heterocystous strain was starved of N_2 , heterocyst frequency increased, but tapering was unaffected and no hairs developed. These responses suggested that hair development and tapering in heterocystous Rivulariaceae are not necessarily directly related phenomena, and that neither is likely to be due solely to nitrogen deficiency in the apical cells.

The influence of phosphate, iron, magnesium, calcium, molybdenum and sulphate deficiencies on hair development was examined. 12 of the heterocystous strains, and one Homoeothrix showed increased hair development under phosphate deficiency; 8 of these (heterocystous) strains also did so under iron deficiency, and one of the 8 did so under magnesium deficiency. The other deficiencies had no marked effect on hair formation. Several other morphological responses to nutrient deficiencies are also described.

Hair development in deficient cultures preceded any degenerative changes, and did not appear to be merely a pathological symptom. The cells of hairs formed under phosphate deficiency were found to be capable of polyphosphate synthesis, indicating metabolic activity. The possibility is considered that the hairs of Rivulariaceae may have a functional significance, perhaps in the uptake of nutrients present at a low concentration.

During the work some strains suddenly developed morphological abnormalities when grown in standard medium. Attempts to elucidate this phenomenon are described in the Appendix.

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List of abbreviations

°C	degrees Celsius
EDTA	ethylenediaminetetra- acetic acid (disodium salt)
g	gram
h	hour
HEPES	<u>N</u> -2-hydroxyethylpiperazine- <u>N'</u> - 2-ethanesulphonic acid
l	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
µg	microgram
µm	micrometre
nm	nanometre
nmol	nanomole
OD	optical density
P	probability
TRIS	2-amino-2-hydroxymethyl- 1,3-propanediol
u.v.	ultra-violet
w/v	weight per volume

1 INTRODUCTION

1.1 Introductory remarks

Blue-green algae show a diversity and complexity of structure greater than that of other prokaryotic organisms. Many filamentous forms show quite marked cellular differentiation, with the potential to develop heterocysts and spores as well as normal vegetative cells. In some blue-green algae a fourth type of cell, the hair cell, may be present, at least during certain stages of the life cycle.

While many studies have been made of the factors influencing the differentiation of heterocysts and spores, there are only scattered comments in the literature concerning the effects of environmental factors on hair development; in fact the hair cells have often been assumed to be merely dead or dying. The aim of the present study was to investigate the effect of different environmental factors on the development of hairs in blue-green algae, with the hope that the results might indicate the function, if any, of this rather marked cellular differentiation.

The study was confined to the Rivulariaceae, which is the family in which hairs most often occur (Geitler, 1932). A particular characteristic of this family is the tapering of the trichomes, which is observed even in forms which do not produce hairs. Since it seemed possible that there might be a relationship between tapering and hair development, the character of tapering was also studied.

While the ultimate hope was to indicate the possible role of hairs in the natural environment, the problem seemed initially more amenable to study by laboratory experiments, and most of the work was done with laboratory cultures. Some experiments were however performed with materials from local field sites, using both crude and unialgal cultures. A wide range of laboratory strains was used; by taking a broad approach it was hoped that it would be possible to make generalizations about the biology of the family.

1.2 Hairs and tapering in blue-green algae

1.21 Distribution of occurrence

Bornet and Flahault (1886a) defined the blue-green algal hair as 'a series of narrow, elongated cells, containing very little protoplasm, and incapable of further growth', formed at the tips of the trichomes. Such hairs are most characteristic of the family Rivulariaceae. Geitler (1932)¹ recognizes seven heterocystous genera (Calothrix, Dichothrix, Gloeotrichia, Isactis, Polythrix, Rivularia and Sacconema) in this family, and five non-heterocystous genera (Ammatoidea², Amphithrix, Homoeothrix, Leptochaete and Tapinothrix); he describes the presence of hairs in at least some species of all but two of the genera (Amphithrix and Tapinothrix). Komárek and Kann (1973) have suggested however that Amphithrix and Tapinothrix, and also Leptochaete, are merely growth forms of Homoeothrix.

A characteristic feature of Rivulariaceae is the tapering of the trichomes from base to apex, even in forms without hairs (in the genus Ammatoidea the trichomes taper towards both ends). In the heterocystous genera the heterocysts are always basal (i.e. at the end distal to the hair), though additional intercalary heterocysts may also be present.

Hairs may also be present in a number of genera not included by Geitler in the Rivulariaceae:

<u>Loefgrenia</u>	(Loefgreniaceae)
<u>Mastigocoleus</u> <u>Nostochopsis</u>	(Nostochopsidaceae)
<u>Brachytrichia</u> <u>Kyrtuthrix</u>	(Mastigocladaceae)

(Geitler does not mention the occurrence of hairs in Kyrtuthrix, but they are described by Ercegović (1930)). All these genera are heterocystous except Loefgrenia. In the Mastigocladaceae the

¹Geitler's (1932) scheme of classification of blue-green algae is used throughout this thesis, unless otherwise indicated.

²This spelling, used by West and West (1897) in their original description of the genus, is used in preference to 'Hammatoidaea' (cf. Starmach, 1959).

heterocysts are intercalary, and in the Nostochopsidaceae they are terminal, intercalary or lateral (Geitler, 1932). Thus unlike the situation in the Rivulariaceae there is no obvious and constant relationship between the position of the heterocyst and that of the hair.

The end cells of trichomes of Aphanizomenon (Nostocaceae) may sometimes be very elongated and vacuolated, much as the hair cells of Rivulariaceae, but this is not a constant feature (Geitler, 1932). The terminal cells at one or both ends of the non-heterocystous Raphidiopsis taper to a fine point, but colourless multicellular hairs are not developed (Bourrelly, 1970).

Geitler's (1932) flora includes several genera, not members of the Rivulariaceae, in which the trichomes taper from base to apex, but which do not form vacuolated hair cells:

<u>Microchaete</u> (= <u>Premyella</u>)	(Microchaetaceae)
<u>Tildenia</u>	(Scytonemataceae)
<u>Leptobasis</u>	(Leptobasaceae)
<u>Sokolovia</u>	(Sokoloviaceae)

Sokolovia is non-heterocystous, but the other three genera have basal and/or intercalary heterocysts. The similarity between Calothrix and Microchaete is very close. The distinction between the Microchaetaceae and Rivulariaceae is based chiefly upon the absence of hairs in the former (Geitler, 1932); there is no obvious criterion by which tapered heterocystous trichomes without hairs would be placed in one family or the other.

Several of the blue-green algae which show true branching have lateral branches which taper quite markedly, but which are not prolonged into a colourless hair:

<u>Hapalosiphon</u>	(Stigonemataceae)
<u>Westiella</u>	
<u>Mastigocladus</u>	(Mastigocladaceae)
<u>Herpyzonema</u>	
<u>Lithonema</u>	

A tapered filamentous morphology is also found in certain colourless gliding bacteria, of the genera Leucothrix and Thiothrix. Filaments of these genera are remarkably similar to those of Homoeothrix, both in their overall structure and in their mode of reproduction (Harold & Stanier, 1955).

Since the Rivulariaceae are the family of blue-green algae which show the most marked tendency toward hair formation, and are also more widespread than the other forms mentioned, the present study was confined to members of this family. The rest of the Introduction deals with the Rivulariaceae only, though comments on genera from other families are included as seems relevant. While hair formation is the topic of primary interest, a fairly detailed account of other aspects of the biology of the family is nevertheless given, to provide a background to the study, since the literature on Rivulariaceae is rather scattered. The ecology of Rivulariaceae was reviewed by Kirkby (1975), and is not dealt with here.

1.22 Characteristics of trichomes with hairs

Hair cells differ chiefly from normal vegetative cells in their highly vacuolated condition, and in their narrow and elongated shape. A loss of normal colour in the cytoplasm of hair cells has also been described (Geitler, 1932). Hair cells lack the granular inclusions of normal vegetative cells, but gas vacuoles may be present (Geitler, 1960). The transition between normal vegetative cells and fully developed hair cells in a trichome may sometimes be very abrupt, with narrowing, elongation and vacuolation occurring over a very short distance, but Geitler's (1932) figures give the impression that a gradual transition is more common.

Several authors have demonstrated cytological gradients along trichomes of Rivulariaceae with hairs. Studies on Gloeotrichia species (Palla, 1893: G. pisum; Poljansky & Petruschewsky, 1929: G. natans, G. intermedia; von Zastrow, 1953: G. pisum) showed that the 'central substance' of the basal cells was a single compact mass, but that in the hair cells it was in the form of many small granules, or even undetectable. The identity of the structures stained in early cytochemical studies is not always certain however, owing to the lack of specificity of the staining methods used (Fuhs, 1968). Ueda (1971b) estimated the DNA content of cells along filaments of Calothrix braunii, using fluorescence microscopy, and found that the DNA content of the hair cells, and of the 'transition' cells immediately below the hair, was about two to five times less than that of the normal vegetative cells.

Two electron microscopic studies have described differences

between cells in different parts of trichomes of Rivulariaceae. Miller and Lang (1971) found a progressive loss of thylakoid integrity with increasing cell age in trichomes of a Gloeotrichia sp. There was an increase in cyanophycin granulation, and a decrease in polyglucoside granulation as the cells aged; in the oldest cells all inclusions except lipid globules and polyhedral bodies were absent. Feldmann and Guglielmi (1973), working with Rivularia mesenterica, also described a gradual decrease in the number of thylakoids, and a loss of granular inclusions; cyanophycin granules were lost before alpha and beta granules. Despite these differences in appearance, it is not clear from either of these reports how the 'age' of the cells, or their position in the trichome, were identified.

1.23 Hair formation as a distinct morphogenetic phenomenon

Although cell vacuolation is perhaps most striking in the hairs of Rivulariaceae, it may also occur in other blue-green algae. The development of vacuoles seems to be a fairly common response to unfavourable conditions, taking place particularly in older cells (Desikachary, 1959). The apical cells of subaerial species are sometimes vacuolated, perhaps as a result of desiccation, since vacuoles are not present in forms from submerged situations (Geitler, 1960). Under some conditions, cells of Oscillatoriaceae may develop numerous small vacuoles, giving the cytoplasm an alveolate appearance (keritomie: Geitler (1960)). Geitler (1932; 1960) considered that this was probably a response to nutrient deficiency, since trichomes showing the condition recover a normal cell content following transfer to nutrient medium. Ueda (1971a) showed that the keritomic vacuoles of Oscillatoria borneti and O. pseudogeminata were caused by swelling of the intrathylakoidal space, and that their development was accelerated by increasing the light intensity. She suggested that the vacuolation of the thylakoids might be related to a reduction in the chlorophyll content of the cells. Geitler (1932) pointed out the distinction between this type of vacuolation, and that seen in the hairs of Rivulariaceae. Keritomie occurs as a pathological symptom in otherwise normal cells, still capable of division, and it is reversible. The vacuolation typical of Rivulariaceae represents a normal stage in their developmental cycle, and is confined to a particular

part of the trichome; after a certain point the process is irreversible. Von Zastrow (1953) found vacuolation of otherwise normal vegetative cells in trichomes of Anabaena cylindrica, A. variabilis, Cylindrospermum alatosporum, C. maius, Scytonema hofmanni and Gloeotrichia pisum, from very old laboratory cultures (in soil-water medium). Palla (1893) also described this phenomenon for G. pisum. In both these cases, the affected Gloeotrichia trichomes lacked normal hairs, thus highlighting the difference between normal and pathological vacuolation.

A paler colour, and a lower level of granulation, may also be observed in cells at the apices of trichomes other than those of Rivulariaceae, in the absence of any vacuolation or any marked change in cell shape. Thurston and Ingram (1971) and Martin and Wyatt (1974a) have described the lower granule content and less pigmented appearance of the cells in the lateral branches of Stigonemataceae as compared with cells from the prostrate part of the thallus; this may have been due to the meristematic nature of the lateral branch cells (Thurston & Ingram, 1971).

Changes in the shape of cells at the tips of the trichomes, in some ways similar to those seen in association with hair development, are also found in blue-green algae other than Rivulariaceae. The end cells of many Oscillatoriaceae, for instance, are quite markedly narrowed, forming a tapered tip (calyptra), though usually without vacuolation (Geitler, 1960). Members of the Scytonemataceae quite often have slightly pointed apices also, sometimes with associated paling and decrease in granulation in the narrower cells at the apex. Bharadwaja (1934) described how the cells in the older parts of subaerial forms of Scytonema and Tolypothrix may become only half as wide, and many times longer than cells in the growing apical region, particularly when conditions are 'acute'.

From the examples described, it will be clear that the various changes in cell morphology associated with the development of hairs are thus not unique to hair-forming species. Nevertheless, hair formation seems to be a quite distinct morphogenetic phenomenon, in which these changes take place simultaneously, and in an apparently co-ordinated fashion.

1.24 Possible functions of hairs in blue-green algae

Despite the rather striking cellular differentiation associated with hair development, no particular role has been demonstrated for hairs in blue-green algae, and the assumption has often been made that the hair cells are dead or dying (Fuhs, 1973). The only speculation regarding the function of hairs appears to be that of Palla (1893) who suggested (without experimental evidence) that the hairs of Gloeotrichia pisum probably had an absorptive role. Fuhs (1973) has also commented that the hairs of Gloeotrichia, which protrude beyond the gelatinous colony, are the part of the trichome least likely to suffer from nutrient limitation.

1.3 Growth and development of Rivulariaceae

1.31 Trichome growth

The growth of Rivulariaceae is typically meristematic, i.e. cell division is confined to a particular part of the trichome, though Polyanskiĭ (1928) commented on the absence of a meristematic zone in Calothrix elenkinii, and Singh (1939) reported the same for Gloeotrichia ghosei. The meristematic cells are recognizable by their short, disc-like appearance, and by their pale colour and lower content of granules (Geitler, 1960).

Most authors have described the meristem of Rivulariaceae as occurring in the region of the trichome just below the hair ('trichothallic growth': Fritsch (1945)). This applies to heterocystous forms (Bornet & Thuret, 1880; Schwendener, 1894; Geitler, 1932, 1960; Weber, 1933; Desikachary, 1959; Darley, 1968; Malmeström, 1972) and to the non-heterocystous Homoeothrix (Komárek & Kann, 1974). The meristem of Ammatoidea normanii is described as being in the centre of the trichome, between the two tapered ends (Golubić, 1966). In some forms showing trichothallic growth, the cells in the meristematic zone are considerably wider, as well as shorter than those at the base of the trichome, which may be quite markedly long and narrow (Schwendener, 1894; Polyanskiĭ, 1930; Friedmann, 1956). In such cases the trichomes do not taper uniformly from base to apex, but show a spindle-shaped swelling below the hair. Schwendener (1894) commented that this phenomenon appeared to be most marked in forms which form rounded colonies, an examination of the

figures of Bornet and Thuret (1880), Geitler (1932) and Desikachary (1959) also gives this impression. Geitler and Ruttner (1935-36; p.440) remarked that the development of long thin cells in the basal region of Rivularia angulosa was characteristic of older trichomes, and resulted from the cessation of growth. Even in the absence of hairs the cells of trichomes of Rivulariaceae may be shorter than those below, and thus presumably meristematic. Lami and Meslin (1959) described this for Calothrix chapmanii, and Darley (1968) for C. prolifera and C. pulvinata.

Localization of cell division in the basal region of the trichomes has also been described, by de Bary (1863a) (Rivularia angulosa), Palla (1893) (Gloeotrichia pisum) and Fay, Stewart, Walsby and Fogg (1968) (Calothrix and Gloeotrichia), among others. Polyanskiĭ (1930) and Serbanescu (1966) found that growth of G. natans was trichothallic in trichomes without spores, but that when spores began to develop the meristematic zone moved to the basal region. In association with this change, the cells below the hair, which had been broad and disc-shaped, became narrow and elongated, while the basal cells became shorter and increased in width. Weber (1933) found that while cell division in Calothrix braunii was normally located below the hair, there was sometimes secondary development of meristematic activity in the basal part of the trichome. This appeared to be an atypical phenomenon, however, since it was associated with displacement of the basal heterocyst, and outgrowth of the trichome from the basal end of the sheath. Desikachary (1945) also observed the secondary development of meristematic activity, in the mid-region of trichomes of G. raciborskii, apparently in response to attack by a parasite. A very similar phenomenon was described by Serbanescu (1966), for G. natans. She attributed it to the germination of intercalary heterocysts, though it is rather difficult to deduce this from her figures.

1.32 Spores and heterocysts

The ability to form spores is a characteristic feature of Gloeotrichia, and several species of Calothrix may also develop spores (Geitler, 1932). Böcher (1946) described spores in Dichothrix gelatinosa. The spores in Rivulariaceae normally develop next to the basal heterocyst, but intercalary spores have also been described, by Steinecke (1931) in C. weberi, by

Böcher (1946) in D. gelatinosa, and by Claus (1957) in G. andrean-szkyana. Several authors (de Bary, 1863b; Gomont, 1895; Polyanskiĭ, 1930; Claassen, 1973) have given fairly detailed accounts of the development of spores. Roelofs and Oglesby (1970) noted that in G. echinulata spores seemed to be produced when the colonies reached a particular size, even when conditions were apparently favourable for growth.

The germination of spores of Gloeotrichia has been described by de Bary (1863b), Bornet and Thuret (1880), Palla (1893) and Polyanskiĭ (1930); all these authors observed the release of a long gas-vacuolate hormogonium from the spore case. Desikachary (1959) describes the germination of spores of Calothrix javanica. Germination of heterocysts of Rivulariaceae has also been observed. Steinecke (1931) described this for C. weberi, and Desikachary (1946) for G. raciborskii and Rivularia mangini. Serbanescu (1966) reported the germination of intercalary heterocysts in G. natans (see p. 22). A very high frequency of heterocyst germination was observed by Singh and Tiwari (1970) in a non-sporulating mutant of G. ghosei, grown in the presence of ammonia.

In trichomes of Rivulariaceae the most typical condition is the possession of a single basal heterocyst, but further basal and/or intercalary heterocysts may also be produced (Geitler, 1932). Weber (1933) described how in Calothrix fusca as many as seven successive basal heterocysts could develop, each time with the death of the preceding heterocyst. Darley (1968) observed that in C. pulvinata, C. prolifera, C. scopulorum, C. crustacea and C. parietina, growing in the field, intercalary heterocysts were present only in the oldest parts (i.e. the basal region) of the longer trichomes. When C. scopulorum and C. pulvinata were grown in liquid culture (Miquel's medium), but not when they were grown on solidified medium, intercalary heterocysts developed in the basal region of longer trichomes following the release of hormogonia, when the hairs were beginning to re-form. Palik (1946) also observed that the appearance of intercalary heterocysts followed hormogonium release in C. weberi. Darley (1968) speculated that the development of intercalary heterocysts was related to depletion of nutrient salts in the medium. Terminal heterocysts (i.e. with a single pore: Fritsch (1945)) may also develop in an intercalary position, particularly in association

with the production of false branches. Schwabe (1960) found that in C. desertica heterocysts of this type usually developed in pairs, and had a width very like that of the adjacent cells.

1.33 Trichome development

Reproduction in the Rivulariaceae takes place by means of hormogonia. These are parallel lengths of cells, which show gliding motility, and develop in the meristematic zone at the apex of the trichome (Bornet & Thuret, 1876, 1880; Geitler, 1932). The hormogonia are released by the production of biconcave separation discs (Fritsch, 1945). Hormogonia may also be released during spore germination (see p. 23). The presence of gas vacuoles in the hormogonia, both preceding their release from the parent filament, and during their motile phase, has been reported for several species, including Calothrix epiphytica (Canabaeus, 1929), Gloeotrichia pisum (Palla, 1893), G. punctulata, Rivularia bullata, R. hospita (Bornet & Thuret, 1876; 1880), 'Rivularia' (Gloeotrichia) angulosa (de Bary, 1863b). (Although the earlier authors describe the hormogonia as being densely granulated, it is clear from their descriptions and figures that the structures they observed were in fact gas vacuoles.)

When hormogonia are released from the parent trichome, the hairs, if present, are shed (Bornet & Thuret, 1876; 1880). This occurs in Brachytrichia and Mastigocoleus as well as in the Rivulariaceae (Desikachary, 1959). Thus there may be considerable variation in the frequency of trichomes with hairs in populations of Rivulariaceae, depending upon their stage of growth. Malmeström (1972) for instance, studying a population of Calothrix scopulorum on the Swedish west coast, found that hairs were present only between June and August, when active growth was taking place. There was a decrease in the number of trichomes with hairs during August, when hormogonium release was at its maximum. In some cases, the apical hair may re-develop following the release of hormogonia (Bornet & Thuret, 1880; Darley, 1968; Malmeström, 1972), but several authors have mentioned that hairs are present only in young trichomes, and that older trichomes are devoid of hairs (Setchell, 1895: Rivularia bornetiana; Steinecke, 1931: Calothrix weberi; Weber, 1933: C. fusca, C. braunii; Starmach, 1966: Homoeothrix crustacea). This may lead to taxonomic confusion; for instance Geitler (1928) describes the similarity of H. varians to a species of Lyngbya, in the absence of

hairs, and Steinecke (1931) found that older filaments of C. weberi resembled Microchaete.

It seems likely that active hormogonium formation is a reflection of favourable growth conditions. Dutein (1962) mentioned that hormogonium production in a population of Calothrix aeruginosa took place during the warm season, and Malmeström (1972) found that C. scopulorum stands only developed hormogonia in the summer months. Jones (1967) found that in cultures of C. scopulorum the alga was in a constantly hormogonial condition during the exponential phase of growth. No detailed work appears to have been done on the factors which stimulate the release of hormogonia, as opposed to their initial development at the trichome apex. Bornet and Thuret (1876), Börzi (1882) and Darley (1968) have all described the prompt release of hormogonia by marine Calothrix species, placed in a drop of liquid shortly after collection from the field. Bornet and Thuret (1876) attributed this to the change of water and the warmer temperature of the laboratory. They suggested that the phase of the tide probably had an important influence on hormogonium release in the marine littoral forms they studied.

Several authors have followed the development of symmetrical, parallel hormogonia of Rivulariaceae into tapered trichomes. The following pattern has been described most often. Shortly after coming to rest, the hormogonium develops a heterocyst at one end, while the other end becomes pointed. In forms with hairs, these are formed by progressive narrowing and elongation of the cells at the pointed end (de Bary, 1863b; Bornet & Thuret, 1876, 1880; Schwendener, 1894; Palik, 1946; Darley, 1968). Wyatt, Martin and Jackson (1973) noted that in Fremyella diplosiphon (see p.17), following the development of the basal heterocyst and pointed apex, the cells next to the heterocyst increased in width from 4-5 μm to 8-10 μm as growth progressed. In some cases, the hormogonia become tapered at both ends, and two heterocysts differentiate in the centre; these subsequently separate, yielding two tapered heterocystous trichomes (Polyanskiĭ, 1928; Geitler, 1932; Fritsch, 1945; Darley, 1968). In their observations on the development of material in culture, de Bary (1863b), Polyanskiĭ (1928), Weber (1933) and Palik (1946) all occasionally saw trichomes which tapered towards both ends, but which had no

central heterocysts, and were considerably longer than hormogonia. Palik (1946) commented on the similarity between these trichomes and those of Ammatoidea, and he suggested that Ammatoidea was merely a growth form of Calothrix. Golubić (1966) has described the development of hormogonia in Ammatoidea: they grow at one or both ends, and in the early stages may resemble Homoeothrix; both ends subsequently develop a hair. This pattern is thus essentially the same as that observed in the heterocystous forms. Schwabe (1960) described what appears to be an unusual pattern of hormogonium development, in C. desertica. Some of the hormogonia developed a heterocyst at each end, and tapered towards the centre of the trichome. The two halves later separated to give normal trichomes. Another rather unusual developmental stage has been described by Serbanescu (1966), in Gloeotrichia natans, and by Glaassen (1973) in G. ghosei. Both authors observed the secondary development of heterocysts at the apices of tapered, heterocystous trichomes. In G. ghosei the cells adjacent to the new apical heterocyst enlarged, and later developed into a spore, thus giving rise to a trichome which tapered towards the centre from both ends.

1.34 Colony development

The genera Gloeotrichia, Rivularia and Isactis, and some species of Homoeothrix and Dichothrix, have a colonial growth form. Many species of Calothrix, while not forming distinct colonies, grow with the trichomes associated to form a macroscopic thallus which may have a characteristic appearance (e.g. C. pulvinata: Bornet & Thuret (1880)). Bornet and Thuret (1880) and Darley (1968) have described how the behaviour of hormogonia following their release contributes to the structure of the thallus. The hormogonia of C. pulvinata, for instance, tended to attach themselves to the parent filaments, giving a bushy, tufted appearance. The development of false branches is also an important factor in colony development. The spherical colonies of Rivularia and Gloeotrichia are produced by successive tiers of false branches (Schwendener, 1894; Geitler, 1932). The texture of the sheath, whether firm or soft, may be important in determining the extent to which displacement of branch filaments can occur (Fritsch, 1945). Darley (1968) found that none of the marine colonial Rivulariaceae she studied (Rivularia spp. and Isactis plana) produced their characteristic thalli when grown in culture. She attributed this

to a failure of the individual sheaths to fuse. Zehnder (1963) similarly noted that the strain of G. echinulata he studied produced only small stellate clusters in culture, and not the colonies typical of natural populations. Bornet and Thuret (1876, 1880) and Darley (1968) noted that the hormogonia of C. scopulorum tended to group together in stellate clusters before beginning to differentiate; de Bary (1863b) and Bornet and Thuret (1876) also observed this in Gloeotrichia. Further development of the trichomes produced a daughter colony. Nevertheless, a single trichome is capable of giving rise to a colony or thallus, by successive false branches, as Darley (1968) noted for C. pulvinata.

Few authors seem to have considered the possibility that colonies of Rivulariaceae might contain more than a single species, though Bornet and Thuret (1880) noted that it was not uncommon to find Calothrix trichomes growing among Rivularia hospita colonies; Bornet and Flahault (1886b) and Dutein (1962) also mention the presence of Calothrix filaments in the thalli of Rivularia spp. Forest and Khan (1972) described the presence of Calothrix-like trichomes in colonies of Gloeotrichia natans and G. echinulata, collected from Conesus Lake, New York State. When the material was cultured, the number of Calothrix-like trichomes increased, sometimes replacing the typical Gloeotrichia completely. Forest and Khan considered that this phenomenon may have been a reflection of the morphological plasticity of a single species, but it nevertheless seems possible that the colonies they studied were genetically heterogeneous. In view of the close association of several species of Rivulariaceae that occurs in certain habitats, such as the marine littoral and supra-littoral (Dutein, 1962; Darley, 1968), and the active dispersion of hormogonia, it seems quite likely that the formation of 'mixed' colonies could easily occur.

1.4 Morphological variation in blue-green algae

1.41 Rivulariaceae

It will be apparent from the preceding section that species of Rivulariaceae may exhibit a wide range of morphological forms as a result of their rather complex life cycle (see, for instance, Polyanskiĭ (1930); Geitler & Ruttner (1935-36; p. 478); Serbanescu (1966)). In addition to this inherent variability, some forms may also show morphological variation not apparently directly

related to the developmental cycle. Such variation has been observed in both field and laboratory studies.

Several authors have reported a reduction in hair development or a complete loss of hairs in species of Calothrix when exposed to frequent drying. Golubić and Marčenko (1965) for example commented that under such conditions (in the spray zone of the sea shore) C. parietina had the appearance of Tolypothrix. Darley (1967; 1968), studying marine littoral forms, found that C. scopulorum, growing in exposed situations, had no hairs, whereas C. crustacea, which was continually submerged, had long hairs. On the basis of their behaviour in culture, Darley concluded that these two species were identical; in liquid medium, both showed extensive hair development, but on agar or silica gel neither produced hairs. Malmeström (1972) similarly found that in a population of C. scopulorum on a rocky shore, the number of trichomes with hairs was lower in the upper part of the Calothrix zone, where the alga was exposed more frequently. Kirkby (1975) reported an effect of chemical environment on hair development in colonies of Rivularia grown in crude laboratory culture, in a modified version of Allen and Arnon's (1955) medium. Hairs formed a higher percentage of the total trichome length when the initial phosphate level was relatively low ($1.55-15.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$) than at higher levels (up to $31 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$).

Starmach (1968), in a study of the morphological forms of Homoeothrix fusca, found that trichomes of this species were longest in fully submerged situations, and shortest in the splash zone, where they were subject to periodic drying. Khan (1969) also reported effects of exposure on trichome length, in Calothrix pilosa and C. scopulorum from a littoral rock pool environment. Filaments of C. pilosa growing subaerially on silt, and exposed to direct sunlight, were shorter than those from crevices, shaded from the direct sun. Trichomes of this species growing at the air-water interface were narrower than those from the subaerial habitat. Trichomes of C. scopulorum growing at the air-water boundary had a greater diameter than those from submerged situations.

Variations in the character of the sheath of Rivulariaceae have been reported several times. A thin, colourless sheath seems to be characteristic of filaments which are growing actively, whereas in conditions unfavourable for growth, the sheath may become thick and brown pigmented, as Bornet and Thuret (1880)

observed for Calothrix scopulorum. Weber (1933) remarked that yellow-brown pigmentation of the sheaths was particularly noticeable in older filaments of C. braunii, and Geitler (1934) found an increase in sheath thickness with increasing trichome age in cultures of a Dichothrix sp. isolated from the lichen Placynthium. Kirkby (1975) tested for correlations between aspects of the morphology of Calothrix species, according to Geitler's (1932) descriptions. She found a slight tendency for species described as having yellow brown sheaths to possess hairs also. Darley (1968) observed that algae growing in exposed situations had thicker sheaths than forms from submerged habitats, and that the sheaths were more often pigmented. Golubić (1966) found that Ammatoidea normanii filaments had yellow brown sheaths in places exposed to light (see also Section 1.42).

Variations in the size and shape of cells, and in the growth form of trichomes, have also been described. Böcher (1946) noted that the cells of Dichothrix gelatinosa were much shorter during periods of active growth than at other times, and Darley (1968) found the same in Calothrix parietina. Pearson and Kingsbury (1966) observed considerable variation in the size and shape of vegetative cells, and in the size and location of heterocysts, in trichomes of C. membranacea cultured in a variety of media. These variations did not seem to be correlated with the conditions of culture. Schwabe (1960) found that in cultures of C. desertica, the secondarily developed 'intercalary' heterocysts were extremely variable in length. Weber (1933) commented on the extreme variability in form and size of the heterocysts in samples of C. fusca from the field, and Claus (1957) noticed the same in Gloeotrichia andreaszkjana. Pearson and Kingsbury (1966) found that C. membranacea sometimes grew as helically twisted 'ropes' in culture, and Lange (1970b; 1975) observed a similar phenomenon in G. echinulata, grown in the medium No. 11 of Zehnder and Gorham (1960). Helically coiled strands were formed only by non-polar (probably hormogonial) trichomes, when the culture reached a certain critical density. Coiling of trichomes inside the sheath, causing irregular bulging of the filament was described by Weber (1933) for C. fusca and C. braunii, and similar observations were made by Darley (1968) for C. scopulorum, and by Golubić (1966) for Ammatoidea normanii. This seems to occur when the rate of trichome growth exceeds that of sheath synthesis (Darley, 1968), or when the presence of 'inter-

calary' (terminal) heterocysts prevents the extension of the growing trichome (Weber, 1933). In her studies of colonies of G. natans, Serbanescu (1966) observed, side by side with normal filaments, ones in which the cells in the basal region had a markedly undulating profile, and in which the trichomes themselves were also curved.

The lichen genera Lichina, Calotrichopsis and Porocyphus contain a Calothrix phycobiont, and Dichothrix is the phycobiont in Placynthium (Ahmadjian, 1967). As is the case with many lichenized algae (Ahmadjian, 1967), the algal trichomes have sometimes been observed to have an atypical morphology. Geitler (1934) and de Puymaly (1949) described the resemblance of the phycobiont of Placynthium nigrum to a species of Nostoc: although heterocysts were present, there was no basal-apical differentiation of the trichomes, and growth was not localized in a meristem. When the alga was cultured in the free living state, however, it showed meristematic growth, and grew as normal tapered trichomes, with basal heterocysts and apical hairs. Henssen (1969) found that the phycobiont of Lichina rosulans consisted of short, contorted chains, and basal heterocysts were rarely observed. In L. polycarpa, basal heterocysts were visible, but no tapering of the tips of the trichomes was seen (Henssen, 1973). In L. willeyi and L. tasmanica, however, the phycobionts retained a normal tapered morphology. Welsh (1965) observed Calothrix trichomes in a preserved sample of an unidentified lichen species; he noted the frequent absence of heterocysts, giving a resemblance to a Homoeothrix sp., but hairs were present on some of the trichomes.

A number of reports indicate quite marked effects of combined nitrogen on the morphology of heterocystous Rivulariaceae. Pearson and Kingsbury (1966) grew Calothrix membranacea in a variety of media containing combined nitrogen, and found that the trichomes often lacked heterocysts and showed no attenuation, with a positive correlation between these characters. Fay, Stewart, Walsby and Fogg (1968) mentioned that when cultured in the presence of ammonia, filaments of Calothrix and Gloeotrichia, which normally have basal heterocysts and apical hairs, became 'of uniform appearance throughout', and were unrestricted in length. On the basis of the results described in this paper, they suggested that the development of hairs in Rivulariaceae might be due to depletion of nitrogenous substances in the cells most distal to the basal heterocyst

(cf. Stewart, 1972). In a later publication (Fogg, Stewart, Fay & Walsby, 1973) these workers mention that Gloeotrichia grown on high levels of combined nitrogen resembles Homoeothrix or Leptochaete, which implies that the trichomes lacked heterocysts, but were still tapered.

Kirkby (1975) studied the effects of combined nitrogen on the morphology of three strains of Calothrix. In medium without added combined nitrogen, all three strains, C. brevissima, C. scopulorum and C. viguieri, were tapered, but none of them had hairs. Kirkby grew C. brevissima and C. viguieri in the presence of $\text{NH}_4\text{-N}$, and found an almost total loss of heterocysts in both strains. Most of the trichomes of C. brevissima, and some of the trichomes of C. viguieri became parallel, but many of the trichomes of C. viguieri tapered towards both ends, and resembled Ammatoidea. Similar results were obtained when C. viguieri was grown in the presence of $\text{NO}_3\text{-N}$. C. scopulorum, grown in medium with $\text{NO}_3\text{-N}$ also lost its heterocysts; some trichomes became parallel, but others had a tapered morphology, though in this case they tapered from base to apex only. Schwabe (1960), while not specifically studying the morphological effects of combined nitrogen, observed rather similar responses in C. desertica, grown in tapwater with urine as nitrogen source. The alga did not develop heterocysts in the early phases of culture, and strongly resembled Homoeothrix. Schwabe also depicts (loc. cit., Fig. 2) trichomes without heterocysts which appear untapered, and resemble Plectonema; he does not, however, mention this in the text.

Wyatt, Martin and Jackson (1973), working with strains of Fremyella (Microchaete) diplosiphon (cf. Section 1.21; p.17), obtained results rather similar to those described above for Rivulariaceae. F. diplosiphon IUC 590 had terminal heterocysts and tapered trichomes when grown in ASM-1 (Gorham, McLachlan, Hammer & Kim, 1964) without combined nitrogen, but in ASM-1 containing nitrate, few heterocysts developed, and the taper was 'almost immeasurable'. F. diplosiphon IUC 481 also grew as long, untapered filaments in the presence of nitrate, and heterocysts rarely occurred. In the absence of combined nitrogen, this strain developed heterocysts, but the overall appearance of the trichomes was unhealthy, apparently due to the inability of the alga to fix atmospheric nitrogen.

1.42 Other blue-green algae

It will be clear from the preceding section that Rivulariaceae may exhibit striking variations in morphology. This must be viewed, however, in the context of the extreme morphological plasticity of blue-green algae as a group. There are numerous reports of morphological variations in members of other families, both as a result of complex life cycles, and in response to environmental conditions. Demeter (1956), Geitler (1957), Lazaroff and Vishniac (1961), Stein (1963), Gorham, McLachlan, Hammer and Kim (1964), Suba-Claus (1965), Pearson and Kingsbury (1966), Pringsheim (1966), Evans, Foulds and Carr (1976) and Jeeji-Bai (1976) have all described examples of such variation. In many cases, the observations made have been purely empirical, with no suggestion as to the underlying mechanisms involved in the morphological changes, or any possible adaptive significance they may have, but some work has been done in this direction.

Fuhs (1958; 1968) for instance, has made detailed cytological studies of the development of the tapered calyptra following trichome breakage in Oscillatoria amoena. The narrow cells at the tip of the trichome contain fewer chromatin elements than the cells of normal width, and Fuhs has speculated that the progressive narrowing of these cells might be related to inhibition of replication of the nuclear equivalents in the new end cells produced when the trichomes fragment. Shukovsky and Halfen (1976) suggested however that in O. princeps the changes in cell shape during calyptra development were probably due to mechanical and/or osmotic stresses. The transverse walls of cells in the apical region of trichomes of this species became thickened following trichome breakage, and Shukovsky and Halfen suggested that the calyptra might thus provide mechanical protection for cells further down the trichome. They also speculated that by preventing the transfer of solutes between the cells, the thickening of the walls might be partly responsible for the breakdown of normal cell structure, and the loss of chlorophyll that were observed in the terminal region.

Ingram and Van Baalen (1970) isolated septate and non-septate filamentous mutants of the normally coccoid blue-green alga Agmenellum quadruplicatum (Strain BGl) following treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Morphologically identical forms could be produced by treatment with sublethal concentrations of chloramphenicol or penicillin G (Ingram, Thurston & Van Baalen, 1972).

The metabolic characteristics of the morphological variants were very similar to those of the parent strains, suggesting that the control of cell shape was independent of gross metabolic processes (Ingram, Thurston & Van Baalen, 1972). Similar filamentous forms were induced in Anacystis nidulans by potassium starvation (Ingram & Thurston, 1976). Ingram and his collaborators have suggested that the physical properties of the plasma membrane may be involved in the regulation of cell division, and thus of cell shape, in these organisms (Ingram & Fisher, 1973a, 1973b; Ingram & Thurston, 1976).

Many studies of morphological variation have been concerned with the differentiation of heterocysts and spores. For recent reviews on heterocysts, see Tyagi (1975), Fay (1973), Fogg, Stewart, Fay and Walsby (1973), Wilcox, Mitchison and Smith (1975). The differentiation of heterocysts may be partly or completely inhibited in the presence of assimilable sources of combined nitrogen. Differences have been found between nitrogen sources as to the extent of suppression of heterocyst development produced: $\text{NH}_4\text{-N}$ usually has the most pronounced effect (see Tyagi, 1975). Under nitrogen starvation, induced by incubation in an atmosphere without N_2 , increases in the frequency of heterocysts have been observed, in Anabaena sp. (Neilson, Rippka & Kunisawa, 1971), A. flos-aquae (Mickelson, Davis & Tischer, 1967) and A. cylindrica (de Vasconcelos & Fay, 1974). These changes in heterocyst frequency in response to the availability of nitrogen are presumably related to the generally accepted role of these cells as the main site of nitrogen fixing activity in aerobically grown filamentous blue-green algae (Stewart, 1973b). Variations in the size of heterocysts have also been observed. Canabaeus (1929) found an increase in the size of heterocysts of Anabaena spp. following the addition of NaCl, though above a certain concentration the effect was reversed. Heterocyst size also increased in cultures incubated anaerobically in the dark. Tyagi (1974) found that in filaments of A. doliolum grown in the absence of molybdenum and tungsten the heterocysts were larger than in filaments grown in the presence of these elements; there was a slight and progressive increase in heterocyst size with increasing concentration of either element.

The precise factors leading to the development of spores (akinetes) are not known, though a deficiency of mineral salts

was suggested by Geitler (1960). More specifically, low phosphate concentration has been found to stimulate sporulation in Anabaena cylindrica (Wolk, 1965) and Aphanizomenon flos-aquae (Gentile & Maloney, 1969). Some species appear to produce spores when the trichomes have reached a certain size (Fritsch, 1945; p. 809).

Changes in the character of the sheath in response to environmental conditions have been described for many blue-green algae. Sheath thickness may increase in response to desiccation (Golubić & Marčenko, 1965), and changes in sheath thickness in response to the composition of the growth medium have also been observed (Foerster, 1964). In addition to polysaccharides localized in a sheath, blue-green algae may also release these materials into their surroundings. It seems likely that in some cases at least, there is no sharp distinction between sheath material and dissolved extracellular polysaccharides, and that the latter derive from the sloughing off of sheath material (Bishop, Adams & Hughes, 1954; Martin & Wyatt, 1974b). Sangar and Dugan (1972) found that production of soluble extracellular polysaccharide by Anacystis nidulans cultures with a range of nitrogen sources was greater in the media where least growth occurred. They suggested that under conditions unfavourable for growth, fixed carbon dioxide may be channelled into extracellular polysaccharide production, rather than into cell material. The sheath may become more deeply pigmented at higher light intensities (Fritsch, 1945; Jaag, 1945), presumably as a protection against excessive light (Golubić & Marčenko, 1965). Extracellular pigments may possibly serve a function other than protection from high light intensity, since Walsby (1974) has shown that the brown pigment-peptide complex released into the culture medium by Anabaena cylindrica forms a strong complex with iron. Though no relationship with iron uptake was demonstrated, it seems likely that such complex formation might well affect the availability of iron to the alga.

1.5 Hairs and similar structures in other organisms

1.51 Hairs in other algal phyla

Colourless hairs, often superficially very similar to those of blue-green algae, are also found in members of the Chlorophyta, Phaeophyta and Rhodophyta. While the only speculation as to a possible (absorptive) function for the hairs of Rivulariaceae appears to be that of Palla (1893) (Section 1.24; p.21), several

authors have suggested roles for the hairs of members of these eukaryotic phyla, sometimes based on experimental observations.

Chlorophyta

In the Chlorophyta, the development of hairs is most marked among the Chaetophorales. The hairs of Chaetophoraceae consist of one to several cells, but in the Coleochaetaceae the colourless 'hairs' (setae) are actually outgrowths of a pigmented vegetative cell (Fritsch, 1935; McBride, 1974). Several authors have noted that hair development of various Chaetophorales was more extensive in low concentrations of nitrate than at higher concentrations (Uspenskaya, 1930: Draparnaldia glomerata; Suomalainen, 1933: D. glomerata; Uspenskaya, 1936: Stigeoclonium tenue; Abbas & Godward, 1963: S. amoenum and D. plumosa; Tupa, 1974: Aphanochaete spp.; Yarish, 1976: Bolbocoleon piliferum, Entocladia viridis, E. flustrae). Yarish (1976) also found that B. piliferum, E. viridis, E. flustrae and E. ramulosa produced more hairs when the phosphate concentration was lowered. J.P.C. Harding and B.A. Whitton (personal communication) similarly found a very marked increase in hair development in S. tenue in cultures deficient in phosphate and nitrate, but not in ones deficient in sulphate. Nielsen (1969) observed that Pheophila dendroides and P. tenuis developed hairs when they were transferred from artificial seawater to natural seawater diluted to 3‰ salinity. Moestrup (1969) similarly found that B. piliferum produced many hairs when cultured in natural seawater diluted to 0.2-0.3‰ salinity, but did not produce hairs in an enriched seawater medium. The observation of Godward (1942) that hairs were more abundant in old cultures of S. amoenum also seems likely to be related to depletion of nutrients. Tupa (1974) quotes the findings of Cholnoky (1929) and Vischer (1933) that Stigeoclonium plants from running water had fewer hairs than those from standing water. This, too could be related to the higher rate of nutrient replenishment in the former situations. Uspenskaya (1930) and Suomalainen (1933) found that hair development in D. plumosa was increased by increasing the carbon dioxide concentration. This could perhaps have resulted from a promotion of growth, causing more rapid uptake of other nutrients from the medium.

Effects of light intensity on hair development have also been observed. Suomalainen (1933) found that Draparnaldia glomerata

produced more hairs at higher light intensities, but Uspenskaya (1936) found that an increase in light intensity suppressed hair development in Stigeoclonium tenue. Yarish (1976) observed that Ectochaete ramulosa and Ochlochaete hystrix produced more hairs at higher light intensities, but that in Bolbocoleon piliferum hairs were more abundant at lower light intensities; all three species showed an increase in the extent of growth with increasing light intensity.

Tupa (1974) suggested that the hairs of Aphanochaete might be an adaptation facilitating the uptake of nitrogen compounds from the surrounding medium, by increasing the surface area of the cells. Cook (1970) speculated that the hairs of Draparnaldia champlainensis might facilitate the exchange of materials with the exterior, since they were not enclosed by mucilage. McBride (1974) observed apparently high Golgi activity in the seta-bearing cells of Coloeochaete scutata, and suggested that the setae might have a secretory role.

In the Oedogoniales, colourless unicellular hairs are characteristic of the genus Bulbochaete (Fritsch, 1935). Fraser and Gunning (1974) have described the ultrastructure of these hair cells, which were found to have a highly developed Golgi apparatus. Fraser and Gunning suggested that the hairs might function in the secretion of mucilage, and its subsequent export through pores in the hair cell walls.

Hairs are produced by some members of the Siphonales. In Acetabularia, successive whorls of branching hairs develop before the reproductive cap appears. These hairs are not completely colourless, but the cytoplasmic contents are progressively reduced with increasing distance from the main axis (Gibor, 1973a). Adamich, Gibor and Sweeney (1975) found that the whorls of hairs of A. mediterranea were longer under low nitrate concentrations, and that nitrogen depletion inhibited the abscission of the whorls, which normally precedes the development of the fertile cap. These workers suggested that the sterile whorls may have an absorptive role, since they greatly increase the surface area of the plant. Gibor (1973a) calculated that the surface area of a single whorl of hairs in A. mediterranea was almost as great as that of the main axis. Head and Carpenter (1975) found that plants of Codium fragile collected from sites with a low level

of combined nitrogen were light green, and covered with whitish hairs, but plants from high nitrogen sites were dark green and did not have hairs. When plants from one low nitrogen site were transferred to enriched seawater without added combined nitrogen, they became paler green and retained their hairs; in the same medium with nitrate or ammonium added the plants lost their hairs and became dark green. The behaviour of this Codium was thus rather similar to that of the Acetabularia studied by Gibor and his collaborators.

Phaeophyta

Fritsch (1945) recognizes nine orders of Phaeophyta, and describes the occurrence of hairs in some genera from each of these. Both colourless hairs and 'assimilatory hairs' (containing chloroplasts) may be present. Fritsch notes that the degree of development of the hairs (in the Ectocarpales) can be very variable, but that little is known about the factors determining this variation. He quotes the speculation of Berthold (1882) that since hairs tend to be better developed in well-illuminated situations, they may function as a light screen. Other authors (Oltmanns, 1923; Wille, 1897; Sauvageau, 1896; see Fritsch, 1945) suggested that the hairs had a role in the absorption of nutrients, or in gas exchange. Fritsch (1945) also held this view; he speculated that the frequent association of groups of hairs with the reproductive organs might be related to a particular need of these organs for nutrients. More recently, Dring and Lüning (1974) have shown that hair formation (and also two-dimensional growth) in Scytosiphon lomentaria (Ectocarpales) can be induced by blue light. The effect was shown to be a specific morphogenetic one, occurring independently of photosynthesis and growth.

Rhodophyta

Hairs are produced by members of the Florideae (Fritsch, 1945), though not all the genera show this character. As in the case of the hairs of Phaeophyta, a role in protection against excessive light has been suggested (Berthold, 1882; Oltmanns, 1923). Kylin (1917) germinated spores of Dumontia filiformis in seawater with and without the addition of 2% KNO_3 . In the medium without added nitrate, the germlings developed hairs, but they were not produced in the nitrate-containing medium. Kylin also found that

if germlings of Stilophora rhizoides and Asperococcus bullosus were left for ten days without a change of nutrient solution, they developed hairs; if the solution was changed daily the germlings showed greater development but did not form hairs. Kylin (1917) speculated that hairs perhaps had a role in the absorption of nutrients, especially nitrogen and phosphorus compounds. He suggested that Berthold's (1882) observation that hairs were more abundant in well-illuminated sites could be due to the more rapid utilization of nutrients under these conditions, rather than to a direct effect of light. Oltmanns (1923) and Rosenvinge (1903) also suggested that the hairs of Rhodophyta had an absorptive role. Fritsch (1945) noted that the prevalence of hairs in the early part of the growing season, and their restriction to the growing parts of the plants, were compatible with such a role.

No recent physiological work appears to have been done on the hairs of Rhodophyta, but at least two descriptions of the ultra-structure of hairs have been published. Chamberlain (1974) noted the elaborate plugged pore at the base of the unicellular hairs of Ceramium rubrum, and also the high Golgi activity. Duckett, Buchanan, Peel and Martin (1974), studying Nemalion helminthoides, also observed many Golgi vesicles in the hair cells, and commented on the unique structure of the pit connections between the hair mother cell and the hair.

The similarity in structure and behaviour between hairs from these different phyla of eukaryotic algae is rather striking. The response most often observed in studies of hair-forming algae seems to be an increase in hair development under conditions of nutrient deficiency, leading to speculations that these structures, with their high surface area to volume ratio, may function in the absorption of nutrients present in low concentration. Tupa (1974) incubated Aphanochaete magna on slides in different field situations (lake, pond and river) and in the laboratory, and found that the greatest hair development (and the poorest growth) occurred in the lake water. She suggested that this might indicate that the lake waters were poorest in nitrogen content. Head and Carpenter (1975) found that Codium plants from sites low in combined nitrogen had many hairs, while those from nitrogen-rich sites did not have hairs. These observations suggest that the

extent of hair development in particular algae might have potential as an indicator of the nutrient status of natural environments. Such a bioassay could have particular value if the increases in hair development occurred only in response to specific nutrient deficiencies.

Despite the relatively numerous reports of effects of nutrient depletion on hair development in eukaryotic algae, little similar work appears to have been done with blue-green algae, apart from that of Kirkby (1975) (see Section 1.41; p. 28). The effects of various mineral deficiencies on members of the Rivulariaceae were therefore examined, to determine whether hair development would increase under nutrient deficiency; these experiments are described in Chapters 4, 5 and 6.

1.52 Bacterial prosthecae

Hairs are not found in bacteria, but some forms possess cellular projections (prosthecae: Staley, 1968) which, like hairs, increase the surface area to volume ratio of the organisms. Experiments on the prosthecae of the Caulobacteriaceae show some interesting parallels with those described above for the hairs of various algal phyla, and it seems worthwhile to mention these here. Members of the caulobacter group have a dimorphic life cycle in which a sessile prosthecate cell produces a motile swarmer cell. This is broadly analogous with the life cycle of Rivulariaceae, in which non-motile trichomes release hormogonia. In the genus Caulobacter, the holdfast of the sessile cell is at the base of the prosthecum, which is thus called a 'stalk'; in Asticacaulis spp., the holdfast and prosthecum are not associated (Poindexter, 1964).

Caulobacteriaceae characteristically grow in environments very low in organic nutrients, and may be unable to grow in rich media (Larson & Pate, 1975). Larson and Pate (1975) found that in a strain of Asticacaulis biprosthecum normal growth could occur only if nutrient concentrations were low. The growth rate could be increased to a certain extent by increasing the concentration of nutrients, but beyond a certain point the growth rate was sharply reduced, and the cells developed an abnormal morphology. This response apparently resulted from the loss of co-ordination, at high metabolic rates, of the obligate sequence of morphogenetic events in the complex growth cycle.

Pate and Ordal (1965) speculated that prosthecae might assist nutrient uptake by increasing the area of membrane in contact with the environment. The observation by Schmidt and Stanier (1966), that in their natural environment, caulobacters often have stalks as long as 20 μm , whereas in rich laboratory media the stalks are typically only 2-3 μm long, provides circumstantial evidence in support of this hypothesis. Schmidt and Stanier (1966) found that Caulobacter crescentus produced long stalks when grown in medium with a low concentration ($< 2 \times 10^{-4}$ M) of inorganic phosphate, with an inverse correlation between stalk length and phosphate concentration. In low phosphate medium, the stalk elongation response occurred before any detectable effects on cell division were seen. Schmidt and Stanier concluded that phosphate limitation de-repressed the synthesis of wall material at the site of stalk formation, but did not affect the regulation of wall synthesis leading to cell division; cessation of cell division apparently occurred only when the net synthesis of cell material had ceased as a result of extreme phosphate starvation. Schmidt (1968) isolated mutants of C. crescentus which produced long stalks even in phosphate-rich medium; in some of these mutants, stalk length could be reduced by further increasing the phosphate concentration (to 10^{-2} M).

Some clues as to possible mechanisms underlying the stalk elongation response in Caulobacter crescentus were obtained by Schmidt and Samuelson (1972). They found that the long-stalked mutants had lower endogenous levels of ATP and GTP than wild-type strains, and that exogenous nucleoside triphosphates (ATP, GTP, CTP and UTP) inhibited stalk elongation in these mutants. Exogenously supplied cyclic GMP (but not cyclic AMP) was found to cause stalk elongation in both mutant and wild-type strains. These results suggested that intracellular levels of nucleotides probably play an important role in the regulation of stalk development.

Jordan, Porter and Pate (1974) compared the activity of various enzymes in isolated prosthecae and in the main cell body of Asticcacaulis biprosthecum. All the enzymes assayed were present in the main body of the cell, but the prosthecae contained only some of the enzymes. Jordan, Porter and Pate (1974) suggested that this might indicate that the prosthecae were specialized biochemically as well as morphologically. In view of

the results obtained with Caulobacter by Schmidt and Stanier (1966), it is of interest that alkaline phosphatase was one of the enzymes shown by Porter and Pate (1974) to be present in the prosthecae of Asticcacaulis biprosthecum. However, Larson and Pate (1975) found that the length of the prosthecae in this strain of Asticcacaulis was not affected by variations in the concentration of phosphate. Further evidence in support of a specialized nutrient absorptive role for prosthecae was found by Porter and Pate (1975), who showed that isolated prosthecae of A. biprosthecum were capable of respiration-linked active transport of glucose and amino acids, but they did not metabolize these solutes further, due to the absence of complete catabolic pathways.

1.6 Mineral nutrition and effects of mineral deficiency in blue-green algae

1.61 General comments

Since the reports reviewed in Section 1.5 indicated that nutrient deficiency may increase the development of hairs in eukaryotic algae, and the length of prosthecae in bacteria, experiments were performed to see whether hair development in Rivulariaceae would respond similarly to deficiency of mineral nutrients. A brief account of relevant aspects of the mineral nutrition of blue-green algae, in particular their response to mineral deficiencies, is therefore given. O'Kelley (1968; 1974), Wolk (1973) and Healey (1973b) have recently reviewed the inorganic nutrition of algae, and only a few comments are necessary here. The account will be restricted to the elements which were varied experimentally in the present study, namely nitrogen, phosphorus, iron, magnesium, calcium, molybdenum and sulphur. Since relatively few reports have dealt specifically with morphological effects of mineral deficiency on blue-green algae, results obtained with other organisms will be mentioned as seems relevant.

Healey (1973b) has pointed out that certain responses of algae (not specifically blue-green algae) to nutrient deficiency are of fairly general occurrence, and may not be specific to particular elements. He mentions the following:

- decrease in the content of photosynthetic pigments;
- accumulation of carbon storage compounds, usually carbohydrate;
- decrease in protein;
- decrease in nucleic acids, RNA usually before DNA;
- increase in cell size.

Tempest (1974) and Ellwood (1975) have indicated the marked changes in the composition of microbial cell walls that may occur in response to different nutrient limitations; such modifications may cause morphological changes (Tempest, 1974). Filamentation of normally unicellular bacteria may occur in response to mineral deficiency; this has been described for Escherichia coli under magnesium (Brock, 1962) and iron (Ratledge & Winder, 1964) deficiencies, and for Lactobacillus bifidus under calcium deficiency (Kojima, Suda, Hotta, Hamada & Sukanuma, 1970). Slater and Schaechter (1974) have pointed out, however, that many conditions may lead to filamentation in bacteria, as a result of cell division being more sensitive to inhibition than overall growth.

1.62 Nitrogen

The most obvious response of blue-green algae to nitrogen deficiency is a marked yellowing of the cells, due to a decrease in the content of the nitrogen-containing pigments phycocyanin and chlorophyll, and retention of carotenoids (Fogg, Stewart, Fay & Walsby, 1973). Loss of phycocyanin under nitrogen starvation has been observed in both non-heterocystous (Allen & Smith, 1969) and heterocystous strains (Neilson, Rippka & Kunisawa, 1971; de Vasconcelos & Fay, 1974). In Anabaena sp. (Neilson, Rippka & Kunisawa, 1971) and in A. cylindrica (de Vasconcelos & Fay, 1974) nitrogen starvation also led to an increase in the frequency of heterocysts and in the activity of nitrogenase (cf. Section 1.42; p.33). In A. cylindrica de Vasconcelos and Fay also observed a gradual disappearance of cyanophycin granules and polyhedral bodies, and the development of intrathylakoidal vesicles in heterocysts and vegetative cells. Within 25 h of the addition of ammonia to nitrogen deficient cultures, the alga had fully recovered, and the cells had synthesized numerous cyanophycin granules.

Many blue-green algae are able to fix atmospheric nitrogen; the majority of these are heterocystous (Fogg, Stewart, Fay & Walsby, 1973). Relatively recently, nitrogenase activity has also been demonstrated in several non-heterocystous blue-green algae. Some of these (e.g. Plectonema boryanum) require micro-aerophilic conditions for nitrogen fixation, but at least one strain of Gloeocapsa shows nitrogenase activity in air (Fogg, 1974). Carpenter and Price (1976) have recently presented evidence that in the non-heterocystous Trichodesmium, its colonial growth

habit may have a protective effect on nitrogenase, enabling activity to be maintained under externally aerobic conditions. They suggest that nitrogen fixation may be confined to certain cells at the centre of the colony, which were found not to incorporate $^{14}\text{CO}_2$, implying that they did not evolve oxygen.

1.63 Phosphorus

Phosphorus has a central role in metabolism, as a component of nucleic acids, lipids and carbohydrates, and through its involvement in high energy transfer reactions. The ratio of phosphorus to other elements tends to be considerably higher in living organisms than in the external environment (Hutchinson, 1957), and its availability has often been implicated as a factor limiting the growth of algae (Kuhl, 1962), particularly nitrogen-fixing forms (Stewart & Alexander, 1971).

Algae vary in their requirements for and tolerance of phosphate; some require less than $20 \mu\text{g l}^{-1}$ P for optimum growth, and are inhibited by higher concentrations (see Fogg, 1973). Allen (1963) grew a marine Calothrix in a range of phosphate concentrations, and observed appreciable growth with 0.001 mM PO_4 (c. $0.03 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$). The yield increased with increasing phosphate concentration up to 0.33 mM PO_4 (c. $10 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$), but 1 mM PO_4 (c. $31 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$) had an inhibitory effect on growth. Fogg (1969) commented that even low concentrations of inorganic phosphate in artificial media were inhibitory to Gloeotrichia.

Phosphate uptake may be influenced by the levels of other ions. Stimulation of phosphate uptake in various micro-organisms has been reported for Na, K, Ca and Mg (see Healey, 1973a). In Anabaena flos-aquae, Healey (1973a) the requirement for Mg for maximum rates of phosphate uptake increased as the phosphate concentration decreased. Birch (1973) found that a Zygnema sp. (Chlorophyta, Conjugales) absorbed no phosphate in the absence of iron.

Many algae, including blue-green algae, have been shown to possess adaptations enabling them to make the best use of available phosphorus. One such adaptation is the ability to store phosphorus in excess of immediate needs, as polyphosphate granules. Polyphosphate may also accumulate under conditions of nutrient imbalance, unfavourable for growth (Harold, 1966). Harold (1963)

found that in phosphate-sufficient Aerobacter aerogenes, polyphosphate accumulated when nucleic acid synthesis ceased as a result of nutrient deficiency. Phosphorus deficient algae show rapid (within minutes) and extensive polyphosphate synthesis following the addition of phosphate (the 'polyphosphate overplus' phenomenon: Jensen & Sicko, 1974). In Phormidium luridum, almost every cell contained a large polyphosphate granule two hours after the addition of phosphate to a phosphate starved culture (Jensen & Sicko, 1974). Polyphosphate synthesis in Chlorella was found to be stimulated by, but not dependent upon light; the stimulation was prevented by inhibitors of photophosphorylation (Fogg, 1973; Kuhl, 1974). The utilization of stored polyphosphate may enable algae to continue growth in the absence of detectable phosphorus in the culture medium (Rhee, 1972; Healey, 1973a). Under severe phosphate deficiency, however, the polyphosphate granules disappear (Stewart & Alexander, 1971; Jensen & Sicko, 1974). Algae show more rapid initial uptake of phosphate under phosphorus deficiency than in phosphorus sufficient conditions (see Healey, 1973a). Activity of surface phosphatases has been shown to increase under phosphorus deficiency in many algae (Fogg, 1973).

Various cytological changes in addition to loss of polyphosphate granules have been observed in phosphate deficient blue-green algae. Yellowing of the cells was observed by Stewart and Alexander (1971) and by Ihlenfeldt and Gibson (1975). The latter workers found that in phosphate deficient Anacystis nidulans, the phycocyanin level fell, and the carotenoid level increased, but the chlorophyll level was not affected (all pigment levels as per mg protein). Healey (1973a) found that in Anabaena flos-aquae the chlorophyll content (per mg dry weight) fell under phosphate deficiency. In an electron microscope study, Jensen and Sicko (1974) found an increase in the number of cyanophycin granules in phosphate starved Plectonema boryanum.

Morphological changes in response to phosphorus concentration have been observed in many micro-organisms. The finding of Kirkby (1975) that hair length in Rivularia was greater at relatively low phosphate concentrations was mentioned in Section 1.41. In some cases, a specific morphogenetic role has been suggested for phosphate. The induction of sporulation in blue-green algae by phosphate deficiency was mentioned in Section 1.42.

Lien and Knutsen (1973) demonstrated that phosphate had a key role in the control of cell division in Chlamydomonas reinhardtii, and Shubert and Trainor (1974) found that phosphate was the only nutrient that induced the formation of unicells in Scenedesmus cultured in a dilute inorganic salts medium (see also Elliot & Conway, 1975). Gezelius (1974) mentions the involvement of inorganic phosphate in morphogenesis of the slime mould Dictyostelium discoideum. The effects of phosphate concentration on the length of bacterial prosthecae were mentioned in Section 1.52. Jensen and Sicko (1974) found that in phosphate deficient Plectonema boryanum, many cells were about three times longer than those in control trichomes. Abnormally short cells were also observed, suggesting some derangement of the division process. Healey (1973a) observed a decrease in heterocyst frequency in Anabaena flos-aquae as it entered phosphorus deficiency, and in severely phosphate limited trichomes, no heterocysts were detectable (a high frequency of heterocysts was present in phosphate sufficient cultures, even though the medium initially contained 1.5 mM NO₃-N). Phosphorus limitation has been found to have marked effects on the composition of microbial (bacterial and yeast) cell walls, phosphorylated polymers being replaced by molecules of a different type (Ellwood, 1975); such changes in composition may lead to quite pronounced changes in cell morphology (Forsberg, Wyrick, Ward & Rogers, 1973; Tempest, 1974).

Several of the responses described above have been used as assays for phosphorus limitation in field populations. The presence of polyphosphate granules, for instance, implies phosphate sufficiency (Stewart, 1971/72). Fitzgerald and Nelson (1966) estimated surplus stored phosphorus in algae by a 60 min boiling water extraction technique, and also used the extent of alkaline phosphatase activity as an assay for phosphorus deficiency. Stewart and Alexander (1971) observed a marked stimulation of acetylene reducing activity following the addition of phosphate to phosphorus starved blue-green algae; this response has been used to estimate the availability of phosphorus in Wisconsin lakes (Stewart, Fitzgerald & Burris, 1970). Healey (1973b) and Fogg (1973) have discussed these and other possible bioassay techniques.

1.64 Iron

Iron is a component of cytochromes and ferredoxin, which are involved in numerous electron transfer pathways, and is also a co-factor in other enzyme reactions (Healey, 1973b). Iron deficiency may therefore be expected to have diverse effects. Under aerobic conditions, the solubility of inorganic ferric iron is very low in non-acid waters (Hutchinson, 1957). Availability of iron has been implicated as a factor limiting algal productivity, particularly in waters of high pH (Schelske, 1962; Allen, 1972). Iron limitation is likely to be more common among nitrogen fixing forms, since iron is a component of nitrogenase (Dalton & Mortenson, 1972). An increased iron demand under nitrogen fixing conditions has been reported for Nostoc muscorum (Carnahan & Castle, 1958; Eyster, 1972) and for Anabaena flos-aquae (Murphy, Lean & Nalewajko, 1976).

An iron requirement for chlorophyll synthesis has been demonstrated in eukaryotic algae and higher plants; it is not certain whether iron is necessary for porphyrin synthesis, or whether iron deficiency disturbs the development of chloroplast lamellae as a result of reduced protein synthesis (Öquist, 1971). Boresch (1921) observed chlorosis of Phormidium retzii as a result of iron deficiency. The cultures changed from dark olive green to violet, and finally became reddish brown in colour. The normal colour could be restored by the addition of iron salts, provided sufficient nitrate was still present in the medium. Boresch found that under iron deficiency there was a decrease in the levels of both chlorophyll and of water-soluble red pigment (presumably phycoerythrin), but that the chlorophyll was affected earlier, thus giving the violet colour of cultures at an intermediate stage of deficiency. These changes in pigmentation contrasted with those seen in nitrogen deficient cultures, in which chlorophyll and phycoerythrin decreased at the same time. Öquist (1971; 1974) observed a reduction in the chlorophyll and phycocyanin content of Anacystis nidulans under iron deficiency, and also a shift in the main red absorption peak of chlorophyll a from 679 to 673 nm, probably due to an altered ratio of different forms of the pigment. In higher plants, changes in the appearance of chloroplast thylakoids have been observed in association with iron chlorosis. In chloroplasts of Tradescantia and maize, fewer grana developed, and intrathylakoidal vesicles were formed;

eventually the chloroplasts degenerated completely (Lamprecht, 1961; Stocking, 1975).

Many bacteria and fungi have been shown to release specific iron chelating molecules under iron deficiency (Lankford, 1973). These compounds presumably enable the organisms to mobilize for their own use whatever iron is present in the environment. Extracellular products of blue-green algae have also been found to interact with iron. Fogg (1952) found that production of extracellular nitrogen (mainly polypeptide) by Anabaena cylindrica increased under deficiency of certain mineral nutrients (particularly iron) and was less in a chelated than in a non-chelated medium. The polypeptide was shown to be capable of chelating iron (Fogg & Westlake, 1955), and it was suggested that such extracellular material might well influence the availability of iron and other metals to the algae (Fogg, 1952; Fogg & Westlake, 1955). Walsby (1974) suggested that at least part of the chelating activity observed by Fogg and Westlake (1955) might have been attributable to the brown pigment-peptide complex that is a major component of the extracellular nitrogen released by A. cylindrica cultures (cf. Section 1.42; p. 34). More recently, evidence has been presented for the production of specific iron chelating molecules by blue-green algae. Estep, Armstrong and Van Baalen (1975) reported that Agmenellum quadruplicatum may produce a compound similar to the bacterial siderochromes, and Simpson and Neilands (1976) describe the isolation of a siderochrome from low-iron cultures of an axenic Anabaena sp. Murphy, Lean and Nalewajko (1976) have similarly reported the isolation of an hydroxamate chelator from an axenic culture of Anabaena flos-aquae. They also found evidence for production of such chelators in field populations of planktonic blue-green algae (though possibly by associated bacteria), and suggested that this enabled the algae to dominate the plankton under conditions of high iron demand.

A further adaptation to growth at low iron concentrations, which has been demonstrated for some algae, is the ability to form phytoflavin. This is a flavoprotein, not containing iron, which can replace ferredoxin in redox reactions in vitro, and it has been extracted from Anacystis nidulans and Anabaena cylindrica (Smillie & Entsch, 1971) and from Chlorella (Zumft & Spiller, 1971). The highest concentrations of phytoflavin are found in iron

deficient cells, and it has been suggested that it may substitute for ferredoxin in iron deficient conditions (Smillie & Entsch, 1971).

1.65 Magnesium

Magnesium is a component of the chlorophyll molecule, and is an essential co-factor in high energy phosphate transfer reactions. It is also involved in the aggregation of ribosome subunits (Healey, 1973b). Magnesium deficiency does not appear to have been studied in blue-green algae; responses observed in eukaryotic algae include a lowered chlorophyll content, accumulation of carbohydrate, and decreases in protein and nucleic acid content (Healey, 1973b).

A strain of Chlorella vulgaris was found to contain no polyphosphate granules in magnesium deficient conditions (Badour, 1961). Magnesium starvation caused an almost total loss of ribosomes in Aerobacter aerogenes (Kennel & Kotoulas, 1967) and in Escherichia coli (Lutsch & Venker, 1969); in E. coli, there was also a marked development of intracytoplasmic membranes. In several strains of Gram-positive bacteria, the teichoic acid content of the walls has been found to increase in magnesium limited chemostat cultures, leading to speculation that this phosphorylated anionic polymer may have a role in the uptake of magnesium (Ellwood, 1975).

1.66 Calcium

Healey (1973b) commented that the function of calcium in algae remains largely unknown. Nitrogen fixing organisms, including blue-green algae, have been found to have a higher calcium requirement when fixing nitrogen than when grown on combined nitrogen (Allen, 1956; Eyster, 1972; Dalton & Mortenson, 1972). This perhaps suggests some function of calcium in the nitrogen fixing process, but no specific role seems to have been identified.

1.67 Molybdenum

Molybdenum is a component of nitrogenase and of nitrate reductase (Healey, 1973b). Anabaena cylindrica (Wolfe, 1954) and Nostoc muscorum (Eyster, 1973) have been shown to have a higher molybdenum requirement when grown with N_2 as nitrogen source than when grown on NO_3^- -N. As might be expected, the

symptoms of molybdenum deficiency have been found to be similar to those of nitrogen deficiency (cf. Section 1.62). Increases in heterocyst frequency have been described, for Anabaena cylindrica (Fogg, 1949) and for A. doliolum (Tyagi, 1974). Fay and de Vasconcelos (1974) found that molybdenum deficient A. cylindrica became yellow in colour, and developed intra-thylakoidal vesicles; the heterocyst frequency increased, but there was a decrease in nitrogenase activity.

1.68 Sulphur

Quantitatively, the chief function of sulphur is as a component of proteins (O'Kelley, 1974). A specific role for sulphate in the cell division of green algae has been described (O'Kelley, 1974), but there are no reports of such a function for sulphur in blue-green algae. Prakash and Kumar (1971) found that Anacystis nidulans and Anabaena variabilis showed progressive yellowing and vacuolation under sulphate starvation, and the cells eventually disintegrated completely. Wolk (1973) suggested that the marked yellowing of sulphur starved cells might indicate that phycocyanin could function as a sulphur reserve. Wolk (1973) mentions that the availability of sulphur does not affect the appearance of polyhedral bodies in Anabaena cylindrica.

Interactions between sulphate and phosphate uptake have been described. Sulphate uptake by phosphorus deficient Scenedesmus is inhibited by the addition of phosphate (O'Kelley, 1968), and sulphur deficient Anacystis nidulans and Microcystis aeruginosa show greatly increased orthophosphate uptake, with accumulation of massive polyphosphate bodies (Lawry, 1976). Harold and Sylvan (1963) similarly observed accumulation of polyphosphate in sulphur starved cells of Aerobacter aerogenes. The response to sulphur deficiency appeared to be due to the lowering of intracellular levels of glutathione, or a closely related compound, which acted as an inhibitor of polyphosphate accumulation.

1.7 Physiology and biochemistry of Rivulariaceae

Few studies have dealt specifically with the physiology and biochemistry of Rivulariaceae, though Stewart (1962; 1963; 1964a; 1964b; 1965) and Jones (1967) have made detailed investigations of nitrogen fixation and liberation of extracellular

nitrogen by Calothrix scopulorum. A number of physiological and biochemical investigations have nevertheless incidentally included members of the Rivulariaceae, and these are summarized in Tables 1.1 and 1.2. Where the strains used are ones also held at Durham, the Durham culture numbers are indicated on the tables (cf. Table 2.1). Fremyella diplosiphon is included in these tables, since at least one strain of this species (Indiana Culture Collection No. 481) is also held as a Calothrix sp. in other culture collections (see Table 2.1).

1.8 Culture of Rivulariaceae

As explained in the Appendix, it proved difficult to find a culture medium suitable for growth of some of the strains of Rivulariaceae. A survey of published work on the culture of Rivulariaceae was made, to see whether other workers had experienced similar problems, and a brief review of this literature is included here.

Judging from the papers listed in Tables 1.1 and 1.2, successful growth of Rivulariaceae has been obtained in most of the media commonly used for blue-green algae. A few examples are summarized in Table 1.3. A few authors have made specific comments on the growth characteristics of strains of Rivulariaceae. Darley (1968), for instance, noted the tendency of the marine strains of Calothrix she studied to grow attached to the flask base, and Wyatt, Martin and Jackson (1973) found a similar tendency in a Fremyella diplosiphon strain. Darley (1968) found that the growth of her cultures was extremely slow, and Lange (1974) found that the Calothrix parietina strain he studied grew relatively slowly. Lange (1974) found that Gloeotrichia echinulata grew poorly in the absence of added chelator in the medium, and concluded that it was probably unable to produce chelating material of its own, unlike some of the other strains of blue-green algae he studied.

Zehnder (1963) made a detailed study of the culture requirements of Gloeotrichia echinulata, and found Medium No. 11 (Hughes, Gorham & Zehnder, 1960) with 1-2 mg l⁻¹ chelated iron, at a pH of 8-9 to be especially favourable. Concentrations of iron greater than 2 mg l⁻¹ were inhibitory, and TRIS buffer was also toxic, the inhibition of growth increasing with increasing concentration between 0.5 and 10 mM.

TABLE 1.1

Use of Rivulariaceae in Physiological and Biochemical Research

BCC = Berkeley Culture Collection; CCAP = Culture Centre for Algae & Protozoa, Cambridge; IUCC = Indiana University Culture Collection.

<u>Organism</u>	<u>Strain</u>	<u>Durham culture no.</u>	<u>Nature of Study</u>	<u>Reference</u>
<u>Amphitrix janthina</u>	Wisc. 1046		Growth characteristics	Gerloff, Fitzgerald & Skoog (1950a)
<u>Calothrix sp.</u>	BCC 7101		ammonia assimilation	Neilson & Doudoroff (1973)
<u>Calothrix sp.</u>	field material		polyphosphate cytochemistry	Talpasayi (1963)
<u>Calothrix sp.</u>			heterocysts' redox properties	Talpasayi (1967)
<u>C. brevisissima</u>	CCAP 1410/7	D156	bioassay of heavy metals	Rana, Gopal & Kumar (1971)
<u>C. brevisissima</u>	Tokyo M-7	D156	heterotrophic growth	Khoja & Whitton (1971; 1975)
<u>C. brevisissima</u>			detection of brominated phenols	Pedersén & DaSilva (1973)
			analysis of sugars	Tsuse & Fujita (1964)
			preservation by lyophilization	Watanabe (1959b)
<u>C. desertica</u>	BCC 7:02	D270	effect on rice plants	Watanabe, Nishigaki & Konishi (1951)
<u>C. epiphytica</u>			fatty acids; physiological properties	Kenyon, Rippka & Stanier (1972)
<u>C. membranacea</u>			gas-vacuole development	Canabaeus (1929)
			rRNA characterization	Payne & Dyer (1972)
<u>C. parietina</u>	IUCC 484	D179	heterotrophic growth	Khoja & Whitton (1971; 1975)
			demonstration of boron requirement	Gerloff (1968)
			effect organic materials on growth	Lange (1971)
<u>C. parietina</u>	Göttingen B1410/3		carotenoid characterization	Stransky & Hager (1970)
<u>C. parietina</u>	CCAP B.182/3		C-phycoerythrin characterization	Kessel, MacColl, Berns & Edwards (1973)
<u>C. parietina</u>	"M.B. Allen's"		DNA characterization	Edelman, Swinton, Schiff, Epstein & Zeldin (1967)
<u>C. parietina</u>	Wisc. 1018		growth characteristics	Gerloff, Fitzgerald & Skoog (1950a)
			viability after lyophilization	Holm-Hansen (1964)
			Co requirement	Holm-Hansen, Gerloff & Skoog (1954)
<u>C. parietina</u>	BCC 6303		fatty acids; physiological properties	Kenyon, Rippka & Stanier (1972)
<u>C. pulvinata</u>	field material		carbohydrate analysis	Payen (1938)
<u>C. scopulorum</u>	"		isolation of sheath pigments	Kylin (1927)
<u>C. scopulorum</u>	"		analysis of carbohydrates, carotenoids & sheath material	Kylin (1943)
<u>C. scopulorum</u>	CCAP 1410/5	D256	liberation extracellular N	Stewart (1963)
			effect NH ₄ & NO ₃ on growth	Stewart (1964)
			mineral nutrition	Maertens (1914)
<u>C. stellaris</u>	ET 71-1	D255	growth & metabolic characteristics	Wyatt, Martin & Jackson (1973)
<u>Fremyella sp.</u>	IUCC 481		billiprotein properties	Bennett & Bogorad (1971)
<u>F. diplosiphon</u>			chromatic adaptation	Bennett & Bogorad (1973)
			isotopic labelling of proteins	Crespi, DaBoll & Katz (1970)
			heterotrophic growth; light quality effects on growth	Diakoff & Sheibe (1975)
			isolation of sterols	Reitz & Hamilton (1968)
			growth & metabolic characteristics	Wyatt, Martin & Jackson (1973)

continued

TABLE 1.1 (continued)

<u>Organism</u>	<u>Strain</u>	<u>Durham culture no.</u>	<u>Nature of Study</u>	<u>Reference</u>
<u>F. diplosiphon</u>	IUCC 590		growth & metabolic characteristics	Wyatt, Martin & Jackson (1973)
<u>Gbeotrichia sp.</u>	field material		polyphosphate cytochemistry	Talpasayi (1963)
<u>G. echinulata</u>	"		lipid & amino acid analysis	Mazur & Clarke (1942)
<u>G. echinulata</u>			culture requirements	Rodhe (1948)
<u>G. echinulata</u>	Wisc. 1052		toxin production	Gorham (1962)
<u>G. echinulata</u>			phosphorus limitation assays	Fitzgerald & Nelson (1966)
			viability after lyophilization	Holm-Hansen (1964)
			effects organic material on growth	Lange (1971)
<u>G. echinulata</u>	Wisc. 1053		culture study	Lange (1970a)
<u>G. echinulata</u>	Wisc. 1054		effect fulvic acid on growth	Lange (1970b)
			effect chelators on growth	Lange (1974)
<u>G. echinulata</u>	CCAP 1432/1	D126	culture study	Zehnder (1963)
<u>Rivularia sp.</u>	field material		heterocysts' redox properties	Talpasayi (1967)
<u>R. bullata</u>	"		carbohydrate analysis	Colin & Payen (1934)
<u>R. bullata</u>	"		"	Payen (1938)
<u>R. bullata</u>	"		"	Quillet (1967)
<u>R. bullata</u>	"		"	Quillet & Lestang-Laisné (1967)
<u>R. polyoriz</u>	"		gas exchange in thallus	Danin (1932)

TABLE 1.2

Demonstrations of Nitrogenase activity in Rivulariaceae

(Papers giving circumstantial evidence of nitrogen fixation are included).

<u>Organism</u>	<u>Nature of Material</u>	<u>Strain</u>	<u>Durham Culture no.</u>	<u>Method of Assay</u>	<u>Reference</u>
(a) <u>Positive Results</u>					
<u>Calothrix</u> sp.	field material (marine)			acetylene reduction	Bunt, Cooksey, Keeb, Lee & Taylor (1970)
<u>Calothrix</u> sp.	Culture (? axenic)			kjeldahl	El-Nawawy, Ibrahim & Aboul-Fadl (1968)
<u>Calothrix</u> sp.	epiphyte on sea-grass			acetylene reduction	Goering & Parker (1972)
<u>Calothrix</u> sp.	field material (coral reef)			"	Mague & Holm-Hansen (1975)
<u>Calothrix</u> sp.	field material			"	Stewart, Fitzgerald & Burris (1967)
<u>C. acruginea</u>	unialgal culture			15N	Stewart (1971)
<u>C. brevissima</u>	axenic culture	M.7	D156	kjeldahl	Watanabe (1951)
<u>C. brevissima</u>	"	M.7	D156	15N	Watanabe (1959a)
<u>C. clavata</u>	"			kjeldahl	Saubert & Grobbelaar (1962)
<u>C. clavata</u>	"			"	Taha & Refai (1963)
<u>C. clavata</u>	field material			15N	Stewart (1971)
<u>C. confaroides</u>	"			acetylene reduction	Van Raalte, Valiela, Carpenter & Teal (1974)
<u>C. confaroides</u>	"			increase in total N	Allen (1963)
<u>C. crustacea</u>	culture			15N	Stewart (1965)
<u>C. crustacea</u>	field material			acetylene reduction	Webb, Dupaul, Wiebe, Solittle & Johannes (1975)
<u>C. crustacea</u>	field material (coral reef)			"	Wiebe, Johannes & Webb (1975)
<u>C. crustacea</u>	"			"	Kenyon, Rippka & Stanier (1972)
<u>C. desertica</u>	axenic culture	BCC7102	D270	15N	Stewart (1965)
<u>C. fasciculata</u>	field material			growth without combined N	Allen (1952)
<u>C. fasciculata</u>	axenic culture			acetylene reduction	Kenyon, Rippka & Stanier (1972)
<u>C. parietina</u>	field material	BCC6303		kjeldahl	Saubert & Grobbelaar (1962)
<u>C. parietina</u>	axenic culture			15N	Schneidder, Bradbeer, Singh, Wang, Wilson & Burris (1953)
<u>C. parietina</u>	cell-free extract			15N	Burris (1953)
<u>C. parietina</u>	axenic culture	Wisc. 1018		kjeldahl	Williams & Burris (1952)
<u>C. scopulorum</u>	axenic culture	CCAP 14:0/5	D256	kjeldahl & 15N	Jones (1967)
<u>C. scopulorum</u>	"			15N	Jones & Stewart (1969)
<u>C. scopulorum</u>	"			acetylene reduction	Kirkby (1975)
<u>C. scopulorum</u>	field material			kjeldahl	Stewart (1962)
<u>C. scopulorum</u>	"			15N	Stewart (1964a; 1964b)
<u>C. scopulorum</u>	"			acetylene reduction	Mague & Lewin (1974)
<u>C. scopulorum</u>	"			15N	Stewart (1965)
<u>C. scopulorum</u>	axenic culture			acetylene reduction	Wärmling (1973)
<u>C. scopulorum</u>	"			"	Kirkby (1975)
<u>C. scopulorum</u>	axenic culture	CCAP 1410/6	D253	"	Carpenter (1972)
<u>C. scopulorum</u>	epiphyte on <u>Sargassum</u>			kjeldahl	Cameron & Fuller (1960)
<u>C. scopulorum</u>	impure isolate	IUC 590		growth without combined N	Wyatt, Martin & Jackson (1973)
<u>C. scopulorum</u>	axenic culture	ET-71		"	"
<u>Dichothrix fucicola</u>					
<u>D. orsiniana</u>					
<u>Fremvella</u> sp.					
<u>F. diploisphon</u>					

continued

TABLE 1.2 (continued)

<u>Organism</u>	<u>Nature of Material</u>	<u>Strain</u>	<u>Durham Culture no.</u>	<u>Method of Assay</u>	<u>Reference</u>
<u>Gloeotrichia</u> sp.	field material			acetylene reduction	Taylor & Pearson (1976)
<u>G. echinulata</u>	"			"	Findley, Findley & Stein (1973)
<u>G. echinulata</u>	"			"	Rusness & Burris (1970)
<u>G. echinulata</u>	non-axenic culture	IUCC 1303		Growth without combined N	Stewart, Fitzgerald, & Burris (1967)
<u>G. echinulata</u>	mixed culture			"	Ogawa & Carr (1968)
<u>G. echinulata</u>	cell-free extract			¹⁵ N	Roelofs & Oglesby (1970)
<u>G. echinulata</u>	non-axenic culture	Wisc. 1052		kjeldahl	Schneider, Bradbeer, Singh, Wang, Wilson & Burris (1960)
<u>G. echinulata</u>	"	CCAP 1432/1	D126	Growth without combined N	Williams & Burris (1952)
<u>Lichina confinis</u>	lichen thallus			acetylene reduction	Zehnder (1963)
<u>L. pygmaea</u>	"			"	Hitch & Millbank (1975)
<u>Placynthium nigrum</u>	"			"	Hitch & Stewart (1973)
<u>P. pannariellum</u>	"			"	Stewart (1971/72)
<u>Rivularia atra</u>	field material			"	Hitch & Millbank (1975)
<u>R. biasolettiana</u>	"			"	Hitch & Stewart (1973)
<u>R. bullata</u>	"			"	Hitch & Millbank (1975)
				¹⁵ N	"
				not given	Warming (1973)
					Stewart (1971)
					Stewart (1973a)
(b) Negative Results					
<u>Dichothrix</u> sp.	field material (Antarctica)			¹⁵ N	Fogg & Stewart (1968)
<u>Fremvella diplosiphon</u>	axenic culture	IUCC 481	D255	acetylene reduction	Wyatt, Martin & Jackson (1973)
<u>Gloeotrichia Ghosei</u>	"	non-sporulating mutant		lack of growth without combined N	Singh & Tiwari (1970)

TABLE 1.3
Culture Media which have been used for Rivulariaceae

<u>Medium and Reference</u>	<u>Rivulariaceae grown in medium</u>	<u>Strain</u>	<u>Durham Culture no.</u>	<u>Reference</u>
Allen's (Allen, 1968)	<u>Calothrix brevissima</u>	BCC 7102 BCC 6303	D270	Rana, Gopal & Kumar (1971)
Allen & Arnon's (Allen & Arnon, 1955)	<u>C. desertica</u> <u>C. parietina</u>			Kenyon, Rippka & Stanier (1972) "
ASH-1 (Gorham, McLachlan, Hammer & Kim, 1964)	<u>Gloeotrichia ghosei</u>			Singh & Tiwari (1970)
Chu-10 (Gerloff, Fitzgerald & Skoog, 1950a)	<u>Fremvella diplosiphon</u>	IUCC 481	D255	Wyatt, Martin & Jackson (1973)
Fogg's (Fogg, 1949)	<u>Calothrix parietina</u> <u>C. braunii</u> <u>Gloeotrichia echinulata</u> <u>Calothrix antarctica</u> <u>C. parietina</u> <u>C. clavata</u>	Wisc. 1018		Gerloff, Fitzgerald & Skoog (1950a) Ueda (1971b) Ogawa & Carr (1969) Saubert & Grobelaar (1962)
Kratz & Myers' (Kratz & Myers, 1955)	<u>Fremvella diplosiphon</u>		D255	Bennett & Bogorad (1973)
Zehnder-Gorham Medium (Medium No. 11) (Zehnder & Gorham, 1960)	<u>Gloeotrichia echinulata</u> <u>G. echinulata</u> <u>Calothrix parietina</u>	CCAP 1432/1 Wisc. 1052 Wisc. 1018	D126	Zehnder (1963) Lange (1970b) "

1.9 Aims of the project

The hairs of blue-green algae represent a rather striking cellular differentiation, and judging from the literature, their formation appears to be a normal part of the developmental cycle. Nevertheless, it has often been assumed that hair cells are simply dead or moribund. Little systematic investigation has been made of the factors influencing the production of hairs, however. The present study was therefore planned with the aim of determining the influence of certain environmental factors on hair development in blue-green algae, in the hope that this might provide clues as to any possible functional significance of hairs. While any speculation as to function must ultimately be based upon observations of algae in their natural environment, it seemed that the problem was initially more amenable to investigation in laboratory culture. A broad experimental approach was planned, using a wide range of strains, including both heterocystous and non-heterocystous forms. The study was confined to the Rivulariaceae, since this is the family in which hairs have most often been described.

The presence of hairs is associated with tapering of the trichomes, and some members of the Rivulariaceae show tapering in the absence of hairs. Although this suggests a possible relationship between tapering and hair development, it was felt that such a relationship should not be assumed to exist. The possibility that hair development and tapering may not necessarily be related phenomena was therefore borne in mind, and it was hoped that the experiments performed might clarify this point.

Several reports in the literature have shown that tapering and hair development are lost in heterocystous trichomes grown in the presence of combined nitrogen (Section 1.41). On the basis of such results, it has been suggested that hair development in Rivulariaceae may be related to a decreasing gradient of fixed nitrogen between the basal heterocyst and the trichome apex (Fay, Stewart, Walsby & Fogg, 1968; Stewart, 1972). It was thus of interest to examine the effects of combined nitrogen on tapering and hair development in heterocystous Rivulariaceae, to see whether these characters were likely to be simply the result of nitrogen depletion in the cells distal to the heterocyst.

The literature on hairs in eukaryotic algae indicates that while many factors may influence hair development, nutrient

deficiency has been most often implicated as a condition tending to promote the formation of hairs (Section 1.51). Experiments to determine the effects of specific mineral nutrient deficiencies on hair development in Rivulariaceae were therefore designed (Chapters 4, 5 and 6). Diverse morphogenetic effects have been described for phosphorus (Sections 1.52 and 1.63), and one report has shown that phosphate concentration may affect hair development in Rivularia (Section 1.41). It was thus decided to study phosphate deficiency in rather more detail (Chapter 4).

Marked morphological plasticity in relation to variations in environmental conditions is a feature of many blue-green algae (Section 1.42), and it seemed worthwhile, in experiments on nutrient deficiency, to record morphological and cytological responses in addition to that of hair formation. It was felt that besides their intrinsic interest, such observations would be of potential value for future taxonomic work. They might also, by any relationship with a hair development response, shed light on the nature of this response.

2 MATERIALS AND METHODS

2.1 Algal cultures

2.11 Origins

As many strains of Rivulariaceae as possible were brought together, in the hope that experiments performed on a wide range of organisms would permit generalization about the biology of the family. Cultures were obtained of all the strains of Rivulariaceae held in the major culture collections of the world. Some strains were given by individual people, and some were isolated by the author. The final collection used for experimental purposes consisted of 36 strains: 29 Calothrix, 2 Rivularia, 2 Gloeotrichia, 1 Dichotrix and 2 Homoeothrix. These cultures are listed, together with their sources, in Table 2.1. Several of the strains are held in more than one culture collection (Table 2.1). Duplicate cultures of some of these strains were obtained, but only one culture of any strain was used experimentally. The specific names used for the cultures are those given by the donors of the strains, except for Calothrix viguieri D253, which is held at Cambridge as Calothrix sp., though it is referred to as C. viguieri in the collection at Třeboň. In some experiments, Anabaena cylindrica was included for comparative purposes; this was Strain 1403/2a of the Cambridge Culture Collection (Durham culture no. D2A). For the sake of brevity, strains are normally referred to in the text by their Durham culture number only, though generic and/or specific names are sometimes given also, for clarity. The names of the algae are given in full in the tables.

An effort was made to obtain any available information on the original habitats of the algae, in the hope that this would assist the interpretation of experimental results. In practice, however, the habitat descriptions were usually rather vague; all available information is included in Table 2.1. For strains isolated in Durham, fuller details of collection sites are given in Table 2.2. Only four strains were axenic (Table 2.1). These were used for experiments whenever possible, but their various characteristics were not always suitable, particularly in the latter part of the work. About halfway through the period of research, the number of strains that could be used experimentally became restricted,

TABLE 2.1
Sources of Cultures of Rivulariaceae

1 Full postal addresses of culture collections are given in Krmárek (1973). When a culture is held at more than one culture collection, the source collection is marked *.

<u>Organism</u>	<u>Durham Culture no.</u>	<u>Source, and other: col- lections where held</u>	<u>Culture no. at source</u>	<u>Whether axenic</u>	<u>Whether definitely clonal</u>	<u>Known details of habitat</u>	<u>References to col- lection and/or isolation</u>
<i>Calothrix</i> sp.	D184	see Table 2.2			+	see Table 2.2	
<i>Calothrix</i> sp.	D251	Oregon	OH-67-c, clone 1		+	Hunter's Hot Springs, Oregon, U.S.A.; 45°C, pH c. 8.5	Castenholz (1970)
<i>Calothrix</i> sp.	D232	Oregon	I-21-c			Hvergerdi, steam (Iceland). Col- lection temperature 30°C	"
<i>Calothrix</i> sp.	D254	see Table 2.2	1410/2			see Table 2.2	
<i>Calothrix</i> sp.	D255	* Göttingen Cambridge } as <i>Fremvella</i> Cöttingen } Indiana } Indiana } New Delhi } Gif-sur-Yvette } Cambridge } * Cambridge } Göttingen } Indiana } * Cambridge } Tokyo } Tokyo } Prof. R. N. Singh, } Banaras } Tokyo } Indiana } see Table 2.2 } * Cambridge } Göttingen } Indiana } New Delhi } " } " } " } Tokyo }	1429/1 1.1429/1a 481 B1827 ARM 142 M13 1410/8 1410/4 1410/4 1319 1410/7 M-7 M-34 - M-61		+	nitrogen-free enrichment culture, Yale University	
<i>Calothrix</i> sp.	D258	Indiana	B1827			India	
<i>Calothrix</i> sp.	D264	New Delhi	ARM 142			rice field soil, India	
<i>Calothrix</i> sp.	D267	Gif-sur-Yvette	M13			soil	
<i>Calothrix</i> sp.	D283	Cambridge	1410/8				
<i>Calothrix</i> sp.	D182	* Cambridge	1410/4			soil, India	Mitra (1951)
<i>C. anomala</i> (nomen nudum)		Göttingen	1410/4				
<i>C. brevis</i>	D156	* Cambridge	1319				
<i>C. brevis</i>	D275	Tokyo	1410/7			paddy field mud, Palau Island	Watanabe (1951)
<i>C. brevis</i>	D270	Göttingen	M-34				
<i>C. desertica</i> Schwabe	D273	Tokyo	-				
<i>C. eLenkinii</i> Kossinskaja	D269	Prof. R. N. Singh, Banaras	M-61			fine sand, near La Portada, Chile	Schwabe (1960)
<i>C. fusca</i> (Kütz.) Bornet & Flahault							
<i>C. gracilis</i> Fritsch	D274	Tokyo	M-55				
<i>C. javanica</i> de.Wilde	D257	Indiana	B1825			India	
<i>C. marchica</i> Lemm.	D202	see Table 2.2				see Table 2.2	
<i>C. membranacea</i> Schmidle	D179	* Cambridge	1410/1		+	soil, India	Mitra (1951)
		Göttingen	B1410/1				
		Indiana	B379				
<i>C. membranacea</i> Schmidle	D259	New Delhi	ARM 92			rice field soil, India	
<i>C. membranacea</i> Schmidle	D260	"	ARM 93			"	
<i>C. membranacea</i> Schmidle	D261	"	ARM 94			"	
<i>C. membranacea</i> Schmidle	D262	"	ARM 108			"	
<i>C. membranacea</i> Schmidle	D263	"	ARM 17a			"	
<i>C. parietina</i> Thuret	D272	Tokyo	M-121				

continued

TABLE 2.1 (continued)

<u>Organism</u>	<u>Durham Culture no.</u>	<u>Source, and other collections where held</u>	<u>Culture no. at source</u>	<u>Whether axenic</u>	<u>Whether Definitively clonal</u>	<u>Known Details of Habitat</u>	<u>References to collection and/or isolation</u>
<i>C. prolifica</i> (nomen nudum)	D265	New Delhi	ARM 87			paddy field, India	
<i>C. scopulorum</i> (Weber & Mohr) Ag.	D256	Cambridge	1410/5	+		supralittoral fringe, Scottish coast	Stewart (1962)
<i>C. thermalis</i> (Schmidle) Hansgird	D266	Gif-sur-Yvette	M13/1			warm water, Dax, France	
<i>C. viguieri</i> Frémy	D253	* Cambridge (as <i>Calothrix</i> sp.)	1410/6	+		subaerial on tree rind, mangrove swamp, Cuba	
<i>Dichothrix</i> sp.	D280	Třeboň	KOM 64/44			periphyton on stones, spring of magnesium water, Cuba	
<i>Gloeotrichia echinulata</i> (J.E. Smith) Richter	D125	Třeboň	HIND 1965/32			Lake Erken, Sweden	
<i>G. Ghosei</i> R. N. Singh	D277	Cambridge	1432/1				
<i>Homoeothrix</i> sp.	D402	Indiana	1920		+	epiphyte on Chara, littoral R. Ganges, India	Singh and Tiwari (1970)
<i>H. crustacea</i> Woronichin	D401	see Table 2.2				see Table 2.2	
<i>Rivularia</i> sp.	D403	"				"	
<i>Rivularia</i> sp.	D404	"				"	

TABLE 2.2
Details of collection sites of strains isolated in Durham

Organism	Durham Culture no.	Date of isolation	Material from which isolated	Site of collection	Comments on site	Reference giving further data
<u>Calothrix</u> sp.	D184	1972	mixed culture of algae in laboratory tank	Durham University	water enriched with Zn	
<u>Calothrix</u> sp.	D254	1973	<u>Gloeotrichia natans</u> colonies	Sigiriya Pond, Sri Lanka	Na, 21.7; Mg, 10.1; Ca 26.8 mg l ⁻¹	
<u>C. marchica</u>	D202	1973	epilithic thallus	small pool (W7), Aldabra Atoll		Donaldson & Whitton (in press)
<u>Homoeothrix</u> sp.	D402	1972	<u>Homoeothrix</u> epiphytic on <u>Gongrosira</u>	"		"
<u>H. crustacea</u>	D401	1974	<u>H. crustacea</u> colonies, epiphytic on crusting stones	Scandal Beck, Cumbria (NY 748109); Durham stream/reach no. 0062/30	high Ca, moderately eutrophic	
<u>Rivularia</u> sp.	D403	1973	<u>Rivularia</u> colonies, epiphytic on <u>Cladium mariscus</u>	Croft Kettle (pond), Co. Durham (NZ 282108)	high Mg and Ca	Hudson, Crompton & Whitton (1971); Section 2.462
<u>Rivularia</u> sp.	D404	1973	"	"	"	"

when several cultures suddenly began to show gross morphological abnormalities when grown in standard medium. This meant that certain strains, including the axenic Calothrix viguieri D235, which were used in early experiments, could not be used in later work. This phenomenon is described in detail in the Appendix.

2.12 Morphology and growth

2.121 Morphology and development

The morphology and development of Rivulariaceae were described in the Introduction (Section 1.3), but a brief account of morphological features of selected strains in their control medium (Section 2.32) is nevertheless given here, as a background to the experimental studies. Much of the thesis is concerned with morphological responses to environmental factors, and such information may assist the interpretation of experimental results.

(i) Hairs

Under standard conditions (Section 2.42) 3 of the 34 heterocystous strains (D126, D277, D404) produced many long hairs. Two strains (D251, D403) developed a few short (2-6 cells) hairs, but the other 29 strains were without hairs. Neither of the two strains of Homoeothrix possessed hairs when growing actively, but Strain D401 developed a few short hairs at the end of the growth period (see ii(b) below, and Section 3.33).

(ii) Tapering

a) Heterocystous strains

In all the heterocystous strains without hairs, most of the tapered appearance was due to the presence of a swelling next to the basal heterocyst, and the trichomes tapered relatively little in the apical region. The cells in the basal region of some of these strains were considerably shorter than broad (e.g. Strain D256; Fig. 2.1a). In all these 29 strains, hormogonia were produced at the trichome apex, and were thus equivalent in diameter to the narrowest part of the tapered trichome. During their subsequent differentiation, the hormogonia formed a terminal heterocyst, and the cells next to the heterocyst enlarged to form a swelling. The initial establishment of a tapered morphology could apparently occur with little or no cell division, since tapered trichomes were frequently observed with only 10 or 12 cells, the same number as in the hormogonia (Fig. 2.1c). During

their further development, the basal diameter and length of the trichomes increased, but the diameter of the apical region remained unchanged, apart from a slight narrowing of the apical one or two cells in some strains. The final tapered morphology evidently otherwise resulted entirely from the increase in diameter of cells towards the base. This type of developmental sequence is illustrated in Fig. 2.1, for Strain D256. In strains of this type, the basal diameter seemed to be the best indicator of trichome age; length was a less reliable criterion, since short trichomes could result from the release of hormogonia by a mature trichome.

In Strains D126 and D277, the trichomes were not markedly enlarged in the basal region (except in association with spore development in Strain D277), and the tapered appearance in each case resulted chiefly from narrowing in the apical region below the hair (Figs 2.2 and 2.3). Hormogonia in these strains derived not from the narrowest region of the trichome, but from a region of uniform diameter beneath the tapering zone of vegetative cells which led to the hair. The differentiation of a terminal heterocyst was accompanied by, or shortly followed by, narrowing and elongation of cells at the other end of the trichome. This type of developmental sequence is illustrated for Strain D277 in Fig. 2.3. Further narrowing and elongation of the apical cells often occurred, with extensive vacuolation, to form a hair. Sometimes, however, increase in trichome length occurred with little change in the cells at the apex, producing a tapered trichome without a true hair (Fig. 2.3d). In these strains much of the tapered appearance was thus the result of a decrease in diameter of the original hormogonium.

Strain D404, which also had many hairs, showed both a basal enlargement and a tapered apical region (Fig. 3.3). In Strains D403 and D251, which had only a few hairs, most trichomes showed the greatest change in diameter in the basal region.

b) Homoeothrix strains

In the original material from which Strain D402 was isolated (Table 2.2), the trichomes had a slight, but distinct taper, from about 3.5 μm to about 2 μm . The greatest change in diameter occurred in the basal region, but there was sometimes a slight narrowing in the apical region also (Fig. 2.5a). In culture, the

trichomes grew to much greater lengths than were seen in the field material, and were often densely interwoven, so that it was often difficult to trace a trichome along its whole length. There was no apparent tendency for the trichome apices to taper, and on superficial examination, the cultured material resembled a Lyngbya sp. However, in young cultures, before the growth became too dense, trichomes with basal enlargements were often observed (Fig. 2.5b). The dimensions of these trichomes were very like those of the field material. Although basal enlargements were less readily apparent in older cultures, they could be detected by careful examination. The cultured alga thus seemed to have a sufficiently tapered morphology to warrant the name Homoeothrix.

Strain D401 was isolated from colonies of Homoeothrix crustacea (Table 2.2). The trichomes were quite heavily calcified, and only their apical parts were readily visible. At the time of collection (Section 3.3), the colonies contained, in approximately equal numbers, trichomes with untapered, rounded apices (Fig. 2.6a), and trichomes with tapered apices, in which the cells were slightly elongated and sometimes somewhat vacuolated (Fig. 2.6b). The rounded apices seemed to arise as a result of hormogonium production (Fig. 2.6a). Short hairs were present on a small proportion of the trichomes (<5%). The few trichome bases that were observed had a slight but distinct basal enlargement (Fig. 2.6c). In culture, Strain D401 showed no tendency to develop macroscopic colonies, and the trichomes were not calcified. However, the morphology of the individual trichomes was very similar to that seen in the field material. As with Strain D402, it was difficult to see the bases of the trichomes clearly, except in the early stages of growth, before they became densely interwoven. In young cultures, however, trichomes with basal enlargements like those in the field population could be seen (Fig. 2.6d). Each of the types of trichome apex seen in the original material also developed in cultures of Strain D401, but the relative frequencies of the different types varied at different stages of growth in batch culture. This is dealt with more fully in Section 3.33.

(iii) Meristems

As explained in the Introduction (Section 1.3), growth of

trichomes of Rivulariaceae is typically meristematic; both basal and apical meristems have been described. As mentioned in (ii) above, several of the strains had very short cells in the region of the basal enlargement. However, judging from the apparent absence of newly formed transverse walls, these cells did not appear to be in a state of active division. In all the strains examined, whenever a zone of most active cell division could be identified (deduced by the presence of many new transverse walls), it seemed to be in the region of uniform diameter towards the apex of the trichome, and below the hair (where present). As described in (ii) above, this was the region from which hormogonia were released. Although the cells in this region were sometimes longer (both absolutely and relative to their width) than those at the base, the presence of numerous apparently recently formed transverse walls did imply a meristematic character, as did their normally lower content of granules (cf. Section 1.31; p.21).

(iv) Heterocysts

Under standard conditions (Section 2.32), most of the strains had only basal heterocysts. Usually only a single basal heterocyst was present; in some strains however a few trichomes developed additional basal heterocysts, particularly in older cultures. In a number of strains, however, the majority of trichomes possessed intercalary heterocysts. This feature was especially marked in Strains D179, D259, D260, D261, D262, D263 and D265. All these strains were of similar morphology, and all except D265 had been named Calothrix membranacea by their donors (Table 2.1). They had only a slight taper, and this, together with the presence of many intercalary heterocysts, gave them an almost Anabaena-like appearance (Fig. 2.4). Strain D251 also formed intercalary heterocysts, but its morphology was otherwise distinct from that of the seven strains mentioned above.

(v) Spores

Only one of the strains of Rivulariaceae, Gloeotrichia ghosei D277, was ever seen to produce spores. They developed at the bases of the majority of trichomes towards the end of the growth period. Anabaena cylindrica D2A also produced many spores in older cultures. The other strain of Gloeotrichia held, G. echinulata D126, did not sporulate under any of the conditions used.

As stated in Section 2.11, a large number of strains of Rivulariaceae was used, in the hope that this would permit generalization about the family. To see whether the cultures held did represent a reasonable cross-section, they were compared with the descriptions given in Geitler's (1932) flora. It was found that the collection of strains covered most of the chief morphological forms of Rivulariaceae described, and also several species of Microchaete (Microchaetaceae: cf. Section 1.2). There were differences in the relative proportions of the different morphological types in Geitler's flora and in the present author's collection, however. In particular, the frequency of forms with hairs was higher among Geitler's descriptions; hairs are described for 52 of the 78 species of Calothrix he includes (Kirkby, 1975), whereas only one of the 29 Calothrix strains had hairs under standard conditions. Whereas only Strains D126 and D277 lacked a basal enlargement, and tapered chiefly in the apical region, Geitler depicts several species of this type (e.g. C. flahaultii, C. scytonemicola). Geitler describes one type of trichome in which the region of maximum width is immediately below the hair, in an intercalary meristematic zone, so that the trichome tapers towards both ends, even though a basal heterocyst is present (e.g. C. pilosa). Trichomes of this type were not represented in the collection of strains held, but there was no other major morphological form not included. The cultures thus seemed to be reasonably representative of the family. In the case of the non-heterocystous genera, the two cultures held were rather less representative of the range of forms included by Geitler (1932). Neither of the Homoeothrix strains possessed a basal enlargement as large as that shown by Geitler for H. juliana, and neither had a long hair under standard conditions. No culture was held of the genus Ammatoidea, in which the trichomes taper towards both ends.

2.122 Growth

(1) Growth form

All but four of the 36 strains of Rivulariaceae had a mat-like growth habit in culture. Soon after subculture into liquid medium, the inoculum material released hormogonia which migrated to the base of the culture flask, and to the surface of the liquid.

Further development in both these zones produced a more or less continuous mat at the air-liquid boundary, and a lawn-like cover over the base of the flask, both consisting of closely interwoven trichomes; there was little or no suspended growth. The texture of the surface mat varied from tough and leathery to soft and gelatinous.

Three of the strains, Rivularia sp. D403, Rivularia sp. D404 and Gloeotrichia ghosei D277, grew as discrete spherical colonies in culture. The production of colonies seemed to result from the hormogonia remaining attached to their parent trichome as a false branch, rather than being released as they were in the mat-forming strains. Further branch production by each successive 'generation' of branch filaments eventually produced a macroscopic colony. In G. echinulata D126, the trichomes tended to form small stellate clusters with the heterocysts at the centre of the cluster, but these clusters were so small that the culture had the appearance of a uniform suspension. A similar growth habit was described for this strain by Zehnder (1963).

(ii) Growth rate

Under the conditions used, the growth of cultures of Rivulariaceae was rather slow in comparison with more commonly used research organisms such as Anabaena. Even the fastest growing strains took 2-3 days to double in dry weight, and it often took 3-4 weeks for a culture to reach full yield.

An evident inability to grow with N_2 as the sole nitrogen source has been reported for two of the strains, Gloeotrichia ghosei D277 (Singh & Tiwari, 1970) and Calothrix sp. D255 ('Fremyella diplosiphon': Wyatt, Lawley & Barnes, 1971; Wyatt, Martin & Jackson, 1973; Table 1.3). However, in the present study, all the heterocystous strains grew well in the absence of combined nitrogen, which was taken as strong evidence of the ability to fix atmospheric nitrogen.

2.2 Scoring of morphological characters

2.21 General microscopic technique

Samples were mounted in a drop of the medium in which they had been growing, so as to avoid osmotic effects, and were gently teased out with needles. It was often difficult to achieve sufficient separation of the filaments without causing them to

break, which presented some problems for trichome measurement.

Most of the measurements of trichomes were made using a x40 objective lens, and an eyepiece of x8 magnification, with a x2 magnifying lens in the light path. With this system, the smallest eyepiece graticule unit of 0.01 mm was equivalent to 1.61 μ m. For the narrow trichomes of Homoeothrix, a x100 lens was used, giving an equivalence of 0.01 mm \equiv 0.65 μ m. Measurements of cell width or length were made to the nearest 0.25 graticule unit; measurements of trichome length were generally made to the nearest 1.0 unit, but this degree of accuracy was not always possible when measuring hairs, which sometimes exceeded 1 mm in length.

2.22 Trichome dimensions and tapering

In most of the experiments, several strains were screened for any obvious morphological response to a particular environmental variable. In such experiments, detailed measurements of trichomes were made only when an obvious change in dimensions had occurred, the aim being to confirm and quantify the subjective observations, rather than to pick up small changes not already observed. A serious problem in obtaining representative measurements of populations of trichomes was the great variation in trichome size, especially length and basal width, which occurred within a single sample (cf. Kirkby, 1975). Much of the variation probably resulted from the presence of trichomes at different stages of development (cf. Section 2.121), since even clonal cultures had a highly variable morphology (Section 2.363). In an attempt to overcome this problem, measurements were often taken only on trichomes of a particular size range. The range was usually defined in terms of the basal width, which was believed to be the best indicator of trichome age (Section 2.121). Thus when comparing an experimental population against a control, the comparison might be restricted to trichomes with basal widths ranging from 6 μ m to 8 μ m, for instance.

Trichomes were measured as far as possible at random, bearing in mind the restricted size categories mentioned above, and the fact that the whole trichome had to be visible. Measurements were normally taken on 20 or 30 trichomes from each treatment. These rather small sample sizes were used mainly because of the time-consuming nature of taking several measurements on trichomes

which often grew closely interwoven, particularly when several strains, and a number of different treatments had to be scored. A second reason was that as pointed out above, the measurements were intended primarily to illustrate differences already observed.

The characters measured are defined below:

(i) Basal width

This measurement was made on the widest vegetative cell in the basal region (this was not necessarily the very basal cell).

(ii) Apical and subapical width

In many strains, the width of the trichomes was more or less constant in the apical region, but in some cases, the apical one or two cells were slightly narrower than those below. In such cases, two apical measurements were taken:

'apical width' - measured on the narrowest apical cell

'subapical width' - measured on the cell immediately below the narrowed apical cell(s), and hair (if present).

The subapical width was considered to be a generally more useful term for the expression of overall trichome shape, since to give only the apical width would overestimate the degree of tapering (cf. (vi) below).

(iii) Heterocyst width and length

The maximum width and length of the basal heterocyst were measured. When more than one heterocyst was present at the base of the trichome, the measurements were taken on the one most recently formed.

(iv) Trichome length

For trichomes without hairs, the length between the basal and apical vegetative cells was measured. When hairs were present, the length was measured from the basal vegetative cell to the most distal cell showing no vacuolation (see (v) below).

(v) Dimensions of hair

For the sake of definition, a hair was considered to be present only when at least one of the cells in the apical region had vacuoles occupying more than 50% of the cell profile. Thus a series of cells with only small vacuoles was not regarded as a hair, even if the cells were narrow and elongated. For the purpose

of measurement, the base of the hair was considered to be the first cell to show vacuolation, regardless of any changes in cell shape. The extent of hair development was expressed as a percentage of the total trichome length (including the hair). This value was either calculated from trichome measurements, or estimated subjectively. The width of the hair was measured two or three cells below its tip.

Throughout this thesis, cells are described as 'vegetative' if they are neither spores nor heterocysts nor part of the hair (as defined above). Thus when reference is made to the vegetative cells, or to the vegetative region of the trichome, this does not include the hair cells. The zone of cells between the normal vegetative cells and the fully developed hair cells is referred to as the transition zone.

(vi) Tapering

The degree of tapering of the vegetative region of trichomes was expressed in terms of the difference between the basal and subapical diameters; trichome length was not considered (cf. Kirkby, 1975). The presence of hairs was not taken into account in making assessments of tapering.

2.23 Heterocyst frequency

Under standard conditions, most of the strains studied had only a single basal heterocyst (Section 2.121 (iv)). Under some experimental conditions, additional heterocysts were developed, sometimes at the base of the trichome, and sometimes in an intercalary position. Increases in heterocyst frequency were expressed in terms of the relative abundance of these 'new' heterocysts, rather than by the more conventional percentage of the total cell population. A semi-quantitative scoring system, explained in Section 2.25, was used.

To avoid any confusion over terminology, the terms used to describe the different types of heterocyst observed are defined below. The types are illustrated schematically in Fig. 2.7.

Basal heterocyst

Heterocyst in a terminal position (Fig. 2.7a).

Secondary basal heterocyst

Heterocyst developed in a basal position, immediately adjacent to original basal heterocyst (Fig. 2.7b). Its development often associated with death of original basal heterocyst.

Intercalary heterocyst

Heterocyst in an intercalary position, with two pores (Fig. 2.7c).

Pseudo-intercalary heterocyst

Heterocyst in an intercalary position, but with only one pore (Fig. 2.7d).

2.24 Cell inclusions

(i) Polyphosphate granules

Polyphosphate granules were identified by the staining method of Ebel, Colas and Muller (1958), as described by Fuhs (1973), but using fresh algal material, without prior fixation. Material was soaked for 15 min in a 10% (w/v) solution of $Pb(NO_3)_2$ in 1.0 M HNO_3 , at a pH of 1.2. After thorough washing with distilled water, the material was treated for 30 seconds with $(NH_4)_2S$ solution (direct from the bottle as supplied), and again rinsed thoroughly. After this treatment, polyphosphate granules were clearly stained dark brown to black.

The extent of polyphosphate granulation was expressed in terms of the estimated percentage of the cell profile which was stained. The degree of staining was expressed by a 0-5 semi-quantitative scale, as follows:

0	no granules visible
+	very occasional granules
1	up to 10% of cell profile stained
2	10-20% of cell profile stained
3	20-50% of cell profile stained
4	50-80% of cell profile stained
5	>80% of cell profile stained

Since the whole of a cell's granulation was expressed in terms of a two dimensional profile, a broad cell might appear more granulated than a narrow one, even though the cytoplasmic density of granules was the same. No attempt was made to correct for this when making the scores.

(ii) Cyanophycin granules

These were identified, without staining, by their characteristic refractive appearance (Fuhs, 1973). In most strains, cyanophycin granules were not very obvious under control conditions; consequently only relatively large increases in the level of

granulation could be scored with confidence. Cyanophycin granules were not estimated in the two strains of Homoeothrix, in which the cells were very small.

2.25 Semi-quantitative estimates of characters

In experiments involving several strains, it was not always practicable to make detailed counts and measurements on every strain, and a semi-quantitative scoring system was often used. The frequency of trichomes with hairs, and the relative abundance of different types of heterocyst were scored in this way, for instance. Characters were scored on a 0-5 scale of abundance:

Score on semi-quantitative scale	Approximate % of trichomes showing the character
0	none
+	very occasional trichomes
1	up to 10%
2	10-20%
3	20-50%
4	50-90%
5	>90%

The frequency of a character scored in this way is referred to as the 'frequency score' of the character.

2.3 Culturing

2.31 Culture vessels

For culturing in liquid media, 100 ml conical flasks or 50 ml boiling tubes were used, both of Pyrex glass. The vessels were plugged with cotton wool. For solid media, pre-sterilized plastic petri dishes were used. Glassware for culture work was cleaned by scrubbing out all algal material and soaking for 24 h in a mixture of six volumes of concentrated H_2SO_4 to one volume of saturated $NaNO_3$ solution. The vessels were then rinsed thoroughly and oven dried at $100^\circ C$.

2.32 Media

Table 2.3a shows the compositions of the media used, in $mg\ l^{-1}$ of each element; the salts used are shown in Table 2.3b. A brief description of each medium is given below.

Most work was done using either AD medium or Chu 10-D medium. AD is a rich medium, without added combined nitrogen, modified from that of Allen and Arnon (1955), and Chu 10-D is a modification

TABLE 2.3a
Composition of Media (mg l⁻¹ of elements)

Element	AD	Medium of Allen & Arnon (1955)	Chu 10-D	Medium of Gerloff et al. (1950a)	SC	ZD	Medium I	ASM-D(-N)	AC(-N)	Protease Agar
N	-	-	6.83	6.83	-	-	-	-	-	27.72
P	44.5	61.9	1.78	1.78	44.5	5.51	26.68	3.1	177.9	3.56
S	26.0	32.0	3.25	3.25	24.0	3.25	26.0	6.4	32.5	2.6
Cl	171.4	177.3	-	-	3336.0	212.15	12.06	99.2	378.1	-
Na	90.5	92.0	8.44	18.1	2102.0	126.1	9.42	48.7	9.04	-
K	112.2	156.0	2.24	4.49	170.0	13.91	67.33	3.9	835.6	-
Ca	18.1	20.0	9.77	9.77	24.0	10.0	6.82	7.91	5.45	-
Mg	19.7	24.3	2.47	2.46	90.0	2.46	19.72	9.73	24.65	1.97
Si	-	-	2.50	5.75	-	-	5.75	-	-	-
Fe	4.0	4.0	0.5	0.5	2.0	0.56	0.41	0.22	2.0	-
Mn	0.12	0.5	0.012	-	0.12	0.044	0.11	0.39	0.5	-
Mo	0.08	0.1	0.0025	-	0.08	0.004	0.16	0.5	0.01	-
Zn	0.01	0.05	0.012	-	0.01	0.005	0.009	0.16	0.05	-
Cu	0.005	0.02	0.005	-	0.005	0.002	0.01	0.0005	0.02	-
Co	0.0005	0.01	0.002	-	0.0005	0.002	-	0.005	0.008	-
B	0.09	0.5	0.125	-	0.09	0.043	0.10	0.42	0.5	-
V	0.004	0.01	-	-	0.004	0.0004	-	-	-	-
W	0.005	0.01	-	-	0.005	0.001	-	-	-	-
N1	0.002	0.01	-	-	0.002	0.002	-	-	-	-
Cr	0.001	0.01	-	-	0.001	0.0004	-	-	-	-
Al	-	-	-	-	-	0.002	-	-	-	-
Tl	-	0.01	-	-	-	-	-	-	-	-
Cd	-	-	-	-	-	-	-	-	-	-
Br	-	-	-	-	-	0.004	-	-	-	-
I	-	-	-	-	-	0.006	-	-	-	-
	-	-	-	-	-	0.005	-	-	-	-

TABLE 2.3b
Composition of Media (mg l⁻¹ of salts)

? = nct specified

Salt	AD	Medium of Allen & Arnon (1955)	Chu 10-D	Medium of Gerloff et al. (1950a)	So	ZD	Medium I	ASM-D(-N)	AC(-N)	Proteose Agar Medium
KNO ₃	-	-	-	-	-	-	-	-	-	200
Ca(NO ₃) ₂	-	-	40.0	40.0	-	-	-	-	-	-
Na ₂ HPO ₄	-	-	-	-	-	-	-	7.0	-	-
K ₂ HPO ₄	250.0	348.4	-	10.0	250.0	31.0	150.0	8.5	1000.0	20
K ₂ SO ₄	-	-	8.0	-	-	-	-	-	-	-
K ₂ SO ₄	-	-	-	-	130.0	-	-	-	-	-
MgSO ₄ ·7H ₂ O	200.0	246.5	25.0	25.0	-	25.0	200.0	49.0	250.0	20
Na ₂ SiO ₃ ·5H ₂ O	-	-	1.0	25.0	-	-	41.4	-	-	-
NaCl	230.0	233.8	-	-	5000	320.5	-	117.0	607.4	-
NaHCO ₃	-	-	16.0	-	-	-	-	-	-	-
Na ₂ CO ₃	-	-	-	20.0	-	21.0	-	-	-	-
CaCl ₂ ·2H ₂ O	66.2	73.5	-	-	88.0	36.8	25.0	29.0	20.0	-
MgCl ₂ ·6H ₂ O	-	-	-	-	750.0	-	-	41.0	-	-
FeCl ₃ ·6H ₂ O	19.4	?	2.42	-	9.7	2.70	2.0	1.08	9.7	-
Na ₂ EDTA·2H ₂ O	25.4	?	3.17	-	12.7	3.72	-	7.44	12.7	-
Molar ratio EDTA:Fe	0.94:1	?	0.94:1	-	0.94:1	1:1	-	5:1	0.94:1	-
Ferric citrate	-	-	-	3.0	-	-	-	-	-	-
Citric acid	-	-	-	3.0	-	-	-	-	-	-
MnCl ₂ ·4H ₂ O	-	-	0.045	-	-	-	0.4	1.39	1.81	-
MnSO ₄ ·4H ₂ O	0.5	2.03	-	-	0.5	0.178	-	-	-	-
NaMoO ₄ ·2H ₂ O	0.19	-	0.007	-	0.19	0.010	0.4	1.26	0.03	-
MoO ₃	-	0.15	-	-	-	-	-	-	-	-
ZnSO ₄ ·7H ₂ O	0.05	0.22	0.056	-	0.05	0.023	0.04	-	0.22	-
ZnCl ₂	-	-	-	-	-	-	-	0.33	-	-
CuCl ₂ ·2H ₂ O	-	-	-	-	-	-	-	0.001	-	-
Co(NO ₃) ₂ ·6H ₂ O	0.02	0.08	0.019	-	0.02	0.01	0.04	-	0.08	-
CoCl ₂ ·6H ₂ O	0.01	-	-	-	0.01	0.009	-	0.019	-	-
Co(NO ₃) ₂ ·6H ₂ O	-	0.04	-	-	-	-	-	-	-	-
CoSO ₄ ·7H ₂ O	-	-	0.010	-	-	-	-	-	0.04	-
H ₃ PO ₄	0.5	2.86	0.72	-	0.5	0.245	0.6	2.48	2.86	-
NH ₄ VO ₃	0.01	0.02	-	-	0.01	-	-	-	-	-
VOSO ₄	-	-	-	-	-	-	-	-	-	-
Na ₂ WO ₄ ·2H ₂ O	0.01	0.018	-	-	0.01	0.002	-	-	-	-
Na ₂ WO ₄ ·6H ₂ O	-	0.045	-	-	-	0.003	-	-	-	-
Na ₂ SO ₄ ·7H ₂ O	0.01	-	-	-	0.01	0.011	-	-	-	-
Cr(SO ₄) ₃ ·K ₂ SO ₄ ·24H ₂ O	0.01	0.96	-	-	0.01	0.004	-	-	-	-

continued

TABLE 2.3b (continued)

<u>Salt</u>	<u>AD</u>	<u>Medium of Allen & Arnon (1955)</u>	<u>Chu 10-D</u>	<u>Medium of Gerloff et al. (1950a)</u>	<u>Sp</u>	<u>ZD</u>	<u>Medium I</u>	<u>ASM-D(-N)</u>	<u>AC(-N)</u>	<u>Protease Agar Medium</u>
$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$	-	-	-	-	-	0.038	-	-	-	-
$Ti(CO_3)_2 \cdot x \cdot yH_2O$	-	?	-	-	-	-	-	-	-	-
$CdCl_2 \cdot 2H_2O$	-	-	-	-	-	0.009	-	-	-	-
KBr	-	-	-	-	-	0.009	-	-	-	-
KI	-	-	-	-	-	0.006	-	-	-	-
Protease peptone pH after autoclaving	7.2-7.5	?	7.2-7.5	8.0-9.5	7.2-7.5	7.0-7.4	7.3	6.8	7.5-7.8	1000

of the formulation of Gerloff, Fitzgerald and Skoog (1950a). A nitrate-free version of Chu 10-D was sometimes used, made by substituting $36 \text{ mg l}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ for the $\text{Ca}(\text{NO}_3)_2$; this nitrate-free version was termed Chu 10-D(-N). The compositions of the original media upon which AD and Chu 10-D are based are also shown in Tables 2.3a and 2.3b.

AD was used for routine subculturing of all the heterocystous strains, and Chu 10-D for the two strains of Homoeothrix.

Whenever 'standard conditions' are referred to in the text, this means cultures grown in these media. Although AD is not a marine medium, the marine strain Calothrix scopulorum D256 showed satisfactory growth in this medium, and it was therefore used for this strain for the sake of consistency. AD was also used as control medium for experiments with heterocystous strains, with the exception that Strain D404 was grown in Chu 10-D(-N) in the experiments described in Chapter 5, and that some field materials were also cultured in this medium (Chapter 10). For experiments with the Homoeothrix strains, either Chu 10-D or AD supplemented with NaNO_3 was used; details are given with each experiment.

In many experiments, the concentrations of components of the media were varied; details are given with individual experiments. For the experiments on mineral deficiencies, described in Chapters 5 and 6, both AD and Chu 10-D media were made up with microelement levels the same as those shown for AD in Table 2.3a, but without V, W, Ni or Cr (see Section 5.22).

The other media shown in Table 2.3 were used only in the attempt, described in the Appendix, to find a medium suitable for growth of Strain D253. All are free of combined nitrogen except the proteose agar medium, which is used for maintenance of cultures at the Cambridge Culture Collection. S_0 is a marine medium, modified from that of Stewart (1962), which is itself based upon the V_{37} medium of Provasoli, McLauchlin and Droop (1957). ZD is a nitrogen-free modification of the Z medium of Staub (1961). Medium I is a formulation supplied by Prof. N. Lazaroff (cf. Lazaroff & Vishniac, 1961). ASM-D(-N) is based on the ASM-1 medium of Gorham, McLachlan, Hammer and Kim (1964); the phosphate concentration is reduced by half, NaCl replaces NaNO_3 and $0.5 \text{ mg l}^{-1} \text{ Mo}$ is added. AC(-N) is a nitrate-free version of a medium modified from the 'C' medium of Kratz and Myers (1955).

Media were made up freshly as required, from stock solutions stored in a refrigerator. Iron and EDTA (ethylenediaminetetraacetic acid) were made up as one solution. The microelements were also added as a single solution, except for the experiments described in Chapter 5, when each element was added separately. Glass-distilled water was used for all media, except for a few experiments described in the Appendix. All volumetric glassware was of Pyrex brand. It was cleaned by thorough rinsing with distilled water, preceded if necessary by soaking overnight in 10% HCl.

Aliquots of 25 or 50 ml of medium were used in 100 ml conical flasks; 10 ml of medium were used in boiling tubes. For solid media, agar (Davis Standard Agar: Davis Gelatin Ltd, Leamington Spa) was used, at a concentration of 0.5 or 1.0% (w/v). All media and glassware used for culturing were sterilized by autoclaving at 121°C (10.35 kN m^{-2} ; 15 lb in^{-2}) for 15 min. In the case of liquid media containing high concentrations of phosphate (AD, S₀, ZD and AC(-N)), the phosphate was autoclaved separately from the rest of the medium and added aseptically to each vessel after the medium had cooled and re-equilibrated with the atmosphere, so as to avoid precipitation. The pH values of media were measured, after autoclaving, using an EIL Model 23A direct reading pH meter, fitted with a combination electrode (Electronic Instruments, Ltd, Chertsey, Surrey).

2.33 Buffering

For most purposes, media were used without added buffer, relying on the buffering capacity of phosphate (AD) or phosphate and silicate (Chu 10-D) in the medium. However, when AD medium was supplemented with a high level of combined nitrogen, as NaNO₃ or as NH₄Cl, the buffering capacity of the medium was insufficient to prevent changes in pH caused by preferential absorption of the nitrogen-containing ion. When the phosphate concentration of either medium was lowered, the consequent reduction in buffering capacity made it desirable to have some additional buffer for adequate pH control.

Experiments were performed to test the suitability of TRIS (2-amino-2-hydroxymethyl-1,3-propanediol) as a buffer for AD+5 mM NaNO₃ and AD+ 5 mM NH₄Cl, with the initial pH adjusted to 7.4 in each case. At the concentration required for adequate buffering (10 mM), TRIS inhibited the growth of the three strains

tested (D126, D202, D253), the extent of inhibition varying between strains. Zehnder (1963) also found an inhibitory effect of TRIS, on Gloeoetrichia echinulata (Section 1.8), and in view of these results, TRIS was not used in the present study. In the absence of a suitable buffer, some experiments in the earlier part of the work were performed in unbuffered media (Section 3.1; Chapter 10); details are given with specific experiments.

In 1974, Smith and Foy reported the successful use of the 'Good' buffers (Good, Winget, Winter, Connolly, Iswa & Singh, 1966) as buffers in a freshwater algal medium. Of the six buffers they tested, N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES) was found to be the most suitable on a variety of criteria. Of particular relevance is its negligible binding capacity for metal ions (tested for Mg, Ca, Mn and Cu (Good et al., 1966), and inferred for Fe (Smith & Foy, 1974)). Tests with HEPES in AD and Chu 10-D media showed it to be a very effective buffer both for low phosphate media and for media containing high levels of combined nitrogen. No inhibition of growth was observed at the highest concentration used (50 mM) in any of the 12 strains tested, but in buffered media containing high levels of combined nitrogen, there was an increased tendency for the trichomes to coil inside their sheaths. No other morphological effect was seen, and the response was attributed to the rapid growth of the trichomes being constrained by their sheaths (cf. Darley, 1968; Section 1.41). This small effect was considered insignificant relative to the advantages of a properly buffered medium.

5 mM HEPES was found adequate for buffering media with reduced levels of phosphate; 10 mM was used for media with added NaNO_3 or NH_4Cl . The HEPES was added to the medium before autoclaving; after autoclaving, and the addition of phosphate (where necessary), the pH of the medium was adjusted to its normal value by the aseptic addition of 0.2 M NaOH to each flask. In this thesis, the names of media containing HEPES are prefixed with an H. Thus AD buffered with HEPES = HAD.

2.34 Subculturing

All subculturing was performed using standard aseptic techniques, in a room separated from the main laboratory, and fitted with u.v. lights. 40 minutes before use, the room was sprayed with ethanol, and the u.v. lights turned on, to reduce

the population of air-borne bacteria and fungi. Before sub-culturing a stock, or inoculating an experiment, the inoculum material was microscopically checked for contamination. In the case of axenic cultures, a further check for contaminants was made by adding sterile sucrose to the culture, to encourage growth of any bacteria present, and examining after a few days. The level of sucrose used gave a concentration of about 2 mM in the medium.

Difficulty was experienced in obtaining a uniform inoculum (cf. Kirkby, 1975). Only one of the 36 strains of Rivulariaceae, Gloeotrichia echinulata D126, grew as a suspension that was sufficiently uniform to be transferred by pipette (Section 2.122). Stewart (1962) and Jones (1967) used glass beads to obtain a uniform inoculum of Calothrix scopulorum (the same strain as D256); this method was attempted, but it was found that very prolonged shaking with glass beads was necessary to obtain a suspension that was both uniform and sufficiently dense for inoculation. The treatment also caused considerable fragmentation of the trichomes, which seemed undesirable since many experiments were concerned with possible morphological modifications of the algae. All inoculation was therefore done with a wire loop, an effort being made to transfer pieces of algal material that were of uniform size (cf. Kirkby, 1975). The variation in the size of inocula prepared in this way did not seem to be excessive, particularly since most of the experiments were concerned with morphological, rather than quantitative effects on the algae. For experimental inocula, algae from stock cultures were inoculated into unmodified AD or Chu 10-D, and incubated under the same conditions as were to be used for the experiment. Experiments were inoculated after 14-21 days, when the inocula had reached an adequate yield, and were growing vigorously.

2.35 Incubation

2.351 Light and temperature

Most experimental cultures were incubated in thermostatically controlled tanks, illuminated continuously from below with warm white fluorescent tubes. A shaking mechanism moved the flasks through a horizontal distance of 20 mm, 72 times per minute. Some experimental cultures, and all stock cultures, were incubated on glass shelves in thermostatically controlled growth rooms,

with continuous illumination from above by white fluorescent tubes. Growth rooms were available at 15°, 25° and 32°C, and tanks at 15°, 20°, 25° and 32°C. The majority of cultures were maintained at 25°C, but stocks of Strains D401, D403 and D404, isolated from local sites, were kept at 15°C, and Strains D251 and D252, isolated from thermal springs, were maintained at 32°C. For experimental purposes, however, a temperature of 25°C was used for all strains except Homoeothrix crustacea D401, which was grown at 15°C. This was because owing to the slow growth of the algae, experiments often lasted for several weeks, during which time considerable evaporation of the medium occurred at 32°C. On the other hand, growth of stocks maintained at 15°C was extremely slow, and an experimental incubation temperature of 25° was preferred for strains that would tolerate it (viz. D403 and D404). Stock cultures were maintained at low light intensities (200-500 lx); under these conditions, it was not necessary to subculture them more than once every 4-6 months. For experimental purposes, light intensities of 2000-3000 lx were used; details are given with individual experiments. Light intensity was measured with an EEL Lightmaster Photometer (Evans Electroselenium Ltd, Halstead, Essex), at the level of the flask base, and perpendicular to the direction of illumination.

2.352 Variations in gaseous atmosphere

When modifications of gaseous atmosphere were required, algae were grown in 100 ml conical flasks with sidearms for introduction of the required gas. The gas inlet was close to the flask base, to ensure distribution throughout the medium. The flasks were connected to the gas supply via a manifold of teflon tubing; gases were passed through a sterile cotton wool filter before they entered the culture vessels.

In the experiment on nitrogen starvation (Section 3.2) the flasks were fitted with gas-tight rubber seals ('Suba-seals'), and vented during gassing with wide gauge (19G) syringe needles. The gas flow rate during flushing was about 2 l min⁻¹. The 'nitrogen-free air' used for this experiment was a gas mixture containing 79.97% Ar:20% O₂: 0.03% CO₂ (by volumes); it was used without further purification. Brill (1975) has pointed out the difficulty of obtaining completely nitrogen-free gas; in practice, however, the nitrogen level was evidently low enough to induce

deficiency.

When cultures were continuously sparged with air (Appendix; Section A3.33), normal cotton wool plugs were used. The gas flow rate was about 0.5 l min^{-1} . To reduce evaporation, the gas was humidified by passing it through distilled water before it entered the flasks.

2.36 Isolation and cloning of algae

2.361 Isolation methods

The following procedure was used for isolation:

a) The field material was carefully examined microscopically, to determine the different organisms present. It often happened that the bulk of growth in culture was due to algae present in very small numbers in the original population, which might be missed in a cursory examination.

b) Material was inoculated into media as soon as possible after collection. If delay was unavoidable, then material was kept cool ($5-10^{\circ}\text{C}$) at a low light intensity (c. 500 lx). When the initial material was in the form of a dried sample (Homoeothrix sp. D402), this was sprinkled directly onto agar.

c) AD and Chu 10-D(-N) were used for isolating heterocystous algae; Chu 10-D was used for the isolation of Homoeothrix. The following modifications to the media were made:

(i) The K_2HPO_4 concentration in AD was reduced to 0.05x the normal level, with an appropriate adjustment of pH. This was based on observations of S.M. Kirkby (personal communication) and the present author, that growth of Rivularia was more successful under these conditions, and that overgrowth by competing organisms was often reduced (cf. Section 10.1).

(ii) The $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ was omitted from the Chu 10-D media, in an attempt to reduce the growth of diatoms.

(iii) Cycloheximide (actidione) was added to the media (Zehnder & Hughes, 1958) to give a concentration of about $10 \mu\text{g ml}^{-1}$. For use in agar media, the cycloheximide was dissolved in distilled water, and 0.1 ml of the solution pipetted into each petri dish before pouring the agar, at about 40°C . No attempt was made to sterilize the antibiotic, since it was used only in crude cultures. Cycloheximide was found to be very effective in suppressing the growth of eukaryotic algae and fungi, but not that of protozoa.

d) Isolation was done by successive transfers on agar plates.

This method had the advantage that the gliding and growth of the algae tended to separate them from one another; the algae could also be directly observed without disturbance, by low power microscopy through the agar. Areas containing the required alga could then be marked and subsequently cut or micro-dissected out for further subculture. It was normally possible to establish unialgal cultures after four or five subcultures, but sometimes the organisms grew in such close association that separation was not possible by this method. A particularly troublesome contaminant of many Rivularia colonies was a 1-2 μ m diameter Phormidium sp. which often advanced across the agar plates before the Rivularia hormogonia, even in medium without combined nitrogen.

e) A suitable incubation temperature, approximating to the temperature of the natural environment, was selected, and very high light intensities avoided.

2.362 Isolation of colonial Rivulariaceae

Gloeotrichia ghosei was the only species of Rivulariaceae with a colonial growth form that was available in the major world culture collections. Attempts were therefore made to isolate colonial forms from local field sites. Only two colonial strains (Rivularia sp. D403 and Rivularia sp. D404) were successfully established, but during several isolation attempts evidence was obtained that colonies of Rivulariaceae may not be genetically homogeneous. A fairly detailed account of these isolation attempts is therefore given.

a) Rivularia from Croft Kettle

Details of this site are shown in Table 2.2. The Rivularia colonies ranged from 1-10 mm in diameter, and consisted of bright bluish green trichomes, moderately calcified. The morphology of the trichomes was fairly uniform, and the variation in trichome diameter that was observed was attributed to differences in trichome age.

Colonies were cut into pieces, and the pieces inoculated onto plates of Chu 10-D(-N) agar. Each agar plate received pieces from a single colony only. When 'haloes' of hormogonia appeared around the colonies, patches were transferred to fresh plates. As the hormogonia began to differentiate, it became apparent that more than one type of tapered trichome was present. The plates were carefully observed as growth continued, and at least three

TABLE 2.4
 Summary of Characters of Rivularia from Croft Kettle and the Three Strains Isolated from it. Cultured Strains Grown in Chu 10-D(-N)

Character	Field Material			Strain D403	Strain D404	Strain D405
	6-12 μ m	slight or absent	2-4 μ m	5-6 (-9) μ m	9-12 (-15) μ m	9-12 μ m
Basal diameter	2-4 μ m	2-3 μ m	10-15 μ m	moderate	moderate	marked
Basal swelling	spherical or subspherical	none seen	spherical or subspherical	2-3 μ m	5-6 μ m	4-5 μ m
Subapical diameter	bright green	bright green	4-6 x 60-100 μ m	1-2 μ m	3-5 μ m	2-4 μ m
Hair diameter	golden brown	golden brown	bright green	6-8 μ m	10-12 μ m	8-12 μ m
Heterocysts: diameter	yes	yes	bright green	spherical or subspherical	spherical or subspherical	hemispherical
Heterocysts: shape	colourless or brown	colourless	bright green	4-6 x 60-100 μ m	8-10 x 100-200 μ m	5-6 x 30-40 μ m
Dimensions of hormogonia	hemispherical or subspherical	hemispherical or subspherical	bright green	irregular colonies up to 15 mm	hemispherical colonies up to 2 mm diameter; daughter colonies spread widely over agar surface	colourless or brown stratum over surface; no macroscopic colonies
Colour of vegetative cells	colonies 1-10 mm diameter, sometimes confluent	colonies 1-10 mm diameter, sometimes confluent	bright green	diameter; daughter colonies remain associated with inoculum	2 mm diameter; daughter colonies spread widely over agar surface	grey-green
Colour of heterocysts			yes	remain associated with inoculum	colonies spread widely over agar surface	green
Sheath divergent			yes	remain associated with inoculum	colonies spread widely over agar surface	no
Sheath colour			colourless	remain associated with inoculum	colonies spread widely over agar surface	no
Growth form: in field or on agar			colourless	irregular colonies up to 15 mm diameter; daughter colonies remain associated with inoculum	hemispherical colonies up to 2 mm diameter; daughter colonies spread widely over agar surface	colourless or brown stratum over surface; no macroscopic colonies
Growth form: in liquid			hemispherical or subspherical colonies 1-10 mm diameter, sometimes confluent	irregular colonies up to 15 mm diameter; daughter colonies remain associated with inoculum	hemispherical colonies up to 2 mm diameter; free-floating and attached; very mucilaginous	mat of trichomes at liquid surface; no macroscopic colonies
Trichomes calcified	yes	yes	yes	spherical colonies up to 2 mm diameter; free-floating and attached	spherical colonies up to 2 mm diameter; free-floating and attached; very mucilaginous	no

distinct morphological types were recognised. Unialgal cultures were established from each of these (Strains D403, D404 and D405), and they maintained their characteristics through successive subcultures. The morphological features of the three strains as they appeared in Chu 10-D(-N) medium are summarized in Table 2.4, with the original field material included for comparison. Trichomes of the different types are shown in Fig. 2.8.

None of the three isolated strains exactly corresponded with the original material. Strain D405 showed the greatest difference; it showed no tendency to form colonies, and seemed closer to a Calothrix than to a Rivularia. It was of course possible that a Calothrix had been picked up as a contaminant, despite the precautions taken to avoid this. However, the frequency with which Calothrix arose in subsequent cultures inoculated with Rivularia colonies (see (b) below) suggested that contamination was unlikely to be responsible.

b) Rivularia and Gloeotrichia from other sites

Attempts were made to establish cultures of colonial Rivulariaceae from samples of Rivularia and Gloeotrichia from five other sites; the results are summarized in Table 2.5. Calothrix trichomes were detected in three of the five field samples. They were readily distinguishable from the Rivularia trichomes, differing from them in much the same way as Strain D405 differed from its inoculum material (trichome bases swollen; heterocysts hemispherical, not spherical; cells discoid, not cylindrical; sheath characters different; Table 2.4). The Calothrix trichomes never formed more than a very small proportion of the total population; perhaps only two or three trichomes would be seen in a slide preparation of a whole crushed colony. In spite of this, Calothrix trichomes grew up in every culture; in fact their growth was always more vigorous than that of the colony-forming types. In three cases, Calothrix was the only tapered form to develop, and no hormogonia appeared to be produced by the Rivularia (or Gloeotrichia) trichomes.

These results suggested that the association of Rivulariaceae of more than one type in a single colony may not be uncommon. The association may be fairly obvious, as in the case of 'epiphytic' Calothrix trichomes, but the heterogeneity may be less apparent, however, as seemed to be the case in the Rivularia colonies from

TABLE 2.5

Isolation attempts with Colonial Rivulariaceae : Origins of Colonies and Forms Observed in Culture

<u>Site of Collection</u>	<u>Durham stream/reach code number</u>	<u>Type of Colony</u>	<u>Calothrix trichomes observed in Field Material</u>	<u>Calothrix developed in Culture</u>	<u>Colonial Forms developed in Culture</u>
Stream, Barras, Cumbria (NY848122)	0064/01	<u>Rivularia</u>	+	+	0
Stream, Helbeck, Cumbria (NY827200)	0063/01	<u>Rivularia</u>	+	+	+
Slapstone Sike, Upper Teesdale (NY814304)	0002/06	<u>Rivularia</u>	0	+	0
Stream, Sunbiggin, Cumbria (NY672077)	0032/02	<u>Rivularia</u>	+	+	+
Sigiriya Pond, Sri Lanka		<u>Gloeotrichia natans</u>	0	+	0

Croft Kettle. In several cases, Calothrix trichomes forming only a small minority of the original population became the dominant form in culture. This emphasizes the care that must be taken to ensure that the form eventually isolated is the same as that forming the bulk of the original population.

2.363 Cloning of cultures

The marked variation that occurred in samples of Rivulariaceae taken from batch cultures was mentioned in Section 2.22. It seemed that the establishment of clonal cultures might indicate whether this variation was simply due to the presence of trichomes at different stages of development, or whether genetic heterogeneity was an important factor. Of the cultures held, only two were unequivocally clonal, Gloeotrichia ghosei D277, and Calothrix sp. D251 (Table 2.1). Attempts were therefore made to clone some of the more useful experimental strains. The following procedure was adopted:

a) Algae from a young, vigorously growing culture were inoculated onto Chu 10-D agar (1%) and incubated under normal conditions. After 2-5 days, there was usually a 10-20 mm zone of hormogonia around the inoculum, with the trichomes at the outer boundary of the zone sufficiently well separated for individuals to be picked off.

b) A suitable area of hormogonia was located under a dissecting microscope, and single hormogonia were picked off and transferred to fresh plates of Chu 10-D agar (0.5%). A medium containing combined nitrogen was used to minimize physiological stresses on the hormogonia, and the low concentration of agar was used since this has been found to favour the survival of single filaments of blue-green algae (S.I. Heaney, personal communication). The lower concentration of agar was not used for the initial plates, since the trichomes were difficult to manipulate on soft agar.

c) Plates were incubated at the normal temperature for the strain, at a low light intensity (c. 300 lx), and examined at weekly intervals. Within 4-8 weeks, micro-colonies had normally developed from about 20% of the inoculated trichomes. These were picked out and transferred to further plates, and thereafter cultured in the normal way.

Three strains were cloned in this way, D184 (1 clone), D253

(2 clones) and D404 (3 clones). Although detailed physiological comparisons were not undertaken, there was no obvious difference between any of the clones and their uncloned parent strains, either in morphological form and variation, or in behaviour under different culture conditions.

2.4 Chemicals and gases

2.41 Chemicals

Apart from the items specified below, all chemicals were of Analar grade, and were obtained from the British Drug Houses Ltd (BDH), Poole, Dorset.

Chemical	Specification	Supplier
$\text{Ca}(\text{NO}_3)_2$	Laboratory Reagent	BDH
K_2HPO_4	Laboratory Reagent	BDH
$\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$	Technical Grade	BDH
HEPES	loss at 110°C	0.2%
	sulphated ash	0.1%
	iron (Fe)	0.0005%
	lead	0.001%
	sulphate	0.01%

cycloheximide

Micro-Bio Laboratories,
146 Pembridge Rd.,
London W 11

2.42 Gases

All gases were supplied by the British Oxygen Company Ltd, except for the pure ethylene standard (99.8% ethylene) that was used to calibrate the gas chromatograph (cf. Section 2.6). This was obtained from BDH Laboratory Gas Service.

2.5 Estimation of yield

2.51 Dry weight

Algal material was separated from the growth medium by centrifugation for 15 min at 5000 x g, and washed twice to remove salts by resuspending in distilled water and centrifuging as before. The washed algal pellet was transferred to a tared Vitreosil crucible, and dried for 48 h at 105°C. On removal from the oven, crucibles were immediately placed in a desiccator to prevent absorption of water as they cooled to ambient temperature.

2.52 Extraction and estimation of chlorophyll a

Chlorophyll was extracted with hot methanol; acetone extraction was attempted, but this failed to give complete extraction with either cultured or field material, even after several extractions using a variety of disruptive techniques (homogenization, sonication and grinding with sand). 90% methanol (Talling & Driver, 1961) was used for field materials (Section 3.3), but 95% methanol was used for cultured material (Chapter 7), since the method followed was that of Marker (1972) (see below). Extractions were performed in 5-10 ml of solvent. Algae and solvent were placed in 30 ml Universal bottles, lids were screwed on firmly, and the bottles incubated for 10 min in a waterbath at 70°C, with occasional shaking. Samples were filtered through Whatman GF/C glass fibre paper, and made up to a standard volume. A single extraction was generally sufficient for cultured material, but a trial second extraction was always performed; two extractions were required for field materials. The chlorophyll peaks were read as soon as possible after extraction (not more than 1 h); extracts were stored in a refrigerator until required. Optical densities were read on a Perkin-Elmer 402 ultraviolet-visible spectrophotometer (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire).

The concentration of chlorophyll in 90% methanol extracts of field populations (Section 3.3) was calculated from the equation of Talling and Driver (1961):

$$\text{chlorophyll } \underline{a} \text{ (mg l}^{-1}\text{)} = 13.9 \times \text{OD}_{665}.$$

In the experiment described in Chapter 7, chlorophyll was extracted from phosphate deficient cultures of Calothrix. It seemed possible that chlorophyll breakdown products, which would contribute to the measured chlorophyll peaks, might be present in this material; allowance was therefore made for the presence of phaeophytin in the extracts. Unfortunately the absorption spectra of chlorophyll and its degradation products are much less well characterized for methanol than for acetone solutions. Marker (1972) describes a method for the estimation of chlorophyll a in methanol, in the presence of its degradation products. It is based upon that of Lorenzen (1967) for acetone, and relies upon the difference in the specific absorption coefficients of chlorophyll a and phaeophytin a in the region of 665 nm. Acidification of a chlorophyll extract converts all the chlorophyll to phaeophytin, with a

consequent drop in the OD₆₆₅. Comparison of the OD₆₆₅ before and after acidification permits calculation of the amount of phaeophytin present in the initial extract. The situation is complicated in the case of methanolic extracts by the fact that the spectrum of phaeophytin in methanol is pH sensitive. The peak in the red region of the spectrum is shifted after acidification, from about 665 nm to about 650 nm, and is slightly reduced in height (Marker, 1972). Marker found that by treating his extracts with 'excess' magnesium carbonate, he was able to shift the peak back to its original position.

Re-neutralization of acidified chlorophyll extracts was attempted, as described by Marker (1972). The peak shifts were found to be as he describes, but the effects on the height of the phaeophytin peak at 665 nm were variable and inconsistent, and were to some extent affected by the amount of magnesium carbonate added. In view of these results, it was felt that it would be safer to use the OD₆₅₀ (shifted phaeophytin peak) rather than the OD₆₆₅ (re-neutralized phaeophytin peak) for estimations of phaeophytin, despite the slight difference in height between the two peaks.

After reading the OD₆₆₅, one drop of 1 N HCl was added to the chlorophyll extract in the optical cell, and carefully mixed in with a pasteur pipette. The OD of the resulting peak at 650 nm was then measured. The concentration of chlorophyll was calculated from an equation modified from that of Marker (1972). Marker's equation is as follows:

$$C_a = 3.0(A_b - A_a) \times 12.5 \times \frac{v}{l}$$

where C_a = μg chlorophyll a in sample
 A_b = OD₆₆₅ before acidification
 A_a = OD₆₆₅ after acidification and re-neutralization
 v = volume of extract (ml)
 l = cell path length (cm).

The factor of 3.0 is derived from an 'acid factor' of 1.5, obtained by Marker for his material. Marker defines the acid factor as:

OD at max. absorbance between 660 and 665 nm before acidification
OD at same wavelength after acidification and re-neutralization

The factor of 12.5 is the reciprocal of the specific absorption coefficient for chlorophyll a in methanol (taken as 76.07 by Marker), reduced by 4.5% to allow for accessory chlorophylls.

The following modifications were made to Marker's equations:

(i) As already explained, the acidified extracts were not reneutralized. Thus in the modified equation:

$$A_b = OD_{665} \text{ before acidification}$$

$$A_a = OD_{665} \text{ after acidification}$$

(ii) For each set of algal material extracted, a value was obtained for the acid factor, and a factor equivalent to Marker's factor of 3.0 derived from it (see Marker (1972) for the derivation). The acid factor used in this calculation was defined as:

$$\frac{\text{OD at max. absorbance between 650 and 665 nm before acidification}}{\text{OD at max. absorbance between 650 and 665 nm after acidification}}$$

(iii) Since with a few recently described exceptions (Lewin, 1976) blue-green algae contain only chlorophyll a, no correction was necessary for accessory chlorophylls. The unmodified reciprocal of the specific absorption coefficient of chlorophyll a, 13.1, therefore replaces the 12.5 in Marker's equation.

The full equations used for particular strains are given in Chapter 7.

2.53 Chlorophyll and dry weight estimation on a single sample

When values for both dry weight and chlorophyll were required from the same sample, the algal material had to be homogenized to obtain a uniform suspension from which accurate aliquots could be taken. The washed algal pellet, obtained as described in Section 2.51, was transferred to a 20 ml glass homogenizer goblet, and a small volume of distilled water added, to give a total volume of about 6 ml. Two minutes homogenization at medium speed, using an MSE homogenizer (Measuring and Scientific Equipment Ltd, Crawley, Sussex) was found to be adequate. The homogenate was washed into a 25 ml measuring cylinder and made up to a standard volume with distilled water. The homogenate was magnetically stirred to maintain a uniform suspension while aliquots were taken with a 10 ml pipette. Aliquots for dry weight estimation were transferred directly to tared crucibles; those for chlorophyll extraction were centrifuged to remove the water, and then treated as described in Section 2.52.

2.54 Semi-quantitative estimation of growth

In most of the experiments, no absolute measurement was made of the yield of the algae under the different treatments,

and a subjective semi-quantitative 0-5 scale was used to express the extent of growth. 5 was taken as the maximum final yield of the alga in the control treatment; thus the maximum yield of a set of flasks scored before the final yield was reached would score less than 5. The scale was approximately logarithmic, i.e. a culture scoring 3 was estimated to contain about twice as much alga as one scoring 2, for instance. The score '+' was used to indicate a very small, but perceptible amount of growth.

2.6 Acetylene reduction assay technique

Acetylene reduction was used as an assay for nitrogenase activity (Dilworth, 1966; Sch8llhorn & Burris, 1966; Stewart, Fitzgerald & Burris, 1967, 1968), in laboratory studies of field populations of Homoeothrix and Rivularia (Section 3.32).

Algae were incubated in 7 ml serum bottles, with a gas phase of 6 ml. Details of the gaseous atmospheres used are given in Section 3.32. The bottles were incubated in racks, in a culture tank at 20°C, with gentle shaking. The light intensity was 2000 lx. After a variable pre-incubation period under the experimental gas atmosphere, 1 ml of acetylene was injected into each bottle. An incubation period of 2 h or 4 h was used, after which 1 ml gas samples were taken and immediately assayed for ethylene on a Varian 1200 gas chromatograph. The 1.2 m long nickel steel column was packed with Porapak-T and maintained at 110°C; the detector temperature was 150°C. Nitrogen was used as carrier gas, at a flow rate of 25 ml min⁻¹. The chromatograph was calibrated with a pure ethylene standard (Section 2.42) on each day of use.

The following controls were employed:

(i) A distilled water blank, to give an estimate of ethylene present as contaminant in the acetylene.

(ii) Bottles to which no acetylene was added, as a test for possible leakage of hydrocarbons from the rubber serum liners (Postgate, 1972).

(iii) Algal samples incubated in the dark with acetylene. A similar rate of acetylene reduction in the light and in the dark would suggest that bacterial activity might be responsible. A large difference between light and dark rates of reduction (which was always observed) was taken as evidence that the activity was

of algal origin.

Results of acetylene reduction assays are expressed as nmol ethylene produced per mg chlorophyll a over a given period.

2.7 Statistics

Statistics were calculated by computer, using programs from the Statistical Package for the Social Sciences (SPSS V6) (Nie, Bent & Hull, 1970). The significance of differences between means was tested using Student's t-test, at the 99.9% level of probability.

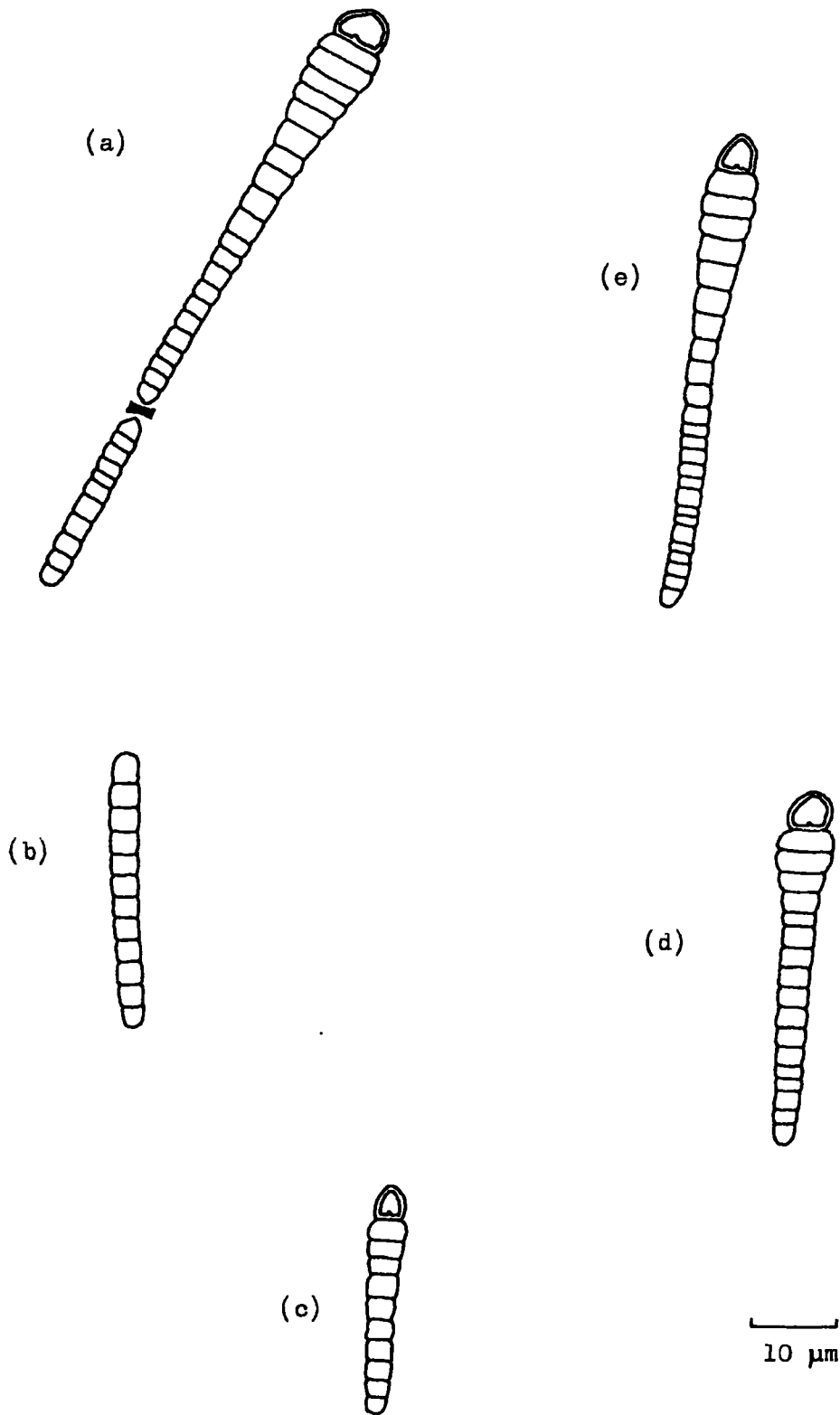


Fig. 2.1 Developmental stages of Calothrix scopulorum D256, grown in AD medium.

(a) Release of hormogonium.

(b) Parallel hormogonium.

(c)-(e) Progressive enlargement of basal region following differentiation of heterocyst.

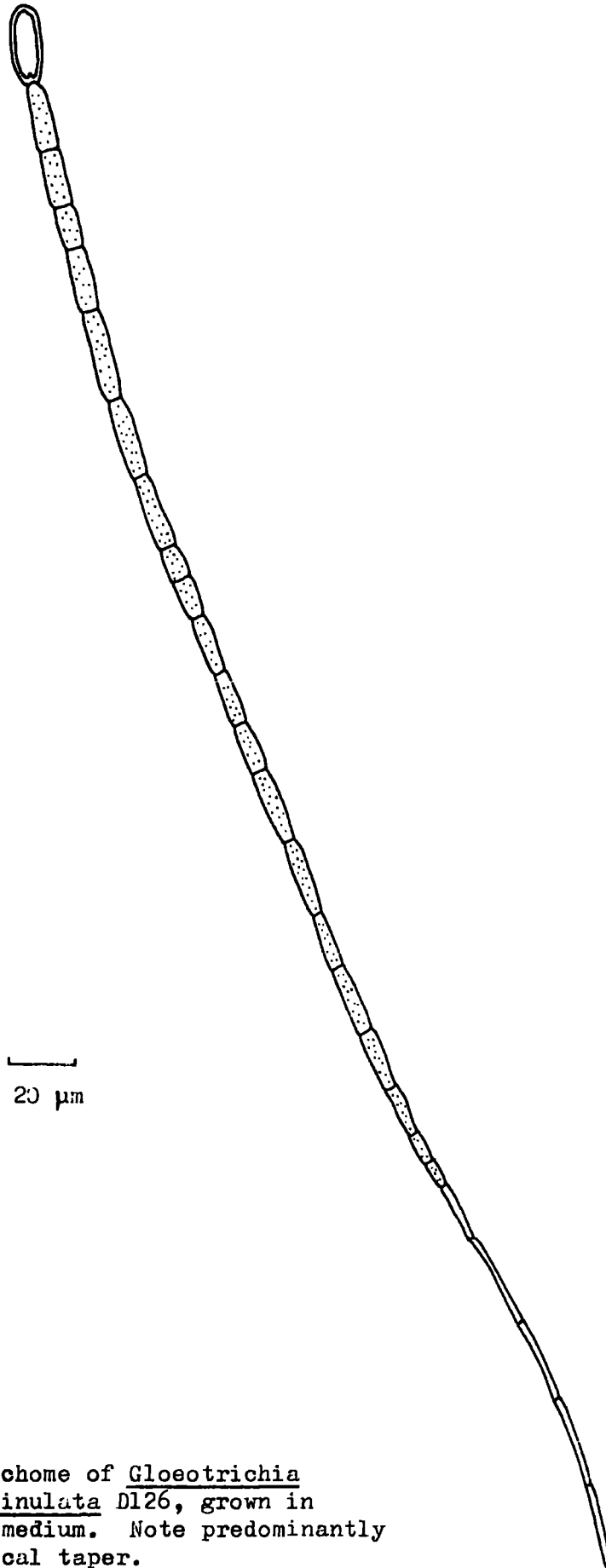


Fig. 2.2 Trichome of Gloeotrichia echinulata D126, grown in AD medium. Note predominantly apical taper.

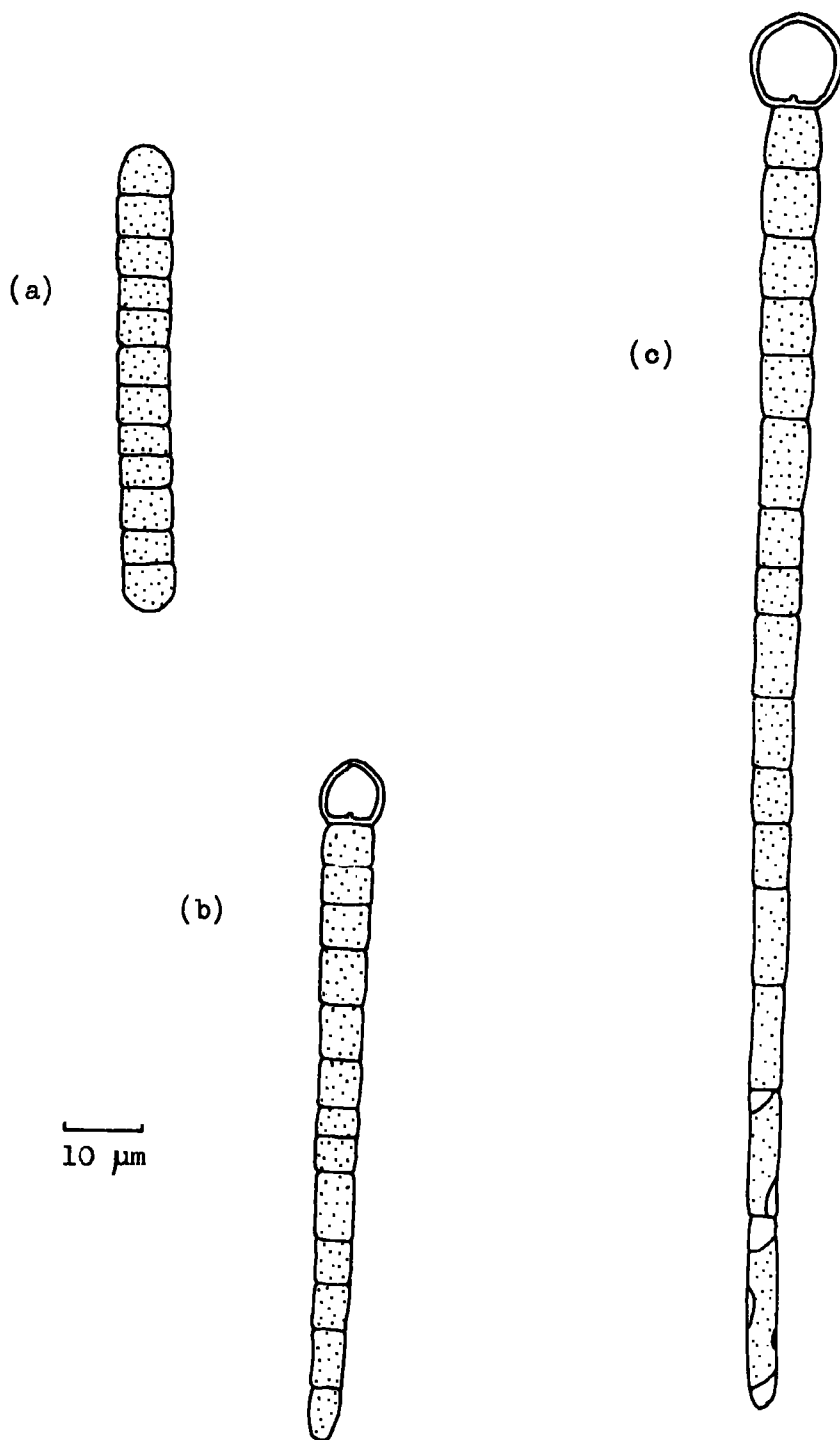


Fig. 2.3 Developmental stages of Gloeotrichia ghosei D277, grown in AD medium.

(a) Parallel hormogonium.

(b)-(c) Progressive narrowing in apical region.

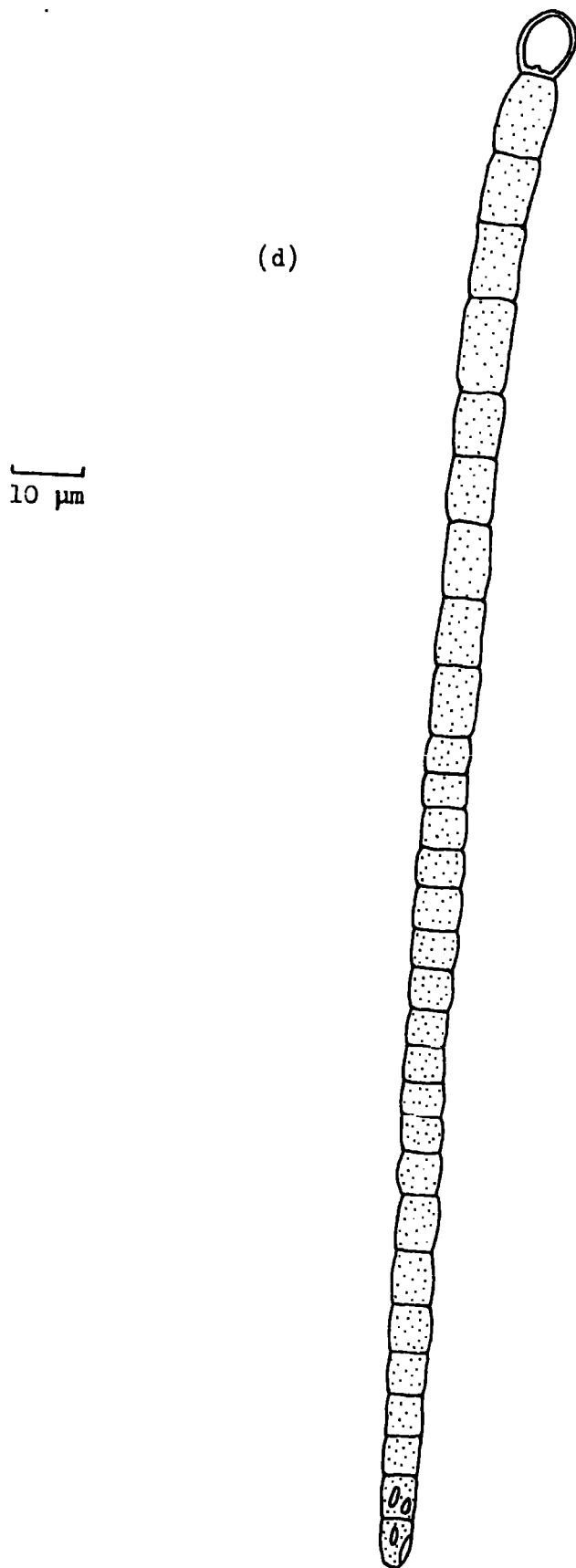


Fig. 2.3(d) Trichome without marked elongation and narrowing in apical region.

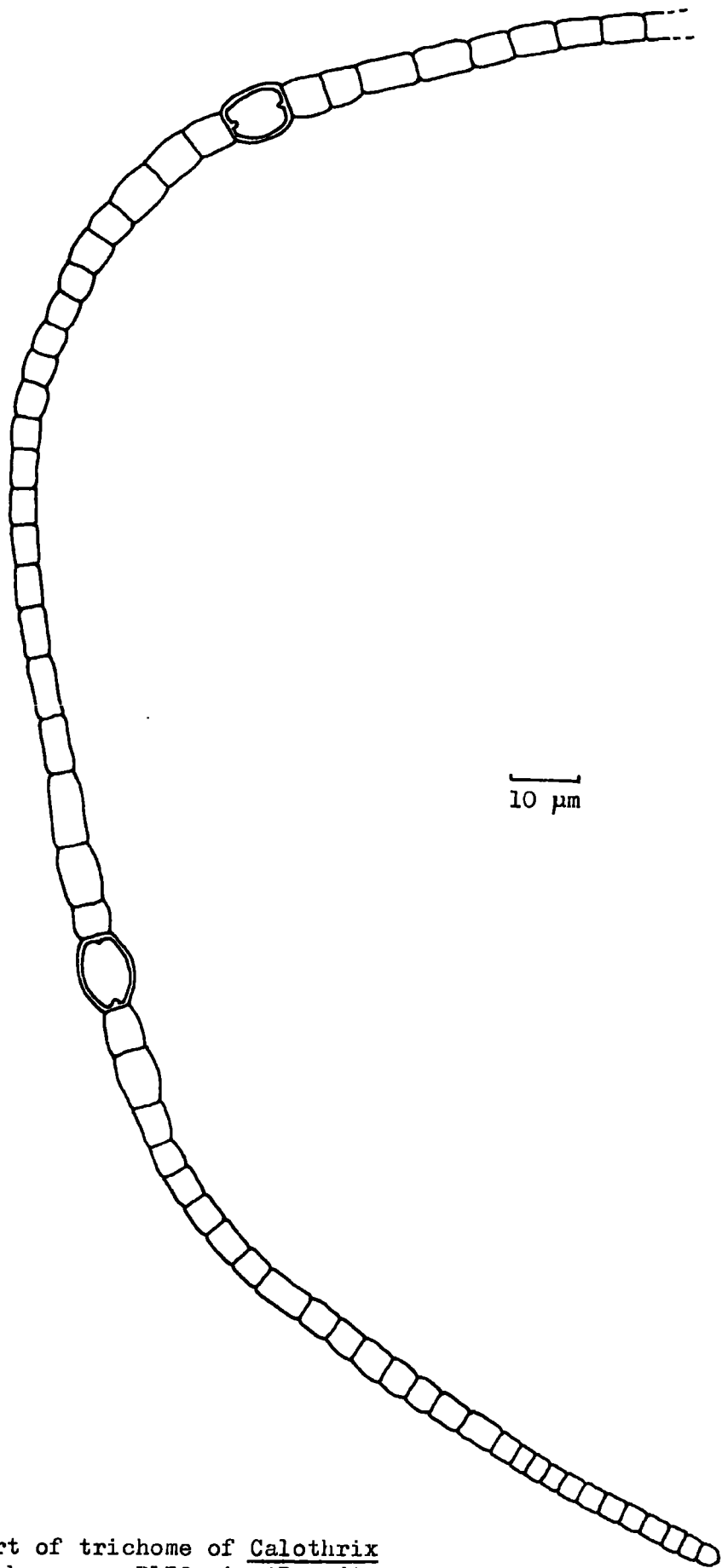


Fig. 2.4 Part of trichome of Calothrix membranacea D179, in AD medium.

Note intercalary heterocysts.

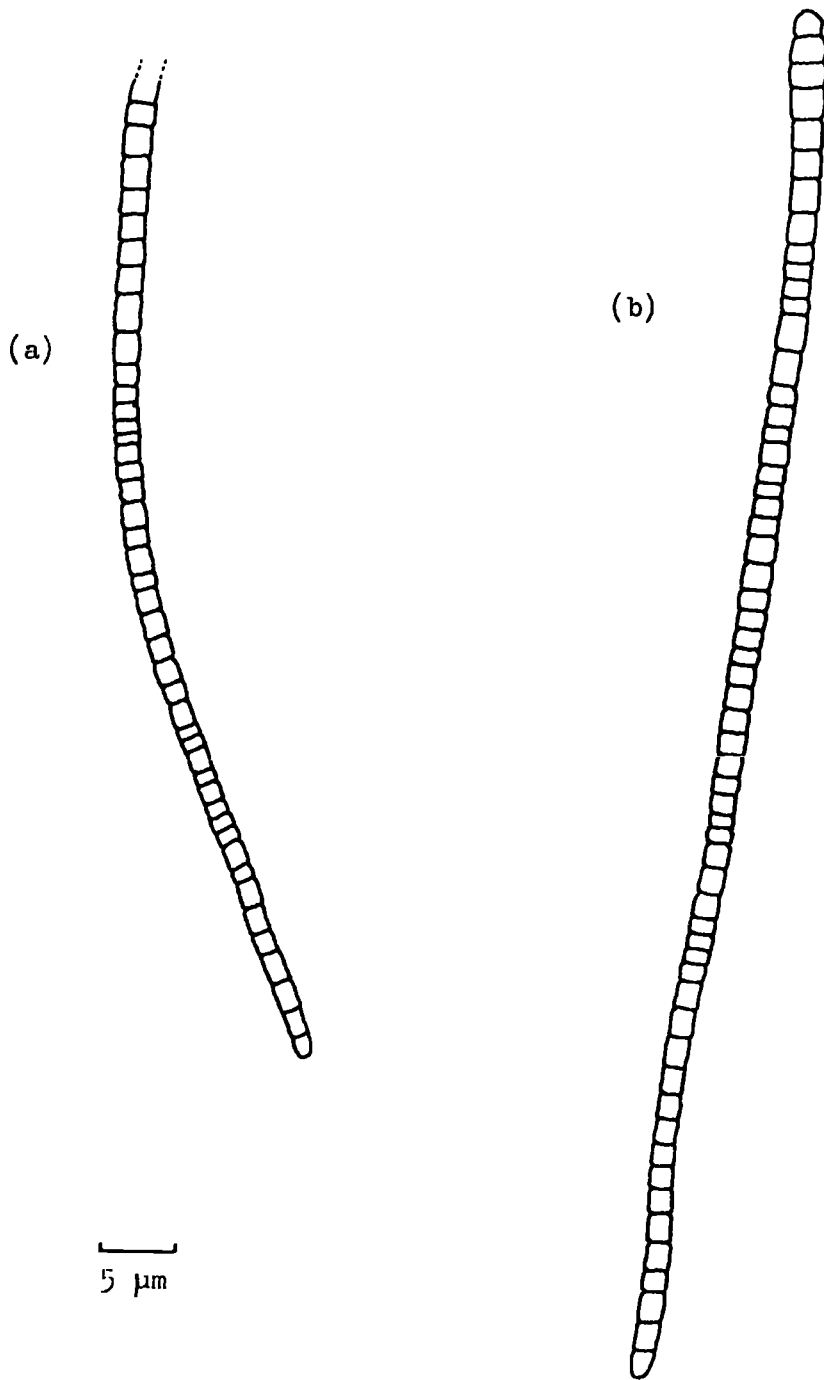


Fig. 2.5 Trichomes of Homoeothrix sp.

(a) Field material.

(b) Cultured material of Strain D402 (in Chu 10-D).

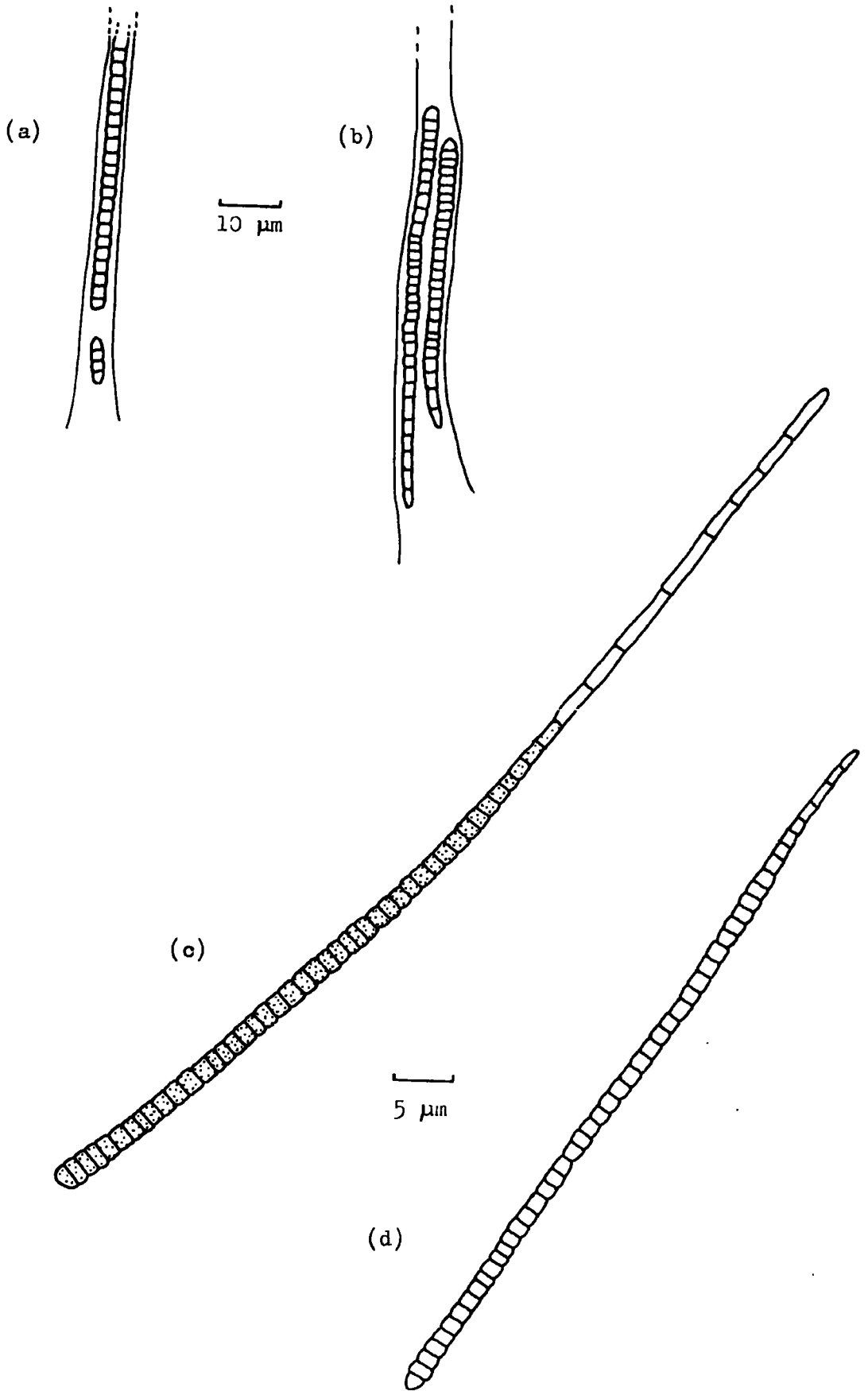


Fig. 2.6 Trichomes of Homoeothrix crustacea.

(a)-(c) Field material.

(d) Cultured material (in Chu 10-D). (see p. 64)

Fig. 2.7 Schematic illustration of different types of secondary heterocyst (see pp. 70-71).



(a) Single (primary) basal heterocyst.



(b) Secondary basal heterocyst.



(c) Intercalary heterocyst.



(d) Pseudo-intercalary heterocyst.

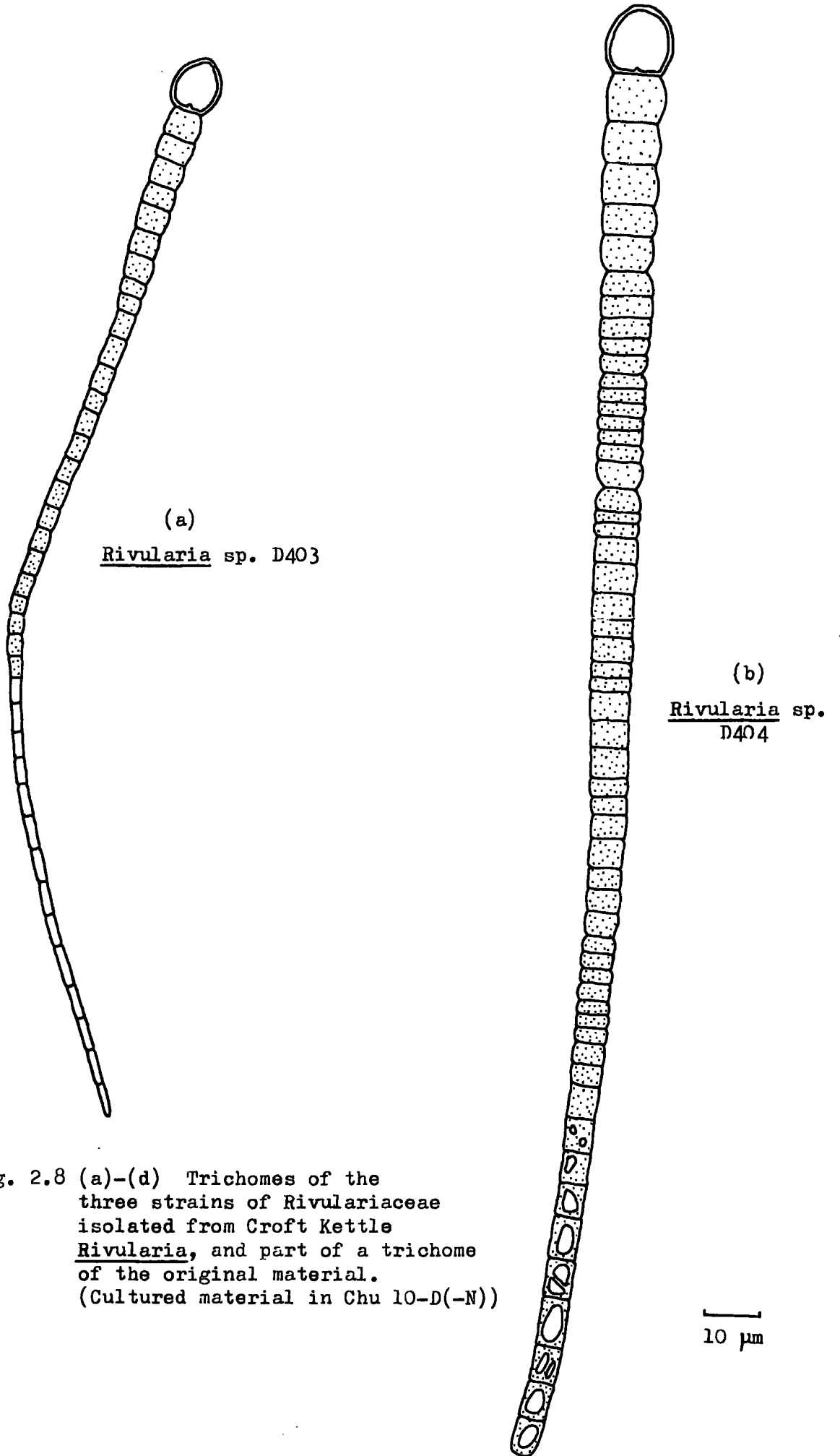
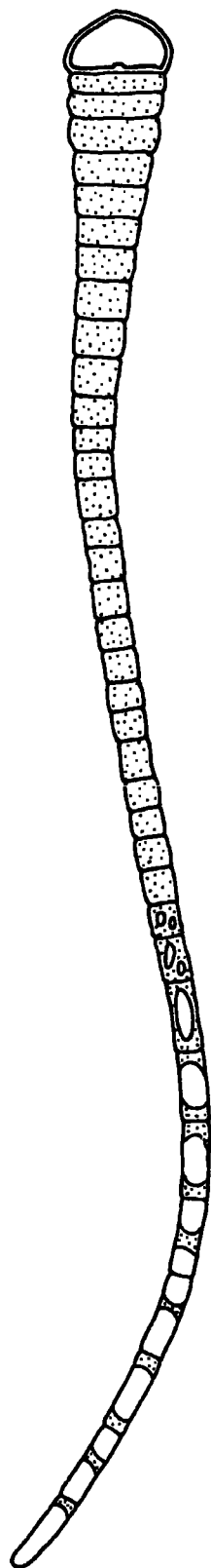
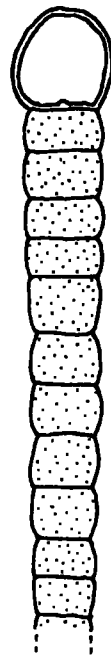


Fig. 2.8 (a)-(d) Trichomes of the three strains of Rivulariaceae isolated from Croft Kettle Rivularia, and part of a trichome of the original material. (Cultured material in Chu 10-D(-N))

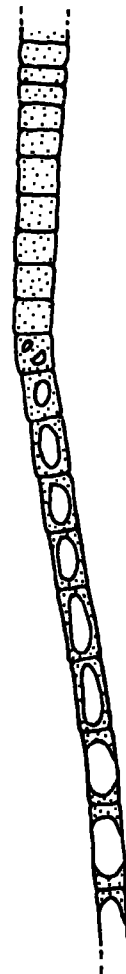


(c) Calothrix sp. D405



c. 100 cells

┌
10 μm



(d) Field material

3 INFLUENCE OF NITROGEN SUPPLY ON MORPHOLOGY OF RIVULARIACEAE

3.1 Influence of combined nitrogen on morphology of heterocystous strains

3.11 Introduction

As mentioned in Section 1.41, several authors have described marked morphological changes in tapered heterocystous blue-green algae grown in the presence of combined nitrogen. In some cases, in addition to the suppression of heterocyst development, a complete loss of trichome polarity has been reported (Pearson & Kingsbury, 1966; Wyatt, Martin & Jackson, 1973), leading to the suggestion that the typical tapered morphology and hair development of Rivulariaceae might be due to a decreasing gradient of fixed nitrogen from the basal heterocyst to the distal apical cells (Fay, Stewart, Walsby & Fogg, 1968; Stewart, 1972). In other instances, however, tapered trichomes of Rivulariaceae have been observed in the presence of high levels of combined nitrogen, even in the absence of heterocysts (Kirkby, 1975).

In an attempt to resolve these slightly conflicting results, all 34 heterocystous strains of Rivulariaceae were screened for their response to combined nitrogen. A preliminary account of this experiment, for 30 strains, was given in Whitton, Kirkby, Peat & Sinclair (1973). After the publication of this report, four more strains were tested, and the results for all 34 strains are described below. During this later work, observations were made on hair development in the presence of combined nitrogen that differ somewhat from the results in the preliminary report (see Section 3.125).

3.12 Influence of $\text{NO}_3\text{-N}$ on morphology of 34 heterocystous strains

3.121 Methods

All 34 heterocystous strains were used (Table 2.1). They were subcultured into AD medium + 10 mM NaNO_3 (AD+N), and into AD medium + 10 mM NaCl as control (AD-N). After 14 days, a second subculture, in duplicate, was made, to avoid any 'hangover' effect from the initial nitrate-free culture. All cultures were incubated in tanks, with shaking, at a light intensity of 2500 lx, and all were grown at 25°C except Strains D251 and D252 (32°C) (Section 2.351).

Nitrate, rather than ammonium, was used as nitrogen source

because at the time, no satisfactory buffering system had been developed (Section 2.33), and growth of algae in AD medium with ammonium salts caused a rapid fall in pH, to lethal levels as low as pH 4. With $\text{NO}_3\text{-N}$ there was a less marked increase in pH, to about pH 8.5-9. While this was clearly rather unsatisfactory, since the pH increase could alter the solubility of salts and carbon dioxide, there was at least no directly lethal effect. The reports reviewed in Section 1.41 indicated that $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ were both able to suppress tapering, and Kirkby (1975) found very similar effects of $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ upon two strains of Calothrix. This suggested that the use of $\text{NO}_3\text{-N}$ was valid, even though $\text{NH}_4\text{-N}$ is generally more effective in suppressing heterocyst development (Section 1.42). The level of NaNO_3 ($140 \text{ mg l}^{-1} \text{ NO}_3\text{-N}$) was taken to be almost certainly well in excess of the total requirement of the algae to achieve maximum yield. Assuming that nitrogen accounts for about 7% of the dry weight of a blue-green alga (Allen & Arnon, 1955), this level of $\text{NO}_3\text{-N}$ allows for the production of 2 g l^{-1} dry weight. Cultures were scored when yields were well below this (estimated $400\text{-}800 \text{ g l}^{-1}$).

The morphology of AD+N and AD-N cultures was scored after 10-15 days' growth of the second subculture, when the algae had grown sufficiently to express any morphological changes, but before any degenerative symptoms were apparent. AD+N and AD-N cultures of each strain were scored at the same time; any difference in growth stage resulting from the generally more rapid growth in AD+N was neglected. The following points were considered in scoring the morphology of the algae in AD+N:

- a) Was there a loss of heterocysts, complete or partial?
- b) Was the tapering of the trichomes lost, or reduced?
- c) Was the length of the trichomes affected? (Fay, Stewart, Walsby & Fogg (1968) reported a loss of length limitation in trichomes of Rivulariaceae grown with ammonia, in comparison with the situation in heterocystous trichomes.)
- d) Was there any effect on the localization of cell division? (Fay, Stewart, Walsby & Fogg (1968) described filaments of Rivulariaceae grown in the presence of ammonia as of 'uniform appearance throughout', implying that their characteristic meristematic pattern of growth (Section 1.31) had been lost.)

- e) Was there any effect on the development of hairs, as distinct from any effects on the vegetative region of the trichomes?

With the large number of strains used, it was not practicable to make detailed measurements of trichome dimensions. For each strain, therefore, an estimate was made of the range of dimensions characteristic of each of the types of trichomes present in AD+N and AD-N (Table 3.2). Any large changes in dimensions were recorded, but smaller effects possibly overlooked. In this Chapter, cell shapes are described in terms of cells' length:width ratio. Thus a trichome is described as having shorter cells than another if this ratio was lower, regardless of the absolute size of the cells.

3.122 Growth characteristics

In the majority of strains, growth was initially more rapid in AD+N than in AD-N. In 10 strains (D156, D184, D258, D265, D266, D267, D272, D273, D280, D403) there was no obvious difference in the rate of growth in the two media. After more than about two weeks in AD+N, most of the strains became unhealthy in appearance. The cultures turned yellow, and large amounts of extracellular material appeared to be released, causing the medium to froth. In AD-N these effects were not observed; the algae remained green, and growth continued for a longer period, to a higher (estimated) final yield. Some of these effects may have been due to unfavourable pH changes in the AD+N cultures (cf. Section 3.13).

AD+N and AD-N cultures differed in macroscopic appearance in all strains except D267. In strains which had a mat-like growth habit in AD medium (Section 2.122), this characteristic was much less apparent in AD+N. The algae tended to grow as a loose floc, distributed throughout the volume of the liquid. In the three colony-forming strains (D277, D403, D404) some degree of colonial structure was maintained, but again much of the growth was in the form of a filamentous floc, not organized into colonies. These changes in growth habit probably resulted from the differences in trichome morphology which are described below (Section 3.142).

3.123 Heterocyst frequency

In one strain, D267, there was no effect upon heterocyst frequency; in fact the morphology of this strain was completely unchanged in AD+N. In the other 33 strains, heterocysts were

almost entirely absent from AD+N cultures at the time of score, though a few heterocysts (frequency score = +/1) were usually observed.

3.124 Tapering

In this section, the presence or absence of hairs is ignored, and the tapering of the vegetative region only is discussed. Effects on hairs are described in Section 3.125.

(i) Relative frequency of tapered and untapered trichomes

As already mentioned, one strain, D267, was morphologically unchanged in AD+N, and this strain is ignored in the following account. The remaining 33 strains fell into two groups on the basis of their morphological response (Table 3.1). In 19 strains, tapering was effectively completely lost in AD+N; although a few tapered heterocystous trichomes were sometimes observed, the great majority of trichomes were parallel. This group included all three strains which had a high frequency of hairs in AD-N (D126, D277, D404). In the remaining 14 strains, similar parallel trichomes also developed, but in addition, many tapered trichomes without heterocysts were present. These tapered either towards one end, and very much resembled trichomes of Homoeothrix, or towards both ends, as in the genus Ammatoidea.

(ii) Morphology of trichomes in AD+N.

The approximate dimensions of each strain in AD+N and AD-N are shown in Table 3.2, and effects on cell shape are shown in Table 3.3. Strain D267 is not included in the following account, which is restricted to the 33 strains in which there was a morphological change in AD+N.

a) Parallel trichomes

As described above, in every strain grown in AD+N some trichomes were parallel. These trichomes differed in several ways from the parallel hormogonia present in AD-N (cf. Figs 3.1b and 3.2b). In each of the 31 strains for which it was possible to compare parallel trichomes in the two media (hormogonia were not observed in Strains D403 or D404) the maximum length reached by these trichomes was greater in AD+N than in AD-N, and often much greater, sometimes exceeding 1 mm (Table 3.2).

In six strains (D179, D182, D255, D259, D262, D274) the parallel trichomes in AD+N did not obviously differ in width from those in AD-N; in the other 25 strains, the maximum diameter reached was

TABLE 3.1

Morphological Responses of 34 Heterocystous Strains grown in AD + 10 mM

NaNO₃

<u>Strain</u>		<u>All trichomes parallel</u>	<u>Tapered and parallel trichomes present</u>	<u>No change in Morphology</u>
<u>Calothrix</u> sp.	D184		+	
<u>Calothrix</u> sp.	D251		+	
<u>Calothrix</u> sp.	D252		+	
<u>Calothrix</u> sp.	D254	+		
<u>Calothrix</u> sp.	D255	+		
<u>Calothrix</u> sp.	D258	+		
<u>Calothrix</u> sp.	D264	+		
<u>Calothrix</u> sp.	D267			+
<u>Calothrix</u> sp.	D283		+	
<u>C. anomala</u>	D182	+		
<u>C. brevissima</u>	D156	+		
<u>C. brevissima</u>	D275	+		
<u>C. desertica</u>	D270		+	
<u>C. elenkinii</u>	D273		+	
<u>C. fusca</u>	D269		+	
<u>C. gracilis</u>	D274		+	
<u>C. javanica</u>	D257	+		
<u>C. marchica</u>	D202		+	
<u>C. membranacea</u>	D179	+		
<u>C. membranacea</u>	D259	+		
<u>C. membranacea</u>	D260	+		
<u>C. membranacea</u>	D261	+		
<u>C. membranacea</u>	D262	+		
<u>C. membranacea</u>	D263	+		
<u>C. parietina</u>	D272	+		
<u>C. prolifica</u>	D265	+		
<u>C. scopulorum</u>	D256		+	
<u>C. thermalis</u>	D266		+	
<u>C. viguieri</u>	D253		+	
<u>Dichothrix</u> sp.	D280		+	
<u>Gloetrichia echinulata</u>	D126	+		
<u>G. ghosei</u>	D277	+		
<u>Rivularia</u> sp.	D403		+	
<u>Rivularia</u> sp.	D404	+		

TABLE 3.2

Approximate dimensions (μm) of trichomes of 34 heterocystous strains grown in AD-N and AD+H

Strain	Maximum diameter		Tapered trichomes		Length		Parallel trichomes		Length (hormogonia)
	AD-N	AD+H	AD-N	AD+H	AD-N	AD+H	Diameter (hormogonia)	AD-N	
Calothrix sp. D184	6.0-12.0	6.0-12.0	3.5-4.5	4.0-6.0	60-500	100-600	3.5-4.5	4.0-8.0	45-60
Calothrix sp. D251	6.0-8.0	6.0-8.0	3.5-4.0	3.5-4.5	300-600	100-450	3.5-4.0	4.5-6.0	30-45
Calothrix sp. D252	4.0-5.0	5.0-6.0	2.0-3.0	3.0-4.0	50-600	80-500	2.0-3.0	3.0-4.0	30-60
Calothrix sp. D254	5.5-6.5	NA	4.5-5.0	NA	90-500	NA	4.5-5.0	4.5-6.0	100-160
Calothrix sp. D258	6.0-7.0	NA	5.0-5.5	NA	90-1000	NA	5.0-5.5	6.0-6.5	40-200
Calothrix sp. D264	5.0-6.0	NA	4.0-4.5	NA	80-500	NA	4.0-4.5	6.0-8.0	20-150
Calothrix sp. D267	6.0-7.0	NA	4.5-5.0	NA	90-500	NA	4.5-5.0	7.0-8.0	100-160
Calothrix sp. D283	5.0-6.0	5.0-6.0	2.5-3.0	2.5-3.0	50-200	50-200	2.5-3.0	2.5-3.0	20-40
Calothrix sp. D287	5.0-6.0	5.0-6.0	2.5-3.0	3.0-4.0	40-300	40-200	2.5-3.0	3.0-5.0	30-45
C. anomala D182	7.0-9.0	NA	4.0-5.0	NA	90-500	NA	4.0-5.0	4.0-5.0	100-350
C. brevissima D156	6.5-8.0	NA	4.5-5.0	NA	100-450	NA	4.5-5.0	6.0-6.5	50-500
C. brevisseri D275	6.5-7.0	NA	4.5-5.0	NA	100-500	NA	4.5-5.0	5.0-7.0	100-500
C. desertica D270	8.0-12.0	8.0-12.0	4.0-4.5	4.0-6.0	150-800	100-300	4.0-4.5	4.0-10.0	30-50
C. elenkinii D273	5.5-6.0	5.5-6.0	4.0-4.5	4.0-4.5	100-250	100-300	4.0-4.5	4.5-6.0	30-60
C. fusca D269	9.0-12.0	9.0-12.0	4.5-5.0	4.5-7.0	150-600	200-400	4.5-5.0	6.0-12.0	30-60
C. gracilis D274	6.0-6.5	6.0-6.5	4.5-5.0	4.5-5.0	100-500	50-200	4.5-5.0	4.5-5.0	30-90
C. javanica D257	3.0-4.0	NA	2.5-3.0	NA	80-400	NA	2.5-3.0	4.0-5.0	40-100
C. marchica D202	6.5-8.0	8.0-11.0	4.0-4.5	4.0-5.0	100-300	90-200	4.0-4.5	6.0-7.0	40-90
C. membranacea D179	4.5-5.5	NA	4.0-4.5	NA	100-800	NA	4.0-4.5	4.0-4.5	60-150
C. membranacea D259	5.0-6.0	NA	4.5-5.0	NA	100-450	NA	4.5-5.0	4.5-5.0	40-300
C. membranacea D260	5.5-6.0	NA	4.5-5.0	NA	100-1200	NA	4.5-5.0	4.5-6.0	40-200
C. membranacea D261	5.0-6.0	NA	4.0-4.5	NA	100-1000	NA	4.0-4.5	4.5-6.0	60-300
C. membranacea D262	5.0-6.0	NA	4.5-5.0	NA	100-600	NA	4.5-5.0	4.5-5.0	50-500
C. membranacea D263	4.5-5.0	NA	3.0-4.0	NA	100-400	NA	3.0-4.0	4.0-4.5	30-400
C. parictina D272	6.0-7.0	NA	3.0-3.5	NA	100-400	NA	3.0-3.5	3.0-5.0	30-100
C. prullifera D265	5.0-6.0	NA	4.5-5.0	NA	100-800	NA	4.5-5.0	4.5-6.0	100-200
C. scppulorum D256	6.0-10.0	6.5-8.0	3.0-3.5	3.5-4.5	45-150	60-300	3.0-3.5	5.0-6.5	30-45
C. thermalis D266	8.0-12.0	8.0-12.0	4.0-5.0	4.0-5.0	100-600	100-400	4.0-5.0	5.0-6.0	20-50
C. viguieri D253	8.0-12.0	8.0-15.0	4.0-4.5	4.0-6.0	100-800	200-600	4.0-4.5	4.0-10.0	50-60
Diclothrix sp. D280	8.0-12.0	8.0-12.0	3.0-4.0	5.5-6.0	150-800	200-600	3.0-4.0	4.0-6.0	50-60
Gloeotrichia echinulata D126	4.0-7.0	NA	3.0-4.0	NA	150-1500	NA	3.0-6.0	5.0-8.0	100-200
G. ghosei D277	7.0-8.0	NA	4.0-5.0	NA	100-1000	NA	4.0-5.0	5.0-9.0	100-800
Rivularia sp. D403	5.0-6.5	4.5-5.0	2.0-2.5	2.5-3.0	40-100	45-60	*	4.5-5.0	*
Rivularia sp. D404	9.0-12.0	NA	6.0-8.0	NA	100-2000	NA	*	10.0-12.0	*

NA = character not applicable * = no hormogonia observed
 'Tapered trichomes' in AD+H includes 'Amatoidea' - and 'Homoeothrix' - types
 The presence of hairs is ignored; dimensions are of vegetative region only

TABLE 3.3

Effects on cell shape in 34 heterocystous strains grown in AD+10 mM NaNO₃

NA = not applicable ; * = no parallel trichomes seen in AD-N

		Tapered trichomes		Parallel trichomes	
		Cell shapes similar in AD-N and AD+N	Many trichomes in AD+N with cells relatively shorter	Cells shapes similar in AD-N and AD+N	Many trichomes in AD+N with cells relatively shorter
<u>Calothrix</u> sp.	D184		+		
<u>Calothrix</u> sp.	L251		+		+
<u>Calothrix</u> sp.	D252		+	+	
<u>Calothrix</u> sp.	D254	NA	NA	+	+
<u>Calothrix</u> sp.	D255	NA	NA		
<u>Calothrix</u> sp.	D258	NA	NA	+	+
<u>Calothrix</u> sp.	D264	NA	NA	+	
<u>Calothrix</u> sp.	D267	+		+	
<u>Calothrix</u> sp.	D283		+		
<u>C. anomala</u>	D182	NA	NA	+	+
<u>C. brevissima</u>	D156	NA	NA	+	
<u>C. brevissima</u>	D275	NA	NA	+	
<u>C. desertica</u>	D270		+		
<u>C. elenkinii</u>	D273	+			+
<u>C. fusca</u>	D263		+		+
<u>C. gracilis</u>	D274	+			+
<u>C. javanica</u>	D257	NA	NA	+	+
<u>C. marchica</u>	D202	+			
<u>C. membranacea</u>	D179	NA	NA	+	+
<u>C. membranacea</u>	D259	NA	NA	+	
<u>C. membranacea</u>	D260	NA	NA	+	
<u>C. membranacea</u>	D261	NA	NA		+
<u>C. membranacea</u>	D262	NA	NA		+
<u>C. membranacea</u>	D263	NA	NA	+	
<u>C. parietina</u>	D272	NA	NA	+	
<u>C. prolifica</u>	D265	NA	NA	+	
<u>C. scopulorum</u>	D256	+			
<u>C. thermalis</u>	D266		+		+
<u>C. viguieri</u>	D253		+		+
<u>Dichothrix</u> sp.	D280	+			+
<u>Gloeotrichia echinulata</u>	D126	NA	NA		+
<u>G. ghosoi</u>	D277	NA	NA		
<u>Rivularia</u> sp.	D403	+		*	*
<u>Rivularia</u> sp.	D404	NA	NA	*	*

greater in AD+N than in AD-N (Table 3.2). As explained in Section 2.121, the hormogonia produced in AD-N typically had a diameter similar to that of the subapical region of the tapered trichomes. By contrast, in AD+N 27 of the 33 strains developed some parallel trichomes that were similar in width to the basal region of tapered heterocystous trichomes; only six strains (D182, D255, D260, D272, D274, D280) showed no such overlap in diameter range.

In 15 strains, the shape of the cells of the parallel trichomes was much the same in AD+N and AD-N (usually with a length:width ratio of about 0.5:1 to 1:1) (Table 3.3). In 16 strains however many parallel trichomes had cells which were relatively shorter than those of hormogonia in AD-N: length:width ratios of about 0.2:1 were not uncommon (Fig. 3.2b). Such disc-shaped cells seemed to occur most frequently in the wider trichomes, and were more common among strains which had cells of a similar shape in the basal region of heterocystous tapered trichomes. In all the parallel trichomes, the cell shape was more or less uniform along the whole trichome, with no evidence of any localization of cell division. Figs 3.1b and 3.2b illustrate the marked differences often observed between parallel trichomes in AD+N and AD-N, for Strain D253.

In every strain, the parallel trichomes in AD+N differed from those in AD-N in that they developed thick, distinct sheaths. Hormogonia in AD-N had either no sheath, or a very thin sheath, formed as they began to differentiate into tapered heterocystous trichomes. Correlated with the presence of thick sheaths was the occurrence of false branches of the type seen in Plectonema.

The parallel trichomes in AD+N were thus quite distinct from the hormogonia in AD-N, which suggested that they were not simply persistent juvenile forms.

b) Tapered trichomes

Tapered trichomes without heterocysts were present in 14 of the strains when grown in AD+N (Table 3.1). As mentioned above, these were of two types, tapering either from base to apex ('Homoeothrix' type: Fig. 3.2a) or towards both ends ('Ammatoidea' type: Fig 2.3d). The diameters of the regions of maximum and minimum width, and the distance between these regions (= 'trichome length' in the following account) were very similar in the two types, and the following comments apply to both types. It seemed possible that the 'Homoeothrix'-like trichomes could have derived

from heterocystous trichomes, by shedding of the basal heterocyst, though no evidence was seen to suggest this. It seemed unlikely, however, that the trichomes which tapered towards both ends had originated from heterocystous tapered trichomes, and the presence of such forms was taken as evidence for the de novo development of tapering.

Besides their lack of heterocysts, the tapered trichomes in AD+N differed from those in AD-N in a number of ways. In 11 of the 14 strains, the apices of the tapered trichomes reached greater widths in AD+N than in AD-N. In the other 3 strains (D266, D273, D274) there was no obvious difference in minimum trichome diameter (Table 3.2). In nine strains (D184, D251, D266, D269, D270, D273, D274, D280, D283) there was no obvious difference in the maximum diameter of tapered trichomes in the two media; in three strains (D202, D252, D253) the basal diameter reached greater values in AD+N, and in two strains (D256, D403) the maximum diameter reached was greater in AD-N. Eleven of the strains produced shorter tapered trichomes in AD+N than in AD-N; in the other three strains (D184, D256, D273) the tapered trichomes were longer in AD+N than in AD-N (Table 3.2).

If the degree of tapering of the trichomes is considered crudely, in terms of the width ranges recorded for the maxima and minima of diameter in the two media (ignoring any differences in length), then eight of the 14 strains (D184, D251, D256, D269, D270, D280, D283, D403) produced less tapered trichomes in AD+N. In six of these, the decrease in tapering was due to an increase in apical diameter only; in two (D256, D403) there was both an increase in apical diameter and a decrease in basal diameter. In three strains (D266, D273, D274) there was no difference in the degree of tapering in the two media. The remaining three strains (D202, D252, D253) showed an increase in the width range of both maximum and minimum diameters, and tapering considered in terms of the width range for the whole population was unchanged. None of the strains produced trichomes in AD+N that were more tapered than those in AD-N. Figures 3.1a and 3.2a illustrate the less tapered appearance, and shorter length typical of the tapered trichomes present in AD+N cultures of Strain D253.

As observed with the parallel trichomes, the tapered trichomes in AD+N tended to have relatively shorter cells than those in AD-N. All eight strains which developed tapered trichomes with shorter

cells in AD+N were ones which also produced parallel trichomes with shorter cells in this medium (Table 3.3). Sometimes the cells in the tapered trichomes in AD+N were very short along their whole length, but more often the cells were relatively shorter (disc-like) in the wider part of the trichome (Fig. 3.2d). However, as mentioned in Section 2.121, the presence of short cells does not necessarily imply meristematic activity, and in the 'Homoeothrix' and 'Ammatoidea' types of trichome there was no obvious localization of cell division, assessed in terms of the frequency of newly formed cross walls.

Sheath development in the tapered filaments was more extensive in AD+N than in AD-N, and as with the parallel trichomes, Plectonema-like false branches were quite common (Fig. 3.2c).

3.125 Hair development

In three of the strains (D126, D277, D404) most of the trichomes (frequency score = 4) had well developed hairs in AD-N. In a further two strains (D251, D403) a few trichomes (frequency score = +/1) in AD-N had short hairs. In all five strains, hairs also developed in AD+N, though there were differences between the hairs in the two media. The presence of hairs at both ends of at least some of the trichomes was taken as a necessary criterion of hair development in AD+N, since trichomes with hairs at only one end might have derived from heterocystous tapered trichomes.

Strains D251 and D403 both grew predominantly as 'Ammatoidea'- and 'Homoeothrix'-like trichomes in AD+N (Table 3.1). In both strains, short hairs were present at much the same frequency in AD+N as in AD-N, and their appearance and length were also very similar. The hairs developed at both ends of the 'Ammatoidea'-like trichomes, and at the apices of the 'Homoeothrix'-like trichomes, but hairs were never seen on any of the parallel trichomes. Strains D126, D277 and D404 all grew almost entirely as parallel trichomes in AD+N (Table 3.1). Most of these trichomes were uniform in appearance throughout their whole length, but a few (frequency score = 1) ended in short hairs. The hairs in AD+N never had more than two or three fully vacuolated cells, whereas in AD-N few of the hairs had less than 10 cells, and they formed a much higher proportion of the total trichome length. The transition from normal vegetative cells to hair cells in AD+N was

often very abrupt, and this, together with the shortness of the hairs, and the great length of the trichomes (Table 3.2) meant that the overall parallel appearance of the trichomes was scarcely affected. Figs 3.3a and 3.3b illustrate the difference between hairs in Strain D404 in AD+N and AD-N. Parallel trichomes with hairs at both ends were observed, but these appeared to fragment into shorter pieces, yielding two trichomes with a hair at one end, and several lengths without hairs. This may have been partly responsible for the observed decrease in the frequency of trichomes with hairs, but it was not possible to say whether all the trichomes without hairs were the product of such trichome breakage.

When AD+N cultures of any of the strains were examined at a late stage in growth, hairs were not observed. The loss of hairs seemed to be associated with the overall degeneration of the algae at this stage (Section 3.122). Failure to observe the algae sufficiently early had previously led to the belief that hairs were not developed in AD+N (Whitton, Kirkby, Peat & Sinclair, 1973). All five strains still produced hairs after a third subculture into AD+N, which strongly suggested that hair development was occurring de novo.

3.13 Influence of NO_3^- -N and NH_4^- -N on morphology of 10 heterocystous strains

After the experiment described in Section 3.12 had been performed, a HEPES buffer system was developed (Section 2.33). It seemed worthwhile to repeat the experiment for selected strains, using NaNO_3 in buffered AD, to confirm that the responses seen had not been due to pH effects. It was also of interest, now that an adequate buffer was available, to see whether NH_4^- -N would elicit the same response as NO_3^- -N.

Strains D126, D156, D179, D182, D184, D202, D256, D259, D267 and D277 were used; the group included strains giving each of the chief responses seen in AD+N (Section 3.12). AD medium was made up with 10 mM HEPES (=HAD), with the addition of 5 mM NaCl, NaNO_3 or NH_4Cl . The pH was adjusted to 7.4 after autoclaving. The level of combined nitrogen was reduced from that used in the earlier experiment (10 mM) since even in the buffered medium the pH of AD+ NH_4Cl tended to fall. The incubation conditions were the same as those used previously (Section 3.121); the algae were

scored after 14 days in the second subculture, and pH values measured at the time of scoring. The highest pH value recorded for cultures +NaNO₃ was 8.0, and the lowest pH recorded for cultures +NH₄Cl was 6.7.

Growth in either medium with combined nitrogen was more successful than it had been in the unbuffered AD+NaNO₃: yellowing of older cultures +NaNO₃ was not observed, and cultures in HAD+NH₄Cl did not turn yellow until about three weeks after inoculation. Production of extracellular material was also less marked than it had been in AD+NaNO₃.

Microscopically, the response of each of the strains in HAD+NH₄Cl was the same as it had been in AD+NaNO₃, except that sheath development was less marked. The morphology of Strain D267 again remained unchanged. There was no apparent difference in morphology between algae from HAD+NaNO₃ and those from HAD+NH₄Cl, except that the latter usually had a higher level of cyanophycin granulation, and tended to be bluer green in colour. Germination of a few of the residual heterocysts of Strain D277 was observed in HAD+NH₄Cl, but not in the other media.

The results of this experiment suggested that the lack of buffering in AD+NaNO₃ had not seriously affected the responses described in Section 3.12, though the extensive sheath development may have been partly due to pH effects. The similarity between results obtained with NaNO₃ and NH₄Cl suggested that differences between these results and those in published reports of experiments using NH₄-N (Fay, Stewart, Walsby & Fogg, 1968) were unlikely to be due entirely to the different source of combined nitrogen used.

3.14 Summary of results

1. Heterocysts were entirely or almost entirely absent in AD with combined nitrogen in all except one of the strains. The lack of heterocyst suppression by NO₃-N or NH₄-N in Strain D267 was not investigated further.

2. Two types of response to NO₃-N were observed. In 19 strains, including the three with many long hairs in AD-N, all the trichomes became parallel in AD+N; in 14 strains, trichomes which tapered at one or both ends were also present.

The tapered trichomes in AD+N were generally wider than those in AD-N, especially in the apical region. In the majority of

strains, the trichomes had a less tapered appearance in AD+N; the degree of tapering was sometimes the same in the two media, but it was never greater in AD+N than in AD-N.

The parallel trichomes developed in AD+N were also often wider than the parallel hormogonia in AD-N; in fact one rather striking difference between parallel trichomes in the two media was that those in AD+N resembled the basal, rather than the apical region of tapered heterocystous trichomes in width.

3. The parallel trichomes that developed in AD+N were generally longer than either parallel or tapered trichomes in AD-N, and were often very long (>1 mm). Tapered trichomes in AD+N however, were either similar in length to those in AD-N, or rather shorter.

4. In many strains the cells in both tapered and parallel trichomes were relatively shorter in AD+N than in AD-N, though it was difficult to say whether this resulted from more rapid cell division. The tendency to develop very short-celled trichomes in AD+N seemed to be most marked among strains in which the cells in the basal region of heterocystous tapered trichomes had similar discoid cells.

In the majority of strains the most active cell division in heterocystous trichomes took place in the subapical region. In AD+N, no clearcut evidence was obtained for such localization of cell division in either parallel or tapered trichomes.

5. All five strains which produced hairs in AD-N also did so in AD+N, though the hairs seemed to be lost as the cultures aged, probably as a reflection of the overall degeneration of the cultures. In the two strains with only few hairs in AD-N, the frequency and appearance of the hairs, which developed at the ends of the tapered trichomes, were very similar in AD+N. In the three strains which developed many hairs in AD-N, hair frequency and length were considerably reduced in AD+N, though part of the apparent decrease in hair frequency may have been due to trichome fragmentation. The hairs in these three strains occurred abruptly at the ends of long trichomes which were otherwise parallel and of uniform appearance.

6. The results summarized above are those obtained with 34 strains grown with $\text{NO}_3\text{-N}$. Ten of these strains were also grown with $\text{NH}_4\text{-N}$; the morphological responses observed were very similar to those of the cultures grown with $\text{NO}_3\text{-N}$.

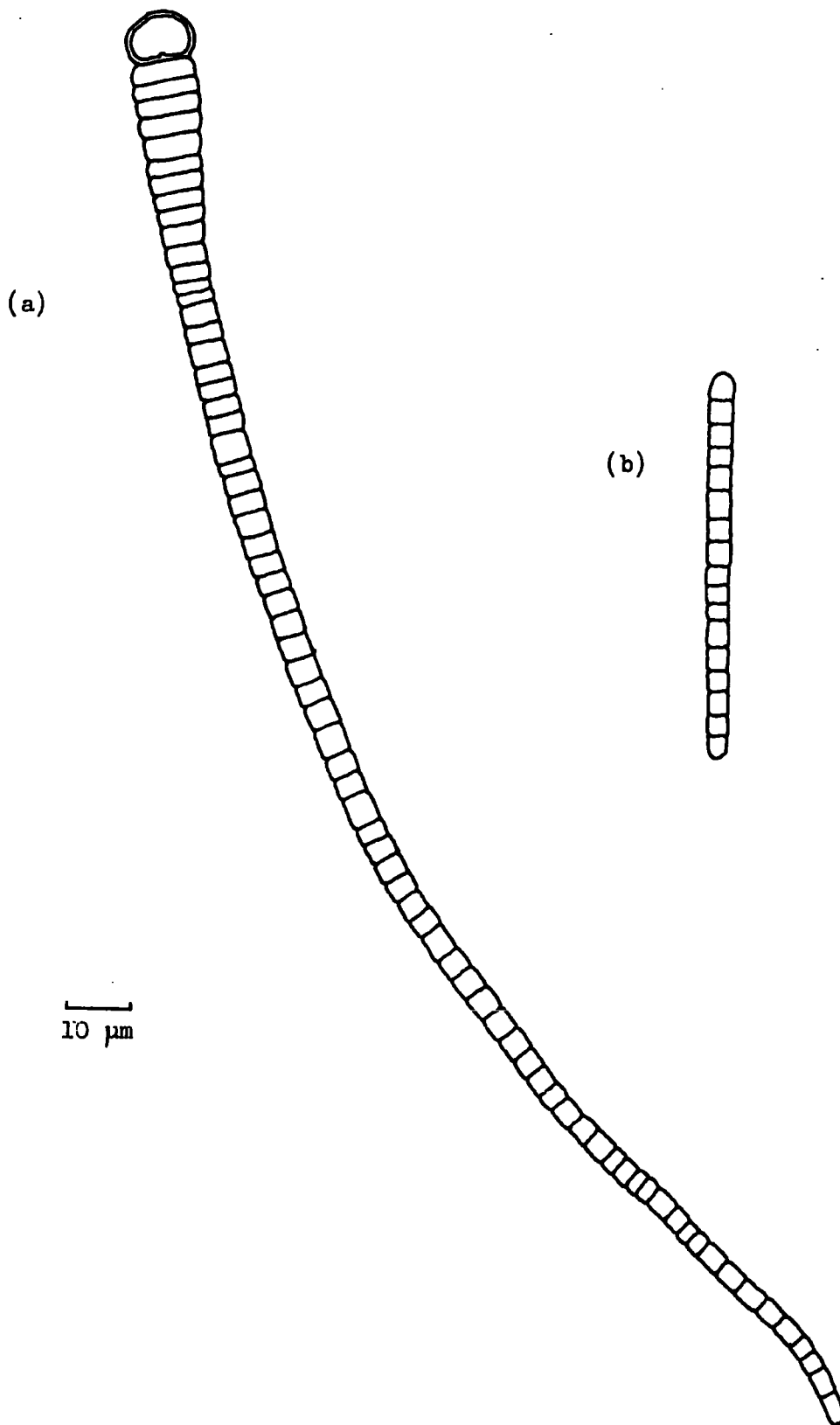


Fig. 3.1 Tapered and parallel trichomes of Calothrix viguieri D253 in AD-N.

(a) Tapered heterocystous trichome.

(b) Parallel hormogonium.

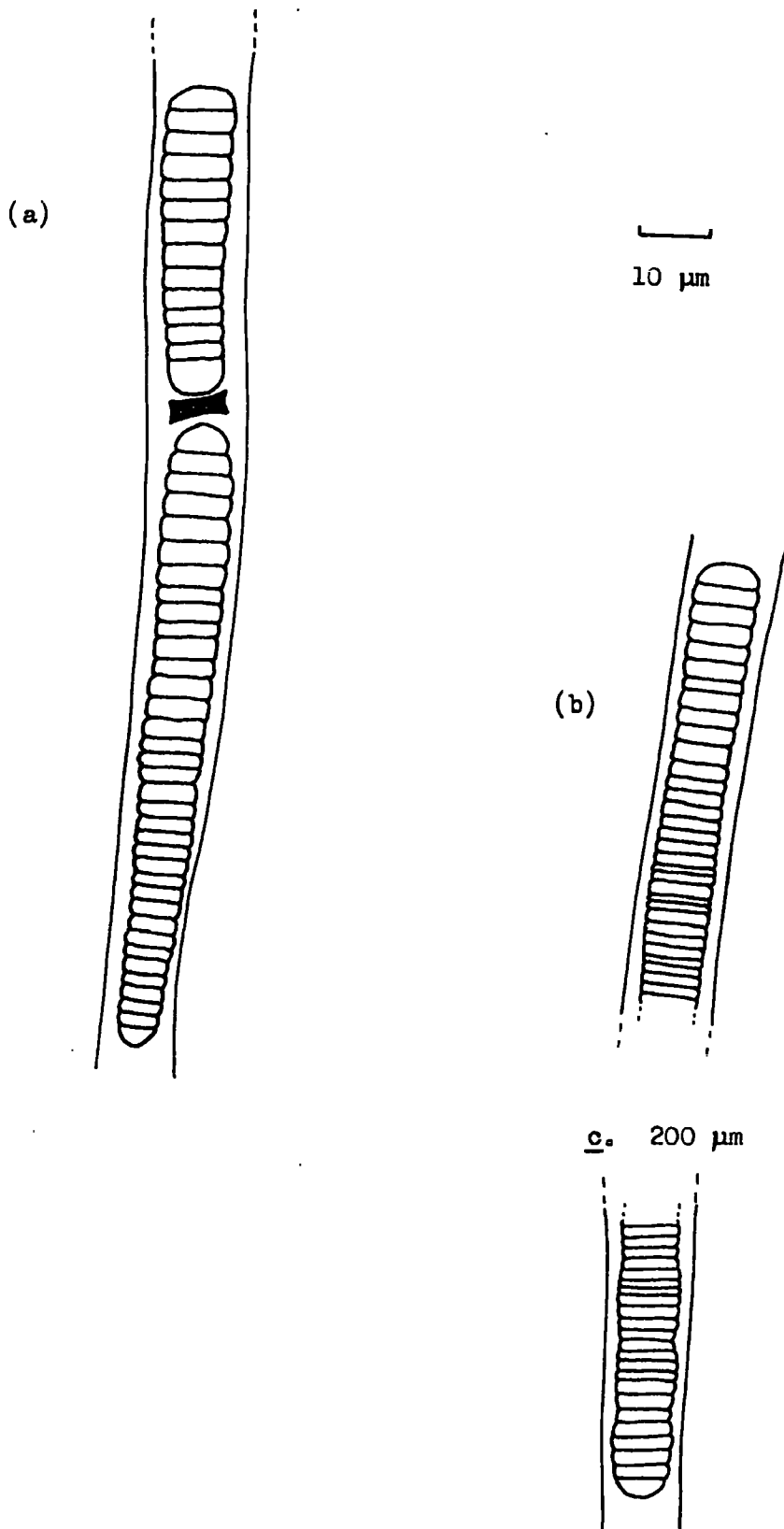
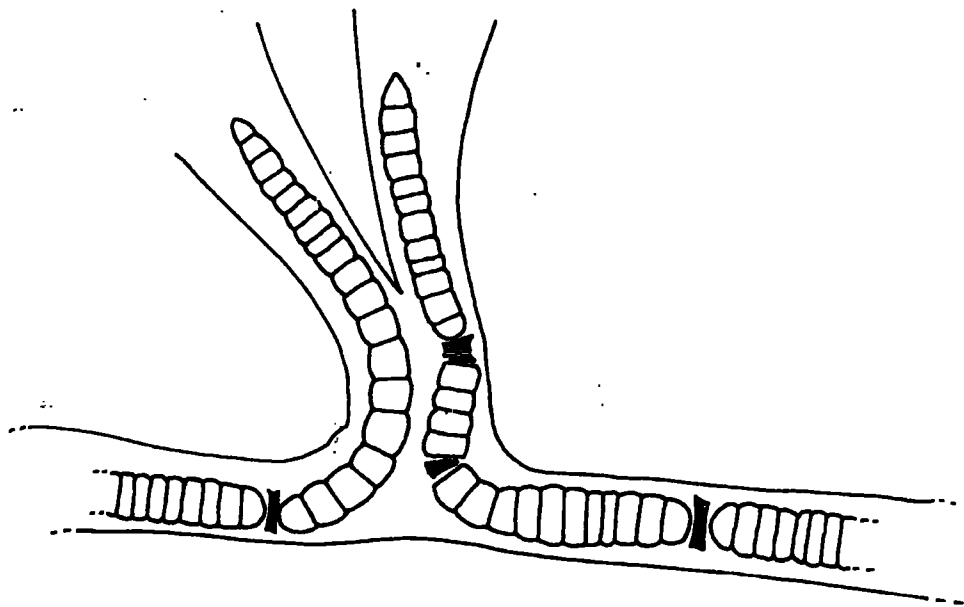


Fig. 3.2 Tapered and parallel trichomes of Calothrix viguieri D253 in AD+N.

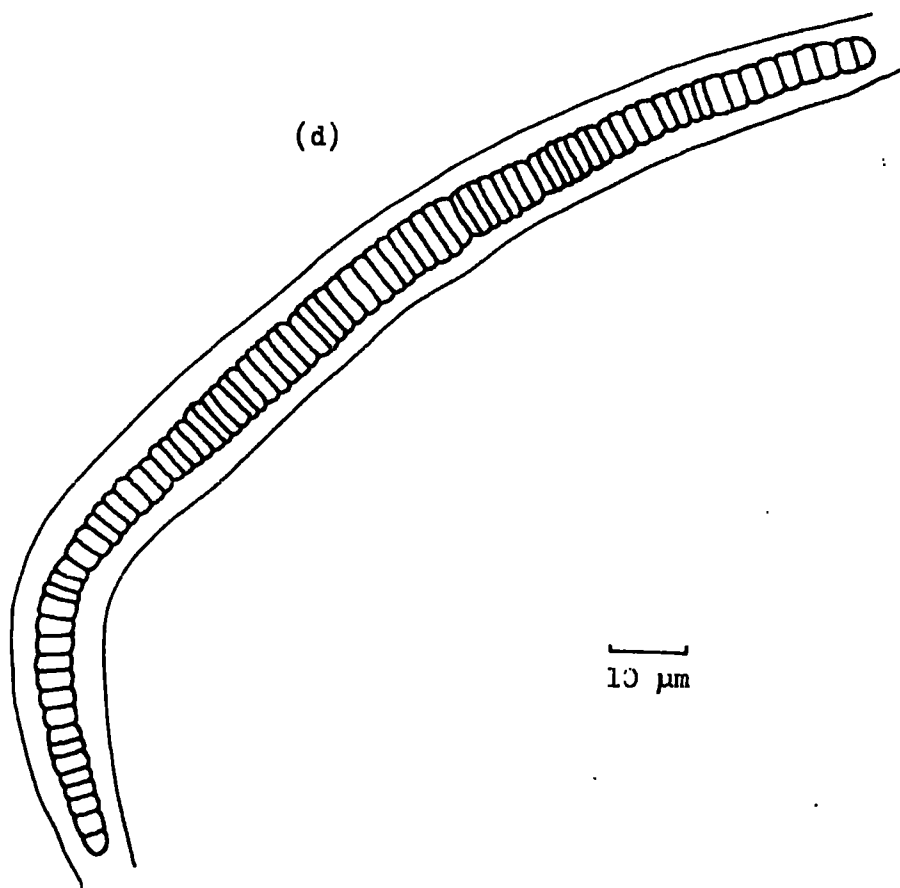
(a) 'Homoeothrix'-like trichome.

(b) Parallel trichome.

(c)



(d)



10 μ m

Fig. 3.2 (c) 'Plectonema'-like false branch.
(d) 'Ammatoidea'-like trichome.

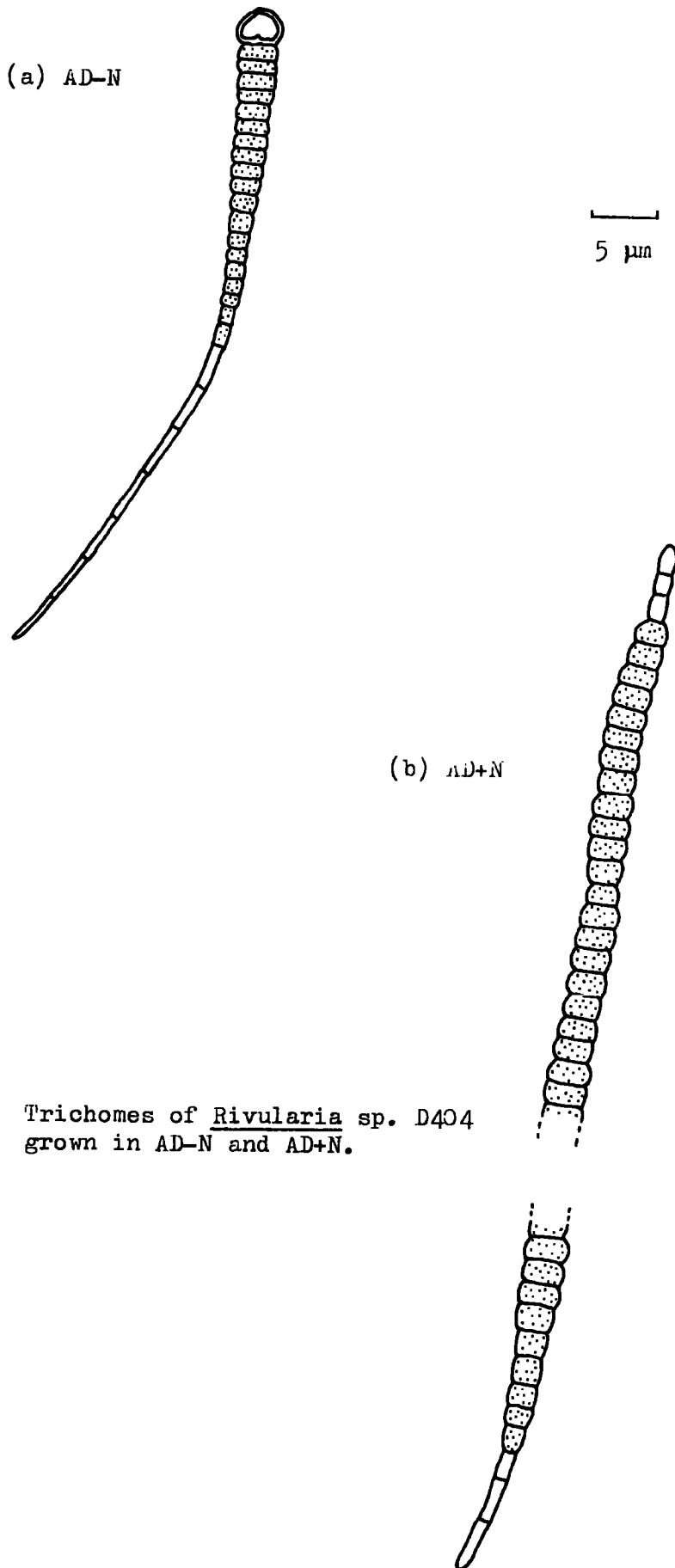


Fig. 3.3 Trichomes of Rivularia sp. D404 grown in AD-N and AD+N.

3.2 Influence of nitrogen starvation on morphology of *Calothrix* sp. D184

3.21 Introduction

The experiments described in Section 3.1 showed that the presence of combined nitrogen could markedly affect the morphology of heterocystous Rivulariaceae, often causing a reduction in, or even a total suppression of tapering, in addition to suppressing heterocyst development. In three of the five strains with hairs in AD medium, the development of hairs was also markedly reduced in the presence of combined nitrogen. It was interesting, therefore, to see whether nitrogen starvation would affect tapering and/or hair production in a heterocystous tapered strain.

3.22 Incubation in an atmosphere of Ar:O₂:CO₂

For practical reasons it was not possible to perform this experiment with more than one strain. The strain used (D184) was not axenic, but it grew more vigorously than the axenic strains that could be grown in AD medium (see Appendix). Strain D184 had no hairs in AD medium, but it was known to be capable of forming them under certain mineral deficiencies (Chapters 4 and 5).

Strain D184 was inoculated into 50 ml aliquots of AD medium in 100 ml conical flasks fitted with sidearms for gassing (Section 2.352). The medium was unbuffered, since preliminary experiments had shown no difference in pH between medium + algae under the different gassing treatments, and it also seemed undesirable to use the nitrogen-containing HEPES buffer. Incubation was in a culture tank at 25°C, with shaking, at a light intensity of 2500 lx. Cultures were allowed to grow for five days before gassing was begun, so that the algae would be in an actively growing state at the start of the experiment. The following treatments were then employed, each in quadruplicate:

- a) Normal incubation, without gassing
- b) Gassing with air
- c) Gassing with 'nitrogen-free air' (a mixture containing Ar:O₂:CO₂ (79.97:20:0.03 by volumes) - cf. Section 2.352)

The flasks were gassed initially for 30 min, and for the next 16 days they were flushed twice daily for 10 min.

The extent of growth, and the macroscopic appearance of the algae after 1, 3, 10 and 16 days are summarized in Table 3.4. After 3 days, trichome dimensions were measured for each of the

TABLE 3.4

Growth and macroscopic appearance of cultures of Calothrix sp. D184 incubated under different gaseous atmospheres

*16 = flasks to which NaNO_3 added on day 10. See section 2.64 for explanation of growth scores.

<u>Days of gassing</u>	<u>Not gassed</u>		<u>Air-gassed</u>		<u>Ar:O₂:CO₂-gassed</u>	
	<u>Growth</u>	<u>Appearance</u>	<u>Growth</u>	<u>Appearance</u>	<u>Growth</u>	<u>Appearance</u>
1	+	green	+	green	+	yellow-green
3	1	green	+ - 1	green	+	brown
10	3	dark green	+ - 1	green-brown	+ - 1	brown
16	5	dark green	1	green-brown	+ - 1	orange
*16	5	dark green	1	green	1	green

treatments; these measurements are shown in Table 3.5. At the time the measurements were taken, obvious morphological changes had occurred, but no serious degenerative changes were apparent.

Table 3.5 Trichome dimensions (μm) of Calothrix sp. D184 incubated under different gaseous atmospheres

Each value is the mean of 20 measurements.

Probabilites are indicated only where $P \leq 0.001$.

	Not gassed	Air-gassed	Ar:O ₂ :CO ₂ -gassed
Basal width	8.5 ± 0.17	8.3 ± 0.19	8.5 ± 0.2
Subapical width	3.6 ± 0.06	3.5 ± 0.05	3.6 ± 0.05
Apical width	3.3 ± 0.06	3.1 ± 0.04	3.2 ± 0.03
Length	202 ± 13	186 ± 15	150 ± 8
Heterocyst width	7.5 ± 0.18 ¹	7.3 ± 0.18 ²	9.3 ± 0.24 ^{1,2}
Heterocyst length	8.1 ± 0.17 ³	7.7 ± 0.21 ⁴	9.3 ± 0.29 ^{3,4}

Both gassing treatments caused considerable inhibition of growth, compared with that in the non-gassed control flasks (Table 3.4), and the inhibition was only slightly greater for the -N₂ than for the +N₂ treatment. There were, however, marked qualitative differences between cultures under the two conditions. After only 24 h, cultures gassed with Ar:O₂:CO₂ had begun to turn yellow; over the next few days, the algae became macroscopically pale brown in colour, and finally orange. Morphological changes also occurred in the -N₂ cultures. The cells became yellow, then brownish, and many trichomes developed numerous vacuoles in every cell. This vacuolation was not associated with any change in cell shape, and there was no apparent tendency for any hair cells to form. The size of the heterocysts increased under Ar:O₂:CO₂ (Table 3.5), but there were no other significant differences in trichome dimensions between the different treatments, and no change in the tapering of the trichomes. Heterocyst frequency continued to increase throughout the period of observation in the Ar:O₂:CO₂ cultures. At first, secondary basal heterocysts (Section 2.23) developed, and in later stages, numerous pseudo-intercalary heterocysts were produced. After 16 days, it was

estimated that about 20% of the cell population consisted of heterocysts. None of these effects were seen in the air-gassed flasks. Although the cells were latterly rather less green than those in control cultures, and occasionally vesiculated, the trichome morphology was otherwise the same as that of the controls.

After 10 days' incubation, NaNO_3 was added to two of the flasks from each treatment, to give a concentration of 10 mM. Algae from both gassed treatments became obviously greener within 24 h of the addition. Over the next six days, a small amount of growth occurred in the $\text{Ar}:\text{O}_2:\text{CO}_2$ -gassed flasks, until their growth extent was similar to that of the air-gassed set (Table 3.4). There was no further growth beyond this level in either treatment, and there was little change in morphology, apart from greening of the cells, and the accumulation of cyanophycin granules. Six days after the addition of NaNO_3 , gassing was discontinued, and the gas-tight seals once more replaced by cotton wool bungs. All the cultures now showed a complete recovery, and grew to final yields comparable with the control. Their morphology also became quite normal.

3.23 Summary of results

1. Effect of gassing

The severe inhibition of growth produced by both gassing treatments was rather puzzling. It was evidently due to some aspect of the gassing procedure, since complete recovery was obtained only when this was discontinued, and the gas-tight seals replaced by cotton wool plugs. CO_2 limitation was a possible reason for the inhibition, but since the flasks were flushed twice daily, this would be expected to cause a slowing, rather than a complete cessation of growth. An inhibitory effect of oxygen could perhaps have been responsible, since blue-green algae as a group seem to be rather sensitive to oxygen (Stewart & Pearson, 1970). Possibly the twice-daily flushing was insufficient to prevent accumulation of oxygen; yet the total algal biomass was only very low, and would not be expected to evolve much oxygen. The flushing process itself may have produced oxygen supersaturation, this being responsible for the inhibitory effect. This problem was not pursued further.

2. Effect of nitrogen deprivation

Although it was not possible to say that growth of Calothrix sp. D184 in an atmosphere of Ar:O₂:CO₂ was limited by a lack of N₂, nevertheless there was a marked morphological response under these conditions. A rapid yellowing of the cells, an increase in heterocyst frequency, and the development of many small vesicles were observed. These changes are very similar to responses to nitrogen starvation described for other blue-green algae (Section 1.62). The re-greening of the cells, and the appearance of abundant cyanophycin granules, following the addition of NaNO₃, was also similar to the responses observed by de Vasconcelos and Fay (1974) on adding NH₄-N to nitrogen starved Anabaena cultures (Section 1.62).

The broad agreement with results in the literature suggested that Strain D184 was in fact nitrogen starved, even though its growth may have been limited by some other factor. Apart from the increase in heterocyst frequency and size, and general degenerative changes in the cells, there was no other obvious effect of nitrogen deprivation on the morphology of this strain. In particular, the tapering of the trichomes was unaffected, and no hairs developed. This could have been due to the lack of overall growth; however, heterocysts continued to differentiate throughout the period of the experiment, which suggested that morphogenetic activity was not entirely prevented.

3.3 Influence of supply of combined nitrogen on morphology of Homoeothrix

3.31 Introduction

The results of Fay, Stewart, Walsby and Fogg (1968) (Section 1.41) suggest that the tapered morphology of heterocystous Rivulariaceae might be due to the lower availability of fixed nitrogen to the cells most distal to the basal heterocyst, though the experiments described in Sections 3.1 and 3.2 suggested that this was unlikely to be the only factor. In the genus Homoeothrix, the trichomes have no heterocysts, but they taper in the same way as those of heterocystous Rivulariaceae, and may also produce hairs; this would seem to argue against the hypothesis that tapering and hair development are due to a gradient of fixed nitrogen along the trichome. However, lack of heterocysts may not necessarily indicate an inability to fix nitrogen, since several non-heterocystous blue-green algae have been found to show nitrogenase activity under suitable conditions, in particular under low oxygen tensions (Fogg, 1974; Section 1.62).

Many species of Homoeothrix have a colonial or thallose growth habit, with the trichome bases closely associated in the basal part of the thallus (Geitler, 1932). It seemed possible that such colonial growth might afford protection of nitrogenase, as suggested for Trichodesmium, by Carpenter and Price (1976) (Section 1.62), thus permitting nitrogenase activity under externally aerobic conditions. It was thus of interest to see whether nitrogenase activity could be detected in colonies of Homoeothrix in which the trichomes had a well developed taper. Acetylene reduction assays on a population of H. crustacea are described in Section 3.32.

The experiments described in Section 3.1 showed that some heterocystous strains of Rivulariaceae could develop trichomes very similar to those of Homoeothrix, when grown in the presence of a high level of combined nitrogen. This suggested the possibility that at least some forms of Homoeothrix might actually be species of Calothrix, in which heterocyst development had been suppressed. To investigate this possibility, two species of Homoeothrix were starved of combined nitrogen, since this condition would be most likely to allow expression of any potential ability

to develop heterocysts. These experiments are described in Section 3.33. An additional reason for performing these experiments was to see whether the availability of combined nitrogen would affect tapering and/or hair development in Homoeothrix, as it had been found to in the heterocystous strains studied.

3.32 Assay for nitrogenase activity in colonies of H. crustacea

3.321 Material

Both the experiments described below were done with a population of H. crustacea from the Scandal Beck, Cumbria (Table 2.2). The colonies were scraped off rocks with a scalpel, and stored on ice in polythene jars until return to the laboratory (2-6 h). The material was carefully examined to see if heterocystous algae were present, but none were observed; associated algae were Gongrosira sp., Scenedesmus spp., Chantrania forms of Rhodophyta, and pennate diatoms. In each experiment, field samples of a Rivularia from a flush near Barras, Cumbria (Table 2.5) were used as a control, to check that the incubation conditions used were actually favourable for nitrogenase activity. Previous tests had shown this alga to be capable of reducing acetylene under normal atmospheric conditions (i.e. in air). Ethylene production was expressed as nmol per mg chlorophyll a, extracted as described in Section 2.52.

3.322 Assay of colonies directly after collection

The algae were collected on 25.6.73. Directly on return to the laboratory (within 4 h of collection) the colonies of Homoeothrix and Rivularia were allowed to equilibrate to 20°C for one hour, and then transferred to 7 ml serum bottles. An estimated 0.5 ml of colonies per bottle was used, together with 0.5 ml of water from the collection site. The air in the bottles was replaced by a mixture of Ar:CO₂ (95.5:0.5 by volumes) by evacuating and refilling three times; each bottle was then injected with 0.3 ml O₂, to give a partial pressure of O₂ of about 5%. This partial pressure of oxygen was used since this had been found to be the level giving most rapid rates of acetylene reduction by colonies of Rivularia from stream sites in Upper Teesdale (M.K. Hughes and B.A. Whitton, personal communication). A pre-incubation period of 30 min was allowed before injecting acetylene. Samples were assayed for ethylene after 2 h and 4 h.

No acetylene reduction by the Homoeothrix colonies was observed after 2 h or 4 h incubation. Under the same conditions, the mean ethylene production by the Rivularia colonies was 175 ± 38 nmol mg chlorophyll a^{-1} after 2h, and 440 ± 104 nmol mg chlorophyll a^{-1} after 4 h (means of 4 replicates). The large standard errors may be partly due to the fact that the volumes of algae per bottle were only approximately equal, with consequent effects on gas pressure; also the partial pressure of oxygen was only crudely achieved.

3.323 Assay of colonies during longer incubation in the absence of combined nitrogen

The absence of demonstrable nitrogenase activity in the experiment described in Section 3.222 could have been due to nitrogen sufficiency of the Homoeothrix. A further attempt was therefore made to detect nitrogenase activity, after a period of nitrogen deprivation.

Colonies of Homoeothrix and Rivularia were collected on 1.7.74. They were stored overnight at 5°C , at a light intensity of 500 lx, and used for experimentation the next day. The colonies were suspended in sterile Chu 10-D(-N) and centrifuged gently, in an attempt to wash away combined nitrogen; this procedure was repeated three times. About 0.5 ml of colonies was used for each 7 ml serum bottle, together with 0.5 ml sterile Chu 10-D(-N). The bottles were evacuated and flushed with either Ar:CO₂ (99.5:0.5 by volumes) or Ar:O₂:CO₂ (79.5:20:0.5 by volumes). The bottles were flushed with the same gas mixtures twice each day (Stewart & Lex, 1970) for the next four days. The atmosphere containing 0% O₂ was based on that used by Stewart and Lex (1970) to obtain nitrogenase activity in cultures of Plectonema boryanum. An atmosphere containing 20% O₂ (approximately the normal atmospheric level) was also used, since if the colonial structure did have a protective effect on any nitrogenase in the trichomes (Section 3.31), then acetylene reducing activity might be expected under externally aerobic conditions. Each day, bottles were incubated for 2 h with acetylene, and assayed for ethylene production (Section 2.6).

The results are shown in Table 3.6 (because the total number of samples was very large, only three replicate bottles per treatment were used, and the results for each replicate are shown individually). There was no detectable ethylene production by the

Homoeothrix colonies under any of the conditions employed. In the Rivularia however, there was clear evidence of nitrogenase activity; the results obtained under Ar:O₂:CO₂ were similar to those of the earlier experiment (Section 3.322), with an evident increase in activity on the third and fourth days of the incubation. Under Ar:CO₂ the rates of ethylene production were consistently lower than those under Ar:O₂:CO₂, and showed a slight fall on the fourth day. Evidently some aspect of the incubation was unfavourable for nitrogenase activity.

The algae were examined microscopically each day. No morphological changes were observed under either gaseous atmosphere, and the algae remained healthy in appearance. By the fourth day however, an obvious increase in the number of bacteria and colourless flagellates had occurred in the bottles containing Homoeothrix. There was no obvious upward or downward trend in the levels of chlorophyll a in either alga during the experiment (Table 3.6).

3.324 Summary of results

1. No acetylene reducing activity was detected in colonies of Homoeothrix crustacea, either when assayed directly after collection, in an atmosphere of Ar:O₂:CO₂ (94.5:5:0.5 by volumes), or when assayed after up to four days' incubation in medium without combined nitrogen, in an atmosphere of Ar:O₂:CO₂ (79.5:20:0.5 by volumes). The negative results of assays carried out under Ar:CO₂ were inconclusive, since acetylene reduction by the Rivularia used as control was adversely affected under this condition.

2. The experiments did not show conclusively that Homoeothrix crustacea was incapable of nitrogenase activity under microaerophilic conditions. They did however demonstrate that colonies of trichomes with a distinctly tapered morphology (see Section 3.33) had no detectable nitrogenase activity in aerobic conditions, even after a period of nitrogen starvation. Since H. crustacea is characteristic of flowing water habitats (Komárek & Kann, 1973), a nitrogenase system which was active only under a low external oxygen level would be of little value. The results thus suggested that H. crustacea was unlikely to possess nitrogenase activity under normal field conditions.

TABLE 3.6

Chlorophyll levels and ethylene production in Rivularia and Homoeothrix incubated in Chu 10-D (-N) under Ar:O₂:CO₂ (79.5:20:0.5) and Ar:CO₂ (99.5:0.5). Incubation period for acetylene reduction assays 2h.

Day of Incubation	Replicate	Rivularia		Homoeothrix					
		Chlorophyll a (mg per bottle)		Ethylene production (nmol mg Chl a ⁻¹)					
		Ar:O ₂ :CO ₂	Ar:CO ₂	Ar:O ₂ :CO ₂	Ar:CO ₂				
0	a	0.133	0.183	143	60	0.283	0.246	not detectable	not detectable
	b	0.114	0.252	368	84	0.275	0.288		
	c	0.218	0.197	309	142	0.354	0.384		
	mean	0.155	0.211	274	95	0.304	0.306		
1	a	0.154	0.178	153	87	0.223	0.272		
	b	0.188	0.137	185	44	0.317	0.286		
	c	0.199	0.142	133	151	0.353	0.297		
	mean	0.180	0.152	157	94	0.345	0.285		
2	a	0.247	0.261	279	60	not estimated	not estimated		
	b	0.190	0.192	232	93				
	c	0.167	0.245	321	67				
	mean	0.201	0.233	278	74				
3	a	0.195	0.114	351	113	"	"		
	b	0.148	0.105	440	47				
	c	0.154	0.119	312	100				
	mean	0.169	0.113	368	87				
4	a	0.172	0.224	483	56	0.292	0.268		
	b	0.229	0.316	777	63	0.364	0.376		
	c	0.202	0.188	513	73	0.374	0.398		
	mean	0.201	0.243	591	64	0.344	0.347		

3.33 Influence of nitrogen deficiency on morphology of two species of Homoeothrix

3.331 Crude cultures of H. crustacea

Colonies were collected from the Scandal Beck (Table 2.2) on 25.6.73. The morphology of the alga at the time of collection was described in Section 2.121 (Fig. 2.6). 10-15 colonies were inoculated into 25 ml aliquots of AD (no combined nitrogen) and AD+10 mM NaNO_3 , both media containing cycloheximide (Section 2.361). Flasks were incubated with shaking at 20°C, at a light intensity of 2000 lx for 10 days, and the morphology of the algae examined every two days. The observations described below were made on the sixth day of culture, which was the first day on which any morphological changes were seen. After six days, growth of bacteria became very heavy, and the colonies were overgrown by other algae, despite the addition of cycloheximide.

In AD+ NaNO_3 , many of the trichomes appeared to have increased in length, giving the colonies a velvety appearance, and numerous motile hormogonia were present. The majority of trichomes (frequency score = 4) had blunt apices like the one shown in Fig. 2.6a, and appeared untapered, since the trichome bases, with their slight enlargements, were not readily visible (cf. Section 2.121). In AD medium, there was no sign that any growth had taken place. The range of morphological forms present was very much the same as it had been in the field material, but there was a rather higher proportion of trichomes in which the apices tapered and had slightly vacuolated cells (cf. Fig. 2.6b). In some trichomes small vacuoles were present in almost every cell, and not just in the tapered apical region. There was no apparent change in the frequency of trichomes with well developed hairs (cf. Fig. 2.6c). No heterocysts were observed, but the trichome bases were highly calcified, and difficult to make out.

3.332 Unialgal cultures of Homoeothrix sp. D402 and H. crustacea D401

Following the isolation of unialgal cultures of two species of Homoeothrix, these were used for further experiments on the effects of nitrogen supply. The morphology of the two strains in culture was described in Section 2.121. In the experiments described below Strain D401 was incubated in a growth room at 15°C, with a light intensity of 2500 lx, and Strain D402 in a growth room

at 25°C, with a light intensity of 1500 lx. 25 ml aliquots of media were used.

(i) Incubation in media without added combined nitrogen

Both strains were inoculated from Chu 10-D into AD and Chu 10-D(-N). Neither strain showed any growth, and within 2-3 days the inocula had turned yellow. This response was obtained each time the experiment was repeated. When a second subculture was made into nitrate-free media, there was no growth, and the algae became almost colourless. There was no sign of heterocyst development in either strain. The morphological responses seen were the same as those described in (ii) below, but they developed very soon after inoculation.

(ii) Incubation in Chu 10-D with a reduced level of NO₃-N

The onset of (presumed) nitrogen deficiency in media without NO₃-N was very rapid, and occurred without apparent growth. From the point of view of determining the morphological response to nitrogen deficiency, it seemed preferable to allow a certain amount of growth to occur before the deficiency developed, so as to avoid any confusion with possible lag effects shortly after inoculation. Full expression of any morphological response might not be possible if the algae were not metabolizing normally as they began to experience the deficiency. Further experiments were therefore carried out using Chu 10-D with 0.2x the normal level of NO₃-N ('low N medium'), with Chu 10-D as control ('full medium'). The initial levels of NO₃-N in the two media were 1.36 mg l⁻¹ and 6.83 mg l⁻¹ respectively. CaCl₂.6H₂O was used to compensate for the lowered level of Ca in the low N medium.

a) Homoeothrix crustacea D401

In the initial stages of growth, the alga was macroscopically and microscopically identical in the two media. During this phase of active growth all the trichomes had untapered apices, and many hormogonia were present. The cells were all short, with length:width ratios of about 0.5:1 (Fig. 3.4a). Growth in the low N medium first began to lag behind that in the full medium after about five days, and the cultures began to turn yellowish as compared with the pinkish brown of the control.

The first morphological changes coincided with the onset of growth limitation: some of the trichomes developed tapered.

apices, with slightly elongated and sometimes vacuolated cells (Fig. 3.4). The degree of vacuolation at this stage was not sufficient to constitute hair development by the criterion explained in Section 2.22. Over the next few days, the number of trichomes with tapered apices increased, until, by about the eighth day of culture, almost all the trichomes had apices of this type, usually with vacuolation of the elongated apical cells. Not more than about six cells at the apex were usually affected; the cells further down the trichome retained their moniliform appearance and were not usually longer than broad. At this stage, the types of trichomes, and their relative abundance, were very similar to those observed in the original field material when collected.

Further changes occurred between days 8 to 12: small vacuoles appeared in many of the cells in the non-apical parts of the trichomes, and these cells increased in length to become about 1.5x longer than broad. In some trichomes (frequency score = 1) short hairs developed (Fig. 3.4c). Throughout this period the algae became progressively yellower, and the sheaths increased in thickness. No evidence of heterocyst development was seen at any stage.

b) Homoeothrix sp. D402

During active growth, this strain was bright blue-green in colour. All the trichomes had untapered apices and short cells, with a length:width ratio of about 0.5:1 (Fig. 3.5a) and hormogonia were abundant. Deficiency symptoms were first seen after about three days in low N medium: growth apparently ceased, and the cultures became green rather than blue-green. They later turned yellow, and eventually orange.

The first morphological change, which occurred at about the same time as the cells turned yellow, was an increase in the average length of the cells. They were seldom shorter than broad, and sometimes rather longer (Fig. 3.5b). The sheaths also increased in thickness. None of the trichomes developed any vacuolation, and the majority had untapered apices without any modifications of the cells. After about eight days, however, some of the trichomes (frequency score = 1-2) showed a slight enlargement of the apical cell, which became both wider and longer than those below (Fig. 3.5c). There were no further morphological

changes, and no vacuolation of the cells. The thickness of the sheaths continued to increase after active growth had ceased. After about 14 days, by which time the cultures were orange in colour, the trichomes began to fragment into short pieces. There was no sign of any heterocyst development at any stage. The enlarged cells at the apices of some of the trichomes could conceivably have been taken for heterocysts, but the lack of differentiation of both cell walls and cell contents argued against this.

If NaNO_3 was added to low N cultures of either strain at any time after the onset of growth limitation, the cells became green within about 12 h, and active cell division and hormogonium production recommenced soon afterwards. This confirmed that the symptoms observed were due to nitrogen deficiency. The morphology of both strains in the full medium followed a sequence of changes very similar to that seen in the low N medium, but each stage was observed three or four days earlier. Nitrogen was probably the growth limiting element in Chu 10-D, since addition of NaNO_3 to full medium cultures in which growth had ceased led to re-greening and a further period of growth; addition of KH_2PO_4 was without effect.

3.333 Summary of results

1. Neither strain of Homoeothrix showed any growth in the absence of combined nitrogen, which strongly suggested that nitrogenase activity was not present under these (aerobic) conditions. This was in agreement with the results of the acetylene reduction assays on colonies of H. crustacea (Section 3.32).

2. Both strains rapidly became yellow in low nitrate medium. This response has been described for other blue-green algae under nitrogen starvation (Section 1.62). A slight increase in average cell length, and increased production of sheath material were also observed in both strains. All these symptoms were lost on the addition of $\text{NO}_3\text{-N}$. Although all the evidence suggested that the growth of the two Homoeothrix strains was limited by shortage of combined nitrogen, neither strain showed any tendency to develop heterocysts. It thus seemed very unlikely that either was merely a growth form of Calothrix or Rivularia.

3. In the presence of sufficient combined nitrogen to allow active growth, trichomes of both strains had untapered apices; this was apparently associated with the release of hormogonia. The trichomes did however have a slight taper under these conditions, due to the enlargement of a few cells in the basal region (Section 2.121).

The tapering of trichomes in Strain D402 was not affected by nitrate deficiency, apart from a nominal decrease in tapering, caused by the curious enlargement of the apical cell in some trichomes. In Strain D401, the trichomes became more tapered as growth began to slow down; the apical few cells became somewhat narrowed and elongated, and also slightly vacuolated. In later stages, a few fully-formed hairs were produced, but these were seldom more than six cells long.

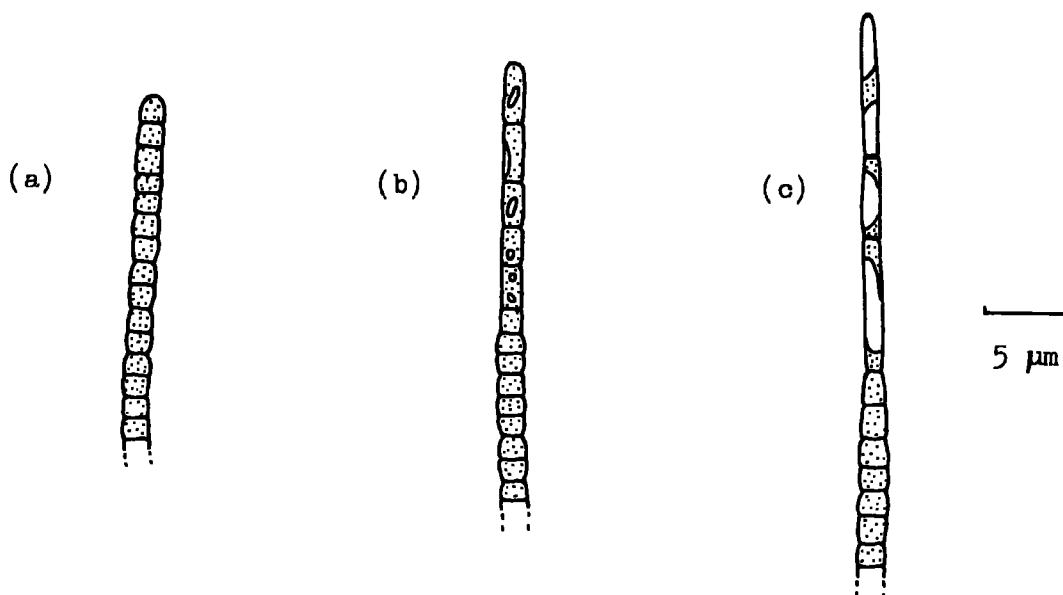


Fig. 3.4 Morphological changes in the apical region of trichomes of Homoeothrix crustacea D401 during onset of nitrate deficiency (see pp. 132-133).

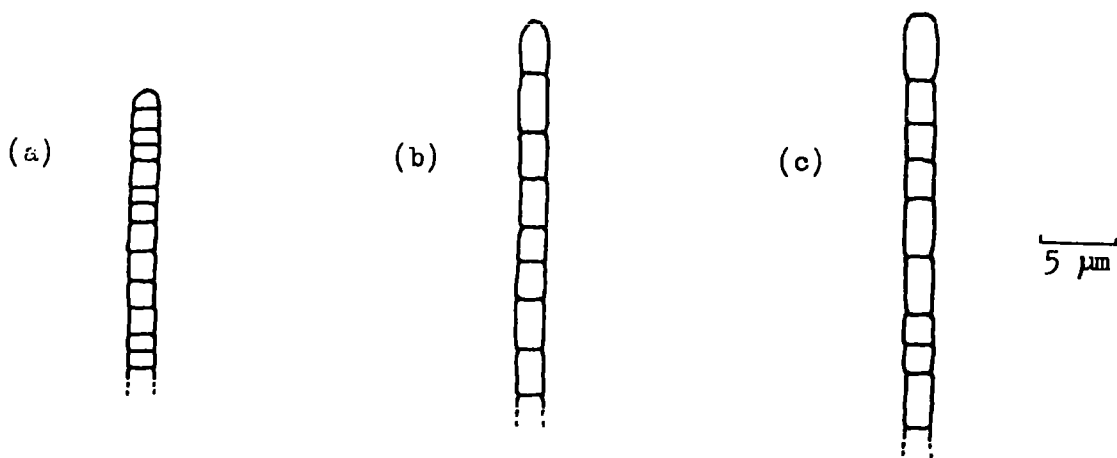


Fig. 3.5 Morphological changes in the apical region of trichomes of Homoeothrix sp. D402 during onset of nitrate deficiency (see pp. 133-134).

4 INFLUENCE OF PHOSPHATE DEFICIENCY ON HAIR DEVELOPMENT IN 36 STRAINS OF RIVULARIACEAE

4.1 Introduction

Deficiency of phosphorus has been found to increase hair development in several eukaryotic algae (Section 1.51), and Kirkby (1975) found that Rivularia also produced longer hairs when incubated at a relatively low level of inorganic phosphate (Section 1.41). It was thus of interest to see whether any of the 36 laboratory strains of Rivulariaceae would similarly show increased hair formation when cultured at a low phosphate concentration.

4.2 Methods Strains D126, D253, D266, D269, D270 and D277 were grown with shaking; the other strains The 36 strains used are listed in Table 2.1. \downarrow were incubated, without shaking, at 2000-2500 lx, at the temperatures given in Section 2.351. For the 34 heterocystous strains, AD was used as the high phosphate control medium ($44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$); the low phosphate version was made up with $0.44 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. Chu 10-D was control medium for the two strains of Homoeothrix; the high and low phosphate versions of this medium contained 1.78 and 0.09 $\text{mg l}^{-1} \text{ PO}_4\text{-P}$ respectively. KCl was used to compensate for the reduced levels of K^+ , and all media were buffered with 5 mM HEPES.

Each strain was taken through two subcultures, in duplicate, in low and high phosphate medium. Morphological scores were taken after 10-15 days in the second subculture, at a stage when growth was clearly limited in the low phosphate cultures, but before obvious degenerative changes had occurred. While the experiment was chiefly intended to determine effects on hair development, some other quite marked morphological responses were seen, and these are also noted below.

4.3 Results

As described in Section 2.121, only three of the strains (D126, D277, D404) had many long hairs in the control medium; two more (D251, D403) had a few short hairs, and Homoeothrix crustacea D401 produced a few short hairs at the end of the growth period. Morphological responses of the algae in low phosphate medium are summarized in Table 4.1.

TABLE 4.1

Morphological responses of 36 strains of Rivulariaceae to phosphate deficiency

* = few, short hairs in control culture ** = many, long hairs in control culture

	<u>Hair development increased</u>		<u>Modifications of apical few cells (other than hair formation)</u>		<u>Sheath bases dark brown</u>
	<u>Hair development increased</u>	<u>Elongation</u>	<u>Narrowing</u>	<u>Loss of granules</u>	
Calothrix sp.					
D184	+				+
D251*	+				+
D252					
D254			+		
D255			+		
D258			*		
D264					
D267			+		
D283		+	+		
D182		+			
D156			+		+
D275			+		+
D270	+				
D273		+			+
D269	+				+
D274					
D257			+		
D202		+			+
D179					
D259		+	+		+
D260		+	+		+
D261		+	+		+
D262		+	+		+
D263			+		+
D272					
D265			+		+
D256	+				+
D266	+				+
D253	+				+
D280	+				+
D126**	+				+
D277**	+				+
D402					
D401*	+				+
D403*	+				
D404**	+				+

- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- Calothrix sp.
- C. anomala
- C. brevissima
- C. brevissima
- C. desertica
- C. elenkinii
- C. fusca
- C. gracilis
- C. javanica?
- C. marchica
- C. membranacea
- C. membranacea
- C. membranacea
- C. membranacea
- C. membranacea
- C. parietina
- C. prolifica
- C. scopulorum
- C. thermalis
- C. viguieri
- Dichothrix sp.
- Gloeotrichia echinulata
- G. ghosei
- Homoeothrix sp.
- H. crustacea
- Rivularia sp.
- Rivularia sp.

Twelve of the heterocystous strains and one strain of Homoeothrix showed a marked increase in the development of hairs in the low phosphate medium. These included the six strains which had at least some hairs in the control medium; the other seven affected strains had no hairs when grown in the high phosphate control medium. In each case hairs were eventually present on at least 90% (estimated) of the trichomes, forming as much as 80% of the total trichome length.

The remaining 22 heterocystous strains and one Homoeothrix strain did not develop hairs, but 15 of the heterocystous strains, and Homoeothrix sp. D402 showed some modifications of the apical 1-3 cells. The chief changes seen in the apical region were narrowing of the cells (7 strains); cell elongation (8 strains); loss of normal granular inclusions (14 strains) and the development of small vacuoles (4 strains). These characters were sometimes, but not always correlated (Table 4.1).

In 12 of the heterocystous strains almost every trichome in low phosphate cultures developed a dark brown sheath. Nine of these were strains in which hair development increased. In control cultures, only five strains (D184, D251, D256, D280, D404) developed brown pigmented sheaths, and then only in the wider-based (presumed older) trichomes, at the end of the growth period. Some responses were common to all the strains. Loss of polyphosphate granulation, and increased production of sheath material, occurred in every case, and the level of cyanophycin granulation was seen to increase in each strain except the two Homoeothrix, for which the estimation was difficult because of the small size of their cells. A more detailed account of the responses of 13 of the strains to phosphate deficiency is given in the following chapter (Section 5.3).

4.4 Summary of results

12 of the 34 heterocystous strains, and one of the two Homoeothrix strains showed a marked increase in hair development under phosphate deficiency. Seven of these strains were ones which produced no hairs when grown in the high phosphate control medium.

5 MORPHOLOGICAL RESPONSES OF 13 STRAINS OF RIVULARIACEAE TO A RANGE OF MINERAL DEFICIENCIES

5.1 Introduction

The results described in Chapter 4 showed that under phosphate deficiency 13 of the 36 strains of Rivulariaceae showed a marked increase in hair development. It seemed possible that this might have been a response to growth limitation per se, rather than a specific response to phosphate deficiency. It was also possible that some of the strains which did not produce hairs under phosphate deficiency might do so under deficiency of other nutrients.

13 of the strains were therefore tested for their responses to deficiencies of iron, magnesium, calcium, molybdenum and sulphur; these experiments are described in Sections 5.4-5.8. Each of the 13 strains was also again grown to phosphate deficiency (Section 5.3), both for comparison with the other deficiencies, and also because with fewer strains it was possible to make more detailed scores than had been the case with the experiment described in Chapter 4. In addition to effects on hair development, a number of other marked morphological responses were recorded also.

5.2 Methods

5.21 Algae

Eleven heterocystous Rivulariaceae, and the two Homoeothrix strains were used. These are listed in Table 5.1, together with their incubation conditions. The strains were selected to provide a cross-section of the range of morphological types in the collection, but the proportion of strains known to be capable of forming hairs under phosphate deficiency (Chapter 4) was higher than that of other forms (D184, D251, D256, D277, D280, D401, D403, D404 but not D156, D179, D267, D283, D402). Two of the strains (D277, D404) had long hairs in the control medium; Strains D251 and D403 had a few short hairs, and H. crustacea D401 produced a few short hairs at the end of the growth period (Section 3.33). Anabaena cylindrica D2A was also included, so that the responses of this widely used research organism could be compared with those of the Rivulariaceae.

5.22 Media

Chu 10-D was used as basal medium for the two strains of

TABLE 5.1

Strains, basal media, and incubation conditions used to test effects of deficiencies of PO_4 , Fe, Mg, Ca, Mo and SO_4

<u>Strain</u>		<u>Basal medium</u>	<u>Light intensity (lx)</u>	<u>Incubation temp. ($^{\circ}C$)</u> (standing culture, unless indicated)
<u>Calothrix</u> sp.	D184	AD	2500	25 $^{\circ}$, shaken
<u>Calothrix</u> sp.	D251	AD	2500	32 $^{\circ}$
<u>Calothrix</u> sp.	D267	AD	2500	25 $^{\circ}$, shaken
<u>Calothrix</u> sp.	D283	AD	2500	25 $^{\circ}$, shaken
<u>C. brevissima</u>	D156	AD	2500	25 $^{\circ}$, shaken
<u>C. membranacea</u>	D179	AD	2500	25 $^{\circ}$, shaken
<u>C. scopulorum</u>	D256	AD	2000	25 $^{\circ}$
<u>Dichothrix</u> sp.	D280	AD	2500	25 $^{\circ}$
<u>Gloeotrichia ghosei</u>	D277	AD	2500	25 $^{\circ}$, shaken
<u>Rivularia</u> sp.	D403	AD	2000	25 $^{\circ}$
<u>Rivularia</u> sp.	D404	Chu 10-D (-N)	2500	25 $^{\circ}$
<u>Homoeothrix</u> sp.	D402	Chu 10-D	2000	25 $^{\circ}$
<u>H. crustacea</u>	D401	Chu 10-D	2500	15 $^{\circ}$
<u>Anabaena cylindrica</u>	D2A	AD	2500	25 $^{\circ}$

TABLE 5.2
Composition of media used to induce nutrient deficiencies

(a) AD-based media

Element	mg l ⁻¹ in Basal Medium	mg l ⁻¹ in Deficient Medium	Deficient Level Relative to Normal	Compensating Salt	mg l ⁻¹ of Compensating Salt
P	44.50	0.445	0.01	KCl	211.80
Fe	4.0	'0.0'	0.0	Na ₂ EDTA.2H ₂ O	6.35
Mg	19.70	0.49	0.025	Na ₂ SO ₄	112.30
Ca	18.10	0.45	0.025	NaCl	51.44
Mo	0.08	'0.0'	0.0	-	-
S	26.00	0.65	0.025	MgCl ₂ .6H ₂ O	160.7
		0.26	0.01		163.2

(b) Chu 10-D-based media (* Chu 10-D; ** Chu 10-D (-N))

Element	mg l ⁻¹ in Basal Medium	mg l ⁻¹ in Deficient Medium	Deficient Level Relative to Normal	Compensating Salt	mg l ⁻¹ of Compensating Salt
P	1.78	0.09	0.05	KCl	4.06
Fe	0.5	'0.0'	0.0	Na ₂ EDTA.2H ₂ O	0.79
Mg	2.47	0.06	0.025	Na ₂ SO ₄	14.04
Ca	9.77	0.24	0.025	*NaNO ₃ **NaCl * NaNO ₃	40.41. 27.48 41.03
Mo	0.0025	'0.0'	0.0	-	-
S	3.25	0.08	0.025	MgCl ₂ .6H ₂ O	20.15
		0.03	0.01		20.46

Homoeothrix, and AD for 11 of the 12 heterocystous strains; experiments on Strain D404, however, were done with Chu 10-D(-N) as base (Table 5.1). This exception was made partly because this strain grew rather poorly in AD, but also as a 'control' to indicate whether any differences in response between heterocystous and non-heterocystous strains were due simply to differences in the basal composition of the media. All the basal media were made up with a simplified version of AD microelements, containing only Mo, Mn, Cu, Co, B and Zn (Table 2.3; Section 2.32). Experiments described in the Appendix (Section A3.31) had shown no obvious differences between this microelement mix and the normal AD microelement solution for growth of Strain D253, and it seemed desirable to simplify the medium as much as possible, to reduce the likelihood of interactive effects between medium components. 25 ml aliquots of media were used, in 100 ml conical flasks, with treatments performed in duplicate or triplicate.

The effects of phosphate, iron, magnesium, calcium, molybdenum and sulphate deficiencies were examined. To induce deficiency of phosphate, sulphate, calcium or magnesium, these nutrients were added to the media at a reduced level that allowed a certain amount of growth, to permit expression of any morphological response; this seemed preferable to omitting the element entirely. To induce deficiency of iron and molybdenum, media were made up without the addition of these elements, relying on traces stored by the algae, or present as contaminants in other chemicals, to permit some initial growth. Table 5.2 shows the composition of the deficient media used for the majority of strains. It was sometimes necessary to use a different level of a particular element to induce deficiency in certain strains; these exceptions to Table 5.2 are pointed out in the text. For media without added iron, EDTA was added at 0.25x the normal level.

Buffering was strictly necessary only for the low phosphate media, but for consistency all media were buffered with 5 mM HEPES. In some strains however it proved difficult to induce iron or molybdenum deficiency in HEPES-buffered media (Section 5.4 and 5.7). It seemed quite likely that impurities in the HEPES could satisfy the requirements of the algae for these elements, and in these cases later subcultures were made into deficient media without HEPES. The pH of unbuffered iron-deficient media was adjusted to

normal with 0.05 M HCl.

Besides inducing nutrient deficiency, these variations in medium composition could have produced secondary effects, as a result of an altered balance of medium components. Reducing the calcium or magnesium concentration, for instance, would increase the ratio of EDTA to other metal ions, possibly thereby altering their availability to the algae. The ratio of monovalent to divalent ions would also be affected. In the present series of experiments, no attempt was made to explore such possibilities, by using more than one compensating salt, or by simultaneously varying the concentration of more than one medium component.

5.23 Scoring of morphological responses

Morphological scores were made when growth was obviously limited in the deficient medium, but before serious degeneration had occurred (usually after 15-20 days). Sometimes several subcultures were necessary, but even when obvious deficiency had been produced by a single subculture, a second subculture was always performed, and a check made for any further morphological changes. After scoring, the deficient element was aseptically added to the culture, to the normal control level. The recovery of a normal morphology and full final yield were taken as evidence that the symptoms seen had developed in response to the particular nutrient deficiency. In the case of phosphate deficient cultures, a rather more detailed study was made of the response to the addition of phosphate, since several of the changes took place within minutes or hours, rather than days (Section 5.31(ix)).

The experiments were primarily intended to determine effects of nutrient deficiency on hair formation, but it seemed worthwhile to record any other well-defined morphological response also. Most of the morphological scores were based on subjective assessment of the differences between deficient and control trichomes, and recorded using the semi-quantitative system described in Section 2.25. This meant that only relatively large morphological changes were recorded; small effects on trichome dimensions may well have been overlooked. In some cases, more detailed measurements of trichome dimensions were made. These were intended primarily to illustrate and confirm morphological changes already observed (cf. Section 2.22). For the heterocystous strains, 20

trichomes were measured from each treatment, usually with a restriction placed on the basal diameter (Section 2.22); for Homoeothrix sp. D402, 30 trichomes were measured.

Responses to the deficiencies are described in Sections 5.3-5.8, taking each nutrient in turn, and considering heterocystous and Homoeothrix strains separately. The responses are summarized in Tables 5.4-5.9, which for ease of reference are placed at the end of this section. The following symbols are used in these tables:

0 = no change
+ = increase
- = decrease
NA = character not applicable
? = doubtful or unknown

Most of the characters scored were explained in Section 2.2, but some additional notes are given here to assist the interpretation of results.

(i) Trichome dimensions and tapering

As explained in Section 2.12, the growth form of the two Homoeothrix strains meant that it was seldom possible to see their trichome bases clearly. Measurements on these strains were therefore confined to the apical and subapical regions of the trichomes. Effects on basal diameter and tapering, and shape of basal cells, are thus scored '?' in Tables 5.5 and 5.6.

When there was a change in the relative proportions of trichomes of different diameters, but without an absolute change in the range of diameters in the culture, this was not considered to affect the degree of tapering (Table 5.5b).

(ii) Shape of vegetative cells

Effects on cell width are considered under the heading of 'trichome dimensions'; changes in cell shape are described as effects on length:width ratio. Distinction is made between absolute changes in cell length, and relative changes in cell proportions resulting from changes in trichome diameter. In Table 5.6, a score of + or - indicates an absolute change in cell length; if the length changed only relative to the width, then (+) or (-) is recorded.

Table 5.6 indicates the region of the trichome in which any change in cell shape occurred:

- a) throughout trichome
- b) basal region (region of basal enlargement)
- c) subapical region (includes whole trichome apart from apical cell and basal enlargement)
- d) apical vegetative cell

A + or - score for (b), (c) or (d) indicates that the change occurred specifically in that region, or to a greater extent than in other regions of the trichome. Thus if (a) is scored + or -, then (b), (c) and (d) score 0, unless there was an additional effect in one of these regions. For instance, Strain D283 showed elongation of cells throughout the trichome under phosphate deficiency, but the apical cell was more markedly elongated.

(iii) Cytological effects other than hair formation

Under this heading are described effects on cell colour, vacuolation of vegetative cells, and changes in the level of cyanophycin and polyphosphate granulation. Under control conditions, most of the 14 strains had vegetative cells of a green or olive green colour; those of Strains D2A and D402 were bright blue-green, and Strains D156, D277 and D401 pinkish-brown. None of the strains had vacuolated vegetative cells. As mentioned in Section 2.24, only relatively large increases in cyanophycin granulation were recorded, and this character was not scored for the two Homoeothrix strains. All the strains possessed polyphosphate granules under control conditions, but there were some differences in the distribution of these granules along the trichomes. A brief account of the patterns of granule distribution in control trichomes of the 14 strains is given here.

Table 5.3 summarizes the density of polyphosphate granulation (Section 2.24) in the vegetative cells in the basal, subapical and middle regions of control trichomes for each strain. These three regions were not rigorously defined, and there was usually a steady gradation in the level of granulation between the different zones. All 13 strains of Rivulariaceae showed a decreasing gradient in the apparent density of polyphosphate granules, between base and apex. In each strain, at least some trichomes had an apparently higher density of polyphosphate in the basal few cells

TABLE 5.3

Density of polyphosphate granulation in different regions of trichomes of 13 strains of Rivulariaceae and Anabaena cylindrica in control medium

The scoring system for polyphosphate granule density is explained in Section 2.24

		<u>Polyphosphate granule density</u>		
		<u>Basal region</u>	<u>Mid-region</u>	<u>Subapical region</u>
<u>Calothrix</u> sp.	D184	4	1	1
<u>Calothrix</u> sp.	D251	1-3	1-2	0-2
<u>Calothrix</u> sp.	D267	3-4	1-2	1-2
<u>Calothrix</u> sp.	D283	3	+ -1	+ -1
<u>C. brevissima</u>	D156	1-2	1	1
<u>C. membranacea</u>	D179	3	1	1
<u>C. scopulorum</u>	D256	4	1-2	0-2
<u>Dichothrix</u> sp.	D280	3	+ -1	+ -1
<u>Gloeotrichia</u> <u>ghosei</u>	D277	3	2-3	0
<u>Rivularia</u> sp.	D403	4-5	3-4	0
<u>Rivularia</u> sp.	D404	4	3	0
<u>Homoeothrix</u> sp.	D402	1-2	1	1
<u>H. crustacea</u>	D401	1-2	1	0-1
<u>Anabaena</u> <u>cylindrica</u>	D2A	NA	1-2	NA

than in the rest of the trichome, though in Strains D156 and D277 only about 20% of the trichomes showed this effect. Part of the apparently greater granule density of the basal cells may have been due to their greater width, since the whole cell content of granules was resolved into a single two dimensional plane (Section 2.24). Nevertheless, this greater granulation at least sometimes represented an absolute increase, since in newly differentiated trichomes the granulation of the 2-4 cells next to the basal heterocyst was sometimes higher than that of the other cells, even though there was little difference in their size. Possibly the lower granulation of the cells in the apical region was related to their meristematic condition (cf. Section 2.121).

In Strains D277, D403 and D404 the granulation decreased to zero towards the apices of all trichomes, whether or not hairs were present. In Strains D251 and D256 granules were absent from the apical 5-10 cells of about 20% of the trichomes; the pattern of granulation in these two strains was not correlated with other aspects of trichome morphology, including the presence or absence of hairs in Strain D251. In Strain D401 polyphosphate decreased to zero at the apices of trichomes which tapered in this region, regardless of whether there was any vacuolation of the cells, but there was no change in the density of granulation at the tips of trichomes with untapered apices. The density of granulation was uniform along the trichomes of Anabaena cylindrica, and showed no gradients in relation to the heterocysts.

There was considerable variation between the strains in the overall level of granulation, taking the trichome as a whole. The strains are arranged below in sequence according to the level of polyphosphate in control trichomes:

D403 > D404 > D256 > D184 > D277 > D267 > D251 >

D2A	D156
D179	D401
D280	D402
D283	

(iv) Sheath characters and extracellular pigment production

Changes in the thickness and pigmentation of the sheaths were recorded (including the diffuse mucilage sheath of Anabaena cylindrica). The majority of strains had colourless sheaths in the control medium, but older cultures of Strains D184, D251, D256,

D280 and D404 normally contained some trichomes with brown pigmented sheath bases. In these strains, a change in sheath pigmentation was scored + (Table 5.7) only if there was an increase above this level.

(v) Effects on heterocysts

The majority of strains had only single basal heterocysts under control conditions, and it was therefore easy to assess changes in frequency when secondary heterocysts developed under particular deficiencies. Control cultures of Strain D251 however had a relatively high frequency of secondary basal, intercalary and pseudo-intercalary heterocysts, making it difficult to estimate further increases in heterocyst frequency. In the two Rivularia strains, D403 and D404, secondary basal heterocyst frequency was again quite high; in addition, many short daughter trichomes remained associated with the parent trichomes (Section 2.121), and it was difficult to distinguish between this phenomenon and the formation of pseudo-intercalary heterocysts. For these three strains, therefore, only very marked increases in heterocyst frequency were recorded.

Since there was usually considerable variation in heterocyst size within a single culture of any of the strains, again only marked effects were scored. Only apparently healthy heterocysts were taken into account when scoring this character.

(vi) Effects on the development of spores

This character was scored only for Strains D2A and D277 (Section 2.121(v)).

TABLE 5.5b

Changes in basal, subapical and apical diameter, and consequent changes in the degree of tapering of the vegetative region of the trichomes, in 13 strains of Rivulariaceae under PO₄, Fe, Mg, Ca, Mo and SO₄ deficiencies

Symbols are explained in Section 5.23.

Degree of taper is assessed in terms of basal and subapical diameter, ignoring hairs, where present. Apical cell diameter is scored NA in cases where many hairs present. (-) indicates an increased frequency of narrow-based trichomes, without change in absolute basal diameter; this is not considered to affect trichome tapering. Diameter of *Anabaena cylindrica* trichomes is scored as 'basal diameter'.

	PO ₄			Fe			Mg					
	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper
<i>Calothrix</i> sp. D184	0	0	NA	0	(-)	-	-	-	-	-	NA	-
<i>Calothrix</i> sp. D251	0	0	NA	0	0	0	0	0	0	0	NA	-
<i>Calothrix</i> sp. D267	0	0	0	0	(-)	0	0	0	-	0	0	-
<i>Calothrix</i> sp. D283	0	0	0	0	(-)	-	-	-	-	0	0	-
<i>C. brevissima</i> D156	0	0	-	0	(-)	-	-	-	-	-	-	-
<i>C. membranacea</i> D179	0	0	0	0	(-)	-	-	-	-	-	-	-
<i>C. scopulorum</i> D256	0	-	NA	+	(-)	-	NA	+	-	0	0	-
<i>Dichothrix</i> sp. D280	0	0	NA	0	0	0	0	0	-	-	-	-
<i>Gloeotrichia ghosei</i> D277	0	+	NA	-	0	0	NA	0	0	0	NA	0
<i>Rivularia</i> sp. D403	0	0	NA	0	(-)	0	NA	0	-	0	0	-
<i>Rivularia</i> sp. D404	0	0	NA	0	0	0	NA	0	-	0	NA	-
<i>Homeothrix</i> sp. D402	?	0	+	?	?	-	-	?	?	-	-	?
<i>H. crustacea</i> D401	?	0	NA	?	?	0	0	?	?	0	0	?
<i>Anabaena cylindrica</i> D2A	0	NA	NA	NA	0	NA	NA	NA	0	NA	NA	NA
	Ca			Mg			SO ₄					
	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper	Basal diameter	Subapical diameter	Apical cell diameter	Degree of taper
<i>Calothrix</i> sp. D184	(-)	+	+	-	(-)	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D251	0	0	0	0	(-)	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D267	0	0	0	0	(-)	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D283	(-)	0	0	0	(-)	0	0	0	0	0	0	0
<i>C. brevissima</i> D156	(-)	0	0	0	(-)	0	0	0	0	0	0	0
<i>C. membranacea</i> D179	(-)	0	0	0	(-)	0	0	0	0	0	0	0
<i>C. scopulorum</i> D256	(-)	0	0	0	(-)	0	0	0	0	0	0	0
<i>Dichothrix</i> sp. D280	0	+	+	-	(-)	0	0	0	0	0	0	0
<i>Gloeotrichia ghosei</i> D277	0	0	NA	0	(-)	0	NA	0	0	0	NA	0
<i>Rivularia</i> sp. D403	0	+	+	0	(-)	0	NA	0	0	0	NA	0
<i>Rivularia</i> sp. D404	(-)	+	NA	-	(-)	0	NA	0	0	0	NA	0
<i>Homeothrix</i> sp. D402	?	0	0	?	?	0	0	?	?	-	-	?
<i>H. crustacea</i> D401	?	0	0	?	?	0	0	?	?	0	0	?
<i>Anabaena cylindrica</i> D2A	0	NA	NA	NA	0	NA	NA	NA	0	NA	NA	NA

TABLE 5.6
Changes in length of vegetative cells in different regions of trichomes of 13 strains of Rivulariaceae, and *Anabaena cylindrica*, under deficiencies of PO_4 , Fe, Mg, Ca, Mo and SO_4

Symbols are explained in Section 5.23.
(+) or (-) = change in length not absolute, but relative to cell width.

	PO_4			Fe			Mg					
	Whole trichome	Basal region	Subapical region	Apical cell	Whole trichome	Basal region	Subapical region	Apical cell	Whole trichome	Basal region	Subapical region	Apical cell
<i>Calothrix</i> sp. D184	0	0	0	NA	0	0	+	0	(+)	0	+	0
<i>Calothrix</i> sp. D251	0	0	0	NA	0	0	0	NA	(+)	0	0	NA
<i>Calothrix</i> sp. D267	+	0	0	0	+	0	0	0	0	(+)	0	0
<i>Calothrix</i> sp. D283	+	0	0	+	0	0	(+)	0	0	0	0	0
<i>C. brevis</i> D156	0	0	0	(+)	+	0	0	0	+	0	0	0
<i>C. membranacea</i> D179	0	0	0	0	+	0	0	0	(+)	0	0	0
<i>C. scopulorum</i> D256	0	0	0	NA	0	0	(+)	0	0	(+)	0	0
<i>Dichothrix</i> sp. D280	0	0	0	NA	0	0	0	0	0	0	0	0
<i>Gloeotrichia ghosei</i> D277	0	0	0	NA	0	0	0	NA	0	0	0	0
<i>Rivularia</i> sp. D403	+	0	0	NA	0	0	0	NA	0	(+)	0	0
<i>Rivularia</i> sp. D404	0	0	0	NA	0	0	0	NA	0	(+)	0	0
<i>Homoeothrix</i> sp. D402	?	?	+	0	?	?	-	0	?	?	(+)	0
<i>H. crustacea</i> D401	?	?	+	NA	?	?	-	0	?	?	0	0
<i>Anabaena cylindrica</i> D2A	+	NA	NA	NA	+	NA	NA	NA	0	NA	NA	NA

	Ca			Mo			SO_4					
	Whole trichome	Basal region	Subapical region	Apical cell	Whole trichome	Basal region	Subapical region	Apical cell	Whole trichome	Basal region	Subapical region	Apical cell
<i>Calothrix</i> sp. D184	-	0	0	0	0	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D251	0	0	-	0	0	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D267	0	0	0	0	0	0	0	0	0	0	0	0
<i>Calothrix</i> sp. D283	0	-	0	0	0	0	0	0	0	0	0	0
<i>C. brevis</i> D156	-	0	0	0	0	0	0	0	0	0	0	0
<i>C. membranacea</i> D179	0	0	0	0	0	0	0	0	0	0	0	0
<i>C. scopulorum</i> D256	0	0	0	0	0	0	0	0	0	0	0	0
<i>Dichothrix</i> sp. D280	-	0	0	0	0	0	0	0	0	0	0	0
<i>Gloeotrichia ghosei</i> D277	0	0	0	0	0	0	0	0	0	0	0	0
<i>Rivularia</i> sp. D403	0	0	(-)	0	0	0	0	0	0	0	0	0
<i>Rivularia</i> sp. D404	-	0	0	0	0	0	0	0	0	0	0	0
<i>Homoeothrix</i> sp. D402	?	?	0	0	?	?	0	0	?	?	-	0
<i>H. crustacea</i> D401	?	?	0	0	?	?	0	0	?	?	0	0
<i>Anabaena cylindrica</i> D2A	-	NA	NA	NA	0	NA	NA	NA	0	NA	NA	NA

TABLE 5.7

Cytological changes in vegetative cells, and development of brown sheath pigment, in 13 strains of Rivulariaceae and *Anabaena cylindrica* under deficiencies of PO_4 , Fe, Mg, Ca, Mo and SO_4

Symbols are explained in Section 5.23.

B = buff; BG = blue-green; G = green; P = pinkish; PG = pale green; Y = yellow.

	PO_4			Fe			Mg			
	Cell colour of vegetative cells	Vacuolation of vegetative cells	Poly-phosphate granules	Cyano-physcin sheath pigment	Brown sheath pigment	Cell colour of vegetative cells	Vacuolation of vegetative cells	Poly-phosphate granules	Cyano-physcin sheath pigment	Brown sheath pigment
<i>Calothrix</i> sp. D184	PG	0	-	+	+	PG	0	-	+	+
<i>Calothrix</i> sp. D251	PG	0	-	+	+	PG	0	0	+	+
<i>Calothrix</i> sp. D267	Y	0	-	0	0	PG	0	0	0	0
<i>Calothrix</i> sp. D283	Y	0	-	0	0	PG	0	0	0	0
<i>C. brevisima</i> D156	PG	+	-	+	0	PG	0	-	+	0
<i>C. membranacea</i> D179	Y	0	-	0	0	PG	0	0	0	0
<i>C. scopulorum</i> D256	PG	0	-	+	+	PG	0	0	0	0
<i>Dichothrix</i> sp. D280	PG	0	-	+	+	PG	0	0	+	0
<i>Gloeotrichia ghosei</i> D277	B	+	-	0	0	PG	0	0	0	0
<i>Rivularia</i> sp. D403	G	0	-	0	0	PG	0	0	0	0
<i>Rivularia</i> sp. D404	PG	0	-	+	+	PG	0	+	0	0
<i>Homoeothrix</i> sp. D402	Y	0	-	?	0	BG	0	0	?	0
<i>H. crustacea</i> D401	Y	+	-	?	0	P	+	0	?	0
<i>Anabaena cylindrica</i> D2A	Y	0	-	+	0	PG	0	+	0	0

	Ca			Mo			SO_4			
	Cell colour of vegetative cells	Vacuolation of vegetative cells	Poly-phosphate granules	Cyano-physcin sheath pigment	Brown sheath pigment	Cell colour of vegetative cells	Vacuolation of vegetative cells	Poly-phosphate granules	Cyano-physcin sheath pigment	Brown sheath pigment
<i>Calothrix</i> sp. D184	Y	0	0	0	0	Y	0	+	?	0
<i>Calothrix</i> sp. D251	G	0	0	0	0	Y	0	+	?	0
<i>Calothrix</i> sp. D267	Y	0	0	0	0	Y	0	+	?	0
<i>Calothrix</i> sp. D283	Y	0	0	0	0	Y	0	+	?	0
<i>C. brevisima</i> D156	Y	0	0	0	0	Y	0	+	?	0
<i>C. membranacea</i> D179	Y	0	0	0	0	Y	0	+	?	0
<i>C. scopulorum</i> D256	G	0	0	0	0	Y	0	+	?	0
<i>Dichothrix</i> sp. D280	Y	0	0	0	0	Y	0	+	?	0
<i>Gloeotrichia ghosei</i> D277	B	0	-	0	0	Y	0	+	?	0
<i>Rivularia</i> sp. D403	Y	0	0	0	0	Y	0	?	?	0
<i>Rivularia</i> sp. D404	Y	0	0	0	0	Y	0	+	?	0
<i>Homoeothrix</i> sp. D402	Y	0	0	?	0	Y	+	+	?	0
<i>H. crustacea</i> D401	Y	+	0	?	0	Y	0	+	?	0
<i>Anabaena cylindrica</i> D2A	Y	0	0	0	0	Y	0	+	0	0

TABLE 5.8

Changes in heterocyst frequency and size in 11 strains of Rivulariaceae, and *Anabaena cylindrica*, under deficiencies of PO₄, Fe, Ca and Mo. (Magnesium and sulphate deficiencies did not affect these characters in any of these strains, and are therefore not included in the table).

Symbols are explained in Section 5.23

Het. = heterocyst; SB = Secondary basal heterocysts; I = intercalary heterocysts; PI = pseudointercalary heterocysts

Strain	PO ₄			Fe			Ca			Mo					
	Het. size	Het. frequency	Het. frequency	Het. size	Het. frequency	Het. frequency	Het. size	Het. frequency	Het. size	Het. frequency	Het. frequency	Het. size	Het. frequency		
	SB	I	PI	SB	I	PI	SB	I	PI	SB	I	PI	SB	I	PI
<i>Calothrix</i> sp. D184	0	0	0	+	0	+	+	+	+	0	0	+	0	0	+
<i>Calothrix</i> sp. D251	0	0	0	0	?	?	?	?	?	0	?	?	+	+	?
<i>Calothrix</i> sp. D267	0	0	0	-	+	+	+	+	0	0	0	0	+	+	+
<i>Calothrix</i> sp. D283	0	0	0	0	0	+	0	+	0	+	+	+	+	+	0
<i>C. brevisima</i> D156	-	0	0	0	+	+	0	+	+	+	+	+	+	+	0
<i>C. membranacea</i> D179	0	0	0	0	+	+	0	+	+	+	+	C	0	0	+
<i>C. scopulorum</i> D256	-	0	0	+	+	+	+	+	+	+	+	+	+	+	+
<i>Dichothrix</i> sp. D280	0	0	0	0	+	+	0	+	+	+	+	0	+	+	0
<i>Gloeotrichia ghosei</i> D277	+	0	0	0	+	+	0	+	+	+	+	+	+	+	+
<i>Rivularia</i> sp. D403	+	0	0	+	0	?	0	0	?	+	+	?	0	0	+
<i>Rivularia</i> sp. D404	0	0	0	0	0	?	0	0	?	0	0	?	0	0	+
<i>Anabaena cylindrica</i> D2A	0	NA	-	0	NA	0	0	NA	+	NA	+	NA	0	NA	+

TABLE 5.9

Changes in extent of spore development in Gloeotrichia ghosei D277 and Anabaena cylindrica D2A under deficiencies of PO₄, Fe, Mg, Ca, Mo and SO₄

+ = increase; - = decrease; 0 = no change

<u>Strain</u>	<u>Deficiency</u>					
	PO ₄	Fe	Mg	Ca	Mo	SO ₄
<u>Gloeotrichia ghosei</u> D277	+	-	-	-	0	-
<u>Anabaena cylindrica</u> D2A	+	+	-	-	0	0

5.3 Phosphate deficiency

5.31 Heterocystous strains

(i) Induction of deficiency

A single subculture into low phosphate medium was sufficient to induce obvious limitation of growth of all strains. Phosphate deficient cultures of Strains D2A, D179, D267 and D283 were yellow in colour, those of Strains D184, D251, D256, D280 and D404 were dark brown, and those of Strains D156 and D277 were buff coloured. Strain D403 differed from the others in that it appeared 'healthier' in low phosphate medium than in full medium. Its growth was initially more rapid in the low phosphate medium, and the cultures were brighter green. Nevertheless, the final yield was lower in the deficient medium, though the difference in yield was not apparent until quite late.

(ii) Effects on hair development (Table 5.4)

As described in Chapter 4, hair development increased in Strains D251, D277, D403 and D404, and Strains D184, D256 and D280 also produced long hairs. In each case, the development of hairs caused an increase in the total trichome length; measurements of hair length in Strains D256, D277, D403 and D404 are given in Tables 5.12, 5.14, 5.16 and 5.17 respectively. Figs 5.1, 5.2 and 5.3 show control and phosphate deficient trichomes of Strains D184, D256 and D403.

In six of the seven strains, the increase in hair frequency was observed before any macroscopic signs of phosphate deficiency; in Strain D256 the first observation of hairs coincided with the onset of obvious growth limitation (within the limits of the spacing of the observations).

In Strains D184, D251 and D256, the hairs which developed in low phosphate medium occasionally contained one to several apparently normal vegetative cells, in either an apical (Fig. 5.4a) or an intercalary (Figs 5.4b and 5.4c) position. The transition between these vegetative cells and the adjacent hair cells was usually abrupt (Fig. 5.4b), but there was sometimes a transition zone of partially vacuolated cells (Fig. 5.4c). This phenomenon was observed in hairs of different lengths and in cultures of various ages, including ones in which growth had apparently ceased. This seemed to reduce the likelihood of its being simply an early stage in hair development. The apparent absence of this phenomenon in

the other strains is not conclusive, since the character was not searched for systematically.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

The dimensions of trichomes from phosphate deficient and control cultures of Strains D156, D184, D256, D267, D277, D283, D403 and D404 are shown in Tables 5.10, 5.11, 5.12, 5.13, 5.14, 5.15, 5.16 and 5.17 respectively.

No effects on basal diameter were detected in any of the strains. The development of hairs by Strains D184, D256 and D280 caused a decrease in the apical diameter of the trichomes as a whole, but ignoring the presence of hairs, and comparing the width of the most distal vegetative cell in the two media, then only Strain D256 showed a decrease in subapical diameter under phosphate deficiency (Table 5.12). There was an increase in subapical width in Strain D277 (Table 5.14), and no apparent change in the other strains with hairs. In Strain D156 the apical one or two cells were often (frequency score = 3) narrower than in the control medium, but there was no significant difference in subapical width (Table 5.10).

The length of the vegetative region of the trichomes (excluding the hair) decreased in Strains D184 (Table 5.11) and D277 (Table 5.14), increased in Strain D403 (Table 5.16) and was unchanged in Strains D251, D256 (Table 5.12), D280 and D404 (Table 5.17).

Apart from changes associated with hair development, there was no effect of phosphate deficiency on tapering of trichomes, apart from a slight decrease in Strain D277 and a nominal increase in Strain D156.

(iv) Effects on the shape of vegetative cells (Table 5.6)

In Strain D403 the vegetative cells were rather longer in phosphate deficient trichomes than in control trichomes (Figs 5.3a and 5.3b). In the other six strains in which hair development had increased or been induced, the cell shapes of trichomes with hairs appeared the same as those of control trichomes, apart from a short transition zone between the vegetative cells and the hair, where the cells were elongated and narrowed. There was an increase in the average length of the vegetative cells in two more strains of Rivulariaceae, D267 (Fig. 5.5) and D283 (Fig. 5.6), and in Anabaena cylindrica D2A. This increase generally occurred throughout the trichome, with no evident polarity of response, but in

Strain D283 the apical cell was sometimes (frequency score = 2) rather more markedly elongated (Fig. 5.6). The slightly narrower apical cells in some trichomes of Strain D156 were mentioned above.

(v) Cytological effects other than hair formation (Table 5.7)

Only Rivularia sp. D403 retained its original (bright green) cell colouration under phosphate deficiency. Cells of the other strains became pale green, yellow or buff in colour (Table 5.7). In older cultures of Strains D2A, D156, D179, D267 and D283 some of the cells lysed, causing fragmentation of many of the trichomes.

In two strains, D156 and D277, the vegetative cells of some of the trichomes (frequency score = 2) became vesiculated. This was quite distinct from hair formation, since the vacuoles never occupied more than about 10% of the cell profile, and there was no marked change in cell dimensions. In both strains this vacuolation generally occurred in cells along the whole length of the trichomes, but in Strain D156 the vesicles were sometimes confined to the apical one or two cells; this was not necessarily correlated with the narrowing of these cells mentioned in (ii) above.

There was a decrease in polyphosphate granulation in every strain. In all the strains of Rivulariaceae the granules were lost from the trichome apices before they disappeared from the basal cells. This was possibly due to the fact that the total amount of polyphosphate was greater in the basal cells (Table 5.3), so that a uniform decrease in granulation along the trichome would affect these cells relatively less than the more sparsely granulated apical cells. In most cases, polyphosphate granules were undetectable in any of the trichomes after a few days' growth in the first subculture to low phosphate medium, but in Strains D277, D403 and D404 polyphosphate granulation was more persistent. Though reduced in number, granules were still apparent after four weeks in the first subculture, even in trichomes with long hairs. In Strain D404 a few (frequency score = 1) of the trichomes were granulated, but in the basal region only; in Strain D277 some trichomes (frequency score = 2) had granules in all the vegetative cells, but at a lower level than in control trichomes; in Strain D403 all the trichomes were granulated, but at an estimated 50% of the control level (which was high in this strain: Table 5.3; p.148). A second subculture into low phosphate medium achieved

complete loss of granules for Strains D277 and D404, but in Strain D403 every trichome was still granulated, though now only in the basal region. Only after a third subculture, scored at three weeks, was total loss of polyphosphate observed for this strain.

There was a marked increase in cyanophycin granulation in all 12 strains (Chapter 4). Generally, all the vegetative cells were granulated, but granules were often, though not always, absent from the apical one or two cells in Strains D156, D267 and D283, whether or not these cells were elongated or narrowed. In Strains D256 and D277 cyanophycin granules were not uncommonly observed in the cytoplasmic strands of the hair cells.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

An increase in sheath thickness was observed in phosphate deficient cultures of all the strains of Rivulariaceae, and in Anabaena cylindrica there was an obvious increase in the production of mucilage.

As mentioned in Chapter 4, almost every trichome of phosphate deficient cultures of Strains D184, D251, D256, D283 and D404 had a dark brown sheath.

(vii) Effects on heterocysts (Table 5.8)

The heterocysts in phosphate deficient cultures of Anabaena cylindrica D2A were rather more widely spaced than those in control trichomes (separated by 25-50 cells, rather than 15-20 cells). There was no obvious effect of phosphate deficiency on the frequency of heterocysts in the other strains examined.

Heterocyst dimensions were measured for seven strains; three of these (D256: Table 5.12; D267: Table 5.13; D283: Table 5.15) showed no significant change in heterocyst size. In Strain D403 (Table 5.16) there was an increase in heterocyst length; in Strain D184 (Table 5.11) there was a decrease in heterocyst length, and in Strains D156 (Table 5.10) and D277 (Table 5.14) there was a decrease in heterocyst width.

(ix) Response of phosphate deficient cultures to the addition of phosphate (Table 5.18)
(Includes responses of Homoeothrix strains)

K_2HPO_4 (for HAD) or KH_2PO_4 (for HChu 10-D) was added to the phosphate deficient cultures to give the normal level of PO_4-P . The pH of the HEPES-buffered media remained steady to within 0.1 unit following the addition. At the time the addition was made,

polyphosphate granules were completely absent from all the cultures. Polyphosphate granules appeared in the cells very soon after the addition of PO_4-P ; since this was such a marked and rapid response, it was studied over a shorter time scale than was used in scoring responses to the addition of other nutrients. Following the addition of phosphate, samples were taken for examination and polyphosphate staining after 5, 10, 20 and 30 min, and 1, 2, 3, 16 and 24 h; thereafter the cultures were examined every 24 h until they had fully recovered and growth had recommenced.

Table 5.18 summarizes the times at which various responses were observed. For ease of comparison, the responses of the two Homoeothrix strains are described in this section also.

a) Granulation of vegetative cells

Polyphosphate granules appeared in the vegetative cells of all the strains within minutes of the addition of phosphate. In the majority of strains granules were visible when the algae were first examined, after 5 min, but they were slower to appear in Strains D251 and D403 (10 min), D277 and D404 (20 min) and D401 (30 min). It seemed possible that the slower response of Strain D401 might have been due to its lower incubation temperature ($15^{\circ}C$; Table 5.1); the experiment was therefore repeated for this strain at $25^{\circ}C$ (with a 24 h pre-incubation at $25^{\circ}C$ before the addition of phosphate), but there was little difference in response between the two temperatures.

In all strains, when granules were first observed, they were present in all the vegetative cells, and the absolute amount of granulation was very similar in each cell along the trichome. The granules increased in size and number during the incubation; the absolute amount of granulation was approximately the same in each cell for the first 30-60 min, but in the Rivulariaceae the total granulation of the larger basal cells later became greater than that of the other cells. This was probably due to their greater size, since the actual cytoplasmic density of granules appeared very similar in all cells.

It was rather difficult to determine the time at which the granulation reached its maximum level, since once the granule density was very high, it was hard to estimate further increases.

However, most of the strains showed little further increase in granulation in the vegetative cells after 2-3 h; Strains D403 and D251 did not reach this endpoint until 16 and 24 h respectively. In all 14 strains, the maximum level of granulation reached was higher than that of control trichomes. Every vegetative cell became densely granulated (scoring 4-5: Section 2.24), and the basal-apical gradients of granule density characteristic of control trichomes (Table 5.3) were not apparent. As active growth recommenced, granulation returned to the normal control level.

b) Granulation of hair cells

In all the strains with hairs, polyphosphate granules also developed in the cytoplasmic strands of the hair cells. There was some variation in the pattern and rate of this response. In Strains D251, D256, D280 and D401 granules were first observed in the hair cells at the same time as in the vegetative cells, but in Strains D184, D277, D403 and D404 the hair cells did not appear granulated until some time after the vegetative cells. In Strain D256, the normal vegetative cells that were occasionally seen in a series of hair cells (cf. (ii) above) showed granulation the same as that of the other vegetative cells (i.e. greater than that of the adjacent hair cells).

In Strains D251, D401 and D403 the majority of the hair cells developed polyphosphate granules, but in Strains D256, D277, D280 and D404 no more than 10% (estimated) of these cells became granulated. Often a single hair contained both granulated and non-granulated cells, with no obvious pattern in their distribution. In Strain D184 only a few hair cells were granulated initially, but after longer incubation the majority had visible granules. There was an increase in the size and number of granules in the hair cells of all strains during the incubation, but only Strain D184 showed an increase in the frequency of hair cells showing granulation.

c) Shedding of hairs

Within about 48 h of the addition of phosphate, the vegetative cells began to divide in all the cultures, but no evidence was obtained for the division of hair cells in any of the strains. Hairs were eventually lost in all strains following the addition

of phosphate; at the time of shedding, the hair cells still contained the maximum level of polyphosphate reached earlier in the incubation. Two distinct types of response were observed in the shedding of hairs. In Strains D184, D251, D256 and D280, hair loss occurred in association with the release of hormogonia, which occurred about two days after the addition of phosphate. The hairs sometimes seemed to lyse completely, but more often appeared to be shed more or less intact, by lysis of only a few cells at the base of the hair. In the early phases of hormogonium release, free hormogonia were quite often observed with a few residual hair cells or transition zone cells still attached.

In Strains D277, D401, D403 and D404 the hairs were lost at a much earlier stage. Obvious fragmentation of the hairs began within 2-3 h of the addition of phosphate, and no intact hairs were visible in the cultures after 16 h. These changes occurred in the absence of any evident cell division of the vegetative cells. In Strains D403 and D404 the hairs were shed by lysis of cells at their base, leaving a short series of transition zone cells at the trichome apex. Hormogonia were not released until some hours later. In Strains D277 and D401 hairs were also lost in this manner, but there was much more extensive lysis of hair cells other than those at the hair base. Many of the vegetative cells also lysed in these two strains, at various points along the trichomes, causing them to fragment into short lengths. The end result was rather similar to a mass development of hormogonia, except that the fragments derived from all parts of the trichomes, not only from the apices, and they did not show gliding motility like the normal hormogonia in control cultures of these strains. Many of the fragments nevertheless later appeared to develop into normal tapered trichomes.

As a check that the very rapid loss of hairs that occurred in these four strains was not due to an osmotic effect of the added phosphate, the experiment was repeated, for Strain D277, with a solution of NaCl of the same ionic concentration as the K_2HPO_4 used in the earlier experiment. The addition of NaCl had no effect on the morphology of the phosphate deficient trichomes during 7 days' incubation, which suggested that osmotic effects were unlikely to be important in causing the lysis of the hair cells.

In Strains D251, D277, D403 and D404, which normally possessed

hairs in the control medium, hairs again developed after the initial phase of hormogonium release, but with a length and frequency characteristic of control cultures. All the cultures reached a normal full yield within 7-10 days of adding phosphate.

Table 5.10 Trichome dimensions (μm) of Calothrix sp. D156, grown for 19 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$
 Measurements were restricted to trichomes with basal diameters $\geq 6.5 \mu\text{m}$.
 Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
basal width	6.8 ± 0.11	6.6 ± 0.07	0.2 ± 0.13	0.083
subapical width	5.3 ± 0.10	5.4 ± 0.07	0.1 ± 0.10	0.741
apical width	5.2 ± 0.11	4.7 ± 0.03	0.5 ± 0.11	0.001
length	191 ± 12	182 ± 11	9 ± 16	0.594
heterocyst width	7.4 ± 0.12	6.6 ± 0.13	0.8 ± 0.15	< 0.001
heterocyst length	7.2 ± 0.14	6.8 ± 0.28	0.4 ± 0.29	0.180

Table 5.11 Trichome dimensions (μm) of Calothrix sp. D184 grown for 24 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$
 Measurements were restricted to trichomes with basal diameters $\geq 9 \mu\text{m}$.
 Each value is the mean of 20 measurements.

(It was not possible to make accurate measurements of hair length in this strain, as the hairs were very long, and densely interwoven.)

	control mean	P-deficient mean	difference	probability
basal width	10.1 ± 0.23	10.7 ± 0.25	0.6 ± 0.33	0.100
subapical width	4.4 ± 0.08	4.3 ± 0.09	0.1 ± 0.10	0.300
hair width	-	2.8 ± 0.08	-	-
trichome length (minus hair)	396 ± 24	144 ± 19	252 ± 32	< 0.001
heterocyst width	8.6 ± 0.25	8.3 ± 0.36	0.3 ± 0.41	0.438
heterocyst length	8.3 ± 0.23	6.3 ± 0.23	2.0 ± 0.30	< 0.001

Table 5.12 Trichome dimensions (μm) of Calothrix scopulorum D256 grown for 20 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$

Measurements were restricted to trichomes of basal diameters 8.0-9.0 μm .

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
basal width	8.3 \pm 0.08	8.4 \pm 0.09	0.1 \pm 0.14	0.258
subapical width	3.4 \pm 0.05	3.0 \pm 0.05	0.4 \pm 0.06	<0.001
hair width	-	2.0 \pm 0.05	-	-
trichome length (minus hair)	101 \pm 7	120 \pm 9	19 \pm 12	0.154
hair length	-	361 \pm 60	-	-
total trichome length	101 \pm 7	481 \pm 63	380 \pm 66	<0.001
hair as % trichome length	-	71 \pm 2.4	-	-
heterocyst width	6.0 \pm 0.25	6.6 \pm 0.16	0.6 \pm 0.24	0.023
heterocyst length	5.1 \pm 0.19	5.1 \pm 0.18	0.0 \pm 0.21	0.853

Table 5.13 Trichome dimensions (μm) of Calothrix sp. D267 grown for 22 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$

No restriction was placed on the basal diameter of the trichomes measured.

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
basal width	5.5 \pm 0.15	5.1 \pm 0.14	0.4 \pm 0.22	0.092
subapical width	2.8 \pm 0.06	3.0 \pm 0.05	0.2 \pm 0.07	0.038
length	109 \pm 7	165 \pm 13	56 \pm 17	0.005
heterocyst width	5.4 \pm 0.16	5.0 \pm 0.28	0.4 \pm 0.38	0.288
heterocyst length	5.8 \pm 0.19	4.8 \pm 0.15	1.0 \pm 0.26	0.002

Table 5.14 Trichome dimensions (μm) of Gloeotrichia ghosei D277 grown for 16 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$

Measurements were restricted to trichomes of basal diameter $6.5 \mu\text{m}$.

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
subapical width	4.4 ± 0.13	5.3 ± 0.59	0.9 ± 0.20	<0.001
hair width	3.0 ± 0.18	2.3 ± 0.13	0.7 ± 0.23	0.007
trichome length (minus hair)	478 ± 38	246 ± 31	232 ± 53	<0.001
hair length	97 ± 13	1816 ± 219	1719 ± 227	<0.001
total trichome length	557 ± 41	2069 ± 231	1492 ± 242	<0.001
hair as % trichome length	17 ± 2	84 ± 3	67 ± 4	<0.001
heterocyst width	10.8 ± 0.28	8.9 ± 0.30	1.9 ± 0.45	<0.001
heterocyst length	12.2 ± 0.34	10.6 ± 0.39	1.6 ± 0.48	0.005

Table 5.15 Trichome dimensions (μm) of Calothrix sp. D283 grown for 32 days in HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$

Measurements were restricted to trichomes of basal diameter $\geq 5.5 \mu\text{m}$.

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
basal width	6.1 ± 0.10	6.0 ± 0.09	0.1 ± 0.11	0.614
subapical width	2.9 ± 0.06	3.0 ± 0.06	0.1 ± 0.08	0.500
length	197 ± 20	204 ± 17	7 ± 24	0.769
heterocyst width	6.2 ± 0.15	6.4 ± 0.14	0.2 ± 0.17	0.464
heterocyst length	6.2 ± 0.23	5.5 ± 0.25	0.7 ± 0.39	0.062

Table 5.16 Trichome dimensions (μm) of Rivularia sp. D403 grown for 17 days in the second subculture to HAD with 1.0x and 0.01x the normal level of $\text{PO}_4\text{-P}$

No restriction was placed on the basal diameter of the trichomes measured, but all measurements were made on trichomes with hairs.

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
basal width	6.4 \pm 0.08	6.0 \pm 0.18	0.4 \pm 0.22	0.038
subapical width	2.1 \pm 0.07	2.1 \pm 0.09	0.0 \pm 0.12	1.000
hair width	1.5 \pm 0.03	1.6 \pm 0.05	0.1 \pm 0.06	0.171
trichome length (minus hair)	83 \pm 5	155 \pm 7	72 \pm 11	<0.001
hair length	14 \pm 1	99 \pm 14	85 \pm 14	<0.001
total trichome length	97 \pm 6	254 \pm 19	157 \pm 20	<0.001
hair as % trichome length	15 \pm 1.2	36 \pm 2.9	21 \pm 3.1	<0.001
heterocyst width	6.2 \pm 0.09	6.4 \pm 0.18	0.2 \pm 0.19	0.541
heterocyst length	5.8 \pm 0.14	8.1 \pm 0.21	2.3 \pm 0.23	0.001

Table 5.17 Extent of hair development in Rivularia sp. D404 grown for 28 days in HChu 10-D with 1.0x and 0.05x the normal level of $\text{PO}_4\text{-P}$ (measurements in μm)

No restriction was placed on the basal diameter of trichomes measured, but all measurements were made on trichomes with hairs.

Each value is the mean of 20 measurements.

	control mean	P-deficient mean	difference	probability
trichome length (minus hair)	338 \pm 29	369 \pm 18	31 \pm 26	0.251
hair length	206 \pm 22	788 \pm 50	582 \pm 57	<0.001
total trichome length	544 \pm 36	1157 \pm 60	613 \pm 64	<0.001
hair as % trichome length	39 \pm 3	67 \pm 1	28 \pm 3	<0.001

TABLE 5.18

Timing of responses following addition of phosphate to phosphate-starved cultures of 13 strains of Rivulariaceae and Anabaena cylindrica
 Samples were examined after 5, 10, 20, 30 min and 1, 2, 3, 16, 24, 72, 96, 120, 144 h
 (Section 5.21 (ix))

	Vegetative cells		Polyphosphate granulation		Hair cells		Morphological changes		
	Granules first seen	Granules at maximum	Granules first seen	Granules at maximum	Frequency at hair cells Initially	At maximum	First loss of hairs	Hairs completely lost	First release of hormogonia
<i>Calothrix</i> sp. D184	5 min	3 h	2 h	24 h	+	5	48 h	120 h	48 h
<i>Calothrix</i> sp. D251	10 min	48 h	10 min	48 h	4	4	72 h	144 h	72 h
<i>Calothrix</i> sp. D267	5 min	3 h	NA	NA	NA	NA	NA	NA	NA
<i>Calothrix</i> sp. D283	5 min	2 h	NA	NA	NA	NA	NA	NA	NA
<i>C. brevissima</i> D156	5 min	3 h	NA	NA	NA	NA	NA	NA	NA
<i>C. membranacea</i> D179	5 min	2 h	NA	NA	NA	NA	NA	NA	NA
<i>C. scopulorum</i> D256	5 min	3 h	5 min	48 h	1	1	48 h	72 h	48 h
<i>Dichothrix</i> sp. D280	5 min	3 h	5 min	48 h	1	1	48 h	72 h	48 h
<i>Gloeotrichia ghosei</i> D277	20 min	2 h	30 min	2 h	+	+	2 h	16 h	*
<i>Rivularia</i> sp. D403	10 min	16 h	2 h	16 h	4	4	2 h	16 h	16 h
<i>Rivularia</i> sp. D404	20 min	3 h	1 h	3 h	+	+	3 h	16 h	24 h
<i>Homoeothrix</i> sp. D402	5 min	2 h	NA	NA	NA	NA	NA	NA	NA
<i>H. crustacea</i> D401	30 min	3 h	30 min	3 h	5	5	3 h	16 h	*
<i>Anabaena cylindrica</i> D2A	5 min	3 h	NA	NA	NA	NA	NA	NA	NA

* = In strains D277 and D401, normal hormogonium release was not observed (Section 5.21 (ix))
 NA = Character not applicable.

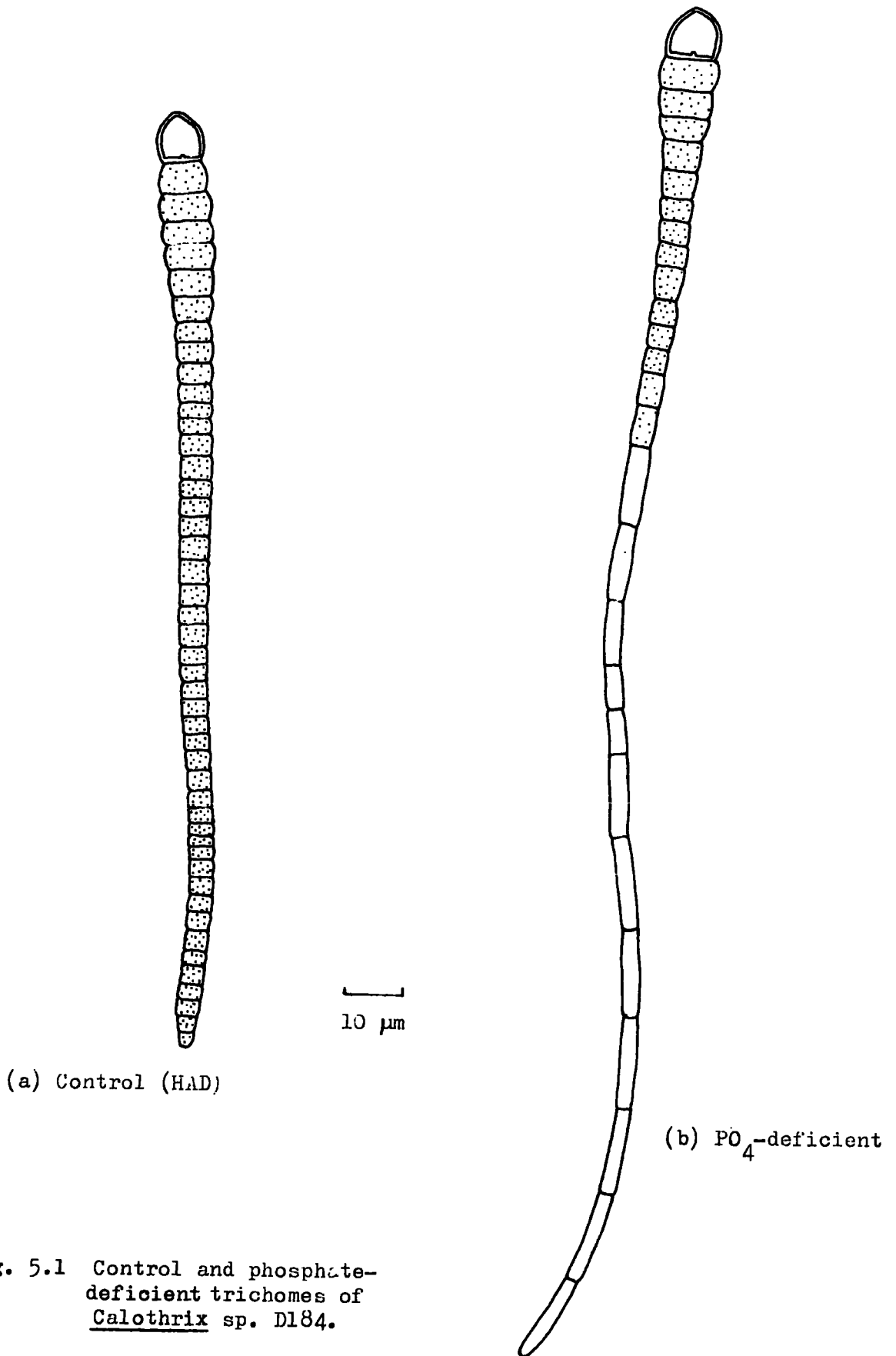


Fig. 5.1 Control and phosphate-deficient trichomes of Calothrix sp. D184.

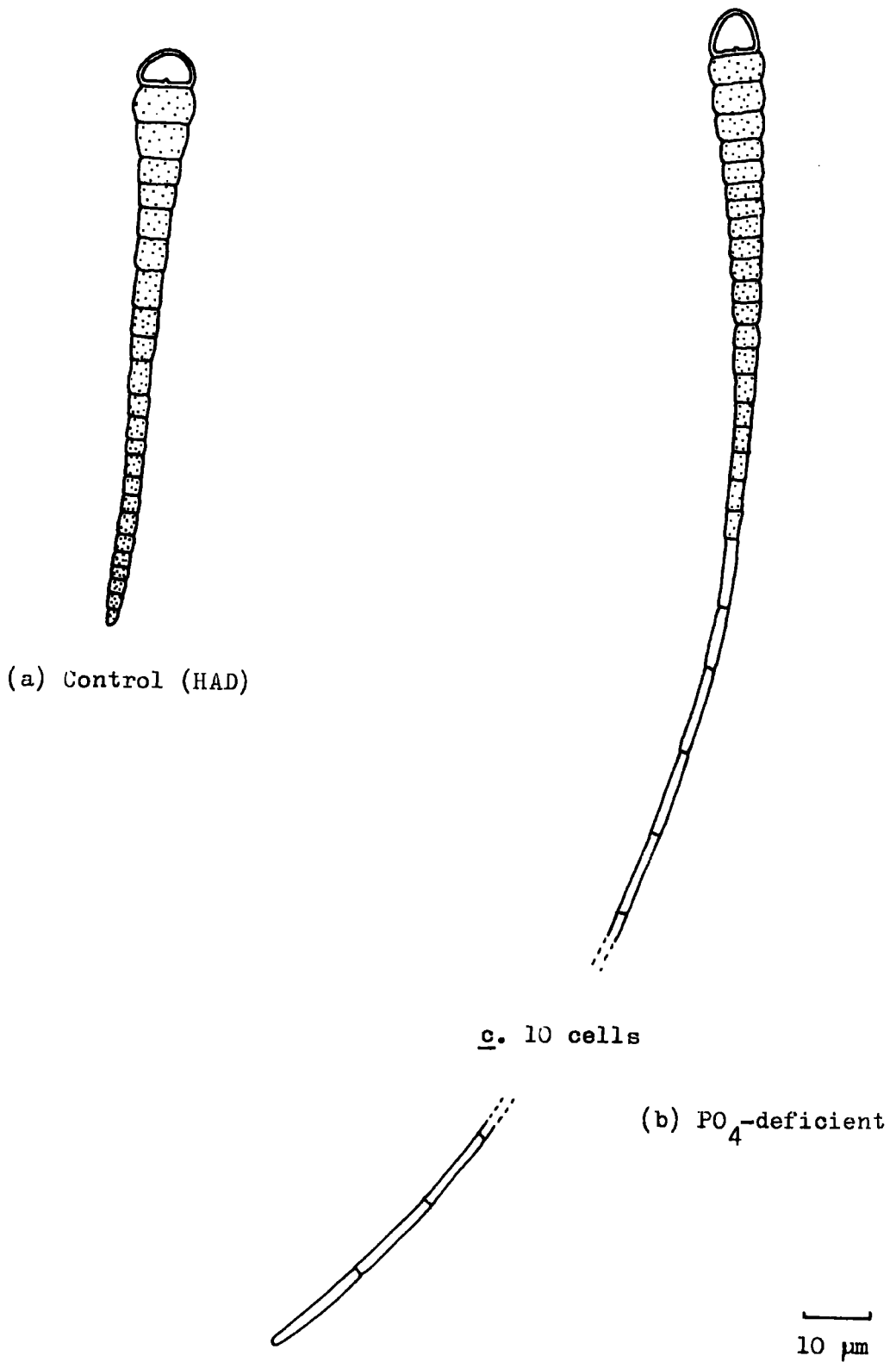


Fig. 5.2 Control and phosphate deficient trichomes of Calothrix scopulorum D256.

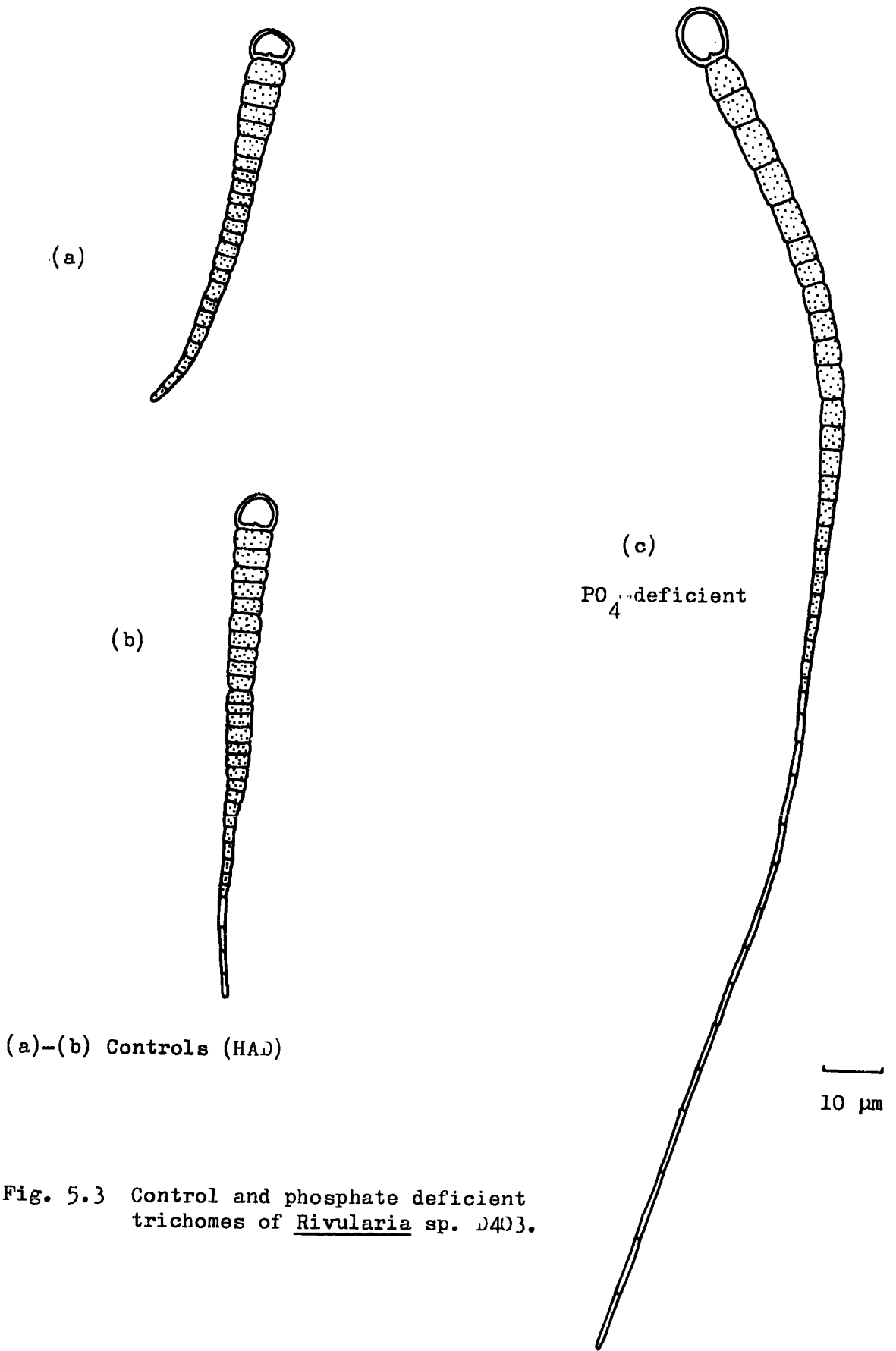
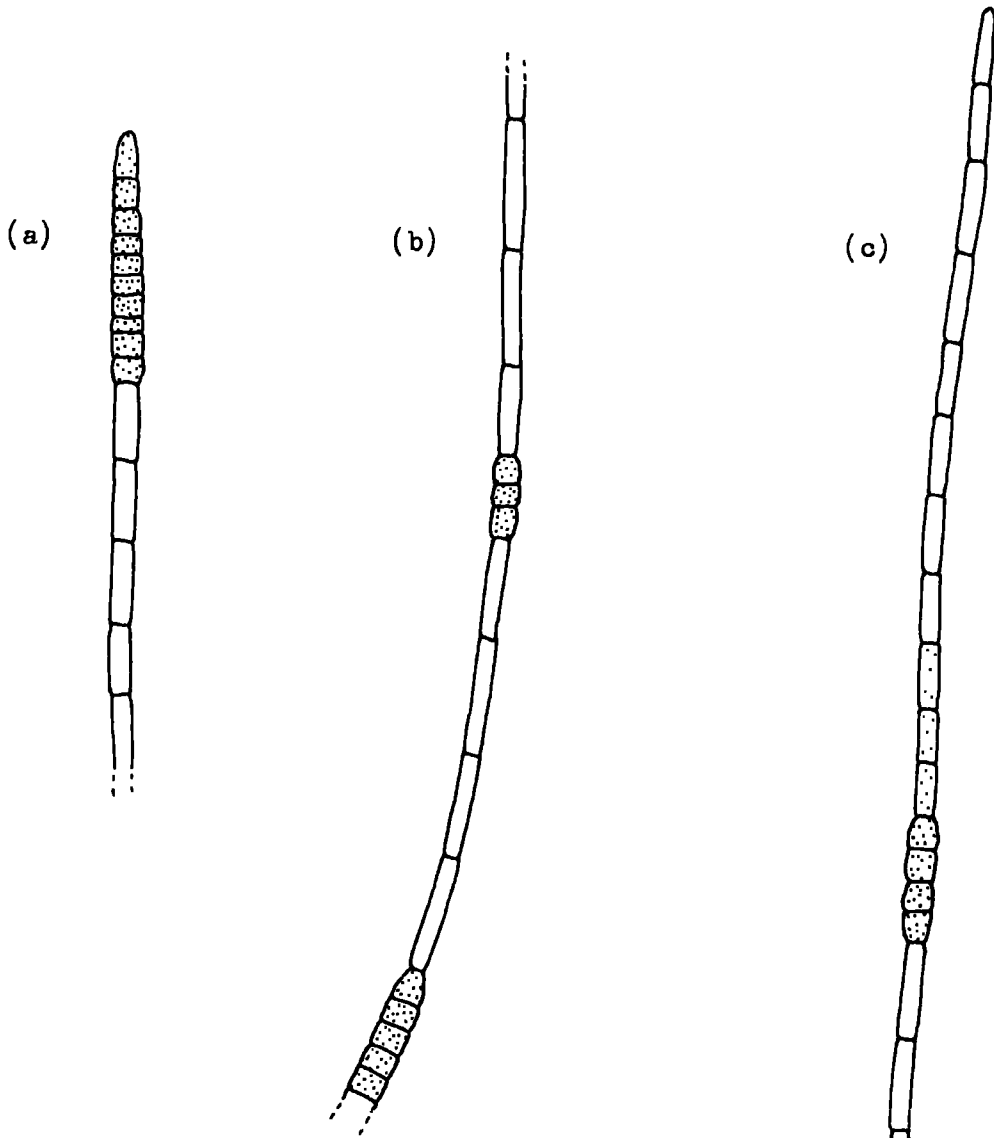


Fig. 5.3 Control and phosphate deficient trichomes of Rivularia sp. D403.



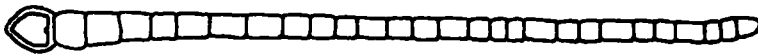
(a)-(b) Calothrix sp. D184

10 μ m

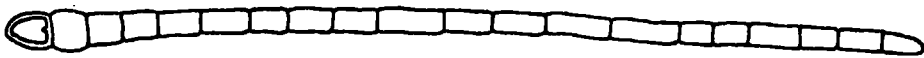
Fig. 5.4 Hairs of phosphate deficient Calothrix sp. D184 and Calothrix sp. D251, showing intercalary vegetative cells.

(c) Calothrix sp. D251

10 μm

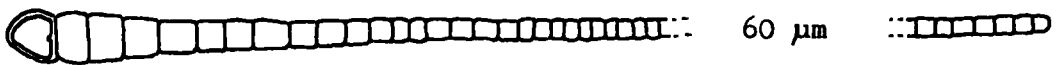


(a) Control (HAD)

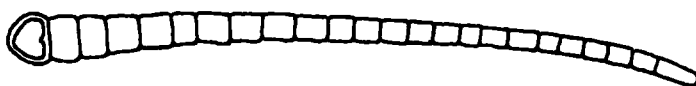


(b) PO_4 -deficient

Fig. 5.5 Control and phosphate deficient trichomes of Calothrix sp. D267.



(a) Control (HAD)



(b) PO_4 -deficient

Fig. 5.6 Control and phosphate deficient trichomes of Calothrix sp. D283

5.32 Homoeothrix strains

(i) Induction of deficiency

Growth limitation and morphological symptoms of deficiency were apparent in both strains after a 'few days' growth in the first subculture to low phosphate medium. Cultures of Strain D401 became brownish yellow in colour, and those of Strain D402 yellow.

(ii) Effects on hair development (Table 5.4)

No hairs were produced by Strain D402, but many long colourless hairs (frequency score = 5) developed in Strain D401 in low phosphate medium, appearing at about the same time as limitation of yield. The cells of these hairs were highly vacuolated, much narrower than normal apical cells, and as much as ten times as long as broad. They were thus quite distinct from the short series of partly vacuolated cells which normally developed at the apices of trichomes under nitrate deficiency (Section 3.33). Fig. 5.8 shows a short hair developed at the apex of a phosphate limited trichome of Strain D401 (cf. Fig. 3.4, which illustrates trichome apices of this strain under nitrate limitation).

(iii) Effects on trichome dimensions (Table 5.5)

There was no effect upon the trichome dimensions of Strain D401, apart from the marked narrowing of the apical cells in association with hair development. In Strain D402 also there was no significant difference in subapical diameter between the two media, but the enlarged apical cells (Section 3.33) were wider in the low phosphate medium (Table 5.19; Fig. 5.7).

Table 5.19 Subapical and apical width (μm) of Homoeothrix sp. D402 grown for 11 days in HChu 10-D with 1.0x and 0.05x the normal level of $\text{PO}_4\text{-P}$

Each value is the mean of 30 measurements.

	control mean	P-deficient mean	difference	probability
subapical width	1.93 \pm 0.01	1.95 \pm 0.01	0.02 \pm 0.01	0.023
apical width	1.95 \pm 0.01	2.21 \pm 0.05	0.26 \pm 0.04	<0.001

(iv) Effects on the shape of vegetative cells (Table 5.6)

Both strains of Homoeothrix showed an increase in the length of the vegetative cells along the whole trichome (in addition to

the changes associated with hair development in Strain D401). In Strain D402 the cells reached length:width ratios of 1.5:1 to 3.0:1; the maximum length reached was greater than that seen in nitrate limited control cultures (cf. Figs 5.7 and 3.5). Enlarged apical cells like those seen in control trichomes also developed in low phosphate cultures of Strain D402; they were both wider and longer than those in the control medium (Table 5.19; Fig. 5.7).

(v) Cytological effects other than hair formation (Table 5.7)

The vegetative cells of both strains became pale green and then yellowish green. In older (10 days) cultures of Strain D402 many trichomes (frequency score = 4) developed one or more small vesicles in the apical 1-4 cells, sometimes giving them a spongy appearance. Apart from the enlargement of the apical cell, these vacuolated cells did not otherwise differ from those immediately below. In Strain D401, small vacuoles developed in the elongated vegetative cells along the whole length of the trichome.

Polyphosphate granules became undetectable in both strains after a few days in low phosphate medium. As in the heterocystous strains, the basal few cells retained their granules for longer than the other cells.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

There was no obvious effect on sheath thickness in either strain, and no sheath pigmentation was observed.

(vii) Response of phosphate deficient cultures to the addition of phosphate (Table 5.18)

Responses to the addition of phosphate were described in Section 5.31(ix), with those of the heterocystous strains.

5.33 Summary of results

a) Heterocystous strains

1. 7 of the 11 strains of Rivulariaceae (D184, D251, D256, D277, D280, D403, D404) showed a marked increase in hair development under phosphate deficiency; 4 of these strains had at least some hairs in full medium, but the other 3 had none. The 4 remaining strains did not develop hairs, but in all except D179 there were slight modifications in the apical one or two cells.

In older phosphate deficient cultures of Strains D2A, D156, D179, D267 and D283 many of the trichomes fragmented as a result of cell lysis. Such fragmentation was not observed in cultures of the strains which developed hairs.

2. There was a loss of polyphosphate granules, and an increase in cyanophycin granulation in every strain (cf. Chapter 4). The vegetative cells became less green in colour in every strain except D403.

3. Large deposits of polyphosphate rapidly formed in the vegetative cells of all the strains following the addition of phosphate to phosphate starved cultures. The granules first appeared rather later in the four strains which normally had at least some hairs in the control medium (D251, D277, D403, D404). In all strains the granules appeared simultaneously in each vegetative cell along the trichome. Granules also formed in the cytoplasmic strands of the hair cells in the 7 strains with hairs.

4. The hairs were lost following the addition of phosphate. In each case the hair cells contained their maximum level of polyphosphate at the time they were shed. In Strains D184, D251, D256 and D280 the loss of hairs took place in association with the release of hormogonia, about 48 h after the addition of phosphate, but in Strains D277, D403 and D404 the hairs were shed within 2-3 h, and in Strain D277 many of the vegetative cells also lysed. All 4 strains in the latter group were ones with hairs in the control medium.

b) Homoeothrix strains

1. Only Strain D401 developed hairs under phosphate deficiency. These were much longer and more frequent than the ones seen in nitrate limited control cultures.

2. The vegetative cells of both strains became rather elongated, and in Strain D401 they developed small vacuoles. Both strains became yellow under phosphate deficiency. The enlargement of the apical cells that occurred in some trichomes of Strain D402 was more marked in low phosphate medium than in control medium, and these cells sometimes developed small vacuoles.

3. Like the heterocystous strains, both strains of Homoeothrix rapidly synthesized polyphosphate granules following the addition of phosphate to the phosphate starved cultures. In Strain D401 the hair cells also developed polyphosphate granules. The hairs of Strain D401 lysed 2-3 h after the addition of phosphate, and as observed in Strain D277, many of the vegetative cells also lysed.

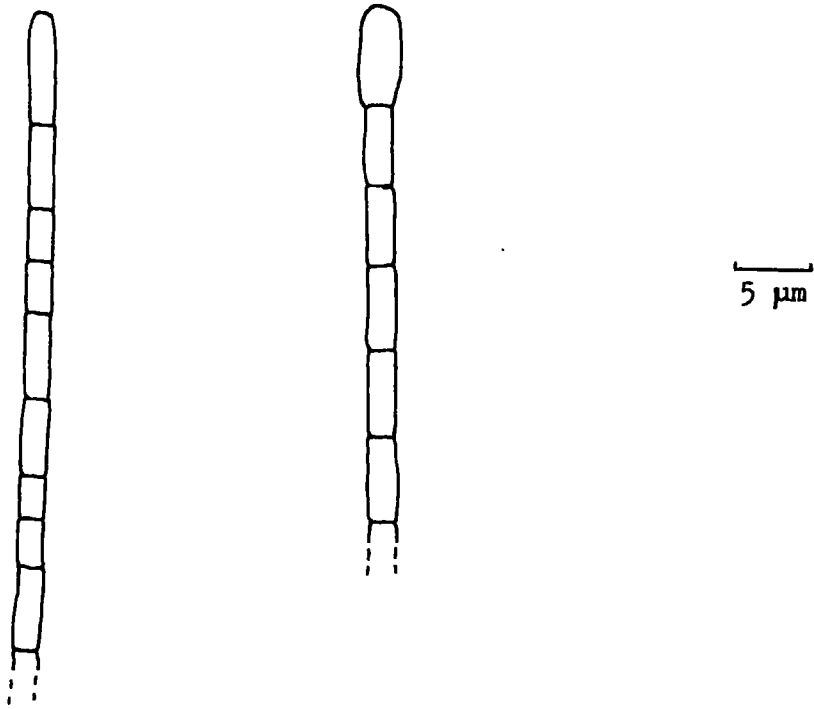


Fig. 5.7 Trichome apices of phosphate deficient Homoeothrix sp. D402 (see pp. 175-176); cf. control trichomes in Figs 2.5b and 3.5.

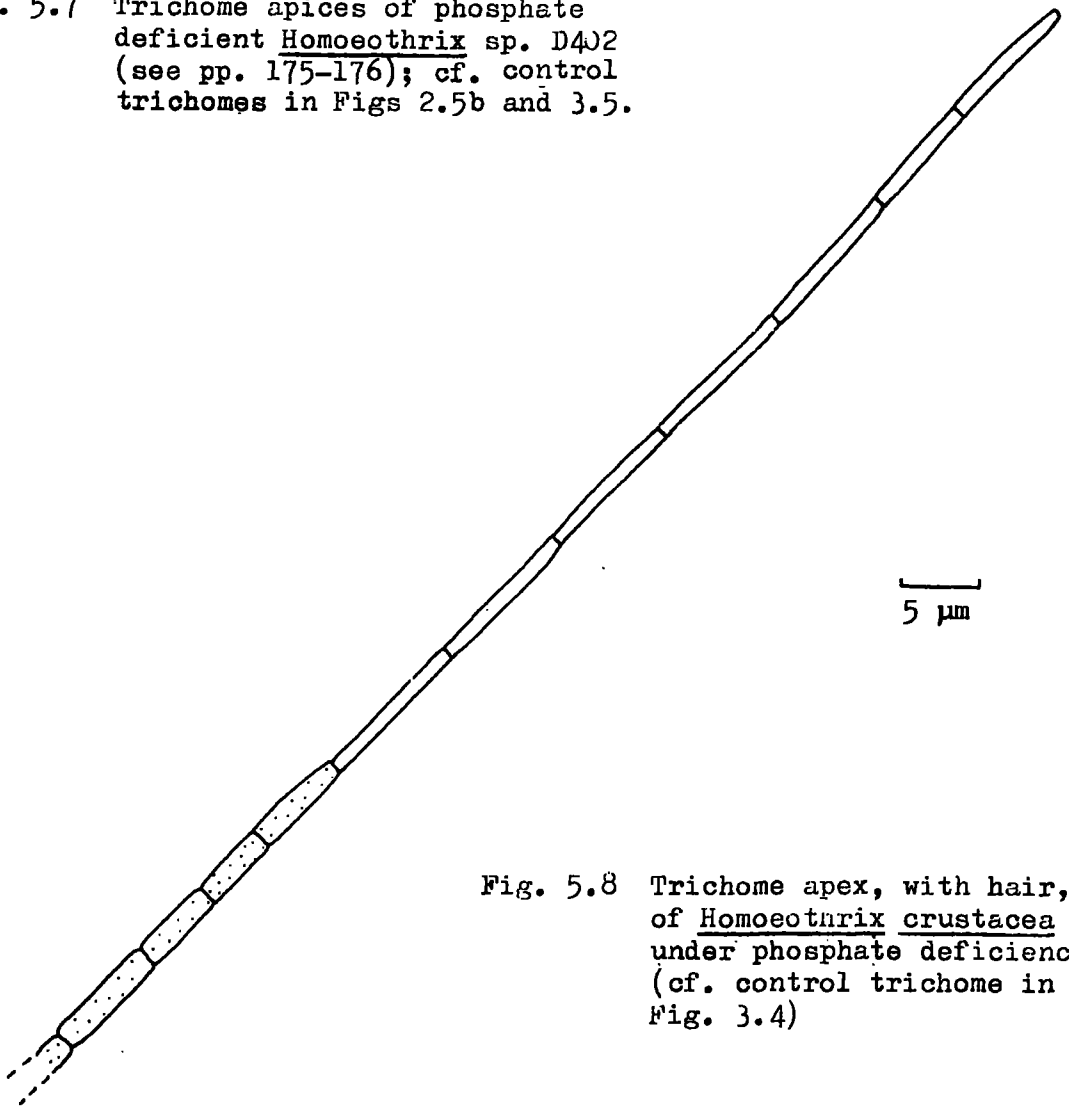


Fig. 5.8 Trichome apex, with hair, of Homoeothrix crustacea D401 under phosphate deficiency. (cf. control trichome in Fig. 3.4)

5.4 Iron deficiency

5.4.1 Heterocystous strains

(i) Induction of deficiency

All strains except one showed growth limitation after a single subculture into HEPES-buffered medium without added iron; Strain D403 required a further subculture. Iron deficient cultures of Strains D2A, D179, D184, D256, D267, D283 and D403 became initially pale bluish green, and latterly yellow; those of Strains D156, D277 and D404 were buff coloured, and those of Strains D251 and D280 were brown.

(ii) Effects on hair development (Table 5.4)

A high proportion of trichomes developed long hairs in Strains D251, D256 and D403. The extent of hair development in Strains D256 and D403 is illustrated by the trichome dimensions given in Tables 5.22a and 5.25; Figs 5.9b and 5.13 show iron deficient trichomes of these strains. In iron deficient cultures of Strain D184, a few trichomes (frequency score = +) developed very short hairs of not more than one or two cells (Fig. 5.10c). There was no increase in the frequency or length of hairs in this strain even after several weeks of culture in the initial medium, and three successive subcultures through iron deficient medium. In Strain D277 some of the trichomes (frequency score = 1) developed hairs which were longer than those of control trichomes (up to 50% of the total trichome length, as against 5-20% for the control), though there was no obvious increase in the total number of hairs.

In Strains D184, D251 and D277 the effects on hair development were first observed at about the same time as growth limitation became apparent. In Strain D403 hairs appeared before any signs of growth limitation, but in Strain D256 they were observed only after the onset of growth limitation. In Strains D251 and D256, vegetative cells were occasionally observed in an intercalary position in the hairs, very much as described for phosphate deficient cultures (Section 5.3(ii); p. 157). This was not observed in the other strains, but no systematic search was made.

There was no effect on the frequency or length of hairs in Strain D404, and none of the remaining six strains produced any hairs.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

In seven strains (D156, D179, D184, D256, D267, D283, D403) there was a decrease in the average basal diameter in iron deficient cultures. The trichomes all had basal diameters that fell within the normal range, but there was an increased proportion of trichomes with narrower bases; this was particularly apparent in the later stages of growth. The lower average basal diameter is illustrated for strains D267 and D403 by the dimensions shown in Tables 5.23 and 5.25 (the measurements on the other strains were restricted to trichomes of a particular basal diameter, so the effect is obscured for these strains).

The main factor contributing to the decrease in basal diameter was the development of pseudo-intercalary heterocysts in many of the trichomes (see (vii) below), giving rise to numerous narrow-based 'daughter' trichomes, which did not seem to develop further (Figs 5.10a and 5.11b). Some of the narrower based trichomes also seemed to result from a failure of recently differentiated trichomes to complete their normal development; thus a higher than normal proportion of immature trichomes was present in the cultures at later stages of the deficiency. There was no evidence that any shrinkage of pre-existing trichome bases occurred; trichomes of normal basal diameter were also observed in all the affected strains. In Strains D251, D277, D280 and D404 there was no effect upon the basal diameter, and the diameter of trichomes of Anabaena cylindrica was similarly unaffected by iron deficiency.

The increases in hair development in Strains D184, D251, D256 and D403 caused a decrease in the average apical diameter of these strains; in Strains D184 and D256 there were also effects on the subapical diameter of trichomes without hairs (Tables 5.21 and 5.22b). In both these strains, the vegetative cells in the subapical region of iron deficient trichomes were narrower than those from the same region in control trichomes (Figs 5.9c and 5.10b); in Strain D184 the cells in this region were even narrower than the very apical cell of HAD-grown trichomes (Table 5.21). There might be 20 or more cells in the narrower apical region in these two strains, but further down the trichome there was always a region where the diameter was the same as that of a normal trichome apex. In Strain D256, in which many (frequency score = 3) of the trichomes did have hairs, the narrowing of the apices of trichomes

without hairs may have been simply an early stage in hair development. However, the cells at the apex were not markedly elongated (Fig. 5.9c), and the frequency of hairs did not increase as the cultures aged, so the two effects may have been separate phenomena. A very similar narrowing of the apices of iron deficient trichomes was seen in Strain D283, which did not produce hairs (Table 5.24); again only relatively few cells in the apical region were affected (Fig. 5.11c). In Strains D156 (Table 5.20) and D179 also, there was a decrease in the subapical diameter of the trichomes, but in these two strains the reduction in diameter took place throughout almost the whole of the trichome, and not merely in the apical few cells; only a few cells in the basal region were not obviously narrowed. The responses just described for Strains D156 and D179 seemed to develop independently of the effects on basal diameter (see above): narrower subapical regions were seen in trichomes of normal basal diameter as well as in those with narrower bases.

The total length of trichomes increased in iron deficient cultures of Strains D256 (Table 5.22a), D403 (Table 5.25) and (slightly) D277, as a result of hair development. In Strain D403 the length of the vegetative part of the trichomes was also greater (Table 5.25). The very variable dimensions of trichomes of Strain D251 made it difficult to estimate whether hair formation increased the average trichome length. The short hairs developed by Strain D104 had a negligible effect on the length of the trichomes. There was no consistent effect on trichome length in the other seven strains. Qualitative observations suggested that in the initial stages of growth, trichome length was similar in control and iron deficient cultures of each strain. In later stages of deficiency, there was a decrease in the average trichome length, since many short trichomes accumulated, as a result of pseudo-intercalary heterocyst development. (The measurements on Strains D156 (Table 5.20), D256 (Table 5.22b) and D283 (Table 5.24) show an increase in trichome length for each strain under iron deficiency. However, these measurements were taken on trichomes of a particular base width category (cf. Section 2.22), and at least part of the apparent increase in length probably actually reflects the reduced basal diameter in these strains. Thus control trichomes of a similar length would be ones with wider bases, and would therefore be excluded from the size category selected.)

It was rather difficult to assess overall changes in the

tapering of the trichomes, since in many strains there was a decrease in both basal and subapical diameter. However, as explained above, the changes in average basal diameter seemed to be due mainly if not entirely to alterations in the proportions of different types of trichomes present, whereas the changes in subapical diameter were absolute decreases, and apparently occurred in trichomes of all basal diameters. Thus, ignoring changes associated with hair development, decreases in subapical diameter caused an overall increase in the degree of tapering in Strains D156, D179, D184, D256 and D283 (Table 5.5).

(iv) Effects on the shape of vegetative cells (Table 5.6)

The narrowing of the apical part of trichomes in Strains D184, D256 and D283 was described above. In each of these strains, the cells in some, but not all of the narrowed apical regions had a greater length:width ratio than those of control trichomes. The absolute length of the cells was not necessarily greater, but the decrease in width gave them a more elongated appearance. (Figs 5.9c and 5.10b). In some trichomes from each of these strains the narrower cells at the apex had the same (usually quadrate) shape as those of control trichomes, and were thus absolutely shorter (Fig. 5.11c). The shape of the cells below the apical region in these strains was not obviously affected.

In Strains D2A, D156, D179 and D267 (Fig. 5.12) there was an increase in the average length of cells along the whole trichome. In Strains D156 and D179 many of the cells were also narrower (see (iii) above).

(v) Cytological effects other than hair formation (Table 5.7)

The vegetative cells of all strains became pale greyish or bluish green at about the same time as other symptoms of deficiency became apparent. In later stages, the cells became brownish yellow. In Strains D156, D184 and D277 the vegetative cells of many trichomes (frequency score = 3) developed small vacuoles. A smaller proportion (frequency score = 1) of trichomes of Strain D283 were similarly affected. There was no apparent polarity in this response: cells were equally affected along the whole trichome. Normal hairs were present on the vacuolated trichomes of Strain D277.

There was no consistent effect of iron deficiency on polyphosphate granulation, but some strains gave quite marked responses.

In Strains D184, D267, D277 and D283 there was a decrease in the level of granulation along the whole trichome: the gradients of distribution (Section 5.23; Table 5.3) remained, but at a lower density. Sometimes there was a complete loss of granules from the apical region, probably because the total granulation of control trichomes was lowest in this region. There was no apparent correlation between the presence or absence of polyphosphate in the apical region, and the changes in dimensions of the cells in Strains D184 and D283, or the presence of hairs in Strain D184. In Strains D156 and D179 the majority of trichomes (frequency score = 4) were completely devoid of granules; the other filaments had a normal level of granulation.

In Strain D404 there was a marked increase in polyphosphate granulation in all the vegetative cells of iron deficient trichomes, but as in the control, the granulation always decreased to zero in the transition zone below the hair. In Anabaena cylindrica D2A there was much variation in response between individual cells, even in a single trichome. In some cells (frequency score = 1) the granulation was normal; in others (frequency score = 3) granules were completely absent, and in the remaining cells the granulation had markedly increased. Taking the population as a whole, there was an overall increase in the level of polyphosphate granulation.

Polyphosphate granulation in the remaining strains (D251, D256, D280 and D403) was not affected by iron deficiency. In Strains D251 and D403 the granulation always decreased to zero in the 5-10 vegetative cells below the transition zone to the hair (this was also the case in control trichomes of Strain D403, but not always in Strain D251); in Strain D256 the cells below the transition zone might or might not contain polyphosphate. On one occasion, a normal vegetative cell was observed in one of the hairs in a sample of Strain D256 stained for polyphosphate (cf. (ii) above). The granulation of this cell was the same as that of the vegetative cells in the subapical region, while the surrounding hair cells were devoid of granules. There was no apparent correlation between the effects on polyphosphate in the different strains, and other aspects of their response to iron deficiency, such as hair formation, or effects on apical diameter.

No obvious effect on cyanophycin granulation was found in any of the strains.

(vi) Sheath characters and extracellular pigment production
(Table 5.7)

Increased production of sheath material was observed in all the strains. The basal part of the wider based trichomes became slightly darker brown pigmented in Strains D184 and D251, and the sheath bases of Strain D280 became dark brown. There was no obvious production of extracellular pigment in the other nine strains.

(vii) Effects on heterocysts (Table 5.7)

There was an obvious increase in heterocyst frequency in Strains D156, D179, D184 (Fig. 5.10a), D256, D267 (Fig. 5.12), D280 and D283 (Fig. 5.11). The type of secondary heterocyst produced (basal, intercalary or pseudo-intercalary) varied somewhat between strains; most strains developed more than one type, and all three were quite often observed in a single strain (Table 5.8).

Intercalary and pseudo-intercalary heterocysts developed in all regions of the trichomes, including the apical region, and whatever their position of development, the heterocysts seemed to retain the same dimensions as the vegetative cells from which they had differentiated (figs 5.10a, 5.11b and 5.12c). There was no apparent tendency for the vegetative cells adjacent to the newly formed heterocysts to enlarge. In Strain D283, about half of the pseudo-intercalary heterocysts formed in pairs, separated by an intercalary disc. This led to the formation of one or more lengths of trichome with a heterocyst at each end, but still retaining a tapered morphology (Fig. 5.11c). The development of single pseudo-intercalary heterocysts often produced many short tapered daughter filaments in the apical part of the original trichomes. This phenomenon was almost equivalent to the differentiation of hormogonia in situ, without prior release from the parent trichome, but the heterocysts also developed in the wider parts of the trichomes, below the zone from which hormogonia were normally released. This is illustrated for Strain D184 in Fig. 5.10a.

Heterocyst dimensions were measured in six strains. Significant changes were found in Strain D403 (heterocysts longer: Table 5.25), Strain D184 (heterocysts shorter: Table 5.21) and Strain D267 (heterocysts narrower: Table 5.23).

(viii) Effects on the development of spores (Table 5.9)

In iron deficient cultures of Anabaena cylindrica D2A spores developed earlier, and in greater numbers, than in the control medium. In Strain D277 very few of the trichomes developed spores in the iron deficient medium, whereas the majority of trichomes in control cultures had produced spores by the end of the growth period.

(ix) Response of iron deficient cultures to the addition of iron

Iron was added to the deficient cultures as a solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, to restore both compounds to their normal level.

In each case, the vegetative cells regained their normal colour in 12-24 hours; cell division and hormogonium release were apparent by 48-72 hours. There was no effect on the hairs in Strains D184, D251, D256 or D403 until hormogonia began to be released, when they were normally shed by lysis of a few cells just above the transition zone. The short hairs of Strain D184 often remained attached to the hormogonia as they were released. There was no apparent tendency for hairs to be shed within 2-3 hours of the addition of iron, as had been seen in Strains D277, D403 and D404 following the addition of phosphate to phosphate deficient cultures (Section 5.31(ix)). No evidence was seen of division of hair cells in any of the cultures.

When cultures of Anabaena cylindrica D2A were examined 36 hours after the addition of FeIII-EDTA, the medium had become a deep reddish brown colour, though the sheath material was not pigmented.

Table 5.20 Trichome dimensions (μm) of Calothrix brevissima D156 grown for 19 days in HAD and HAD-Fe

Measurements were restricted to trichomes of basal diameter $\geq 6.5 \mu\text{m}$.

Each value is the mean of 20 measurements.

	control mean	Fe-deficient mean	difference	probability
basal width	6.8 ± 0.11	7.0 ± 0.15	0.2 ± 0.18	0.505
subapical width	5.3 ± 0.10	4.6 ± 0.06	0.7 ± 0.10	<0.001
trichome length	191 ± 12	258 ± 22	67 ± 26	0.019
heterocyst width	7.4 ± 0.12	7.2 ± 0.19	0.2 ± 0.24	0.614
heterocyst length	7.2 ± 0.14	7.9 ± 0.40	0.7 ± 0.45	0.103

Table 5.21 Trichome dimensions (μm) of Calothrix sp. D184 grown for 24 days in HAD and HAD-Fe

Measurements were restricted to trichomes of basal diameter $\geq 9 \mu\text{m}$.

Each value is the mean of 20 measurements.

	control mean	Fe-deficient mean	difference	probability
basal width	10.1 ± 0.23	10.0 ± 0.18	0.1 ± 0.31	0.612
subapical width	4.4 ± 0.08	3.3 ± 0.05	1.1 ± 0.10	<0.001
apical width	3.9 ± 0.13	3.3 ± 0.05	0.6 ± 0.14	0.001
trichome length	396 ± 24	315 ± 32	81 ± 41	0.059
heterocyst width	8.7 ± 0.25	7.9 ± 0.19	0.8 ± 0.31	0.026
heterocyst length	8.3 ± 0.23	6.6 ± 0.19	1.7 ± 0.26	<0.001

Table 5.22 Trichome dimensions (μm) of Calothrix scopulorum D256 grown for 20 days in HAD and HAD-Fe

Each value is the mean of 20 measurements.

(a) Measurements were restricted to trichomes of basal diameter 8-9 μm , and only trichomes with hairs were measured in the HAD-Fe culture

	control mean	Fe-deficient mean	difference	probability
basal width	8.3 \pm 0.08	8.2 \pm 0.08	0.1 \pm 0.09	0.666
subapical width	3.4 \pm 0.05	2.6 \pm 0.08	0.8 \pm 0.10	<0.001
hair width	-	2.1 \pm 0.06	-	-
trichome length (minus hair)	101 \pm 7	110 \pm 6	9 \pm 10	0.367
hair length	-	60 \pm 5	-	-
total trichome length	101 \pm 7	170 \pm 8	69 \pm 11	<0.001
hair as % trichome length	-	35 \pm 2.4	-	-
heterocyst width	6.0 \pm 0.25	6.4 \pm 0.19	0.4 \pm 0.33	0.235
heterocyst length	5.1 \pm 0.19	5.4 \pm 0.14	0.3 \pm 0.25	0.192

(b) Measurements were restricted to trichomes of basal diameter 8-9 μm , and only trichomes without hairs were measured in the HAD-Fe culture

	control mean	Fe-deficient mean	difference	probability
basal width	8.3 \pm 0.05	8.3 \pm 0.08	0.0 \pm 0.10	1.000
subapical width	3.4 \pm 0.05	2.8 \pm 0.08	0.6 \pm 0.09	<0.001
trichome length	101 \pm 7	155 \pm 8	54 \pm 11	<0.001
heterocyst width	6.0 \pm 0.25	6.8 \pm 0.15	0.8 \pm 0.2	0.012
heterocyst length	5.1 \pm 0.19	5.9 \pm 0.16	0.8 \pm 0.23	0.004

Table 5.23 Trichome dimensions (μm) of Calothrix sp. D267 grown for 22 days in HAD and HAD-Fe

No restriction was placed on the basal diameter of the trichomes measured.

Each value is the mean of 20 measurements.

	control mean	Fe-deficient mean	difference	probability
basal width	5.5 \pm 0.15	4.8 \pm 0.10	0.7 \pm 0.16	< 0.001
subapical width	2.8 \pm 0.06	2.9 \pm 0.07	0.1 \pm 0.10	0.629
trichome length	110 \pm 7	63 \pm 3	47 \pm 7	< 0.001
heterocyst width	5.4 \pm 0.16	4.5 \pm 0.10	0.9 \pm 0.19	< 0.001
heterocyst length	5.8 \pm 0.19	4.8 \pm 0.19	1.0 \pm 0.27	0.039

Table 5.24 Trichome dimensions (μm) of Calothrix sp. D283 grown for 21 days in the second subculture to HAD and HAD-Fe

Measurements were restricted to trichomes of basal diameter \approx 5.5 μm .

Each value is the mean of 20 measurements.

	control mean	Fe-deficient mean	difference	probability
basal width	6.2 \pm 0.10	6.1 \pm 0.10	0.1 \pm 0.12	0.657
subapical width	3.1 \pm 0.03	2.9 \pm 0.06	0.2 \pm 0.07	0.014
apical width	3.1 \pm 0.03	2.1 \pm 0.07	1.0 \pm 0.09	< 0.001
trichome length	119 \pm 8	287 \pm 17	168 \pm 19	< 0.001
heterocyst width	5.5 \pm 0.12	5.7 \pm 0.14	0.2 \pm 0.15	0.162
heterocyst length	5.1 \pm 0.26	5.4 \pm 0.20	0.3 \pm 0.25	0.240

Table 5.25 Trichome dimensions (μm) of Rivularia sp. D403 grown for 17 days in the second subculture to HAD and HAD-Fe

No restriction was placed on the basal diameter of the trichomes measured, but all measurements were made on trichomes with hairs.

Each value is the mean of 20 measurements.

	control mean	Fe-deficient mean	difference	probability
basal width	6.4 \pm 0.08	5.5 \pm 0.18	0.9 \pm 0.23	0.001
subapical width	2.1 \pm 0.07	2.0 \pm 0.07	0.1 \pm 0.12	0.328
hair width	1.5 \pm 0.03	1.5 \pm 0.05	0.0 \pm 0.06	0.789
trichome length (minus hair)	83 \pm 5	121 \pm 5	38 \pm 7	<0.001
hair length	14 \pm 1	49 \pm 4	35 \pm 5	<0.001
total trichome length	97 \pm 6	171 \pm 8	74 \pm 8	<0.001
hair as % trichome length	15 \pm 1.2	28 \pm 1.9	13 \pm 2.1	<0.001
heterocyst width	6.2 \pm 0.09	6.0 \pm 0.11	0.2 \pm 0.15	0.098
heterocyst length	5.8 \pm 0.14	7.9 \pm 0.24	2.1 \pm 0.28	<0.001

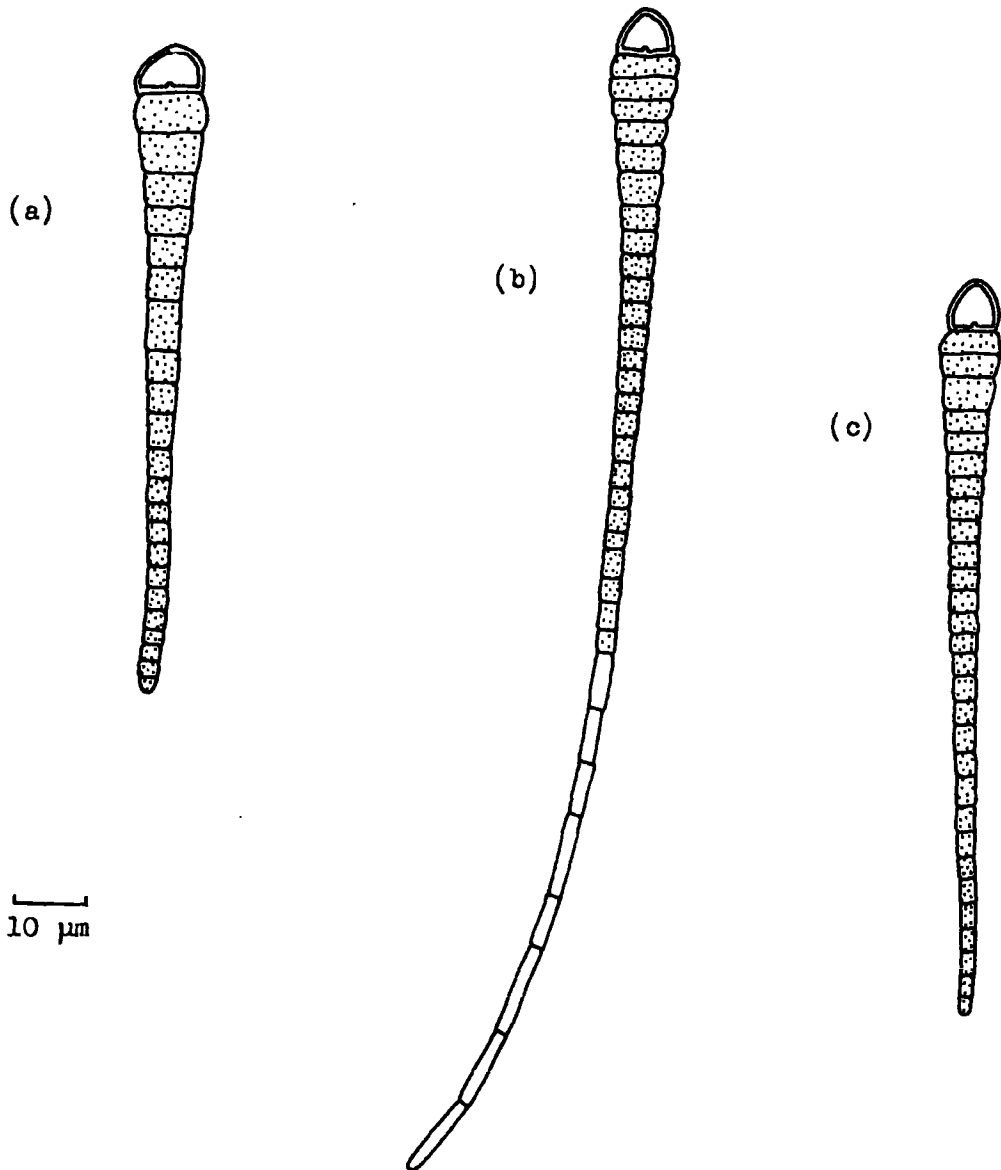


Fig. 5.9 Control and iron deficient trichomes of Calothrix scopulorum D256.

(a) Control (HAD).

(b) Fe-deficient trichome with hair (cf. Fig. 5.2).

(c) Fe-deficient trichome with narrowing of apical cells.

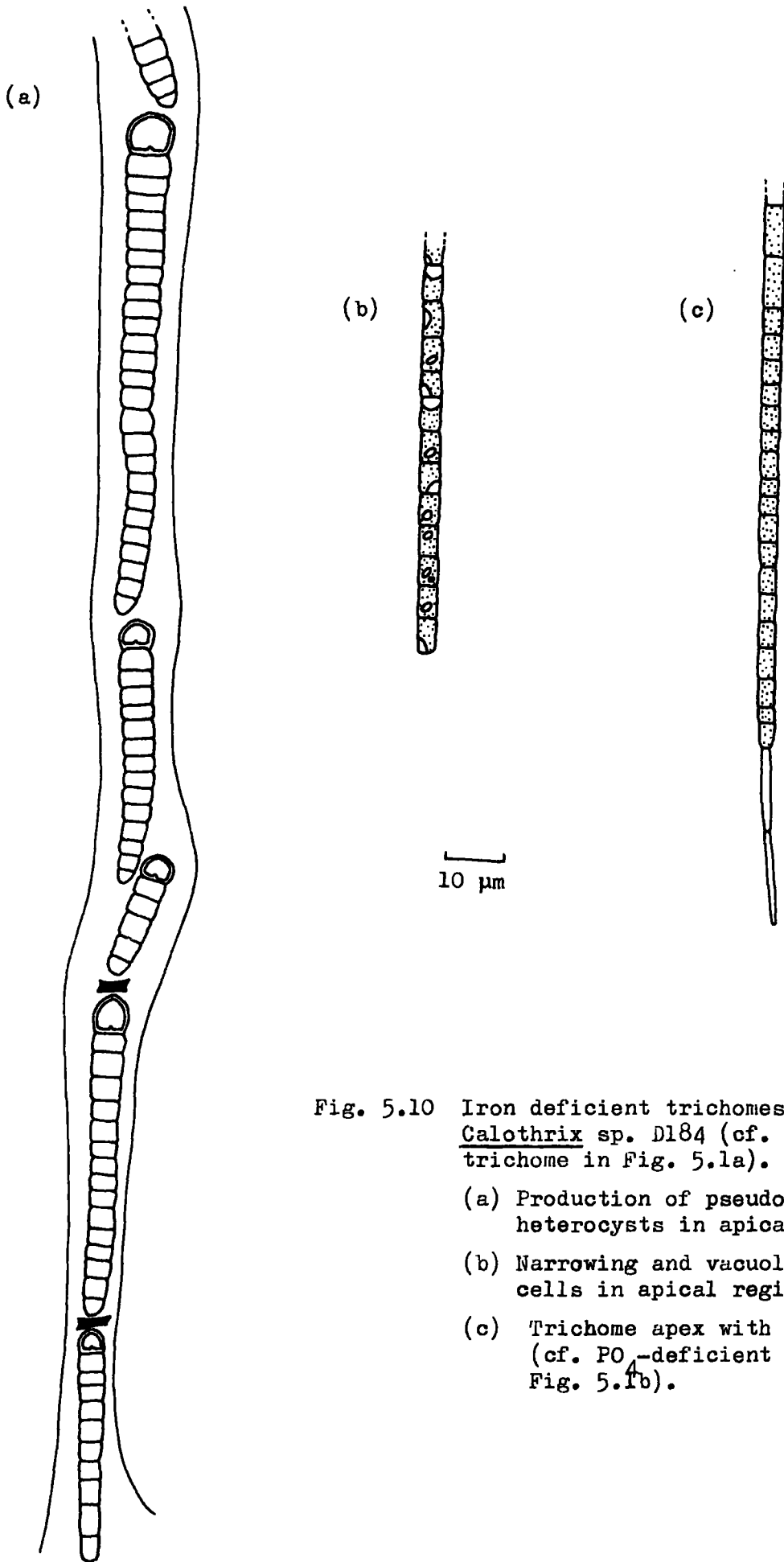
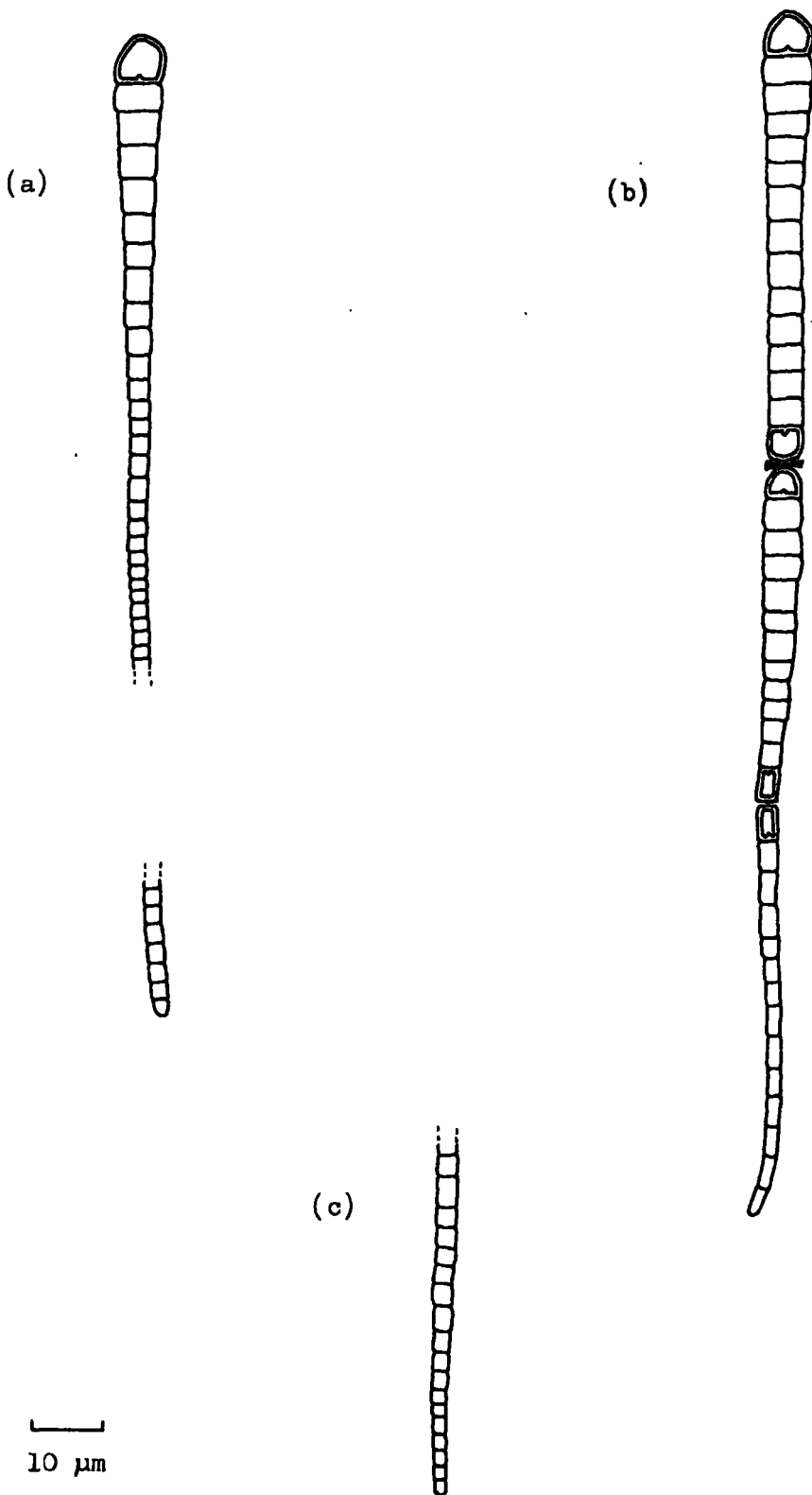


Fig. 5.10 Iron deficient trichomes of Calothrix sp. D184 (cf. control trichome in Fig. 5.1a).

- (a) Production of pseudo-intercalary heterocysts in apical region.
- (b) Narrowing and vacuolation of cells in apical region.
- (c) Trichome apex with short hair (cf. PO_4 -deficient trichome in Fig. 5.1b).

Fig. 5.11 Control and iron deficient trichomes of Calothrix sp. D283



(a) Control (HAD).

(b) Fe-deficient trichome with pseudo-intercalary heterocysts, and narrowing and elongation of cells in apical region.

(c) Apex of Fe-deficient trichome with narrowing, but not elongation of cells.

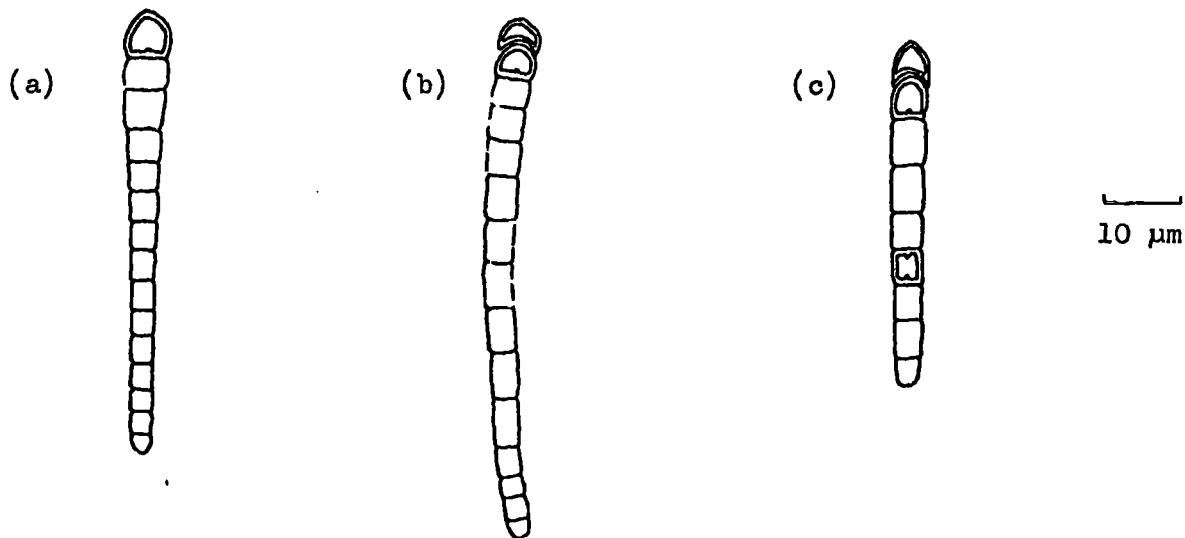


Fig. 5.12 Control and iron deficient trichomes of Calothrix sp. D267.

(a) Control (HAD).

(b) Fe-deficient trichome with secondary basal heterocyst and elongation of vegetative cells.

(c) Fe-deficient trichome with secondary basal and intercalary heterocysts.



Fig. 5.13 Iron deficient trichome of Rivularia sp. D403.

(cf. control and PO_4 -deficient trichomes in Fig. 5.3)

10 µm

5.42 Homoeothrix strains

(i) Induction of deficiency

Neither strain developed any signs of deficiency after three successive subcultures through HEPES-buffered medium without added iron. Further subcultures were therefore made into medium without HEPES (Section 5.22). Strain D401 developed symptoms of deficiency after a single subculture to this low iron medium; a second subculture was required before growth of Strain D402 was limited.

Iron deficient cultures of Strain D401 were pinkish in colour, and Strain D402 became a pale bluish green. Both strains retained these colours even at the end of the growth period, by which time the control cultures had turned yellow.

(ii) Effects on hair development (Table 5.4)

Iron deficiency had no effect on hair formation in either strain.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

In Strain D401 the trichomes in iron deficient cultures developed tapering apices, sometimes with vacuolated cells, a few days earlier than in control cultures. In both media the appearance of tapering apices coincided with the cessation of growth. The trichomes did not obviously differ in dimensions in the two media.

The diameter of trichomes of Strain D402 decreased under iron deficiency (Table 5.26). The effect was first seen at about the same time as the onset of growth limitation. The decrease in diameter took place along the whole visible length of the trichomes, but the trichome bases were seldom visible.

Table 5.26 Subapical and apical width (μm) of Homoeothrix sp. D402 grown for 11 days in the 5th subculture to HChu 10-D and Chu 10-D(-Fe)

Each value is the mean of 30 measurements.

	control mean	Fe-deficient mean	difference	probability
subapical width	1.93 \pm 0.01	1.62 \pm 0.01	0.31 \pm 0.11	<0.001
apical width	1.95 \pm 0.01	1.74 \pm 0.03	0.21 \pm 0.03	<0.001

(iv) Effects on the shape of vegetative cells (Table 5.5)

The development of tapered apices in Strain D401 was mentioned above. The changes in cell shape in the apical region were very much the same as those described for nitrate limited cultures in Section 3.33. In iron limited trichomes however, the cells below the apical region did not elongate like those in nitrate limited trichomes: they retained a length:width ratio of about 1:1.

(v) Cytological effects other than hair formation (Table 5.7)

Cells of both strains became less green, and correspondingly appeared pinkish (D401) and bluish (D402) respectively. A few (frequency score = 1) of the iron deficient trichomes of Strain D401 developed small vacuol. in every cell.

Polyphosphate granulation increased in Strain D401 under iron deficiency; all the cells were affected to a similar extent, but as in the control, the granulation decreased to zero at the apices of trichomes with an apical taper. Polyphosphate granulation was unchanged in Strain D402.

(vi) Sheath characters and extracellular pigment production
(Table 5.7)

There was no obvious effect upon sheath thickness or pigmentation in either strain of Homoeothrix.

(vii) Response of iron deficient cultures to the addition of iron

Both cultures regained their normal colour within one day of the addition of FeIII-EDTA, and hormogonia began to be produced shortly afterwards. The trichomes of Strain D401 were all quite normal in appearance, but some of the trichomes of Strain D402 remained rather narrower than normal. After a brief period of growth, both strains developed the symptoms shown by control cultures at the end of the growth period (Section 3.33).

5.43 Summary of results

a) Heterocystous strains

1. Five strains (D184, D251, D256, D277, D403) showed increased hair development under iron deficiency, though in Strains D184 and D277 the increase was only slight. All these strains were ones which produced long hairs in low phosphate medium. Fewer hairs were produced under iron deficiency than under phosphate deficiency, and the hairs were not as long. Two of the strains which showed increased hair production under phosphate deficiency (D280, D404) did not do so in low iron medium.

2. 7 of the 11 strains of Rivulariaceae showed a decrease in the average basal diameter in iron deficient cultures, but this seemed to result mainly from the production of pseudo-intercalary heterocysts, and the range of basal diameters was the same as in control cultures. In five strains (D156, D179, D184, D256, D283) there was an absolute decrease in subapical diameter that was distinct from changes associated with hair development. In three of the five strains (D184, D256, D283) the decrease in diameter was confined to the trichome apices, but in the other two, the decrease occurred in all but the very basal region of the trichome. In all these five strains the cells in the narrower region (but not elsewhere in the trichome) were sometimes relatively longer than those of control trichomes. In a further two strains (D2A, D267) the length:width ratio of the cells increased along the whole trichome.

3. In all strains the cells became less green in colour under iron deficiency, and in the early stages the colour of accessory phycobilin pigments was correspondingly more apparent.

In Strains D156, D184, D277 and D283 many of the vegetative cells developed vacuoles, without any marked change in cell shape. Some of the affected trichomes of Strains D184 and D277 had hairs as well, illustrating the difference between this response and true hair development.

4. Production of sheath material increased in all the strains under iron deficiency, and three strains (D184, D251, D280) showed increased production of brown sheath pigment; two of these (D184,

D251) also developed hairs in iron deficient medium. In iron deficient cultures of Anabaena cylindrica D2A the sheath and medium were colourless, but following the addition of iron to the culture the medium became brown in colour.

5. There was a marked increase in heterocyst frequency in iron deficient cultures of all strains except D251, D403, D404 and Anabaena cylindrica D2A.

The production of spores increased in Anabaena cylindrica D2A, but decreased in Gloeotrichia ghosei D277.

6. Hairs were lost following the addition of iron to the deficient cultures, but only in association with hormogonium release. There was no rapid lysis of the hair cells like that seen following the addition of phosphate to phosphate deficient cultures of certain strains.

b) Homoeothrix strains

1. The morphology of Strain D401 was unaffected by iron deficiency, apart from the development of small vesicles in some of the trichomes. Tapering apices developed at about the same time as growth ceased in iron deficient cultures, as it did in control cultures.

Trichomes of Strain D402 decreased in diameter under iron deficiency.

2. In both strains of Homoeothrix, the cells remained short under iron deficiency, whereas the average length of cells in control cultures increased as they became nitrate limited.

Like the heterocystous strains, both strains of Homoeothrix became less green in iron deficient culture.

5.5 Magnesium deficiency

5.51 Heterocystous strains

(i) Induction of deficiency

All the strains except D403 showed growth limitation after two to three weeks' growth of the first subculture to medium with 0.025x the normal level of magnesium. The limitation was especially marked in Strain D256, which showed very little growth in this medium. Strain D403 developed symptoms after a second subculture.

In most cases, the cultures became progressively paler green, and later pale yellow in colour. In Strain D156, control cultures of which were a deep mulberry colour, the magnesium deficient cultures became pale brown.

(ii) Effects on hair development (Table 5.4)

Only one strain, D251, showed a marked increase in the production of hairs under magnesium deficiency (Table 5.4). In magnesium deficient cultures of Strain D277, some of the trichomes (frequency score = 1) had hairs which were rather longer than those in control cultures, reaching 50% of the total trichome length, as against 5-20% in the control. This response was very like that seen in iron deficient cultures (Section 5.4(ii)). In both the affected strains, the changes in hair development were observed at much the same time as obvious growth limitation became apparent, but before any degenerative changes were seen. In the later stages of deficiency there was extensive fragmentation of the trichomes in all the strains, and very few intact hair cells were seen; this seemed to reflect the overall degeneration of the cultures, rather than being any specific response.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

There was a marked decrease in trichome diameter in all strains except D2A and D277, in which the dimensions were unaffected. The decreases in diameter occurred only at a late stage of deficiency, when growth had apparently ceased. In five strains (D256, D267 (Table 5.28), D277, D283, D403) the decrease in diameter seemed to occur in the basal region only. Sometimes the decrease was relatively slight, so that the trichome still had a tapered morphology, but many trichomes were almost parallel, with a diameter like that of the apical region of normal trichomes. Fig. 5.14a shows a trichome

of Strain D256 in which the basal swelling is much less pronounced than in a control trichome of similar length (Fig. 5.9). The five remaining strains (D156, D179, D184 (Table 5.27), D251, D280) showed a decrease in diameter along the whole length of the trichome. At least some trichomes of each of these strains retained a tapered morphology, but some appeared parallel, with a diameter less than that of the subapical diameter of control trichomes. In all strains, the decrease in trichome diameter was observed at the same time as the apparent death of the basal heterocyst (see (vii) below), and the trichomes with the most markedly narrowed vegetative cells were ones in which the heterocysts were colourless and collapsed, and sometimes detached from the vegetative cells.

Magnesium deficient cultures of Strains D184, D251, D256, D280, D403 and D404 contained short trichomes without heterocysts, which tapered towards both ends, and were surrounded by a very thick sheath (Figs 5.14b, 5.14d). In Strains D251 and D404 some of these trichomes had short hairs at both ends. In all six strains the diameter of the central part of these trichomes was similar to that of normal hormogonia, and they were at first believed to be derived from hormogonia which had failed to differentiate heterocysts and complete a normal development. Evidence was obtained, however, that at least some were probably derived from heterocystous trichomes.

Fig. 5.14b shows a trichome of Strain D256, tapered at both ends, and contained within a sheath which has a colourless basal heterocyst attached. The sheath surrounding the trichome in Fig. 5.14c has no heterocyst attached but its shape suggests that it once contained a trichome with a basal enlargement. One end of the trichome appears to have broken through the basal part of the sheath. Fig. 5.15 shows three heterocystous trichomes from a magnesium deficient culture of Strain D404, in which the trichome bases are narrowed to an increasing extent, with associated elongation of the terminal cells. These observations, and similar ones in other strains, suggested that in some cases one of the two tapered ends actually derived from the basal enlarged region of a heterocystous trichome.

It appeared that all the observed changes in trichome diameter took place by actual shrinkage of existing trichomes, rather than

by de novo development of narrower trichomes. As mentioned above, the changes occurred after growth had apparently ceased, and in old magnesium deficient cultures, no trichomes of normal width were observed. Table 5.27b, in which the dimensions of magnesium deficient trichomes of Strain D184 are compared with those of (immature) control trichomes of similar basal diameter, indicates that the smaller diameter of the magnesium deficient trichomes was not simply due to a failure of young trichomes to mature.

While growth was taking place, the length of the trichomes in most strains was apparently unaffected by magnesium deficiency. Trichomes of Strain D184 however were considerably longer in magnesium deficient cultures (Table 5.27), due at least partly to the elongation of cells in the apical region of the trichomes (see (iv) below). In later stages, in association with the decrease in trichome diameter just described, many of the trichomes of all strains fragmented into short lengths, most of which had no heterocysts.

Magnesium deficiency had considerable effects upon the tapering of the trichomes in every strain of Rivulariaceae except D277. The reduction in the basal diameter in Strains D256, D267, D283, D403 and D404 caused a decrease in the degree of tapering; similarly, although a reduction in diameter took place throughout the trichomes of the other strains (D156, D179, D184, D251, D280), the effect was generally more marked in the basal region, so that again the tapering was reduced. In all these ten strains, at least some of the trichomes became virtually parallel under magnesium deficiency. An additional effect on tapering (not scored in Table 5.5) was the development of trichomes which tapered at both ends, in Strains D184, D251, D256, D280, D403 and D404.

(iv) Effects on the shape of vegetative cells (Table 5.6)

The narrowing of the cells in many of the strains, and the elongation of the cells in the trichome bases of some strains were mentioned in (iii) above. Apart from these effects there was little apparent change in the absolute cell length in the majority of strains, though the narrowing of cells sometimes gave them a more elongated appearance. In two strains, however, D156 and D184, there was a marked increase in the absolute length of the cells. In Strain D156 the increase took place along the whole trichome:

the cells were 3-3.5(-5) μm wide by 9-20 μm long, against 4.5-5.5 μm by 4-6 μm in the subapical region of control trichomes. These elongated cells often had a rather distorted shape, with an undulating profile. In Strain D184 cell length increased in the apical region only. The cells in this region were 2-3 μm wide by 8-10 μm long, against 4.5-5 μm by 3-4.5 μm in control trichomes (compare Figs 5.16 and 5.1a). The apical cells of magnesium deficient trichomes of Strain D184 resembled hair cells in their dimensions, but there was no sign of vacuolation.

(v) Cytological effects other than hair formation (Table 5.7)

The vegetative cells of all strains became initially pale green; some trichomes of Strain D156 also had brownish cells. In all strains the cells later became yellowish, and sometimes eventually colourless, and there was some cell lysis, causing the trichomes to fragment. No vacuolation of the cells was seen, except in the secondary development of tapering trichome ends in Strains D251 and D404 (see (iii) above).

In the majority of strains, polyphosphate granulation was unaffected by magnesium deficiency. In Strain D184 the granulation in each trichome was reduced to about 50% of the control level, the decrease occurring uniformly along the trichome. In Strain D156 the majority (frequency score = 4) of the trichomes were devoid of granules, but the remainder had a normal level of granulation. The response of Anabaena cylindrica D2A was very like that observed for this strain under iron deficiency (Section 5.41(v)): some cells had no granules, some had a normal level of granulation, and some had an increased level; overall there was an estimated (slight) increase for the whole population. In Strain D404 granulation increased slightly along the whole trichome, but it still decreased to zero at the trichome apices, as it did in the control.

An increase in the level of cyanophycin granulation was observed in Strains D156, D184, D251 and D280 under magnesium deficiency, but there was no obvious effect in the other eight strains.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

In each strain, the thickness of the sheaths increased markedly in magnesium deficient cultures. In strains with clearly defined

sheaths, these could be seen to be 4-5 μm in thickness, whereas control trichomes usually had sheaths of 1 μm or less (see Figs 5.14 and 5.16). A marked increase in the brown pigmentation of the sheath bases occurred only in Strains D184 and D251.

(vii) Effects on heterocysts (Table 5.8)

In each of the strains studied, the heterocysts appeared to degenerate in magnesium deficient conditions. They became pale, then colourless, and often collapsed slightly, usually longitudinally rather than laterally. The trichome dimensions of Strain D184 (Table 5.27) and D267 (Table 5.28) illustrate the decrease in heterocyst size caused by this collapse. There was no other obvious effect on heterocyst size, apart from that associated with their apparent degeneration. There was no tendency for secondary heterocysts to differentiate following the death of the original ones; at some stage in the degenerative sequence described, the heterocysts usually became detached from the vegetative cells (Figs 5.14b and 5.15c).

(viii) Effects on the development of spores (Table 5.9)

Very few spores were produced by either Strain D2A or Strain D277 under magnesium deficiency; in both strains the spore frequency was lower than that in control cultures.

(ix) Response of magnesium deficient cultures to the addition of magnesium

In all strains, the vegetative cells had regained a normal colour one or two days after the addition of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$. Re-greening of colourless heterocysts was not observed, but many of the lengths of vegetative cells produced by trichome lysis differentiated a basal heterocyst, and their morphology returned to normal.

The hairs in Strain D251 were lost during the hormogonium release that occurred about three days after the addition of magnesium; there was no rapid lysis of the hairs like that observed in some strains following the addition of phosphate to phosphate deficient cultures (Section 5.31(ix)).

Table 5.27 Trichome dimensions (μm) of Calothrix sp. D184 grown for 24 days in HAD with 1.0x and 0.025x the normal level of Mg. Each value is the mean of 20 measurements.

(a) Measurements in the control culture were restricted to trichomes of basal diameter $\geq 9 \mu\text{m}$; no restriction was placed on trichomes measured in the magnesium deficient culture, but none had basal diameters $> 8 \mu\text{m}$.

	control mean	Mg-deficient mean	difference	probability
basal width	10.1 ± 0.23	7.8 ± 0.16	2.3 ± 0.27	< 0.001
subapical width	4.4 ± 0.08	2.9 ± 0.07	1.5 ± 0.12	< 0.001
apical width	3.9 ± 0.13	2.9 ± 0.07	1.0 ± 0.14	< 0.001
trichome length	396 ± 24	650 ± 35	254 ± 34	< 0.001
heterocyst width	8.6 ± 0.25	7.4 ± 0.33	1.2 ± 0.35	0.002
heterocyst length	8.3 ± 0.23	5.5 ± 0.27	2.8 ± 0.34	< 0.001

(b) Only trichomes of basal diameter $\leq 8 \mu\text{m}$ were measured from the control culture; the measurements from the magnesium deficient culture are the same as those in (a)

	control mean	Mg-deficient mean	difference	probability
basal width	7.7 ± 0.11	7.8 ± 0.16	0.1 ± 0.20	0.905
subapical width	4.4 ± 0.07	2.9 ± 0.07	1.5 ± 0.11	< 0.001
apical width	3.7 ± 0.12	2.9 ± 0.07	0.8 ± 0.14	< 0.001
trichome length	244 ± 22	650 ± 35	406 ± 48	< 0.001
heterocyst width	7.0 ± 0.22	7.4 ± 0.33	0.4 ± 0.44	0.326
heterocyst length	7.4 ± 0.24	5.5 ± 0.27	1.9 ± 0.34	< 0.001

Table 5.28 Trichome dimensions (μm) of Calothrix sp. D267 grown for 22 days in HAD with 1.0x and 0.025x the normal level of Mg

No restriction was placed on the basal diameter of the trichomes measured in either culture.

Each value is the mean of 20 measurements.

	control mean	Mg-deficient mean	difference	probability
basal width	5.5 ± 0.15	3.6 ± 0.11	1.9 ± 0.20	<0.001
subapical width	2.8 ± 0.06	2.7 ± 0.06	0.1 ± 0.09	0.409
trichome length	110 ± 7	73 ± 4	36 ± 8	<0.001
heterocyst width	5.4 ± 0.16	3.9 ± 0.12	1.5 ± 0.18	<0.001
heterocyst length	5.8 ± 0.19	5.2 ± 0.16	0.6 ± 0.24	0.039

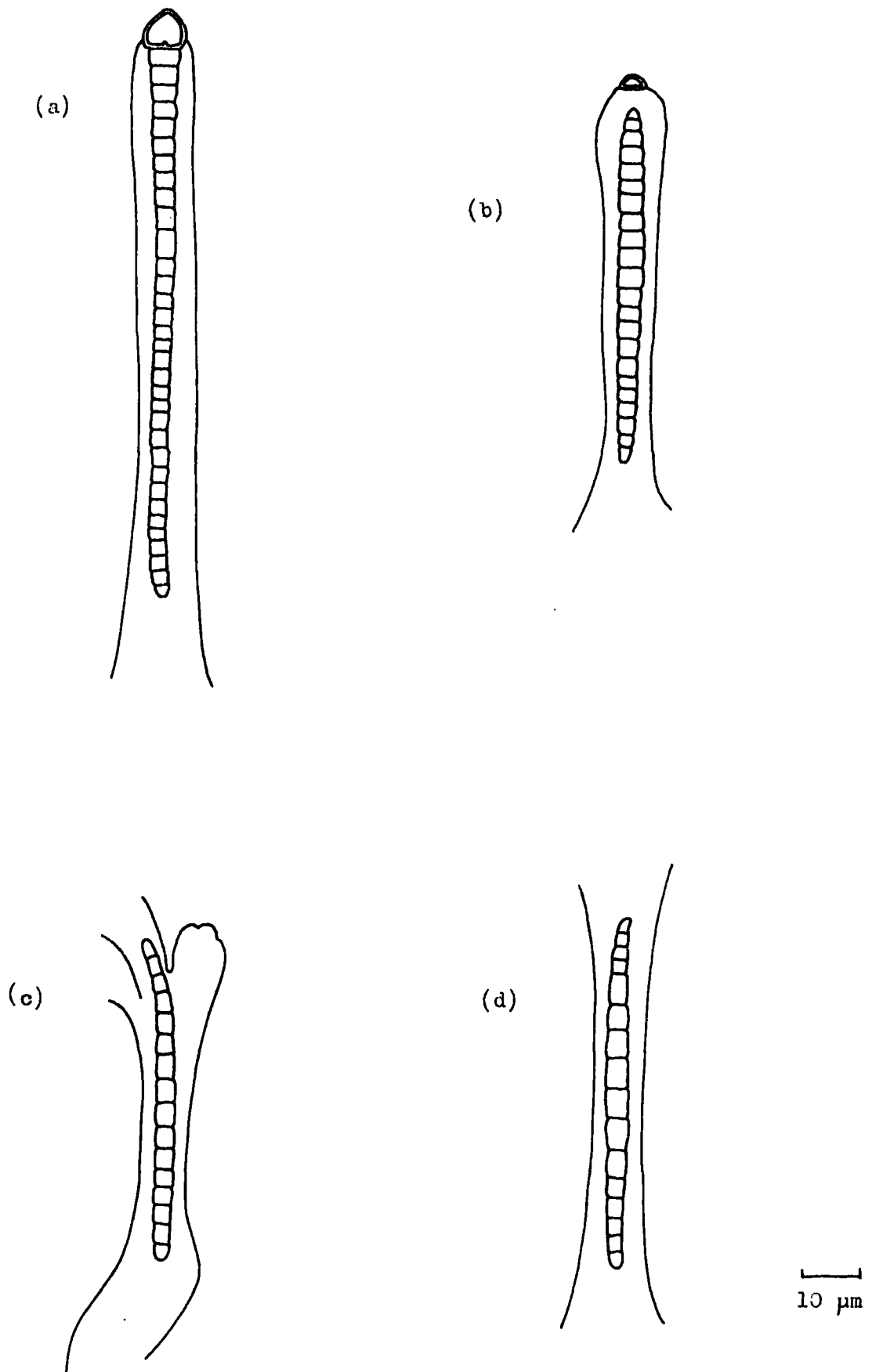


Fig. 5.14 Trichomes from magnesium deficient cultures of Calothrix scopulorum D256, showing tapering in the basal region (see pp. 199-200). Compare control trichome in Fig. 5.9a.

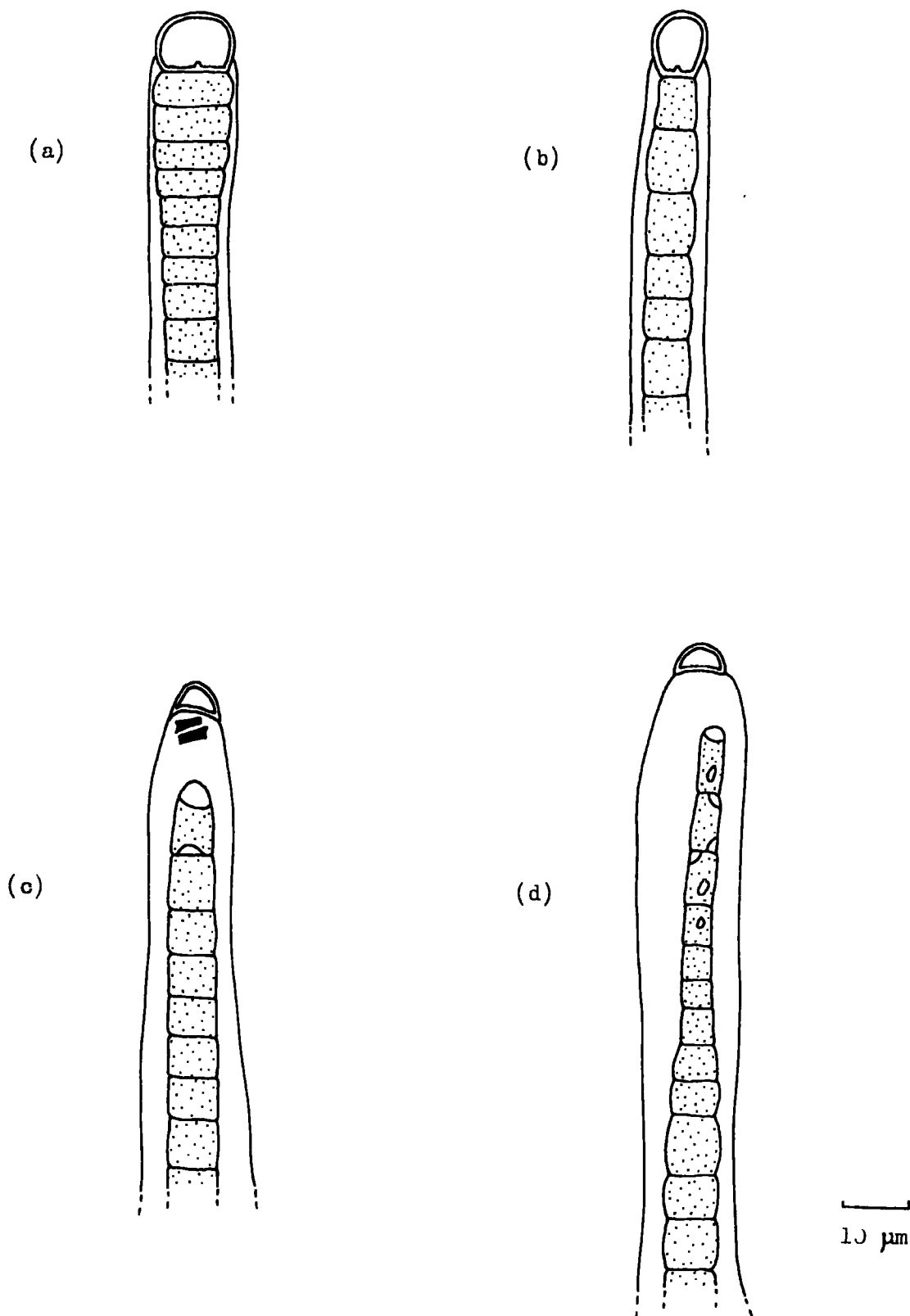


Fig. 5.15 Basal region of control and magnesium deficient trichomes of *Rivularia* sp. D404, showing apparent development of hairs in basal region.

(a) Control (HChu 10-D(-N)).

(b)-(d) Mg-deficient (see pp. 199-200).

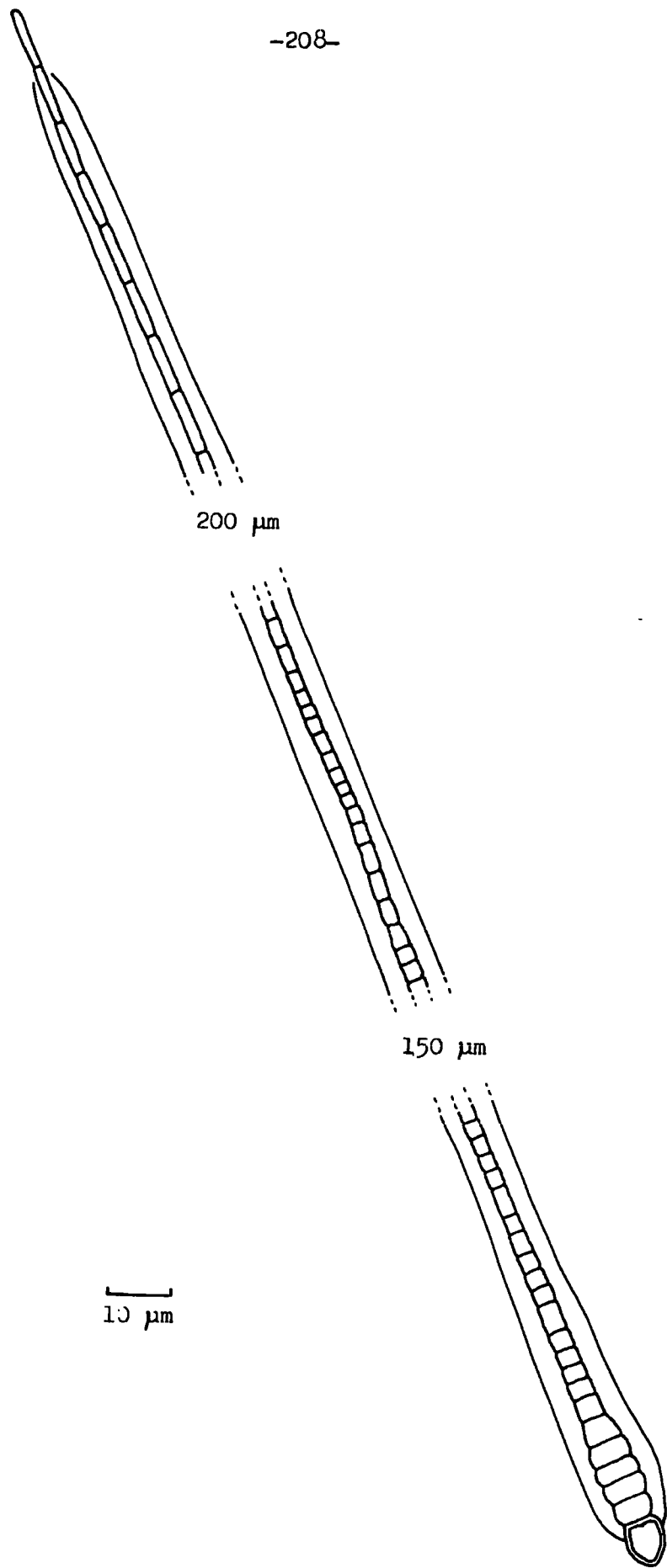


Fig. 5.16 Magnesium deficient trichome of *Calothrix* sp. D184.
(Compare the control trichome in Fig. 5.1a)

5.22 Homoeothrix strains

(i) Induction of deficiency

Growth of Strain D402 became limited after a single subculture into HChu 10-D with 0.025x the normal level of magnesium, but a second subculture was required for Strain D401. Magnesium deficient cultures of Strain D401 were pinkish; those of Strain D402 became pale green and latterly yellow.

(ii) Effects on hair development (Table 5.4)

Neither strain produced long hairs under magnesium deficiency; a few short hairs developed in Strain D401, at the same frequency as in control cultures, as growth came to a halt.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

The trichome dimensions of Strain D401 were not affected by magnesium deficiency, but tapering apices developed earlier than in control cultures, though in both media this occurred at the same time as growth began to slow down.

Trichomes of Strain D402 became markedly narrowed under magnesium deficiency (Table 5.29). The decrease in diameter occurred in all visible parts of the trichomes.

Table 5.29 Subapical and apical width (μm) of Homoeothrix sp. D402 grown for 11 days in the fifth subculture to HChu 10-D with 1.0x and 0.025x the normal level of Mg
Each value is the mean of 30 measurements.

	control mean	Mg-deficient mean	difference	probability
subapical width	1.93 \pm 0.01	1.51 \pm 0.02	0.42 \pm 0.02	<0.001
apical width	1.95 \pm 0.01	1.51 \pm 0.02	0.44 \pm 0.02	<0.001

(iv) Effects on the shape of vegetative cells (Table 5.6)

The shape of cells of Strain D401 was not affected, apart from the development of tapered apices mentioned above. There was no absolute increase in the length of the cells in magnesium deficient trichomes of Strain D402, but the narrowing of the trichomes gave the cells a more elongated appearance. The enlarged apical cells that developed in older control cultures of this strain (Section 3.33) were not observed in magnesium deficient cultures.

(v) Cytological effects other than hair formation (Table 5.7)

Cells of Strain D401 were initially pale greyish pink under magnesium deficiency; those of Strain D402 were pale green. Later, trichomes of both strains became yellowish, and some cells became colourless, and lysed, causing trichome fragmentation. Some trichomes (frequency score = 2) of Strain D401 developed small vacuoles in every cell. The level of polyphosphate granulation was not affected by magnesium deficiency in either strain.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

The thickness of the sheaths was not obviously affected by magnesium deficiency in either strain, and no sheath pigmentation was observed.

(vii) Response of magnesium deficient cultures to addition of magnesium

Strain D402 recovered its normal colour one day after the addition, and Strain D401 after two days. Cell division and hormone release occurred shortly afterwards in both strains. Both strains later developed symptoms of nitrate deficiency, like the control cultures (Section 3.33).

5.53 Summary of results

a) Heterocystous strains

1. Only one strain (D251) showed a marked increase in hair development under magnesium deficiency; this strain also produced more hairs in response to iron and phosphate deficiencies. Strain D277 produced a few longer hairs under magnesium deficiency: the response was very like that observed for this strain under iron deficiency.

In Strain D184 the cells in the apical region became very elongated and narrowed, resembling hair cells in their dimensions, but showing no vacuolation. Marked elongation of the vegetative cells also occurred in Strain D156, throughout the length of the trichomes, and some of the elongated cells had a distorted shape.

2. Magnesium deficient trichomes decreased in diameter in all but two strains (D277, D2A). Sometimes the whole trichome was affected, but sometimes apparently only the basal region. The change seemed to result from actual shrinkage of pre-existing cells. Five strains (D184, D251, D256, D280, D403, D404), all of which had a marked basal enlargement in control medium, developed trichomes which tapered at both ends, and in at least some cases one of the tapered ends apparently derived from the basal region of an originally heterocystous trichome.

3. All the strains became much paler green. Very marked production of sheath material occurred in all strains, but only D251 and D184 showed increased production of brown sheath pigment.

4. In all 12 strains the heterocysts degenerated under magnesium deficiency, with no tendency for further heterocysts to develop.

5. The hairs developed by Strain D251 were lost following the addition of magnesium, as hormogonia were released. There was no rapid lysis of the hair cells.

b) Homoeothrix strains

The morphology of Strain D401 was not affected by magnesium deficiency, but tapered apices developed as growth became limited. Trichomes of Strain D402 became narrower, like those of many of the heterocystous strains. Both Homoeothrix strains became less green.

5.6 Calcium deficiency

5.61 Heterocystous strains

(i) Induction of deficiency

All 12 strains showed signs of deficiency after a single subculture into medium with 0.025x the normal level of calcium. In the majority of strains the calcium deficient cultures became pale green, and later yellow, but cultures of Strains D251 and D256 retained a bright green colour despite obvious limitation of growth. The colour of algae in calcium deficient cultures of Strain D156 was also the same (mulberry) as in control cultures, but the medium became red-brown in colour.

Qualitative changes in the cultures were generally observed at about the same time as effects on yield, but the macroscopic appearance of Anabaena cylindrica D2A showed one change within 12 h of inoculation into low calcium medium. The inoculated filaments appeared to clump together, and settled to the base of the flask, instead of remaining suspended throughout the medium as they did in the control and other media.

(ii) Effects on hair development (Table 5.4)

The extent of hair development was unaffected in all strains except D277. In this strain some of the trichomes (frequency score = 1) had rather longer hairs than those of control trichomes. This response was very like that seen in iron and magnesium deficient cultures of this strain (Sections 5.41(ii) and 5.51(ii)). Many of the hairs (frequency score = 3) in calcium deficient cultures of Strain D277 had spindle-shaped cells (Fig. 5.17), with a diameter as great as 8 μm in the mid-region. In the control medium, and in the other deficient media, the hair cells were cylindrical, with a diameter of 2-4 μm .

(iii) Effects on trichome dimensions and tapering (Table 5.5)

In Strains D156, D184, D256, D277 and D283 there was a decrease in the average basal diameter, as a result of the development of pseudo-intercalary heterocysts in the narrower parts of the trichomes (see (vii) below). Basal width was not affected in the other strains, and the diameter of Anabaena cylindrica D2A was also unchanged.

In Strains D184 (Table 5.30), D280, D403 (Fig. 5.18) and D404 there was an increase in the subapical width of calcium deficient

trichomes. The observed increases did not seem to be simply the result of trichome fragmentation, with loss of the narrowest apical region, since characteristic modifications of the apical cells were still present (Fig. 5.18) and the sheaths did not extend beyond the trichome apex. The other seven strains of Rivulariaceae showed no change in apical or subapical width.

Trichome length was not obviously affected by calcium deficiency, except when the development of pseudo-intercalary heterocysts (see (vii) below) gave rise to shorter daughter trichomes.

Four strains became less tapered under calcium deficiency, as a result of the increase in subapical width mentioned above. An additional effect on tapering was observed in Strain D403. Most of the taper of control trichomes was due to the enlargement of cells in the basal region, but in calcium deficient trichomes the taper was more gradual, with a diameter characteristic of the basal enlargement of control trichomes maintained for as much as two thirds of the trichome length (Fig. 5.18).

(iv) Effects on the shape of vegetative cells (Table 5.6)

In five strains (D2A, D156, D184, D280, D404) cells along the whole length of the trichomes were shorter under calcium deficiency. The cells in the affected strains had length:width ratios of 0.25:1 to 0.5:1, against about 1:1 in the controls (compare Figs 5.19 and 5.1a). In Strain D283 the cells in the basal one third of calcium deficient trichomes were shorter than those of control trichomes, but cells in other regions were not affected; in Strain D251 only cells in the apical region became shorter under calcium deficiency. The increase in trichome width in Strain D403 (see (iii) above) made the cells appear relatively shorter, but there was no absolute change in length.

Cell shape was not obviously affected in Strains D179, D267 or D277.

(v) Cytological effects other than hair formation (Table 5.7)

Vegetative cells of Strains D251 and D256 remained green even after several weeks in low calcium medium, and trichomes of Strain D156 also retained their normal (mulberry) colour. In the other nine strains the cells became progressively paler, and finally yellow (buff in the case of Strain D277).

In Strain D277 the level of polyphosphate granulation decreased

in all the vegetative cells, to about 50% of the control level, but there was no apparent effect of calcium deficiency on polyphosphate in the other strains. No change in the level of cyanophycin granulation was observed in any of the strains.

It seemed that the clumping of trichomes of Anabaena cylindrica D2A (see (i) above) might have been due to some effect on trichome motility. However, the gliding motility of the alga in slide preparations did not obviously differ between trichomes from low calcium and control cultures.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

Production of sheath material increased in all 12 strains, but there was no increase in sheath pigmentation. The red-brown colouration of the medium in calcium deficient cultures of Strain D156 was mentioned in (i) above.

(vii) Effects on heterocysts (Table 5.8)

There was an obvious increase in heterocyst frequency in all strains except D251 and D404 (cf. Section 5.23(v); p. 149). Secondary basal heterocysts developed in each of the nine strains of Rivulariaceae showing an increase, and intercalary and/or pseudo-intercalary heterocysts were also present in most cases. Pseudo-intercalary heterocysts often occurred in pairs, and secondary heterocysts of different types were quite often observed in a single trichome (Fig. 5.20). In calcium deficient trichomes of Anabaena cylindrica D2A the heterocysts were separated by 5-8 cells, as against 15-25 cells in the control at the time of score. The increases in heterocyst frequency occurred relatively late, when growth had apparently ceased.

An obvious increase in heterocyst size was observed only in Strain D256 (Table 5.31a). Detailed measurements of heterocysts were made for only two other strains; they revealed a significant increase in heterocyst width in Strain D267 (Table 5.31b), but no significant effect on heterocyst size in Strain D184 (Table 5.30).

(viii) Effects on the development of spores (Table 5.9)

Very few spores were produced by either Strain D2A or Strain D277 in low calcium medium; in both cases spore frequency was less than that of control cultures.

(ix) Response of calcium deficient cultures to addition of calcium
Morphological changes following the addition of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to the cultures were not followed in detail, but all the strains grew to a normal final yield within a week of the addition. Their morphology also returned to normal, though secondary heterocysts were still apparent in some trichomes.

5.62 Homoeothrix strains

Neither strain showed any signs of deficiency after three successive subcultures through HChu 10-D with 0.025x the normal level of calcium. Both strains were then subcultured from this medium into HChu 10-D with 0.01x the normal level of calcium, and growth limitation and yellowing of the cultures were apparent after a few days.

(ii) Effects on hair development (Table 5.4)

Calcium deficiency had no effect on hair development in either strain.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

Trichomes in calcium deficient cultures of Strain D401 developed tapered apices earlier than those in control cultures; in both media this change coincided with the cessation of growth. There was no other effect on trichome dimensions in either strain.

(iv) Effects on the shape of vegetative cells (Table 5.6)

When growth limitation became apparent, the cell shapes of both strains were similar to those of the nitrate limited control cultures at the end of the growth period, i.e. slightly longer than broad, but not markedly elongated.

(v) Cytological effects other than hair formation (Table 5.7)

Cells in both strains became pale green, then yellow, and some trichomes (frequency score = 2) of Strain D401 developed small vacuoles in every cell. Polyphosphate granulation was not affected in either strain.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

There was no obvious effect on sheath development or pigmentation in either strain.

(ix) Response of calcium deficient cultures to addition of calcium

Both strains regained a normal appearance within two days of the addition, and grew to a normal yield before developing the morphological symptoms characteristic of nitrate limited control cultures (Section 3.33).

5.63 Summary of results

a) Heterocystous strains

1. Hair development was unaffected by calcium deficiency in all strains except D277, in which a few trichomes developed rather longer hairs, a response also given by this strain under iron and magnesium deficiencies.

2. In five strains (D156, D184, D280, D404, D2A) all the vegetative cells were markedly shortened under calcium deficiency, and in a further two strains (D251, D283) cells in a part of the trichome were similarly affected.

Four strains of Rivulariaceae (D184, D280, D403, D404) showed an increase in subapical diameter; all four were strains which had a marked basal enlargement in the control medium, and which had shorter cells in calcium deficient medium.

3. All but two strains (D251, D404) showed a marked increase in heterocyst frequency.

4. Trichomes of Anabaena cylindrica D2A clumped together shortly after inoculation into calcium deficient medium. The motility of the trichomes in the clumps was not obviously affected.

b) Homoeothrix strains

Neither strain showed any change in the dimensions of cells or trichomes under calcium deficiency. As observed in iron and magnesium deficient cultures, trichomes of Strain D401 developed tapered apices earlier than they did in the controls, the change coinciding with the cessation of growth in both media.

Cells of both strains became yellowish, and in Strain D401 some of them developed small vacuoles.

Table 5.30 Trichome dimensions (μm) of Calothrix sp. D184 grown for 24 days in HAD with 1.0x and 0.025x the normal level of Ca

Measurements were restricted to trichomes of basal diameter $\geq 9 \mu\text{m}$.

Each value is the mean of 20 measurements.

	control mean	Ca-deficient mean	difference	probability
basal width	10.1 \pm 0.23	10.8 \pm 0.26	0.7 \pm 0.31	0.030
subapical width	4.4 \pm 0.08	6.0 \pm 0.22	1.6 \pm 0.23	< 0.001
apical width	3.9 \pm 0.13	6.0 \pm 0.22	2.1 \pm 0.25	< 0.001
trichome length	396 \pm 24	248 \pm 16	148 \pm 29	< 0.001
heterocyst width	8.6 \pm 0.25	9.1 \pm 0.41	0.5 \pm 0.48	0.330
heterocyst length	8.3 \pm 0.23	8.2 \pm 0.27	0.1 \pm 0.34	0.906

Table 5.31 Heterocyst dimensions (μm) of Calothrix scopulorum D256 and Calothrix sp. D267 in HAD with 1.0x and 0.025x the normal level of Ca

Each value is the mean of 20 measurements.

(a) Calothrix scopulorum D256 (21 days' growth)

Measurements were restricted to trichomes of basal diameter $6.5 \mu\text{m}$.

	control mean	Ca-deficient mean	difference	probability
heterocyst width	5.6 \pm 0.10	6.2 \pm 0.08	0.6 \pm 0.12	< 0.001
heterocyst length	5.3 \pm 0.13	6.4 \pm 0.19	1.1 \pm 0.25	< 0.001

(b) Calothrix sp. D267 (22 days' growth)

Measurements were restricted to trichomes of basal diameter $4.8 \mu\text{m}$.

	control mean	Ca-deficient mean	difference	probability
heterocyst width	4.9 \pm 0.12	5.6 \pm 0.14	0.7 \pm 0.18	< 0.001
heterocyst length	5.5 \pm 0.20	5.9 \pm 0.20	0.4 \pm 0.24	0.134



(a) Control (HAD)



(b) Ca-deficient

10 μ m

Fig. 5.17 Hair cells of control and calcium deficient trichomes of Gloeotrichia ghosei D277.

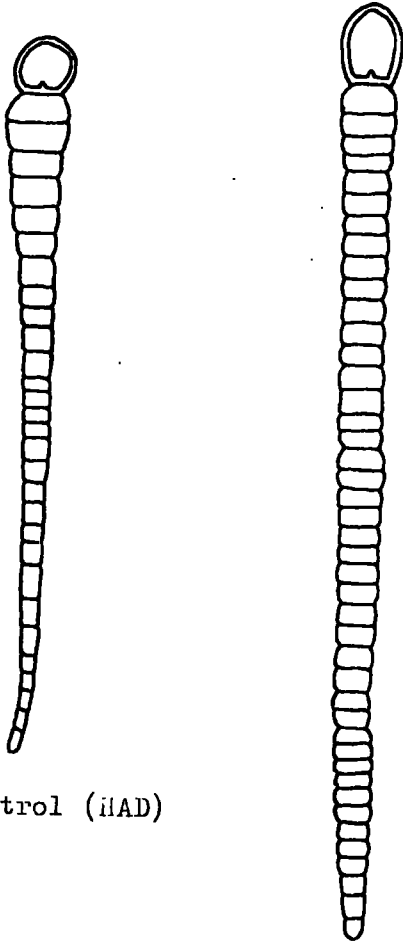


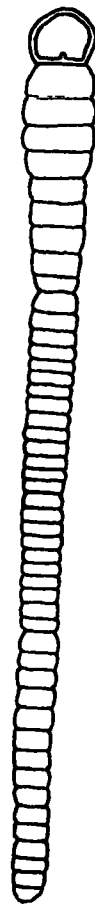
Fig. 5.18 Control and calcium deficient trichomes of Rivularia sp. D403.

(a) Control (HAD)

(b) Ca-deficient

—
10 μ m

Fig. 5.19 Calcium deficient trichome of Calothrix sp. D184 (compare control trichome in Fig. 5.1a).



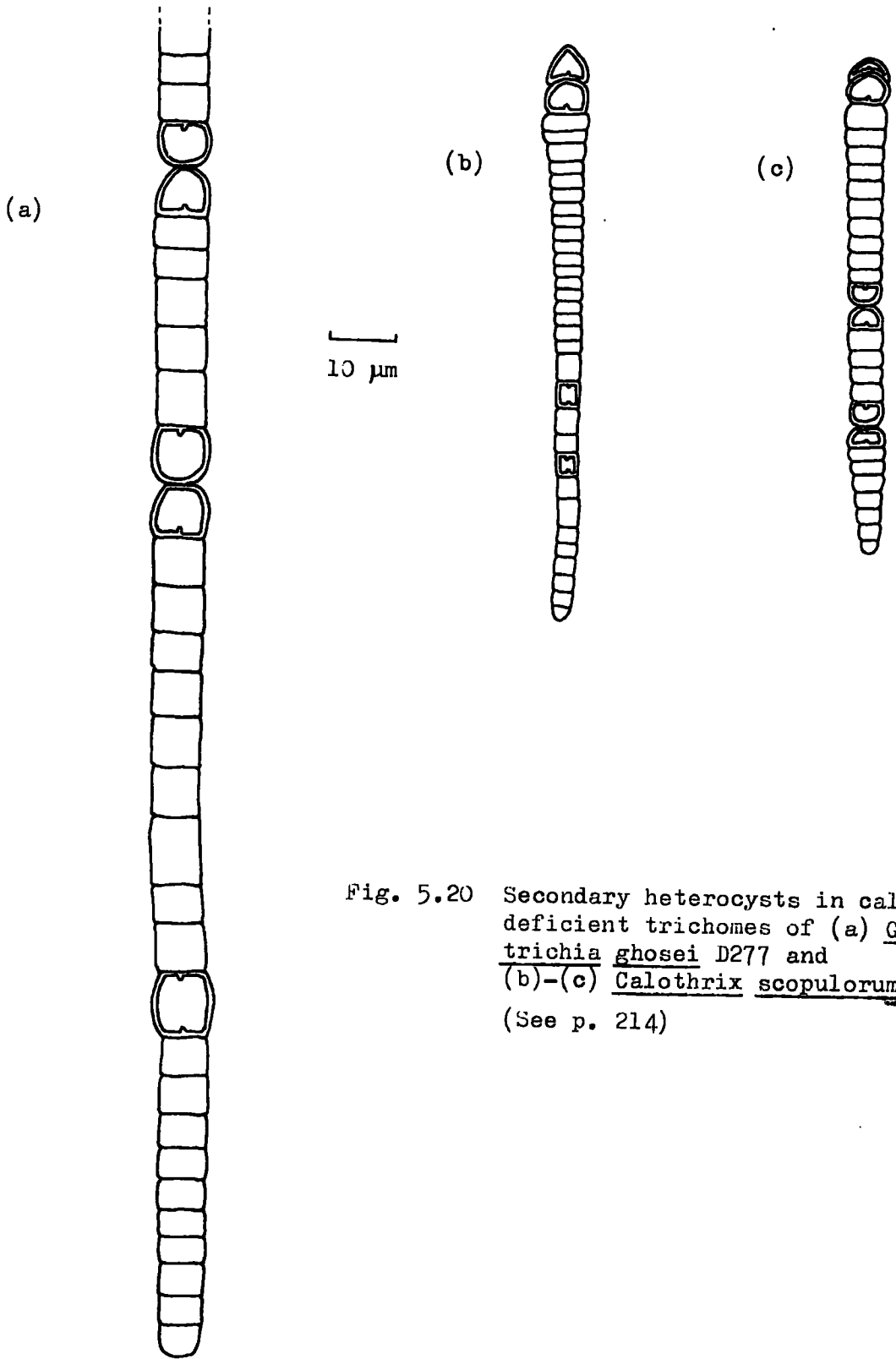


Fig. 5.20 Secondary heterocysts in calcium deficient trichomes of (a) Gloeotrichia ghosei D277 and (b)-(c) Calothrix scopulorum D256. (See p. 214)

5.7 Molybdenum deficiency

5.71 Heterocystous strains

(i) Induction of deficiency

Strains D156, D179, D184, D267, D277, D280 and D404 became yellow in the first subculture into medium without added molybdenum; Strains D2A, D251 and D283 required two subcultures, and Strains D256 and D403 three subcultures. Yellowing normally coincided with obvious reduction in yield, but in Strains D184 and D251 cultures turned yellow after the first and second subcultures respectively, but two (D184) and four (D251) subcultures were required before yield was obviously reduced. All subcultures after the second were made into medium without HEPES (Section 5.22).

(ii) Effects on hair development (Table 5.4)

There was no effect of molybdenum deficiency on hair development in any of the strains, apart from effects related to the inhibition of overall trichome development (see (iii) below).

(iii) Effects on trichome dimensions and tapering (Table 5.4)

In all the strains of Rivulariaceae the development of hormogonia seemed to be inhibited as molybdenum deficiency developed. They differentiated a basal heterocyst, but there was little or no cell division or increase in length, and basal enlargements did not develop. In Strain D277 hairs also failed to develop on the young trichomes, but in Strains D403 and D404 some short hairs did form.

The average length and basal diameter of the trichomes decreased in all the strains as a result of this inhibition of development, but in all cases trichomes of normal dimensions formed a high proportion of the population. Subapical and apical width were unaffected in all the strains, and the dimensions of Anabaena cylindrica D2A were also unchanged.

The young trichomes whose development was stunted were less tapered than 'average' control trichomes, due to their failure to develop basal enlargements, or, in Strain D277, to produce hairs. However, this seemed merely to reflect their inhibited growth, rather than being a specific response.

(iv) Effects on the shape of vegetative cells (Table 5.6)

Cell shape was unaffected by molybdenum deficiency in all the strains.

(v) Cytological effects other than hair formation (Table 5.7)

The vegetative cells became yellow in all 12 strains; this effect was seen first in the short trichomes whose development appeared to be stunted, but the longer trichomes later became yellow also. Some trichomes (frequency score = 2) of Strains D184, D267 and D277 developed small vacuoles in every cell.

There was no obvious effect on the level of polyphosphate or cyanophycin granulation in any of the strains.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

Sheath thickness increased in all strains. In the Rivulariaceae this was most marked in the short, stunted trichomes; these had distinct sheaths, whereas control trichomes at an equivalent stage of development had only very thin sheaths.

(vii) Effects on heterocysts (Table 5.8)

An obvious increase in heterocyst frequency was observed in all the strains. In most strains secondary heterocysts of more than one type were produced, often in a single trichome. In Anabaena cylindrica D2A the heterocysts in molybdenum deficient trichomes were separated by 8-15 cells, as against 20-30 in control trichomes at the time of scoring.

In Strains D256, D267, D277 and D283 there was a marked increase in the size of the (primary) basal heterocysts (Table 5.32; Fig. 5.21).

(viii) Effects on the development of spores (Table 5.9)

Molybdenum had no effect on spore development in either Strain D2A or Strain D277.

(ix) Response of molybdenum deficient cultures to addition of molybdenum

All cultures regained their normal colour within 48 h of the addition of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and cell division and hormogonium release were apparent shortly afterwards. Normal yields were achieved 5-10 days after the addition, and morphologies also returned to normal, apart from the presence of residual intercalary heterocysts.

5.72 Homoeothrix strains

(i) Induction of deficiency

No deficiency symptoms were apparent in either strain until the fifth subculture into Chu 10-D (without HEPES) without added molybdenum, and even then, the difference in yield between +Mo and -Mo cultures was only slight.

(ii) Morphological response

There was no change in the morphology of either strain in the deficient medium, but the symptoms characteristic of nitrate limited cultures developed two or three days earlier than they did in control cultures.

(iii) Response of cultures to the addition of molybdenum

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ was added to the cultures one day after they had begun to turn yellow. No re-greening or growth was observed in either strain, and the same result was obtained when the addition was repeated after a further subculture to the deficient medium. This suggested that growth of the algae was not in fact limited by shortage of molybdenum. As mentioned above, the yield in molybdenum deficient medium was only slightly less than that in full medium. It seemed possible that there was insufficient $\text{NO}_3\text{-N}$ remaining in the molybdenum deficient medium to permit full recovery of the algae following the addition of molybdenum.

5.73 Summary of results

a) Heterocystous strains

1. Molybdenum deficiency led to stunting of normal trichome development in all strains, but there was no specific effect on hair development or trichome dimensions.

2. Heterocyst frequency increased in all strains, and in Strains D256, D267, D277 and D283 there was also an increase in heterocyst size. All strains became markedly yellowed.

b) Homoeothrix strains

It was difficult to obtain symptoms of molybdenum deficiency in either strain. The symptoms observed were the same as those of nitrate limited cultures, but appeared rather earlier.

Table 5.32 Heterocyst dimensions (μm) of Strains D256, D267, D277 and D283 in HAD and HAD-Mo

Each value is the mean of 20 measurements.

(a) Calothrix scopulorum D256 (21 days in 3rd subculture)

Measurements restricted to trichomes of basal width 8-10 μm .

	control mean	Mo-deficient mean	difference	probability
heterocyst width	6.4 \pm 0.15	8.4 \pm 0.18	2.0 \pm 0.22	<0.001
heterocyst length	5.2 \pm 0.24	6.4 \pm 0.13	1.2 \pm 0.23	<0.001

(b) Calothrix sp. D267 (22 days in 2nd subculture)

Measurements restricted to trichomes of basal width 4.8 μm .

	control mean	Mo-deficient mean	difference	probability
heterocyst width	4.9 \pm 0.12	6.4 \pm 0.16	1.5 \pm 0.19	<0.001
heterocyst length	5.5 \pm 0.20	6.3 \pm 0.22	0.8 \pm 0.31	0.026

(c) Gloeotrichia ghosei D277 (16 days in 2nd subculture)

Measurements restricted to trichomes of basal width 6.5-8.0 μm .

	control mean	Mo-deficient mean	difference	probability
heterocyst width	9.7 \pm 0.18	11.6 \pm 0.48	1.9 \pm 0.52	<0.001
heterocyst length	12.2 \pm 0.50	13.8 \pm 0.40	1.6 \pm 0.73	0.039

(d) Calothrix sp. D283 (21 days in 2nd subculture)

Measurements restricted to trichomes of basal width 5.5 μm .

	control mean	Mo-deficient mean	difference	probability
heterocyst width	5.5 \pm 0.12	7.6 \pm 0.17	2.1 \pm 0.22	<0.001
heterocyst length	5.1 \pm 0.26	6.9 \pm 0.24	1.7 \pm 0.39	<0.001

(a)



Control (HAD)



Mo-deficient

(b)



Control (HAD)



Mo-deficient

10 μ m

Fig. 5.21 Secondary heterocysts and increased heterocyst size in (a) Calothrix sp. D267 and (b) Calothrix sp. D283. under molybdenum deficiency.

5.8 Sulphate deficiency

5.81 Heterocystous strains

(i) Induction of deficiency

Strains D179, D184, D256 and D280 showed a reduction in yield after a single subculture into medium with 0.025x the normal level of $\text{SO}_4\text{-S}$; Strains D156, D277, D404 and D2A required a second subculture. The four strains which still showed no limitation of growth were then subcultured into HAD with 0.01x the normal level of $\text{SO}_4\text{-S}$. Growth of Strains D251 and D403 was limited in the first subculture, but growth of Strains D267 and D283 not until the second.

In several strains qualitative effects on the low sulphate cultures appeared in the apparent absence of effects on yield. Thus in early subcultures, Strains D156, D251, D277 and D403 each grew to the same (estimated) final yield as the control, but then turned yellow, while the control remained normal in colour. A further subculture of each strain was required before yield was obviously reduced. Cultures of all the strains eventually became yellow under sulphate deficiency.

(ii) Effects on hair development (Table 5.4)

Hair development was unaffected by sulphate deficiency in all strains except D277. In this strain, some of the trichomes developed rather longer hairs, a response very like that given by this strain under deficiencies of iron, magnesium and calcium.

(iii) Effects on trichome dimensions and tapering (Table 5.5)

None of the strains of Rivulariaceae showed any obvious alteration of trichome shape or dimensions, except in association with the cell lysis and trichome fragmentation which occurred in the most advanced stages of deficiency. In Anabaena cylindrica D2A some of the trichomes in sulphate deficient cultures (frequency score = 3) were rather wider than control trichomes, measuring 5-6.5 μm in diameter, against 3.5-5 μm in the control.

(iv) Effects on the shape of vegetative cells (Table 5.6)

No modifications of cell shape were observed in any of the strains.

(v) Cytological effects other than hair formation (Table 5.7)

In all 12 strains there was marked yellowing of the cells at the end of the growth period in sulphate deficient medium. The cells became progressively paler, and finally almost colourless, and in the later stages of this sequence, many cells lysed, causing the trichomes to fragment. As mentioned in (i) above, this sometimes occurred when the final yield appeared the same as that of the control.

A marked increase in the level of polyphosphate granulation was observed in all strains except D403. The very high control level of granulation in this strain (Table 5.3) made it difficult to estimate whether further increases had occurred. In Strain D277 granules were present in the cytoplasmic strands of most of the hair cells. In the other strains with hairs (D251, D403, D404) the granules extended as far as the transition zone, but were not seen in the hairs themselves. In the majority of strains, the increase in polyphosphate granulation occurred before any other symptoms, but in Strains D2A, D179, D184 and D256, the increase was observed only after the yield was obviously reduced.

The high levels of polyphosphate in the cells made it impossible to determine effects on cyanophycin granulation.

(vi) Sheath characters and extracellular pigment production (Table 5.7)

Sheath thickness increased in all strains, but no effects on sheath pigmentation were observed.

(vii) Effects on heterocysts (Table 5.8)

Heterocyst size and frequency were unaffected in all strains, but like the vegetative cells, the heterocysts became yellow in colour.

(viii) Effects on the development of spores (Table 5.9)

Spore frequency decreased in Strain D277, but it was not affected in Strain D2A.

(ix) Response of sulphate deficient cultures to addition of sulphate

All strains regained a normal colour within 2-3 days of the addition of Na_2SO_4 , and began to produce hormogonia. Changes in polyphosphate were not followed in detail, but the level of granules had returned to normal by the time a full yield had been reached.

5.82 Homoeothrix strains

(i) Induction of deficiency

The first two subcultures were made into HChu 10-D with 0.025x the normal level of sulphate. The second subculture produced yellowing of both strains, but there was no apparent effect on yield; further subcultures were therefore made into medium with 0.01x the normal level of sulphate, and growth of both strains became limited in the second subculture to this medium.

(ii) Effects on hair development (Table 5.4)

Sulphate deficiency had no effect on hair development in either strain.

(iii) Effects on trichome dimensions (Table 5.5)

Trichome dimensions in Strain D401 were not obviously affected by sulphate deficiency, but tapered apices developed earlier than in control cultures, coinciding with the cessation of growth. Trichomes of Strain D402 decreased in diameter (Table 5.33), the decrease occurring along the whole visible part of the trichomes.

Table 5.33 Subapical and apical width (μm) of Homoeothrix sp D402 grown for 11 days in the fifth subculture to HChu 10-D with 1.0x and 0.01x the normal level of SO_4 . Each value is the mean of 30 measurements.

	control mean	S-deficient mean	difference	probability
subapical width	1.93 \pm 0.01	1.71 \pm 0.02	0.21 \pm 0.02	< 0.001
apical width	1.95 \pm 0.01	1.74 \pm 0.02	0.21 \pm 0.02	< 0.001

(iv) Effects on the shape of vegetative cells (Table 5.6)

Despite the narrowing of the trichomes of Strain D402, the cell proportions remained similar to those in the control; thus their absolute length was smaller. As in control trichomes, there was a slight increase in absolute cell length as the cultures aged. Strain D401 showed no changes in cell shape other than those associated with the development of tapered apices.

(v) Cytological effects other than hair formation (Table 5.7)

The cells of both strains became yellow before any effects on yield were seen; in older cultures the trichomes became almost

colourless, and fragmented into short sections. In Strain D401 some trichomes (frequency score = 2) developed small vacuoles in each cell.

Polyphosphate granulation increased in both strains, though in Strain D401 the granulation still decreased to zero at the apices of tapered trichomes, as in the control.

(vi) Sheath characters and extracellular pigment production (Table 5.7).

No modification of sheath characters was observed in either strain.

(vii) Response of sulphate deficient cultures to addition of sulphate

Both strains regained their normal colour, and recommenced growth 2-3 days after the addition of Na_2SO_4 .

5.83 Summary of results

a) Heterocystous strains

1. Sulphate deficiency had little morphological effect on any of the strains, though Strain D277 developed a few longer hairs, as it did under deficiencies of iron, magnesium and calcium.

2. All strains except D403 showed a marked increase in polyphosphate granulation. In Strain D277 granules were present in the hair cells, which normally lacked visible granulation.

3. All strains eventually became yellow, and the trichomes fragmented. In several cases this occurred in cultures which showed no limitation of growth, and after growth had ceased. This seemed to imply that sulphate was required for the maintenance of cell integrity, even in a non-growing state.

b) Homoeothrix strains

The two strains of Homoeothrix responded in much the same way as the heterocystous strains, showing an increase in polyphosphate granulation, and yellowing and fragmentation of the trichomes. In Strain D402 the trichomes also decreased in diameter.

In Strain D401 trichomes in sulphate deficient cultures developed tapered apices earlier than those in control cultures; in both cases the change occurred at the same time as growth became limited.

6 FURTHER EXPERIMENTS ON HAIR DEVELOPMENT UNDER DEFICIENCIES OF PHOSPHATE, IRON AND MAGNESIUM

6.1 Response of a further five strains to deficiencies of iron and magnesium

Eight of the 13 strains of Rivulariaceae studied in the experiments described in Chapter 5 showed increased hair development under phosphate deficiency; four of these also produced more hairs under iron deficiency, and one did so under magnesium deficiency. Calcium, molybdenum and sulphate deficiencies had little effect on hair development in any of the strains. Five of the strains which were known to be capable of forming hairs under phosphate deficiency (Chapter 4) were not included in the experiments described in Chapter 5, and it was of interest to see whether any of these five strains would also show increased hair development under iron or magnesium deficiency.

The five strains (D126, D253, D266, D269, D270) were inoculated into AD and into iron and magnesium deficient versions of this medium (Table 5.2). The methods followed were the same as those described in Section 5.2; incubation was at 25°C, 2500 lx, without shaking. The deficient cultures were scored for the following characters: increased hair development; changes in heterocyst frequency; development of brown sheath pigment, and changes in polyphosphate granulation.

Strains D253, D266 and D269 produced many long hairs in iron deficient medium, and Strain D270 produced a few short hairs; there was no change in hair frequency or length in Strain D126. Magnesium deficiency had no effect on hair formation in any of the strains. All the iron deficient cultures showed a marked increase in heterocyst frequency, and Strain D253 developed brown sheath pigmentation under this condition. Polyphosphate granulation decreased in Strain D269 under iron deficiency, and in Strains D253, D266 and D269 under magnesium deficiency; it was unchanged in the other strains in both conditions. Table 6.1 summarizes some of the responses to phosphate, iron and magnesium deficiencies of the 13 strains known to be capable of hair formation, incorporating results from Chapters 4 and 5 as well as the results just described.

Of all the treatments tested, phosphate deficiency had the most marked effect on hair development. None of the strains which

TABLE 6.1

Effects of deficiencies of PO_4 , Fe and Mg deficiencies on hair development, sheath pigmentation, heterocyst frequency and polyphosphate granulation in the 13 strains of Rivulariaceae known to be capable of forming hairs

+ = increase; (+) = slight increase; - = decrease; blank indicates no change; * = few, short hairs in control cultures; ** = many long hairs in control cultures.

	Hair development		Brown sheath pigment		Heterocyst frequency		Polyphosphate granulation	
	PO_4 deficient	Fe-Mg deficient	PO_4 deficient	Fe-Mg deficient	PO_4 deficient	Fe-Mg deficient	PO_4 deficient	Fe-Mg deficient
<i>Calothrix</i> sp. D184	+	(+)	+	+	+		-	-
<i>Calothrix</i> sp. D251*	+		+	+			-	-
<i>C. desertica</i> D270	+	(+)	+		+		-	-
<i>C. fusca</i> D269	+	+	+		+		-	-
<i>C. scopulorum</i> D256	+	+	+		+		-	-
<i>C. thermalis</i> D266	+	+	+		+		-	-
<i>C. viguieri</i> D253	+	+	+	+	+		-	-
<i>Dichothrix</i> sp. D280	+		+	+	+		-	-
<i>Gloeotrichia echinulata</i> D126**	+		+		+		-	-
<i>G. ghosei</i> D277**	+	(+)					+	+
<i>Homoeothrix crustacea</i> D401*	+						-	-
<i>Rivularia</i> sp. D403*	+	+					-	-
<i>Rivularia</i> sp. D404**	+		+				+	+

had been found to develop hairs did not show an increase in hair formation under this condition. Further experiments on hair development were therefore concentrated on its relationship with the supply of phosphate; these experiments are described in Chapters 7-10.

6.2 Hair development by 13 strains under nutrient deficiencies in the presence of $\text{NO}_3\text{-N}$

6.21 Heterocystous strains

The experiments described in Section 3.1 showed that a high level of combined nitrogen caused marked suppression of hair development in heterocystous strains which normally developed many hairs in AD medium (D126, D277, D404), though not in strains which produced only a few short hairs (D251, D403). It was of interest, therefore, to see whether the heterocystous strains which had shown increased development of hairs under particular deficiencies would also do so in the presence of combined nitrogen.

The heterocystous strains which had shown marked increases in hair development under deficiencies of phosphate, iron and magnesium are shown in Table 6.1 (the slight increase in hair length of some trichomes of Strain D277 under iron, calcium, magnesium and sulphate deficiencies is ignored). Each of these strains was inoculated into AD + 10 mM HEPES, with the addition of either 10 mM NaCl (= HAD-N) or 10 mM NaNO_3 (= HAD+N). These cultures were used as inocula for HAD-based deficient media, made up as described in Section 5.22 (Table 5.2), but with 10 mM NaCl or NaNO_3 added, to make -N and +N versions. Each strain was inoculated into the -N and +N version of the deficient medium or media which had given increased hair formation in the earlier experiments, and into complete medium controls. The incubation conditions were those given in Table 5.21 and Section 6.1. Cultures were scored for the presence of hairs when growth was obviously limited, with a second subculture if necessary.

In HAD+N the morphological response of each strain was the same as that described in Section 3.12. Strains D126, D277 and D404 produced predominantly parallel trichomes, and the other nine strains predominantly tapered trichomes. The overall morphology of the trichomes developed in each of the deficient media +N was very similar

to that in HAD+N, though modified by the presence of hairs. Each of the deficient media which had induced increased hair development in a particular strain in the HAD-N version also did so in the HAD+N version. In Strains D126, D277 and D404 the frequency of hairs was lower, and the hairs were shorter, in the HAD+N control as compared with the HAD-N control (cf. Section 3.12). In phosphate deficient HAD+N, the frequency of hairs was very similar to that in full HAD+N, but the hairs were longer (though not as long as those in phosphate deficient HAD-N). As in full HAD, the hairs were present at the ends of long (up to 2 mm) trichomes, which were otherwise parallel. In the other nine strains, the frequency and appearance of the hairs that developed in deficient cultures were very similar in the presence and absence of $\text{NO}_3\text{-N}$. Thus all the strains produced many long hairs under phosphate deficiency, and the hairs developed in iron and magnesium deficient media were rather shorter. Strains D184 and D270, which produced only a few, very short hairs under iron deficiency in HAD-N, also gave this response in HAD+N without added iron. The hairs in all nine strains developed at one end of the 'Homoeothrix'-like trichomes, and at both ends of the 'Ammatoidea'-like trichomes.

Thus in each strain the characteristic responses to the presence of $\text{NO}_3\text{-N}$ and to particular nutrient deficiencies occurred simultaneously. In the three strains with long hairs in full AD medium, fewer hairs were produced in the media + NO_3 , and the hairs were shorter. However, the length of the hairs increased under phosphate deficiency in the presence and absence of NO_3 . The seven strains that had no hairs in AD medium, and the two strains that had only a few short hairs all showed an increase in hair development under the various deficiencies, and there was no obvious suppressive effect of $\text{NO}_3\text{-N}$ on the production of hairs.

6.22 Homoeothrix crustacea D401

Strain D401 did not produce hairs in full Chu 10-D medium in the presence of sufficient $\text{NO}_3\text{-N}$ to allow active growth, but tapered apices, and some short hairs, were developed as growth became limited by the availability of nitrate (Section 3.33). However, from the results described in Chapter 5, it seemed that the development of apices of this type was a relatively non-specific response to growth limitation, rather than a specific effect of

nitrogen deficiency. Long hairs were observed only in low phosphate medium. The relatively low level of $\text{NO}_3\text{-N}$ in Chu 10-D (6.83 mg l^{-1}) meant that control cultures began to develop symptoms of nitrate deficiency after about a week of incubation. Although the long hairs characteristic of phosphate deficient cultures appeared at a time when the control trichomes were still predominantly untapered, it did seem possible that incipient nitrate deficiency might have had some influence in producing the response.

Strain D401 was therefore inoculated into HAD+N and HAD+N with $0.01\times$ the normal level of $\text{PO}_4\text{-P}$ (Table 5.2). The initial level of $\text{NO}_3\text{-N}$ in HAD+N (140 mg l^{-1}) was about $20\times$ that in Chu 10-D. The incubation conditions were those shown in Table 5.1.

In HAD+N, the trichome apices remained untapered for about 3-4 weeks, and only then began to show any evidence of growth limitation, with the development of some tapered apices, and a few short hairs. Growth was obviously limited after six days in the low phosphate medium, and many long hairs had been produced. The appearance of the trichomes was very much as described for phosphate deficient Chu 10-D cultures (Section 5.32). This result suggested that the development of long hairs by Strain D401 in low phosphate medium was unlikely to be due to nitrogen deficiency.

7. MORE DETAILED STUDY OF ONSET OF PHOSPHATE DEFICIENCY IN CALOTHRIX STRAINS D184 AND D267

7.1 Introduction

In the experiments described in Chapter 5, detailed morphological scores were generally made at a time when hairs, if produced, were fully developed and present at a high frequency. Relatively few observations had been made on cultures at the time hairs first developed, but a general impression had been gained that hair formation seemed to be one of the earliest responses, preceding changes such as increased cyanophycin granulation, or production of brown sheath pigment. Hairs also seemed to appear at an earlier stage of growth than the morphological changes seen in strains which did not develop hairs.

In order to confirm or refute these impressions, a more detailed study was made of the appearance of symptoms of phosphate deficiency in two strains, one of which (D184) developed hairs under this deficiency, and one of which (D267) did not, relating the morphological changes to the growth curves of the algae. The development of long, highly vacuolated hairs by Strain D184 would be expected to lead to a fall in overall chlorophyll content under phosphate deficiency, and a loss of their normal bright green colour was a symptom of phosphate deficiency in both strains. In an attempt to quantify these changes, chlorophyll a was also estimated.

7.2 Methods

Strains D184 and D267 were selected for this experiment, since both grew relatively rapidly, and Strain D267, though not producing hairs, underwent a fairly clear-cut morphological change (elongation of the vegetative cells) under phosphate deficiency (Section 5.31). The algae were inoculated as uniformly as possible (Section 2.34) into 25 ml aliquots of AD + 5 mM HEPES (= HAD), with K_2HPO_4 to give 1.0x and 0.02x the normal level of $44.5 \text{ mg l}^{-1} PO_4\text{-P}$. The low phosphate level used was higher than that used for the earlier experiments (Chapter 4; Section 5.3), with the aim of delaying the onset of deficiency until measurable growth had taken place. The incubation conditions were those shown in Table 5.2; the flasks were moved around the culture tanks each day, to minimize effects of variation in light intensity between the sides and centre.

Cultures were harvested every 2-3 days for 28 days, taking four replicate flasks for each treatment; the replicates were pooled for the first three harvests (days 0-5). Dry weight and chlorophyll a determinations were made as described in Section 2.5. Since it seemed possible that chlorophyll degradation products might be present in the low phosphate cultures, allowance was made for the presence of phaeophytin in the extracts when calculating the chlorophyll values (Section 2.52). For Strain D184, the equation used was:

$$C_a = 2.10(Ab - Aa) \times 13.1 \times \frac{V}{I}$$

and for Strain D267:

$$C_a = 2.12(Ab - Aa) \times 13.1 \times \frac{V}{I} \quad (\text{cf. Section 2.52}).$$

The morphology of the algae was scored from a set of flasks which were not used for quantitative harvests.

7.3 Results

Dry weight and chlorophyll values for the two strains are plotted in Figs 7.1 and 7.2, with the times at which the chief morphological changes were observed indicated on the graphs (see also p. 239).

a) Calothrix sp. D184

There was no change in the morphology of Strain D184 during the first four days of culture in low phosphate medium, and polyphosphate and cyanophycin granulation also remained at the control levels. Hairs were first observed on day 5, with a frequency score of 2, and forming about 20-50% of the total trichome length. The majority of trichomes (frequency score = 4), including those with hairs, still contained polyphosphate granules, but at an estimated 50% of the control level, with granules visible in the basal one third of the trichomes only. The rest of the trichomes had no visible polyphosphate granulation. There were no other morphological or cytological changes, and the macroscopic appearance of the low phosphate and control cultures was the same.

When the algae were next examined, on day 7, the frequency of trichomes with hairs had increased to 4, and the hairs were longer, forming about 50-80% of the total trichome length. There was no

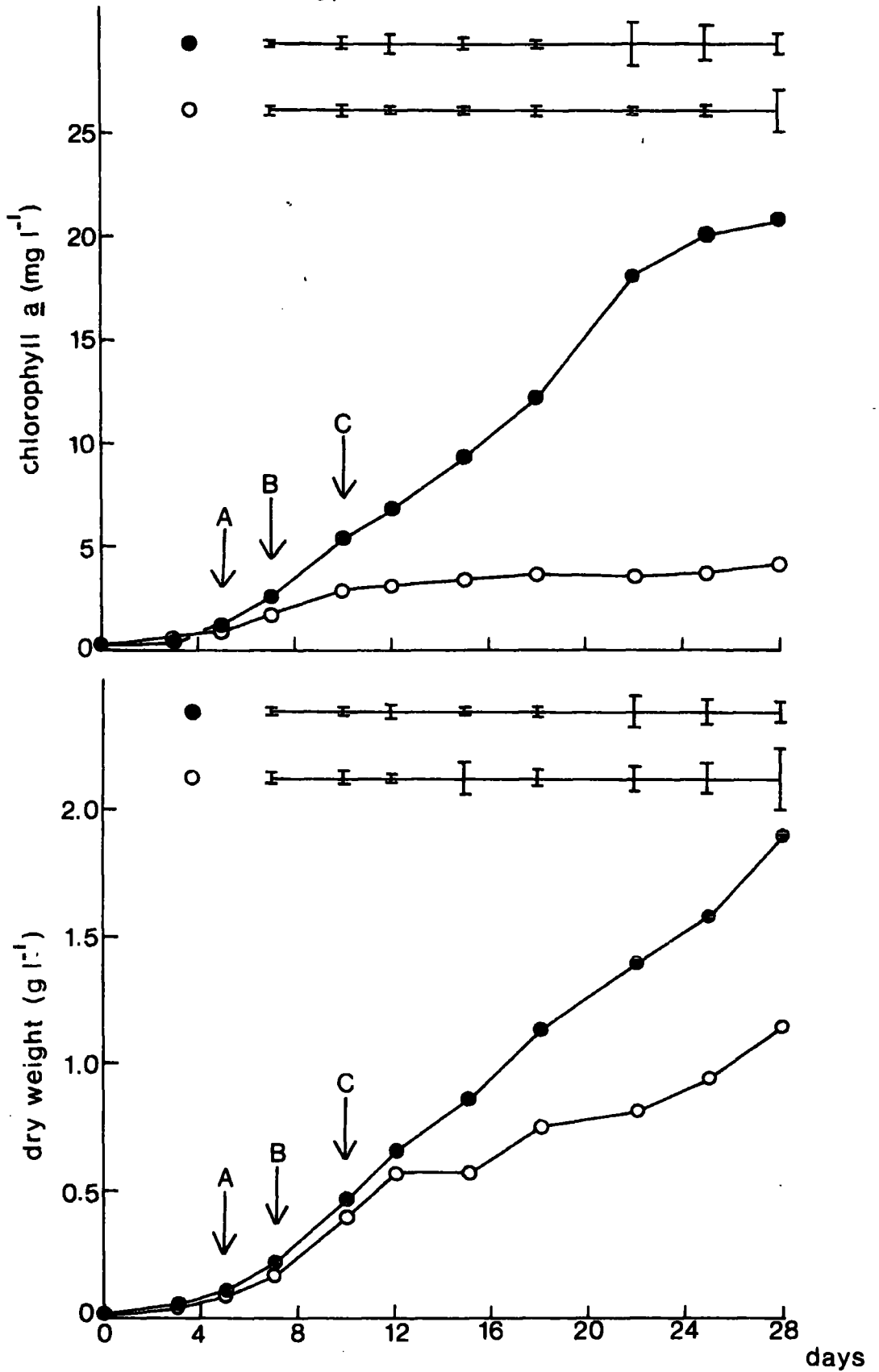


Fig. 7.1 Growth of *Calothrix* sp. D184 in HAD with initial levels of 44.5 (●) and 0.89 (○) mg l⁻¹ PO₄-P

Morphological responses at the times indicated by arrows are shown on p. 239.

For clarity, standard error bars are shown separately.

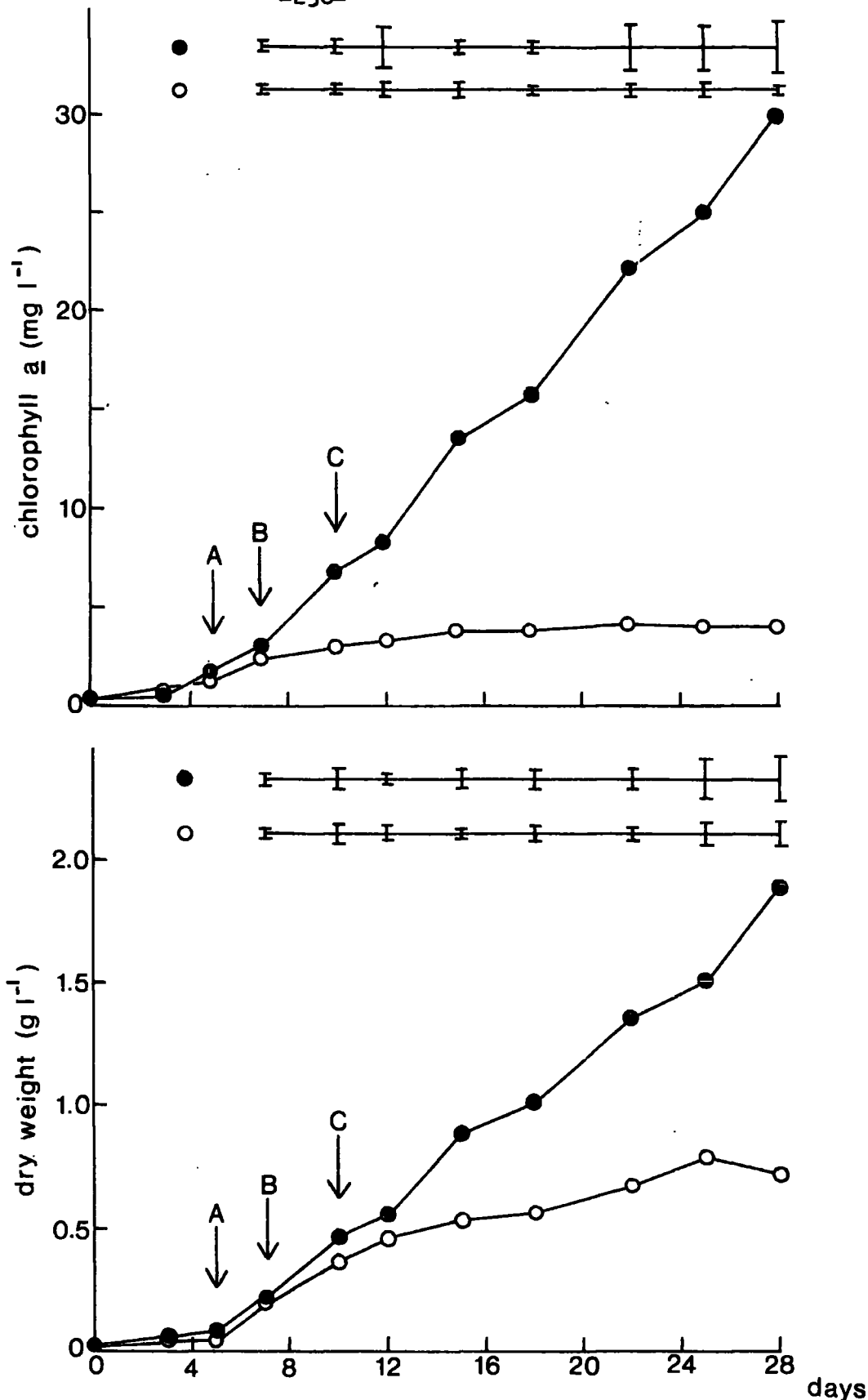


Fig. 7.2 Growth of *Calothrix* sp. D267 in HAD with initial levels of 44.5 (●) and 0.89 (○) mg l⁻¹ PO₄-P

Morphological responses at the times indicated by arrows are shown on p. 239.

For clarity, standard error bars are shown separately.

Key to Figs 7.1 and 7.2 (morphological changes in low phosphate cultures)

a) Calothrix sp. D184 (Fig. 7.1)

	A	B	C
Hairs	few	many	many
Polyphosphate	reduced	absent	absent
Cyanophycin	normal	increase	large increase
Brown sheaths	normal	increase	large increase
Hormogonia	many	many	few

b) Calothrix sp. D267 (Fig. 7.2)

	A	B	C
Cell elongation	none	slight	marked
Polyphosphate	reduced	absent	absent
Cyanophycin	normal	normal	increase
Hormogonia	many	many	few

detectable polyphosphate granulation in any of the trichomes, but cyanophycin granulation had increased slightly. Some of the trichomes (frequency score = 2) had slightly brown pigmented sheaths. This gave the cultures a faint brown colour when viewed macroscopically, but there was no other difference from the control cultures. Despite the presence of hairs in the cultures, many hormogonia were still present, and hormogonium release still appeared to be taking place.

On day 10, almost every trichome in the low phosphate cultures had a hair, and the hairs were very long, often exceeding 80% of the total trichome length. Cyanophycin granulation had now markedly increased, and most of the trichomes had brown sheaths. A slight paling in the colour of vegetative cells was also apparent at this time. Very few hormogonia were observed in the low phosphate cultures at this stage, but they were still abundant in the control cultures.

There was little apparent change in the overall morphology of the alga after this time, but the very great length of the

hairs made it difficult to estimate whether further increases in hair length occurred. The thickness and pigmentation of the sheaths, and the level of cyanophycin granulation, all showed slow increases throughout the experiment. The morphology of the alga in control cultures was essentially unchanged during the experiment, apart from an increase in the average basal diameter (cf. Section 2.121) and a reduction in the frequency of hormogonia.

b) Calothrix sp. D267

In this strain, the first change that was observed in low phosphate cultures was a decrease in the level of polyphosphate granulation, on day 5. The granulation was unaffected in about half the trichomes, but in the rest of the population the level of granulation was an estimated 50% of that of control trichomes, and granules were present only in the basal few cells. There was no change in the morphology of the alga until day 7, when many of the trichomes (frequency score = 3) had developed rather elongated cells (cf. Section 5.31(iv)). Polyphosphate granules were now undetectable in all the cells; there was no increase in cyanophycin granulation. The algae were still normal in colour.

By day 10, the low phosphate cultures were yellow macroscopically, and almost all the trichomes had yellowish, elongated cells. An increase in the level of cyanophycin granulation was also now apparent. For the first time, the frequency of hormogonia was observed to be well below that in control cultures, and the sheaths of trichomes in the low phosphate medium were now obviously thicker than those of control trichomes. No other specific changes in trichome morphology were observed, but partial fragmentation of the trichomes was seen in low phosphate cultures on day 15, and this increased towards the end of the experiment (frequency score = 2 by day 28). As in Strain D184, there was little change in the appearance of control cultures during the experiment.

In both strains, chlorophyll showed little increase in low phosphate cultures after days 7-10, but dry weight continued to increase up to the end of the experiment, on day 28 (Figs 7.1 and 7.2). Much of the increase was probably due to the production of sheath material; certainly the sheaths of phosphate deficient trichomes increased in thickness during the experiment. Adsorption of ions from the medium by the sheaths could perhaps also have

contributed to the increase in dry weight. In the high phosphate cultures, chlorophyll and dry weight were still increasing in both strains on day 28. There was no marked change in chlorophyll as percent dry weight (Table 7.1) in either strain, which suggested that balanced growth was still taking place. It was possible, however, that the increases in chlorophyll took place in response to self-shading effects, and that the increases in dry weight were due to increased sheath development and/or adsorption of ions.

There was a fall in % chlorophyll content in both strains in low phosphate medium (Table 7.1). In Table 7.1 the percentage chlorophyll values for the low phosphate cultures on days 7, 10, 15, 22 and 28 are also expressed as a percentage of the high phosphate value for that strain on day 10, to give an indication of the extent to which chlorophyll content was reduced. The percentage fall in chlorophyll content was not significantly different ($P \leq 0.05$) between the two strains, despite the presence of hairs in Strain D184, but the comparison is complicated by the production of abundant sheath material, which would contribute to the dry weight value, by both strains.

7.4 Summary of results

1. The results confirmed the impression that at least in Strain D184, the appearance of hairs was the first clear-cut response to a low concentration of phosphate, and that hairs developed before growth became limited. When hairs first developed, polyphosphate granules were often present in trichomes with hairs, though they were usually present in the basal cells only. This seemed to imply that hairs were produced before the trichomes were completely phosphate starved. Other symptoms of phosphate deficiency in Strain D184 did not appear until 2-5 days later, when almost all the trichomes had long hairs.
2. In Strain D267, no change in trichome morphology was observed before the polyphosphate granules had disappeared completely; a partial loss of polyphosphate was the first detectable symptom.
3. In both strains, the initial responses in the low phosphate medium were observed before there was any divergence in dry weight or chlorophyll level between the high and low phosphate cultures.

TABLE 7.1

Chlorophyll as % dry weight for *Calothrix* sp. D184 and *Calothrix* sp. D267 in HAD with 1.0x and 0.02x the normal level of PO₄-P, after 7, 10, 15, 22 and 28 days of culture

For each strain, the low phosphate % chlorophyll is also expressed as a % of the high phosphate % chlorophyll for that strain on day 10 ($= \frac{\text{low}}{\text{high}} \times 100$). This last value is compared between the two strains, and the probabilities that the differences are significant are shown.

Day	Calothrix sp. D184			Calothrix sp. D267			low high x 100(D267) - high x 100(D184)	Pro- bability
	PO ₄ x 1 4	PO ₄ x 0.02 4	low high x 100	PO ₄ x 1 4	PO ₄ x 0.02 4	low high x 100		
7	1.21±0.04	0.94±0.05	80.6±4.3	1.34±0.09	1.00±0.05	69.4±3.8	- 11.2±5.7	0.1-0.2
10	1.16±0.08	0.75±0.06	64.1±4.8	1.45±0.06	0.83±0.08	57.6±5.2	- 6.5±7.1	0.4-0.5
15	1.09±0.03	0.61±0.05	52.6±4.3	1.53±0.03	0.72±0.06	49.7±4.1	- 2.9±6.0	0.6-0.7
22	1.31±0.10	0.44±0.01	37.8±1.3	1.64±0.06	0.62±0.01	42.7±1.0	4.9±1.6	0.05-0.1
28	1.10±0.03	0.38±0.04	32.7±3.6	1.60±0.01	0.58±0.02	40.5±1.5	7.8±3.9	0.1-0.2

Despite the obvious limitation of growth in the low phosphate cultures, they continued to show increases in dry weight throughout the experiment. This seemed likely to be due mainly to the production of sheath material.

4. There was a fall in chlorophyll content (as % dry weight) in both strains under phosphate deficiency, and the percentage fall was very similar in the two strains, despite the development of long vacuolated hairs by Strain D184. However, the comparison was complicated by the contribution of sheath material to the dry weight value in both strains.

8 GROWTH AND HAIR DEVELOPMENT IN 6 STRAINS OF RIVULARIACEAE IN AD MEDIUM WITH INCREASED LEVELS OF $\text{PO}_4\text{-P}$

8.1 Introduction

The experiment described in Chapter 4 showed that seven of the heterocystous strains would produce hairs when the initial concentration of phosphate in AD medium was reduced one hundredfold, to $0.44 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$, but their hair development was suppressed in the full medium, with an initial phosphate concentration of $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. Five heterocystous strains did produce hairs in full AD medium; all these also showed increased hair development in the low phosphate medium. By analogy with the former group of strains, it seemed possible that the development of hairs in these five strains might be suppressed by increasing the phosphate concentration above the normal AD level.

8.2 Methods

AD medium was made up with 10 mM HEPES (= HAD), and K_2HPO_4 was added to give 1.0x, 2.0x, 10.0x and 20.0x the normal level of $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. Since these increases considerably increased the total ionic concentration of the medium, a series of controls was run, containing NaCl at the same ionic concentration as the K_2HPO_4 . In retrospect, controls containing KCl would clearly have been more suitable. The pH of all media was adjusted to 7.0, instead of the usual 7.4, to reduce precipitation at the highest phosphate concentrations. There was no apparent precipitation in media with 1.0x, 2.0x or 5.0x the normal level of phosphate; at 10.0x the normal level, the medium appeared clear, but small crystals could be seen associated with the algal filaments on microscopic examination; at 20.0x the normal level, a fine white precipitate was visible in the flasks.

Six strains were used for the experiment: Strains D126 and D277, which produced many hairs in AD medium; Strains D251 and D403, which produced a few short hairs, and Strains D184 and D267, which had no hairs in AD, though Strain D184 produced them at lower levels of $\text{PO}_4\text{-P}$ (Chapter 4). The last two strains were included as controls, to indicate whether any responses shown by the strains with hairs were specific to this group, or were more general effects. Strain D404, which also produced long hairs in AD medium, was not

used, because of its poor growth in AD medium (cf. Section 5.22). Cultures were incubated, with shaking, in culture tanks at 25°C or 32°C (D251) (cf. Section 2.351), with a light intensity of 2500 lx. Growth and morphology were scored after 7 and 21 days.

8.3 Results

The extent of growth in control and experimental media after 21 days is shown in Table 8.1.

Increasing the concentration of K_2HPO_4 to more than twice the normal level was inhibitory to all the strains except D267, and Strain D126 was inhibited by twice the normal level. In all strains, a decrease in growth below a score of 3 (Table 8.1) was associated with yellowing of the algae; the yellowing increased progressively as phosphate concentration increased, and growth extent decreased. The strains varied in their apparent sensitivity to increased phosphate concentration; they are arranged below in sequence according to sensitivity:

D126 > D403 > D277 > D251 > D184 > D267

The strains with hairs in AD medium were the ones most sensitive to increased phosphate concentration, whereas those without hairs were relatively tolerant, showing perceptible, though rather poor growth at 10x the normal level of K_2HPO_4 . The behaviour of Strain D403 was more similar to that of the two strains (D126, D277) with many hairs in AD than to that of Strain D251, which it more closely resembled in hair frequency and length. The differences in growth extent that were observed after 21 days were still apparent after a further 21 days' culture, which suggested that total growth, and not merely growth rate, was inhibited by the high K_2HPO_4 levels.

Increased ionic concentration in the medium did not appear to be responsible for the growth inhibition, since most strains showed no response to increased NaCl concentration until at least the concentration equivalent to 10x the normal level of K_2HPO_4 (Table 8.1). Growth of Strain D403 was reduced at NaCl levels equivalent to 5x the normal level of K_2HPO_4 and above, but the colonies remained green in colour.

Since some precipitation occurred at the highest levels of K_2HPO_4 , some of the effects seen could have been due to changes

TABLE 8.1

Growth (0-5) of six strains of Rivulariaceae in HAD with K_2HPO_4 at 1x, 2x, 5x, 10x and 20x normal.

At each phosphate level a control with added NaCl was used; growth in control flasks is also shown.

* = few, short hairs with $PO_4 \times 1$

** = many, long hairs with $PO_4 \times 1$

Strain	Initial K_2HPO_4 level				
	1x (44.5 $mg\ l^{-1}$)	2x (89 $mg\ l^{-1}$)	5x (222 $mg\ l^{-1}$)	10x (445 $mg\ l^{-1}$)	20x (890 $mg\ l^{-1}$)
<u>Calothrix</u> sp. D184 (control)	5	5	3	2	+
<u>Calothrix</u> sp. D251* (control)	5	5	3	1	+
<u>Calothrix</u> sp. D267 (control)	5	5	5	5	5
<u>Gloeotrichia echinulata</u> D126** (control)	5	1	+	0	0
<u>G. ghosei</u> D277** (control)	5	5	5	5	4
<u>Rivularia</u> sp. D403 (control)	4	4	0	0	0
			2	2	2

in the medium resulting from co-precipitation of other components. However, differences in response between the algae were obvious even in media without apparent precipitation, so it seemed unlikely that the effects were wholly due to this effect.

In the strains with hairs, the overall morphology and the extent of hair development were the same in each of the media in which reasonable growth had occurred (score 3 and above), and the cultures were more or less normal in colour. In media with higher levels of K_2HPO_4 , the algae were yellowish, and many of the trichomes were extensively fragmented. There was a decrease in the number of hairs that were seen in such cultures, but this seemed likely to be a consequence of the general fragmentation of the trichomes, rather than a specific effect upon the hairs. In the two strains without hairs, the morphology was unaffected at 2x and 5x the normal level of K_2HPO_4 . At levels above this, the trichomes were yellowish, and fragmented, but there was no other effect upon morphology. In Strains D184, D251 and D267 many undifferentiated hormogonia were present in the media with higher concentrations of K_2HPO_4 , presumably reflecting the inhibition of growth in these media. None of the strains showed any morphological response to increased NaCl concentration, even when growth was slightly inhibited.

Thus although phosphate deficiency caused marked increases in the development of hairs in the strains which possessed them in AD medium, it was not apparently possible to suppress hair development entirely by providing very high levels of phosphate, except as a result of the fragmentation of trichomes at the highest levels of phosphate. Some other factor presumably controlled hair development in these strains.

8.4 Summary of results

Increasing the K_2HPO_4 concentration of AD medium to more than 2-5x the normal level of $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$ inhibited the growth of all six strains tested. The two strains with many hairs in AD (D126, D277), and one of the strains with few hairs in AD (D403) were more inhibited than the other three strains (D184, D251, D267).

Hair frequency and length were not affected by increases in phosphate concentration, apart from reductions in frequency associated with trichome fragmentation. Thus it was not apparently possible to suppress hair formation by raising the phosphate concentration.

9 FURTHER STUDY OF DEVELOPMENT OF POLYPHOSPHATE GRANULES IN VEGETATIVE CELLS AND HAIR CELLS OF CALOTHRIX SP. D184

9.1 Introduction

The results described in Section 5.31(ix) showed that hair cells as well as vegetative cells were able to develop polyphosphate granules following the addition of phosphate to a phosphate starved culture. Polyphosphate synthesis is an energy-requiring process (Harold, 1966; Section 1.63), and it seemed that this response was a potential means of comparing the physiology and energy requirements of hair cells and vegetative cells. As a simple experiment, it seemed of interest to compare the effects of dark incubation and pre-incubation on polyphosphate granule formation by hair cells and vegetative cells.

9.2 Methods

Calothrix sp. D184 was grown for 21 days in quadruplicate flasks of AD + 5 mM HEPES, with 0.01x the normal level of PO_4-P (Table 5.2). After this time, polyphosphate granules had completely disappeared and almost every trichome had a long hair. For four days preceding the addition of phosphate, two of the flasks were covered with aluminium foil, with the aim of depleting stored energy reserves. At the end of the four days, the algae had become bluer green in colour, but their morphology was unchanged. Immediately before the addition of phosphate, the aluminium foil was removed from one of the flasks, and one of the two flasks pre-incubated in the light was wrapped in foil. The four different experimental treatments were designated as follows:

pre-incubation condition	condition during incubation + PO_4	
light	light	= LL
dark	light	= DL
light	dark	= LD
dark	dark	= DD

K_2HPO_4 was added to each flask, to give the normal level of $44.5 \text{ mg l}^{-1} PO_4-P$. Samples were examined and stained for polyphosphate after 5, 10, 20 and 30 min, and 1, 2, 3, 5, 8 and 24 h,

and every 24 h thereafter until no further changes in granulation were seen. The experiment was performed three times.

9.3 Results

The times at which various responses occurred are shown in Table 9.1. The results were very similar each time the experiment was performed.

Granules were visible in the vegetative cells after 5 min in all the treatments except DD, where they were not seen until 10 min after the addition of phosphate. The granulation of the DD treated trichomes was slightly less than that of the other treatments for the first 30 min, but after this time there was no discernible difference in the granulation of the vegetative cells between the four treatments. Granulation in all treatments increased during the first 3 h of the incubation, but there was little apparent increase after this time.

Granules were first visible in a small proportion of the hair cells of the LL, DL and LD treated cultures after 2, 3 and 8 h respectively. The size of the granules increased up to about 24 h; the frequency of granulated hair cells also increased in the LL and DL treatments, but not in the LD treatment. Granulation of the hair cells was never observed in the DD treatment.

After 48 h, trichomes in the LL and DL flasks had begun to produce typical gas-vacuolate hormogonia, and the hairs, still containing polyphosphate, were shed as described in Section 5.31(ix). No hormogonium production was observed in the LD or DD cultures, even after 10 days' incubation with phosphate (one experiment only), and the hairs remained intact. When the aluminium foil was removed from the LD and DD flasks after three days' incubation, and the cultures examined after 24 h, the granulation of the hair cells had reached the same high frequency as observed in the LL and DL flasks, and hormogonia had begun to form in the subapical region. The subsequent hormogonium release and hair shedding were the same as observed in the LL and DL incubations.

9.4 Summary of results

1. Polyphosphate synthesis was able to occur in the vegetative cells of Strain D184 even after 4 days' dark pre-incubation, so presumably its stored energy reserves were not fully depleted by

TABLE 9.1

Timing of responses of phosphate starved cultures of *Calothrix* sp. D184, following the addition of PO₄^{-P} under different incubation conditions

<u>Response</u>	<u>Treatment</u>			
	LL	DL	LD	DD
Polyphosphate granules first seen	5 min	5 min	5 min	10 min
	2 h	2 h	8 h	-
Polyphosphate granulation at maximum level	3 h	3 h	3 h	3 h
	24 h	24 h	24 h	-
Frequency of hair cells and granules	+	+	+	-
	5	5	+	-
Hair fragmentation first seen	48 h	48 h	-	-
Complete loss of hair	120 h	120 h	-	-
Hormogonia first released	48 h	48 h	-	-

this time. This may have been related to the fact that the cultures were not growing actively at the time of the experiment, with a corresponding reduction in their energy requirements. The slower rate of granule development in DD cultures as compared with LD cultures suggests that some depletion of the energy reserves of the vegetative cells did occur during the dark pre-incubation.

2. Extensive production of visible polyphosphate granules ^{by the hair cells} required light during the incubation with phosphate. Although the DD treated vegetative cells evidently had sufficient energy reserves to synthesize large amounts of polyphosphate, there was apparently no transfer of such energy reserves to the hair cells under this condition.

3. The production of gas vacuoles and the release of hormogonia did not occur in the dark. This was probably due to the (presumed) high energy requirements of hormogonium production and release, though the possibility of a photomorphogenetic response is not excluded.

10 EXPERIMENTS WITH FIELD MATERIALS

10.1 Growth and hair development in Rivularia in crude culture with different initial levels of phosphate

The results described in Chapters 4 and 5 showed that phosphate concentration had a marked influence on hair development in various laboratory strains of Rivulariaceae. From the limited data available, it also appeared that very high levels of phosphate in the medium were more inhibitory to growth of strains which had hairs in AD medium than to that of strains which did not have hairs (Chapter 8). Several experiments were performed on the effects of phosphate concentration on growth and hair development in Rivularia from field populations, to compare with those using laboratory cultures. These experiments are described below.

The media used for these experiments contained no added buffer, since at the time they were performed, a HEPES buffering system had not yet been developed (Section 2.33). The pHs of the low phosphate media were adjusted to normal with 0.05 M NaOH (after autoclaving for liquid media; before autoclaving for agar media). Once adjusted, the pH remained fairly steady throughout the period of growth, and did not fall below 7.0 in any of the (liquid) media. In all media with reduced levels of K_2HPO_4 or KH_2PO_4 , the lower level of K^+ was compensated for by the addition of KCl. Chu 10-D was made up without the addition of silicate, to reduce the growth of diatoms.

10.11 Rivularia from Croft Kettle in liquid media

A description of this material was given in Section 2.362; information on the site is shown in Table 2.2.

Freshly collected colonies were inoculated into AD medium with 1.0x, 0.1x, 0.05x, 0.025x and 0.005x the normal level of $44.5 \text{ mg l}^{-1} PO_4\text{-P}$, and into Chu 10-D(-N) with 20.0x, 2.0x, 1.0x and 0.2x the normal level of $1.78 \text{ mg l}^{-1} PO_4\text{-P}$. The absolute levels of $PO_4\text{-P}$ are shown in Table 10.1. 25 ml aliquots of medium were used, in 100 ml conical flasks; incubation was in a growth room at 15°C , with a light intensity of 2000 lx. Growth and morphology were scored after 21 days (Table 10.1a).

Homogonia were released by the colonies in all the different media, forming 'haloes' around the colonies on the flask base.

Table 10.1a Growth of Rivularia (Croft Kettle) and associated algae, and extent of hair development in Rivularia, in AD and Chu 10-D(-N) with different initial levels of $\text{PO}_4\text{-P}$
 Scored +, ++, +++; where +++ = maximum observed.

X normal	Initial $[\text{PO}_4]_{\text{L}}$ mg l ⁻¹	Extent of growth			Hair development
		<u>Nostoc</u>	<u>Phormidium</u>	<u>Rivularia</u>	(<u>Rivularia</u>)
(i) <u>AD</u>					
1.0	44.50	+++	+++	+	+
0.1	4.45	++	+++	+++	++
0.05	2.22	+	+++	+++	++
0.025	1.11	0	++	+++	+++
0.005	0.22	0	+	++	+++
(ii) <u>Chu 10-D(-N)</u>					
20.0	35.60	+++	+++	+	+
2.0	3.56	+	+++	++	++
1.0	1.78	+	+++	++	++
0.2	0.36	0	+	++	+++

Table 10.1b Extent of hair development in Rivularia trichomes from AD cultures with initial phosphate concentrations of 44.5 and 1.11 mg l⁻¹ $\text{PO}_4\text{-P}$
 Each value is the mean of 20 measurements (μm).
 All differences between means are significant ($P \leq 0.001$).

Initial $[\text{PO}_4]$ (mg l ⁻¹)	Total trichome length	Hair length	Trichome length minus hair	Hair as % total length
44.50	256 ± 18	20 ± 4	236 ± 16	7.8 ± 1.5
1.11	1008 ± 88	630 ± 90	378 ± 29	59.6 ± 3.4

The three morphological forms described in Section 2.362 were all observed, but the majority of trichomes had a morphology corresponding to that described for Rivularia sp. D404. In several of the media, there was heavy growth of other blue-green algae, chiefly a 2 μm wide Phormidium sp. and a Nostoc sp. Both these algae had been observed in the original field material, but forming only a very small proportion of the total population. The extent of growth of these contaminants is also shown in Table 10.1a. The most vigorous growth of the two contaminants, particularly the Nostoc, occurred at the highest levels of phosphate used. Growth of the Rivularia was very poor at these high levels, and its maximum development occurred in media with initial phosphate concentrations of about 0.2-0.5 $\text{mg l}^{-1} \text{PO}_4\text{-P}$. The poor growth of the Rivularia at the highest phosphate concentrations did not seem to be entirely due to competitive growth of the contaminant algae, since at intermediate phosphate levels there was vigorous growth of all the forms.

The extent of hair development in the Rivularia daughter colonies also varied with the initial phosphate concentration (Tables 10.1a and 10.1b). Almost all the trichomes had hairs in all the media, but there was considerable variation in hair length. At the highest levels of phosphate, the hairs seldom had more than about six cells, and formed about 5-10% of the total trichome length, whereas at their maximum development, in media with initial levels of 1.11 $\text{mg l}^{-1} \text{PO}_4\text{-P}$ and below, the hairs formed as much as 80% of the total trichome length.

10.12 Rivularia from Croft Kettle on agar

Freshly collected colonies were inoculated onto plates of 0.5% (w/v) agar in AD medium, containing $\text{PO}_4\text{-P}$ at 0.5x, 0.05x, 0.005x and 0.0x the normal level of 44.5 mg l^{-1} . The normal level was not used, since the experiment described in Section 10.11 had shown growth to be very poor at this concentration of phosphate (Table 10.1a). Growth and morphology were scored after 4 weeks' incubation at 15°C, with a light intensity of 2000 lx (Table 10.2).

In each medium, 'haloes' of about 15 mm radius had developed around the inoculum colonies; these consisted of numerous daughter colonies 0.2-0.5 mm in diameter, all containing trichomes similar to those of Rivularia sp. D404. As observed in the experiment

Table 10.2 Growth and hair development in Rivularia (Croft Kettle) incubated on AD agar with different initial levels of PO_4-P .

Each value is the mean of 35 measurements (all measurements in μm).

Differences between means are all significant ($P \leq 0.001$) except where indicated. (The mean values for each character were compared in pairs, between phosphate concentrations.)

X normal	Initial $[PO_4]_{-1}$ mg l	Growth	Trichome length (+ hair)	Hair length	Trichome length (- hair)	Hair as % length	
	0.5	22.25	++	146 ± 6^a	39 ± 6^b	107 ± 5	25.0 ± 3.0^d
	0.05	2.22	+++	187 ± 13^a	42 ± 5^b	145 ± 9	21.2 ± 2.1^d
	0.005	0.22	+	633 ± 44	355 ± 38	278 ± 19^c	51.9 ± 3.3^e
	0.0	0.0	+	1006 ± 65	670 ± 61	335 ± 16^c	62.6 ± 2.7^e

- a) $P = 0.011$
- b) $P = 0.718$
- c) $P = 0.030$
- d) $P = 0.336$
- e) $P = 0.016$

in liquid media (Section 10.11), the best growth occurred with a relatively low initial level of PO_4-P , with less growth at the highest and lowest levels used. At the two higher levels of phosphate (Table 10.2), the trichomes in the daughter colonies were profusely false branched, usually with two or three successive tiers of branches. At the two lower phosphate levels, the daughter 'colonies' often consisted only of one trichome with perhaps 2-6 false branch trichomes associated.

Hair development was again most extensive in the media with lower initial levels of PO_4-P (Table 10.2). The absolute length of the hair and of the whole trichome, and the hair length as a percentage of the total trichome length, all increased with decreasing phosphate concentration. There was also an increase in the length of the vegetative part of the trichomes with decreasing phosphate concentration. This may have been related to the lack of hormogonium production under these conditions, since as mentioned above, the daughter colonies were much less extensively false branched.

10.13 Rivularia from Barras flush on agar

Details of the site from which this material was collected are given in Table 2.2. Colonies were inoculated onto plates of 0.5% (w/v) agar in AD medium with 1.0x, 0.1x, 0.025x and 0.005x the normal level of $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. The extent of growth and hair development were scored after four weeks at 15°C , with a light intensity of 2000 lx; the results are summarized in Table 10.3a.

As did the Rivularia from Croft Kettle (Section 10.12) these colonies produced haloes of hormogonia, but there was no apparent tendency for colonies to form, and the outgrowing trichomes seemed to correspond to a Calothrix sp. Trichomes like these had been observed in the inoculum colonies, but as a very small proportion of the total population (cf. Section 2.362). Unlike the Rivularia from Croft Kettle, this Calothrix did not show growth inhibition at high levels of phosphate; in fact its growth was most vigorous at the highest concentrations of phosphate used. However, as in the experiments described in Sections 10.11 and 10.12, the hairs produced at low phosphate levels were longer, both absolutely and as a percentage of total trichome length (Table 10.3b).

Table 10.3a Growth and hair development in trichomes from colonies of Rivularia (Barras) incubated on AD agar with different initial levels of $\text{PO}_4\text{-P}$

Initial $\left[\text{PO}_4\right]$ X normal mg l^{-1}	Growth	Hair development
1.0 44.50	+++	+
0.1 4.45	++	++
0.05 2.22	+	+++
0.025 1.11	+	+++
0.005 0.22	+	+++

Table 10.3b Extent of hair development in trichomes from plates with initial concentrations of 44.5, 4.45 and 1.11 $\text{mg l}^{-1} \text{PO}_4\text{-P}$

Each value is the mean of 40 measurements (all dimensions in μm).

Differences between means are significant ($P \leq 0.001$) except where indicated.

Initial $\left[\text{PO}_4\right]$ (mg l^{-1})	Total trichome length	Hair length	Trichome length minus hair	Hair as % total length
44.50	111 ± 12^a	18 ± 4^b	$93 \pm 10^{c,e}$	11.9 ± 2.1
4.45	162 ± 20^a	60 ± 13^b	$102 \pm 12^{c,d}$	27.4 ± 4.2
1.11	417 ± 51	350 ± 45	$90 \pm 8^{d,e}$	71.1 ± 2.8

a) $P = 0.025$
 b) $P = 0.002$
 c) $P = 0.559$

d) $P = 0.296$
 e) $P = 0.757$

10.2 Short-term incubation of Rivularia from Croft Kettle in medium with a high level of PO_4-P

Experiments with laboratory cultures showed that phosphate starved algae rapidly synthesized polyphosphate following the addition of PO_4-P . When hairs were present, the hair cells also developed polyphosphate granules, and the hairs were eventually shed (Section 5.31(ix)). It was of interest to determine whether providing a high level of phosphate to a field sample of Rivularia would elicit a similar response. This would confirm that the responses of the laboratory strains were not simply artefacts of culture, and might also indicate whether the hairs present in the field material had developed in response to phosphate deficiency.

Details of the material are given in Section 2.362, and of the site in Table 2.2. Long hairs were present on almost all the trichomes at the time of collection. Trichomes from several colonies were stained for polyphosphate within one hour of collection. The granulation of trichomes from each colony was very similar. Polyphosphate granules were visible in every vegetative cell of almost every trichome. The level of granulation was quite low, scoring 1-2 in the basal few cells, and 1 in cells further up the trichome (cf. Section 2.24); the granulation always decreased to zero in the cells below the transition zone. A single colony was cut into pieces which were incubated in 6 ml vials of AD medium at 15°C, with a light intensity of 1500 lx. Samples were taken for examination at time intervals, as described in Section 5.31(ix).

An unequivocal increase in the level of polyphosphate granulation in the vegetative cells was observed after 20 minutes' incubation. The presence of granules in the cells in the initial material made it difficult to estimate whether any increase occurred earlier than this. The level of granulation continued to increase up to 24 h after the start of the incubation, and all the vegetative cells reached granule densities scoring 4-5 (Section 2.24), with no apparent polarity of response along the trichome. Granules were first observed in the hair cells after 2 h incubation, and these granules also increased in density during the experiment, reaching a maximum at much the same time as the vegetative cells. Less than 5% of the hair cells (estimated) were affected, and the percentage did not increase during the incubation.

There was no apparent loss of hairs until hormogonia began to

be released, after about 48 h. The hairs then became detached, usually by lysis of one or several cells at the base of the hair. After their release, the hormogonia usually remained associated with the parent trichomes, producing small bunches of daughter trichomes at the top of most of the filaments. None of this second generation of trichomes had developed hairs after 7 days, when the experiment was terminated because of overgrowth by other algae. Overall, the response of this alga was very similar to that of the phosphate starved laboratory cultures following the addition of phosphate (Section 5.31(ix)).

11 DISCUSSION

11.1 Introductory outline

The aim of the work described in this thesis was to determine the influence of certain environmental factors on hair development in the Rivulariaceae. In view of a possible relationship between hair development and tapering, the latter character was also studied.

Several reports in the literature have described marked effects of combined nitrogen on tapering in heterocystous trichomes (Section 1.41), and the results of Fay, Stewart, Walsby and Fogg (1968) suggested that both tapering and hair development might be related to a decreasing gradient of fixed nitrogen between the basal and apical cells. Effects of combined nitrogen and of nitrogen starvation on tapering and hair formation in heterocystous and non-heterocystous strains were therefore studied (Chapter 3; Section 11.3).

A review of the literature indicated that hair development in members of several algal phyla has been found to increase under conditions of nutrient deficiency (Section 1.51). The effects of various mineral deficiencies on hair development were therefore examined (Chapters 4-6); the results are discussed in Section 11.4. Some other quite marked morphological responses to nutrient deficiency were observed, and these are also discussed in Section 11.4. Phosphate deficiency was found to have the most marked effect on hair formation, and further experiments were performed on the relationship between hair development and phosphorus concentration, with laboratory cultures (Chapters 7 and 8; Sections 11.4 and 11.5), and with field materials (Chapter 10; Section 11.6). Several results suggested the possibility that strains which produced long hairs even at high phosphate levels were better adapted to growth at low phosphate concentrations; this evidence is discussed in Section 11.7.

During the research, general observations were made on the morphology and development of Rivulariaceae which seemed sufficiently relevant to be included in the thesis (Section 2.12), and these observations are discussed in Section 11.2.

11.2 General observations on morphology and growth

As a background to the experimental studies, observations were made on the morphology and development of the 36 strains of Rivulariaceae held in culture (Section 2.12), and also on some field materials (Section 2.362). Although tapering of the trichomes was a character common to all the strains, there were qualitative as well as quantitative differences in this character between strains. In the majority of cases, most of the tapered appearance seemed to be due to enlargement of the trichome next to the basal heterocyst during hormogonium development, rather than to narrowing in the apical region. Marked decrease in apical diameter apparently occurred only in association with hair development. In the rich AD medium used as control for the 34 heterocystous strains, only three strains (D126, D277, D404) had many long hairs; two more (D251, D403) had a few short hairs. The proportion of forms with hairs in the collection of strains was considerably less than that among the descriptions given by Geitler (1932) (cf. Section 2.121). Only one of the two strains of Homoeothrix developed hairs in control conditions (in Chu 10-D medium), and then only as its growth became (nitrate) limited.

In the majority of strains studied, the most active cell division appeared to take place in the apical region, below the hair (where present). Such meristematic growth is typical of Rivulariaceae (Section 1.31). Although in some strains the cells in the basal region were short and disc-like, the apparently low frequency of newly formed transverse walls suggested that they were not actively dividing. No clear evidence of a basal meristem, as described by de Bary (1863a), Palla (1893) and Fay, Stewart, Walsby and Fogg (1968) (Section 1.31) was found. The nature of the developmental pattern in Rivulariaceae meant that batch cultures of a single strain contained trichomes of very variable morphology. This was observed even with clonal strains (Section 2.363), indicating that it was not merely the result of genetic variation.

Another potential source of morphological variation in field populations was suggested by observations made during attempts to isolate colonial Rivulariaceae (Section 2.362). Evidence was obtained that colonies of Rivularia may contain tapered filaments of more than one type. In several instances trichomes of Calothrix

were found among those of Rivularia; such associations have also been described in the literature (Bornet & Thuret, 1880; Bornet & Flahault, 1886b; Dutein, 1962; Section 1.34). Though present in only small numbers, or sometimes not even detectable in the field material, such Calothrix trichomes often became the dominant form in culture. A very similar phenomenon was described by Forest and Khan (1972), in Gloeotrichia echinulata and G. natans (Section 1.34).

Some general points emerged during routine culture work. The strains all showed rather slow rates of growth, with an estimated doubling time of not more than 2-4 days. Darley (1968) and Lange (1971) have also commented on the slow growth of Rivulariaceae in culture (Section 1.8). The growth form of the algae was also rather inconvenient for experimental purposes, consisting in most cases of a mat of densely interwoven filaments (Section 2.122).

11.3 Influence of nitrogen supply on hair development and tapering

11.31 Response of heterocystous strains to combined nitrogen

As described in Section 1.41, tapered heterocystous blue-green algae have been found to lose their taper when grown in the presence of combined nitrogen (Pearson & Kingsbury, 1966; Fay, Stewart, Walsby & Fogg, 1968; Wyatt, Martin & Jackson, 1973); this has led to speculations that tapering and hair development in Rivulariaceae may be related to a gradient of combined nitrogen along the trichomes (Fay, Stewart, Walsby & Fogg, 1968; Stewart, 1972). Kirkby (1975) found, however, that of the three strains of Calothrix she studied, only one became completely parallel in the presence of combined nitrogen. The other two strains produced trichomes which still tapered, towards one or both ends, resembling Homoeothrix or Ammatoidea (Section 1.41). This result implied that tapering in these two strains was unlikely to be due simply to shortage of combined nitrogen in the trichome apices. To find out whether suppression of tapering by combined nitrogen was widespread among Rivulariaceae, 34 heterocystous strains were grown in AD medium (Section 2.32) with and without the addition of 10 mM NaNO_3 (AD+N and AD-N) (Section 3.12).

(i) Tapering and heterocyst development

In all except one of the strains, which was completely unaltered in morphology, there was an almost total loss of heterocysts in AD+N. Such suppression of heterocyst development by combined nitrogen has been reported many times (Section 1.42).

19 of the strains, including the three which produced many long hairs in AD-N, grew almost entirely as parallel trichomes in AD+N; the remaining 14 strains all produced some parallel trichomes, but many tapered trichomes without heterocysts, and resembling Homoeothrix or Ammatoidea, were also present. As described in Section 1.33, several earlier workers have noted the occurrence of occasional Ammatoidea-like trichomes in cultures of heterocystous Rivulariaceae (de Bary, 1863b; Polyanskiĭ, 1928; Weber, 1933; Palik, 1946); Schwabe (1960) observed Homoeothrix-like filaments in a culture of Calothrix desertica (Section 1.41). Though the media used by these workers were not clearly defined, it seems quite possible that the effects they describe were responses to combined nitrogen, in view of the similarity to the present author's results.

The parallel and the tapered trichomes which developed in the AD+N cultures differed from those in AD-N in several respects besides the absence of heterocysts. In 16 of the 33 cases in which parallel trichomes developed in AD+N, and in 8 of the 14 cases in which tapered trichomes developed, the cells were relatively shorter in the trichomes from AD+N, and were often discoid in shape. The trichomes grown in the presence of combined nitrogen also tended to be wider: in 25 of the 33 strains with parallel trichomes in AD+N these were wider than those in AD-N, and in 11 of the 14 strains which developed tapered trichomes in AD+N their apical diameter was greater than that of heterocystous tapered trichomes.

Fay, Stewart, Walsby and Fogg (1968) described the growth of Rivulariaceae as being 'restricted to cells near the basal heterocyst', whereas trichomes cultured in the presence of ammonia were 'of uniform appearance', and unrestricted in length, implying that under these conditions all the cells were capable of growth and division, like those at the base of heterocystous trichomes. In the present study, the parallel trichomes which developed in AD+N

showed no apparent limitation in length, and there was no evidence that cell division was localized in any particular region of the trichome; in this respect the results agreed with those of Fay, Stewart, Walsby and Pogg (1968). The present author was however unable to detect an obvious meristematic zone at the base of trichomes of any of the strains examined (Section 2.121). Nevertheless, there did seem to be a tendency for the cells of trichomes in AD+N to resemble those close to the basal heterocyst of trichomes in AD-N, in their width and overall shape. The similarity was most marked in strains in which the basal region of heterocystous trichomes was quite markedly enlarged, with short, discoid cells.

The fact that in 19 of the 34 strains tapering was completely lost in the presence of combined nitrogen suggests that a gradient of combined nitrogen along the trichome may well be an important factor in determining the development of a tapered morphology. However, since 14 strains still produced tapered trichomes in the presence of sufficient combined nitrogen to suppress heterocyst development, it seems likely that other factors may be involved in at least some cases. The similarity in cell shape and dimensions between trichomes which developed in AD+N, and the basal region of heterocystous trichomes, perhaps suggests that the characteristic appearance of cells adjacent to the heterocyst may be at least partly due to effects of combined nitrogen from the heterocyst. This raises the possibility that the influence of heterocysts on tapering may be to cause enlargement of the basal cells, rather than narrowing of the apical cells. As noted in Section 2.121, in most of the strains studied the tapered morphology developed largely as a result of enlargement of cells at one end of the hormogonium. Nevertheless, as discussed below (Section 11.33), the two Homoeothrix strains both possessed basal enlargements in the absence of heterocysts.

(ii) Hair development

In the two strains which had only a few short hairs in AD-N, the hair length and frequency were very similar in AD+N. The hairs in these two strains developed at the ends of the tapered trichomes in AD+N. The three strains which produced many long hairs in AD-N all grew almost entirely as long parallel trichomes in AD+N. Hairs were still developed in AD+N, however, though they were

reduced in frequency and length. The hairs occurred abruptly at the ends of the long parallel trichomes, which were of otherwise uniform appearance. The results suggested that hair development was unlikely to be due solely to nitrogen deficiency at the trichome apex. Furthermore, the occurrence of hairs at the ends of otherwise parallel trichomes suggested that tapering and hair development may not necessarily be directly related phenomena.

Most of the observations discussed above were made on cultures grown with $\text{NO}_3\text{-N}$. Ten of the strains, including two of those with long hairs in AD-N, were also grown in HEPES-buffered AD with 5 mM NH_4Cl ; the results were very similar to those obtained with NaNO_3 (Section 3.13).

The taxonomic implications of the morphological modifications of members of the Rivulariaceae grown in the presence of combined nitrogen have been pointed out by Fogg, Stewart, Fay and Walsby (1973). Certainly, the similarity between many of the forms observed in AD+N, and members of the genera Lyngbya, Plectonema, Homoeothrix and Ammatoidea was often very striking. Palik (1946) (Section 1.41) considered that Ammatoidea was probably merely a growth form of Calothrix, and the results of the present study suggest that this could be true in some cases.

11.32 Response of Calothrix sp. D184 to nitrogen starvation

In a further study of the relationship between hair development (and tapering) and the supply of nitrogen, Calothrix sp. D184 was starved of nitrogen by incubating it under $\text{Ar:O}_2\text{:CO}_2$ (Section 3.2). Under these conditions, the alga rapidly became yellow in colour, and developed numerous small vacuoles in the cells. The frequency of heterocysts increased markedly, to an estimated 20% of the cell population. Similar responses have been described for Anabaena spp. under nitrogen starvation (Neilson, Rippka & Kunisawa, 1971; de Vasconcelos & Fay, 1974; Sections 1.42 and 1.62). Since the air-gassed control in this experiment also showed little growth in comparison with the non-gassed control cultures, it was not possible to state unequivocally that growth of the alga under $\text{Ar:O}_2\text{:CO}_2$ was limited by lack of nitrogen. Nevertheless, the similarity of its responses to those observed by other workers did suggest that the alga was nitrogen starved.

There was no marked change in the shape of the Calothrix trichomes under nitrogen starvation, apart from an increase in heterocyst size: the degree of tapering was unaltered, and there was no hint of hair formation, even though this strain was known to be capable of developing hairs under certain mineral nutrient deficiencies (Chapter 5). This was taken as further evidence that tapering and hair development in heterocystous Rivulariaceae are unlikely to be due solely to shortage of combined nitrogen in the apical region. The secondary heterocysts which developed in Strain D184 under nitrogen deprivation were formed in all regions of the trichomes, implying that the cells in the apical region had the potential to develop into heterocysts when starved of nitrogen. This suggested that in normal tapered heterocystous trichomes, with a single basal heterocyst, the apical cells were probably not in a severely nitrogen starved condition.

It could be argued that the failure to develop hairs was due to the overall inhibition of growth in the nitrogen starved cultures. However, the fact that the alga was still able to differentiate numerous heterocysts implies that it was capable of morphogenetic change under these conditions.

11.33 Response of Homoeothrix to the supply of combined nitrogen

Trichomes of Homoeothrix are tapered, and hairs may be present, but the trichomes do not possess heterocysts (Geitler, 1932). Superficially, at least, this would suggest that hair formation and/or tapering in this genus is unlikely to be the result of a gradient of fixed nitrogen along the trichomes, as has been suggested for heterocystous Rivulariaceae (Section 1.41). Despite the absence of heterocysts, it nevertheless seemed possible that Homoeothrix trichomes might be able to fix nitrogen under suitable conditions, since nitrogenase activity has been demonstrated in several non-heterocystous blue-green algae, particularly under microaerophilic conditions (Fogg, 1974; Section 1.62). In the case of a colonial form, such as H. crustacea, it seemed possible that the colonial growth habit might have a protective effect on nitrogenase, enabling it to function under externally aerobic conditions. Such a protective effect has been recently suggested by Carpenter and Price (1976), for Trichodesmium (Section 1.62).

Colonies of Homoeothrix crustacea from the field were

therefore assayed for acetylene reduction activity (Section 3.32). Assays were made both directly after collection, under Ar:O₂:CO₂ (94.5:4.5:0.5), and after 1-4 days' incubation in medium without combined nitrogen, under Ar:O₂:CO₂ (79.5:20:0.5) and under Ar:CO₂ (99.5:0.5). A Rivularia sp. known to be capable of acetylene reduction was included as control. No acetylene reduction activity was detected in the Homoeothrix colonies under any of these conditions. Since the Rivularia control showed a fall in acetylene reduction during prolonged incubation under Ar:CO₂, it was not possible to conclude that the H. crustacea colonies were incapable of acetylene reduction under this condition. The results suggested, however, that the colonies were not able to reduce acetylene under aerobic conditions, and thus probably did not fix nitrogen in the field, since they came from a well oxygenated environment (cool, flowing water).

The results obtained with heterocystous strains of Rivulariaceae grown in the presence of combined nitrogen (Sections 3.1 and 11.31) suggested the possibility that at least some forms of Homoeothrix might actually be growth forms of Calothrix or Rivularia in which heterocyst development had been suppressed. To examine this possibility, and to determine effects of combined nitrogen on tapering and hair development, two species of Homoeothrix were incubated in medium with and without the addition of NO₃-N (Section 3.33). In medium with combined nitrogen, the apices of both strains remained untapered as long as active growth and hormogonium release continued. Both still possessed a tapered morphology, however, insofar as the basal few cells of the trichomes were slightly enlarged. This was clearly apparent only in young cultures, since the trichomes became densely interwoven at higher culture densities. Neither strain showed any growth in the absence of combined nitrogen, and both rapidly became yellow, a common response of blue-green algae to nitrogen deficiency (Section 1.62). There was no change in the degree of tapering of Homoeothrix sp. D402, but in nitrate limited cultures of H. crustacea D401, most of the trichomes developed tapered apices, and a few short hairs were also formed. However, later experiments (Chapter 5) indicated that this response of Strain D401 was a relatively non-specific response to growth limitation (cf. Section 11.4).

Neither strain of Homoeothrix showed any evidence of heterocyst

development, even when obviously nitrogen starved. It thus appeared unlikely that either was simply a growth form of Calothrix or Rivularia.

11.4 Morphological responses to deficiency of inorganic nutrients

11.41 General remarks

Several published reports have described increases in hair formation in eukaryotic algae under conditions of nutrient deficiency (Section 1.51). Experiments were therefore performed to determine effects of nutrient deficiency on hair development in Rivulariaceae. The deficiencies examined were those of phosphate, iron, magnesium, calcium, molybdenum and sulphate. Kirkby (1975) found that Rivularia trichomes produced longer hairs when cultured with relatively low levels of inorganic phosphate (Section 1.41), and phosphate deficiency was therefore studied in rather more detail. In addition to effects on hair development, several other marked morphological responses to these deficiencies were also recorded, both for their inherent interest, and because of possible relationships with hair development.

11.42 Hair development in response to nutrient deficiency

Following Kirkby's (1975) findings on hair development in Rivularia at different phosphate concentrations (Section 1.41), all 34 heterocystous strains, and the two strains of Homoeothrix were screened for the effects of phosphate deficiency on hair formation (Chapter 4). Twelve of the heterocystous strains and one Homoeothrix strain showed a marked increase in hair development (Table 4.1). Three of these strains (D126, D277, D404) had many hairs in the high phosphate control medium, and two more (D251, D403) had a few short hairs; H. crustacea D401 also developed a few hairs, but only at the end of the growth period, in control medium. The remaining seven strains (D184, D253, D256, D269, D270, D280) had no hairs in full AD medium. The hairs that developed in low phosphate medium were eventually present on at least 90% of the trichomes in each affected strain, and formed as much as 80% of the total trichome length.

To determine whether the development of hairs in low phosphate medium was a specific response to phosphate deficiency, or was an effect of growth limitation per se, 13 strains were subjected to

iron, magnesium, calcium, molybdenum and sulphate deficiencies, with phosphate deficiency again included for comparison (Chapter 5). The 13 strains included eight of those which showed increased hair development under phosphate deficiency (D184, D251, D256, D277, D280, D401, D403, D404) and five which did not (D156, D179, D267, D283, D402); Anabaena cylindrica D2A was included, as a control. Three of the 13 strains (D251, D256, D403) developed many long hairs under iron deficiency, and one (D184) developed a few short hairs. One strain (D251) also produced long hairs under magnesium deficiency. All these strains were ones which also showed increased hair development in low phosphate medium. In Strain D277, an estimated 10-20% of the trichomes developed rather longer hairs under deficiencies of iron, magnesium, calcium and sulphate, but there was no increase in the number of trichomes with hairs; this response was thus distinct from that seen in phosphate deficient cultures. Homoeothrix crustacea D401 developed a few short hairs as growth became limited in each of the deficient media, as it did in the nitrate limited control (cf. Sections 3.33 and 11.33); again this response was much less marked than that seen in phosphate deficient trichomes. The responses of Strains D277 and D401 to deficiencies other than that of phosphate were so slight that they are not treated as increases in hair development in the following account. (The development of short hairs by Strain D184 under iron deficiency is included however, since this strain had no hairs in the control medium.)

During this experiment, what appeared to be normal vegetative cells were occasionally observed among a series of fully vacuolated hair cells in deficient cultures (Sections 5.31(ii) and 5.41(ii)). This did not appear to be simply an early stage in hair development. The occurrence of these cells perhaps implies that not all the cells in the apical region were equally susceptible to whatever stimulus led to the hair development response.

Of the deficiencies, other than that of phosphate, that were tested in this experiment with 13 strains, only those of iron and magnesium led to appreciable increases in hair development, and all the affected strains were ones which also showed increased hair development under phosphate deficiency. Five of the strains which had shown increased hair development in low phosphate medium (Chapter 4) had not been tested for the effects of other deficiencies.

These strains (D126, D253, D266, D269, D270) were therefore screened for effects of iron and magnesium deficiencies on hair formation (Section 6.1). Three of the five strains (D253, D266, D269) produced many long hairs under iron deficiency, and one (D270) produced a few short hairs. Magnesium deficiency did not affect hair formation in any of the five strains.

Thus overall, phosphate deficiency led to increased hair development in 13 of the 36 strains; eight of these 13 also showed increased hair development under iron deficiency, and one of the eight did so under magnesium deficiency (Table 6.1; p. 231). In 23 of the strains examined, no hairs were ever observed, and it seemed probable that these strains did not have the genetic ability to form hairs. Nine of the 12 heterocystous strains which developed long hairs under phosphate deficiency were ones which had no hairs or very few hairs in the control medium; these strains all had trichomes with a marked basal enlargement, and were also ones which retained a predominantly tapered morphology in the presence of combined nitrogen (Sections 3.1 and 11.3). All three (heterocystous) strains which had numerous long hairs in the control medium showed a marked increase in hair development in low phosphate medium, but their hair development was unaffected by iron or magnesium deficiencies.

During these experiments on nutrient deficiency, the impression was gained that hair development was one of the first morphological responses to occur in low nutrient media. This was confirmed, for one strain, when a detailed study was made of the onset of phosphate deficiency in Calothrix sp. D184 (Chapter 7). Hairs were found to appear before growth was limited in the low phosphate cultures, and while polyphosphate granules were still present in the basal cells of many trichomes. This implied that hair development took place before the trichomes were fully phosphate starved. In Calothrix sp. D267, the non-hair forming strain used as control in this experiment, there was no change in trichome morphology in low phosphate cultures until all the polyphosphate granules had disappeared.

Each of the 12 heterocystous strains which had shown increased hair development under phosphate, iron or magnesium deficiencies in the absence of combined nitrogen was also grown to the appropriate deficiency or deficiencies in AD medium with 10 mM NaNO_3 (Section 6.2), a level sufficient to suppress heterocyst development

(Section 3.1). In the nine strains which did not have hairs in the complete medium, hair development in the deficient media +NO₃ was very similar to that in deficient cultures without NO₃; the hairs formed at the ends of both 'Homoeothrix'-like and 'Ammatoidea'-like trichomes. In Strains D126, D277 and D404, which produced long hairs in AD medium, hair development was considerably less in the (phosphate) deficient medium +NO₃ than in the same medium without NO₃, but it was nevertheless greater than that in full AD+NO₃. The hairs in these three strains developed at the ends of long trichomes that were otherwise parallel. These results were taken as further evidence that hair formation was unlikely to be due simply to a gradient of combined nitrogen along the trichomes (cf. Section 11.3).

Hair development caused an increase in total trichome length whenever it occurred (except in Strains D184 and D270 in iron deficient medium), and there was thus a considerable increase in surface area of the trichomes (cf. Kirkby, 1975). For any particular strain, the hairs developed under iron or magnesium deficiency were not as long as those found in phosphate deficient cultures, and fewer trichomes were affected (Table 5.4). This difference held good after variable incubation periods, and several subcultures. The greater length of the hairs formed in low phosphate cultures may have been related to the fact that hairs seemed to develop at an earlier stage of growth in such cultures. Possibly an inability to store large quantities of iron or magnesium meant that the onset of deficiency of these metals was more abrupt than that of phosphate deficiency. If deficiency developed very suddenly, then the algae might not be able to express fully their potential for hair development.

11.43 Other morphological responses of Rivulariaceae to mineral deficiencies

During experiments on effects of nutrient deficiencies on hair development (Chapters 4-6), several other quite marked responses were observed. Some of these seemed relevant to the problem of trichome tapering, and some appeared to be correlated with the ability to develop hairs. Most of the responses discussed below were scored only from the 13 strains used for the experiments described in Chapter 5 (see Table 5.1); data from other strains

are included where available. The morphological responses of Anabaena cylindrica D2A are compared with those of the Rivulariaceae in Section 11.45.

(i) Trichome dimensions and tapering

a) Heterocystous strains

Several deficiencies caused changes in the basal and/or apical diameter that were not apparently related to hair development, with consequent effects on the tapered appearance of the trichomes (Table 5.5).

In a number of cases a decrease in the average basal diameter of trichomes in the culture was observed, while the range of diameters remained unchanged. This was seen in Strains D156, D179, D184, D256, D267, D283 and D403 under iron deficiency, in Strains D156, D184, D256, D277 and D283 under calcium deficiency, and in all 11 strains under molybdenum deficiency. This effect appeared to be due to a combination of inhibited trichome development, and the production of pseudo-intercalary heterocysts, rather than being a specific effect on basal diameter. An absolute decrease in basal diameter occurred only under magnesium deficiency; all the strains except D277 were affected (Section 5.51(iii)). In five strains (D256, D267, D283, D403, D404) the decrease in diameter took place in the basal region only, but in the other five (D156, D179, D184, D251, D280) it was part of a decrease that took place along the whole length of the trichome. The decrease was associated with apparent death of the heterocysts, and seemed to involve actual shrinkage of pre-existing cells. In some cases the shrinkage in the basal region was so marked that the trichomes became tapered at both ends (Figs 5.14 and 5.15; pp. 206-207). A rather similar narrowing of cells in the basal region of trichomes of Rivularia angulosa was described by Geitler and Ruttner (1935-36), in colonies from the field; they commented that this phenomenon was characteristic of older trichomes (Section 1.31).

Taking the trichome as a whole, the hair development that occurred in certain strains under phosphate, iron and magnesium deficiencies caused a decrease in apical diameter, and thus an increase in the degree of tapering. However, the tapering of the vegetative region was not necessarily affected by hair development; in fact a change in subapical diameter (of vegetative cells) in

association with hair formation was detected in only two strains, D256 (decrease) and D277 (increase), both under phosphate deficiency (Table 5.5b). There were several instances of changes in subapical diameter that were not associated with hair development. Five strains (D156, D179, D184, D256, D283) showed a decrease in subapical diameter in iron deficient medium (in D156 and D179 the decrease was not specific to the apical region, but took place along the whole trichome). Five strains (D156, D179, D184, D251, D280) had narrower apices under magnesium deficiency, but in each case the decrease in diameter took place throughout the trichome. An increase in apical diameter occurred in five strains (D184, D256, D280, D403, D404) under calcium deficiency.

Overall, the effects on trichome dimensions illustrated the distinction between hair formation, and tapering per se, and indicated that a change in one of these characters is not necessarily associated with a change in the other.

b) Homoeothrix strains

The growth form of the two Homoeothrix strains made observation of their trichome bases difficult (Section 2.121), and detailed measurements on these strains were therefore confined to the apical region of the trichomes (Section 5.23).

The diameter of trichomes of Strain D401 was not obviously affected by any of the deficiencies tested, apart from a decrease in apical diameter in association with hair development in low phosphate medium. However, under deficiencies of iron, magnesium, calcium, molybdenum and sulphate, the transition from trichomes with untapered apices to those with tapered apices (and occasionally with short hairs) occurred at an earlier time than it did in the nitrate limited control. In each medium, this change occurred at the same time as growth became limited; it appeared to be related to the cessation of hormogonium production, and a relatively non-specific response to growth limitation.

Trichomes of Strain D402 decreased in diameter under deficiencies of iron, magnesium and sulphate; the decrease apparently took place throughout the length of the trichomes. Magnesium deficiency had an additional effect upon the apical cells of this strain, which did not enlarge as they characteristically did in many of the control trichomes. Under phosphate deficiency, on the other

hand, these enlarged apical cells were wider than those in the control, though the diameter of the rest of the trichome was not affected.

(ii) Changes in the shape of vegetative cells

(cf. Section 5.23(ii), p. 145; Table 5.6)

a) Heterocystous strains

The effect of each deficiency on cell length was consistent between all the heterocystous strains that were affected. Thus under phosphate deficiency there was an absolute increase in cell length in three strains, and a relative increase in one strain; under iron deficiency an absolute increase in cell length occurred in four strains, and a relative increase in two strains; under magnesium deficiency an absolute increase in cell length occurred in two strains, and a relative increase in nine strains; under calcium deficiency six strains showed an absolute decrease in cell length, and one strain showed a relative decrease (Table 5.6). No effects on cell shape were seen under deficiencies of sulphate or molybdenum. Increases in cell length were seen more often than decreases, and changes more often occurred along the whole length of the trichomes than in a particular region.

In general, the changes in cell shape were due to changes in length and width only, and the cells remained more or less cylindrical. However, in Strain D156 under magnesium deficiency, some of the cells, in addition to being markedly elongated, also had a rather distorted shape, with an irregular, undulating profile. Cells of a very similar appearance were described by Serbanescu (1966) in colonies of Gloeotrichia natans from the field (Section 1.41). The shape of the vegetative cells of Strain D277 was unaffected by any of the deficiencies, but in calcium deficient cultures some of the hair cells were spindle-shaped, rather than cylindrical (Section 5.61(ii); Fig. 5.17).

b) Homoeothrix strains

In the two strains of Homoeothrix, the determination of effects on cell shape was complicated by the fact that the average length of the cells increased towards the end of the growth period in nitrate limited control cultures (Section 3.33). In both strains under phosphate deficiency, however, there was an absolute increase in cell length that was greater than that seen in the controls;

it took place without a change in trichome diameter. In Strain D401 under iron deficiency the cells did not elongate at the end of the growth period as they did in control cultures, and were thus absolutely shorter. Cells of Strain D402 became absolutely shorter under deficiencies of iron and sulphate; in each case this was associated with narrowing of the trichomes, so that the length:width ratio of the cells was much the same as that of control trichomes.

Thus considerable changes in the shape of vegetative cells occurred in some strains in response to certain deficiencies. Elongation and/or narrowing of the cells were the responses most often observed, but these changes were quite distinct from those seen during hair development. Nevertheless, the deficiencies which most often produced cell elongation and/or narrowing were those (phosphate, iron and magnesium) which also had most effect upon hair development. The observed changes in cell length presumably resulted from an altered balance between cell division and overall cell growth. As mentioned in Sections 1.42 and 1.61, cell division in various micro-organisms, including blue-green algae, has been found to be more susceptible to inhibition than growth as a whole. In one of the few reports of morphological effects of nutrient deficiency on blue-green algae, Jensen and Sicko (1974) described marked increases in cell length in phosphate starved Plectonema boryanum, and speculated that the cell division process was deranged under this condition (Section 1.63). Cell elongation similar to that they described was observed in phosphate deficient trichomes of Strains D267, D283, D401, D402 and D403 in the present study.

(iii) Cytological changes

a) Cell colour

Most of the strains became less green, and eventually yellow, under each of the deficiencies tested. In strains with obvious phycobilin pigmentation, this was usually reduced or lost. There were exceptions to this yellowing response, however: Strain D403 remained bright green in phosphate deficient medium, and Strains D251 and D256 did so under calcium deficiency, even though growth of all three strains was obviously limited. Although the yield of Strain D403 was reduced in the low phosphate medium, its initial growth was more rapid, and its overall appearance more healthy

than that of control cultures; it seemed quite likely that the phosphate level in full AD medium was supra-optimal for this strain (cf. Section 1.9). The response of Strain D256 to calcium deficiency may have been related to its marine origin (Table 2.1), since the Ca:Mg ratio of seawater is higher than that of most freshwaters. Strain D251 was isolated from a thermal spring (Table 2.1), where the water chemistry may have been atypical also. Reduced chlorophyll content is a common response of algae to nutrient deficiency (Healey, 1973b; Section 1.61), and many of the colour changes seen probably represented non-specific effects of growth imbalance. In the case of iron and magnesium deficiencies, however, there may have been a more direct effect upon chlorophyll, since both these elements are required for chlorophyll synthesis (Sections 1.64 and 1.65).

Iron deficient cultures of the two strains of Homoeothrix, D401 and D402, remained pinkish and bluish grey respectively, and did not turn yellow like the nitrogen limited controls. This was presumably because the algae were nitrogen sufficient when their growth became limited, and did not use their phycobilin pigments as a nitrogen source (cf. Allen & Smith, 1969; Section 1.62). A very similar retention of phycobilin pigments under iron deficiency was observed by Boresch (1921) in Phormidium retzii (Section 1.64). The heterocystous strains on the other hand, all eventually became yellow under iron deficiency (Section 5.41(i)). This may have been because they were dependent upon nitrogen fixation as a source of combined nitrogen, since this process requires iron (Section 1.64). The very marked yellowing of the cells, with eventual cell lysis, that occurred in all the strains under sulphate deficiency (Section 5.8), was very similar to that described by Prakash and Kumar (1971) in Anacystis nidulans and Anabaena variabilis under this condition (Section 1.68).

b) Cell vacuolation

Vacuolation of vegetative cells, taking place without marked changes in cell shape, and distinct from that associated with hair formation, was observed in Strains D156, D277, D401 and D402 under phosphate deficiency; in Strains D156, D184, D277, D283 and D401 under iron deficiency; in Strain D401 under magnesium and calcium deficiencies; in Strains D184, D267 and D277 under molybdenum

deficiency, and in Strain D402 under sulphate deficiency (Table 5.7). The vacuolation affected all the vegetative cells equally, with no evident polarity of response, except in Strains D156 and D402 under phosphate deficiency, in which the apical cells were more often affected. Vacuolation occurred most often in iron deficient cultures. This might have been related to an effect upon chlorophyll synthesis, since vacuolation of chloroplast thylakoids has been observed in higher plants under iron chlorosis (Lamprecht, 1961; Stocking, 1975; Section 1.64). In phosphate deficient Strains D277 and D401, and in iron deficient Strain D184, normal hairs were present on many of the vacuolated trichomes. This result illustrated the difference between vacuolation as a pathological symptom, and that associated with hair development (cf. Geitler, 1932; Section 1.23).

c) Polyphosphate granulation

Changes in polyphosphate granulation that occurred in 13 strains under different deficiencies are summarized in Table 5.7; effects on additional strains tested under iron and magnesium deficiencies are shown in Table 6.1.

Polyphosphate granules became undetectable under phosphate deficiency in all 36 strains examined (Chapters 4 and 5). This response is well known for micro-organisms (Harold, 1966), including blue-green algae (Stewart & Alexander, 1971; Jensen & Sicko, 1974; Section 1.63). In most strains, polyphosphate became undetectable within a few days of the first subculture to low phosphate medium, but in Strains D126, D277, D403 and D404 more than one subculture was required to deplete the cells of granules. All four strains were ones with at least some hairs in the control medium.

Molybdenum deficiency had no apparent effect upon polyphosphate granulation in any of the 13 strains tested, and calcium deficiency affected the granulation only in Strain D277, which showed a decrease. There was a very marked increase in polyphosphate granulation in 12 of the 13 strains tested under sulphate deficiency: in sulphate deficient Strain D277, polyphosphate granules were even present in the cytoplasmic strands of the hair cells, though it was not certain that the granules had been synthesized after the hair cells had developed. The results obtained with sulphate deficient cultures resemble those of Lawry (1976), who described

accumulation of polyphosphate by sulphur starved cells of Anacystis nidulans and Microcystis aeruginosa.

Iron and magnesium deficiencies had more variable effects on polyphosphate in the 18 strains tested. Under iron deficiency, 7 strains showed decreased granulation, one strain showed an increase, and in 10 strains granulation was unaffected (Tables 5.7 and 6.1). Polyphosphate granulation decreased in 5 strains under magnesium deficiency, increased in one strain, and was unchanged in 12 strains. In 12 of the 18 strains, the effects of iron and magnesium deficiencies on polyphosphate granulation were the same. The level of polyphosphate was less often affected by iron deficiency in strains which showed increased hair development under this condition. Thus 6 of the 8 strains which developed hairs under iron deficiency showed no change in polyphosphate, and 2 showed a decrease, whereas only 4 of the 10 strains which showed no change in hair development did not show a change in polyphosphate (5 showed a decrease, and one an increase in granulation).

When there was a decrease in the level of polyphosphate granulation, the cells in the apical region often became devoid of granules while they were still present in the basal cells. This seemed likely to be due to the fact that the total amount of polyphosphate in the basal cells was greater, and possibly also to the more active metabolic state of the cells in the apical region. In cells which developed hairs in low phosphate medium, polyphosphate was always undetectable in the apical part of the trichomes, but in the early stages of deficiency granules were sometimes present in the basal few cells. In the case of strains showing increased hair development under iron or magnesium deficiency, polyphosphate granules were always undetectable in the hair cells and in the transition zone immediately below the hair, but in all cases except Strains D251 and D403 under iron deficiency, polyphosphate granules extended to the base of the transition zone in at least some trichomes.

d) Cyanophycin granulation

A marked increase in cyanophycin granulation occurred in all 34 heterocystous strains tested under phosphate deficiency (Chapter 4), and in 4 of the 16 heterocystous strains (D156, D184, D251, D280) tested under magnesium deficiency (Sections 5.51 and 6.1). The response observed with phosphate deficient cultures

is in agreement with that described by Jensen and Sicko (1974), for Plectonema boryanum. In an electron microscope study, they observed an increase in the number of cyanophycin granules in phosphate starved cultures (Section 1.63). Simon (1973) has shown that (in Anabaena cylindrica) this nitrogen reserve polymer accumulates under conditions which prevent protein synthesis.

In two strains (D256, D277) cyanophycin granules were quite often observed in the cytoplasmic strands of hair cells of phosphate deficient trichomes. Since in Strain D256 at least, the hairs developed in the cultures before changes in cyanophycin were observed, it seemed quite possible that the granules had been formed by the hair cells after they had differentiated. In 14 of the 23 strains which did not develop hairs under phosphate deficiency, cyanophycin granules were reduced in number or even completely undetectable in the apical one or two cells. In 9 of the 14, the apical cells of many trichomes were slightly elongated and/or narrowed, but the change in shape and the lack of granules were not necessarily correlated (Chapter 4).

e) Sheath characters and extracellular pigment production

An increase in sheath thickness was observed in all the heterocystous strains under each of the deficiencies tested. The most marked effects were seen under deficiencies of phosphate, iron and magnesium. An obvious increase in sheath thickness in the two Homoeothrix strains was observed only under phosphate deficiency, but the small size of their filaments made it difficult to assess smaller changes that may have occurred. Accumulation of carbohydrate appears to be a common response of algae to nutrient deficiency (Healey, 1973b; Section 1.61). Under conditions unfavourable for growth, fixed CO₂ may be used to form polysaccharides instead of cell components (Sangar & Dugan, 1972; Section 1.42). The increase in sheath development by the strains studied in the present work was probably a similar phenomenon.

In control cultures of Strains D184, D251, D256, D280 and D404 some of the filaments developed brown pigmentation of the sheath bases as the cultures aged, but the sheaths were colourless in the other strains. Several strains, however, developed brown sheath pigment under some of the deficiency conditions tested, and the five strains just mentioned also showed increases in sheath

pigmentation. Dark brown sheaths were developed by 12 strains under phosphate deficiency; 9 of these strains also showed increased hair development. 4 strains developed brown sheaths under iron deficiency; 3 of these developed hairs under this condition, and all 4 were ones which also produced brown sheath pigment (and hairs) under phosphate deficiency. 3 strains showed increased production of brown sheath pigment under magnesium deficiency; one of these strains also produced long hairs, and all three were ones which showed increases in both hair development and sheath pigmentation under deficiencies of phosphate and iron (Table 5.7). Thus the development of brown sheath pigment was broadly correlated with increased hair formation. The culture medium did not become coloured in any of the cases mentioned, but this was observed in calcium deficient cultures of Strain D156. In this instance, the medium became deep red-brown, though the trichomes and sheaths were unchanged in colour.

Development of brown sheath pigmentation by blue-green algae has been described as a response to high light intensities (Fritsch, 1945; Jaag, 1945; Golubić & Marčenko, 1965; Sections 1.41 and 1.42). It has also been observed that sheaths may become pigmented under conditions unfavourable for growth (Bornet & Thuret, 1880), and brown sheaths seem to occur more frequently in older trichomes (Weber, 1933; Geitler, 1932; Section 1.41). It would be interesting to know whether the brown sheath pigment produced by the Rivulariaceae studied had any metal-complexing capacity, similar to that described by Walsby (1974) for the brown extracellular pigment-peptide complex produced by Anabaena cylindrica (Sections 1.42 and 1.64).

(iv) Effects on heterocysts

There was an obvious increase in heterocyst frequency under iron deficiency in all but 3 strains (D251, D403, D404) of the 16 tested (Tables 5.8 and 6.1). All 11 strains tested under molybdenum deficiency showed increased heterocyst production, and all but 2 of these (D251, D404) also gave this response under calcium deficiency (Table 5.8). The apparent lack of response by Strains D251, D403 and D404 may have been due to the fact that their growth habit made it difficult to estimate changes in heterocyst frequency (Section 5.23). There was no obvious difference in effects on

heterocysts between strains which developed hairs under iron deficiency, and strains which did not do so. In strains that did develop hairs, the changes in heterocyst frequency were observed some time after the first appearance of hairs. Phosphate, magnesium and sulphate deficiencies did not affect heterocyst frequency, but magnesium deficiency produced collapse and apparent death of the heterocysts in all 18 strains examined.

Usually, development of secondary heterocysts of more than one type (basal, intercalary, pseudo-intercalary; Section 2.23) contributed to the increases in heterocyst frequency, and often more than one type was produced by a single trichome. Intercalary and pseudo-intercalary heterocysts could apparently form in any part of a trichome, from the basal enlargement to the narrow apical region. The heterocysts usually remained the same size as the cells from which they had differentiated; the adjacent vegetative cells were also unchanged in size, and did not enlarge like those next to the primary basal heterocyst. Schwabe (1960) made similar observations on 'intercalary' (terminal) heterocysts in Calothrix desertica (Strain D270 of the present study) (Section 1.41). The development of intercalary heterocysts observed by Palik (1946) and Darley (1968) in older cultures of Calothrix, following the release of hormogonia (Section 1.41) may well have been due to a mineral deficiency. Darley (1968) in fact suggested that this response might be related to depletion of salts from the medium (Section 1.41).

The observed effects of iron, calcium and molybdenum deficiencies on heterocyst frequency seem likely to be related to the involvement of these elements in the process of nitrogen fixation (Sections 1.63, 1.64 and 1.67). The response of Strain D184 to molybdenum deficiency was in fact very like that given by this strain when starved of nitrogen (Sections 3.2 and 11.32). Increased heterocyst frequency has been described for Anabaena cylindrica grown in medium without molybdenum (Fogg, 1949; Fay & de Vasconcelos, 1974; Section 1.67), and for A. doliolum in medium with a low level of the element (Tyagi, 1974). Changes in heterocyst frequency in response to iron or calcium deficiency do not seem to have been reported.

Quite marked effects on heterocyst size were observed in several strains (Table 5.8). Increases in heterocyst size were most often seen under deficiencies of iron, calcium or molybdenum, and it seems quite likely that these changes also were related to effects

on nitrogen fixation. Changes in heterocyst size in response to differences in culture media were described by Canabaeus (1929) and by Tyagi (1974) (Section 1.42).

Although the evidence thus suggested that nitrogen fixation was impaired under deficiencies of iron, molybdenum and calcium, iron deficiency was the only one of the three treatments that led to increased hair development, or to increased tapering. This was taken as further evidence (cf. Sections 11.3 and 11.42) that hair development and tapering are unlikely to be solely the result of nitrogen starvation in the apical cells of trichomes of Rivulariaceae.

(v) Effects on spores

Spores were normally produced by Gloeotrichia ghosei D277 and Anabaena cylindrica D2A in control cultures. The effects of different deficiencies on sporulation in these two strains are summarized in Table 5.9. Both strains produced more spores in low phosphate medium. This response has been reported for A. cylindrica (Wolk, 1965) and Aphanizomenon flos-aquae (Gentile & Maloney, 1969) (Section 1.42). An increase in spore frequency was also observed in iron deficient Anabaena cylindrica cultures in the present study, but no other treatment increased spore production in either strain. There were several instances of decreased spore frequency; these probably reflected the overall degeneration of the cultures in the deficient condition.

11.44 Responses of deficient cultures to addition of the limiting nutrient

In the majority of cases the cultures regained a normal colour within 24-48 h of the addition of the limiting nutrient, and hormogonium release recommenced within 48-72 h. When hairs were present, these were usually shed in association with the release of hormogonia, by lysis of a few cells at the hair base. In the case of phosphate deficient cultures, however, four of the 13 strains (D277, D401, D403, D404) showed much more rapid lysis of the hairs, this occurring 2-3 h after the addition of phosphate. In Strains D277 and D401 some of the vegetative cells also lysed, causing the trichomes to fragment into short lengths. All four strains were ones which had at least some hairs at some stage of growth in

the full medium.

An additional response of the 14 phosphate starved cultures tested was the synthesis of large deposits of polyphosphate in the cells following the addition of phosphate. This response is widespread among micro-organisms (Harold, 1966), including blue-green algae (Stewart & Alexander, 1971; Jensen & Sicko, 1974; Section 1.63). In the majority of strains, polyphosphate granules were visible in the vegetative cells when the algae were first examined, 5 min after the addition of phosphate, but in Strains D251, D277, D401, D403 and D404 they were not observed before 10-30 min. These strains were again all ones with some hairs in the control medium. In all 13 strains of Rivulariaceae, the granules appeared simultaneously in the vegetative cells along the whole length of the trichome. In all eight strains which developed hairs in low phosphate medium, polyphosphate granules also appeared in the cytoplasmic strands of the hair cells. The size and number of the granules in the hair cells increased during the incubation; at the time the hairs were shed, they still contained the maximum level of polyphosphate reached earlier in the incubation.

In one strain, Calothrix sp. D184, a more detailed study was made of the energy requirements for polyphosphate granule development by vegetative cells and hair cells (Chapter 9), comparing the effects of light and dark incubation and pre-incubation. The vegetative cells of Strain D184 were able to develop polyphosphate granules after 4 days in the dark, even in the absence of light during the incubation with phosphate, though exposure to light resulted in rather more rapid appearance of granules (Table 9.1). In the hair cells, no extensive granulation occurred without light during the incubation, and no visible granules developed in hair cells of cultures with a dark pre-treatment as well as a dark incubation. Cultures pre-incubated in the light and then transferred to the dark formed a few granules; these appeared later than those of light incubated cultures.

The results suggested that some of the hair cells had small amounts of energy reserves, but that these were depleted by the dark pre-incubation. An alternative explanation might be that the hairs derived their energy supplies from the vegetative cells, and that no transfer of energy occurred in the dark. It was additionally

observed during this experiment that no hormogonium release, and no shedding of hairs, occurred in cultures incubated in the dark.

11.45 Comparison of responses of different strains to nutrient deficiencies

(i) Anabaena cylindrica D2A and the heterocystous Rivulariaceae

The responses of Anabaena cylindrica D2A were broadly similar to those of the heterocystous strains of Rivulariaceae, particularly in the case of cytological effects. The dimensions and morphology of the trichomes of A. cylindrica were much less affected than those of the Rivulariaceae, however. An obvious change in trichome width was observed only in sulphate deficient medium. None of the Rivulariaceae showed altered dimensions in this medium, but at least some were affected by all the other deficiencies except that of molybdenum (Table 5.5). The greater morphological plasticity of the Rivulariaceae probably reflects their more complex developmental cycle.

Heterocyst frequency increased in calcium and molybdenum deficient cultures of Anabaena cylindrica D2A, as it did in most of the strains of Rivulariaceae, but no increase was observed in iron deficient cultures of this strain. There was a decrease in heterocyst frequency in phosphate deficient Anabaena cultures, an effect not seen in any of the Rivulariaceae. Healey (1973a) described an increase in heterocyst frequency in A. flos-aquae under phosphate deficiency (Section 1.63). Like those of the Rivulariaceae, the heterocysts of A. cylindrica D2A became colourless under magnesium deficiency, but the marked shrinkage of the vegetative cells that was seen in all but one of the Rivulariaceae did not occur. Cell length increased in A. cylindrica D2A under iron and phosphate deficiencies, and decreased under calcium deficiency. These effects were also seen in several of the strains of Rivulariaceae.

Anabaena cylindrica D2A gave two responses that were not observed in any of the Rivulariaceae. In low calcium medium, the filaments clumped together to form a floc at the bottom of the flask, within hours of inoculation, and growth continued with the alga in this form. There was no obvious effect upon trichome motility; possibly some change in the charge properties of the sheath was responsible for this response. In iron deficient

cultures of A. cylindrica D2A, the medium was colourless, but it became brown following the addition of FeIII-EDTA. The colour change was not instantaneous, but had taken place when the cultures were examined 36 h after the addition. It seemed possible that the iron formed a coloured complex with some chelating material present in the medium, though this would have been expected to occur immediately.

(ii) Strain D404 and the other heterocystous Rivulariaceae

Experiments on mineral deficiency in Strain D404 were done in Chu 10-D(-N) medium, whereas AD was used for the other heterocystous strains (Section 5.22), but its responses did not differ markedly from those of the strains grown in AD medium. Strain D404 showed an increase in hair development under phosphate deficiency, but not in any of the other deficient media. Strain D403, which was isolated from the same field material as Strain D404 (Table 2.2) did show increased production of hairs in iron deficient medium. However, the morphology of the two strains differed in that most of the trichomes of Strain D404 already had long hairs in the control medium, whereas only a few short hairs were developed in control cultures of Strain D403. Strain D404 differed from all the other strains of Rivulariaceae in showing an increase in the level of polyphosphate granulation under deficiencies of iron and magnesium (this response was also given by Anabaena cylindrica D2A).

The overall similarity between the responses given by Strain D404 and those given by the other heterocystous Rivulariaceae suggested that differences in composition between AD and Chu 10-D were not such that they would markedly alter the effects of lowering the levels of particular components.

(iii) Homoeothrix strains and heterocystous strains of Rivulariaceae

Only one of the two Homoeothrix strains (H. crustacea D401) was ever seen to develop hairs. A marked increase in hair development by this strain was seen only under phosphate deficiency, which was also the condition with most effect on hair formation in the heterocystous strains. Under all the other deficiencies tested, Strain D401 developed tapering apices earlier than it did in the control medium, the change taking place as active growth and hormogonium production ceased. Such an effect was not seen in any of the heterocystous strains.

There was a decrease in trichome diameter in Strain D402 under iron and magnesium deficiencies; this was also observed for several heterocystous strains. Trichome diameter also decreased in sulphate deficient cultures of Strain D402, but this was not observed in the heterocystous strains. Under iron, magnesium and sulphur deficiencies, the cells of the narrower trichomes of Strain D402 were either the same length as those in the control, or absolutely shorter, whereas iron and magnesium deficiencies led to cell elongation in several of the heterocystous strains. Under phosphate deficiency, both strains of Homoeothrix showed cell elongation, with occasional vacuolation of the apical cells in Strain D402. A similar response was also observed in some of the heterocystous strains.

It was more difficult to induce deficiencies of iron, calcium and particularly molybdenum in the Homoeothrix strains than it was in the heterocystous strains, and in fact the response obtained in low molybdenum medium was not very satisfactory. This presumably reflected the greater requirement of the nitrogen fixing heterocystous strains for these elements (Dalton & Mortenson, 1972; Sections 1.64, 1.66 and 1.67).

11.46 Consideration of hair development in the light of other morphological responses to nutrient deficiency

Hair formation in 13 of the 36 strains of Rivulariaceae studied was found to increase under conditions of nutrient deficiency. This result was in agreement with similar reports for eukaryotic algae (Section 1.51). Of the deficiencies tested (phosphate, iron, magnesium, calcium, molybdenum and sulphate) only those of phosphate, iron and magnesium had a marked influence on hair development. Phosphate deficiency affected more strains than iron or magnesium deficiencies, and all the strains in which hair formation increased under the latter two deficiencies also gave this response under phosphate deficiency. Morphogenetic effects of phosphate concentration have been described for several micro-organisms (Section 1.63). One response that is strikingly similar to that seen in the Rivulariaceae studied is the increase in the length of prothecae that has been observed in certain Caulobacteriaceae in media with low levels of phosphate (Schmidt & Stanier, 1966; Section 1.52).

It seemed possible that the hair development response that

occurred in low magnesium and low iron media were actually the result of a secondary deficiency of phosphate, induced by some interactive effect. Birch (1973) found that a Zygnema sp. absorbed no phosphate in the absence of iron, and Healey (1973a) reported that Anabaena flos-aquae required magnesium for maximum rates of phosphate uptake (Section 1.63). However, polyphosphate granules were still present in trichomes of all the strains which produced hairs in iron or magnesium deficient cultures, and only two of the eight strains showed any reduction in the level of granulation. This seemed to suggest that the trichomes were unlikely to be deficient in phosphate, unless they were unable to use the stored polyphosphate.

Under the different mineral deficiencies studied, there was considerable variation in trichome morphology quite apart from that associated with hair development. Phosphate, iron and magnesium deficiencies produced the most pronounced morphological changes: these were also the conditions which most affected hair development. Calcium, molybdenum, and particularly sulphate deficiency had much less effect upon trichome morphology. Some strains gave responses that were similar to certain aspects of hair development. Narrowing and elongation of cells were quite frequently observed, and the cells in the apical region seemed to be affected more often than those at the base. Sometimes the apical one or two cells lost the normal granular inclusions, and occasionally developed vacuoles. Vacuolation of cells along the whole length of the trichome was sometimes observed also. Such responses were often seen in the absence of hair formation, even in strains known to be able to develop hairs. This seemed to emphasize the distinction between these individual responses, and that of hair development, in which several changes occurred in a co-ordinated manner (cf. Section 1.23).

The responses to nutrient deficiencies provided further evidence (cf. Section 11.3) that tapering and hair formation are not necessarily correlated. There were several instances in which trichome tapering increased in the absence of any hair development, even in strains known to be capable of hair formation. When hairs did develop, this did not necessarily increase the taper of the vegetative region of the trichomes (though the trichome as a whole became more tapered). Each of the strains examined showed increased

heterocyst frequency under at least one of the treatments which seemed likely to have impaired nitrogen fixing activity (iron, calcium or molybdenum deficiency), and the heterocysts often developed in the apical region of the trichomes. Thus evidently each strain had the potential to develop additional heterocysts if these were required. It thus seemed unlikely that narrowing of the cells in the apical region of normal trichomes was simply the result of extreme nitrogen starvation. (It was of course possible, however, that the threshold level of nitrogen to induce heterocyst development was lower than that which would influence tapering or hair development.)

In general, the strains which developed hairs under particular deficiencies showed other symptoms (cytological changes, increases in heterocyst frequency, etc.) that were very similar to those of the other strains under the same conditions. One response, however, that of increased brown sheath pigmentation, was observed more often among strains which developed hairs than among strains which did not. Kirkby (1975) found that according to descriptions given by Geitler (1932), based upon samples from the field, there was a tendency for species of Calothrix and Rivularia with yellow-brown sheaths to possess hairs also (Section 1.41). This might suggest that in natural conditions the factors tending to promote these two characters are similar.

When hairs developed in nutrient deficient media, they appeared at an earlier stage than other deficiency symptoms such as increased heterocyst frequency or cyanophycin granulation, and certainly before any marked degenerative changes took place. This seemed to suggest that the development of hairs was not merely a sign of senescence or impending death. In fact in phosphate deficient medium, strains which developed hairs seemed to retain trichome integrity for longer than those which did not produce hairs: cultures in the latter category showed trichome lysis and fragmentation at a relatively early stage. In each of the eight strains tested, the hair cells of phosphate starved cultures developed polyphosphate granules following the addition of phosphate, which indicated that they retained at least some metabolic activity.

Overall, the results suggested that hair development was not simply a symptom of trichome degeneration, but that it was a specific morphogenetic process, and it seems not unreasonable to speculate

that hairs in Rivulariaceae may in fact have some particular function. Hair development increased in nutrient deficient media (though not under every deficiency tested), and this resulted in a considerable increase in the surface area of the trichomes, apparently with relatively little increase in total cytoplasmic volume. These observations are compatible with a role for hairs in the absorption of nutrients, as speculated by Palla (1893) for the hairs of Gloeotrichia pisum (Section 1.24). Similar suggestions have been made for the hairs of eukaryotic algae (Section 1.51) and for the prosthecae of Caulobacteriaceae (Section 1.52). Kirkby (1975) noted that according to descriptions of Calothrix and Rivularia in the literature, there was a greater tendency for species with spherical or hemispherical colonies to possess hairs. She commented that such colonies have a relatively small surface area:volume ratio, and that the development of hairs was likely to aid in absorption.

One might speculate that phosphate and iron deficiencies had the most marked effects upon hair development because these nutrients are ones most often likely to be limiting under natural conditions (Sections 1.63 and 1.64). Differences between strains in their hair development response under particular deficiencies might perhaps be related to the water chemistries of their original habitats. Strain D251 was the only one to form long hairs in magnesium deficient medium. This strain was isolated from a thermal spring (Table 2.1), but the level of magnesium in this spring is not especially high (R.W. Castenholz, personal communication). Furthermore, Strain D256, which is from a littoral marine environment (Table 2.1), where the Mg:Ca ratio was presumably high, showed no hair development in low magnesium cultures. The same was true for Strain D280, isolated from 'a spring of magnesium water' (Table 2.1). However, the origins of the strains are too vaguely defined to permit any firm conclusions on this point.

Despite these circumstantial indications, no direct evidence was obtained for the involvement of hairs in nutrient uptake. When the development of polyphosphate granules was studied, following the addition of phosphate to phosphate starved cultures, the granules were found to appear no earlier in strains with hairs than in those without hairs. None of the strains showed any gradient along the trichomes in the rate at which polyphosphate granules

appeared. Though the appearance of polyphosphate is a very crude measure of phosphate uptake by the cells, some detectable effects might nevertheless be expected if the hairs were in fact involved in phosphate absorption. However, the level of phosphate following the addition was very high (44.5 mg l^{-1}), and abundant phosphate was presumably available to every cell. The results are thus not incompatible with the hairs' having a nutrient absorptive role under conditions of low nutrient concentration. The fact that the hairs were shed, still with their full complement of polyphosphate granules, following the restoration of a high level of phosphate, could be interpreted as an indication that they are redundant under phosphate sufficient conditions. By analogy with the results of Schmidt and Stanier (1966) for Caulobacter prosthecae (Section 1.52) it seems not unlikely that the presence of long hairs is the natural condition in Rivulariaceae with the genetic ability to form them, and that the absence of hairs in the rich AD medium used as control was a laboratory artefact. Thus the nutrient deficiencies should perhaps be described as removing an inhibition of hair development, rather than as stimulating an increase in hair development.

11.5 Effect of increased phosphate concentration on hair development

The hair development that occurred in seven of the heterocystous strains in AD with $0.44 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$ was suppressed when they were grown in AD with $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. Five strains which produced hairs in low phosphate AD, however, also did so in the medium with $44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$, though the extent of hair development was much less. It seemed possible that for each strain there was a level of phosphate above which hair development would not occur, and that by further increasing the phosphate concentration of AD it would be possible to suppress hair development in these five strains. When this was attempted, for Strains D126, D251, D277 and D403 (Chapter 8), it was found that raising the initial concentration of phosphate inhibited growth, but did not affect the frequency of hairs, except indirectly, as a result of trichome fragmentation. This suggested that the external phosphate level was not the sole factor controlling hair development in these strains.

This experiment additionally showed that the strains which had many hairs in AD medium appeared to be more sensitive to a raised level of phosphate than those that did not have hairs (cf. Section 11.7).

11.6 Experiments with Rivularia from the field

Experiments were performed on Rivularia colonies from two local sites, to compare their responses to variations in phosphate concentration with those of the laboratory cultures (Section 10.1). In both the materials studied, hair development was greatest in media with the lowest initial levels of phosphate, as had been observed with the laboratory strains. Similar results were obtained by Kirkby (1975), with Rivularia colonies from two other sites (Malham and Upper Teesdale) (Section 1.41). Since the trichomes examined in these experiments were derived directly from field colonies, and had not been subject to possible selective change during prolonged subculture in artificial media, it seemed unlikely that the responses observed in the laboratory strains were simply an artefact of culture.

These experiments also indicated some apparent differences in the preferred phosphate concentration for growth of different algae present in the Rivularia colonies. Growth of the Rivularia from Croft Kettle was poor at the highest concentrations of phosphate used ($> 3.5-4.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$), but abundant growth of associated algae (Phormidium and Nostoc) occurred at these high levels of phosphate. At the lower phosphate concentrations, there was good growth of the Rivularia, but little growth of associated forms. No daughter colonies were produced by the Rivularia from Barras, but there was outgrowth of a Calothrix, occasional trichomes of which had been observed in the original colonies. Unlike the Rivularia from Croft kettle, this alga grew most vigorously at the highest levels of phosphate used, thus behaving like the 'contaminants' of the other Rivularia colonies. A difference in optimum phosphate concentration might perhaps be one factor involved in determining the relative abundance of the different algae in their natural habitat, and also the relative success of their growth in artificial media. The behaviour of the Croft Kettle Rivularia was rather similar to that of the two strains of Rivularia (D403 and D404) that were isolated from the same

site. Both grew rather slowly in AD medium ($44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$), and showed more vigorous growth in media with a lower level of phosphate (Sections 5.22 and 5.31). It seems quite likely that the levels of phosphate in many blue-green algal media may be supra-optimal for many Rivulariaceae (cf. Section 11.7).

A study was also made of short-term responses of Rivularia from Croft Kettle to incubation in medium with a high concentration of phosphate. When collected, the trichomes had long hairs, and polyphosphate granules were present (at a low density) in the basal cells of most trichomes (Section 10.2). Although this suggested that the trichomes were not severely phosphate starved; nevertheless massive deposits of polyphosphate formed in the vegetative cells during incubation in AD medium, and some of the hair cells also became granulated. Thus, despite the presence of polyphosphate, the trichomes were presumably not fully phosphate sufficient at the time of collection. Similar behaviour was observed in Strains D277, D403 and D404, which were found to retain some polyphosphate granules despite obvious growth limitation in the low phosphate medium (Section 5.31). Strains D403 and D404 were in fact isolated from Rivularia colonies from Croft Kettle (Section 2.362).

The Croft Kettle Rivularia shed its hairs about 48 h after inoculation into AD medium, in association with the release of hormogonia. Rapid lysis of the hairs, as observed in some of the laboratory cultures, was not observed. Since AD medium contains high levels of several nutrients, it was not possible to say which, if any, particular component of the medium caused this response. The results obtained with this field material suggested that the responses given by cultured strains (in particular the development of polyphosphate granules by the hair cells, and the shedding of the hairs) were not merely laboratory artefacts.

11.7 Relationship between ability to develop hairs, and overall response to phosphate concentration

13 of the 36 strains studied developed many long hairs in media with low phosphate concentrations, and the same was observed with the two field materials that were examined. In eight of the laboratory strains, the development of hairs could be suppressed by growth in medium with a high level of phosphate ($44.5 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$). Five strains (D126, D251, D277, D403, D404) still

developed hairs under such conditions, however, and in three of these (D126, D277, D404) the hairs were of considerable length. Four of these strains were cultured at still higher levels of phosphate, but hair development was not suppressed (Chapter 8; Section 11.5). Some other responses were found to be broadly correlated with the persistence of hairs at high phosphate concentrations; all these suggested a preference for low levels of inorganic phosphate.

Growth of Strains D126, D277 and D403 was inhibited in media with phosphate concentrations above that of AD; this inhibition was greater than that observed for Strains D134 and D267, which had no hairs in AD medium (Chapter 8). Growth of Strain D403 was in fact more vigorous in AD medium with 0.01x the normal level of $PO_4^{3-}P$, although its final yield was lower (Section 5.31). The Rivularia from Croft Kettle (from which Strains D403 and D404 were isolated) also grew better at phosphate concentrations below the normal AD level, and was overgrown by associated algae at higher phosphate levels (Section 10.1).

Strains D126, D277, D403 and D404 all showed lysis of the hairs within 2-3 h of the addition of phosphate to phosphate starved cultures (the Croft Kettle Rivularia did not give this response), and in Strain D277 many vegetative cells also lysed. In the other heterocystous strains hairs were not shed until about 48 h later, when hormogonia were released (Section 5.31(ix)). Strains D277, D403 and D404 all showed prolonged retention of polyphosphate granules during subculture through low phosphate medium (Section 5.31), though the length of the hairs increased at the same time. The Rivularia from Croft Kettle also possessed polyphosphate granules in the presence of long hairs (Section 10.2).

The correlation between these responses, and the presence of hairs in AD medium was not complete, however, since Strain D251 behaved more like the strains without hairs in AD, and the Calothrix from the Barras Rivularia colonies, which also developed hairs in AD, showed an apparent preference for high phosphate media (Section 10.1). Nevertheless, taken together, the results do suggest that the ability to produce hairs even at high phosphate concentrations is correlated with sensitivity to high phosphate levels. From the limited data available, it seems that such algae may be adapted to growth in low phosphate environments, and

their ability to compete against growth of other algae correspondingly reduced at higher phosphate concentrations. As mentioned in Section 1.62, Allen (1963) and Fogg (1969) have both noted inhibitory effects of inorganic phosphate on Rivulariaceae.

The high level of polyphosphate granulation in several of the strains with hairs perhaps indicates a very efficient phosphate scavenging mechanism, adapted to function at low phosphate concentrations. On the hypothesis that hairs may have some role in nutrient uptake (Section 11.46), algae from environments with consistently low nutrient levels would have no need for a mechanism to suppress hair development, nor would they need the physiological capacity to deal with abundant supplies of phosphate. The situation in such algae may be similar to that described by Larson and Pate (1976) for a strain of Asticcacaulis biprosthecum (Caulobacteriaceae). This bacterium appeared to be adapted to growth in dilute environments, and was inhibited by nutrient levels which promoted rapid metabolic rates. Algae with the ability to grow with hairs or without hairs perhaps have a more flexible physiology, enabling them to make efficient use of high levels of phosphate if these are available, but also to compete successfully at low phosphate concentrations.

11.8 Concluding remarks

This study has illustrated the wide range of morphological variation that can occur in members of the Rivulariaceae. In addition to changes in the extent of hair development and tapering, many strains showed other quite marked responses to variations in the culture media.

14 of the 34 heterocystous strains still produced tapered trichomes in the presence of combined nitrogen at a level sufficient to suppress heterocyst development. All 5 heterocystous strains with hairs in the control medium without combined nitrogen still developed them in the presence of combined nitrogen, though in three strains hair length and frequency were reduced. In these three strains, the hairs in +N medium developed at the ends of long trichomes that were otherwise parallel. The results suggested that tapering and hair development may not necessarily be related phenomena, and also that neither is likely in every case to be simply due to nitrogen deficiency in the cells remote from the

basal heterocyst. It was suggested that one contribution of heterocysts to trichome tapering might be to cause the basal cells to enlarge.

13 of the 36 strains studied were found to be capable of producing hairs. Hair development in all these strains was found to increase in phosphate deficient cultures; in 8 of these, iron deficiency also led to increased hair development, and in one strain, magnesium deficiency did so also. Deficiencies of calcium, molybdenum or sulphate had no marked effects upon hair production in any of the strains. Hair development in deficient media apparently occurred before other morphological responses, and it preceded any obvious degenerative changes. Many of the hair cells developed under phosphate deficiency were able to synthesize polyphosphate following the addition of phosphate, indicating that they were capable of metabolic activity. It was concluded that hair development was not simply a symptom of trichome degeneration, but a specific morphogenetic phenomenon.

The fact that increased development of hairs occurred under conditions of nutrient deficiency is compatible with their having a role in nutrient absorption, since their formation would considerably increase the surface area of the trichome, with relatively little increase in cytoplasmic volume. Such an absorptive role was suggested by Palla (1893) for the hairs of Gloeotrichia pisum, and similar speculations have been made about the hairs of eukaryotic algae, and also the prosthecae of the Caulobacteriaceae. Of the conditions studied, deficiencies of phosphate and iron had most effect upon hair development. These nutrients are ones which may often be limiting in natural environments, perhaps especially to nitrogen fixing organisms. This perhaps supports the hypothesis that hairs may have some functional significance under natural conditions. Some of the strains studied produced a few hairs under all the conditions tested, including very high levels of nitrogen and phosphorus. Limited evidence suggested that these forms were sensitive to very high levels of phosphorus, and had a preference for low concentrations of this nutrient.

Most of the experiments were performed with laboratory cultures, but tests with Rivularia from field sites suggested that the results were unlikely to be simply laboratory artefacts. It is to be hoped that further work may lead to greater clarification of the role of hairs in the natural environment.

APPENDIX

OCCURRENCE OF MORPHOLOGICAL ABNORMALITIES IN CERTAIN STRAINS OF RIVULARIACEAE IN AD MEDIUM, AND ATTEMPTS TO IDENTIFY THEIR CAUSE

A1 Introduction

A brief mention was made in Section 2.11 of the sudden inexplicable appearance of gross morphological abnormalities in certain heterocystous strains of Rivulariaceae, when grown in the AD medium normally used for their maintenance. This occurred about halfway through the period of research, following experiments on morphological effects of combined nitrogen (Section 3.1) and preliminary experiments on hair development under nutrient deficiency (Chapter 4 and part of Chapter 6), but before more detailed studies on morphological effects of a range of mineral deficiencies (Chapter 5) or further experiments on the relationship between phosphate concentration and hair development (Chapters 7, 8 and 9).

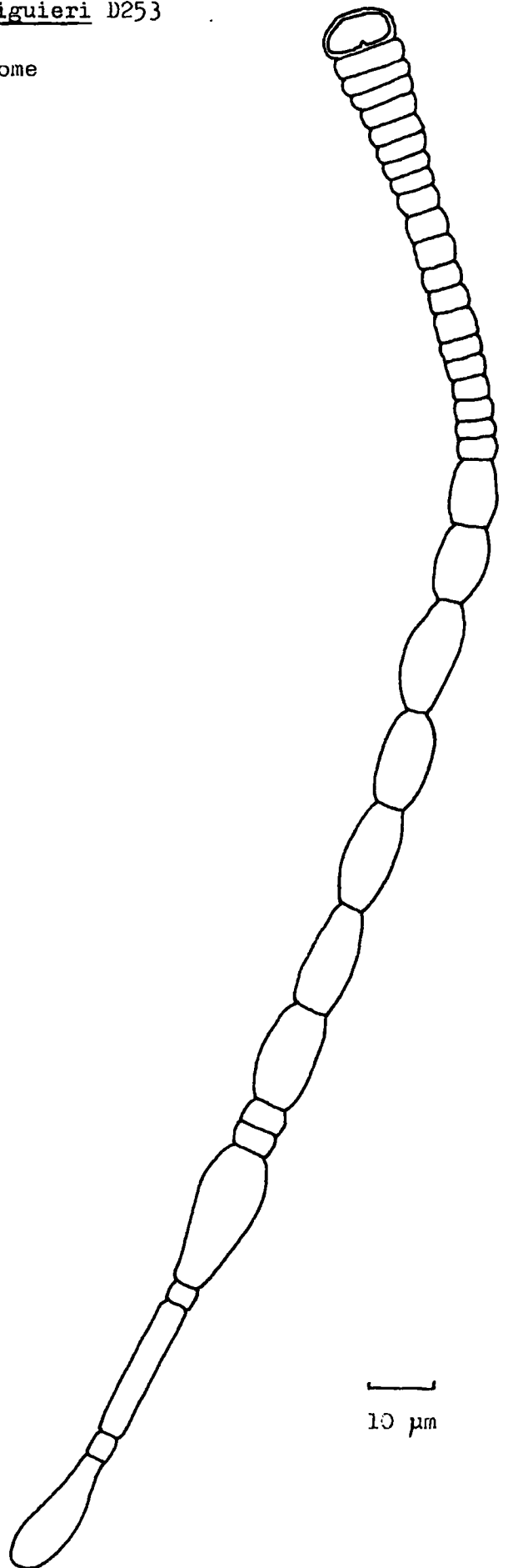
A considerable effort was made to identify the cause of this phenomenon, and this work is described below. Although no firm conclusions were reached, the results are included here as a further example of a marked morphological modification shown by certain Rivulariaceae, but not apparently by the limited number of blue-green algae from other families that were examined. In addition, the results had a bearing on much of the other work performed, insofar as they determined the strains and media which could be used for experimental purposes.

A2 General description of the phenomenon

A2.1 Initial occurrence of abnormalities in Calothrix viguieri D253

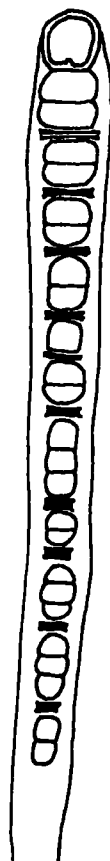
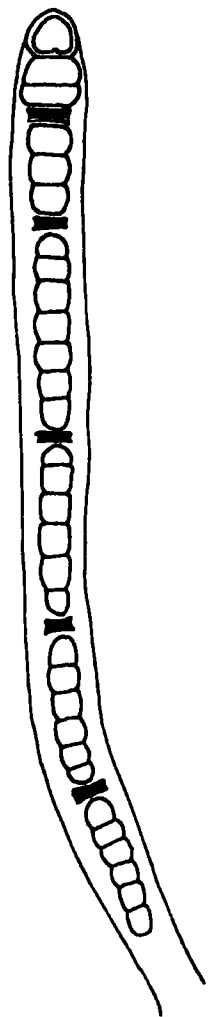
Abnormalities were first observed in cultures of Strain D253, grown for 21 days in AD medium at 25°C, 6000 lx, as an experimental inoculum. The most striking abnormality was the presence of greatly enlarged cells in the mid-region of an estimated 80% of the trichomes. The enlarged cells sometimes extended as far as the apex of the trichomes, but the heterocysts, and the cells in the basal region, had an apparently normal structure. Examples of abnormal trichomes are shown in Fig. A1 (compare the normal morphology of this strain shown in Fig. 3.1). The contents of all the cells appeared green and normal, and there was no vacuolation.

Fig. A1 Abnormally enlarged cells in trichomes of Calothrix viguieri D253 grown in AD medium. Compare the normal trichome in Fig. 3.1a.



10 μ m

Fig. A2 Trichomes of Calothrix viguieri
D253 grown in AD medium, showing
numerous separation discs, and
thick sheaths.



10 μ m

This was an entirely new phenomenon. Enlarged cells, of rather different structure, had occasionally been seen in Calothrix membranacea D179, and in Gloeotrichia echinulata D126, but never on such a large scale. A morphology like that described had never been observed in collections of field material.

The morphological features described (Fig. A1) were found in algae from the surface mat growth (cf. Section 2.122); they also occurred in the much sparser growth over the base of the flask, but other abnormalities were also observed in trichomes from this zone, particularly as the cultures aged (4-6 weeks). Numerous biconcave separation discs developed along the filaments, dividing them up into short lengths, sometimes of as few as two cells (Fig. A2). In normal cultures, production of hormogonia took place by the development of separation discs, but this was confined to the apical part of the trichomes; in the abnormal cultures, the separation discs were often produced in the enlarged basal region, only a few cells from the basal heterocyst (Fig. A2). Unlike normal hormogonia, the short trichome sections showed no apparent tendency to migrate from the parent filament. Sometimes each section began to differentiate a basal heterocyst, producing a series of short, semi-differentiated trichomes in a common sheath; these trichomes never seemed to complete a normal development. More usually, however, the production of separation discs was followed by bleaching and death of the trichome sections. The apical part of the original trichomes was generally more affected than the base, which often remained green when the rest of the trichome had died.

The growth over the base of the flasks also contained (in the later stages) large masses of short trichomes showing various degrees of degeneration. The following sequence of events was inferred from examination of trichomes at different stages. The moribund trichomes evidently derived from hormogonia which had differentiated a basal heterocyst, but had failed to develop any further. The first sign of degeneration was the appearance of small vacuoles in a number of the cells. The vacuolation of the cells became more extensive, with associated paling of the cell contents. Some of the cells along the trichome then seemed to die and lyse completely, often leaving only two or three apparently normal cells in the sheath. There was no apparent pattern to the sequence in which the cells died. Ultimately, all the vegetative

cells in the trichome became completely colourless and collapsed. The heterocysts retained their shape, but they became colourless also. Filaments in the later stages of this sequence had rather thick, distinct, but colourless sheaths. Such trichomes, showing actual lysis of the cells, were much less common in the surface skin growth, where generally only abnormalities of cell shape were found.

Despite these morphological abnormalities, the total yield of the cultures was not obviously affected. Their macroscopic appearance was also healthy, though the large number of dead trichomes in older cultures gave the growth on the flask base a whitish colour. It was clearly not practicable to use this strain for experimental purposes while it exhibited these abnormalities, particularly since most of the experiments were to be concerned with morphological effects. An effort was therefore made to identify the cause of the phenomenon.

A2.2 Preliminary investigations

Since AD medium had been successfully used for maintenance and experimentation for this and other strains for the previous 15 months (but see Section A3.31(iii)), the effect was attributed to some factor other than the growth medium. At the time, the most likely causes seemed to be a bacterial or viral attack, or mutation of the strain.

A series of subcultures of Strain D253 was made into AD medium from various stock flasks of different subculturing lineages; each stock was stringently tested for the presence of bacteria (Section 2.34). In addition, a replacement stock was obtained from the culture centre at Cambridge (Table 2.1). When received, on a slope of proteose-peptone agar (Table 2.3), this culture had a normal morphology. It was subcultured both into AD medium, and onto further slopes of proteose-peptone agar.

All the AD cultures developed abnormalities like those described in Section A2.1; the first abnormalities appeared as soon as the newly grown trichomes had achieved a mature morphology. No bacteria were detected in any of the cultures. There were no abnormalities in the algae subcultured to proteose-peptone agar, though many of the trichomes were of the 'Homoeothrix' or 'Ammatoidea' type characteristic of this strain when grown in medium with

combined nitrogen (Section 3.1). The absence of these forms from the slope received from Cambridge was presumed to be due to depletion of combined nitrogen over the longer period of culture.

These results seemed to eliminate the possibility of bacterial effects, and mutation also seemed unlikely, since the Cambridge stock behaved in the same way as the one maintained at Durham for the previous 15 months. An electron microscopic examination of Strain D253 with abnormal cells failed to show any evidence of viral attack (D.B. Douglas and A. Peat, personal communication). It seemed probable, therefore, that some factor associated with the medium, or with culturing or incubation techniques, was responsible.

A2.3 Occurrence of abnormalities in other strains

Since it now seemed likely that the morphological abnormalities seen in Strain D253 were due to some effect of the medium, all 34 heterocystous strains of Rivulariaceae held (Table 2.1) were checked for the presence of similar symptoms when grown in AD medium. The results are summarized in Table A1. 9 of the 34 strains were affected, though only 6 showed marked enlargement of the cells, and 2 were only slightly affected. 5 of the 6 strains with enlarged cells were ones which had no hairs in AD, but which developed them under deficiencies of phosphate and iron (Table 6.1). The Homoeothrix strains did not grow in AD medium (Section 3.33); in AD + 10 mM NaNO_3 they showed no morphological abnormalities. All the strains that were affected responded in very much the same way as Strain D253. This further reduced the possibility that a mutation was responsible for the affect.

Four of the strains which developed many hairs only in low nutrient media (D251, D256, D280, D403) showed no abnormalities in AD medium, and one strain of this type (D184) was only slightly affected. There was no obvious feature in common between these strains. Strain D251 was isolated from a hot spring, Strain D280 from a spring of magnesium water, and Strain D256 is of marine origin (Table 2.1). These habitat origins might perhaps imply a relative tolerance of high concentrations of dissolved salts. Strain D184 was isolated from a zinc-enriched culture tank (Table 2.2). None of the strains with many hairs in AD showed any morphological abnormalities.

TABLE A1

Occurrence of morphological abnormalities in heterocystous Rivulariaceae grown in AD medium

(+) = slight effect

<u>Strain</u>		<u>Enlarged cells</u>	<u>Cell lysis and many separation discs</u>	<u>No abnormalities</u>
<u>Calothrix</u> sp.	D184	(+)	(+)	
<u>Calothrix</u> sp.	D251			+
<u>Calothrix</u> sp.	D252	+	+	
<u>Calothrix</u> sp.	D254			+
<u>Calothrix</u> sp.	D255			+
<u>Calothrix</u> sp.	D258			+
<u>Calothrix</u> sp.	D264			+
<u>Calothrix</u> sp.	D267		(+)	
<u>Calothrix</u> sp.	D283			+
<u>C. anomala</u>	D182			+
<u>C. brevissima</u>	D156			+
<u>C. brevissima</u>	D275			+
<u>C. desertica</u>	D270	+	+	
<u>C. elenkinii</u>	D273			+
<u>C. fusca</u>	D269	+	+	
<u>C. gracilis</u>	D274			+
<u>C. javanica</u>	D257		+	
<u>C. marchica</u>	D202		+	
<u>C. membranacea</u>	D179			+
<u>C. membranacea</u>	D259			+
<u>C. membranacea</u>	D260			+
<u>C. membranacea</u>	D261			+
<u>C. membranacea</u>	D262			+
<u>C. membranacea</u>	D263			+
<u>C. parietina</u>	D272			+
<u>C. prolifica</u>	D265			+
<u>C. scopulorum</u>	D256			+
<u>C. thermalis</u>	D266	+	+	
<u>C. viguieri</u>	D253	+	+	
<u>Dichothrix</u> sp.	D280			+
<u>Gloeotrichia echinulata</u>	D126			+
<u>G. ghosei</u>	D277			+
<u>Rivularia</u> sp.	D403			+
<u>Rivularia</u> sp.	D404			+

Several strains of blue-green algae from other families, maintained in AD medium by colleagues in the laboratory, were also examined. (Anabaena cylindrica, Anabaenopsis circularis, Fischerella muscicola, Microchaete diplosiphon, Westiellopsis prolifica). No unusual morphological effects were seen in any of these cultures.

A3 Experiments to determine the cause of the abnormalities

A3.1 Rationale

Since Strain D253 was not the only culture to develop an abnormal morphology in AD medium, it seemed most probable that some component of the medium, or some aspect of culturing or incubation was responsible for the effect. A systematic attempt was therefore made to test all factors which it was thought might possibly produce such an effect, or which might shed light on the nature of the effect. Before describing the results of the experiments, it may be useful to summarize the general features of the phenomenon, and the factors which were considered most likely to be involved before experimentation was begun.

Several Calothrix strains developed severe morphological abnormalities when grown in AD medium. The majority of these were ones which developed hairs in low phosphate and low iron medium, but did not do so in full AD. Preliminary investigations suggested that the effect was not due to mutation, nor to bacterial or viral attack. Some component of the medium seemed the most likely cause, though an aspect of the culturing technique could have been responsible. There was a difference in response between algae growing on the surface of the medium, and those growing on the base of the same flask. The former showed distortions of cell shape, but were otherwise apparently healthy; the latter exhibited extensive fragmentation and lysis of the trichomes.

It was not clear why there should be this difference in response between the two zones, but two tentative hypotheses were made. The first was that the effect was due to a difference in time of development between the algae at the top and bottom of the liquid layer. The initial growth in culture flasks generally took place on the base of the vessel, with the surface skin developing later. The first-formed filaments would presumably be exposed to the maximum concentration of any toxic material, and would perhaps

reduce its concentration, by absorption or adsorption. Similarly, if the (presumed) toxic effect were a cumulative one, the algae on the flask base would have a longer time of exposure to the toxic material. The second hypothesis was that a difference in micro-environment between the surface and bottom of the liquid layer was responsible. A difference in gaseous atmosphere between the two zones seemed very likely to exist, since gas equilibration across the 50 ml liquid layer would probably be too slow to keep pace with changes effected by the algae, particularly when the surface of the medium was more or less covered by a mat of trichomes (Section 2.122). In standing cultures under continuous illumination, the bottom of the flasks could well be deficient in carbon dioxide and supersaturated with oxygen, whereas algae on the surface of the medium would probably experience more normal atmospheric conditions. Since high oxygen tensions may be unfavourable to the metabolism of blue-green algae (Stewart & Pearson, 1970), particularly under low carbon dioxide concentrations (Lex, Silvester & Stewart, 1972), it did seem possible that oxygen was involved in a toxic reaction, perhaps in combination with some other factor. Certain metal ions have been shown to affect oxygen toxicity (in animal systems) (Haugaard, 1968). In addition, carbon dioxide depletion might be expected to affect the pH of the medium, perhaps with still further complications of micro-precipitation, or modification of response to a toxic material.

At this stage, it was thought most probable that the effects seen were caused by a toxic factor in the medium, possibly with a cumulative effect, and possibly modified by other environmental factors. Some heavy metal ion, introduced as a contaminant, seemed the most likely candidate. It was with this general speculative hypothesis in mind that experimentation was begun. Although, for clarity, the investigation of each variable is described separately in the account that follows, the order is not strictly chronological. Thus some experiments may appear to be based on an hypothesis already discounted by previously described observations.

A3.2 Summary of factors tested

1. Chemical components of the medium
 - (i) Use of other media
 - (ii) Stock solutions of the major salts
 - (iii) Microelement stock solutions
 - (iv) Iron, manganese and EDTA
 - (v) Presence or absence of combined nitrogen
 - (vi) Molybdenum concentration
 - (vii) Phosphate concentration
 - (viii) NaCl concentration
 - (ix) Buffering

2. Factors involved in making up media
 - (i) Distilled water
 - (ii) Washing of glassware
 - (iii) Sterilization
 - (iv) Cotton wool plugs

3. Factors associated with incubation
 - (i) Gas exchange
 - (ii) Light regime
 - (iii) Solid versus liquid media

A3.3 Experimentation

Calothrix viguieri D253 was used as experimental organism throughout, since this was an axenic strain, and showed severe morphological abnormalities. It was also hoped to use this strain as the main experimental organism in further work; thus it was especially important to find a medium suitable for its growth.

During the period of the experiments described here, stocks of Strain D253 were kept on slopes of proteose-peptone agar (Table 2.3). When inocula were required, subcultures were made into flasks of AD medium. Thus the inoculum for each experiment was of algae from a first 'generation' AD culture. This precaution was taken in view of the possibility that a progressive accumulation

of some component of AD medium was causing the abnormalities, and that the effect might increase with successive subcultures. Unless otherwise stated, experiments were performed in duplicate 100 ml conical flasks, with 50 ml of medium, incubated unshaken at 25°C, 2500 lx (white fluorescent tubes). AD medium was made up as shown in Table 5.3, unless otherwise specified. The growth extent of algae in these experiments was scored using the subjective 0-5 scale explained in Section 2.54.

A3.31 Chemical components of the medium

(i) Use of other media

Several other media which had in the past been successfully used by the author and colleagues were tested for their effects. It seemed that this might indicate whether the alga was sensitive to some factor common to all the media, rather than to AD specifically. If a favourable medium were found, then differences in composition between it and AD might give clues as to the cause of the effects seen in AD.

Strain D253 was taken through three successive subcultures in the following media: Chu 10-D(-N), ASM-D(-N), AC(-N), S₀, ZD and Medium I (Table 2.3; Section 2.32). AD medium was included as control, and algae from each medium were finally subcultured back into AD to see whether the typical symptoms would recur.

The results are summarized in Table A2. All the morphological effects seen developed in the first subculture from proteose-peptone agar. It thus seemed unlikely that a cumulative toxic effect, increasing over successive subcultures, was responsible for the abnormalities. The morphology of the alga in Chu 10-D(-N) was essentially similar to that seen in AD, but there were some differences. One feature, not seen in AD, was rounding off of the cells in the apical region, giving a moniliform appearance. This was sometimes associated with enlargement of the apical 4-10 cells. As in AD, algae from the basal growth were the most badly affected, though there was less outright death of the trichomes. ZD medium produced a morphology very like that seen in Chu 10-D, with the additional feature that almost every cell had a single large vacuole.

Only the marine medium S₀ yielded algae with a normal tapered morphology. This differed slightly from that seen in AD before the

TABLE A2

Growth and morphology of Calothrix viguieri D253 after 21 days in various nitrogen-free media

<u>Medium</u>	<u>Growth extent (0-5)</u>	<u>Morphology</u>	<u>Morphology on subculturing back to AD</u>
AD	5	typically abnormal	typically abnormal
AC (-N)	0	died	"
ASM-D (-N)	1	as AD	"
Medium I	5	as AD	"
Chu 10-D (-N)	5	abnormal (see text)	"
ZD	3	abnormal (")	"
S ₀	3	satisfactory (")	"

abnormalities occurred, in that the sheaths of the older trichomes were brown pigmented, and there was a tendency for the apical region of an estimated 20% of the trichomes to separate into one- or two-celled fragments, which became detached from the trichome. The growth rate and final yield of the alga in S_0 were lower than in AD (estimated \approx 80%).

S_0 differs from AD chiefly in its much greater salinity, and in the lower level of FeIII-EDTA. It contains 23.5x the Na, 19.5x the Cl, and 4.6x the Mg of AD (Table 2.3); the FeIII-EDTA level is half that of AD. The levels of the other medium components, in particular the microelements, are rather similar to those of AD, more so than for the other media tested. The lower iron level of the successful S_0 medium suggested that the iron concentration in AD might be supra-optimal, and possibly involved in the response seen. It also seemed possible that the very high level of Na^+ in S_0 was masking some toxic effect of a metal ion present at a lower concentration, by a competitive effect. Yet in spite of these indications, the results showed that abnormalities could occur in media other than AD, with widely differing levels of iron and other components. Further experiments were performed to determine the effects of NaCl and of FeIII-EDTA concentration on the morphology of Strain D253. These are described in Sections A3.31(viii) and (iv) respectively.

(ii) Stock solutions of the major salts

It seemed possible that one or more of the stock chemicals and/or solutions could have become contaminated. All the stock solutions other than the microelements (see (iii) below) were made up afresh, using chemicals from sources other than the normal laboratory stock bottles. Solutions were also made up using double distilled water, as well as the normal single distilled (see also Section A3.32(i)).

Strain D253 showed the same morphological abnormalities in AD medium made from these stocks as it had in the original. Thus contamination of the chemical stocks seemed unlikely to be responsible.

(iii) Microelement stock solution

For a few weeks previous to the first observation of abnormalities in Strain D253, AD medium had been made up using a microelement

solution the same as that in Allen and Arnon's (1955) original formulation (though without Ti), instead of the Durham version used previously (Table 2.3). This change had been made because some earlier experiments on the morphology of Calothrix had been performed by other workers in the laboratory, using the Allen and Arnon formulation, and it was hoped that a comparison could be made between these results and the author's. The concentration of each element in the Allen and Arnon formulation is higher than that in the Durham version, and it seemed quite likely that one or more elements might be present at a supra-optimal level.

AD medium was made up with the normal Durham AD microelements, and with the original Allen and Arnon formulation, each at 1.0x, 0.5x, 0.2x, 0.1x, 0.05x and 0.02x the normal concentration. A third microelement stock solution, that normally used with AC(-N) medium (Table 2.3) was also used, at the normal level. Strain D253 was taken through three successive subcultures in each of these media. There was no difference in growth or morphology between any of the treatments. Fresh stocks of each of the AD microelement formulations were then made up. Media containing these new solutions (at the normal level) still yielded algae with an abnormal morphology.

The results suggested that contrary to what had been suspected, a component of the microelements was not responsible for the modified morphology of Strain D253. The lack of growth limitation in media with microelements at 0.02x the normal level suggested that the concentrations normally used were well in excess of the alga's requirements.

(iv) Iron, manganese and EDTA

As mentioned in Section A3.1, it had been hypothesized that some element present at a supra-optimal level was producing a toxic effect. Iron seemed quite a likely candidate, in view of its high biological activity (cf. Section 1.64), and the fact that the level in the medium (4 mg l^{-1}) almost certainly exceeded the total requirement of the alga. The results with S_0 medium (Section A3.1(i)) had also indicated that lowering the iron concentration might be beneficial. Gorham, McLachlan, Hammer and Kim (1964) reported morphological effects of iron upon Anabaena flos-aquae, and an interactive effect of iron and manganese in producing the effects seen. It therefore seemed worthwhile to test the effects of

varying the iron:manganese ratio. The concentration of EDTA in the medium is likely to affect the availability of metal ions, and it might therefore be expected to modify the effects (including toxic effects) of these ions on the algae. The level of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ was therefore varied also.

a) Variation of the level of FeIII-EDTA

Strain D253 was inoculated into AD medium containing FeIII-EDTA at 0.1x, 0.2x, 0.5x, 1.0x and 2.0x the normal level ($4 \text{ mg l}^{-1} \text{ Fe}$). Since variations in the level of FeIII-EDTA affect the pH of the medium, pH adjustment was made after autoclaving, with 0.05 M NaOH or HCl, to a standard pH of 7.4. This procedure was followed in each of the experiments involving variations in Fe or $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ concentration.

The morphology and growth of the alga in the different media are summarized in Table A3. Differences between the treatments were only small, but there was an indication that a twofold increase in FeIII-EDTA slightly reduced the extent of the morphological abnormalities, whereas symptoms were rather more severe with 0.1x and 0.2x the normal level. There was no apparent effect on final yield, but the initial growth was rather slower with FeIII-EDTA at 0.1x and 0.2x.

b) Independent variation of the levels of iron and $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$

It was not possible to say whether the slight differences seen in the first experiment were due to variations in the iron or in the $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ concentration. These two components were therefore varied independently, and the range of concentrations also increased, in the hope of obtaining a more clear-cut response.

AD medium was made up with $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ at 0.5x, 1.0x and 2.0x the normal level, and, at each level, with iron (as $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$) at 0.0x, 0.01x, 0.05x, 0.1x, 0.5x, 1.0x, 2.0x and 4.0x the normal concentration. This experiment was performed in boiling tubes, using 10 ml aliquots of media. Growth and morphology were scored after four weeks. The results are shown in Tables A4a and A4b.

At each level of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, there was a reduction in yield when iron concentration was reduced. The highest iron concentration at which limitation was apparent increased with increasing $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ concentration. With $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ at 1.0x and 0.5x, yield was also decreased by an increase in iron concentration above the normal; in fact the optimum growth at these levels of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ was at

TABLE A3

Growth and morphology of Calothrix viguieri D253 in AD medium with different levels of Fe III-EDTA

<u>Fe III-EDTA relative to normal level</u>	<u>2 weeks</u>		<u>4 weeks</u>	
	<u>Growth (0-5)</u>	<u>Morphology</u>	<u>Growth</u>	<u>Morphology</u>
1.0	4	typically abnormal	5	typically abnormal
2.0	4	as control	5	abnormal, but fewer dead trichomes
0.5	4	as control	5	as control
0.2	3	as control, but more subdivision of trichomes	5	as control, but more dead trichomes present
0.1	3		5	

TABLE A4

Growth and morphology of Calothrix viguieri D253 after 4 weeks in AD medium with different levels of iron and Na₂EDTA.2H₂O

(a) Growth (0-5)

EDTA concentration relative to normal	Fe III concentration relative to normal							
	0	0.01	0.05	0.1	0.5	1.0	2.0	4.0
0.5	3	4	5	5	5	4	4	4
1.0	4	4	4	5	5	4	4	4
2.0	3	4	4	4	5	5	5	5

(b) Presence of (assumed) morphological symptoms of iron deficiency
(See text)

EDTA concentration relative to normal	Fe III concentration relative to normal							
	0	0.01	0.05	0.1	0.5	1.0	2.0	4.0
0.5	++	++	0	0	0	0	0	0
1.0	++	++	+	0	0	0	0	0
2.0	++	++	++	++	0	0	0	0

0 = no response ; + = slight response ; ++ = marked response

levels of iron less than normal. This effect was not seen with $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ at 2.0x normal. Increasing the level of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ appeared to raise the concentration of iron required to produce a particular response (limitation or inhibition). This presumably reflected a reduction in availability of iron to the alga.

None of the media yielded algae of normal morphology. In some of the media with lower iron levels, many of the trichomes had brownish cells, often with small vacuoles, and these trichomes showed a greater tendency to fragment than ones from the control medium. The symptoms correlated in general with growth limitation, and were presumed to be due to iron deficiency (Tables A5a and A5b). No hairs were observed in the cultures, even though this strain had previously been found to develop hairs under iron deficiency (Chapter 6).

The differences in response were only slight, but there was some indication that levels of iron of 0.1x or 0.5x the normal, and also an increased EDTA:Fe ratio, were more favourable for growth, though not for morphology. The results suggested that the concentration of iron (and possibly that of other chelatable ions) in AD medium was supra-optimal for this strain, but the absence of any marked effects upon the morphological abnormalities suggested that this was not important in producing the response.

A further experiment was performed using a still wider range of concentrations of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, with parallel variations in iron concentration. The compositions of the media are given in Table A5, together with the resulting growth yields and morphologies, scored after 3 weeks. Three subcultures were made; the results were the same for each subculture.

The only media with any noticeable effect on the growth or morphology of the alga were those containing 10x the normal level of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$. The growth rate and final yield were considerably reduced in these media, particularly when the iron concentration was also 10x normal. The frequency of abnormal trichomes in these media was also reduced, from an estimated 80% to an estimated 20% of the population. Again, however, no entirely normal morphology was obtained.

c) Variations in iron:manganese ratio

A range of different manganese concentrations was included in the experiment described in (b) above (Table A5). In AD medium

TABLE A5

Growth and morphology of Calothrix viguieri D253 after 3 weeks in AD medium with further variations in iron and Na₂EDTA.2H₂O concentration

<u>EDTA relative to normal level</u>	<u>Fe III relative to normal level</u>	<u>Yield (0-5)</u>	<u>Morphology</u>
1.0	1.0	5	typically abnormal
	0.5	5	"
	0.2	5	"
2.0	1.0	5	"
	2.0	5	"
	0.5	5	"
5.0	1.0	5	"
	5.0	5	"
10	1.0	2	fewer abnormal trichomes
	10	1	"
0.5	1.0	5	characteristic abnormalities
	0.5	5	"
0.2	1.0	5	"
	0.2	5	"
0.1	1.0	5	"
	0.1	5	"

with $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ at the normal level, and at selected iron levels, the following variations in manganese concentration were made:

Fe concentration		Mn concentration	
X normal	mg l ⁻¹	X normal	mg l ⁻¹
1.0x	4.0	1.0x	0.5
		4.0x	2.0
		10.0x	5.0
0.05x	0.2	1.0x	0.5
		4.0x	2.0
		10.0x	5.0
2.0x	8.0	1.0x	0.5
		4.0x	2.0
		10.0x	5.0
4.0x	16.0	1.0x	0.5
		4.0x	2.0
		10.0x	5.0

The growth and morphology of Strain D253 at the various iron levels with the normal level of manganese were described in (b) above (Table A5). No detectable effect of varying manganese concentration was found at any of the iron levels.

The experiments failed to show any marked effect of iron, manganese or $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ concentration on the morphology of Strain D253 in AD medium, despite the wide range of levels and ratios used. Although there was some suggestion that the normal iron level of 4 mg l⁻¹ was supra-optimal for the growth of this strain, it seemed unlikely that iron was involved in producing the abnormalities. The improved morphology in S_0 medium (Section A3.31(i)) was thus presumably due to some factor other than iron concentration.

The only significant improvement in morphology (and this was not a complete recovery) was seen in media with Na_2EDTA at 10x the normal level, with iron at either 10x or 1.0x the normal level. This was correlated with a decrease in the growth rate and yield to an estimated 20% of the control. It seemed rather unlikely that the improvement in morphology was directly due to increased chelation, since the results were very similar when the extra $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ was balanced by extra iron. It was possible that $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ (by chelation) and iron (by competitive effects) were blanketing the effect of some other, toxic ion in these media. The

Na^+ contributed by the $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ might also have contributed to such a masking effect. However, it was taken to be more probable that the improvement in morphology was related to the much slower growth of the alga in these media. It was hypothesized that some damaging metabolic reaction might be slowed down under these conditions.

(v) Presence or absence of combined nitrogen

As mentioned in Section A2.2, Strain D253 grown on proteose-peptone agar produced trichomes of a typical '+N' morphology (Section 3.1), with no abnormalities. This medium contains 27.7 mg l^{-1} $\text{NO}_3\text{-N}$ as KNO_3 , in addition to the combined nitrogen supplied by the peptone. It was of interest to see whether the improvement in morphology was due to the presence of combined nitrogen. The abnormal excess production of heterocysts by some of the AD-grown trichomes (Section A2.1) did suggest that the nitrogen fixing process might be deranged.

AD and S_0 (both liquid) and proteose-peptone agar, were each made up with and without the addition of $\text{NO}_3\text{-N}$. AD+ NO_3 and S_0 + NO_3 each contained 10 mM NaNO_3 ; the corresponding - NO_3 media were supplemented with 10 mM NaCl . The '- NO_3 ' version of proteose-peptone agar contained KCl in place of KNO_3 , but the combined nitrogen supplied by the peptone was still present. The pH of the proteose-peptone media was adjusted to 7.0 before autoclaving, but they were not buffered. The other four media were made both with and without the addition of 10 mM HEPES (section 2.33). Strain D253 was inoculated into each of these media, and the morphology scored after two weeks' growth.

There was no difference between the alga grown on the + NO_3 and - NO_3 versions of proteose-peptone agar: the morphology in each case was characteristic of a medium containing combined nitrogen. Presumably the combined nitrogen in the peptone was sufficient to suppress heterocyst formation. AD- NO_3 and S_0 - NO_3 both yielded algae with the characteristic morphologies already described (Sections A2.1 and A3.31(i)). There was no difference between cultures with HEPES and those without HEPES. Algae from the + NO_3 versions of both media were very like those from proteose-peptone agar, with many Homoeothrix- and Ammatoidea-like trichomes present (cf. Section 3.1); no abnormalities of the type seen in

AD-NO₃ were observed. Growth and yield in the +NO₃ media was rather better in the presence of HEPES, but the only difference in morphology between algae from buffered and unbuffered media was the presence of more highly coiled trichomes in the former (cf. Section 2.33).

Thus the presence of a high level of NO₃-N in AD medium completely suppressed the morphological abnormalities, as well as the characteristic heterocystous morphology. This gave support to the hypothesis that a derangement of the nitrogen fixation process might be involved in producing the abnormalities.

(vi) Molybdenum concentration

In view of the results described in Section (v) above, it seemed possible that the molybdenum concentration in AD (0.08 mg l⁻¹) might be unsuitable for nitrogen fixation. Strain D253 was therefore taken through two subcultures in AD containing Mo at 0x, 1.0x, 2.0x, 5.0x, 10.0x and 20.0x the normal level.

Increasing the molybdenum concentration had no effect on either growth or morphology. Omitting molybdenum from the medium reduced the yield, and led to premature yellowing of the culture. There was no alleviation of the morphological abnormalities, but the alga showed additional symptoms: the heterocysts were enlarged, and development of young trichomes was stunted. (Similar responses were later observed in molybdenum deficient cultures of several other strains, studied in the experiments described in Section 5.71.)

Thus the molybdenum concentration in AD was not apparently limiting nitrogen fixation, and seemed unlikely to be involved in producing the abnormal morphology of Strain D253.

(vii) Phosphate concentration

Before the appearance of morphological abnormalities, Strain D253 developed many long hairs when grown in AD medium with a reduced concentration of phosphate (Chapter 4). It was of interest to see whether this morphological response could still be elicited. The basal level of phosphate in AD is very high (44.5 mg l⁻¹ PO₄-P), and it seemed possible that this itself could be producing some deleterious effect.

AD medium, buffered at pH 7.4 with 5 mM HEPES, was made up with PO₄-P at 1.0x, 0.5x, 0.25x, 0.1x, 0.05x, 0.025x and 0.01x

TABLE A6

Growth and morphology of Calothrix viguieri D253 after 3 weeks in AD medium with different levels of $\text{PO}_4\text{-P}$

<u>K_2HPO_4 relative to normal AD level</u>	<u>mg l^{-1} $\text{PO}_4\text{-P}$</u>	<u>Growth (0-5)</u>	<u>Morphology</u>	<u>Hairs present</u>
1.0	44.5	4	typically abnormal	0
0.5	22.25	4	"	0
0.25	11.12	4	"	0
0.1	4.45	3	"	0
0.05	2.22	2-3	"	+
0.025	1.11	2	"	+
0.01	0.44	2	"	+

the normal level. K^+ concentrations were made up by adding KCl. Growth and morphology of Strain D253 were scored after three weeks' incubation in the different media (Table A6).

None of the media yielded algae with an entirely normal morphology. At the lower phosphate concentrations, in association with growth limitation, the majority of trichomes were the same as those in the control, but a small proportion had developed hairs in an apparently normal manner. These trichomes showed no abnormalities of cell shape. A second subculture to the same media gave this result again. Thus reducing the phosphate to a yield-limiting level elicited some hair formation, but this was much less marked than the response obtained in earlier experiments (Chapter 4). It thus appeared that whatever was causing the morphological abnormalities in Strain D253 was also preventing the hair development response which had come to be regarded as characteristic of the phosphate deficient alga. A supra-optimal level of phosphate was not evidently responsible for the abnormal morphology, however.

(viii) NaCl concentration

It had been found that Strain D253 grew with a normal morphology in the marine S_0 medium (Section A3.31(i)). It was of interest to see whether this was related to the much higher NaCl concentration, which is the most obvious difference between S_0 and AD (Table 2.3). Strain D253 was inoculated into AD medium with a range of increased NaCl concentrations, and into S_0 medium with a range of decreased NaCl concentrations. The range used in each medium included the normal concentration in the other medium (Table A7). Growth and morphology in the different media were scored after 3 weeks (Tables A7a and A7b).

Trichomes of entirely normal morphology developed only in media containing NaCl at the basal S_0 level of 5000 mg l^{-1} . In AD as well as in S_0 , these trichomes had brown sheath bases, and showed separation of single cells from their apices (cf. Section A3.31(i)). In S_0 with 0.5x its normal level of NaCl, most of the trichomes had a typical ' S_0 morphology', but trichomes with enlarged cells, characteristic of AD, were also present. In each medium, the improvement in morphology was associated with a

TABLE A7 (a)

Growth and morphology of Calothrix viguieri D253 after 3 weeks in AD medium with different levels of NaCl

<u>NaCl relative to normal AD level</u>	<u>mg l⁻¹ NaCl</u>	<u>Growth (0-5)</u>	<u>Morphology</u>
1 x	230	4	typically abnormal
2 x	460	4	"
5 x	1,150	4	"
10 x	2,300	4	"
21.7 x	5,000	2	normal: as in S ₀ medium

TABLE A7 (b)

Growth and morphology of Calothrix viguieri D253 after 3 weeks in S₀ medium with different levels of NaCl

<u>NaCl relative to normal S₀ level</u>	<u>mg l⁻¹ NaCl</u>	<u>Growth (0-5)</u>	<u>Morphology</u>
1.0 x	5,000	2	normal
0.5 x	2,500	3	intermediate
0.2 x	1,000	4	abnormal: as in AD medium
0.1 x	500	4	"
0.046 x	230	4	"

reduction in growth rate and final yield. It was possible, therefore, that the effects seen were related to a reduction in metabolic rate, rather than being a direct effect of NaCl.

(ix) Buffering

During the experiments on the effects of nitrate and phosphate on Strain D253, described in Sections A3.31 (v) and (vi) respectively, it became apparent that there was no difference in its growth or morphology in unbuffered AD, and in AD buffered with 5 mM or 10 mM HEPES. pH fluctuations thus did not seem likely to be important in producing or modifying the abnormal morphological response of this strain.

A3.32 Factors involved in making up media

(i) Distilled water

It was possible that some contaminant was present in the normal supply of distilled water. AD medium was therefore made up from stock solutions in double distilled water, using single distilled water, double distilled water, and deionized distilled water. There was no difference in growth or morphology of Strain D253 in any of these media, suggesting that the distilled water was not responsible for the abnormalities.

(ii) Washing of glassware

The normal procedure for washing glassware was to use $\text{H}_2\text{SO}_4/\text{NaNO}_3$ wash acid (Section 2.31). In case some toxic factor were being introduced during acid washing (though this seemed unlikely), a set of flasks was washed by overnight soaking in a 2% (w/v) solution of Quadralene Laboratory Detergent (supplied by Fisons Scientific Equipment Ltd, Loughborough), followed by thorough rinsing. Strain D253 incubated in AD medium in these detergent washed flasks developed the same abnormalities as before. Thorough HCl washing of all the volumetric glassware and pipettes used for making up the medium was similarly without effect.

(iii) Sterilization

Marler and Van Baalen (1965) reported inhibition of growth of Anacystis nidulans in autoclaved medium containing citrate and manganese. The inhibitory effect was shown to be due to H_2O_2

produced by reaction between the citrate and manganese during sterilization. It seemed conceivable that a similar reaction might occur during autoclaving of AD medium, perhaps involving some contaminant organic molecule. A batch of AD was therefore sterilized by passing it through a 0.45 μ m membrane filter (Millipore Filter Corp., Bedford, Mass., U.S.A.). Though the sterilization was apparently effective, there was no improvement in the morphology of Strain D253 grown in the membrane filtered medium.

(iv) Cotton wool plugs

It seemed possible, though unlikely, that some toxic factor was being introduced from the cotton wool plugs used to close the flasks. To test this possibility, Strain D253 was grown up in 10 ml aliquots of AD medium in boiling tubes stoppered either with cotton wool bungs or with metal caps with spring clips ('Morton closures'). There was no difference in growth or morphology between algae from the different incubations.

A3.33 Factors associated with incubation

(i) Gas exchange

To test the theory that inadequate gas exchange was responsible for the differences in response between algae from the upper and lower parts of the liquid layer (Section A3.1) Strain D253 was grown up in flasks of AD medium continually sparged with air (Section 2.352). The gassing caused some evaporation of the medium; this was compensated for by the daily addition of sterile distilled water.

Algae in the aerated medium grew rather faster than those in non-aerated control flasks. The formation of a surface mat of trichomes was also less marked, probably because of the agitation of the liquid surface by the gas stream. Growth over the bases of the aerated flasks was correspondingly greater, forming a thick tufted 'lawn'. The morphology of most of the trichomes in this basal growth was like that of algae from the surface skins of the non-aerated flasks, with marked enlargement of cells in the mid-region. These trichomes were mixed with ones more typical of the basal growth of non-aerated flasks (with numerous intercalary discs, and showing death and lysis of many cells).

Thus aeration of AD medium did not produce a uniform morphological response throughout the flask, and this suggested that the death and lysis of trichomes in the basal growth of standing cultures was not due to a difference in gaseous micro-environment between the top and bottom of the liquid layer. Further evidence to support this conclusion was obtained from cultures of Strain D253 incubated in shake-tanks, illuminated from below. Here also, the bulk of the growth was attached to the flask base, probably due to a combination of the agitation of the medium, causing dispersal of the hormogonia, and the direction of illumination, causing the hormogonia to migrate to the bottom of the vessel. As with the aerated cultures, the basal growth contained abnormal trichomes of both the types described above.

(ii) Light regime

a) Light intensity

All the cultures in which abnormalities had been seen had been grown at light intensities of 2500-6000 lx. It seemed possible that a photo-oxidative process might be involved in producing the effects, and it was thus of interest to see whether abnormalities would develop under lower light intensities. Strain D253 was therefore incubated in flasks of AD medium at light intensities ranging from 300-6000 lx (Table A8). The lower light intensities were achieved by wrapping layers of black gauze around the flasks. Growth and morphology were scored after two weeks and after five weeks (Table A8).

Growth rate was reduced at light intensities of 2000 lx and below; this was particularly marked at 800 lx and below, where there was also an obvious increase in the depth of chlorophyll and phycocyanin pigmentation. Light intensities above 3000 lx caused yellowing of older cultures. There was little change in morphology between the two week and five week scores. All the cultures incubated at 1500 lx and above contained the same high proportion of abnormal trichomes. The algae grown at 300 and 500 lx had a rather different, though not entirely normal morphology. Greatly enlarged cells in the mid-regions of the trichomes were not observed, though trichomes with enlarged, rounded cells at the apex, similar to those seen in Chu 10-D(-N) (Section A3.31(i)) were quite common. Subdivision of the trichomes into numerous

TABLE A8

Growth of Calothrix viguieri D253 in AD medium at various light intensities

Light intensity (lx)	<u>2 weeks</u>		<u>5 weeks</u>	
	Growth (0-5)	Colour of culture	Growth (0-5)	Colour of culture
6000	3	green	5	yellowish
4000	3	"	5	"
3000	3	"	5	green
2000	2	"	5	"
1500	2	"	5	} darker, bluish green; the depth of colour increasing with decreasing light intensity.
800	1-2	dark blue-green	4	
500	1	"	3	
300	1	"	3	

short heterocystous lengths, by production of many separation discs, occurred at about the same frequency as it did in cultures at higher light intensities, but there was much less death and lysis of the cells. The separation discs in these cultures were dark brown, whereas in cultures at higher light intensities they were colourless; no sheath pigmentation was observed in any of the cultures. No explanation can be offered for this effect. The algae at 800 lx were intermediate in morphology, with a proportion of trichomes with enlarged cells in the mid-region, as well as the forms just described.

Thus growth at low light intensities did reduce the extent of the morphological abnormalities, but it did not restore an entirely normal morphology. This could imply that some photo-activated process was involved in producing the abnormalities; however, as observed in experiments with iron and EDTA (Section A3.31(iv)) and with NaCl (Section A3.31(vii)), the improved morphology was associated with a considerably reduced rate of growth, and it was possible that this itself was the more important factor.

b) Effect of a light:dark cycle

It was hypothesized that if some light-activated process were generating toxic products, then an alternating light:dark regime might be beneficial, by providing a recovery period. Strain D253 was grown for three weeks both under continuous illumination, and with a daily cycle of 10 h light : 14 h dark. Growth was rather slower in the light:dark incubated cultures, but there was no improvement in morphology.

c) Light quality

No systematic experimental variation of light quality was made. There was no difference in morphology between cultures grown under white and warm white illumination, the two conditions routinely used (Section 2.351).

(iii) Solid versus liquid media

It was of interest to see whether abnormalities would develop in algae grown on agar media, since many of the strains were of subaerial origin (Table 2.1), and it seemed that conditions on

agar might be closer to the natural condition of the algae. The problems of gas exchange through a thick layer of medium (Section A3.1) would also be reduced. In the experiment described in Section A3.31(i), when Strain D253 was grown in a range of different media, each medium was also made up as a 1% (w/v) agar. There was no difference in morphology between liquid and agar cultures with any of the media (cf. Table A2).

A4 Discussion

The abruptness with which the abnormalities developed suggested the likelihood that some sudden change had occurred in the media or incubation conditions, but no such change could be identified. A mutation or bacterial effect seemed unlikely to be responsible, and the possibility of a viral infection was also rejected, though with rather less certainty (Section A2.2). A cumulative toxic effect, building up over successive subcultures, was ruled out by the fact that abnormalities appeared after a single subculture from proteose-peptone slopes obtained from the culture centre at Cambridge.

A wide range of factors, associated with culture media and techniques (Section A3.2), was tested in an attempt to identify the cause of the abnormalities, but no solution to the problem was reached. The experiments showed that there were media in which Calothrix viguieri D253 could grow with a normal heterocystous tapered morphology, but they failed to provide any explanation of what was causing the altered morphology in AD medium. No toxic substance was identified in the medium, and contamination of stock chemicals, distilled water or glassware were ruled out. The normal levels of iron, manganese and other microelements (medium components considered most likely to have a toxic effect) were evidently not responsible for the abnormalities. No explanation was found for the difference in response between the surface and basal growths of the alga in culture flasks; one theory, that a difference in gaseous atmosphere was responsible, was effectively eliminated, however (Section A3.33(i)).

Few clear-cut results emerged from the experiments. One was that the morphological abnormalities did not develop in the presence of combined nitrogen at a level sufficient to suppress heterocyst

formation. This might perhaps imply that some interference with the nitrogen fixation process was involved in producing the morphological effects. It could be speculated that the morphogenetic processes involved in the development of a highly tapered heterocystous morphology might be more susceptible to derangement than those operative when combined nitrogen is equally available to each cell, and a less marked basal-apical differentiation occurs.

In addition it was found that a variety of apparently unrelated conditions reduced the frequency of abnormal trichomes in AD medium without combined nitrogen. These were: a tenfold increase in the concentration of $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, with or without a tenfold increase in iron concentration (Section A3.31(iv)); an increase of 21.7x in NaCl concentration, to an effectively marine level of 5000 mg l^{-1} (Section A3.31(viii)); and a light intensity of less than 800 lx for incubation (Section A3.33(ii)). The high level of NaCl completely eliminated the characteristic abnormalities, but under the other conditions, their occurrence was only reduced in frequency. It is possible to speculate that each of these conditions could act by reducing the effect of a toxic ion: the NaCl by a competitive 'masking' effect, the $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ by chelation, and the low light intensity by reducing some photo-activated reaction. However, as already pointed out, no source of any toxic material was discovered, and this theory is purely speculative.

One feature in common between the three different conditions was that each caused a reduction in the growth rate of the alga, though there was no obvious correlation between the extent to which the growth rate was slowed, and the reduction of abnormalities. It seemed possible that this slowing of growth, rather than a direct effect of each factor, might be important. Perhaps some damaging metabolic reaction would be slowed down under such conditions, to a rate which the alga could tolerate. This again is only speculation; experiments on the effect of slowing the growth rate by other means, perhaps by lowering the incubation temperature, could have been helpful in clarifying this point.

An examination of the limited literature on culture work with Rivulariaceae (Section 1.8) failed to reveal any reports of morphological forms like those found in Calothrix viguieri D253.

Pearson and Kingsbury (1966) described the occurrence of enlarged, irregularly shaped cells in cultures of C. membranacea; there was no apparent correlation with medium or culture conditions. Cells like those they describe were occasionally observed in C. membranacea D179 (which is the same strain as that used by Pearson and Kingsbury), but these differed from the type seen in C. viguieri; and were not associated with trichome lysis. Wyatt, Martin and Jackson (1973) described adverse effects on a Fremyella diplosiphon strain (= Calothrix sp. D255; Table 2.1), grown in ASM-1 medium (Gorham, McLachlan, Hammer & Kim, 1964) free of combined nitrogen: '. . . almost every filament . . . develops terminal heterocysts, pleomorphic cells abound, and most filaments begin localized degeneration'. The filaments depicted by these authors show extensive vacuolation of the cells, but do not resemble the abnormal trichomes of C. viguieri. Calothrix sp. D255 in AD medium showed none of the symptoms described by Wyatt, Martin and Jackson, nor did it develop abnormalities like C. viguieri (Table A1).

Formation of misshapen cells has been described in blue-green algae other than Rivulariaceae. Pearson and Kingsbury (1966) observed 'enlarged and greatly distorted cells' in soil-water cultures of Anabaena cylindrica; Lange (1970) described the occurrence of cells five times longer than normal, and thickened in the centre, in A. circinalis, grown in Zehnder and Gorham's (1960) medium with 100 mg l⁻¹ fulvic acid, or with 0.112 or 1.112 mg l⁻¹ Fe. Martin and Wyatt (1974a) found that misshapen cells, often much larger than normal, were not uncommon in cultures of Stigonematales, and that such cells became more abundant in older cultures and under dark growth conditions. Martin and Wyatt suggested that the abnormal cells might be the result of a failure of normal cell division mechanisms under adverse growth conditions. Apart from illustrating the extreme variability in cell morphology that can occur in blue-green algae, without any obvious relation to particular culture conditions, these observations do not really shed any light on the phenomena observed in the strains of Rivulariaceae studied.

Larson and Pate (1975) have described work with the prosthecate bacterium Astiocaulis biprosthecum which it may be relevant to consider here (cf. Section 1.52). They found that growth of A. biprosthecum was inhibited by conditions which favoured rapid

metabolism (e.g. high levels of carbon sources). A marked decrease in growth rate occurred at nutrient levels above those required to achieve maximum growth rate, and this was associated with morphological distortion of the cells. Irregular 'branched' cells were produced, and few prosthecate or swarmer cells developed. Larson and Pate suggested that the inability of this bacterium to tolerate rapid metabolic rates was due to a derangement of the obligate sequence of morphogenetic events involved in its normal life cycle. In its natural, low nutrient environment, the bacterium would not encounter conditions leading to rapid metabolism. The similarity between certain Rivulariaceae and prosthecate bacteria, in their life cycles and in their response to phosphate concentration, was mentioned in Sections 1.52 and 11.46.

While there are clearly many differences between the two systems, it is nevertheless tempting to speculate that the development of abnormalities in some strains of Calothrix may have been an effect analogous to that described by Larson and Pate (1975). Most of the strains which developed an abnormal morphology in AD medium were ones which produced hairs under phosphate and iron deficiencies (though not all such strains developed abnormalities). On the hypothesis that hairs may have a functional significance (Section 11.46), it would seem reasonable to suppose, since hair formation seems to be more frequent under conditions of nutrient deficiency, that potentially hair-forming strains are adapted to growth in relatively nutrient-poor conditions, and are perhaps therefore correspondingly less well adapted to growth in a rich culture medium. Five of the strains which produced hairs in low phosphate medium, but not in AD, showed no, or only slight abnormalities in AD medium (Section A2.3). The original habitats of these strains were ones which might suggest a tolerance of relatively high levels of dissolved salts, and perhaps, therefore, a corresponding tolerance of the rich AD medium. Nevertheless, none of the three strains with long hairs in AD medium showed abnormalities, though evidence discussed in Section 11.7 suggested that these strains were relatively intolerant of phosphate-rich media.

Despite these speculations, there nevertheless remained the problem of why the cultures suddenly began to show abnormalities,

after several months of normal growth under apparently identical conditions. None of the experiments provided any clues as to the reason for this.

A5 Selection of strains for further work

The attempt to identify the cause of the altered morphology of certain of the Calothrix strains was abandoned after the experiments described had been performed, even though no explanation had been found for the phenomenon. While this was clearly unsatisfactory, it was felt that all the more obvious possibilities had been explored, and that to follow the investigation further was too great a deviation from the studies on hair formation that were the main object of the work.

It now became necessary to select strains suitable for further experimental work. It was unfortunate that the most badly affected strains were ones which showed increased hair development under phosphate and iron deficiencies. Many of the experiments performed before the abnormalities had appeared had used Calothrix viguieri D253, in AD medium; clearly this was no longer feasible. The two alternatives seemed to be either to continue to use Strain D253, but with the marine S_0 as basal medium, or to continue to use AD, with a strain or strains capable of normal growth in this medium. The latter alternative was selected, since AD had been the basal medium for many experiments, not only with Strain D253, and including those of other workers in the laboratory (Kirkby, 1975); valuable comparative data would therefore be lost by changing the medium. Furthermore, it seemed likely that not all the strains held would tolerate the marine S_0 medium; in fact a preliminary survey showed that many of the strains grew very poorly in S_0 . This would clearly limit its usefulness. Further experiments were therefore performed using AD medium, with the strains which grew with a normal morphology in this medium (Table A1).

SUMMARY

A study was made of factors influencing hair development in the Rivulariaceae. Effects on tapering were also examined, since it seemed that the two characters might be related. Experiments were performed using a large number of cultures (34 heterocystous and 2 non-heterocystous), which had a broad range of morphological characteristics.

The 34 heterocystous strains were grown in the presence and absence of combined nitrogen ($140 \text{ mg l}^{-1} \text{ NO}_3\text{-N}$) and their morphological responses described. One strain was unchanged in morphology. The remaining 33 strains lost their heterocysts: 19 became parallel, but 14 produced many trichomes which tapered towards one or both ends, resembling Homoeothrix or Ammatoidea. The development of hairs was not diminished in the 2 strains which had only few short hairs in medium without $\text{NO}_3\text{-N}$, but hairs were considerably reduced in length and number in the 3 strains which had many long hairs in the medium $-\text{NO}_3$. In these 3 strains, the hairs in $+\text{NO}_3$ medium were present on trichomes which were otherwise parallel. These results indicated that hair development and trichome tapering are not necessarily directly related phenomena. They also suggested that while availability of combined nitrogen is undoubtedly an important factor influencing tapering, nevertheless tapering and/or hair development of heterocystous trichomes is unlikely to be due solely to nitrogen deficiency in the apical region in every case. In many cases the cells of the parallel and tapered trichomes in $+\text{NO}_3$ medium closely resembled cells adjacent to the basal heterocyst of trichomes in $-\text{NO}_3$ medium in shape and dimensions. This suggested the possibility that one influence of heterocysts on tapering might be to promote enlargement of the cells in the basal region.

One heterocystous strain known to be capable of hair formation, though not possessing hairs in the nutrient-rich control medium, was starved of nitrogen by incubation under $\text{Ar}:\text{O}_2:\text{CO}_2$ (79.97:20:0.03 by volumes). The alga became yellow and vacuolated, and many secondary heterocysts developed in all parts of the trichomes, but tapering was unaffected, and no hairs developed. These

results showed that cells in the apical region of tapered heterocystous trichomes had the potential to develop into heterocysts if severely nitrogen starved. This suggested that these cells were not normally in this condition.

The influence of nitrate deficiency on tapering and hair development was also examined in two strains of the non-heterocystous genus Homoeothrix. Neither strain grew in media without combined nitrogen, and when H. crustacea colonies from the field were assayed for nitrogenase activity, using the acetylene reduction technique, the results were negative. Even under apparent nitrogen starvation, neither strain showed any sign of heterocyst development, which strongly suggested that neither was merely a growth form of Calothrix, a possibility suggested by the responses of the heterocystous strains to combined nitrogen. One strain of Homoeothrix became more tapered, and developed a few short hairs, when nitrate-limited, but this was found to be a relatively non-specific response to growth limitation.

The influence of deficiencies of phosphate, iron, magnesium, calcium, molybdenum and sulphate on hair development was also examined. 34 heterocystous and 2 Homoeothrix strains were grown to phosphate deficiency: 12 heterocystous strains, and H. crustacea showed marked increases in hair development. 5 of the 12 heterocystous strains, and H. crustacea, had at least some hairs in the high phosphate control medium, but the other 8 strains did not. The 13 strains that showed increased hair development in low phosphate medium were also grown to iron and magnesium deficiencies. 8 of these developed hairs under iron deficiency, and one of the 8 also did so under magnesium deficiency. All 8 strains were ones without hairs in the full control medium. The increases in hair development in these 12 heterocystous strains in response to deficiencies of phosphate, iron and magnesium also occurred in the presence of $140 \text{ mg l}^{-1} \text{ NO}_3\text{-N}$, a level sufficient to suppress heterocyst development. This again suggested that hair development was unlikely to be due only to nitrogen deficiency in the apical cells.

8 of the strains which showed increased hair development under phosphate deficiency (7 heterocystous + 1 Homoeothrix), and 5 strains (4 heterocystous + 1 Homoeothrix) which did not

do so were tested for the effects of calcium, molybdenum and sulphate deficiencies. These deficiencies had no marked effect upon hair formation in any of the strains. Homoeothrix crustacea in fact developed a few short hairs under each of the deficiencies (other than that of phosphate) that were examined; this response was much less pronounced than that of phosphate deficient cultures, and appeared to be a non-specific response to growth limitation.

In addition to hair formation, a number of other morphological and cytological responses to nutrient deficiencies were observed. Heterocyst frequency increased markedly in all 11 heterocystous strains grown under molybdenum deficiency, in 13 of the 16 heterocystous strains grown under iron deficiency, and in 9 of the 11 heterocystous strains grown under calcium deficiency; under each deficiency, heterocysts developed in all regions of the trichomes. Although each of these deficiencies presumably impaired nitrogen fixation, iron deficiency was the only one of the three which also affected hair development. This seemed to be further evidence that hair formation in heterocystous trichomes was not simply due to nitrogen deficiency in the apical cells. The sheath bases of 12 strains became dark brown under phosphate deficiency. 4 of these strains also gave this response under iron deficiency, and 3 did so under magnesium deficiency; 9 of the affected strains were ones which showed increased hair development under phosphate deficiency. Polyphosphate granulation became undetectable in all 36 strains tested under phosphate deficiency, increased markedly in 12 of the 13 strains tested under sulphate deficiency, and was unaffected by molybdenum deficiency in any of the 13 strains tested. Calcium deficiency affected polyphosphate granulation in only one of the 13 strains tested; this strain showed a decrease. The effects of magnesium and iron deficiencies on polyphosphate granulation were more variable. None of the strains which developed hairs under the latter two deficiencies showed more than a slight decrease in polyphosphate granulation, which reduced the likelihood that hair development under these conditions was due to a secondarily induced deficiency of phosphate. All 34 heterocystous strains showed a marked increase in the level of cyanophycin granulation under phosphate deficiency. Several strains showed

changes in the shape of vegetative cells, and in trichome dimensions and tapering, that were distinct from, though sometimes similar to, those that occurred during hair development. The morphological variations of the Rivulariaceae in response to nutrient deficiencies were greater than those shown by Anabaena cylindrica under the same conditions. This perhaps reflected the more complex growth pattern of the Rivulariaceae.

In all strains showing hair development in response to nutrient deficiency, this response was one of the first morphological changes to occur, and preceded any obvious degenerative symptoms. When hair development in response to phosphate deficiency was studied in more detail in one strain, hairs were found to develop when polyphosphate granules were still present in many of the vegetative cells, implying that the trichomes were not fully phosphate starved. In each of the 7 heterocystous strains tested, and in Homoeothrix crustacea, many of the hair cells which developed under phosphate deficiency were found to be able to synthesize polyphosphate granules when phosphate was added to the cultures, indicating that the hair cells were capable of at least some metabolic activity. This fact, together with the observations that hairs only developed in response to certain deficiencies, and that they appeared before any degenerative changes in the cultures, suggested that hair development was not simply a pathological phenomenon, but a specific morphogenetic process. The fact that hair development increased under low nutrient conditions, and led to a considerable increase in trichome surface area, is compatible with a role for hairs in the uptake of nutrients present at low concentration.

4 of the 5 heterocystous strains which produced hairs even in the phosphate-rich control medium were cultured at still higher phosphate levels, in an attempt to suppress hair development, but hairs were apparently lost only as a symptom of trichome degeneration at the highest phosphate concentrations. Several pieces of evidence suggested that strains which retained hairs even at high phosphate levels were more sensitive to high concentrations of phosphate than strains in which hair development was suppressed by phosphate levels of the order of $45 \text{ mg l}^{-1} \text{ PO}_4\text{-P}$. It was speculated that the former type of strains may be adapted

to growth in low nutrient environments.

Some experiments were also done with field materials. Trichomes of Rivularia and Calothrix from two field sites were found to show greater hair development in low phosphate media than at higher phosphate concentrations, and in the one field Rivularia studied, polyphosphate granules developed in the hair cells during incubation in high phosphate medium, in much the same way as phosphate-starved laboratory cultures. The similarity between the responses of the field materials and the laboratory strains suggested that the results obtained with the latter were unlikely to be simply artefacts of culture.

During the research some of the strains suddenly began to show morphological abnormalities when grown in the standard control medium. The most seriously affected strains were ones which produced hairs in low phosphate and low iron media, but did not do so in the nutrient-rich control medium. An experimental investigation failed to reveal the cause of this phenomenon, though it was speculated that a preference of the affected strains for a low nutrient environment might have been one factor influencing their sensitivity.

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