STUDIES IN THE BIOLOGICAL FIXATION OF NITROGEN

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G E N E R A L

I N T R O D U C T I O N

Analyses of living plant tissues show that the four most abundant elements present are usually carbon, hydrogen, oxygen and nitrogen. As a result vigorous plant growth is mainly dependent on an adequate supply of these elements. Carbon, hydrogen and oxygen are normally available to the plant in more than ample supply from atmospheric or soil sources. Nitrogen on the other hand is in a somewhat different category in that while an abundant supply is present in the elemental form in the atmosphere, the majority of plants are unable to assimilate it, being entirely dependent on combined nitrogen available generally in the form of nitrate or ammonium-nitrogen.

However it is now well established that a small minority of plants are capable of assimilating elemental nitrogen, such species falling into two main groups as follows:-

I. Free living forms

Certain bacteria e.g. Clostridium, Azotobacter and Chromatium.

Certain blue-green algae e.g. Nostoc, Anabaena and Mastigocladus.

II. Symbiotic forms

Legumes with root nodules.

Non-legumes with root nodules.

Certain lichens, bryophytes and ferns, with blue-green algal constituents e.g. Peltigera, Blasia and Azolla.

In addition to the above there is some evidence that other groups may also fix nitrogen. Of the free-living forms fixation has been reported in certain soil yeasts (Metcalfe and Chayen, 1954). In symbiotic systems claims of fixation have been more widespread. In the family Podocarpaceae, where the plants bear large numbers of root nodules inhabited by a fungus, evidence of fixation has been obtained by Nobbe and Hiltner (1899) although Bond (1959) obtained negative results both in sand culture and in ^{15}N experiments. In certain members of the Cycadaceae, for example Ceratozamia and Encephalartos, which again bear root nodules but this time having a blue-green alga as endophyte, evidence of fixation has been obtained (Bond, 1959). Certain species belonging to the family Rubiaceae bear leaf nodules which have been suspected of fixing nitrogen (Bremekamp, 1933), although the results are far from conclusive.

It is a reasonable assumption that in the early stages of the evolution of the plant kingdom species capable of utilising atmospheric nitrogen would be at an advantage

over forms which depended on the limited quantities of combined nitrogen available. Palaebotanical evidence although fragmentary does in fact offer some evidence which suggests that nitrogen-fixing organisms may have been important classes in the past. The oldest group discovered in which present day members are known to fix nitrogen is the blue-green algae. This group was discovered in the Rhynie chert of the Upper Devonian among the primitive land plants, the Rhyniales, by Kidston and Lang (1921) and by Croft and George (1959). It is of interest to note that three of the four species recorded have been placed in the order Stigonematales, of which the present day nitrogen-fixing species Mastigocladus laminosus is a member.

Evidence of the presence of possible symbiotic nitrogen-fixing systems in the past is less forthcoming, although as suggested by Bond (1959) it is possible that the cycad-type plants so prevalent in the Jurassic and Cretaceous fixed nitrogen. Pollen analyses have shown that much more recently Coriaria was prominent in many parts of the world (Good, 1947), while in the post-glacial era Alnus and Hippophaë are believed to have been very abundant in Britain (Tansley, 1939) and Europe (Walker, 1955) respectively. It is thus probable that in the pre-agricultural era nitrogen-fixing organisms were of more extensive occurrence in Nature than at the present time.

It has been shown in a 1936 balance sheet of the

agricultural lands in the United States (in Fogg, 1955) that approximately sixteen million tons of combined nitrogen per annum are made available to the soil by the process of biological fixation of nitrogen and it is obvious that any system contributing so much to soil fertility will be the subject of much research. Such research has been carried out and at present is proceeding along two main lines. In the first instance further elucidation of the physiology and biochemistry of nitrogen fixation is being undertaken in known nitrogen-fixers; secondly searches for new nitrogen fixing species are being carried out not only to gain a more complete picture of the importance of biological nitrogen fixation but also in the hope of discovering species more suitable for study of the processes involved in fixation.

In the present thesis studies along both the above lines have been carried out; in Section I experiments on the physiology of nitrogen fixation in certain well established non-legume fixers will be described, while Section II deals with studies on some newly discovered nitrogen fixing blue-green algae.

The following are the non-legume genera of angiosperms normally characterised by the presence of nodules on their roots their families, according to Engler and Diels (1936) also being shown:

Genus	Family
Myrica	Myricaceae
Alnus	Betulaceae
Hippophae	Elaeagnaceae
Elaeagnus	
Shepherdia	
Casuarina	Casuarinaceae
Coriaria	Coriariaceae
Ceanothus	Rhamnaceae
Discaria	

The last genus, Discaria, has only been shown recently to bear root nodules (Morrison and Harris, 1958).

The above plants although bearing nodules do differ from the legumes in the nature of the endophyte. Its identification however is uncertain, due to the inability of workers to isolate an organism which on re-inoculation will cause nodule formation. The general consensus of opinion gained from cytological investigations is that the organism is an actinomycete (Roberg, 1934; von Plotho, 1941). This conclusion is supported by Pommer (1959) who claims to have isolated from Alnus an organism capable of nodule formation on re-infection and considers it to be of an actinomycetal nature.

Studies on the physiology of these plants have been carried out mainly in Glasgow University and data have been accumulated which show that the above species fix nitrogen

(Bond, 1949, 1951; Bond, Fletcher and Ferguson, 1954; Quispel, 1954; Bond, 1955; Bond, MacConnell and MacCallum, 1956; Bond, 1957a, 1957b, Gardner and Bond, 1957; Gardner, 1958; Morrison, 1961). The plants resemble the legumes in many other respects including that the nodules are the site of fixation (Bond, 1955, 1957b) and that combined nitrogen affects nodulation (Bond et al., 1954; Bond et al., 1956; MacConnell and Bond, 1957 and Quispel, 1958). In addition it is clear that these non-legume plants are as efficient nitrogen fixers as their leguminous counterparts and evidence is accumulating which suggests that they are more suitable than the legumes for certain types of experiments associated with nitrogen fixation. As a result it is clearly desirable to obtain information on such systems and Section I of this thesis has been concerned with aspects of the nitrogen nutrition of the two commonest British species, Alnus glutinosa and Myrica gale.

Although extensive fixation in legumes and non-legume nodulated plants is now well established less attention has been paid to other groups of nitrogen fixers, among them the blue-green algae. Up to the present time over twenty species of blue-green algae have been shown to fix nitrogen (Fogg and Wolfe, 1954), and the physiology of certain of those have been considered in detail particularly by Fogg's school in London.

However all species have so far been isolated from fresh water and terrestial habitats although a marked blue-

green algal flora exists in the marine supralittoral fringe in many temperate regions. Fremy (1936) for example records 267 species from the European coasts. Allen (1958) in attempts to test for marine nitrogen fixation found difficulty in culturing the algae, and resorted to carrying out tests on known fresh water nitrogen fixers which she adapted to high salinities. It is of importance however to know whether species from marine habitats do fix nitrogen and Section II of this thesis will be concerned with the culture and nitrogen-fixing ability of certain blue-green algae from the marine supralittoral fringe.

S E C T I O N I

STUDIES ON THE FIXATION OF ELEMENTAL NITROGEN BY *ALNUS GLUTINOSA* (L.) GAERTN. AND *MYRICA GALE* L.

P A R T I

The effect of added ammonium-nitrogen on nitrogen fixation by *Alnus glutinosa* and *Myrica gale*

I N T R O D U C T I O N

The ability of nodulated plants of Alnus and Myrica to fix atmospheric nitrogen at a rate sufficing to sustain vigorous growth in rooting media free of combined nitrogen is well-established in respect of plants growing in artificial media in the greenhouse (Bond, Fletcher and Ferguson, 1954; Bond, 1958), and there is no doubt that if a suitable arrangement could be devised it would be possible to grow for example an alder tree to maturity without access at any stage to external combined nitrogen. It cannot be assumed that equally extensive fixation is associated with plants of these genera in the field, since new factors may operate there. One such factor is the usual presence in soils of some level of available combined nitrogen, for it is well-known that in other nitrogen-fixing organisms combined nitrogen is used preferentially over the elemental form, fixation being reduced or even completely suppressed. In the particular nitrogen-fixing system which in general characteristics resembles that presented by Alnus or Myrica, namely the legume, the work of numerous previous workers has shown that in the presence of substantial amounts of combined nitrogen in the rooting medium, nodule formation is depressed; this at least partly explains the reduction in fixation. As an example of such previous work reference may be made to the

experiments of Giobel (1926). In those experiments soya bean plants were grown in sand-culture in glazed earthenware pots, there being two pots each of which contained three plants, at each nitrogen treatment. The results which are summarised in Table I, show that in an eleven-week growth period both nodule number and fresh weight are greater in the presence of 5.6 mg. nitrogen per litre than at the zero nitrogen level. Higher levels of combined nitrogen cause a depression in nodule number and in fresh weight. This is one of the first experiments in which the beneficial effect of small quantities of combined nitrogen on nodulation was noted, earlier workers (Hiltner, 1900; Zipfel, 1911) stating that nodulation was better in the absence of nitrogen. The reasons for these effects of combined nitrogen on nodule formation will be discussed in Part II of this Section.

Where nodule formation is largely suppressed as a result of the presence of combined nitrogen in the rooting medium it is obvious that fixation of nitrogen likewise must be to a considerable extent extinguished, but the question remains as to how active in fixation are those nodules which form in the presence of moderate amounts of combined nitrogen. Here again there is in respect of the legumes an extensive literature, relating mainly to the study of pot cultures. Among experiments along such lines those of Giobel (1926) may again be noted first. In his experiments soya bean plants were grown in sand-culture moistened with culture solution

TABLE 1

The effect of sodium nitrate on nodulation in soya bean

(Data reproduced from Giobel, 1926)

Mg. nitrogen added (at commencement of experiment) per litre of culture solution	Mean number of nodules per plant	Mean fresh weight of nodules per plant
0	128	1.35
5.6	151	2.10
11.2	77	1.78
22.4	57	1.53
33.6	16	0.49
44.8	18	0.22
67.2	7	0.07

containing various known quantities of nitrate-nitrogen, and analyses of both inoculated and uninoculated plants grown in the presence of similar quantities of combined nitrogen carried out after twelve weeks growth. From the data available it is possible to convert Giobel's nitrogen levels to mg. nitrogen supplied per litre of culture solution; 100 lb. nitrate-nitrogen per acre being equivalent to 5.6 mg. nitrogen per litre. It is thus seen from the results which are reproduced in Table 2 that plants supplied with approximately 130 mg. nitrate-nitrogen per litre still fixed approximately 15 per cent of their total nitrogen.

Later Hopkins, Wilson and Peterson (1932) grew inoculated red clover plants aseptically in the presence of varying concentrations of nitrate-nitrogen and analysed both substrate and plants for nitrogen after a five-week growth period. The results again suggested that a certain proportion of plant nitrogen was fixed even in the presence of the highest quantity of combined nitrogen supplied (approximately 80 mg. nitrate-nitrogen per litre of agar substrate per five-week growth period).

More recently the simpler and more accurate isotopic method has become available and was first used by Norman and Krampitz (1946) who supplied labelled inorganic nitrogen to soya bean plants grown in pot culture. The results showed that fixation although much reduced was not entirely inhibited. It was estimated that in prairie soils of average

TABLE 2

The effect of combined nitrogen on nitrogen fixation by soya bean. (Four plants were available at each nitrogen level)

(Reproduced from Giobel, 1926)

Nitrate added per acre incre-mentally (lb.)	Total N in four inoculated plants (gm.)	Total N in four uninoculated plants (gm.)	Total N fixed (gm.)	Fixed N as %age of total N
100	0.7168	0.0770	0.6398	89.25
500	0.7441	0.2410	0.5031	67.61
900	0.6997	0.3322	0.3675	52.53
1300	0.6997	0.3920	0.3077	43.97
1700	0.6855	0.5905	0.0950	13.86
2100	0.7367	0.6282	0.1085	14.73

fertility soya bean derives 25-30 per cent of its nitrogen from the atmosphere.

Thornton (1947) in experiments with soya bean found by using ^{15}N that plants growing in the presence of combined nitrogen still fixed appreciable quantities of atmospheric nitrogen. In plants harvested after a five week growth period for example approximately 50 per cent of their total nitrogen was fixed even although a near ample supply of combined nitrogen was available.

Allos and Bartholomew (1955) grew several legumes, among them soya bean, alfalfa, ladino clover (Trifolium repens (giganteum)), and birdsfoot trefoil (Lotus corniculatus) for a period of ten weeks in vermiculite pot culture supplied with labelled combined nitrogen in the form of ammonium-nitrogen. There were two pots at each nitrogen level. The results obtained are summarised in Table 3. From such data it is evident that in legumes a decrease in the percentage nitrogen fixed occurs with increase in combined nitrogen. It is to be regretted that the authors fail to give any indication of pot size, or of number of plants per pot.

A fair statement of the position in legumes seems be that in the presence of sufficient combined nitrogen for

TABLE 3

The effect of various levels of combined nitrogen on nitrogen fixation in certain legumes

(Reproduced from Allos and Bartholemew, 1955)

Plant used		Nitrogen added in ten week (mg. per pot)			
		0	108	216	432
Soya bean					
a)	Dry matter (gm. per pot)	9.7	11.4	9.4	9.1
b)	Total N uptake (mg. per pot)	310	349	290	320
c)	N from fertiliser (mg. per pot)	0	60	95	185
d)	Mean fixation (mg. per pot)	316	289	195	135
e)	Percentage N fixed	100	83	67	42
Alfalfa					
a)		4.6	6.2	8.4	8.2
b)		141	195	286	285
d)	As above	0	56	158	220
d)		140	139	128	65
e)		100	71	45	23
Trifolium repens (giganteum)					
a)		1.5	3.5	5.1	5.7
b)		44	112	166	227
c)	As above	0	73	120	210
d)		44	39	46	27
e)		100	35	28	11
Lotus corniculatus					
a)		1.2	3.1	4.1	6.4
b)		34	91	142	259
c)	As above	0	64	113	236
d)		34	27	29	23
e)		100	30	20	9

vigorous plant growth, fixation per plant is reduced but is usually still quite substantial, and the legume grows only partly at the expense of the supplied combined nitrogen. The reduction in fixation is due mainly to the tendency for the total nodule mass per plant to decrease in the presence of combined nitrogen; whether in addition there is a reduction in fixation per unit nodule mass is an aspect to which little attention has been paid.

For certain of the nodule-bearing non-legumes information is available concerning the effect of combined nitrogen on nodulation. The earliest work appears to be that of Hiltner (1896) on Alnus glutinosa. In his experiment four seedlings were set up in nitrogen-containing culture solution and after inoculation nodules developed. On subsequent transferance of two plants to a nitrogen-free solution and two to a nitrogen-containing solution, Hiltner observed that there was little further nodule development in the plants growing in the presence of nitrogen. The significance of these observations is reduced by the author's failure to state the level of nitrogen supplied.

Bjorkman (1942) carried out an experiment in which Alnus glutinosa plants growing in a humus-sand mixture were subjected to nitrogen concentrations (in the form of ammonium nitrate) of 100, 200 or 400 mg. per litre of added culture solution. The effects directly attributable to the presence of nitrogen were complicated by the fact that varying

phosphate levels were also applied, but in instances of similar phosphate and varying nitrogen levels it was noticed that nodulation was almost entirely inhibited at the very high levels of 400 and 200 mg. nitrogen; at the 100 mg. level the volume of nodule tissue compared with that for the nitrogen-free plants was reduced to approximately 50 per cent.

Uemura (1952) in Japanese field experiments with Alnus firma and A. multinervis observed that when supplied with nitrogen fertiliser in the form of 1 lb. of ammonium sulphate per square metre of soil surface, only 50 per cent of the former and 20 per cent of the latter had developed nodules after six months, while control plants free from nitrogen-fertiliser showed 100 per cent nodulation. It should be noted that the concentration of ammonium sulphate employed was equivalent to an application of 36 cwt. per acre, a quantity far in excess of that normally applied in agricultural practice (1 - 2 cwt. per acre).

More recently experiments on the effect of combined nitrogen on nodulation in non-legumes have been carried out in Glasgow, the earliest being those of Bond, Fletcher and Ferguson (1954) who investigated the effect of combined nitrogen in Alnus and Hippophaë. In their experiments the plants, which were grown in water culture, were subjected from the time of inoculation to four different levels of combined nitrogen in the form of ammonium-nitrogen, namely 0, 10, 50 and 100 mg. nitrogen per litre. In Alnus it was observed

after a twelve-week growth period that plants growing in a medium supplied with combined nitrogen were much larger than the nitrogen-free plants and that nodule weight per plant increased up to some level of combined nitrogen between 10 and 50 mg. and afterwards fell. Nodule development failed to keep pace with greater plant growth and as a percentage of total plant weight it decreased from 6.1 per cent in the 0 nitrogen level to 1.0 per cent in the 100 mg. nitrogen level. Significant differences from Alnus were noted in the Hippophaë results where stronger growth of the plants in solutions containing combined nitrogen was accompanied not by an increase but by a decrease in absolute weight of nodules, while nodulation was completely inhibited in the presence of 50 mg. combined nitrogen per litre.

Further work on the effect of combined nitrogen on Alnus was published by Quispel (1954) who found that nodulation was completely suppressed in the presence of 50 or more mg. combined nitrogen per litre. These results are in marked contrast to those of Bond et al. who observed no significant difference between nodule dry weight in the presence of 0 and 50 mg. combined nitrogen per litre.

The discrepancies between the Alnus results of the Glasgow experiments and those of Quispel led to the experiments of MacConnell and Bond (1957) with Alnus and Myrica. In their work with Alnus two series of nitrogen treatments were set up. In one, inoculation of the seedlings was carried out prior to

the establishment of the differential nitrogen levels while in the other seedlings were grown for a five-week period after transplanting in the presence of 50 mg. ammonium-nitrogen prior to the establishment of the differential nitrogen treatments and inoculation. Nodule dry weight was in both cases greatest in the 50 mg. nitrogen level and decreased at higher nitrogen levels, the decrease however being much more marked in the series in which the plants were originally grown in the presenc of combined nitrogen. These results on Alnus confirm those of Bond et al. described above and differ sharply from those of Quispel, thus lending support to the suggestion forwarded by MacConnell and Bond (1957) that Quispel's results were perhaps due to the effect of unfavourable pH levels. In a later publication Quispel (1958) agrees that this in fact was probably the case.

In the Myrica experiment the plants were again subjected to 0, 10, 50 and 100 mg. combined nitrogen immediately before inoculation and those levels were maintained during a ten-week growth-period. The number of plants harvested in this experiment was small owing to considerable losses of plants during the growth period and in addition growth of surviving plants was rather poor. The results which are reproduced in Table 4 show that the plants grown in the presence of combined nitrogen were much larger than those in a nitrogen-free medium, attaining maximum growth at some level between 50 and 100 mg. nitrogen. Mean nodule dry weight

TABLE 4

The effect of added combined nitrogen on nodulation and growth of Myrica plants *

(Reproduced from MacConnell and Bond, 1957)

Mg. NH_4-N added per litre of culture solution	Mean height of shoot in cm.	Mean dry weight per plant in mg.		Mean value for nodule weight as percentage of whole plant weight
		Nodules	Whole plant	
0	3	4	33	12.2
10	8	17	304	5.4
50	12	24	561	4.2
100	10	19	488	3.6

* The numbers of plants harvested at the different nitrogen levels, starting with zero nitrogen, were: 9, 12, 6, and 5 respectively.

remained fairly constant over the various levels of combined nitrogen but as a percentage of total plant weight it decreased, this decrease being greatest between the zero and 10 mg. nitrogen levels after which it was more gradual.

Quispel (1958) described two further experiments with Alnus in which the effect of combined nitrogen (in the form of calcium or potassium nitrate) on nodulation was investigated when supplied prior to inoculation, at inoculation, or following inoculation. In the first experiment no difference in nodule number was observed (as compared with the plants grown in the absence of nitrogen) when the nitrogen was supplied prior to inoculation but nodule number was decreased when the nitrogen was supplied during or after inoculation. In a repeat experiment carried out later no significant difference in nodule number was observed whether the plants were supplied with combined nitrogen or not. Quispel suggested that the difference between the two sets of data may be due to the concentration of nitrogen used (3.75 mg. nitrate-nitrogen per litre of solution) being at the border of the inhibiting concentration and that inhibition depended on other physiological conditions.

Data on the effect of combined nitrogen on actual fixation in non-legumes are limited to those of Bond (1955) who, using isotopic methods, showed that appreciable fixation was still associated with Alnus and Myrica plants in the presence of 50 mg. and 140 mg. combined nitrogen per litre

of culture solution respectively. To gain more information on the effect of combined nitrogen on fixation the experiments now to be described were set up. For comparative purposes data were obtained on the legume Ulex europaeus.

M E T H O D S

The principle of the isotopic method used in this study is as follows. Ammonium-nitrogen, suitably enriched with ^{15}N, is supplied at different levels to nodulated plants over a period of some weeks, starting at an early stage in growth. After harvest the total nitrogen content of the plants is determined, and also the degree of enrichment in ^{15}N shown by this plant nitrogen. It is then possible to calculate how much of the plant nitrogen has been obtained by uptake of the supplied combined nitrogen, and by difference to discover the amount of atmospheric nitrogen concurrently fixed by the plants. The plant cultures in this study were not of aseptic type, but it can be stated that over a good many years' experience of non-legume and legume cultures set up in the way now to be described, no evidence whatever has emerged to indicate that such incidental micro-organisms as gain access to the rooting medium have any effect on the nitrogen nutrition of the plants concerned.

Seed of Alnus glutinosa and Myrica gale collected locally in the field was sown in vermiculite (Alnus) or peat (Myrica) after cold storage for some weeks at 2°C. The Ulex seed was obtained from Thompson and Morgan (Ipswich) Ltd., MacConnell (1956) having shown that seed collected locally

germinated poorly, and in addition bore fungal contamination which could not be removed by surface sterilisation. This Ulex seed, before sowing was shaken in concentrated sulphuric acid for thirty minutes, followed by ten rinses in sterile distilled water over a period of forty minutes. This procedure not only sterilised the seed coat but softened it, thus facilitating germination. Seedlings were transplanted into water culture when one or two leaves had emerged. Containers for culture consisted of 2-litre glazed earthenware jars covered by 7" squares of black polythene sheet (Alnus and Ulex) or waxed teak (Myrica) bored with holes for the plants. The culture solution was that of Crone (nitrogen-free formula, Bond, 1951), used at full strength and natural pH (6.2) for Alnus, full strength and pH 6.4 for Ulex, but initially at one-quarter strength and with pH lowered to 5.4 for Myrica. A minor element supplement was added to the solution. At this stage there were six plants per jar. The greenhouse was lit by daylight.

Inoculation

Inoculation was effected with the plants in the solution without combined nitrogen, except that a single, uniform addition of unlabelled ammonium sulphate was made to the Alnus and Myrica plants, providing 5 mg. nitrogen per litre of culture solution, in order to assist the very small plants at this early stage. For Alnus and Myrica a drop of inoculum prepared by grinding field nodules in water with a little sand

added (10 gm. nodules with 100 ml. water for Alnus, 6 gm. with Myrica) was applied to the root system of each plant, a further 2 ml. of the inoculum being added to the culture solution of each jar. This procedure was repeated two days later. In the case of Ulex the procedure was similar except that the inoculum was prepared by suspending in water four effective strains of gorse rhizobia which had been isolated by MacConnell (1956) and maintained in pure culture on agar slants in the laboratory. The plants in a small number of jars were left uninoculated; these remained without nodules, neither did they receive any deliberate addition of combined nitrogen except that noted above. These plants showed negligible growth, and when harvested at the same time as the nodulated plants they showed a nitrogen content of 0.3 mg. (Alnus), 0.2 mg. (Ulex) or 0.1 mg. (Myrica) per plant confirming that no unsuspected sources of combined nitrogen were available to the plants of the present investigation.

Preparation and application of labelled ammonium-nitrogen

This was prepared in two separate lots, one of which was used for addition to the Alnus and Myrica plants, the other for addition to the Ulex plants. The procedure employed was similar in both instances. An appropriate quantity of ammonium-nitrate with 34 atom per cent excess ^{15}N in the ammonium radical was made alkaline and the ammonia distilled over into dilute sulphuric acid. After neutralisation of the excess acid, ordinary ammonium sulphate was added in amount calculated to reduce the excess ^{15}N to approximately 1.5 - 2.5 atom per cent. The actual excess as determined by subsequent

spectrometric assay proved to be 1.561 atom per cent for the Alnus-Myrica stock and 2.631 atom per cent for the Ulex stock. The ammonium-nitrogen was brought into the form of a stock solution containing 20 mg. nitrogen per ml. by dilution with distilled water.

The application of the labelled ammonium-nitrogen to the plants was commenced 7 weeks (Alnus), 5 weeks (Myrica) and 4 weeks (Ulex) after inoculation. The formation of nodules had naturally begun by this time but the plants were still very small, the height of the shoot being 2 to 3 cm., the dry weight (as determined on spare plants) of the order of 20 mg. per plant, and the mean nitrogen content 0.3 mg. (Alnus), 0.8 mg. (Myrica) or 0.2 mg. (Ulex) per plant. Four levels of ammonium-nitrogen were established, namely 0, 10, 50 and 100 mg. nitrogen per litre of culture solution. Two jars, each with six plants, were set up at each nitrogen level for each species.

Subsequent treatment

The plants were grown for 10 to 12 weeks after commencement of treatment with the labelled nitrogen. After some two weeks the number of plants per jar was reduced from six to three in order to prevent eventual overcrowding, plants of average size being retained. Daily checks of the pH of the culture solution were made, and appropriate additions of NaOH made where necessary to restore the pH to the original level. The exact amount of NaOH to be added was determined by

titrating small quantities of solution from each jar with standardised N/100 sulphuric acid. The level of solution in the jars was restored daily by addition of distilled water; in the later stages of growth water uptake was considerable, with the result that those nodules situated on the upper parts of the roots were not constantly bathed by solution.

Complete renewals of the culture solution in the jars were made on three occasions in the case of Alnus and Myrica and on five occasions in the case of Ulex during the period of treatment with labelled nitrogen, the strength of the Crone's solution being increased to one third of full-strength at the first renewal with Myrica. The levels of ammonium-nitrogen were restored to their original value at each renewal. In partial compensation for uptake by the plants, on a few occasions further additions of ammonium-nitrogen were made during the periods between dates of renewal, the magnitude of these being decided on the basis of estimations by a distillation method of residual ammonium-nitrogen in samples of the culture solution. The harvest data (later) will provide further information on this aspect.

Harvest and analysis

At harvest the nodules were separated from each plant, counted, and their dry weight and also that of the combined root and shoot determined. 'Nodule' in this thesis refers in the case of the non-legumes to the branched, clustered structure which eventually arises from an original infection point. The dry matter from the plants of each jar, including the nodules,

was then combined and milled. Triplicate samples of this were taken for Kjeldahl analyses, allowing the calculation of total nitrogen per plant. In the case of Alnus and Myrica the neutralised distillates from the replicate Kjeldahl analyses were combined, re-acidified and evaporated down to a small volume, a sample of the concentrate being then sent to Dr. R.H. Burris, Department of Biochemistry, University of Wisconsin, for mass spectrometer assay. With Ulex the procedure was slightly different in that the concentrates of the two jars at each ammonium level were bulked before mass spectrometer analyses.

D A T A O B T A I N E D

ALNUS GLUTINOSA

Seedlings from seed sown on 14th March were transferred to water culture on 18th April and first inoculated two days later. Treatment with labelled nitrogen was commenced on 8th June and continued for some ten weeks, harvest being on 17th August. Six plants, in two jars, were available for harvest at each level of ammonium-nitrogen.

All plants were of healthy appearance as indicated by Plate 1. Typical root systems from each nitrogen level are shown in Plate 2. Growth and dry weight data are presented in Table 5, with statistical treatment (based on analyses of variance) appended. The figures for whole plant dry weight (column 7) and further data given in columns (2), (3) and (4) show that the provision of ammonium-nitrogen at initial levels of 10 mg. and 50 mg. per litre resulted in successive large increases in growth, confirming that nodule action alone was incapable of satisfying the full nitrogen requirements of the plants. The dry weight with 100 mg. ammonium-nitrogen initially provided is not, however, significantly greater than at the 50 mg. level, and it is fair to conclude that probably at both these levels, and certainly at the higher one, the nitrogen requirements of the plants were fully satisfied under the prevailing

conditions. In agreement with previous Glasgow experience with Alnus the greater growth of plants receiving 10 mg. or 50 mg. combined nitrogen per litre was accompanied by increased absolute weight of nodules per plant (column 6). Taking into account the data for number of nodules (column 5) it is clear that nodules tended to be larger in the presence of ammonium-nitrogen. The additional formation of nodule tissues did not, however, keep pace with the increase in plant growth, so that in the relative sense (column 8) nodule development was continuously depressed.

Mean nitrogen data are provided in Table 6 with statistical and other information appended. As already explained, separate assays of ^{15}N content (column 4) were made on the plant nitrogen of the two jars of plants at each ammonium level. Agreement within each pair of figures was close, the maximum deviation of a single result from the mean being 8 per cent of the latter. An example will indicate the method by which the values in columns (5), (6) and (7) were calculated. In one of the jars of plants at the '10 mg.' level of ammonium-nitrogen, the excess ^{15}N in the plant nitrogen was 0.383 atom per cent as compared with 1.561 atom per cent present in the supplied ammonium-nitrogen (p. 21). The mean total nitrogen per plant in this jar being 123 mg. it follows that the amount of ammonium-nitrogen taken up during growth was 0.383/1.561 x 123, or 30 mg. per plant. By difference it is learned that the fixation of atmospheric nitrogen was 93 mg. per plant. It is important that the

figure for total nitrogen per plant (column 3) used in the calculation should represent the nitrogen accumulated by the plants during the period of treatment with labelled nitrogen. This requirement is adequately satisfied, since as noted on p. 21 the nitrogen content at the commencement of the ^{15}N treatment was less than 1 mg. per plant. The assay figure for the zero nitrogen plants is not significantly different from the normal value.

The data in column (5) together with information appended to the Table show that at the '10 mg.' level a large proportion of the supplied ammonium-nitrogen was eventually absorbed by the plants. The data in column (6) show that fixation per plant was significantly enhanced in the '10 mg.' plants as compared with those at zero nitrogen, but was depressed in the presence of larger amounts of ammonium-nitrogen. Nitrogen fixed as a percentage of total uptake of nitrogen by the plants (column 7) fell continuously as ammonium-nitrogen was supplied in greater amount, but it is notable that at the '100 mg.' level about one quarter of the over-all uptake of nitrogen was still due to fixation, despite the presence of a considerable amount of unabsorbed ammonium-nitrogen in the rooting medium.

Plate 1

Typical Alnus plants shortly before harvest. From left to right the jars contained 0, 10, 50 and 100 mg. ammonium-nitrogen per litre.

(x 1/11)

Plate 2

The root systems of typical Alnus plants from each nitrogen level shortly before harvest. From left to right the nitrogen levels employed were 0, 10, 50 and 100 mg. nitrogen per litre

(x 1/3)

TABLE 5

Growth and dry weight data for Alnus glutinosa

(1) Mg. NH_4-N added per litre of culture solution	(2) Mean height of shoot (cm.)	(3) Mean number of side shoots per plant	(4) Mean number of leaves per plant	(5)* Mean number of nodules per plant	(6)* Mean dry wt. of nodules per plant (mg.)	(7)* Mean dry wt. of whole plant (mg.)	(8)* Mean value for nodule wt. as %age of whole plant wt.
0	34	2	19	64	144	3123	4.6
10	52	7	38	69	243	6677	3.6
50	52	10	62	32	230	9257	2.5
100	56	11	62	25	158	10634	1.5

* Appropriate statistical treatment of the original data indicates that the differences between means necessary for significance at $P = 0.05$ are as follows:- Column (5), 30; Column (6), 75 mg.; Column (7), 2,024 mg.; Column (8), 0.9 per cent.

TABLE 6

Nitrogen data for Alnus glutinosa ⊕

(1) Mg. NH_4-N added per litre of culture solution Ø	(2) Mean per cent total N in plant dry matter	(3)* Mean total N per plant (mg.)	(4)* Mean excess ^{15}N in plant N, atom per cent	(5)* Mean mg. NH_4-N absorbed per plant	(6)* Mean mg. atmos-pheric N fixed per plant	(7)* N fixed as %age of total uptake of N by plant
0	2.01	63	0.006	0	63	100
10	1.94	130	0.371	31	99	77
50	1.87	174	0.950	105	69	39
100	1.96	208	1.193	159	49	24

⊕ It should be noted that at each level of ammonium-nitrogen two independent values were available for columns (2) to (7), each referring to a jar of three plants. For simplicity only the means of these pairs of figures are shown in the Table. Calculations made from the mean figures may not yield exactly the same results as those based on the original, separate data.

Ø The ammount of ammonium-nitrogen supplied per plant during the period of the experiment was approximately as follows:-

'10 mg.' series..... 37 mg.
'50 mg.' series..... 127 mg.
'100 mg.' series..... 247 mg.

* Differences between means necessary for significance at $P = 0.05$ are as follows:- Column (3), 37 mg.; Column (4), 0.172 atom per cent; Column (5), 12 mg.; Column (6), 35 mg.; Column (7), 6 per cent.

M Y R I C A G A L E

Seedlings from a sowing of 17th February were transplanted into water culture on 20th April and first inoculated two days later. Treatment with labelled nitrogen was commenced on 29th May and continued for some eleven weeks until harvest on 12th August. Six plants, in two jars, were again available for harvest at each level of ammonium-nitrogen.

These plants were also of very healthy appearance. Examples of complete plants and of typical root systems are shown in Plates 3 and 4 respectively, while growth and dry weight data are provided in Table 7. Compared with some earlier Glasgow studies on Myrica (MacConnell and Bond,1957) growth was greatly improved, especially in respect of the plants in nitrogen-free solution, this being due chiefly to the use of diluted rather than full-strength Crone's solution (Gardner, 1958). There was, however, appreciable variation in size from plant to plant, with the result that the differences between means required for statistical significance are rather large. Thus although the various data suggest that the addition of ammonium-nitrogen increased growth, actually the changes in dry weight (column 7) fail to attain significance. The beneficial effect, if any, is however clearly smaller than in Alnus. The data also suggest that the '50 mg.' level of ammonium-nitrogen, together with such

fixation as occurred (below), did completely satisfy the nitrogen requirement. A comparison of columns (5) and (6) suggests that the nodules formed in the presence of ammonium-nitrogen tended to be larger but the differences between the various means do not attain statistical significance. The relative weight of nodules formed (column 8) was significantly depressed by ammonium-nitrogen.

The nitrogen data are provided in Table 8. Those directly dependent on absolute size of plant (columns 3, 5 and 6) show rather low statistical significance. It is probable that almost all the ammonium-nitrogen was eventually absorbed by the plants at the '10 mg.' level, with lower proportions absorbed at the higher levels than with Almus. Also, fixation per plant almost certainly fell as ammonium-nitrogen was increased. The figures for excess ^{15}N in the plant nitrogen of duplicate jars, on the other hand, again agreed satisfactorily, the maximum deviation of a single result from the corresponding mean being 9 per cent of the latter, indicating that at a given level of combined nitrogen plant size is not an important factor governing the relative importance of uptake of nitrogen in the ammonium form against that in the elemental form. Correspondingly the data in column (7) have good significance, and show a significant fall in the proportion of nitrogen fixed for each increase in ammonium-nitrogen supplied.

However, in the '100 mg.' plants, fixation still accounted for a third of the total uptake of nitrogen.

Plate 3

Typical Myrica plants shortly before harvest. From left to right the jars contained 0, 10, 50 and 100 mg. ammonium-nitrogen per litre.

(x 1/11)

Plate 4

The root systems of typical Myrica plants shortly before harvest. From left to right the nitrogen levels employed were 0, 50 and 100 mg. nitrogen per litre, the 10 mg. plants being similar to those at the 0 nitrogen level.

(x 1/3)

TABLE 7

Growth and dry weight data for Myrica gale

(1) Mg. NH_4-N added per litre of culture solution	(2) Mean height of shoot (cm.)	(3) Mean number of side shoots per plant	(4) Mean number of leaves per plant	(5) Mean number of nodules per plant	(6)* Mean dry wt. of nodules per plant (mg.)	(7)* Mean dry wt. of whole plant (mg.)	(8)* Mean value for nodule wt. as %age of whole plant wt.
0	40	5	60	243	197	3273	6.0
10	47	6	57	238	191	3884	4.9
50	50	6	102	195	163	5447	3.0
100	52	6	76	189	162	4587	3.5

* Difference between means necessary for significance at $P = 0.05$ are as follows:- Column (5), 98; Column (6), 90 mg.; Column (7), 2,533 mg.; Column (8), 1.5 per cent.

TABLE 8

Nitrogen data for Myrica gale ⊕

(1) Mg. NH_4-N added per litre of culture solution ø	(2) Mean per cent total N in plant dry matter	(3)* Mean total N per plant (mg.)	(4)* Mean excess ^{15}N in plant N, atom per cent	(5)* Mean mg. NH_4-N absorbed per plant	(6)* Mean mg. atmospheric N fixed per plant	(7)* N fixed as %age of total uptake of N by plant
0	2.59	84	0.004	0	84	100
10	2.64	101	0.496	32	69	68
50	2.80	150	0.867	82	69	45
100	2.62	121	1.041	81	40	33

⊕ The footnote in Table 6 referring to the method of calculation applies to the above Table.

ø The amount of ammonium-nitrogen supplied per plant during the period of the experiment was approximately as follows:-

'10 mg.' series..... 36 mg.
'50 mg.' series..... 135 mg.
'100 mg.' series..... 264 mg.

* Differences between means necessary for significance at P = 0.05 are as follows:- Column (3), 84 mg.; Column (4), 0.299 atom per cent; Column (5), 38 mg.; Column (6), 55 mg.; Column (7), 11 per cent.

ULEX EUROPAEUS

Seedlings from a sowing of 27th March, 1959 were transplanted into water culture on 6th April. Inoculation was first carried out four days later, and differential nitrogen treatment established on 7th May. Harvesting of the plants commenced sixteen weeks after transplanting (3rd August), there being six plants in two jars available for harvest at each ammonium-nitrogen level. Examples of plants from each level just before harvest are shown in Plate 5.

Over the greater part of the growth period these gorse plants were as healthy as those of alder and bog myrtle already considered, though it appeared that combined nitrogen had depressed nodulation to a greater extent than in the experiments of Bond and MacConnell. However, in the last two weeks of the present experiment a rather heavy growth of a fungal contaminant appeared in the high nitrogen (50 and 100 mg.) culture solution, especially in the vicinity of the roots and nodules, followed by a bleaching of some parts of the shoots, though this is not apparent in Plate 5. This occurrence, never experienced previously, was attributed to the unusually high greenhouse temperatures (95°F. and higher) resulting from the prevailing heat-wave; such temperatures, it was concluded, caused extreme oxygen depletion in the culture solution, leading to deterioration and decay in the

root tissues. The effect of this fungal contamination was obviously to reduce the growth of the plants concerned during the last stage of the experiment, some loss of dry matter from roots and nodules being a possible further effect, but it was felt that the isotopic composition of the plants would not be greatly affected. The results of this experiment must be considered as somewhat tentative but do afford a basis of comparison with the results obtained for the non-legumes.

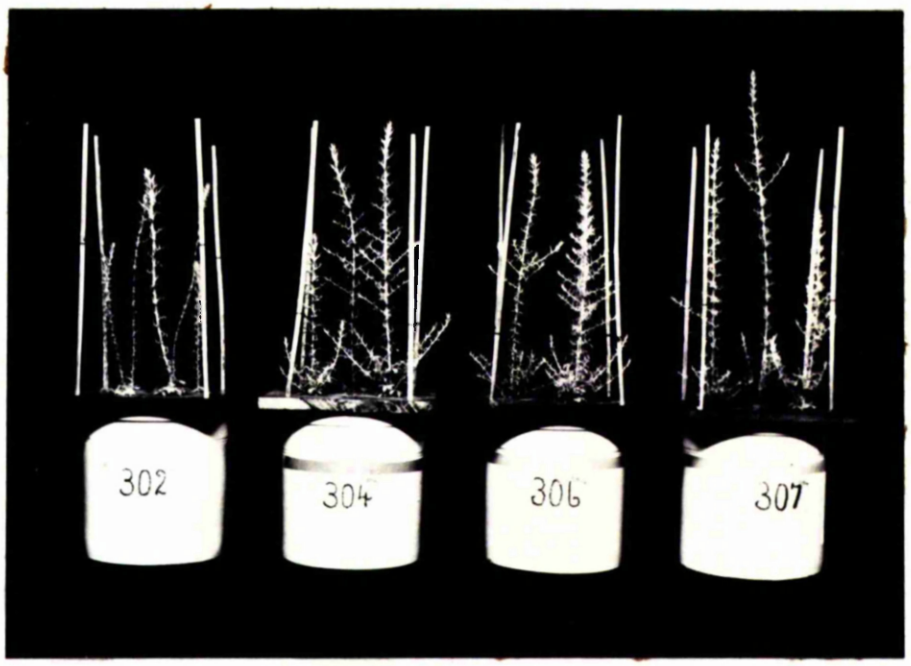
Data for total plant dry weight which are presented in Table 9 (column 6) indicate clearly that plant growth is significantly improved in the presence of supplied ammonium-nitrogen; there is however, no significant difference in total dry weight over the range 10 - 100 mg. combined nitrogen. It would thus appear that in Ulex the nitrogen requirements of the plant are fully satisfied by the presence of 10 mg. combined nitrogen per litre. On the other hand mean nodule dry weight (column 5) fell significantly with increase in combined nitrogen. In addition to a fall in nodule dry weight a significant decrease in nodule number occurred in the presence of combined nitrogen although over the range 10 to 100 mg. nitrogen there was no significant difference.

Mean nitrogen data are presented in Table 10 along with a summary of statistical analyses. In this experiment prior to assaying for ^{15}N the plants from each nitrogen level were combined and a single ^{15}N assay carried out on each. The figure for excess ^{15}N in the zero nitrogen plants suggests that very

slight contamination of these plants with labelled nitrogen must have occurred at some stage. Data on mean total nitrogen fixed show clearly that a marked decrease occurs in the presence of combined nitrogen. Furthermore mean mg. nitrogen fixed was lower in the presence of 100 mg. than in the presence of 10 or 50 mg. nitrogen.

Mean percentage nitrogen fixed likewise fell with increase in combined nitrogen, a reduction in the order of 80 per cent occurring in the presence of 10 or more mg. ammonium-nitrogen. Within the range 10 to 100 mg. nitrogen however there appears to be little variation in the mean percentage fixed. From the results of this particular experiment it can be seen that fixation in Ulex, although appreciable in the presence of quantities of combined nitrogen ranging from 10 - 100 mg. per litre is much less than that accomplished by Alnus and Myrica in the presence of similar quantities of combined nitrogen.

Plate 5



Typical *Ulex* plants shortly before harvest. From left to right the jars contained 0, 10 50 and 100 mg. ammonium-nitrogen per litre.

(x 1/10)

TABLE 9

Growth and dry weight data for Ulex europaeus

(1) Mg. NH_4-N added per litre of culture solution	(2) Mean height of shoot (cm.)	(3) Mean number of side shoots per plant	(4)* Mean number of nodules per plant	(5)* Mean dry wt. of nodules per plant (mg.)	(6)* Mean dry wt. of whole plant (mg.)	(7)* Mean value for nodule wt. as %age of whole plant wt.
0	23	1	126	19	361	5.3
10	30	9	73	10	1059	0.99
50	29	8	37	6	1016	0.58
100	34	8	24	3	1068	0.28

* Appropriate statistical treatment of the original data indicates that the differences between means necessary for significance at $P = 0.05$ are as follows:- Column (4), 68; Column (5), 5.0 mg.; Column (6), 300 mg.; Column (7), 0.9 per cent.

TABLE 10

Nitrogen data for Ulex europaeus

(1) Mg. NH_4-N added per litre of culture solution	(2) Mean per cent total N in plant dry matter	(3)* Mean total N per plant (mg.)	(4) Mean excess ^{15}N in plant N, atom per cent	(5) Mean mg. NH_4-N absorbed per plant	(6) Mean mg. atmospheric N fixed per plant	(7) N fixed as %age of total uptake of N by plant
0	1.88	6.8	0.026	0.01	6.7	99
10	1.59	16.8	2.101	13.4	3.5	20
50	1.84	19.2	2.121	15.5	3.8	20
100	1.59	17.0	2.201	14.2	2.8	16

The amount of ammonium-nitrogen supplied per plant during the period of the experiment was approximately as follows:-

'10 mg.' series.... 43 mg.
'50 mg.' series.... 103 mg.
'100 mg.' series.... 237 mg.

* Difference between means necessary for significance at $P = 0.05$ is 4.81 mg.

D I S C U S S I O N

It has been borne in mind that it was not possible to maintain steady levels of ammonium-nitrogen in the rooting medium; thus it is clear from the data furnished that in the non-legumes the culture solution in the '10 mg.' series must have been at times almost devoid of combined nitrogen, especially in the later stages of the growth period. In the '50 mg.' series there was almost certainly some ammonium-nitrogen present at all stages, while at the '100 mg.' level there was a considerable quantity. Obviously the mean ammonium-levels during the growth period were substantially below those provided initially and at each occasion of replenishment, but the data indicate that with each species at least the highest level more than satisfied the requirements of the plants.

As shown, marked nodule formation occurred in Alnus, Myrica and Ulex in the presence of various levels of ammonium-nitrogen in the rooting medium. In Alnus and Myrica as the ammonium level was increased, nodules eventually became fewer but larger, the weight per plant in the presence of an excess of ammonium-nitrogen being still of the same order as in zero nitrogen. In Ulex a similar tendency for decrease in nodule number occurred, but due to the adverse effect of fungal

contamination on nodule dry weight the reliability of the data on this latter attribute is uncertain. It is true that in this particular study, in order to reduce the consumption of labelled nitrogen, the first application of ammonium-nitrogen was delayed until nodule formation had actually started, but as already emphasised the plants were still extremely small, and it is obvious that practically all growth of plants and nodules occurred subsequently to the commencement of the ammonium treatment. The above data on Alnus agree with previous experience (Bond, Fletcher and Ferguson, 1954; Bond and MacConnell, 1957). The new data on Myrica (though somewhat tentative), are to be preferred to earlier ones since plant growth was so much improved. A fuller study of the effect of combined nitrogen on nodulation in Myrica will be reported in Part II of the present Section.

The object of the present study was to find how the role of the nodules forming in the presence of combined nitrogen is affected as compared with those on plants in nitrogen-free solution. They are obviously of reduced importance to the plant, which now bases its nitrogen metabolism to an increasing extent on the supplied ammonium-nitrogen. Moreover, especially in Alnus and Ulex, the plant grows more strongly and acquires a higher nitrogen content than in nitrogen-free solution, so that the relative significance of a given contribution from the nodules is steadily diminished. The data have shown that the nodules

continue to fix atmospheric nitrogen. Whether they fix it as efficiently as in nitrogen-free solution is indicated when, from data already provided for the three species, the mg. nitrogen fixed per gm. nodule dry matter is calculated for each treatment. Owing to the unreliability of the data on mean nodule dry weight, the Ulex data at the 50 and 100 mg. levels are not considered. The results are as follows:-

Mg. NH_4-H added per litre of culture solution

	0	10	50	100
Alnus	438	410	315	310
Myrica	429	366	414	247
Ulex	358	350	-	-

The difference required for significance between these values are 101 and 112 for Alnus and Myrica respectively. No data on significant differences are available for Ulex as the concentrates of each nitrogen level were bulked before assaying Thus there is evidence of a certain reduction in nodule efficiency, and this reduction could conceivably be due to a curtailment of metabolic supplies to the nodules, e.g. of acceptor substances for the fixed nitrogen, resulting from the more vigorous nitrogen metabolism of the plant itself. Another possible reason is that there is a partial substitution of ammonium-nitrogen for free nitrogen at the nitrogen-fixing centres in the nodule. This is an aspect seldom discussed even in connection with legume nodules. As noted already, in

nitrogen-fixing systems in general, ammonium-nitrogen is used preferentially over elemental nitrogen. There is no obvious reason for believing that this will not also be true of fixation in root nodules, and it is intended to test the matter by means of isotopic experiments with detached nodules or with homogenised nodules. The fact that fixation particularly in non-legumes, as shown by the figures given above, is not greatly reduced suggests that the ammonium-nitrogen in the rooting medium does not penetrate freely into the nodules. However, it may be noted that for structural reasons direct uptake of solutes from the rooting medium by these nodules is unlikely to be extensive (Bond, 1956). For this and other reasons the circumstance that in the later stages of the present experiments some nodules were not constantly bathed by culture solution (p.22) is not thought to be important. Finally the incomplete data on Ulex in the above Table indicate that in nitrogen-free solution the efficiency of legume and non-legume nodules is of the same order.

The differences in the effect of ammonium-nitrogen on the three species call for a few observations. The data show that the over-all growth of the plants was much more stimulated in Alnus and Ulex than in Myrica; also in Alnus there was at low levels of ammonium-nitrogen a substantially increased weight of nodules per plant, while associated with this was a greater fixation per plant. These effects are obviously due to the Alnus nodules in nitrogen-free solution

failing by a larger margin than those of Myrica to satisfy the plant's requirements for nitrogen and to permit maximum nodulation. This could be due to an intrinsic inferiority on the part of Alnus nodules. An alternative and more probable explanation is that the cultural conditions were in some unknown respect less optimal for Alnus nodule activity than for those of Myrica.

From the field aspect it is most important to consider in the light of the above results whether substantial fixation is likely to occur in Nature. Few data are however at present available on the mineral nitrogen contents of soils in which Alnus and Myrica grow. In general, peaty soils tend to have higher mineral nitrogen contents than other types, values as high as 350 and 500 parts per million being reported by Kaila et al. (1953) and Rheinwald (1953) respectively. The mean water contents of acid peat under birch-alder woodland, and in peat of a similar nature to where Myrica grows, have been reported by Dadd, Fowden and Pearsall (1953) as 415 and 470 per cent of the soil dry weight respectively. On the basis of the above figures it appears that the mineral nitrogen contents of soils in which Alnus and Myrica grow may possibly be of the order of 85 - 120 and 75 - 110 mg. mineral nitrogen per litre of soil solution. These tentative estimates suggest that the level of mineral nitrogen in the soil is unlikely to prevent substantial fixation in such nodules as

are present in Alnus and Myrica. Although fixation will then only account for part of the total nitrogen uptake by the plant, its magnitude per plant may be of the same order as in a soil destitute of combined nitrogen.

S U M M A R Y

1) Plants of *Alnus glutinosa*, *Myrica gale*, and *Ulex europaeus* grown in water culture with different levels of ammonium-nitrogen labelled with ^{15}N present in the culture solution showed substantial nodule formation, particularly in the case of the non-legumes. The nodules tended to be fewer than on plants in solution free of combined nitrogen and in *Alnus* and *Myrica* this was accompanied by an increase in nodule size.

2) The nodules continued to fix atmospheric nitrogen despite the presence of ammonium-nitrogen in the rooting medium though fixation per unit weight of nodule tissues formed was somewhat lower than in nitrogen-free solution. Among other possible reasons this could have been due to a substitution of ammonium- for elemental nitrogen at the nitrogen fixing centres of the nodule, but evidently this does not occur to any great extent.

3) In *Alnus* but not in *Myrica* fixation per plant was considerably enhanced in the presence of a low level of ammonium-nitrogen, owing to a greater nodule development. At higher ammonium levels, in excess of the plants' requirements, fixation per plant was still of comparable order to that in nitrogen-free solution in the case of *Alnus* and *Myrica*, but now only represented some 24 to 45 per cent

of the total nitrogen accumulated by the plants. In Ulex fixation per plant was decreased with increase in combined nitrogen, the fixed nitrogen accounting for 16 - 20 per cent of the total nitrogen accumulated.

4) The results suggest that under field conditions some fixation of atmospheric nitrogen will always be associated with nodules present.

P A R T II

The effect of added ammonium-nitrogen on nodulation in *Myrica gale*.

I N T R O D U C T I O N

Although the typical presence of nodules on the roots of Myrica gale has been recognised since late in the 19th century (Brunchorst, 1886-87) little attention was paid to the physiology of these nodules until the work of Bond (1949, 1951) who showed unequivocally that nitrogen fixation is associated with the nodules.

Much investigation into the effect of the presence of combined nitrogen in the rooting medium on nodulation has been undertaken in the legumes, as this is an aspect of considerable agricultural importance. For comparative reasons similar experiments have been carried out with certain non-legumes. From the review of the literature presented in Part I of this Section, it can be seen that although there are satisfactory data on the effect of combined nitrogen on nodulation in the non-legumes Alnus glutinosa and Hippophaë rhamnoides, in Myrica gale the picture is less complete. This has been due mainly to the difficulty that in earlier experiments with the last species in Glasgow University, growth of the plants was variable, some growing strongly, others rather feebly, while a proportion usually died.

In the earliest experiment on the effect of combined nitrogen on nodulation in Myrica (Bond, Fletcher and Ferguson,

1954) no attempt to obtain dry weight data was made because of the small number of plants available, but it appeared that the effect of combined nitrogen on nodulation in Myrica was similar to that in Alnus, where nodulation was depressed by the presence of high quantities of combined nitrogen. This received confirmation in the experiments of MacConnell and Bond (1957) where dry weight data were obtained, though the plants harvested were again small in number and size.

Since those early experiments however, the inability to obtain vigorous growth of Myrica has been overcome, Gardner (1958) showing that the plants grew very much more vigorously if quarter-strength rather than full-strength Crone's solution was employed.

As a result it was thought desirable to set up the experiment now to be described, with the following aims in mind:-

1) To investigate the effect of ammonium-nitrogen on growth and nodulation in Myrica gale now that much improved cultural conditions were available, a larger number of plants to be included than was possible in Part I of this Section where labelled ammonium-nitrogen was employed.

2) To compare the results of the present experiment, in which nodulation was initiated before the start of the differential nitrogen treatments, with those of MacConnell and Bond (1957) in which differential nitrogen treatments were set up at the time of inoculation.

M E T H O D S

Raising of nodulated Myrica plants

In this experiment the procedure employed was in many respects similar to that described in the *Myrica* experiments of Part I of this Section. Seed which had been collected at Stockiemuir, Stirlingshire, in October, 1959, was sown on 16th March, 1960, in trays of horticultural peat moistened with tap water, after having been stored for eight weeks prior to this at 2°C

On 29th April the seedlings, which at this stage were showing the first true leaf, were transplanted into water culture in 2½-litre jars containing quarter-strength Crone's solution adjusted to pH 5.4. A single addition of 5 mg. ammonium-nitrogen was made at this early stage to assist the plants over the period of nodule development. Inoculation of the seedlings was carried out on 6th and 8th May as detailed previously, the inoculum being prepared from 6 gm. fresh weight of nodules per 100 ml. of distilled water.

Establishment of differential nitrogen levels

On 18th June, 1959, the five nitrogen levels 0, 10, 50, 100 and 150 mg. per litre were established, there being three jars, each of which contained six plants, at each level. As in the previous experiments the nitrogen was dispensed as

ammonium-nitrogen from a stock solution of ammonium sulphate which contained 20 mg. nitrogen per ml. In this experiment the stock solution was not enriched with ^{15}N.

Subsequent management of cultures

During the ensuing ten-week growth period the culture solutions and nitrogen levels were renewed at fourteen-day intervals and on the basis of the nitrogen estimates carried out on the culture solutions in the ^{15}N experiment an additional 5 mg. combined nitrogen per litre was added per week to help keep the 10 mg. nitrogen level near to the original. As renewal of the culture solution was carried out more frequently in this experiment than in the ^{15}N experiment, the nitrogen levels were therefore maintained nearer to the original. A tendency for decrease in pH to occur, particularly in the high nitrogen levels, was corrected daily by the addition of calculated amounts of 2N sodium hydroxide.

Increase in the size of the plants necessitated a reduction from six to three per jar on 30th July, two extra jars each of which contained three plants being set up at each nitrogen level to accommodate this reduction. Increase in the culture solution from quarter-strength to third-strength was also effected on this date.

To compensate for any variation in conditions in the greenhouse the jars were changed round in systematic manner at weekly intervals.

Harvesting of the plants

Harvesting of the plants was begun on 20th August and completed by 24th August. Dry weight data on root plus shoot and on nodules were obtained by overnight drying in a 95°C oven. Counts on nodule number, as indicated by the number of infection points, were also carried out.

R E S U L T S

By the start of the differential nitrogen treatments nodulation had commenced, while the shoots of the plants had reached a height of 4 - 5 cms. As the differential treatment proceeded it became evident that the plants supplied with combined nitrogen were showing more rapid growth than those in a nitrogen-free medium, and that the nodules particularly in the combined nitrogen pots were clothed with a very prominent mass of large white upwardly-growing nodule roots. The root-systems of typical plants at harvest are shown in Plate 6, and harvest data are presented in Table 11 along with a summary of statistical analyses.

Data on total plant weight indicate clearly that the plants growing in the presence of combined nitrogen were significantly larger than those in a nitrogen-free medium and although there was no significant difference in total plant weight at nitrogen levels ranging from 10 - 100 mg. it is noticeable that mean shoot height, mean number of side shoots and mean leaf number were greatest at the 50 mg. nitrogen level. From the data on mean nodule dry weight per plant there is a strong suggestion that nodule dry weight was greater in the presence of 10 mg. nitrogen than in a nitrogen-free solution, although the difference

observed just fails to attain significance. At higher levels of combined nitrogen mean nodule dry weight per plant was depressed although only at the 150 mg. level was it significantly lower than at the zero nitrogen level.

Nodule number on the other hand showed a significant decrease with each increase in combined nitrogen from 10 - 100 mg. Between 100 and 150 mg. nitrogen the decrease although not significant was very nearly so. Nodule dry weight as a percentage of total plant weight decreased with increasing combined nitrogen up to the 150 mg. level. Above 50 mg. the decrease is less marked so that although the decrease between the 50 and 150 mg. nitrogen level was significant there was no significant difference between the 50 and 100, and the 100 and 150 mg. combined nitrogen levels. Data on mean dry weight per individual nodule indicate clearly that with increase in combined nitrogen the mean dry weight per nodule also increased, the increases being significant between the 0 and 50, and the 50 and 150 mg. nitrogen levels.

TABLE 11

The effect of added ammonium nitrogen on growth and nodule development of Myrica gale.

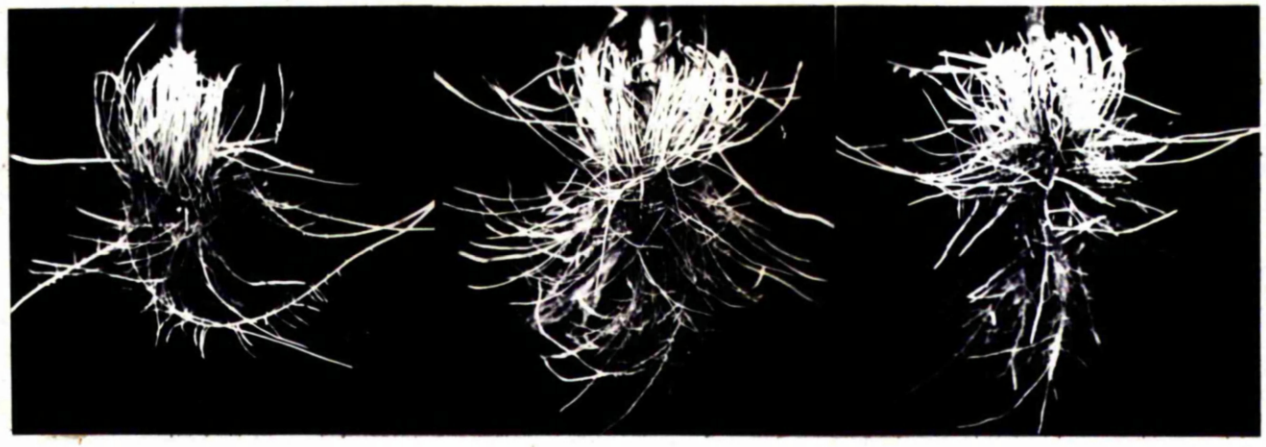
(1) ø Mg. N supplied per litre of culture solution	(2) Mean height of shoot (cm.)	(3) Mean No. of side shoots per plant	(4) Mean No. of leaves per plant	(5) + Mean No. of nodules per plant	(6) Mean dry wt. of root plus shoot per plant (mg.)	(7) +* Mean dry wt. of nodules per plant (mg.)	(8) +* Mean total dry wt. per plant (mg.)	(9) +* Mean nodule wt. as percentage of total plant wt.	(10) +* Mean dry wt. per nodule (mg.)
0	32	1	31	122	1173	84	1257	7.25	0.669
10	35	3	41	115	1979	106	2085	5.26	0.917
50	40	6	45	84	2361	97	2458	3.96	1.157
100	37	2	41	59	1947	78	2025	3.74	1.453
150	35	2	35	41	1687	50	1737	5.48	1.629

ø 15 plants harvested at each nitrogen treatment.

* The term "nodule" in this table does not include nodule roots.

+ Analyses of variance have shown that the differences required for significance ($P = 0.05$) are as follows:- Col.5, 19; Col.7, 24 mg.; Col.8, 470 mg.; Col.9, 0.409 per cent; Col.10, 0.316 mg.

Plate 6



The root systems of typical Myrica plants shortly before harvest. From left to right the nitrogen levels employed were 0, 50 and 100 mg. nitrogen per litre.

(x 1/3)

D I S C U S S I O N

In this study on the effect of combined nitrogen on nodulation much improved cultural techniques have enabled the production of plants which, though not quite as large as those obtained in Part I of the present Section, due to a later transplanting, are very much larger than those obtained by previous authors. Thus the mean dry weights per plant obtained by MacConnell and Bond (1957) at the 0 mg. and 50 mg. nitrogen levels are 33 and 561 mg. respectively while the comparable dry weights from the present study are 1257 and 2458 mg. respectively. Furthermore, in the present experiment a larger number of plants was available at each treatment. The data thus presented must be preferred to those previously available.

Total dry weight data show clearly that the plants growing in the presence of combined nitrogen were larger than those in a nitrogen-free medium. However, it is evident that the greatest mean total weight was obtained not in the highest nitrogen level but rather in the presence of some level near to 50 mg., the higher levels causing a decrease so that the plants growing in the presence of 150 mg. were significantly smaller than those at the 50 mg. nitrogen level. This finding agrees in general with the results of MacConnell and

Bond (1957) and with those described in Part I of this Section. It thus appears that the nitrogen requirements of well nodulated Myrica plants are, as previously stated, fully satisfied by the presence of approximately 50 mg. combined nitrogen per litre.

As noted already, the data obtained suggest strongly that nodule dry weight per plant is enhanced in the presence of the lower levels of combined nitrogen, a feature shown also in Alnus (Part I). The significant depression in mean nodule weight in the presence of 100 mg. combined nitrogen is similar to that detected in the Myrica experiment described in Part I of this Section. It is of interest to note that while the present results on nodule dry weight agree with those obtained in the Myrica ^{15}N experiment they differ from those of MacConnell and Bond loc. cit. who observed much better nodule growth in the presence of all nitrogen levels up to 100 mg. than they did in the 0 nitrogen level.

It thus appears that the effect of large quantities of combined nitrogen in depressing nodulation is typical of non-legume nodulated plants as similar results have been recorded for Alnus by Bond, Fletcher and Ferguson (1954), MacConnell and Bond (1957), and for Hippophae by Bond, MacConnell and MacCallum (1956). However, although a tendency for decrease does occur in all species so far examined, the amount of combined nitrogen necessary to cause this reduction varies considerably. In Hippophaë

for example Bond *et al.* (*loc. cit*) observed complete inhibition of nodules in the presence of 50 mg. nitrogen, while this is certainly not the case in *Alnus* and *Myrica*.

Increase in combined nitrogen resulted in the present experiment, in a significant decrease in nodule dry weight as a percentage of total plant weight with each increase in combined nitrogen from 0 - 50 mg., after which the decrease becomes less marked. This decrease is obviously due to the presence of combined nitrogen promoting root and shoot growth, while nodule growth is not correspondingly increased but is actually eventually decreased.

Apart from the studies on *Myrica* described in Part I of this Section, the only other attempt to estimate nodule numbers in a non-legume has been that of Quispel (1954, 1958) who counted nodule lobes in *Alnus* grown in the presence of different amounts of combined nitrogen. His data showed that the number of lobes per plant was decreased with increase in combined nitrogen. In the non-legumes each infection point produces a branched clustered structure which is equivalent to a single legume nodule; thus for comparative purposes it is more informative to count nodule clusters rather than individual lobes. The results of the present experiment show that as in legumes the number of nodules formed per plant decreases significantly with increase in combined nitrogen, although even in the presence of 150 mg. nitrogen a mean of 41 nodules per plant were formed. From such results on

nodule number it is further possible to calculate the mean dry weight per nodule, a feature on which there are no available data apart from those presented in the ^{15}N Myrica experiment. The results show that with increase in combined nitrogen the mean dry weight per nodule increases so that in the presence of 100 and 150 mg. nitrogen mean nodule weight is twice as great as in the zero nitrogen level. It thus appears that as in the legumes, increase in combined nitrogen results in an increase in dry weight of such nodules as form.

In the present experiment nodulation had commenced before the setting up of the differential nitrogen treatments but both nodules and total plant growth was very small at this stage, the mean shoot height being 4 - 5 cm. In the experiment of MacConnell and Bond (1957) with Myrica the nitrogen levels were established at the time of inoculation and this difference in procedure may account for certain of the differences observed in the two experiments. In the present experiment the plants grown in the absence of combined nitrogen would not be so severely handicapped, since they were already capable of fixing nitrogen at the time when the addition of combined nitrogen to the remaining cultures was commenced. As a result total plant growth in the absence of nitrogen should approach more nearly that of the combined nitrogen plants than in the experiment of MacConnell and Bond loc. cit., so also should mean nodule weight. The results show that this is in fact the case.

From the data obtained in this experiment it is clear that in the presence of combined nitrogen both mean nodule weight as a percentage of total plant weight and also nodule number are significantly decreased. Several theories have been advanced to account for comparable effects in legumes, the earlier ones being reviewed by Allison and Ludwig (1934). These authors also put forward the fresh theory that the presence of combined nitrogen reduced root formation as a whole, thus resulting in a reduction in nodulation also. It is a well known fact that in the presence of large quantities of combined nitrogen in the rooting medium a decrease in root growth does in fact occur. Thornton and Nicol (1936) however showed that in legumes although this reduction in root tissue may account in part for the decrease in nodulation, other factors must also be operative, their results showing clearly that the depressing effect of combined nitrogen is more marked on the nodules than on the roots.

More recently it has been suggested (Wilson, 1940) that the carbohydrate-nitrogen balance in the plant is the main factor affecting nodule development. According to this theory a large proportion of the carbohydrates formed in photosynthesis would be incorporated in the plant proper, into nitrogenous compounds in the presence of large quantities of supplied nitrogen, thus resulting in a reduction in the carbohydrates available for nodule development. Evidence in support of this theory was found in the results of several workers who showed

that nodule development although depressed by the presence of combined nitrogen in the medium was stimulated when a carbohydrate supply was added (Georgi, Orcutt and and Wilson, 1933; Ludwig and Allison, 1935). Supporting evidence along other lines has also been presented; for example Hopkins (1935) showed that by increasing the length of day nodulation was stimulated as a result of the increase in carbohydrates manufactured. Although this theory has been built up from evidence obtained with legumes there is no reason to suppose that it does not similarly apply to non-legumes, which, as has been shown, are physiologically very similar to the legumes.

From the data thus presented on the effect of combined nitrogen on nodulation and from the data on soil nitrogen presented in Part I of this Section it appears unlikely that nodulation of Myrica gale will be substantially reduced by the levels of mineral nitrogen likely to be present in Nature.

S U M M A R Y

1) The effect of combined nitrogen in the form of ammonium sulphate on nodulation in Myrica gale has been investigated using cultural methods which allowed the growth of much larger plants than has been previously possible, apart from those grown in Part I of the present Section.

2) The different nitrogen treatments were set up shortly after nodule initiation when both plants and nodules were very small.

3) The presence of combined nitrogen resulted in better growth of the plants compared with those grown in a nitrogen-free solution. Greatest plant dry weight however was not attained at the highest nitrogen level but at some level near to 50 mg. nitrogen. Although nodule dry weight compared with the zero nitrogen plants was significantly depressed only at the highest nitrogen level applied, nodule dry weight as a percentage of total dry weight decreased with increase in combined nitrogen.

4) The present experiment is the first large scale attempt to count nodule numbers (as denoted by infection points) and results show that increase in combined nitrogen results in a decrease in nodule number although mean dry weight per nodule increases.

P A R T III.

A quantitative study of fixation and transfer of nitrogen in first-year Alder plants.

I N T R O D U C T I O N

I N T R O D U C T I O N

Now that the occurrence of extensive fixation of nitrogen in various nodule-bearing non-legumes has been firmly established, it has become desirable from various standpoints that quantitative information regarding the fixation process should be secured. Particular aspects on which data are required include the stage in plant development at which fixation commences, and the extent of the fixation during later phases of development. Considerable interest is also attached to the activity of the nodule tissues in fixation relative to their weight or nitrogen content, at different stages in development and at different times of the year. Another important aspect is the transport of fixed nitrogen from the site of fixation (the nodules) to other parts of the plant, a process to which the term 'transfer' will be applied, for reasons to be explained later. It can be concluded, merely from the rather rapid growth of plants in nitrogen-free rooting medium once nodules have formed, that such transfer of fixed nitrogen does occur on a considerable scale, but without further study it cannot be ascertained how quickly the transfer follows on fixation, or how the two processes are quantitatively related.

Data on the above aspects, which in the present study have been secured in respect of alder plants during the first year of their development, are likely to throw new light on the symbiotic system presented by the nodulated plant. Thus, for example, data on the immediacy or otherwise with which transfer of nitrogen follows on fixation may permit tentative conclusions regarding the actual site of fixation within the infected cells of the nodules. They may also allow an assessment of the importance, as a means of conveyance of nitrogen, of the eventual degeneration of the endophyte in the nodule cells, a feature which has been detected by cytological students of non-legume nodules and which has frequently been attributed to a process of digestion. Data on nodule activity in fixation will permit interesting comparisons with legume nodules, and viewed in relation to the nitrogen requirements of the endophyte, may allow certain conclusions regarding the metabolic significance of the fixation in the life of the endophyte.

From a technical point of view, data on the intensity of fixation at different stages of plant development and at different times of the year are urgently required in connection with other studies on the non-legumes, for example, experiments in which fixation in detached nodules is studied by the ^{15}N technique. Such experiments are ideally carried out with nodule material showing maximum activity in fixation.

It will be of interest to review comparable studies

on legumes. The earliest major experiments on the rates of fixation and translocation of nitrogen in the legumes appear to be those of Bond (1936) who questioned the belief held by earlier workers (Prazmowski, 1890; Frank, 1891) that release of fixed nitrogen from the nodules occurred only when the ageing bacteria were digested by the nodule cells. In his experiments Bond grew large populations of soya bean plants in nitrogen-free sand culture under greenhouse conditions and sampled these populations at intervals. At each sampling dry weight and nitrogen data were obtained on root plus shoot, and on nodules. Mean total nitrogen fixed between each sampling was obtained by subtracting the nitrogen content of the complete plants at each harvest from the corresponding figure at the following harvest. The amount of nitrogen transferred from the nodules to the plant was calculated by subtracting the total nitrogen content of the plants less nodules at each sampling from the corresponding figure at the following sampling. The results which are reproduced in Table XII show that throughout the period from the opening of the second leaf until the development of the pods (October 4th) the percentage nitrogen transferred from nodules to remainder of plant ranged from 78 to 92 per cent of that fixed, after which it increased sharply to 350 per cent.

Wilson and Umbreit (1937) in further studies on the symbiosis in soya bean confirmed in the main the results of Bond, but in addition recognised three main phases in the

TABLE 12

Fixation of nitrogen and transfer of fixed nitrogen from nodules into plants during successive periods of development (1932 plants). The data refer to eight plants.

(Reproduced from Bond, 1936)

Period (days from sowing)	Fixation of N* (mg.)	Amount of N transferred from nodules to plants+ (mg.)	N transferred as percentage of N fixed	Efficiency (mg. N fixed per gm. dry wt. of nodules
35-43	31.9 (4.0)Ø	25.8 (3.2)Ø	80	27.6
43-49	27.6 (4.6)	21.6 (3.6)	78	18.4
49-63	98.4 (7.0)	81.8 (5.8)	83	15.4
63-70	60.5 (8.6)	46.9 (6.7)	78	12.0
70-84	154.7 (11.1)	136.4 (9.7)	87	10.3
84-99	192.0 (12.8)	162.6 (10.9)	85	7.8
99-108	156.0 (17.3)	138.5 (15.4)	89	8.1
108-125	184.7 (10.9)	171.5 (10.1)	92	4.5
125-141	6.5 (0.4)	22.1 (1.4)	350	0.2

* Calculated by subtracting the nitrogen content of eight plants and their nodules at each sampling from the corresponding figure at the following sampling.

\+ Calculated by subtracting the nitrogen content of eight plants, less nodules, at each sampling from the corresponding figure at the following sampling.

Ø The figure in brackets represent fixation (second column) or transfer (third column) per day, obtained by dividing the total fixation or transfer for the period by the number of days in the period.

growth of the plant. The first was a short initial stage in which a large proportion of the nitrogen fixed was retained by the nodules. This was followed by a long period of vigorous growth in which a rapid transfer of fixed nitrogen to the rest of the plant occurred. Finally, a phase occurred when nitrogen fixation was slowed down and in which transfer of nitrogen from the nodule to the plant was highest.

Virtanen and his collaborators (1947) showed that in several legumes the rate of fixation of nitrogen was paralleled by the development of haemoglobin in the nodules. Further studies along similar lines have since been carried out by Jordan and Garrard (1951) and Neumann (1952).

The most recent experiments on the development of the nodule symbiosis and on the rates of fixation and transfer of nitrogen in legume plants have been carried out by Pate (1958a, 1958b). In his first paper Pate recorded data obtained from studies on two strains of Pisum arvense. The plants were sown under field conditions in plots of low nitrogen content to which a nitrogen-free fertiliser was added prior to sowing. Inoculation of the seedlings was ensured by the addition of a seed-applied rhizobia supplement and random sampling of the plants was carried out at 3 - 7 day intervals. At each sampling it was necessary to harvest fifty plants owing to variation caused by the lack of controlled conditions. The data showed that fixation of nitrogen commenced twenty-two

days after sowing and continued for approximately forty days until the beginning of fruiting. Data on nitrogen fixed showed that per gm. of fresh nodules it increased during the growth phase from 7.0 mg. to a maximum of 65 mg. per day. Of the total nitrogen fixed it was observed that approximately 82 per cent was transferred from the nodules to the remainder of the plant at the twenty-two day stage, increasing to 100 per cent after approximately fifty-five days.

In his second paper Pate (1958b) reported that in Vicia sativa the development of the symbiotic system was very similar to that of P. arvense whether the species was grown as a summer or a winter annual. Although no results were available on the rate of transfer of fixed nitrogen it was observed that the nodules became most active in fixation just before flowering. It was also observed that when Vicia was grown as a winter annual fixation ceased temporarily during the cold weather of the months of December and early January.

In the non-legumes no large-scale experiment on the rates of fixation and transfer of fixed nitrogen has been carried out, and a few data only are available. In order to secure the desired data it is necessary to sample a population of nodulated plants growing in a nitrogen-free rooting medium periodically through their growth season, and determine the dry weight and total nitrogen content of the various parts of the plants. As indicated, this has now been done for a population of alder plants in the first year of their development.

M E T H O D S

Raising of nodulated alder plants.

The procedure followed in this experiment was essentially similar to that employed in the raising of alder plants in Part I. Alder seed which had been collected at Milngavie, Dunbartonshire in October, 1958, was used. On 4th March, 1959, 8 gm. of this seed which had been cleaned to remove scales and plant debris was sown approximately half an inch below the surface in vermiculite moistened with quarter-strength Crone's solution after having been stored prior to this for several months at +2°C. Experiments showed that germination in the case of cold-treated seed was 54 per cent better than that of seed stored throughout at room temperature.

Transplanting of seedlings was carried out at the two-leaf stage, thirty-one 2½-litre jars being set up. The seedlings were inserted in 1/4" thick rings of thick-walled rubber tubing, five of these being fitted into regularly spaced holes in five-inch square teak tops which covered each jar. The culture solution employed was full strength Crone's nitrogen-free solution. Nodule formation was initiated by inoculating seedlings on 23rd and 24th April, the method and amount of inoculum being exactly similar to that employed in

the alder experiment described in Part I. In order to assist the plants during the period of nodule formation an addition of 4 mg. combined nitrogen (as ammonium sulphate) per jar was made at this stage. Within fifteen days of inoculation developing nodules were visible as red swellings on the root surfaces.

Subsequent management of cultures.

During growth the plants were kept in a cool well-lit greenhouse and although it is certain that no substantial variation in conditions occurred along the length of the bench, the precaution was taken of changing the position of the jars in systematic manner at weekly intervals. Between fortnightly renewal of the solution no attempt was made to maintain the original pH level of 6.4 in the jars, for although a drop in pH did occur, at no time did it reach a critical level. Prior to the commencement of the periodic harvests any obviously atypical plants were weeded out, giving a starting total of 124 plants of approximately similar size and vigour at this stage. A reduction to three plants per jar was necessary at the twelve-week stage in order to prevent overcrowding, additional jars being set up to allow for this reduction. It may be noted that the construction of the teak tops permitted transplanting at a fairly late stage in growth.

Sampling and harvesting of plants.

As growth of the plants proceeded a certain variation in size became evident and to compensate for this the plants were classified prior to each sampling into the three

following groups:- large, medium and small. Plants in each group were temporarily distinguished by means of a loop of coloured wool round the base of each shoot. On most sampling occasions the numbers of plants in these three categories proved to be in the approximate ratio:- 1 large, 1 small, 8 medium. The sample of ten plants harvested was on each occasion made up by selecting plants at random from each category in that ratio. Sampling was begun on 9th June, 1959, the plants at this stage being well-nodulated and already fixing nitrogen as indicated by comparison with the control plants (see below). Subsequent samples were taken at twelve-day intervals.

At harvest, data on leaf number and length of main root and shoot were obtained, while separate dry weight determinations were made of nodules and of root plus shoot in respect of each plant. Dry weights were determined by overnight drying in a 95°C oven. The roots and shoots of the ten plants comprising each sample were then combined and ground up, similar treatment being accorded to the nodules. Replicate Kjeldahl analyses were then carried out on weighed samples of such ground material.

As noted, the culture solution and general procedure in this experiment were essentially identical with those of Part I, so that the control plants already referred to in Part I (p.20) serve also to show that in the present experiment the plants had no unintended access to combined nitrogen.

R E S U L T S

After the usual lag of period of 2 - 3 weeks, due to the fact that the nodules require time to become fully functional, the plants began to grow vigorously, and favoured by an unusually fine summer, continued in this condition until the approach of autumn and the termination of the experiment.

Data on plant growth and dry weight at successive harvests are presented in Table 13, typical plants being shown in Plate 7. As noted below the Table, 't' tests showed that the mean dry weights of root plus shoot at successive harvests were significantly different, except in the case of the last pair. The data show that although new leaf formation ceased after the seventh harvest, the plants continued to increase in dry matter well after this stage; during this later period there was a vigorous expansion of the younger leaves. Dry weights per plant at successive harvests are plotted in Figure 1, the expected sigmoid curve being obtained.

In Table 14 the percentage nitrogen contents of the root and shoot material and of the nodules are shown, and also the absolute nitrogen contents per plant calculated from the percentage data together with the dry weight data. A continuous fall is shown in both columns of percentage figures,

doubtless as a result of the accretion of products of photosynthesis outstripping that of the products of fixation. In Figure 2 is plotted the absolute nitrogen content of root and shoot at successive harvests and also that of the complete plant (derivable from the data in Tables 13 and 14), and it is clear from this figure that throughout the growth season the bulk of the nitrogen fixed was quickly transferred from the nodules to the remainder of the plant, a conclusion which is further substantiated below.

In Table 15 are presented data on the amount of nitrogen fixed per plant over successive twelve-day periods and on the amount and proportion transferred from the nodules to the rest of the plant. Fixation per plant attained a maximum of some 2.5 mg. per day during late August, a marked decrease occurring fairly soon thereafter. The transfer data show that at a very early stage, when fixation per plant per day was only of the order of 0.1 mg., already some 90 per cent was being currently transferred from the nodules to the rest of the plant. The proportion transferred tended to rise in the later stages, and the data suggest that ultimately transfer equalled or exceeded fixation.

The efficiency of the nodule tissues in fixation as measured by nitrogen fixed per unit weight of prevailing dry matter, are shown in Table 16. It is clear that young nodules are the most active in fixation. Efficiency subsequently falls somewhat, but then remains fairly steady during July and August, before falling sharply in September.

TABLE 13

Harvest and dry weight data at successive harvests

Harvest number	Date	Mean no. of leaves per plant	Mean height of shoot (cm.)	Mean dry wt. of root + shoot per plant *	Mean dry wt. of nodules per plant
1	9th June	7	6	50	3
2	21st June	9	9	112	8
3	3rd July	10	12	254	17
4	15th July	12	15	465	30
5	27th July	12	18	833	50
6	8th Aug.	17	26	1627	117
7	20th Aug.	24	35	3468	218
8	1st Sept.	24	33	6148	273
9	13th Sept.	24	39	8750	310
10	25th Sept.	23	34	10459	359
11	7th Oct.	22	34	11288	419

* To test the significance of the data, a 't' test was made on the data for dry weight of root + shoot, in respect of each pair of adjacent harvests. For the difference between means to be significant at $P = 0.05$, t should be at least 2.09. The actual values, in chronological order were: 3.0, 3.7, 8.5, 3.1, 3.2, 3.8, 4.0, 3.7, 2.2 and 1.3.

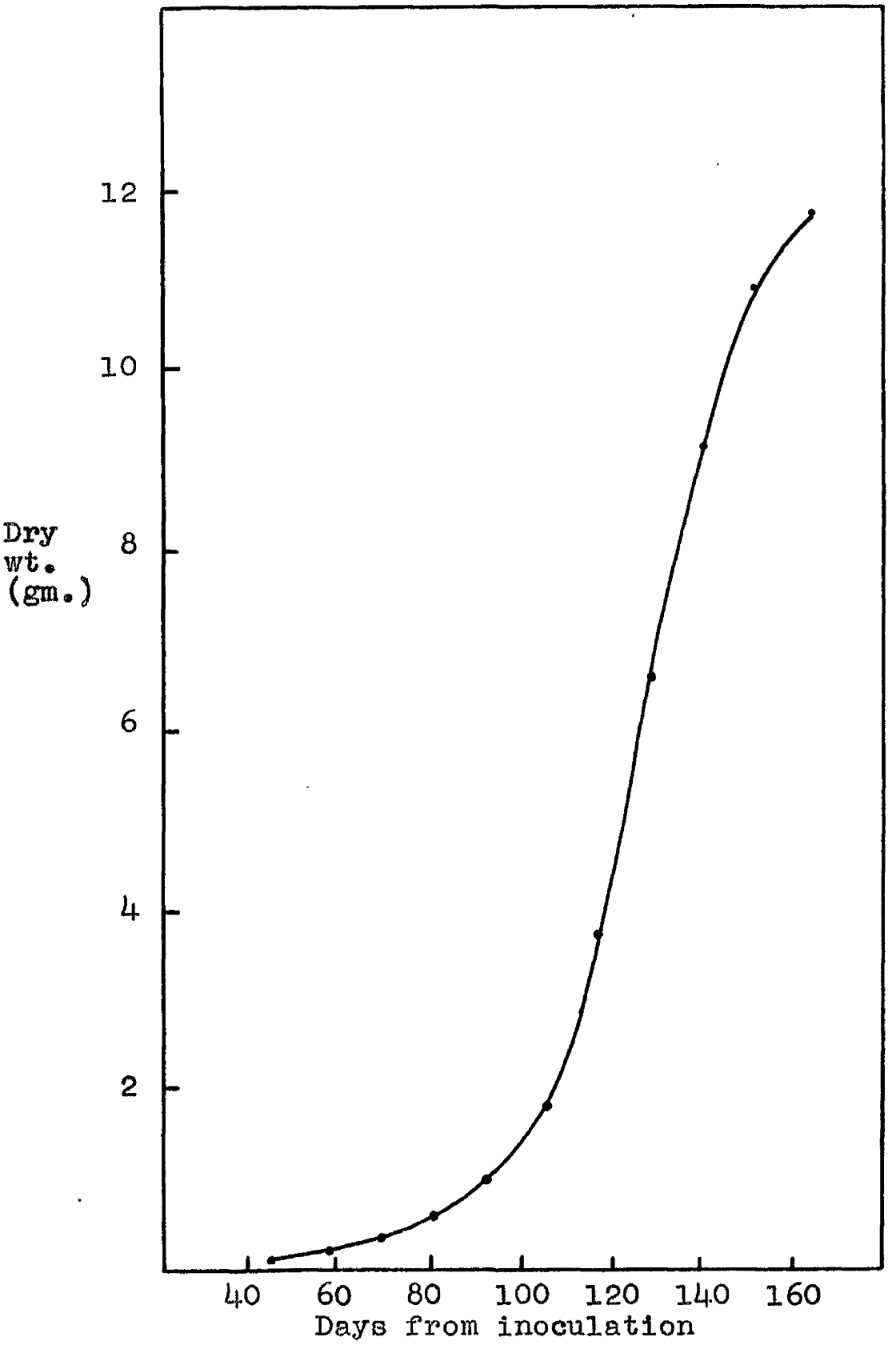
Plate 7

45 68 93 (x 1/4)

128 152 (x 1/5)

Typical Alnus plants at successive harvests. Figures below each jar of plants represent the number of days after inoculation.

Figure 1



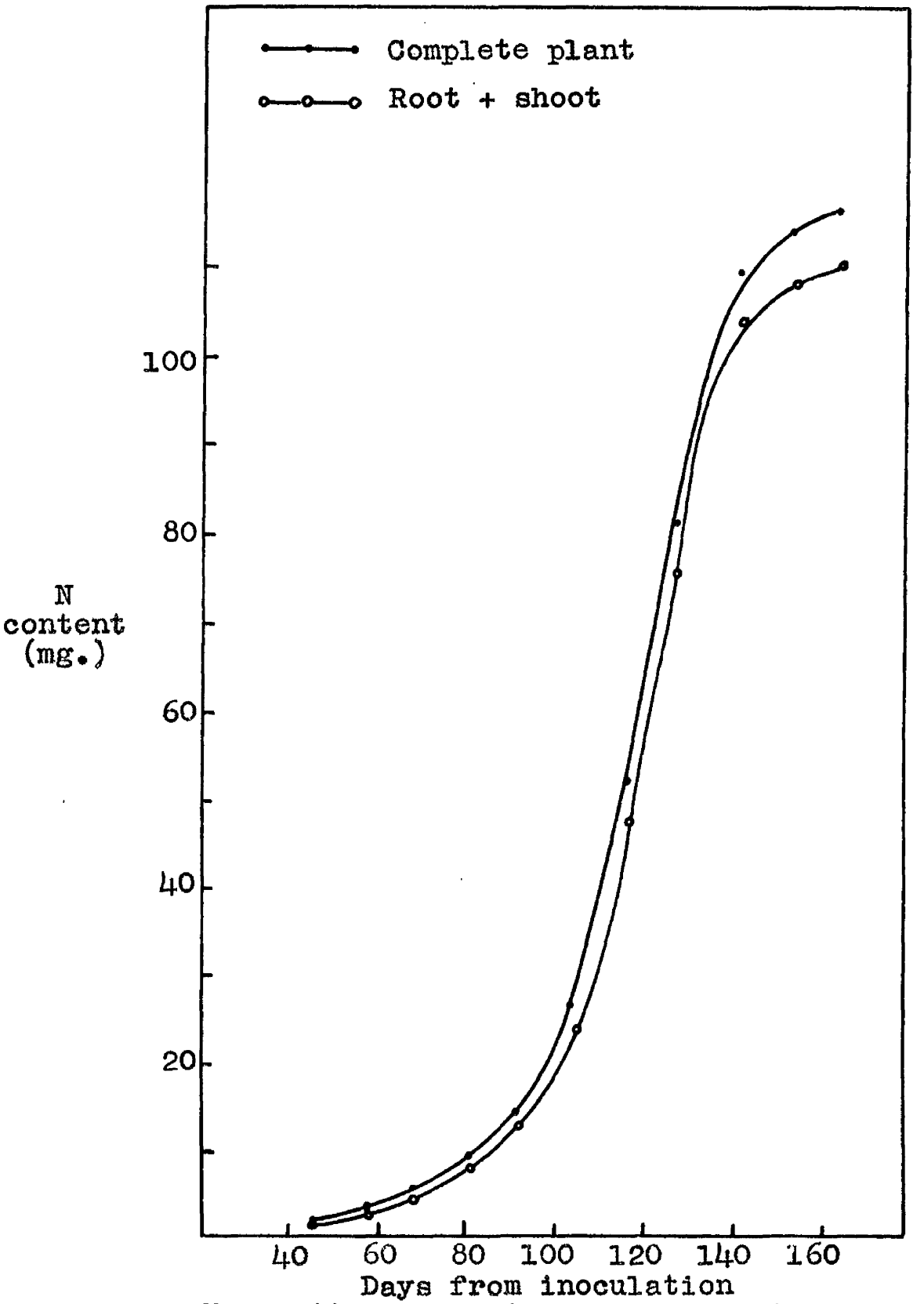
Mean total dry weight per Alder plant (including nodules) at successive harvests.

TABLE 14

Nitrogen data at successive harvests

Harvest number	Date	Mean %age N content of root + shoot	Mean %age N content of nodules	Mean N content of root + shoot per plant (mg.)	Mean N content of nodules per plant (mg.)
1	9th June	2.03	2.84	1.01	0.07
2	21st June	1.82	2.68	2.04	0.21
3	3rd July	1.68	2.50	4.27	0.43
4	15th July	1.59	2.38	7.39	0.71
5	27th July	1.52	2.31	12.66	1.16
6	8th Aug.	1.45	2.11	23.59	2.57
7	20th Aug.	1.32	1.99	46.12	4.33
8	1st Sept.	1.23	1.92	75.62	5.34
9	13th Sept.	1.11	1.85	103.04	5.64
10	25th Sept.	1.02	1.57	107.68	5.64
11	7th Oct.	0.97	1.32	109.49	5.53

Figure 2



Mean nitrogen content per complete Alder plant and per plant minus nodules at successive harvests

TABLE 15

Nitrogen fixed and nitrogen transferred from nodules to remainder of plants during successive periods of growth

Days after inoculation	Dates	Mean N fixed per plant (mg.) *	Mean N transferred from nodules to rest of plant (mg.) ø	N transferred as %age of N fixed
45-56	9 - 21 June	1.17	1.03	88
57-68	21 June-3 July	2.45	2.23	83
69-80	3 - 15 July	3.40	3.12	92
81-92	15 - 27 July	5.72	5.27	92
93-104	27 July-8 Aug.	12.34	10.93	89
105-116	8 - 20 Aug.	24.29	22.53	93
117-128	20 Aug-1 Sept.	30.51	29.50	96
129-140	1 - 13 Sept.	27.42	27.42	98
141-152	13 - 25 Sept.	4.64	4.64	100
153-164	25 Sept-7 Oct.	1.70	1.81	106

* Calculated by subtracting the mean nitrogen content per plant including the nodules, at each sampling, from the corresponding figure at the following sampling.

ø Calculated by subtracting the nitrogen content of each plant less nodules, at each sampling, from the corresponding figure at the following sampling.

TABLE 16

Efficiency of fixation by the nodules at successive periods of development

Days after inoculation	Dates	Mean dry wt. of nodules per plant (mg.)	Efficiency (mg. N fixed per day per gm. dry wt. of nodule tissue)	Efficiency (mg. N fixed per day per 100 mg. nodule N)
45-56	9 - 21 June	6	16.3	70
57-68	21 June-3 July	13	15.6	64
69-80	3 - 15 July	24	11.8	50
81-92	15 - 27 July	40	11.9	51
93-104	27 July-8 Aug.	84	12.3	55
105-116	8 - 20 Aug.	168	12.0	57
117-128	20 Aug-1 Sept.	246	10.3	51
129-140	1 - 13 Sept.	292	7.9	42
141-152	13 - 25 Sept.	335	1.2	7
153-164	25 Sept-7 Oct.	389	0.4	3

D I S C U S S I O N

In the first place it may be remarked that this experiment provides a further testimony of the ability of nodulated alder plants to grow luxuriantly without access to combined nitrogen, for in the space of not more than 100 days from nodule development the plants accumulated individually over 11,00 mgm. dry matter, while some 115 mgm. nitrogen was fixed by the nodules. It is hoped that this property of alder (and of the other nodulated non-legumes), so long thought to be unique to the legumes among Angiosperms, will become more widely appreciated.

As noted, the data (Table 15) show that the rate of fixation per plant rose steadily through June, July and August, attaining a maximum of some 2.5 mgm. per day towards the end of the latter month. A high rate was maintained during the first half of September, but was then followed by a sharp fall. The attainment of the maximum per plant at the time indicated, was doubtless because the continued increase in nodule weight per plant more than compensated for the tendency for metabolic activity in general to slow up in late August, on account of shortening days and falling light intensity. The depression of fixation later in September was obviously due to a drastic change in these factors. Some

other aspects of fixation are considered later.

As indicated in the Introduction, a central aspect of this study is the transfer of fixed nitrogen from the nodules to the rest of the plant. In this connection the term 'transfer' is retained mainly for historic reasons, since it was used by Bond (1936) in his study of the comparable movement of fixed nitrogen in legumes. This movement is probably effected by normal processes of translocation, and strictly speaking a distinctive or special term is unnecessary; it is however in the interests of brevity to speak of 'transfer' rather than 'translocation from the nodules to the rest of the plant'.

Although, as noted in the Introduction, it was already obvious from mere contemplation of the growth of the young alder plants in nitrogen-free solution that transfer of fixed nitrogen begins early and is on a considerable scale, it is only on the basis of data such as those now available that the full extent of the process can be seen. It is clear that from the very commencement of fixation some 90 per cent of the nitrogen fixed was transferred without delay from the nodules, even higher proportions being transferred in later stages of the experiment.

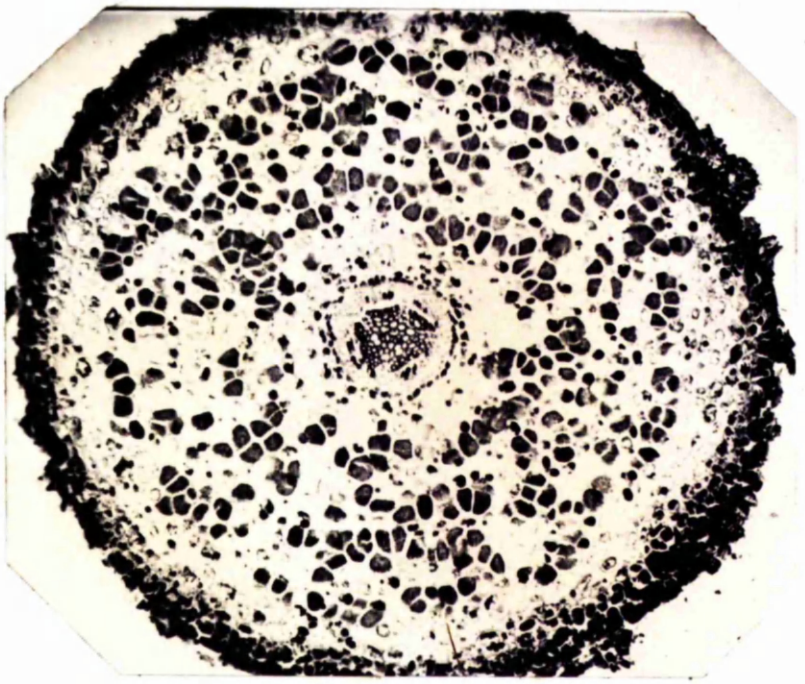
These findings focus attention on two questions, namely the precise site or locus of the fixation process, and the mechanism by which fixed nitrogen is conveyed from the endophyte to the host cells (see also below). In legumes it has tended to be assumed that fixation occurred within the

bacteroids, although a difficulty attendant on this view was that rhizobia isolated and grown in pure culture showed no ability to fix nitrogen. Taking such a view, the conveyance of fixed nitrogen from rhizobia to the nodule cells has to be attributed to a continual lysis of bacteria, for which there is no evidence, or to a process of excretion by the bacteria. However Bergerson, Wilson and Burris (1959) have presented different conclusions as to the site of fixation. Electron microscope study of sections of soya bean nodules showed the bacteroids to be enclosed in groups within membranes probably derived from the protoplasts of the host cells. The physical fractionation of nodules exposed to ^{15}N showed that while the bacteroid fraction remained unlabelled unless the exposure exceeded one hour, enrichment was shown after only ten minutes by acid-soluble material extracted from the fraction containing the above membraneous envelopes. Thus, the fixation appears to be an extra-cellular event so far as the bacteroids are concerned. Virtanen (1959), on general evidence, also believes that fixation in legume nodules occurs outside the bacteroids, the products being passed to the host plant without being used in protein synthesis by the bacteria.

There is at present no comparable evidence regarding the site of fixation in non-legume nodules. The endophytes have not yet been isolated into pure culture with certainty, let alone tested for nitrogen-fixing properties. To assume that here also fixation is extra-cellular to the endophyte assists in the understanding of the available data. Thus it

enables one to comprehend how transfer of fixed nitrogen can occur rapidly - within an hour or two of fixation (Bond, 1956), as is also implied by the present data. It also facilitates understanding of the fact that the scale of fixation is attuned to the growth requirements of the host plant rather than to those of the endophyte. This point is brought out more clearly when a comparison is made of the relation of fixation to growth in the case of the Alnus endophyte with that in a free-living nitrogen-fixing organism such as Azotobacter. In Alnus during the period of maximum fixation per plant (20th August - 1st September) calculations show that for every 100 mg. of new nodule dry matter formed during the period, there was fixed a total of 55 mg. nitrogen. It is reasonable to postulate that growth of the actual endophyte accounted at the most for 50 per cent of the new nodule dry matter, and on this basis it is concluded that the formation of 100 mg. of new endophyte dry matter was attended by the fixation of 110 mg. nitrogen. That the endophyte comprises 50 per cent of the nodule dry matter is almost certainly a considerable over-estimate, allowing for the contribution of tissues such as xylem and cork (Plate), so that the figure of 110 mg. is probably correspondingly too low. From the data of Lee and Burris (1943) on Azotobacter, it can be calculated that during the period of maximum fixation, for every 100 mg. of new Azotobacter cell dry matter produced, there was fixed 32 mg. nitrogen. These calculations show that, relative to its own growth requirements, the Alnus endophyte

Plate 8



Transverse section of a nodule lobe of Alnus.

(The unmodified stele lies in the centre and is surrounded by the enlarged cortex with dark stained infected cells. A peripheral cork layer is present.)

(x 55)

(Photograph by courtesy of Dr. G. Bond)

is indeed several times as active in fixation as is the free-living Azotobacter.

Reference was made in the Introduction to a degeneration of the endophyte in older parts of alder nodules, attributed by some cytological observers to digestive processes comparable with those appearing in mycorrhiza. If the view is taken, contrary to that adopted above, that fixation is intra-cellular, it might be suggested that the digestion is important in the movement of fixed nitrogen from endophyte to host. However, information provided by Pommer (1956) on the time-scale of these changes in the appearance of the endophyte in alder nodules indicate that little or no digestion could have occurred in the nodules of the plants of the present author's experiment, even by the end of the growth period; hence no importance can be attributed to this digestion in connection with the transfer of nitrogen.

Although the present study is concerned with the magnitude of the transfer rather than its route, it may be noted that Bond (1956) showed that the nitrogen fixed ascended the plant in the transpiration stream; the route of actual passage from the nodule to the xylem of the root is uncertain.

Turning now to the efficiency of the nodule tissues in fixation (Table 16) young nodules are clearly the most active relative to their dry weight and nitrogen content. With increasing age of the plant the efficiency falls somewhat but then remains fairly steady around 12 mg. per day per gm. of

nodule dry matter until the second week of August. The early reduction of efficiency may simply be due to the development or an increasing proportion of tissues such as cork and wood in the nodule. These tissues contribute nothing to fixation. The subsequent period of stable efficiency probably arises from the fact that, although the earliest formed parts of the nodules were continuing to decrease in efficiency the constant formation of new nodules and new nodule lobes compensated for that effect. The marked reduction in efficiency during September was doubtless due to reduced photosynthesis by the host plant. When these data for efficiency of alder nodules are compared with those for soya bean (Table 12), considerable similarity is apparent, indicating that weight for weight non-legume nodules will in Nature contribute as much to soil fertility as do those legumes.

Now that reliable data are available for the efficiency of nodules in their normal condition of attachment to the plant it is possible to appreciate more precisely the drastic effect which detachment or excision has on fixation. Isotopic tests in July and August by Bond (1957, 1960) with detached alder nodules indicated that in the twenty-four hours following detachment, total fixation amounted to 0.5 - 1.0 per cent of the total nodule nitrogen. Data in Table 16 show that over the same period fixation by normal attached nodules is of the order of 50 per cent of the total nodule nitrogen per twenty-four hours. The most likely explanation is that, just as

there is a constant export of fixed nitrogen from the nodules, there is also a constant import of metabolites, with no reserves of these within the nodules. Separation from the source of those metabolites is thus attended by a rapid curtailment of fixation.

In the nodulated Alnus plant therefore there is presented an efficient nitrogen-fixing symbiotic system. In such a system a cyclic state of affairs can be conceived in which rapid transfer of fixed nitrogen occurs from the nodules to satisfy the requirements of the root plus shoot; in the shoot this allows further growth, thus increasing the supply of carbohydrates, which in turn appears to control nodule efficiency in fixation.

S U M M A R Y

1) A study of nitrogen fixation and nitrogen transfer in first-year Alnus plants has been made by periodic sampling of a population of plants grown in water culture.

2) The results showed that the nodulated plants were capable of vigorous growth in the absence of combined nitrogen, a mean dry weight of approximately 11,000 mg. being accumulated per plant in 100 days.

3) Maximum fixation per plant occurred in late August but fell rapidly with the onset of autumn.

4) Data on nitrogen transfer show that throughout the growth season approximately 90 per cent of the nitrogen fixed is immediately transferred from the nodules to the remainder of the plant, a view which is incompatible with the suggestion that fixed nitrogen becomes available to the plant only when digestion of the endophyte occurs.

5) The immediacy of transfer of fixed nitrogen suggests that the site of actual fixation must be extracellular to the endophyte. Evidence has been put forward by other workers that this is the case in legume nodules.

6) The magnitude of the nitrogen fixed indicates that the amount fixed is governed not by the requirements of the endophyte but by the requirements of the entire symbiotic

system.

7) Efficiency in fixation is greatest in the young nodules, the efficiency data comparing favourably with those available for legume nodules.

8) Evidence is put forward which shows that the efficiency of detached nodules in fixation is greatly reduced; this has been suggested as being due to a shortage of metabolites normally available to the nodules from the shoot.

S E C T I O N II

STUDIES ON NITROGEN FIXATION BY MARINE SUPRALITTORAL BLUE-GREEN ALGAE.

P A R T I

The isolation in pure culture of certain supralittoral blue-green algae and tests upon them for fixation of elemental nitrogen.

INTRODUCTION

In nature most abundant growth of marine blue-green algae occurs on the supralittoral fringe of the seashore, an inhospitable habitat for most forms of plant life owing to great variations in conditions such as salinity, temperature and dessication. The relatively few plants present, apart from the well-defined band of blue-green algae, a few lichens (Verrucaria spp., Lichina confinis), one or two members of the Chlorophyceae (Prasiola stipitata) and of the Rhodophyceae (Porphyra linearis, Bangia atropurpurea) may be due in part to a lack of mineral nitrogen for, in such a zone, coverage by the sea occurs only at very high tide and soil nitrogen must be present only in small quantity. It might be expected that if nitrogen-fixing marine blue-green algae did occur they would occupy such a habitat. Selected species from this region were chosen and tested for the fixation of elemental nitrogen.

Although the cultural requirements of many fresh water blue-green algae have been studied in detail (Gerloff, Fitzgerald and Skoog, 1950a, 1952; Kratz and Myers, 1955) few studies exist on the culturing of marine Myxophyceae. Allen (1958), in a short paper on the problems of marine nitrogen fixation, stated that few physiological studies on marine blue-green algae had been carried out due to difficulty in

culturing the algae.

Kinne-Diettrich (1955) reported on the nitrogen nutrition of two non-nitrogen-fixing marine species, Lyngbya confervoides and L. maiscula, and showed that growth in medium containing nitrate-nitrogen was better than in that containing ammonium-nitrogen; there was no evidence that amino acids acted as a nitrogen source. Pintner and Provasoli (1958) investigated the cultural requirements of Phormidium persicinum, a red-pigmented blue-green, and found it capable of growth in several different artificial sea-water media so long as an exogenous source of vitamin B_{12} was available.

Isolation of blue-green algae in pure culture is a matter of some difficulty, due to the fact that the thick gelatinous sheaths harbour numerous bacteria. The earliest successful method was that of Pringsheim (1914) who obtained pure cultures by the very tedious method of repeated subculturing. The longevity and unreliability of this method led to the methods of Allison and Morris (1930), Allison, Hoover and Morris (1937), and Bortels (1940) who isolated pure cultures by exposing the algae to ultra-violet irradiation for short periods. This method has been elaborated on by Gerloff, Fitzgerald and Skoog (1950b) to obtain pure cultures of eight Myxophyceae. In their experiments Gerloff et al. placed a dilute algal suspension in a quartz windowed chamber and irradiated with ultra-violet light. Samples were withdrawn at five minute intervals and dilution cultures made. The results

showed the algae to be slightly more resistant to ultra-violet irradiation than the bacteria, and as a result, certain samples which just showed algal growth were found to be pure. Using a similar method many other workers have obtained pure cultures of blue-green algae.

De (1939) and Watanabe (1951) obtained pure cultures by repeated subculturing on nutrient solution solidified with silica gel. The inert nature of this substrate largely prevented bacterial growth and pure cultures were eventually obtained. Fogg (1942) obtained pure cultures of Anabaena cylindrica by immersing small portions of algal material in a dilute solution of chlorine water and plating out after washing in water. This method proved unsuccessful with other species.

Provasoli, Pintner and Packer (1951) obtained evidence that three antibiotics, Polymyxin-B-sulphate, Bacitracin, and Aureomycin, were bactericidal at concentrations which did not entirely inhibit the growth of blue-green algae, while Pintner and Provasoli (1958) obtained pure cultures of the red-pigmented blue-green Phormidium persicinum after treatment with a mixture of Penicillin and Streptomycin. These latter antibiotics are however toxic in low concentrations to normal blue-green pigmented Myxophyceae. Galloway and Kraus (1959) obtained evidence that certain chemical agents: Phenyl Mercuric Triethanol Ammonium Lactate, Cetyl Trimethyl Ammonium Bromide, Dichloronapthoquinone, Copper sulphate, Bacitracin and Polymyxin may be helpful in obtaining pure cultures of Myxophyceae.

The first tests for fixation of nitrogen by blue-green

algae in pure culture were those of Pringsheim (1914). He obtained negative results and thus, although earlier workers had obtained evidence of fixation in impure cultures, it became generally accepted that blue-green algae did not fix nitrogen.

In 1928 Drewes however obtained pure cultures of Anabaena variablis, an unidentified Anabaena, and Nostoc punctiforme by repeated subculturing and showed that these species could in fact fix nitrogen. Tests for purity of the cultures were made by inoculation to bacteriological media and in no instance did growth of bacteria occur.

Vouk and Wellisch (1931) and Copeland (1932) gave short accounts of fixation of elemental nitrogen by certain blue-green algae. No details of their tests for purity of the cultures were given however and the results therefore cannot be considered reliable.

Allison and Morris (1932) observed that in a pure culture of Anabaena variablis an average fixation of 5 mg. nitrogen per 100 ml. of medium occurred in nitrogen-free stagnant cultures after a period of seventy-five days, higher rates being attained when the cultures were aerated or supplied with sucrose as a carbohydrate source. In 1935 Winter reported fixation of nitrogen by a species of Nostoc.

De (1939) published reliable evidence that three species of Anabaena, A. variablis, A. naviculoides and A. gelatinosa

were capable of nitrogen fixation while Phormidium faveolarum was not. In his experiments the algae were grown in 250 ml. Erlenmeyer flasks and increase in nitrogen determined by Kjeldahl analyses of alga plus medium from each flask. Evidence of fixation by several species of Anabaena, Nostoc, and Cylindrospermum was presented by Bortels (1940) while Singh (1942) reported nitrogen fixation by certain of the commonest blue-green algae from Indian rice fields: Anabaena ambigua, Nostoc paludosum, and Cylindrospermum gorakporense.

Fogg (1942) produced evidence that another Anabaena species, Anabaena cylindrica, fixed nitrogen after carrying out detailed tests to ensure that the cultures were pure. Total nitrogen fixed was determined as the increase in combined nitrogen present in cultures from which all contaminating sources of combined nitrogen had been removed. The culture vessels consisted of four 250 ml. Pyrex culture flasks arranged in series in a water bath. The air supply drawn through the flasks was freed from combined nitrogen by passing through a 1 per cent sodium bicarbonate solution to remove oxides of nitrogen, through 25 per cent sulphuric acid to remove traces of ammonia, and finally through a sterile cotton wool filter to remove bacterial and fungal contamination, before passing into the culture flasks which had been assembled under aseptic conditions. Contamination via the outlet was prevented by a sterile cotton wool filter and a vessel containing sulphuric acid. The efficiency of the gas washing system was tested,

the results showing that all traces of combined nitrogen had been efficiently removed from the air. The results showed that in a nitrogen-free medium an average of 1.562 mg. nitrogen was fixed per 100 ml. of medium by A. cylindrica in 50 days, approximately 5 per cent of this being exuded by the alga into the medium.

Using essentially the procedure described above, Fogg (1951) obtained evidence that Mastigocladus laminosus fixed nitrogen, a mean total of 2.48 mg. combined nitrogen being fixed per 100 ml. of medium. In the same paper much improved rates of fixation by Anabaena cylindrica (approximately 17.5 mg. per 100 ml. of medium) were reported.

Watanabe (1951) reported nitrogen fixation in pure cultures of Tolypothrix tenuis, Calothrix brevissima, Anabaenopsis sp. and Nostoc sp. although no details of his tests for fixation or for purity were given. In the same year Henriksson reported fixation by a Nostoc isolated from the lichen Collema tenax. Allen (1952) in a survey of several blue-green algae for fixation obtained evidence of fixation only in a species of Nostoc, while Herisset (1952) reported nitrogen fixation by Nostoc commune.

In the above experiments evidence of fixation depended on determining the increase in combined nitrogen in media initially free from combined nitrogen, but in recent years the more convincing method of proving nitrogen fixation by use of the isotope ^{15}N, such as was employed in Section I

of the present thesis, has been used. Evidence in this instance depends on an accumulation of ^{15}N within the algal tissue after exposure of the alga to an atmosphere enriched with the heavy isotope. Using this method Burris, Eppling, Wahlin and Wilson (1942, 1943) detected fixation of nitrogen in Nostoc muscorum while Williams and Burris (1952) demonstrated nitrogen fixation in Calothrix parietina, N. muscorum and Nostoc species. More recently Bond and Scott (1955) obtained evidence of nitrogen fixation by the lichens Collema granosum and Leptogium lichenoides and the liverwort Blasia pusilla. Scott (1956) using similar methods reported fixation in the lichen Peltigera praetextata. Evidence was put forward by Bond and Scott (loc. cit.) which showed that fixation was due to the Nostoc present as a symbiont in all these species.

The most recent species reported to possess the property of nitrogen fixation is Chlorogloea fritschii. This has been reported by Fogg (1960) in a summary of papers presented at the New Delhi symposium on Algae, December, 1959.

THE ALGAE UNDER INVESTIGATION

Identification

The organisms used in the experiments to be described have been identified as follows:-

1) Calothrix scopulorum (Weber et Mohr) Ag. ex Bornet et Flahault. This species has been included under Calothrix crustacea Thur. by Fan (1956) in his unfinished monograph on the genus Calothrix.

2) Nostoc entophytum Bornet et Flahault.

3) Oscillatoria brevis (Kutz.) Gom. ex Bornet et Flahault.

Occurrence in nature

1) Calothrix scopulorum

In north temperate regions Calothrix scopulorum is probably the dominant blue-green alga on the rocks of the supralittoral fringe of the seashore. In thirty 1" square samples taken during winter and early spring, from the supralittoral fringe at each of five localities on the west coast of Scotland: (1) West Kilbride, Ayrshire; (2) Portencross, Ayrshire; (3) Isle-of-Barra, Inverness shire; (4) Port Ellen, Argyllshire; and (5) Kilchoman, Isle-of-Islay, Argyllshire, microscopic examination showed clearly, on the basis of percentage coverage, that C. scopulorum was the dominant alga in all instances.

Literature on the blue-green flora of other British localities shows that the species is abundant in the

supralittoral (Newton, 1931). Fremy (1936) and Lindstedt (1943) record the alga as dominant in the supralittoral fringe at many localities on the coast of France and on the west coast of Sweden respectively. Den Hartog (1959) records a marked C. scopulorum association in the supralittoral fringe in Holland, providing further evidence that this is one of the commonest marine blue-green algae.

2) Nostoc entophytum

This alga though not so abundant as C. scopulorum is widespread in occurrence. On examination of samples from the above five localities N. entophytum was recorded from (1) and (3) although never in large quantity. At Port Ellen the alga occurred epiphytically and endophytically on filaments of Rhodocorton floridulum taken from near the top of the Fucus spiralis zone. At Portencross the species was frequent among the gelatinous colonies of Rivularia atra in the supralittoral fringe. The literature shows that the species occurs among and in the cells of other algae (Newton, 1931).

3) Oscillatoria brevis

This species is fairly common in the supralittoral fringe, particularly in sheltered habitats where it forms marked wefts among other blue-green algae. In samples taken from the five localities detailed above the species was recorde at (1), (3), (4) and (5). It has been recorded from Cumbrae (Newton, 1931), while Fremy (1936) and Lindstedt (1945)

record it from the French and Swedish coasts respectively.

Isolation

O. scopulorum and O. brevis were isolated from samples of blue-green algae from the supralittoral fringe of the seashore at West Kilbride, Ayrshire, while N. entophytum was isolated from among filaments of Rhodocorton floridulum collected at Port Ellen, Argyllshire. The samples were shaken up with glass beads (Fogg, 1942) and sea water. When a fine suspension was obtained, one drop was plated to medium solidified with 2 per cent agar in glass petri dishes.

Initial attempts at culturing the algae were unsuccessful, the media employed being modified Bristol (Henriksson, 1951); Allen's artificial sea water (in Jones, 1930); and Erdschreiber-sea water (Foyn, 1934). This was probably due in the first two instances to unfavourable salt concentrations, while in the third instance the high organic content promoted heavy bacterial growth which adversely affected the algae. Several other media were then employed, the most successful being: media ASP2 and V37 (Provasoli, MacLaughlin and Droop, 1957); medium S50 (Droop, 1958); and medium S66 (Droop, personal communication). The formulae of these media are reproduced in Table 17.

Best growth was initially achieved on medium V37, but in later experiments set up in an attempt to further

TABLE 17

Culture media employed

Medium	ASP 2 [1]	V37 [2]	V37 (mod.1) [3]	V37 (mod.2) [3]	S50 [4]	S66 [4]
NaCl	18.0gm.	5.0gm	5.0gm.	5.0gm.	15.0gm.	3.0gm.
$MgCl_2.6H_2O$	-	0.75gm.	0.75gm.	0.75gm.	2.5gm.	0.5gm.
$MgSO_4.7H_2O$	5.0gm.	-	-	-	-	-
KCl	0.6gm.	-	-	-	0.4gm.	80.0mg.
Ca (as Cl)	0.1gm.	24.0mg.	24.0mg.	24.0mg.	-	-
$CaSO_4.2H_2O$	-	-	-	-	0.5gm.	-
Ca (as SO_4)	-	-	-	-	-	24.0mg.
$NaNO_3$	50.0mg.	-	-	-	-	-
KNO_3	-	0.1gm.	0.1gm.	-	0.1gm.	0.1gm.
K_2HPO_4	5.0mg.	10.0mg.	2.5gm.	2.5gm.	10.0mg.	10.0mg.
K_2SO_4	-	0.13gm.	0.13gm.	0.13gm.	-	-
$Na_2SiO_3.9H_2O$	0.15gm.	-	-	-	-	-
TRIS	1.0gm.	0.5gm.	-	-	-	-
B_{12}	2.0ug.	0.1mg.	0.1mg.	-	0.1ug.	0.1ug.
Vitamin Mix S3 [5]	10.0ml.	-	-	-	-	-
Thiamine	-	1.0mg.	1.0mg.	-	1.0mg.	0.1mg.
Glycl-glycine	-	-	-	-	0.5gm.	0.5gm.
Glycine	-	40.0mg.	40.0mg.	-	0.25mg.	0.2gm.
Uric Acid	-	4.0mg.	4.0mg.	-	-	-
Guanine	-	10.0mg.	10.0mg.	-	-	-
Fe Citrate	-	-	0.01gm.	0.01gm.	-	-
Citric Acid	-	-	0.01gm.	0.01gm.	-	40.0mg.
Na_2EDTA	30.0mg.	-	-	-	50.0mg.	-
Dist. H_2O	1000ml.	1000ml.	1000ml.	1000ml.	1000ml.	1000ml.

[1] Trace element supplement added: Fe(as Cl), 0.08mg; Zn(as Cl), 15.0ug; Mn(as Cl), 0.12mg; Co(as Cl), 0.3ug; Cu(as Cl), 0.12ug. B(as H_3BO_3), 0.6mg.

[2] Minor and Trace element supplement added: Br(as K), 2.2mg; Sr (as Cl), 0.38mg; Al(as Cl), 2.8ug; Rb(as Cl), 6.1ug; Li(as Cl), 0.6ug; I(as K), 2.0ug; Fe(as Cl), 0.01mg; Zn(as SO_4), 0.23mg; Mn(as SO_4), 65.0ug; Mo(as $NaMoO_4$), 0.02mg; Co(as SO_4), 0.63ug; Cu(as SO_4), 0.13ug.

[3] Trace element supplement added: Fe(as Cl), 0.4mg; Mn(as Cl), 0.1 mg; Mo(as Na salt), 0.1mg; B(as H_3BO_3), 0.1mg; Cu(as SO_4), 0.01 mg; Zn(as SO_4), 0.01 mg.

[4] Over/...

4 Trace element supplement added: Br, 22mg; Sr, 3.8mg; Al, 28ug; Rb, 61ug; Li, 6.0ug; I, 20ug; Fe, 500ug; Mn, 50ug; Zn, 5.0ug; Cu, 5.0ug; Co, 500mug; Mo, 500mug.

5 1 ml. of Vitamin Mix S3 contains: thiamine.HCl, 0.2mg; nicotinic acid, 0.01mg; Ca pantothenate, 0.01mg; p-amino-benzoic acid, 1.0ug; biotin, 0.1ug; inositol, 0.5mg; folic acid, 0.2ug; thymine, 0.3mg.

improve growth it was observed that deletion of TRIS (tris (hydroxymethyl) aminomethane) from the medium achieved this objective. The buffer action of TRIS was replaced by a phosphate/citric acid buffer and in addition the trace element supply of Fogg (1949) was substituted for the original V37 supplement. This much modified medium (V37 mod.1) allowed more vigorous algal growth.

In attempts to isolate the nitrogen-fixing species a nitrogen- and vitamin-free modification was used (V37 mod.2). On the latter medium the three species showed vigorous growth initially, but it later became evident that O. brevis would not grow in the absence of combined nitrogen; a supplement in the form of 0.25 gm. sodium nitrate per litre of culture solution was therefore added for this alga.

By repeated subculturing unialgal cultures were isolated within approximately three months. Separate strains were than obtained by micropipetting single filaments to fresh medium. Growth from a single filament was very poor in the case of Oscillatoria, only two out of a total of twenty developing, but in the case of the other species growth readily occurred.

Isolation of pure cultures

The method used was similar for all species. Using aseptic techniques throughout, samples of the alga in the hormogonial stage were homogenised with 2 ml. of medium in

a glass homogeniser until a very fine suspension was obtained. This suspension was diluted to 4 ml. with culture solution and filtered through glass wool (Fogg, 1944). The filtrate was then pipetted into a 10 ml. capacity quartz flask fitted with a cotton wool stopper and placed on a flask shaker at a distance of twenty inches from an ultra-violet lamp and the vigorously shaken suspension irradiated for one to eight minutes. Samples withdrawn at thirty-second intervals were plated to a nitrogen-free (Nostoc and Calothrix) or nitrogen-containing (Oscillatoria) medium solidified with silica gel, prepared by the ion exchange method of Smith (1952). The silicic acid and concentrated nutrient solution, to which an appropriate quantity of 2N sodium hydroxide had been added to bring the pH of the final medium to 7.0 - 7.5, were autoclaved separately at 15 lbs. p.s.i. for twenty minutes. The cooled solutions were mixed immediately before pouring into 9 cm. diameter petri dishes. To reduce bacterial contamination still further, two antibiotics, Aureomycin and Polymyxin-B-sulphate in concentrations of 4 and 30 units per ml. respectively were also added to the medium before pouring.

The irradiation time which just allowed growth of the algae was 4.5 minutes for C. scopulorum and O. brevis and 5 minutes for N. entophytum.

Using the above techniques the three algae, C. scopulorum, N. entophytum and O. brevis were obtained in pure culture on 23rd November, 1959, 8th May, 1959, and 4th February, 1960

respectively. Rigorous tests to ensure the purity of the cultures were carried out, samples being inoculated to the following media and incubated at 20°C and 30°C for a period of thirty days:-

1) Peptone-dextrose-yeast agar (bacteriological peptone, 2.0 gm.; dextrose, 5.0 gm.; yeast extract, 2.0 gm.; agar, 20.0 gm.; water, 1000 ml.).
2) Potato-dextrose-agar (fresh potatoes, 200 gm.; dextrose, 20 gm.; agar, 20 gm.; water, 1000 ml.).
3) Aleem's Azotobacter agar (Aleem, 1953).
4) Bullion broth (NaCl,5gm.; peptone, 10 gm.; "Lab Lemco", 5 gm.; water, 1000 ml.).
5) Bullion agar. As medium 4 but solidified with 2 per cent agar.
6) Malt-yeast-peptone-glucose-agar (malt extract, 3 gm.; yeast, 3 gm.; peptone, 5 gm.; glucose, 10 gm.; water, 1000 ml.).
7) Soluble-starch-agar (soluble starch, 40 gm.; "Marmite", 5 gm.; agar, 25 gm.; water, 1000 ml.).
8) Ashby's mannitol-phosphate medium (Salle, 1948).
9) Casein sea water (Spencer, 1952).
10) Peptone-sea-water agar (Spencer, 1952).

Media 1 - 8 inclusive were prepared both with distilled water and with natural sea water. Tests on each medium were carried out in triplicate. In addition microscopic examination of the algal material under a phase contrast

microscope indicated that all three cultures were pure.

Stock pure cultures of the three algae were maintained in culture solution in 1 oz. bottles sealed with screw-on aluminium caps with rubber liners. Under such conditions the cultures can be maintained at room temperature for periods of 6 - 12 months without subculturing.

Cultural characteristics

1) Calothrix scopulorum

In pure culture this alga exhibits a variety of forms depending particularly on the stage of growth. Good growth occurs both in liquid and on solid media, the young filaments being normally unbranched but occasionally with false branches (Plate 9a). Basal heterocysts are always present and in the absence of combined nitrogen intercalary heterocysts may occur. The protoplasm is normally bright blue-green with dense contents but with age becomes more yellow in colour.

The organs of vegetative reproduction, the hormogonia, are 3 - 12 times longer than broad, 4 - 9u in diameter and composed of square cells or cells slightly shorter than broad (Plate 9b). Formation of hormogonia normally occurs immediately after subculturing. They grow out in all directions from the point of inoculation and on coming to rest develop in either of two ways. In the first instance a terminal or subterminal cell becomes depleted of protoplasm and forms a basal heterocyst resulting in a single filament. In the second instance the hormogonia enlarge slightly in the

PLATE 9

a) Filament of *Calothrix* showing false branches (×500 approx)

b) Hormogonium of *Calothrix* (×500 approx)

c) Two *Calothrix* filaments formed from a hormogonium in which two adjacent intercalary heterocysts had developed (×500 approx)

d) Mature *Calothrix* filament (×500 approx.)

middle, with the formation of two intercalary heterocysts. These heterocysts are formed adjacent to each other or are separated by a vegetative cell which then breaks down with the formation of two Calothrix filaments (Plate 9c). At this stage the filaments are normally 7 - 10u in diameter, slightly thickened at the base, and composed of cells 1.0 - 4.0 times shorter than broad. In certain instances the filaments at this stage resemble those of Calothrix aeruginea Thur. If conditions are favourable hormogonium formation may occur again immediately, the hormogonia being produced terminally on the main filaments or sometimes on false branches. Under less favourable conditions the young filaments enlarge considerably attaining lengths of up to 0.75 mm., the cells becoming two or four times broader than long and the trichomes mainly 8 - 12u broad (Plate 9d).

In old cultures an extremely thick brown pigmented sheath up to 7u broad may be formed. On transference of such cultures to fresh culture medium hormogonium formation is again initiated and the reproduction process is repeated.

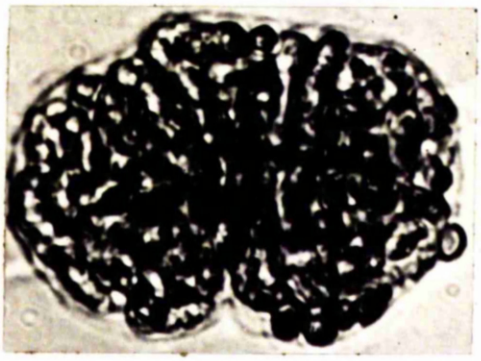
2) Nostoc entophytum

This alga exhibits vigorous growth in the laboratory, either in liquid culture where large gelatinous masses are formed or on solid media where more diffuse colonies are produced. On subculturing to fresh medium hormogonia are formed within twenty-four hours, and move rapidly over the surface of the medium. They exhibit positive phototaxis, at

PLATE 10

a) Hormogonium of Nostoc (×500 approx.)

b) Contorted filament of Nostoc showing the presence of heterocysts (×500 approx.)



c) Adult colony of Nostoc (×500 approx)

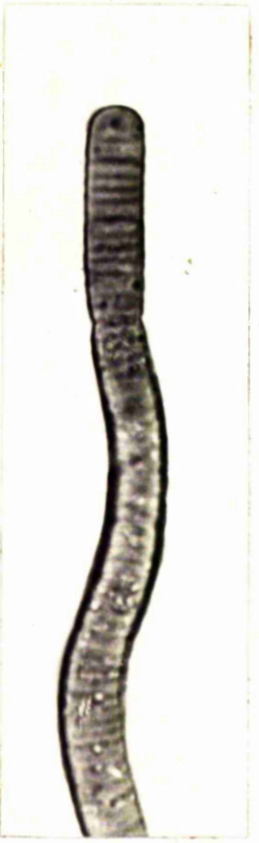
least towards light intensities of 150- 300 foot candles. They measure 15 - 130u long and are 2 - 2.5u in diameter (Plate 10a). On coming to rest the hormogonia divide mainly in the transverse, but on occasion in the longitudinal plane, resulting in contorted filaments. Heterocysts form at this stage. In the adult filaments the cells are barrel shaped or broader then long (Plate 10b). Further divisions result in colonies in which the cells become polygonal in shape, 2.0 - 3.0u in diameter and enclosed in a colourless or brown-pigmented sheath (Plate 10c). In old cultures akinetes may form. These are round or flattened and 5 - 6u in diameter. On transference to fresh medium these germinate with the formation of hormogonia.

3) Oscillatoria brevis

This alga grows best on solid media or in stagnant liquid culture. The adult filaments are 3.5 - 6.5u in diameter, have rounded or uncinate tips and thin but distinct sheaths. No narrowing of the filaments occurs at the septa, each of which is lined by several protoplasmic granules (Plate 11). In liquid culture the bright blue-green filaments oscillate vigorously.

The hormogonia, produced terminally, are 3.0 - 18.0 times longer than broad. Several are often formed in a row. They grow out in all directions and on coming to rest cell division occurs resulting in the formation of a long adult filament. This may divide by means of an oblique division resulting in the formation of two daughter filaments each with an uncinate tip. The two newly formed filaments then enlarge and the process is repeated with further hormogonium formation.

PLATE 11



Adult filament of Oscillatoria

(×900 approx.)

TESTS ON CALOTHRIX SCOPULORUM, NOSTOC ENTOPHYTUM AND OSCILLATORIA BREVIS FOR THE FIXATION OF ELEMENTAL NITROGEN

M E T H O D S

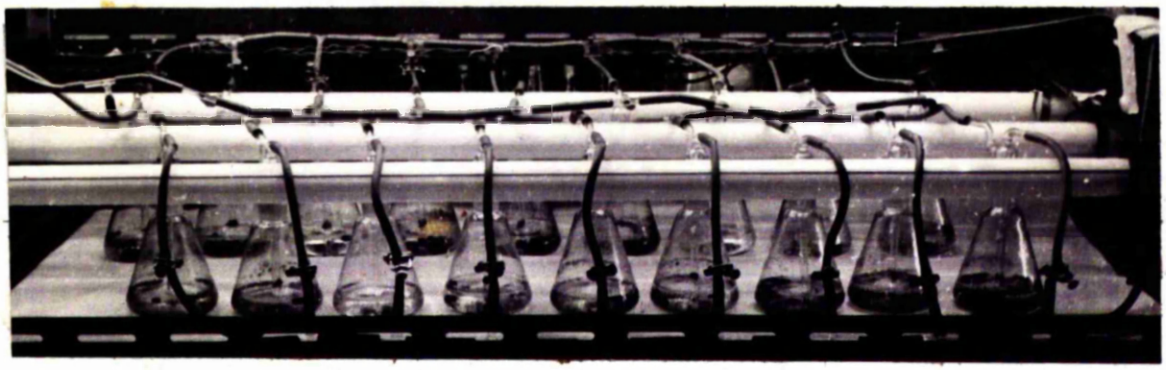
The ability of the algae to fix nitrogen was tested by inoculating filaments to a medium from which all forms of combined nitrogen had been removed and determining increase in combined nitrogen after a period of days.

Culture apparatus and air supply

The culture apparatus consisted of eighteen 250 ml. conical flasks fitted with dreschel heads, the inlets of which passed into the culture medium. The flasks were arranged in series in two rows of nine beneath a bank of three fluorescent tubes, whigh gave a mean light intensity of 390 foot candles. Each flask contained 100 ml. of medium. Air, supplied from an electric pump, was passed through the flasks, the rate of flow being regulated by a screw clip on each inlet tube (Plate 12).

Purification of the air supply was based on the method of Fogg (1942). Combined nitrogen was removed by successive passage through 1.5 litres of 2 per cent sodium bicarbonate (to remove oxides of nitrogen) and 500 ml. of 50 per cent sulphuric acid (to remove ammonia). Both containers (dreschel bottles) were fitted with sintered glass bubblers, resulting in a greater dispersion of the air through the reagents, thus facilitating the removal of combined nitrogen. An air trap

PLATE 12



The arrangement of the culture apparatus in a typical test for fixation. (×⅛)

separated the vessels containing acid and bicarbonate.

Bacterial and fungal contamination via the inlet was eliminated by passing the scrubbed air through a 24-inch-long sterile cotton wool filter. The air was then moistened by passage through 1.5 litres of distilled water in a dreschel bottle fitted with a sintered glass bubbler, before entering the culture flasks. Gas outlets from the flasks were united into one main stream. Contamination via the outlet was prevented by the passage of the outlet tube into a 1 per cent solution of mercuric chloride. An air trap separated the mercuric chloride from the culture flasks.

Before use the glassware was cleaned by immersion in chromic acid for forty-eight hours, then together with all rubber tubing connections, thoroughly washed in boiling water and rinsed with distilled water.

The apparatus was assembled under aseptic conditions; flasks, media, distilled water and traps were autoclaved at 15 lbs. p.s.i. for twenty minutes.

The efficiency of the method of removing combined nitrogen from the air was tested by setting up the apparatus as in a test for nitrogen fixation, but employing only six flasks, in three of which the culture solution was replaced by Nessler's reagent and in the other three by N/50 sodium hydroxide. After twenty days each flask was analysed for combined nitrogen. In the case of Nessler's reagent the colour density of the solution was determined on a Unicam

S.P.600 spectrophotometer at 490mu using a blank of distilled water. In the case of the N/50 sodium hydroxide the contents were analysed by a microkjeldahl method. No increase in combined nitrogen, compared with controls tested at the commencement of the experiment, was detected in either case.

The culture medium

Medium V37 mod.2 (detailed on page 83a) prepared with distilled water and as far as possible "Analar" reagents was employed. With Calothrix and Nostoc the medium was free from combined nitrogen; in the Oscillatoria experiment nine flasks contained nitrogen-free medium and nine contained medium enriched with 0.25 gm. sodium nitrate per litre.

Inoculation and controls

The inoculum was prepared by shaking algal filaments from a fifteen day old culture with 25 ml. of the appropriate sterile medium and glass beads until a homogeneous suspension was obtained (Fogg, 1942). Aliquots of this suspension were inoculated aseptically into each of sixteen flasks by means of specially graduated pipettes. Two control flasks in each experiment were left uninoculated, allowing nitrogen estimations of the medium at the end of the experiment to be carried out. Replicate kjeldahl analyses of inoculum samples were carried out for each experiment.

Subsequent management of the cultures

During each experiment no attempt to maintain a constant temperature was made but thermograph readings showed

that throughout the course of the experiments, temperature varied within the ranges 18.5 - 23.5°C (*Calothrix* and *Nostoc*) and 19.5 - 25.5°C (*Oscillatoria*).

An air flow of approximately 2 c.c. per flask per second was achieved by suitable adjustment of screw clips present on the inlet tubes.

Tests for purity of the cultures

Tests for bacterial and fungal contamination were made immediately before harvesting. One drop of the contents of each flask was inoculated to triplicate samples of the ten media described on page 86, and incubated at 20°C and 30°C for thirty days. A further drop was used for examination under a phase-contrast microscope.

Harvesting of the algae

Harvesting was carried out after twenty days' growth and was completed within a few days, each flask being treated separately. Alga and medium were separated by filtration through previously weighed Whatman No.50 filter papers fitted into a Seitz vacuum filter. Previous microscopic examination showed that this filter paper prevented the passage through it of any algal cells. Dry weights were determined by weighing alga plus filter paper after overnight drying in a 95°C oven.

Nitrogen determinations of the alga plus filter paper, filtrates, and control medium were carried out in a semi-microkjeldahl apparatus using 100 ml. flasks. In the

digestion, 5 ml. of a concentrated sulphuric acid-salicylic acid mixture (30 gm. salicylic acid per 1000 ml. sulphuric acid) plus one B.D.H. selenate kjeldahl catalyst tablet were employed. Digestion was continued for two hours after clearing. The ammonia evolved was absorbed into N/40 hydrochloric acid, and estimated by back titration with N/40 sodium hydroxide, using methyl red as indicator.

DATA OBTAINED

CALOTHRIX SCOPULORUM

In this experiment algal growth was evident by the third day stage, the filaments being dispersed throughout the medium in small, bright, blue-green colonies. A little growth occurred on the base and on the sides of the flasks.

As the experiment neared completion the medium in the inoculated flasks developed a very slight yellow colouration. This was assumed to be due to substances exuded by the alga. In the uninoculated flasks the medium remained colourless for the duration of the experiment.

Tests on the purity of the cultures were carried out at the end of the experiment. These indicated that all cultures had remained pure.

The dry weight and nitrogen data presented in Table 18 show clearly that this species is capable of fixing atmospheric nitrogen. Over a period of twenty days a mean of 2.35 mg. nitrogen was accumulated per 100 ml. of medium. There was no significant increase in the uninoculated control flasks. Of the total nitrogen fixed approximately 12.9 per cent was present in the medium showing that this alga liberates a proportion of the nitrogen fixed. This extra-cellular nitrogen cannot be accounted for by cell autolysis,

since all the cultures appeared extremely healthy and showed no signs of senescence. Mean increase in algal dry weight was 39.7 mg. per flask. The mean percentage nitrogen content of the alga was 5.15 per cent of the dry weight. Over the growth period of twenty days the total nitrogen fixed per 1600 ml. of medium was 37.6 mg.

TABLE 18

Dry weight and nitrogen data for Calothrix scopulorum after twenty days growth in a nitrogen-free medium

Flask No.	Dry wt. of alga (mg.)	N in alga (mg.) ⊛	N in filtrate (mg.) ø	Total N fixed (mg.)	N content of alga as %age of dry wt.	Extra-cellular N as %age of total N fixed
1	35.5	1.844	0.293	2.137	5.19	13.71
2	52.5	2.680	0.416	3.096	5.10	13.44
3	50.0	2.613	0.387	3.000	5.23	12.90
4	35.5	1.832	0.302	2.134	5.16	14.16
5	34.5	1.756	0.282	2.038	5.09	13.84
6	34.5	1.776	0.256	2.032	5.15	12.60
7	40.0	2.110	0.317	2.427	5.16	13.06
8	44.5	2.318	0.273	2.591	5.21	10.54
9	43.0	2.177	0.298	2.475	5.06	12.04
10	35.5	1.837	0.294	2.131	5.17	14.11
11	37.0	1.943	0.266	2.209	5.25	12.04
12	34.0	1.753	0.237	1.990	5.16	11.91
13	45.0	2.293	0.361	2.654	5.10	13.60
14	38.5	2.011	0.298	2.309	5.22	12.90
15	46.0	2.282	0.342	2.624	4.96	13.03
16	29.5	1.514	0.207	1.721	5.13	12.03
17*	0	0	0.008	0	0	0
18*	0	0	0.006	0	0	0

N in inoculum = 0.054 mg.

* Uninoculated controls.

⊛ In this column a deduction of 0.054 mg. N has been made from flasks 1 - 16 inclusive to account for combined nitrogen added in the inoculum.

ø In this column a deduction of 0.007 mg. N has been made from flasks 1 - 16 inclusive to account for nitrogen detected in the uninoculated control flasks.

NOSTOC ENTOPHYTUM

In the inoculated flasks algal growth was visible after three days and the filaments soon became aggregated in floating gelatinous colonies. A proportion of growth occurred on the sides of the culture flasks and on the gas inlet tubes; this was prevented as far as possible by manual shaking of the flasks. A yellow colouration was noticeable in the medium of the inoculated flasks a few days before harvest. Tests for contamination after twenty days growth showed that the cultures had remained pure. The alga showed no sign of senescence at the end of the experiment.

Harvest data presented in Table 19 show that Nostoc entophytum also exhibits the property of nitrogen fixation. During the growth period a total of 2.93 mg. nitrogen was fixed per 100 ml. of medium. Approximately 7.9 per cent of this was present in the medium. Mean increase in algal dry weight per flask was 51.2 mg. and taking into account the size of inoculum it is evident that the present cultural conditions are more suitable for the growth of Nostoc than for Calothrix. The nitrogen content of the alga accounted for 5.30 per cent of the total dry weight. In twenty days a total of 46.9 mg. nitrogen was fixed per 1600 ml. of medium.

TABLE 19

Dry weight and nitrogen data for Nostoc entophytum after twenty days growth in a nitrogen-free medium

Flask No.	Dry wt. of alga (mg.)	N in alga (mg.) ⊕	N in filtrate (mg.) ⌀	Total N fixed (mg.)	N content of alga as %age of dry wt.	Extra-cellular N as %age of total N fixed
1	52.0	2.773	0.229	3.002	5.332	7.63
2	60.0	3.088	0.221	3.309	5.146	6.68
3	50.0	2.650	0.210	2.860	5.298	7.34
4	55.5	2.948	0.226	3.174	5.314	7.12
5	44.0	2.344	0.204	2.548	5.326	8.01
6	48.0	2.599	0.192	2.791	5.414	6.88
7	51.0	2.717	0.218	2.935	5.329	7.43
8	54.5	2.895	0.245	3.140	5.311	7.60
9	47.0	2.480	0.242	2.722	5.278	8.89
10	51.5	2.659	0.201	2.860	5.164	7.03
11	61.0	3.265	0.244	3.509	5.332	6.95
12	52.0	2.751	0.237	2.988	5.288	7.93
13	45.0	2.419	0.195	2.614	5.318	7.46
14	53.0	2.821	0.221	3.042	5.324	7.26
15	45.0	2.371	0.217	2.588	5.269	8.39
16	48.5	2.588	0.228	2.816	5.337	8.10
17*	0	0	0.008	0	0	0
18*	0	0	0.008	0	0	0

N in inoculum = 0.036 mg.

* Uninoculated controls.

⊕ In this column a deduction of 0.036 mg. N has been made from flasks 1 - 16 inclusive to account for combined nitrogen added in the inoculum.

⌀ In this column a deduction of 0.008 mg. N has been made from flasks 1 - 16 inclusive to account for nitrogen detected in the uninoculated control flasks.

OSCILLATORIA BREVIS

In this experiment abundant growth occurred only in those inoculated flasks containing a source of combined nitrogen. The alga was healthy in appearance and deep blue-green in colour. Unlike the two previous algae this species formed a delicate mat of filaments over the surface of the liquid and on the sides of the flasks.

Nitrogen determinations in the case of the nitrogen-containing flasks consisted of separate analyses per flask of the filtrates, and of the alga plus filter paper. In the case of the nitrogen-free series, alga and filter paper were combined before Kjeldahl analyses.

Dry weight and nitrogen data are presented in Table 20. These show clearly that in the nitrogen-containing series although a mean of 48 mg. dry weight of alga was formed per flask there was no significant increase in the total amount of combined nitrogen. The percentage nitrogen content of this alga was approximately 5.6 per cent. In the flasks free from combined nitrogen no increase in algal dry weight or in nitrogen content occurred. These results offer convincing proof that this species unlike Calothrix scopulorum and Nostoc entophytum does not fix nitrogen.

TABLE 20

Dry weight and nitrogen data for Oscillatoria brevis after twenty days growth with and without combined nitrogen

Series	Flask No.	Dry wt. of alga (mg.)	Mg. N in alga	Mg. N in filtrate	Total N
Nitrogen-containing	1	50	2.809	5.356	8.165
	2	46	2.572	5.614	8.186
	3	40	2.242	5.922	8.164
	4	52	2.901	5.317	8.218
	5	45	2.525	5.565	8.090
	6*	0	0.000	8.152	8.152
	7	50	2.826	5.312	8.138
	8	48	2.701	5.501	8.202
	9	52	2.929	5.274	8.203
Nitrogen-free	10	1	0.046	0.000	0.046
	11	1	0.050	0.000	0.050
	12	1	0.053	0.000	0.053
	13	1	0.056	0.000	0.056
	14	1	0.050	0.000	0.050
	15	1	0.053	0.000	0.053
	16	1	0.053	0.000	0.053
	17	1	0.046	0.000	0.046
	18*	0	0.000	0.000	0.000

N in inoculum = 0.053 mg.

* Uninoculated controls.

D I S C U S S I O N

In the present experiments, although the isotopic method using ^{15}N was not employed, the vigorous growth of Calothrix scopulorum and Nostoc entophytum in a nitrogen-free medium offers convincing proof that fixation is associated with both these species. As far as is known this is the first evidence that algae isolated from marine habitats fix nitrogen.

In the experiments described no detailed attempt was made to obtain optimum cultural conditions and it is probable that conditions realising more vigorous growth of all three species can be achieved. However, the mean increase in total nitrogen compares favourably with data obtained by other workers with different species. Fogg (1942, 1951) records a fixation of 1.562 mg. nitrogen per 100 ml. medium in fifty days for Anabaena and 2.450 mg. nitrogen per 100 ml. medium in twenty days for Mastigocladus. Compared with later figures quoted by Fogg (1951) for Anabaena cylindrica (17.5 mg.

quoted by Fogg (1951) for Anabaena cylindrica (17.5 mg. rate, at least under the present conditions, is much lower. In the experiment with Oscillatoria brevis no growth occurred in a nitrogen-free medium and, although there was vigorous growth in the presence of combined nitrogen, no significant increase in the nitrogen content of the flasks occurred. It

is therefore certain that this species is incapable of fixing atmospheric nitrogen.

Analyses of the medium after twenty days growth show clearly that both Calothrix and Nostoc liberate a certain proportion of the fixed nitrogen to the medium. As the cultures were all of healthy appearance at the end of the experiment there is no evidence that liberation is due to cell autolysis. This feature has been observed by other workers for fresh water blue-green algae, for example Watanabe (1951) and Fogg (1952). Fogg suggests that this production of extracellular nitrogen is a normal concomitant of growth. The present results support this suggestion.

On considering the proportion of extracellular nitrogen it is seen that in Nostoc approximately 7 per cent of the total nitrogen fixed was liberated over a period of twenty days while the corresponding figure for Calothrix was approximately 13 per cent.

While a close comparison with the data obtained on other algae cannot be made, as it has been shown by Fogg (1952) that the amount of extracellular nitrogen produced varies depending on the conditions, general trends can be considered. It is seen that the amount produced by N. entophytum is rather similar to that obtained for Nostoc muscorum by Magee and Burris (1954) and for Anabaena cylindrica by Allen and Arnon (1955). Fogg however quotes much higher figures in his experiments with Anabaena and

Mastigocladus as does Henriksson (1951) with Nostoc (approximately 24 per cent). The suggestion has also been put forward (Fogg, 1956) that the lower rates of exudation observed in the experiments of Magee and Burris loc. cit. and Allen and Arnon loc. cit. may have been due to a better supply of trace elements than in his own experiments. In the present experiments however the micronutrient solution used was similar to that employed by Fogg and lower results in the case of Nostoc were still obtained suggesting that factors other than micronutrient supply are important in determining the proportion of exuded nitrogen.

The percentage nitrogen content of the algae show that, compared with other groups of plants except the bacteria, these species have relatively high nitrogen contents. There appears to be no significant difference in the nitrogen content irrespective of whether or not the species fixes nitrogen.

The fact that fixation of nitrogen was observed only in Nostoc and Calothrix, members of the families Nostocaceae and Rivulariaceae respectively while not in Oscillatoria, a member of the family Oscillatoriaceae, agrees with data obtained by recent workers, for example Allen (1952), Williams and Burris (1952). So far there has been no unequivocal proof that fixation of nitrogen is associated with members of the Oscillatoriaceae, although several species have been reported as possessing the property

(Copeland, 1932). It is possible that species belonging to this family will yet be shown to be fixers.

The nitrogen fixing ability of C. scopulorum may be one of the factors accounting for the prominence of the species in the algal flora of the rocks of the supralittoral fringe. In such a habitat where it is probable that a shortage of nitrogen exists, colonisation by C. scopulorum and other nitrogen-fixing species could rapidly occur while non nitrogen-fixers, such as Oscillatoria brevis would possibly be handicapped by lack of nitrogen. In the supralittoral fringe the mat of blue-green algae present is often completely peeled off the rocks in the warm summer months and when re-colonisation occurs in the autumn it is probable that C. scopulorum is one of the early colonisers.

N. entophytum, while not so abundant, is widespread in occurrence and its nitrogen-fixing ability may be an advantage in certain micro-environments. Very often N. entophytum occurs on and within the cells of other algae and in such circumstances it is possible that a relationship somewhat similar to the nitrogen-fixing system of Blasia pusilla exists.

In the present study it is evident that two of the three species critically examined for nitrogen fixation possess the property. This proportion cannot be considered as representative however, for, in addition to the fact that very few species were examined, it must be remembered that the media employed in isolating the algae were nitrogen-free and thus nitrogen-fixing species tended to be selected. Williams and

Burris loc. cit. in a survey of fixation by fresh water species observed that out of eleven species isolated on nitrogen-containing media eight possessed the property of nitrogen fixation. Whether such a high proportion exists in marine habitats is as yet unknown.

S U M M A R Y

1) Three blue-green algae Calothrix scopulorum, Nostoc entophytum and Oscillatoria brevis, isolated from the marine supralittoral fringe, have been obtained in pure culture.

2) A short account of the occurrence and of the cultural characteristics of these algae has been given.

3) Tests to determine whether these species are capable of fixing atmospheric nitrogen have been carried out by inoculating filaments into medium free from combined nitrogen and determining increase in combined nitrogen after a period of days by semi-microkjeldahl analyses.

4) The data provided show that Calothrix scopulorum and Nostoc entophytum fix atmospheric nitrogen while Oscillatoria brevis does not. As far as is known, this is the first evidence that algae isolated from marine habitats fix nitrogen.

P A R T II

Studies in the production of extracellular organic substances by *Calothrix scopulorum*, *Nostoc entophytum* and *Oscillatoria brevis*

I N T R O D U C T I O N

It has been shown, in Part I of this Section, that the nitrogen-fixing blue green algae Calothrix scopulorum and Nostoc entophytum exude a considerable proportion of the nitrogen fixed into the medium. In the light of such evidence it becomes desirable to obtain data on various aspects of exudation by these nitrogen-fixing species. For comparative purposes data should also be obtained on the non-nitrogen-fixing species Oscillatoria brevis.

The first study of the nature of nitrogen liberated by blue-green algae was that of Watanabe (1951), although the presence of extracellular nitrogen in cultures of Myxophyceae had been recorded by various authors (Allison and Morris, 1932; De, 1939; Fogg, 1942, 1951; Henriksson, 1951). In his experiments Watanabe observed the percentage of the total nitrogen fixed which was exuded by Tolypothrix tenuis, Calothrix brevissima, Anabaenopsis sp. and Nostoc sp. during two months of growth to be 13.5, 42.3, 20.6 and 19.4 per cent respectively. When paper partition chromatography of concentrated filtrates from the algae was carried out, free amino acids were detected only in Calothrix brevissima; of these, aspartic acid was the most abundant, glutamic acid, alanine, valine, methionine and leucine following in that order.

Fogg (1952) carried out detailed experiments on the production of extracellular nitrogen by Anabaena cylindrica. The effects of various factors on the total amount of extracellular nitrogen produced were studied. It was found that the percentage exudation was independent of the volume of culture medium, the presence or absence of nitrogen or of glucose, or of light intensity. The most important factor governing exudation appeared to be the age of the culture. In healthy cultures exudation was greatest during the early stages of growth and decreased with age, a minimum being reached towards the end of the exponential phase, after which it tended to increase. In one experiment, for example, it was observed that the extracellular nitrogen in a four day culture represented 21 per cent of the total nitrogen fixed. This figure decreased to 9.7 per cent at the fourteen day stage and later increased to 15.2 per cent at the thirty-six day stage. It was also shown that exudation was affected by the concentrations of certain elements.

Filtrates from twenty-eight day old cultures contained traces of free amino acid probably glutamic acid and alanine. After hydrolysis of the filtrates, serine and threonine were detected together with smaller quantities of glutamic acid, glycine, tyrosine and traces of alanine, valine and leucine. Fogg suggested that the latter amino acids originated from polypeptide present before hydrolysis. In filtrates from twelve day old cultures grown in the presence of ammonium-nitrogen, glutamic acid, alanine, valine, leucine, glutamine,

glycine and serine were present in the free form. After hydrolysis, there was an approximate five-fold increase in the total amino acids; tyrosine, phenylalanine, aspartic acid and the basic amino acids were also present.

Magee and Burris (1954) in experiments with Nostoc muscorum did not detect free amino acids in the medium in which the alga was grown but hydrolysed filtrates showed 0.3 millimoles of ninhydrin-positive material. This corresponded to approximately 5 per cent of the total nitrogen fixed. The composition of the ninhydrin-positive fraction was not determined. In one experiment in which the culture medium contained nitrate a considerable quantity of nitrite also accumulated.

Investigations have also been carried out on the exudation products of marine algae. Fogg and Boalch (1958) found that medium from eleven week old pure cultures of the brown alga Ectocarpus confervoides was yellow-brown in colour, and that filtrates from younger cultures showed the presence of approximately 0.15 mg. of amino-nitrogen per litre of culture solution.

Armstrong and Boalch (1960) showed that filtrates from cultures of E. confervoides, Enteromorpha sp., Phaeocystis pouchetti and Phaeodactylum tricornatum, and sea water showed a higher absorption of ultra-violet light than did distilled water. The nature of the substances causing this absorption was uncertain.

In addition to the production of nitrogenous substances by the blue-green algae a few records exist of the production of carbohydrates. Fogg (1952) observed that Anabaena cylindrica liberated pentose into the medium during healthy growth, the amounts increasing as the cultures aged. Bishop, Adams and Hughes (1954) and Biswas (1957) recorded the presence of polysaccharides in the medium from Anabaena cylindrica and Nostoc muscorum respectively. In A. cylindrica the polysaccharide contained glucose, xylose, galactose, rhamnose, arabinose and uronic acid and was identical in composition with that of the sheath.

The exudation of substances by algae has been considered to be biologically important; Lucas (1955) suggested that extracellular products act as antimetabolites while Lefevre (1952) provided evidence that extracellular substances may sometimes be growth promoting. Fogg and Westlake (1955) showed that the exuded polypeptides from Anabaena cylindrica acted as chelating agents while there is some evidence that vitamins are exuded by certain algae (Brown, Cuthbertson and Fogg, 1956). More recently research on dissolved organic matter has been reviewed by Saunders (1957) and by Vallentyne (1957).

In view of the possible biological importance of extracellular algal products the experiments to be described were carried out. The data obtained will, it

is hoped, help in our understanding of exudation by marine algae and will afford a useful comparison with previous data for both marine and fresh water species.

M E T H O D S

Culturing of the algae

The methods employed were similar in all respects to those described in Part I of the present Section except that a faster air flow (3.5 c.c. per flask per second) was employed. The inocula were prepared aseptically as before by shaking up algae from pure liquid cultures with sterile medium and glass beads until a homogeneous suspension was obtained. In each experiment aliquots of inoculum were added to seventeen flasks, one flask remaining uninoculated as a control. Replicate Kjeldahl analyses of samples of the inocula were carried out as described previously.

The rate of exudation of fixed nitrogen by Calothrix scopulorum and Nostoc entophytum

In these experiments the progress of exudation of fixed nitrogen was studied by harvesting the contents of three inoculated flasks every fourth day. At each harvest the alga and medium were separated by filtration through Whatman No.50 filter paper. Dry weight and nitrogen data on the algal samples and on the filtrates were obtained by a method exactly similar to that described in Part I of the present Section.

Spectrophotometric examination of algal filtrates

The absorption spectra of filtrates from three flasks of each algal species harvested at the twenty-four day stage were determined. Absorption in the wave lengths 220 - 360mu and 360 - 680mu was determined on Unicam S.P.500 and S.P.600 spectrophotometers respectively.

At four day intervals the absorption by filtrates of Calothrix and Nostoc were recorded in the region 360 - 680mu to determine whether any relationship existed between colouration of the filtrates and growth of the alga or exudation of nitrogen.

Chromatographic analyses of algal filtrates for the presence of exuded amino acids

The method used was that of semi-quantitative paper partition chromatography (Heilmann, Barrollier and Watzke, 1957-58). 100 ml. samples of filtrates from twenty-four day old cultures were filtered through Whatman No.50 filter paper, through Ford's SB6 bacteria-proof sterimats and concentrated in vacuo to a volume of 25 ml. Duplicate 5 ml. samples of this filtrate were analysed for total nitrogen by the microkjeldahl method using mercury as a catalyst in the digestion. Distillation was carried out in a Markham still.

Samples of the filtrates were then desalted by the electrolytic method of Stevens, Smith and Jepson (1954). Aliquots of desalted solution were concentrated in vacuo and

applied either directly to paper, or hydrolysed for twenty-four hours with 6N hydrochloric acid at 119°C before application. Whatman No.1 chromatography paper was used throughout. The solvent system employed in the first dimension was butanol/acetic acid/water (4:1:5 by volume), followed by water saturated phenol in the presence of ammonia vapour and potassium cyanide in the second dimension. The paper was then dried and freed from ammonia using the method of Mead (1948).

The amino acids were detected by dipping in a cadmium acetate-ninhydrin solution followed by development over concentrated sulphuric acid for twenty-four hours. The spots were then cut out and the colour eluted with absolute methanol for two hours at 37°C. Optical density readings of the solutions were carried out on a Unicam SP600 spectrophotometer at 500mu. From the extinction co-efficients derived by Heilmann et al. (1957-58) the quantities of nitrogen present in the form of each amino acid were determined. The percentage recovery of nitrogen after desalting was determined by Kjeldahl analyses, using Nostoc filtrate and an aspartic acid solution as test substances. These tests showed that approximately 40 per cent of the total nitrogen present in each test sample was lost in the desalting process.

Analyses of filtrates for the presence of exuded carbohydrates

Preliminary tests for the presence of reducing sugars, pentoses and polysaccharides were carried out using Benedict's test, the glacial acetic acid-aniline furfuranol

test and precipitation in absolute alcohol respectively. The test for polysaccharide is not however specific since proteinaceous compounds may also be precipitated.

On the basis of the above tests, quantities of filtrate were desalted by passing through a 6" x 1" column of "Activated Carbongrade S.C.120/240" charcoal and after eluting the salt with distilled water, monosaccharides and disaccharides were eluted in 5 per cent and 10 per cent ethanol respectively. These fractions were concentrated in vacuo then applied to one dimensional paper chromatograms together with marker sugars. Whatman No.1 chromatography paper was used throughout. The solvent systems used were butanol/acetic acid/water (4:1:5 by volume) and water saturated phenol. Polysaccharide, precipitated by adding the algal filtrate to three times its own volume of absolute alcohol was separated by centrifugation. The precipitate was then hydrolysed by refluxing for thirty hours with 2N sulphuric acid, the sulphate being removed by precipitation with barium hydroxide. The solution was then concentrated in vacuo and applied to one dimensional paper chromatograms as above.

D A T A O B T A I N E D

I. THE RATE OF EXUDATION OF FIXED NITROGEN

CALOTHRIX SCOPULORUM

In this experiment vigorous algal growth was noted after three days. The alga remained healthy throughout the twenty-four day growth period and tests on the purity of each flask showed that no contamination had occurred.

The relative data are presented in Table 21 and Fig. 3. The mean dry weight data show that the first sixteen days of this experiment coincided with the exponential growth phase of the alga. During the succeeding eight day period there was a continued increase in dry weight resulting in a mean total of 69 mg. per flask. Data on the proportion of nitrogen exuded as a percentage of the total fixed during each successive four day period show that a high initial exudation rate occurred, followed by a progressive decline, so that towards the end of the exponential phase exuded nitrogen accounted for approximately 3 per cent of the total amount fixed. This period of low exudation was followed by a phase in which the percentage exuded nitrogen increased sharply to approximately 34 per cent and to approximately 45 per cent during the final four days.

The production of large quantities of extracellular

TABLE 21

The rate of exudation of fixed nitrogen

by Calothrix scopulorum

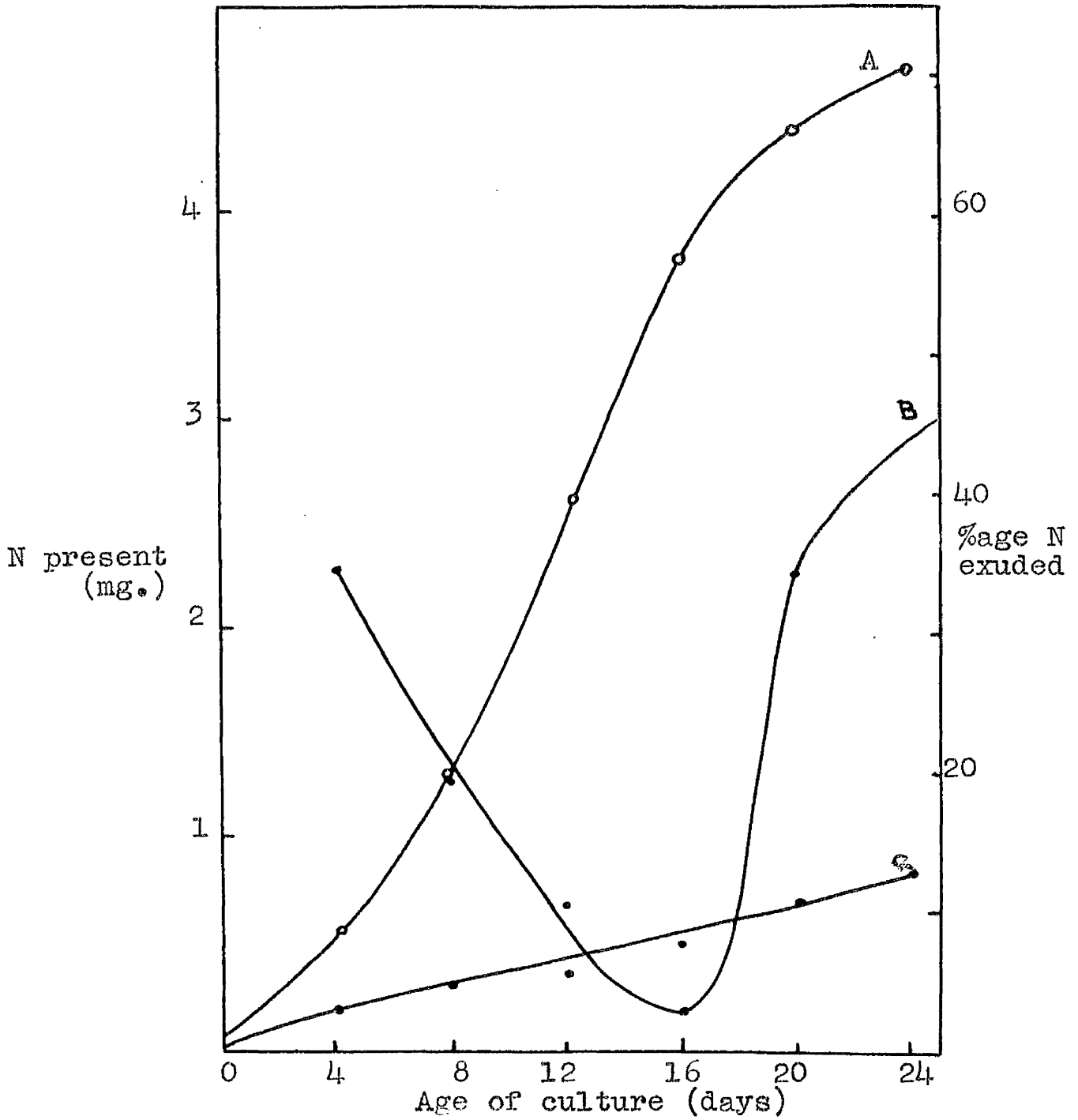
Age of alga (days)	Mean dry wt. of alga (mg.)	Mean N content of alga (mg.)	Mean N content of medium (mg.)	Mean total N present (mg.)	Mean N exuded as %age of N fixed over four day periods	%age N content of alga
4	6.6 (6.0, 6.0, 8.0)	0.40 (0.36, 0.38, 0.47)	0.18 (0.16, 0.17, 0.21)	0.58	34.6 *	6.1
8	17.0 (17.0, 18.0, 16.0)	0.99 (1.00, 1.03, 0.95)	0.32 (0.32, 0.33, 0.30)	1.31	19.2	5.8
12	38.7 (38.0, 38.0, 40.0)	2.20 (2.22, 2.13, 2.24)	0.46 (0.47, 0.46, 0.45)	2.66	10.4	5.7
16	57.3 (60.0, 58.0, 54.0)	3.26 (3.35, 3.34, 3.10)	0.49 (0.50, 0.49, 0.48)	3.75	2.8	5.7
20	64.0 (65.0, 62.0, 65.0)	3.66 (3.78, 3.56, 3.63)	0.70 (0.68, 0.70, 0.72)	4.36	34.4	5.7
24	69.3 (69.0, 69.5)	3.84 (3.81, 3.88)	0.85 (0.85, 0.85)	4.69	45.5	5.5
≡	-	-	0.00	-	-	-

N in inoculum = 0.062 mg.

* The N present in inoculum has been taken into account before calculating this result.

≡ Uninoculated control flask.

Figure 3



The rate of exudation of fixed nitrogen by C. scopulorum. Curve A represents the mean total N fixed; curve B represents the N exuded during four day periods as a %age of the total N fixed during the same period; curve C represents the mean amount of exuded N.

nitrogen was not due to senescence as the cultures remained healthy throughout. That this is the case is supported by the fact that the data on percentage nitrogen content of the alga remained relatively constant throughout.

NOSTOC ENTOPHYTUM

The alga in this experiment showed vigorous growth by the three day stage, the filaments being dispersed throughout the medium in small gelatinous colonies. Tests showed that no contamination occurred throughout the length of the experiment.

Dry weight and nitrogen data are presented in Table 22 and Fig.4. Over the twenty-four day growth period a mean of 272 mg. dry matter per 100 ml. of medium was accumulated. The data show that the alga was still in the exponential growth phase at the termination of the experiment. Data on the percentage nitrogen exuded during successive four day periods show that exudation was greatest during the first four days. This initial period of high exudation was followed by a period during which a marked decline in the percentage exudation occurred until, over the greater part of the exponential phase, exuded nitrogen accounted for only 2 - 3 per cent of the total amount fixed. Data on the percentage nitrogen content of the alga show that a very slight decrease occurred as the culture aged. As there was no increase in the proportion of nitrogen exuded this decrease was not due to autolysis but was probably due to the development of a thick mucilaginous sheath.

TABLE 22

The rate of exudation of fixed nitrogen by Nostoc entophytum

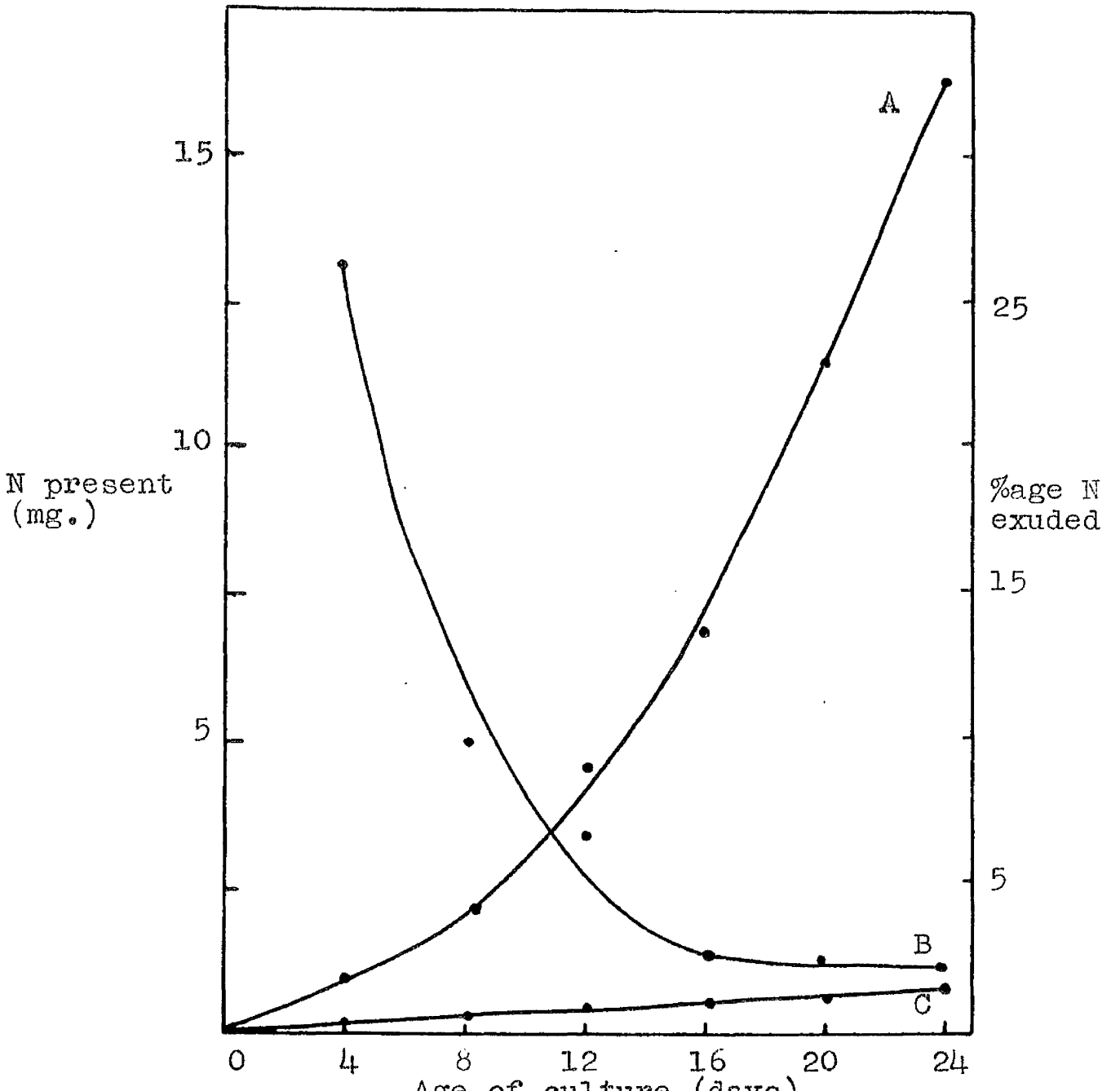
Age of alga (days)	Mean dry wt. of alga (mg.)	Mean N content of alga (mg.)	Mean N content of medium (mg.)	Mean total N present (mg.)	Mean N exuded as %age of N fixed over four day periods	%age N content of alga
4	12.0 (11.0, 12.5, 12.5)	0.72 (0.65, 0.73, 0.77)	0.25 (0.20, 0.22, 0.26)	0.95	26.1*	6.0
8	28.0 (27.5, 28.0, 29.0)	1.74 (1.76, 1.80, 1.76)	0.34 (0.33, 0.32, 0.35)	2.08	9.8	6.2
12	66.6 (69.0, 66.5, 64.5)	4.05 (4.20, 4.06, 3.92)	0.50 (0.49, 0.49, 0.51)	4.55	6.6	6.1
16	102.8 (100.5, 110.5, 97.5)	6.23 (6.05, 6.70, 5.93)	0.56 (0.55, 0.57, 0.55)	6.79	2.8	6.1
20	188.0 (192.5, 183.0, 186.0)	10.76 (9.56, 11.19, 11.53)	0.67 (0.66, 0.68, 0.69)	11.43	2.5	5.7
24	272.0 (288.0, 264.0)	15.50 (15.80, 15.20)	0.78 (0.79, 0.78)	16.28	2.3	5.7
#	-	-	0.00	0.00		

N in inoculum = 0.072 mg.

* The N present in inoculum has been taken into account before calculating this result.

Uninoculated control flask.

Figure 4



The rate of exudation of fixed nitrogen by N. entophytum. Curve A represents the mean total N fixed; curve B represents the N exuded during four day periods as a %age of the total N fixed during the same period; curve C represents the mean amount of exuded N.

II. SPECTROPHOTOMETRIC EXAMINATION OF ALGAL FILTRATES

CALOTHRIX SCOPULORUM

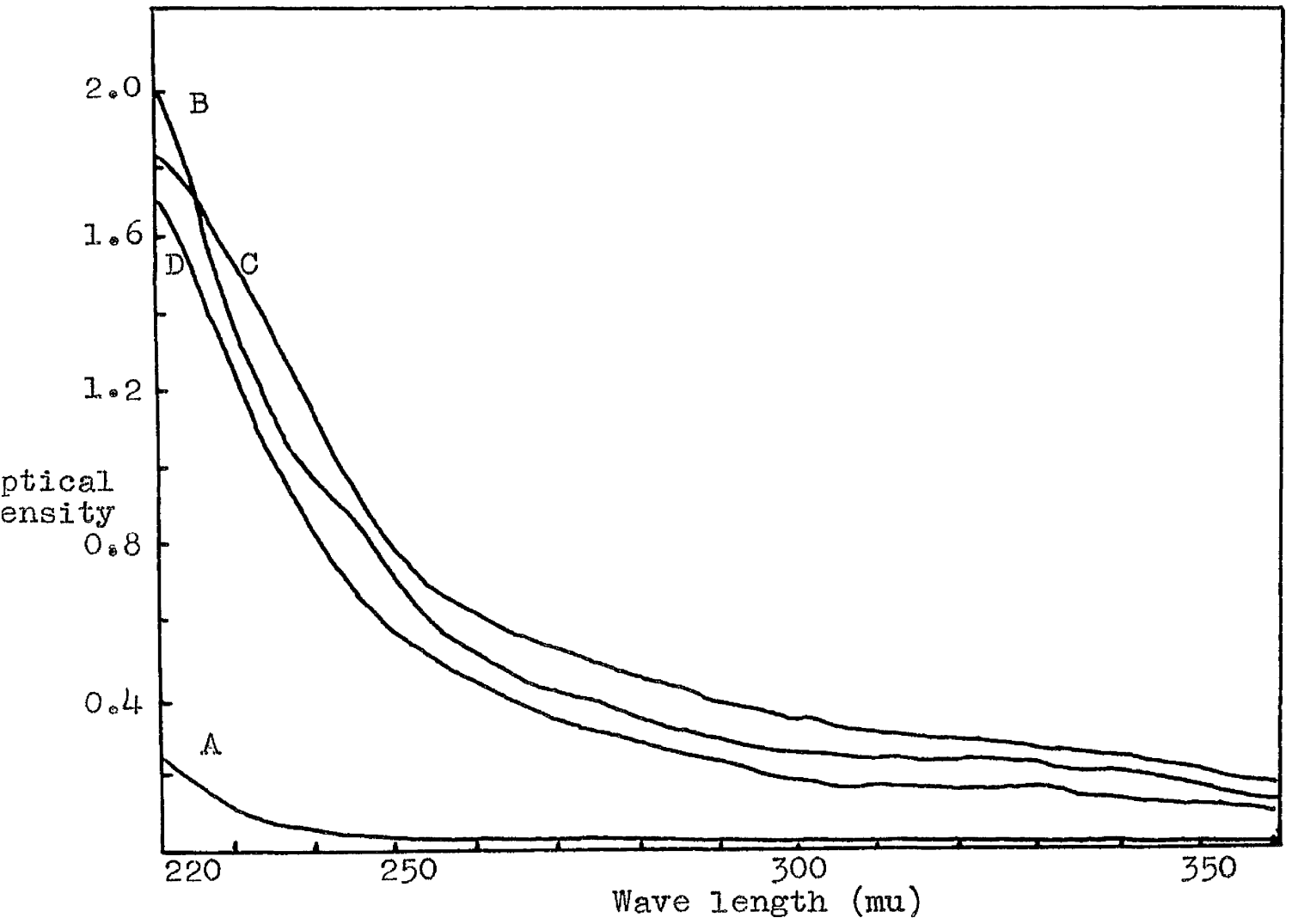
The absorption spectra of filtrates from twenty-four day old cultures are presented in graph form in Figures 5 and 6. These show that absorption is maximum in the ultra-violet wave-band (220mu), and decreases steadily throughout the visible light region. Determinations of absorption at successive four day intervals over the wave-band 360 - 680 mu revealed no significant change in the shape of the absorption curve although a marked increase in absorption occurred after the twelve day stage. This can be seen from Fig.7 in which absorption at 360mu and 500 mu is plotted at successive four day periods, as a typical example of the increase in absorption with age.

The nature of the substances causing absorption in the ultra-violet region is uncertain. These are undoubtedly produced by the alga however as the control culture solution showed very little absorption (Fig.5).

NOSTOC ENTOPHYTUM

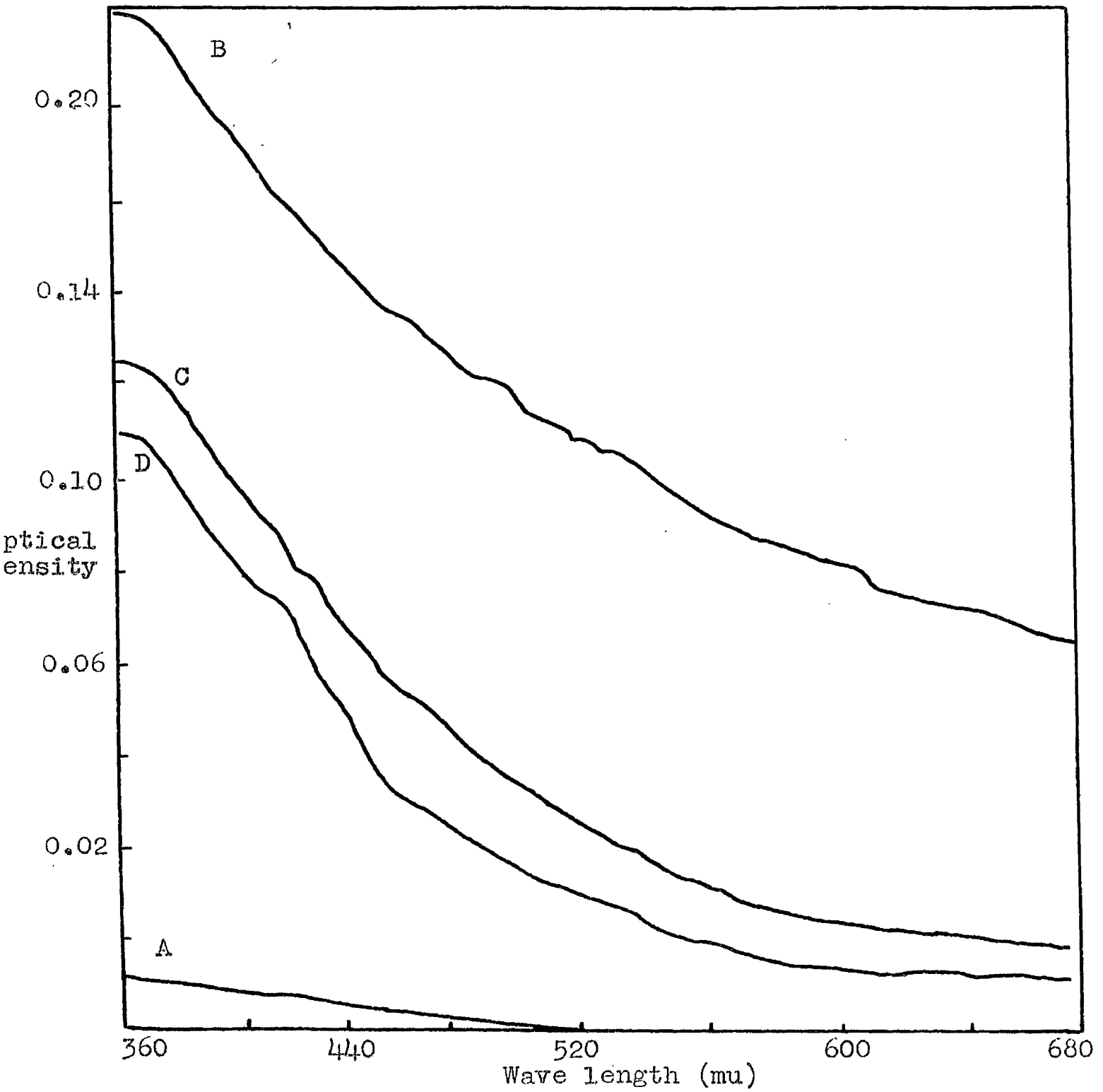
The absorption spectrum of filtrates from twenty-four day old cultures of Nostoc entophytum in the wave-band 220 - 680mu is very similar to that of Calothrix. Maximum absorption occurs in the region of 220mu and is followed by a decrease with increase in wave-length up to 680mu. Data on the absorption in the wave-band 360-680mu show that little change

Figure 5



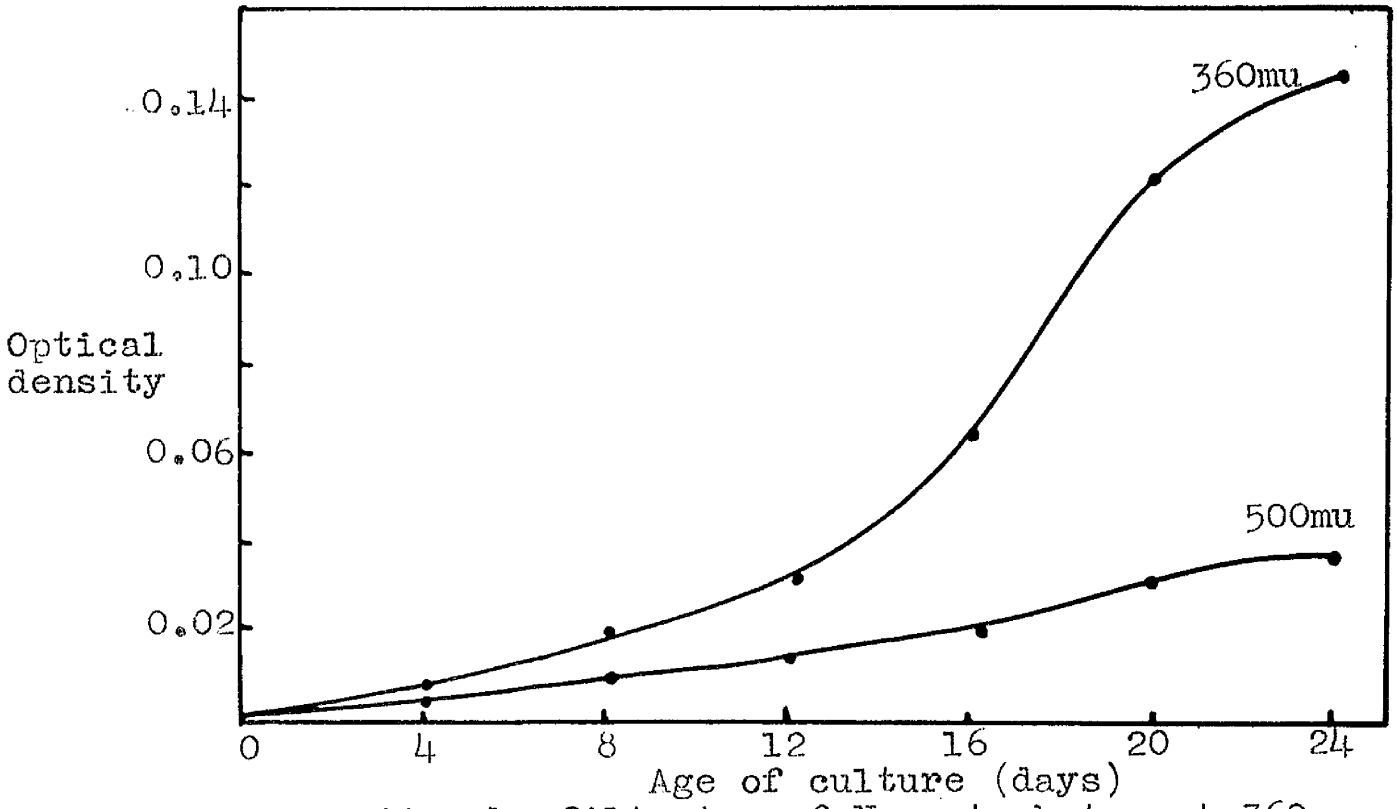
Absorption spectra in the wave-band 220-360mu by culture medium (A), and filtrates from 24 day old cultures of Calothrix (B), Nostoc (C) and Oscillatoria (D) read against a blank of distilled water

Figure 6

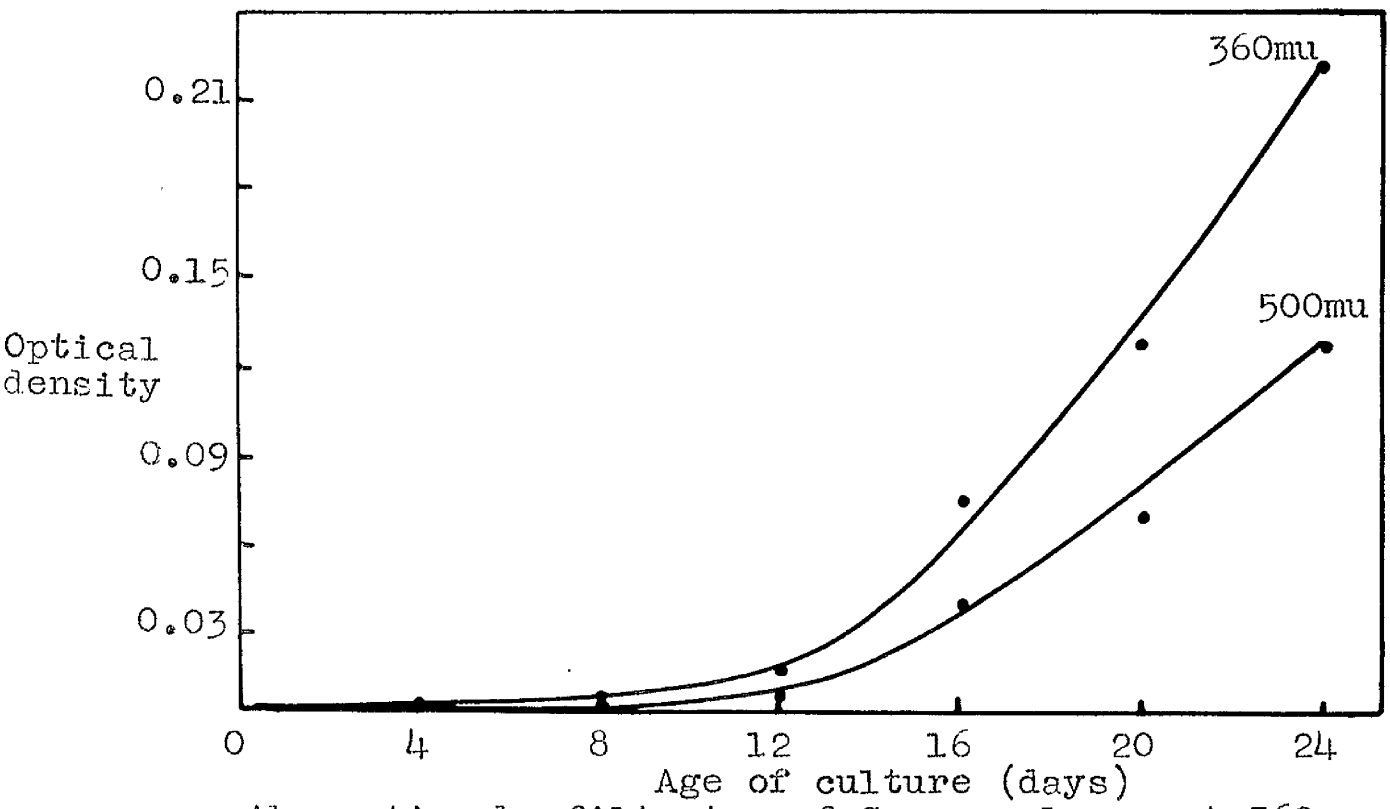


Absorption spec a in the wave-band 360-680mu by culture medium (A) an iltrat s f 24 day old cultures of Caloth ix 3) s Oscillatoria (D) read against

Figure 7



Absorption by filtrates of N. entophytum at 360mu and 500mu over successive four day periods



Absorption by filtrates of C. scopulorum at 360mu and 500mu over successive four day periods

occurs in the shape of the absorption curve with increase in age of the cultures. Fig. 8 however, in which absorption at 350 and 500mu has been plotted against age of the culture, shows that there is an increase in density with age.

OSCILLATORIA BREVIS

The absorption spectrum of filtrates from twenty-four day old cultures of this alga closely resembles those of the two nitrogen-fixing species. Absorption is greatest at 220mu although here it shows signs of levelling off. The similarity of the spectrum to those of the nitrogen-fixing species suggests that the substances responsible for high absorption in the ultra-violet band are of wide occurrence in filtrates from blue-green algae.

III. CHROMATOGRAPHIC ANALYSES OF ALGAL FILTRATES FOR THE PRESENCE OF AMINO ACIDS

CALOTHRIX SCOPULORUM

The data obtained are presented in Table 23. These show that both free and bound amino acids are liberated by this alga during healthy growth. Alanine, threonine and leucine are present in largest quantity in the free state. Data on the total amino acids show that after hydrolysis proline and valine are also present and that alanine, glutamic acid and leucine are in largest quantity. On a quantitative basis it is evident (after taking into account the nitrogen

lost on desalting) that approximately 39 per cent of the total nitrogen liberated is present in the form of amino acid nitrogen. This comprises 5 per cent in the free form and 34 per cent in the bound form (Table 24).

NOSTOC ENTOPHYTUM

The data obtained in this experiment (Table 23) show that as in Calothrix both free and bound amino acids are liberated during healthy growth. In the free state the histidine-arginine-lysine fraction was present in highest quantity together with smaller amounts of threonine, glutamic acid, serine and traces of aspartic acid and alanine. After hydrolysis however there was an increase both in the quantity and number of the acids detected and it was also possible to separate the basic amino acids into histidine and lysine. On a quantitative basis approximately 57 per cent of the nitrogen exuded by the alga was present in the form of amino acid nitrogen. This comprised 11 per cent in the free state and 46 per cent in the bound form (Table 24).

OSCILLATORIA BREVIS

The results of this experiment (Table 23) show that like the two nitrogen fixers O. brevis liberates both free and combined amino acids during healthy growth. The number of amino acids detected was lower however, only glutamic acid, serine, alanine and glycine being present in the free state. In hydrolysed filtrates glutamic acid was present in the highest quantity with smaller amounts of threonine, leucine, serine

TABLE 23

The production of extracellular amino acid-nitrogen by Calothrix, Nostoc and Oscillatoria

(ugm amino acid-N per 100ugm extracellular N)

	Calothrix		Nostoc		Oscillatoria	
Amino Acid	Free	Total	Free	Total	Free	Total
Aspartic acid	0.06	2.66	0.15	5.07	-	-
Glutamic acid	0.13	14.83	0.32	9.81	*	*
Histidine	-	-		12.25	-	-
Lysine	-	-	8.82	10.93	-	-
Arginine	-	-		-	-	-
Leucine	0.96	5.97	-	5.14	-	*
Valine	-	3.94	-	3.86	-	*
Threonine	1.42	2.02	1.15	6.42	-	*
Phenyl-alanine	0.22	1.06	-	-	-	-
Serine	0.21	0.72	0.38	2.54	*	*
Alanine	1.76	7.30	0.07	0.85	*	*
Glycine	*	*	*	*	*	*
Proline	*	*	-	-	-	-

* Present but not estimated quantitatively.

TABLE 24

Nitrogen data on filtrates of Calothrix

Ni

and Nostoc

and Nostoc

Alga	Calothrix	Nostoc
Amino acid-nitrogen as a percentage of total extracellular nitrogen	38.5	56.8
Percentage free amino acid-nitrogen	4.8	10.9
Percentage bound amino acid-nitrogen	33.7	45.9

and traces of valine and alanine. It was not possible to determine quantitatively the percentage of the exuded nitrogen which was in the form of amino acid nitrogen, as it was impossible to distinguish between nitrate-nitrogen present in the medium originally and nitrogen exuded by the alga.

IV. ANALYSES OF FILTRATES FOR THE PRESENCE OF EXUDED CARBOHYDRATES

Preliminary tests on filtrates of all three species showed that pentoses and possibly other reducing sugars were liberated in all instances. Precipitates indicative of the presence of polysaccharides were very marked in Calothrix, less so in Nostoc, while in Oscillatoria little occurred. Replicate one-dimensional chromatograms showed that the filtrates from all three species contained glucuronic acid, glucose and arabinose in small quantities in the free state. The above three substances together with rhamnose and possibly traces of galactose in the case of Calothrix, were present in hydrolysates of the polysaccharides.

D I S C U S S I O N

In the present experiments a greater yield of algae was achieved than in the initial tests for fixation. This may be attributed to two factors; firstly the increase in rate of air flow through the flasks (approximately 3.5 c.c. per flask per second) resulting in vigorous agitation of the algae and, secondly, the slightly larger inocula used.

Data on the nitrogen exuded as a percentage of the total nitrogen fixed during successive four day periods show that in both Calothrix and Nostoc a high proportion was exuded during the first four days of growth. In the Calothrix experiment this was followed by a marked decrease during the early exponential phase until at the twelve to sixteen day stage only 2.8 per cent of the total nitrogen fixed was exuded. During the sixteen to twenty day stage the proportion exuded increased very markedly to 34 per cent, this increase coinciding with the decrease in growth rate at the end of the exponential phase. This proportion further increased to 45 per cent during the twenty to twenty-four day stage. The pattern of exudation noted in Calothrix is somewhat similar to that recorded by Fogg (1952) for Anabaena cylindrica although he expressed his results in terms of extracellular nitrogen as a percentage of the total nitrogen fixed while the present results are expressed in terms

of nitrogen exuded as a percentage of total nitrogen fixed during successive periods of growth.

In the Nostoc experiment the alga was still in the exponential phase at the twenty-four day stage. A marked decrease occurred in the percentage nitrogen exuded during successive four day periods until the twelve to sixteen day stage when only 2.8 per cent was exuded. This low exudation rate then remained more or less constant until the termination of the experiment. It is probable that an increase in percentage nitrogen exuded, similar to that noted for Calothrix would have occurred if the experiment had been continued past the exponential phase.

Although the general pattern of the rate of exudation by Calothrix is rather similar to that recorded by Fogg (1952) for A. cylindrica, a valid comparison of the amounts of nitrogen exuded cannot be made since different cultural conditions were employed. It is of interest to note however that, in the present experiments, the nitrogen exuded as a percentage of the total nitrogen fixed at the twenty day stage, by Calothrix and Nostoc, was 16.1 per cent and 5.9 per cent respectively while the corresponding figures in the tests for fixation were 13.5 per cent and 7.9 per cent respectively. This suggests that neither the size of inoculum nor the rate of aeration materially affect the rate of exudation.

Data on the absorption spectra of the filtrates of all three species show a marked similarity. On comparing the

absorption by the three filtrates in the ultra-violet region with the absorption spectra of natural sea water and filtrates from four other marine algae, obtained by Armstrong and Boalch (1960), it is seen that the curves are very similar in general form.

In the visible wave-lengths, the absorption noted in the present experiments resembles that of natural sea water as observed by Clarke and James (1939) and by Burt (1953). As a result of the similarity of the present absorption curves to that of natural sea water, tests of the medium for fluorescence in ultra-violet light were carried out, as this phenomenon had been noted for natural sea water by Kalle (1949) and Johnston (1955). All three filtrates showed fluorescence in ultra-violet light while the control media did not. These results suggest that the shape of the absorption curves of natural sea water is perhaps due, in part at least, to the extracellular products of algae and other micro-organisms.

Chromatographic analyses showed that much of the nitrogenous material present was in the form of amino acid nitrogen. The fact that amino acids are present in the filtrates of all three species shows that their liberation occurs whether or not the alga is a nitrogen-fixer.

Data presented in Table 24 show that the percentage nitrogen present in the form of amino acid nitrogen ranges from approximately 34 per cent in Calothrix to approximately 57 per cent in Nostoc. These values are in fact slightly low

since it was not possible, with the method employed, to estimate the glycine (and proline in the case of Calothrix) quantitatively. In all instances over 80 per cent of the total amino acids liberated are in the bound form. However the amino acids are by no means uniform. In the free state only glutamic acid, serine and glycine are common, while in the bound form the common amino acids are glutamic acid, leucine, valine, serine, threonine, glycine and alanine. The presence of the basic amino acids in Nostoc filtrates is of interest as this fraction accounts for a high proportion of the exuded nitrogen.

Data on the carbohydrate material present are of interest in that all three species appear similar. In the free form, arabinose, glucose and glucuronic acid were present. Pentose has been recorded in filtrates from Anabaena by Fogg (1952) but was not identified further. The component sugars of the polysaccharides are identical with the free sugars, but in addition rhamnose is present and in Calothrix traces of galactose also. The presence of polysaccharide in the medium leads to speculation as to its origin. It is difficult to conceive the passage of polysaccharide through the algal cell wall so that its probable derivation is from the mucilaginous sheath. Polysaccharides, identical with those in the sheaths have been detected in the medium by Bishop et al. (1954) and Biswas (1957) working with other blue-green algae. It is probable that in these cases also they are derived from the sheaths. Further support for this theory is given in the present

experiments by the fact that the largest quantity of polysaccharide was detected in the medium from Calothrix where the algae has a very marked sheath which often sloughs off, while in Oscillatoria an alga with an extremely thin sheath, only small quantities were detected.

If, as has been suggested above, the polysaccharide is derived from the sheath, an interesting comparison of the present data can be made with data on the cell wall composition of the Chlorophyceae, Rhodophyceae and Phaeophyceae as presented by Preston (1958). This comparison shows that, as regards the chemical nature of the cell wall, the Myxophyceae resemble the Chlorophyceae more closely than the other classes. It must be borne in mind however that the blue-green algae also show affinity with the Rhodophyceae on account of the requirement of certain species for vitamin B_{12} (Fries, 1960) and there is no conclusive evidence that the blue-green algae are more closely related to any one particular present day algal class.

In Nature extracellular algal products may be of distinct biological importance. From the point of view of nutrition, experiments have shown that certain micro-organisms utilise amino acids as a nitrogen source while in some instances the amino acids are essential for growth. Droop (1958) observed for example that Helmiselmis virescens, a supralittoral cryptomonad required glycine for growth. It is possible that in the case of Helmiselmis the glycine in Nature may be supplied by the Calothrix, Nostoc or Oscillatoria which are themselves

present in the supralittoral fringe. Certain of the carbohydrates liberated by these algae are also known to be assimilated by various micro-organisms. Beckwith (1933) for example showed that several Chlorella species could utilise arabinose, glucose and rhamnose as carbohydrate sources, while glucose is a well known carbohydrate source for many micro-organisms.

In addition to acting as nutritional sources extracellular nitrogenous substances have been shown to act as chelating agents. Fogg and Westlake (1955) have emphasised the possible importance as chelating agents of the peptides and polypeptides exuded by Anabaena cylindrica in to fresh water. There is no evidence to suggest that in marine environments the nitrogenous substances of marine blue-green algae would not exert a similar function.

The results of the present analyses show that the three blue-green algae, Calothrix, Nostoc and Oscillatoria exude nitrogenous and carbohydrate substances into the medium during normal growth, the nitrogenous substances being derived from elemental nitrogen in the case of Calothrix and Nostoc. The two latter species are thus capable of converting an inorganic nitrogen-free medium into one containing substances suitable for the growth of many heterotrophic species. Whether other species can grow in the presence of exudates from these blue-green algae is as yet undetermined, for the possible production of toxins by the algae cannot be overlooked.

S U M M A R Y

1) A study on the production of extracellular substances by the two nitrogen-fixing species Calothrix scopulorum and Nostoc entophytum has been carried out. For comparative purposes data has also been obtained on the non-nitrogen-fixing species Oscillatoria brevis.

2) The results showed that in Calothrix and Nostoc the rate of exudation of fixed nitrogen varied considerably depending on the stage of growth of the alga. A high initial exudation rate occurred which decreased to a minimum during most of the exponential phase. Evidence obtained in the Calothrix experiment showed that as growth slowed down towards the end of the exponential phase exudation increased markedly.

3) The absorption spectra of filtrates from all three species were determined in the ultra-violet and visible wave-bands. These show a close similarity to those obtained by other workers for natural sea water and filtrates from other classes of algae.

4) Experiments showed that an appreciable proportion of the nitrogen liberated by Calothrix and Nostoc was in the form of amino acid nitrogen. The amino acids were mainly in the bound form. Amino acids were also liberated by Oscillatoria although quantitative data was not obtained for this species.

5) Certain reducing sugars and polysaccharides were also liberated by the three algae.

P A R T III

Studies on the effects of salinity and hydrogen ion concentration on the growth of *Calothrix scopulorum*, *Nostoc entophytum* and *Oscillatoria brevis*.

INTRODUCTION

Data presented in Parts I and II of this Section show clearly that certain marine blue-green algae are capable of fixing atmospheric nitrogen. It is therefore now important to consider the distribution of these algae in nature and to examine factors likely to affect this distribution. In the first instance the dominance of the blue-green algae in the supralittoral fringe is undoubtedly due to these plants being among the few capable of withstanding the adverse conditions referred to on p. 74. It has already been suggested that one of the factors responsible for the dominance of Calothrix scopulorum may be its capacity to fix elemental nitrogen, but this is certainly not the only factor and does not account for the presence of many non-nitrogen-fixing species such as Oscillatoria brevis. The factor which is probably most important in determining the distribution of marine species is salinity and in the supralittoral fringe large variations in this factor occur, due to the influx of fresh water, the state of the tide, and the time of the year.

In addition to salinity, hydrogen ion concentration probably influences growth of the algae. The effect of pH on growth of marine blue-green algae has, like salinity, received little attention in the past. Chapman (1946) stated that pH was probably unimportant in the distribution of

marine plants although he gave no evidence to support his statement and it is probable that, in the constantly changing habitats of the upper littoral and supralittoral fringe, fluctuations in pH will occur and possibly affect algal growth.

The frequent occurrence of certain Myxophyceae in habitats of high salinity (salt marshes and solar evaporation works) was recorded in the late 19th century by Hansgirg (1887), although the earliest experimental data on the effect of salinity on growth appear to be those of Cavara (1902) who investigated the growth of an Oscillatoria and Microcoleus species isolated from salt works. These experiments showed that, although both species were resistant to extremely high salt concentrations, Microcoleus only could multiply in 0.7 - 1.9 molar sodium chloride.

Woronichin (1929) described many algae growing in salt lakes of salt concentrations 11 - 21 per cent, among them Spirulina fragile and Chroococcus turgidus, two algae normally occurring in the marine supralittoral fringe. In culture experiments Woronichin observed that the highest molarity of sodium chloride at which Aphanocapsa marina, Nodularia harveyana Nodularia sphaerocarpa, and Nostoc linckia could grow was 1.0, 0.8, 1.0 and 0.8 respectively. These four algae have also been recorded from marine habitats.

Hof and Fremy (1933), who provide an excellent review of the earlier literature, described the Myxophyceae living in certain strong brines and distinguish between halophilic

species, i.e. species capable of growth in solutions more concentrated than 3 molar sodium chloride and halotolerant species, i.e. species which can withstand, but not multiply in high salinities. In their studies Hof and Fremy observed Microcoleus chtonoplastes, Lyngbya aestuari and Microcoleus tenerrimus to be the most frequent species, but in culture Microcoleus appeared only at low sodium chloride concentrations (5.85 per cent) and did not grow at 2 molar sodium chloride.

From the point of view of the ecology of the blue-green algae of the upper littoral and supralittoral fringe, the observations of Ercegovic (1930) are of more interest. He determined the salinity at various points on the Dalmatian coast at various times of the year and recorded the blue-green algae present. In the open coast the salinity varied little, being generally between 37.54 and 37.60 parts per thousand. At such high salinities the dominant species were Calothrix scopulorum and numerous Chroococcaceae. At a second locality near the mouth of a river, salinity varied over the range 4.33 at low tide to 33.19 at high tide. In this instance Calothrix scopulorum was again very common, as it was in salinities 6.42 and 9.63. In such low salinities although Calothrix scopulorum persisted, other algae typical of higher salinities, e.g. Hyella and Mastigocoleus were absent. Observations on the flora of the upper rock pools, where salinity varied from 0 - 283

depending on dilution or evaporation, showed the dominant algae to be Solentia faveolorum and S. stratosum. Calothrix scopulorum was absent.

More recent experiments with blue-green algae have been mainly concerned with mineral nutrition rather than the overall effect of salinity. Wollenweider (1950) has studied the interaction of Ca and Mg on the growth of Oscillatoria rubescens. During the initial stages of growth high Ca levels were found to promote growth while in the later stages the Mg concentration became the important factor.

Allen (1952) observed that, in the presence of Na, twenty-three Myxophyceae were capable of growth in a K-free medium, while in the same year Gerloff, Fitzgerald and Skoog reported better growth of Microcystis aeruginosa on the addition of Na to the culture. In 1955, Na was found to be essential for growth of Anabaena cylindrica (Allen and Arnon) and for Gerloff's strain of Nostoc muscorum (Kratz and Myers). These experiments were the first to show that Na was necessary for the growth of certain photosynthetic organisms. Kratz and Myers (1955) also showed that the Na/K ratio had little effect on the growth of N. muscorum G. Zhender and Gorham (1960) observed that with Microcystis aeruginosa NRC1 the concentrations of sodium nitrate, di-potassium hydrogen phosphate and magnesium sulphate were widely interchangeable without affecting growth. A low K tolerance was also noted which appeared to be dependent on the Na/K ratio in the medium.

In studies on the effect of salinity on growth of marine organisms two main methods have been employed. In the first instance varying salinities have been obtained by diluting (with distilled water) or by concentrating natural sea water. This method has been used by such workers as Braarud (1951) and Braarud and Pappas (1951) to determine the optimum salinity concentrations for several neritic species. In the second instance the effects and inter-effects of different concentrations of the ions chiefly responsible for variations in salinity have been investigated. Work along such lines has been mainly carried out by Provasoli's team at the Haskins Laboratories, New York and by Droop at Millport and necessitates the use of artificial sea water media.

In the studies to be described, although the main aim was to investigate the effect of total salinity rather than the effect of various ions on growth, it was considered preferable not to use natural sea water as growth in the absence of combined nitrogen would also supply data on the nitrogen-fixing capacities of the algae. Artificial sea water was therefore used. As the ions chiefly responsible for variations in salinity are: Na^{+}, Mg^{2+}, Ca^{2+}, SO_4^{2-}, Cl^{-} and K^{+}, the artificial sea water should be compounded so that variations occur in these ions only; it can then be stated with certainty that the salinity variations are caused by the ions chiefly responsible for variations in natural sea water and

not by limiting concentrations of minor or trace elements.

The effect of hydrogen ion concentration on growth of blue-green algae has received little detailed study although various records exist of species living in very acid and very alkaline habitats. Geitler and Ruttner (1936), for example, described a Cyanidium from certain acid solfataras while Prat (1929) recorded a species of Oscillatoria from hot springs rich in carbonic acid. Blue-green algae have however been recorded mainly from alkaline (Fritsch and John, 1932) and calcarious soils (Jones, 1935). Lime boring forms, which are abundant in marine habitats have received a certain amount of attention. Bachmann (1915) and Koster (1939) suggested that their penetration of calcarious substrates was due to the action of a solvent secreted by the filaments. Chodat (1904) recorded an alkaline reaction in the neighbourhood of the penetrating filaments.

Experimental studies on the effect of pH appear to be limited to fresh water species. The earliest work is probably that of Allison and Hoover (1935) who concluded that Nostoc muscorum would only grow at pH 5.7 - 9.0 in nitrogen-free medium. Growth was best at 7.0 - 8.5 and decreased markedly below 6.5. In this paper few details of experimental procedure were given but in the experiments of Allison, Hoover and Morris (1937) where similar results for maximum, minimum and optimum levels were obtained, more details are available. In these experiments growth was measured as total nitrogen fixed

over a period of forty-four days. No attempt was made to maintain the cultures at a constant pH level although a buffer of K_2HPO_4 was employed. The results showed that an upward drift in pH occurred until the maximum limit of growth was reached, after which all cells died and pH again fell. The data are thus of limited value as regards the pH for optimum growth of the alga as no indication was given as to the effect of various pH levels at different stages of growth.

Further experiments using Nostoc muscorum were carried out by Walp and Schopbach (1942) who measured growth as increase in cell number. pH levels were controlled by the use of mono-, di-, or tri-potassium phosphate buffers which kept the pH constant at all desired levels except 4.5 at which a rise in pH occurred. In such media the concentrations of the other ions were maintained constant.

In this series Nostoc muscorum was grown to determine the effect of the presence or absence of combined nitrogen on cell proliferation. In a nitrogen-containing medium proliferation was best at 6.9 and was greater at 7.8 than at 5.4. These data accord in general with the findings of Allison et al. except that growth occurred at 5.4. In a nitrogen-free medium Walp and Schopbach arrived at the conclusion that growth was similar whether the medium was neutral, acid or alkaline. Experiments in which Allison's strain and Walp's strain were tested for growth in nitrogen-free medium at low pH levels, showed that Allison's strain

could in fact grow at 5.0 while Walp's strain showed growth between 4.0 and 4.9.

More recently Olsen (1951), Gerloff, Fitzgerald and Skoog (1952) and Zehnder and Gorham (1960) recorded optimum values of 8, 10, and 8 - 11 for their respective strains of Microcystis aeruginosa although in their experiments no serious attempts were made to maintain constant pH levels. Kratz and Myers (1955) in experiments with Nostoc muscorum (Gerloff's strain) observed that in a nitrogen-containing medium growth occurred in the range 6.9 - 9.0, there being little difference in growth of the alga over this range.

In view of the limited data available for Myxophyceae from marine habitats the experiments to be described were set up with the following aims in mind:-

1) To obtain data on the effect of various salinity concentrations on the growth of nitrogen-fixing marine algae in a nitrogen-free medium.
2) To obtain data on the minimum, maximum and optimum pH levels for growth of nitrogen-fixing blue-green algae in nitrogen-free media.
3) To compare the results obtained for the nitrogen-fixing algae with those for Oscillatoria brevis grown in a nitrogen-containing medium.
4) To correlate such findings with the distribution of the algae on the seashore and to compare the results where possible with available data on fresh water species.

M E T H O D S

I. SALINITY EXPERIMENTS

Inoculation from pure cultures of the respective species were made to various salinity concentrations and the growth studied, on the basis of dry weight, over a twenty day period.

Culture apparatus and media employed

The algae were grown in 100 ml. Pyrex conical flasks stoppered with cotton wool, each flask containing 25 ml. of medium. The flasks were placed beneath a bank of three fluorescent tubes which gave a mean light intensity of 395 foot candles. In the Calothrix and Nostoc experiments the flasks were shaken at 100 - 120 oscillations per minute, the amplitude of each oscillation being 1/2". In the Oscillatoria experiment stagnant cultures were employed. No attempt was made to maintain a constant temperature and during the experiments temperature varied within the range 22 - 27°C.

The media employed were based on the following formulae:-

Solution I (normal strength)		Solution II	
NaCl.....	24.80 gm.	K_2HPO_4.....	0.25 gm.
$MgCl_2 6H_2O$.....	10.89 gm.	Fe Citrate.....	0.01 gm.
Ca (as chloride)...	0.43 gm.	Citric Acid.....	0.01 gm.
K_2SO_4.....	0.95 gm.	Fe (as chloride).....	0.40 mg.
Distilled water....	999 ml.	Mn (as chloride).....	0.10 mg.
		Mo (as Na salt).....	0.10 mg.
		B (as boric acid)...	0.10 mg.
		Cu (as sulphate).....	0.01 mg.
		Zn (as sulphate).....	0.01 mg.
		Distilled water.....	1 ml.

In all experiments the proportion of Solution II in the final medium was maintained at 1 ml. per litre irrespective of the extent of dilution or concentration of the components of Solution I. Different salinity levels were achieved by varying the proportions of Solution I salts i.e. of the ions Na, K, Ca, Cl, and SO_4.

Solution I is based on medium U formulated by Droop (in Provasoli, McLaughlin and Droop, 1957) but different concentrations of certain salts have been employed. Solution II is based on the micronutrient solution of Fogg (1949) to which additions of K_2HPO_4, ferric citrate and citric acid have been made. Using these two media eight salinity levels were set up, the Na concentrations of which were as follows:- 250, 500, 1000, 2000, 4000, 8000, 16000 and 32000 mg. per litre. These Na concentrations as can be

seen from Table 25, corresponded to total salinity concentrations of 1.21, 2.03, 3.69, 6.99, 13.50, 26.83, 53.27, and 106.16 parts per thousand. In the case of Oscillatoria where an addition of 0.4 gm. sodium nitrate per litre was made the salinity concentrations were in fact slightly higher than those above. To prevent precipitation, Solutions I and II were autoclaved separately at 10 lbs. pressure p.s.i. for ten minutes. Before inoculation the pH of the media was adjusted to 7.8 with NaOH or HCl as necessary.

The inocula were prepared aseptically from fifteen day old cultures which had been growing in V37 Mod.2 medium (p.83a), 0.25 gm. sodium nitrate being added in the case of Oscillatoria. To prevent the intervention of carry-over effects the algae were homogenised with glass beads (Fogg, 1942), after which they were allowed to settle out and the supernatant liquid pipetted off. The algae were then added to 200 ml. of distilled water in a 500 ml. Pyrex flask, thoroughly shaken for ten minutes and the liquid again withdrawn after settling of the filaments. This procedure was repeated through five changes of distilled water, the alga finally being added to 80 ml. of distilled water. This final suspension was used as the inoculum, 0.5 ml. of inoculum being added to each of 120 flasks, of which there were fifteen at each salinity level. The mean dry weight of inoculum was estimated in each experiment by filtering duplicate 5 ml. samples through weighed Whatman No.50 filter papers and re-weighing after

TABLE 25

Salinity levels

Na concentration parts per thousand	Salinity as total solids (parts per thousand)	Salinity (as determined by method of Ercegovic, 1933) (parts per thousand)
0.25	1.21	0.84
0.50	2.03	1.67
1.00	3.69	3.33
2.00	6.99	6.66
4.00	13.50	13.31
8.00	26.83	26.62
16.00	53.27	53.24
32.00	106.16	106.47

over-night drying at 95°C.

During the experiments increase in salinity, caused by evaporation, was prevented by the addition of sterile distilled water as necessary. At each sampling the pH of the harvested flasks was checked and on the basis of the results obtained, the pH of the remaining flasks were adjusted to uniformity with NaOH or HCl.

Harvesting of the algae

The contents of each flask were filtered through weighed Whatman No.50 filter papers, after having been diluted as necessary to re-dissolve any salts which may have precipitated. Dry weights were obtained by over-night drying at 95°C

II. pH EXPERIMENTS

In this series of experiments the procedure consisted of growing the algae in pure culture at various pH levels and estimating growth over a period of days either by dry weight or optical density measurements.

The optimum pH levels for growth

In these experiments the algae were inoculated into 25 ml. replicates of medium at each of the pH levels, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 in 100 ml. Pyrex conical flasks stoppered with cotton wool. The cultural conditions as regards temperature, light intensity and shaking were similar to those employed with the respective species in the salinity experiments described above. The medium

employed was V.37 mod.2 (Calothrix and Nostoc) or V.37 mod. 2 with the addition of 0.25 gm. sodium nitrate per litre (Oscillatoria). In the present experiments the K_2HPO_4 concentration was increased to 0.5 gm. per litre. The buffering capacity of the medium was further enhanced by the addition of 1 ml. of the pH 10.0 buffer solution of Theorell and Stenhagen (1939) per 24 ml. of culture solution. Media of different pH levels were autoclaved at 15 lbs. p.s.i. for twenty minutes in 1000 c.c. bottles and after twenty-four hours the pH of each solution was adjusted to the desired level by the aseptic addition of calculated quantities of N/20 NaOH or N/20 HCl. Determinations of pH were in all instances made with a Dorran pH meter. The media were then dispensed aseptically into each of 119 conical flasks, there being 17 at each pH level.

The inocula were prepared from pure cultures of Calothrix, Nostoc or Oscillatoria in a manner similar to that employed in the salinity experiments, 0.5 ml. being added to each flask. In each experiment two flasks from each salinity level were tested at least once daily for pH, and on the basis of these determinations and those of the harvested flasks, additions of sterile N/60 NaOH or HCl were made to the remaining flasks to maintain the appropriate pH levels.

Harvesting of the algae

Three flasks at each pH level were harvested at three

day intervals over a fifteen day period. Those continually used for determining pH levels were not harvested. Methods of harvesting the algae and of obtaining dry weight data were similar to those employed in the salinity experiments above.

The pH limits of growth

In these experiments pH levels 0.2 units apart were set up within the ranges, 5.2 - 6.2 and 9.0 - 10.0 (Calothrix), 5.2 - 6.2 and 9.4 - 10.8 (Nostoc), and 5.2 - 6.2 and 9.4 - 10.4 (Oscillatoria). The algae were grown in 16 mm. diameter Pyrex test tubes stoppered with cotton wool and containing 10 ml. of medium, there being four tubes at each pH level.

The culture medium employed at the low pH levels was V37 mod.2 in which K_2HPO_4 was replaced by KH_2PO_4. At the high pH levels it was V37 mod.2 enriched with 0.1 per cent $NaHCO_3$ and 0.1 per cent Na_2CO_3. This carbonate-bicarbonate buffer had no apparent effect on algal growth and was more efficient in maintaining the desired pH levels than the buffers used in the experiments on the optimum pH levels. In the Oscillatoria experiments the medium was enriched with 0.25 gm. $NaNO_3$ per litre. Media at different pH levels were prepared and dispensed as in the previous experiments.

The inocula used were prepared as follows, as it was necessary in the present instance to obtain very fine suspensions: algae from fifteen day old stock cultures were homogenised with 0.5 ml. distilled water in a sterile homogeniser until a very fine uniform suspension was obtained.

This was then pipetted into a 100 ml. conical flask containing 20 ml. distilled water and shaken thoroughly for five minutes. On settling of the algae, the supernatant liquid was pipetted off. This washing procedure was repeated once more, after which the alga was suspended in 5 ml. of sterile distilled water. This suspension was used as the inoculum. To each tube 0.1 ml. of inoculum was added and after shaking to obtain a uniform suspension the density of each tube was immediately determined on a S.P.600 spectrophotometer at a wave length of 450mm.

The light intensity and temperature at which the algae were grown were similar to those described above. To prevent the formation of algal clumps the tubes were manually shaken twice daily.

After seventy-two hours the cotton wool stoppers were replaced by sterile rubber bungs and the contents of each tube vigorously shaken until a homogeneous suspension was obtained and the density again determined on the spectrophotometer. The pH level in each tube was determined at the end of this growth period. The tubes were again stoppered with cotton wool and kept for an additional seven days under conditions similar to those described above, and further growth determined macroscopically.

DATA OBTAINED

I. SALINITY EXPERIMENTS

CALOTHRIX SCOPULORUM

Growth was noted in all flasks at salinity levels ranging from 1.21 to 53.27 parts per thousand after a period of three days. At the salinity level of 106.16 there was no evidence of growth, the alga becoming colourless. At the other levels the alga formed healthy colonies floating freely in the liquid and also adhering a little to the sides of the flasks. There was no macroscopic differences in the type of growth at salinity levels from 3.69 to 26.83, but at the lower salinities the alga formed very loose flocculent colonies while at 53.27 the colonies although more numerous were very much smaller, the filaments being closely packed together.

Harvest data are presented in Table 26, and in histogram form in Fig. 8. Typical cultures from each salinity level are shown in Plate 13a. It is evident that from the eight day stage onwards growth at 13.50 was significantly better than at the levels 1.21, 2.03, 26.83 and 53.27. It did not however differ from that at 6.99. Growth at 3.69 although appearing less than at 13.50 was not significantly so until after the sixteen day stage. At the sixteen and

TABLE 26

The effect of various salinity levels on the growth of Calothrix scopulorum in the absence of combined nitrogen

Days after inoculation	Mean dry weight of alga (mg.)				
	Ø 4	⊕ 8	⊕ 12	⊕ 16	⊕ 20
Salinity					
1.21	2.2	4.3	8.3	15.7	19.7
2.03	2.5	5.3	10.8	17.0	23.6
3.69	2.5	5.7	11.8	19.7	24.0
6.99	2.7	6.8	15.0	21.8	27.3
13.50	3.0	7.7	15.8	24.8	32.8
26.83	2.4	5.0	10.2	19.5	24.6
53.27	2.2	3.5	6.8	12.2	15.3
106.16	1.8	1.8	1.5	1.5	1.8*

Mean dry weight of inoculum = 1.8 mg.

Ø Algal contents of three flasks combined nitrogen before harvesting.

⊕ Differences between means necessary for significance at $P = 0.05$, are 1.9, 4.9, 5.2 and 6.9 mg., at the 8, 12, 16 and 20 day stages respectively.

* One flask only harvested.

and twenty day stage it is seen that growth at 26.83 was significantly greater than at 53.27.

The ability of Calothrix scopulorum to survive prolonged immersion in very high salinity was tested by reducing the salinity of two flasks from 106.16 to 10.62. No growth was evident after fifteen days and it is concluded that this alga is incapable of withstanding prolonged exposure to salinities in the region of 106.16 parts per thousand.

NOSTOC ENTOPHYTUM

In this experiment visible growth was evident after three days in all cultures at salintiy levels 1.21 - 26.83. There was no evidence of algal growth at salinities of 53.27 and 106.16 throughout the length of the experiment. Macroscopically there was no variation in cultural characteristics of the alga at salinity levels 1.21 - 6.99 where large flocculent colonies were formed. At 13.50 and 26.83 the colonies were smaller and more numerous.

Harvest data are presented in Table 27 and Fig.8 while typical cultures from each salinity level are shown in Plate 13b. The data show that growth appeared greatest throughout at the 6.99 level but became significantly better than at 1.21 - 3.69 only at the twenty day stage, although a significant difference from the 26.83 and 13.50 levels was noted by the twelve and sixteen day stages respectively.

TABLE 27

The effect of various salinity levels on the growth of Nostoc entophytum in the absence of combined nitrogen

Days after inoculation	Mean dry weight of alga (mg.)				
	Ø @	Ø @	Ø @	Ø @	Ø @
	4	8	12	16	20
Salinity					
1.21	2.0	4.7	12.7	22.2	34.8
2.03	2.0	5.0	12.3	23.2	35.0
3.69	2.3	5.2	12.5	22.3	35.0
6.99	2.4	5.3	12.7	24.7	39.5
13.50	2.2	5.0	11.3	17.2	29.0
26.83	2.1	3.8	8.0	14.2	25.8
53.27	1.5	1.7	1.5	1.5	1.7 *
106.16	1.4	1.5	1.4	1.8	1.5 *

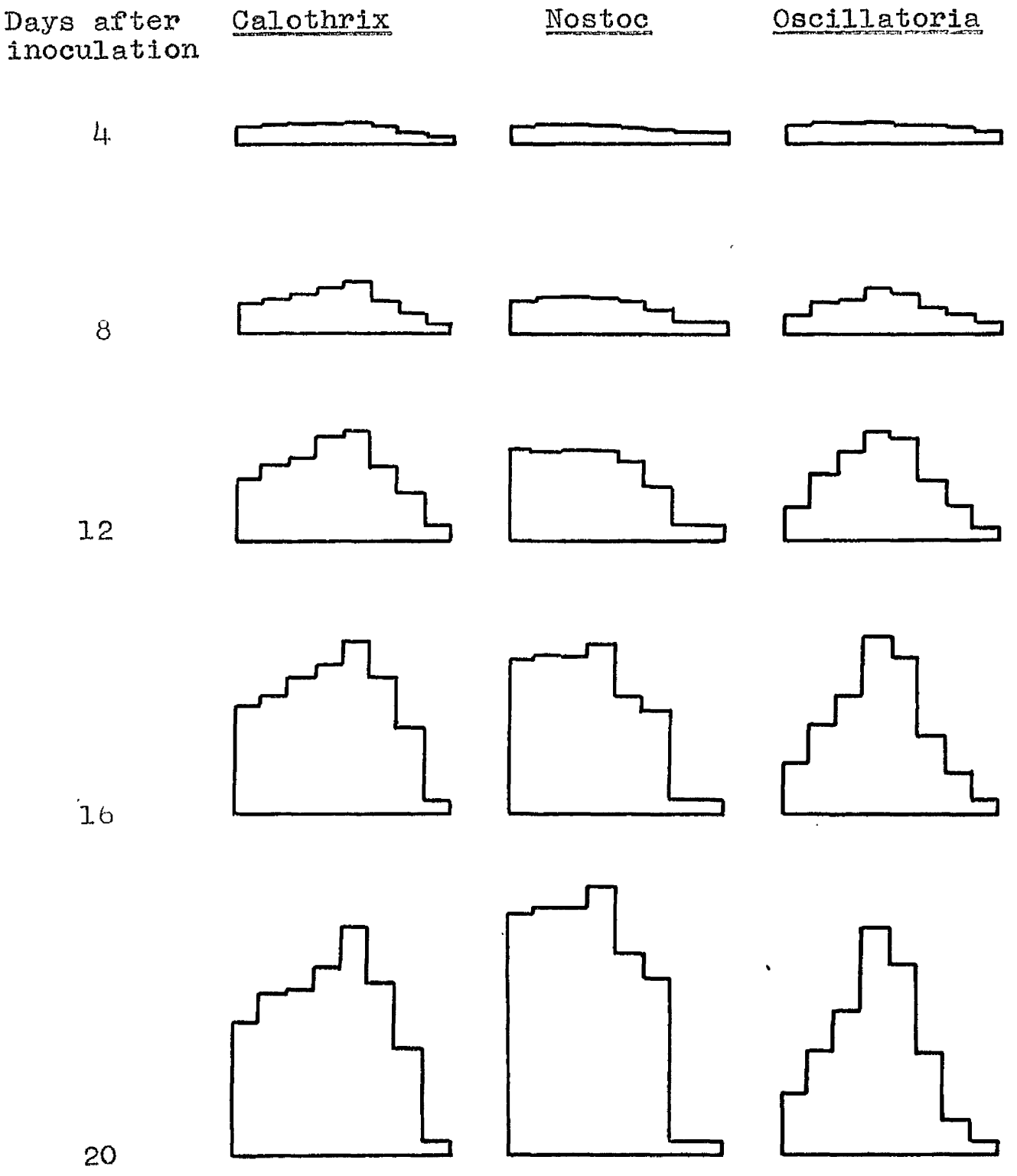
Mean dry weight of inoculum = 1.4 mg.

Ø Algal contents of three flasks combined before harvesting.

@ Differences between means necessary for significance at P = 0.05 are 1.9, 3.0, 3.8 and 2.8 mg., at the 8, 12, 16 and 20 day stages respectively.

* One flask only harvested.

Figure 8



The effect of various salinity concentrations on the growth of Calothrix, Nostoc, and Oscillatoria. Salinity concentrations from left to right = 1.21, 2.03, 3.69, 6.99, 13.50, 26.83, 53.27, and 106.16 parts per thousand.

Over the twenty day growth period there was no difference in growth at the levels 1.21 - 3.69, although it is evident that from the twelve day stage growth at these levels was significantly better than at 26.83. Better growth was also achieved at 13.50 than at 26.83 although at the latter level it was still quite considerable.

Two flasks from each of the salinity levels 53.27 and 106.16 were reduced to a salinity of 10.62 after twenty days. Growth was resumed only in those reduced from 53.27, with the production of 12 mg. dry matter per flask in fifteen days.

OSCILLATORIA BREVIS

Growth occurred during the twenty day period at salinity levels 1.21 - 53.27 parts per thousand. In the range 2.03 - 26.83 growth was vigorous, the alga being deep blue-green in colour. At salinities of 3.69, 6.99 and to a lesser extent 13.50 the alga formed spreading wefts of filaments while the other levels showed more compact colonies. At the 1.21 level the alga at first appeared healthy but by the sixth day had become much paler and it was evident that relatively little growth was occurring.

Harvest data are presented in Table 28 and Fig.8 while typical cultures from each salinity level are shown in Plate 13c. The data show that throughout, best growth was achieved in the region 6.99 - 13.50. Growth at the 6.99 level was significantly greater than at all levels except

TABLE 28

The effect of various salinity levels on the growth of Oscillatoria brevis

Days after inoculation	Mean dry weight of alga (mg.)				
	Ø	Φ	Φ	Φ	Φ
	4	8	12	16	20
Salinity					
1.21	2.2	3.0	5.3	7.3	9.0
2.03	2.3	5.0	9.8	12.5	15.2
3.69	2.3	5.0	12.5	17.8	21.0
6.99	3.0	6.7	16.0	26.0	33.8
13.50	2.2	6.0	14.7	22.7	28.2
26.83	2.2	4.0	8.3	11.2	15.8
53.27	2.0	3.3	5.2	5.7	5.3*
106.16	1.5	1.7	1.7	1.5	1.7*

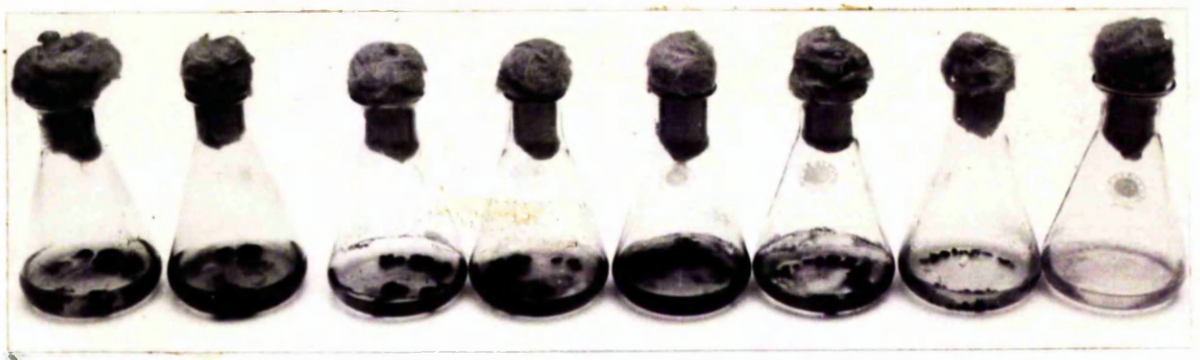
Mean dry weight of inoculum = 1.5 mg.

Ø Algal contents of three flasks combined before harvesting.

Φ Differences between means necessary for significance at P = 0.05 are 1.4, 3.2, 4.8 and 4.9 mg., at the 8, 12, 16 and 20 day stages respectively.

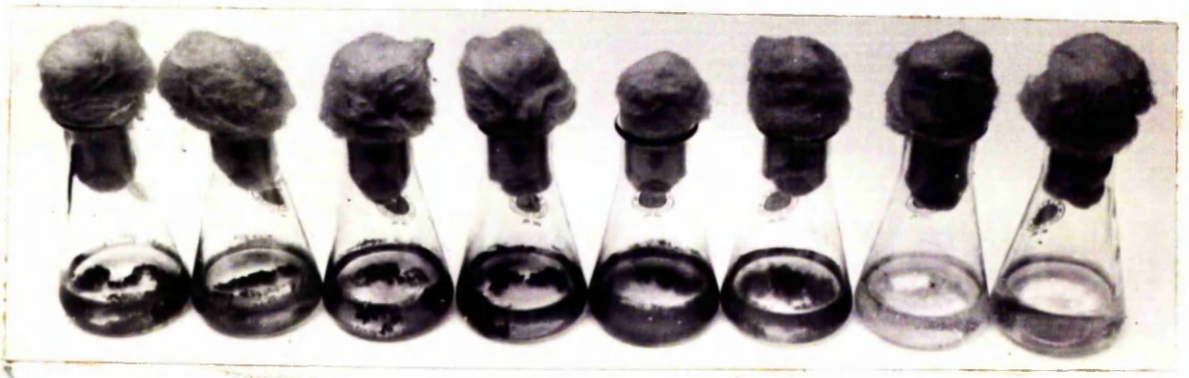
* One flask only harvested.

Plate 13



a)

Calothrix



b)

Nostoc

c)

Oscillatoria

Typical twenty day old cultures of Calothrix, Nostoc and Oscillatoria grown at different salinity levels. Levels from left to right are, 1.21, 2.03, 3.69, 6.99, 13.50, 26.83, 53.27 and 106.16. ($\times\frac{1}{4}$)

13.50, 2.03 and 3.69 from the eight day stage onwards, but at 13.50 it became significantly greater only at the twelve day stage. At 1.21 the mean dry weight was markedly lower than at 2.03. Growth at 2.03 was comparable throughout with that at 26.83, while at 53.27 growth occurred during the first twelve days only. As in the two previous experiments two flasks from cultures at 53.27 and 106.16 were reduced in salinity to 10.61 after twenty days. No growth occurred in those reduced from 106.16, but was marked in those reduced from 53.27, 20 mg. dry weight being obtained after fifteen days.

II. pH EXPERIMENTS

CALOTHRIX SCOPULORUM

The optimum pH level for growth

Harvest and dry weight data are presented in Table 29 and Fig.9. Typical cultures from each level are shown in Plate 11a. Optimum growth from the nine day stage onwards occurred at 7.0 and 8.0, the alga at these levels being deep green, very healthy and aggregated in tufts floating freely in the liquid. It is evident that growth at 6.0 and 9.0 was significantly less after nine days than that at 7.0 and 8.0. No significant difference was noted between growth at 6.0 and 9.0 until the last harvest when growth at 9.0 had fallen off considerably. Throughout the experiment the pH levels did not vary outwith the ranges, 5.0 - 5.2 (pH 5.0), 6.0 - 6.3 (pH 6.0), 7.0 - 7.4 (pH 7.0), 7.9 - 8.3 (pH 8.0),

TABLE 29

The effect of various pH levels on the growth of Calothrix scopulorum in a nitrogen-free medium

Days after inoculation / pH level	Mean dry weight of alga (mg.)*				
	3 Ⓘ	6 Ⓘ	9 Ⓘ	12 Ⓘ	15 Ⓘ
5	2.7	2.5	2.6	2.7	2.6
6	3.5	6.7	11.7	17.0	22.2
7	4.8	8.6	15.0	20.5	24.8
8	5.3	10.2	17.0	22.5	26.8
9	4.3	7.3	11.0	14.5	18.3
10	3.8	5.7	5.8	5.3	6.8
11	2.3	2.7	2.5	2.4	2.7

Mean dry weight of inoculum = 2.6 mg.

* Each value represents the mean of the algal contents of three flasks.

Ⓘ Differences between means necessary for significance at $P = 0.05$ at the 3, 6, 9, 12 and 15 day stages are 3.9, 3.5, 2.3, 3.1 and 2.3 mg. respectively.

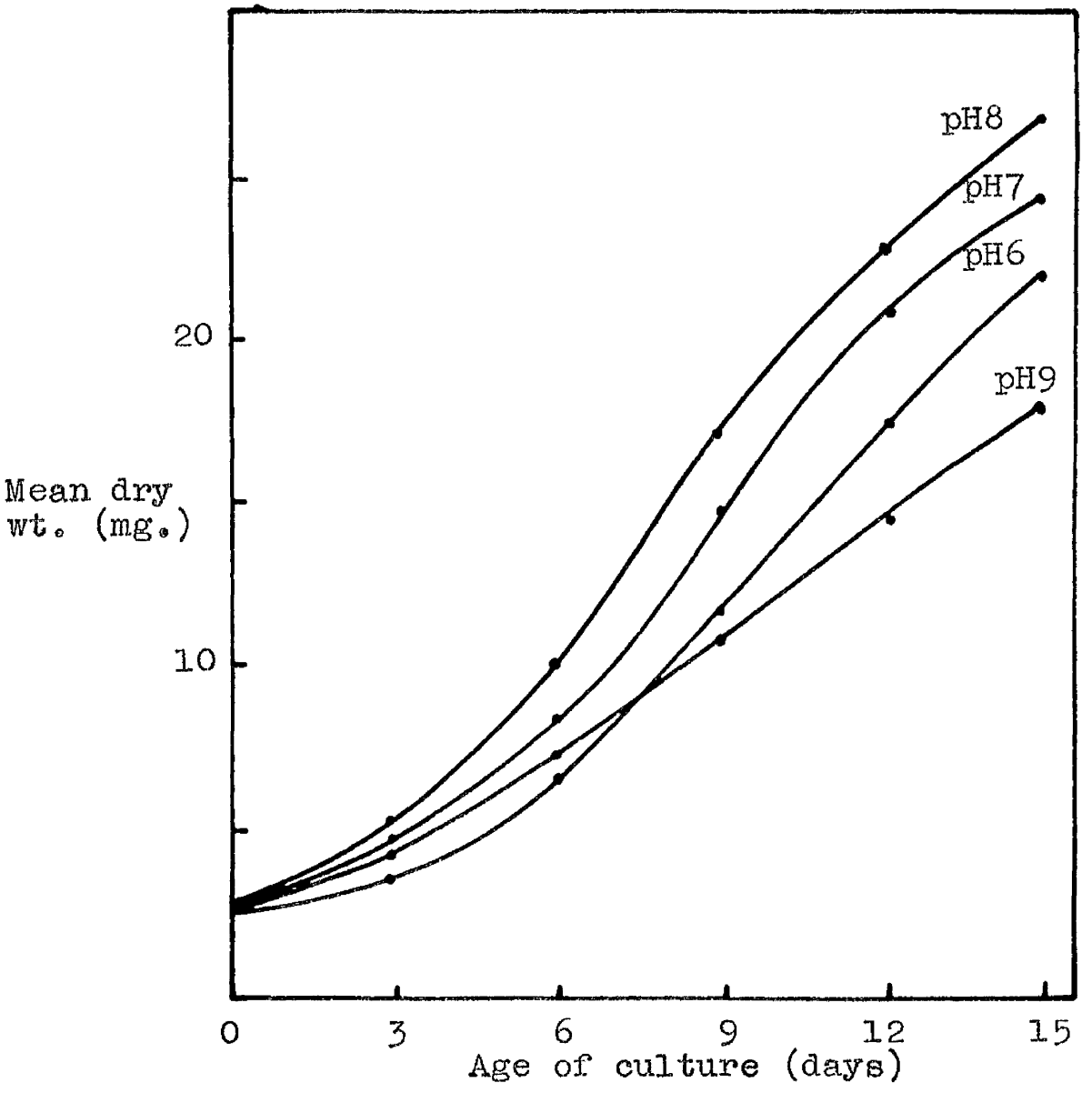
TABLE 30

The pH limits of growth of Calothrix scopulorum

Density (x100) increase after 72 hours

Lower Limit				Upper Limit			
pH	Tube	Increase	Mean Increase	pH	Tube	Increase	Mean Increase
5.2	1	2	1	9.0	1	16	17
	2	2			2	17	
	3	0			3	19	
	4	-2			4	17	
5.4	1	3	0	9.2	1	12	14
	2	0			2	13	
	3	-1			3	11	
	4	-1			4	19	
5.6	1	8	7	9.4	1	10	8
	2	6			2	7	
	3	6			3	6	
	4	9			4	8	
5.8	1	10	11	9.6	1	2	1
	2	13			2	2	
	3	11			3	-2	
	4	11			4	0	
6.0	1	15	16	9.8	1	-3	0
	2	15			2	2	
	3	17			3	-1	
	4	17			4	1	

Figure 9



The effect of various pH levels on the growth of Calothrix scopulorum in a nitrogen-free medium.

9.0 - 8.5 (pH 9.0), 10.0 - 9.4 (pH 10.0) and 11.0 - 9.8 (pH 11.0).

The upper pH limit of growth

The results, reproduced in Table 30, show that growth did not occur above pH 9.4. The cultures at 9.6 retained their colour but at higher levels rapidly became colourless and died. Microscopic examination showed numerous short hormogonia at 9.4. At 9.6 no hormogonia were formed. Tests on the pH of each tube after seventy-two hours showed that no drift had occurred. After a further period of seven days there was no evidence of growth above 9.4. Growth therefore occurred only at those levels which allowed growth at the seventy-two hour stage.

The lower pH limit of growth

In this experiment the lowest pH level at which growth was detected was 5.6 (Table 30). Below 5.6 the cultures became colourless within eighteen hours. As with the upper pH limit no drift occurred during the seventy-two hours of the experiment. On re-examining after a further seven days, growth was noted only in those tubes which showed growth at the seventy-two hour stage.

NOSTOC ENTOPHYTUM

The optimum pH level for growth

The data for this experiment are presented in Table 31 and Fig.10. Typical cultures at the fifteen day stage are shown in Plate 14b. Growth at pH 9.0 was significantly better than at the other levels from the twelve day stage onwards. It also appeared greater during the earlier stages of the experiment although the figures fail to attain significance.

TABLE 31

The effect of various pH levels on the growth of Nostoc entophytum in a nitrogen-free medium

Days after inoculation	Mean dry weight of alga (mg.)*				
	⊕ 3	⊕ 6	⊕ 9	⊕ 12	⊕ 15
pH level					
5	1.5	1.8	1.5	1.7	1.7
6	2.2	3.7	4.3	5.2	6.0
7	3.2	5.2	9.2	17.7	25.3
8	3.5	7.1	12.8	19.5	24.3
9	4.3	8.2	15.8	24.7	32.7
10	3.2	7.5	12.0	15.2	17.7
11	1.7	1.8	2.0	1.5	1.7

Mean dry weight of inoculum = 1.7 mg.

* Each value represents the mean of the algal contents of three flasks.

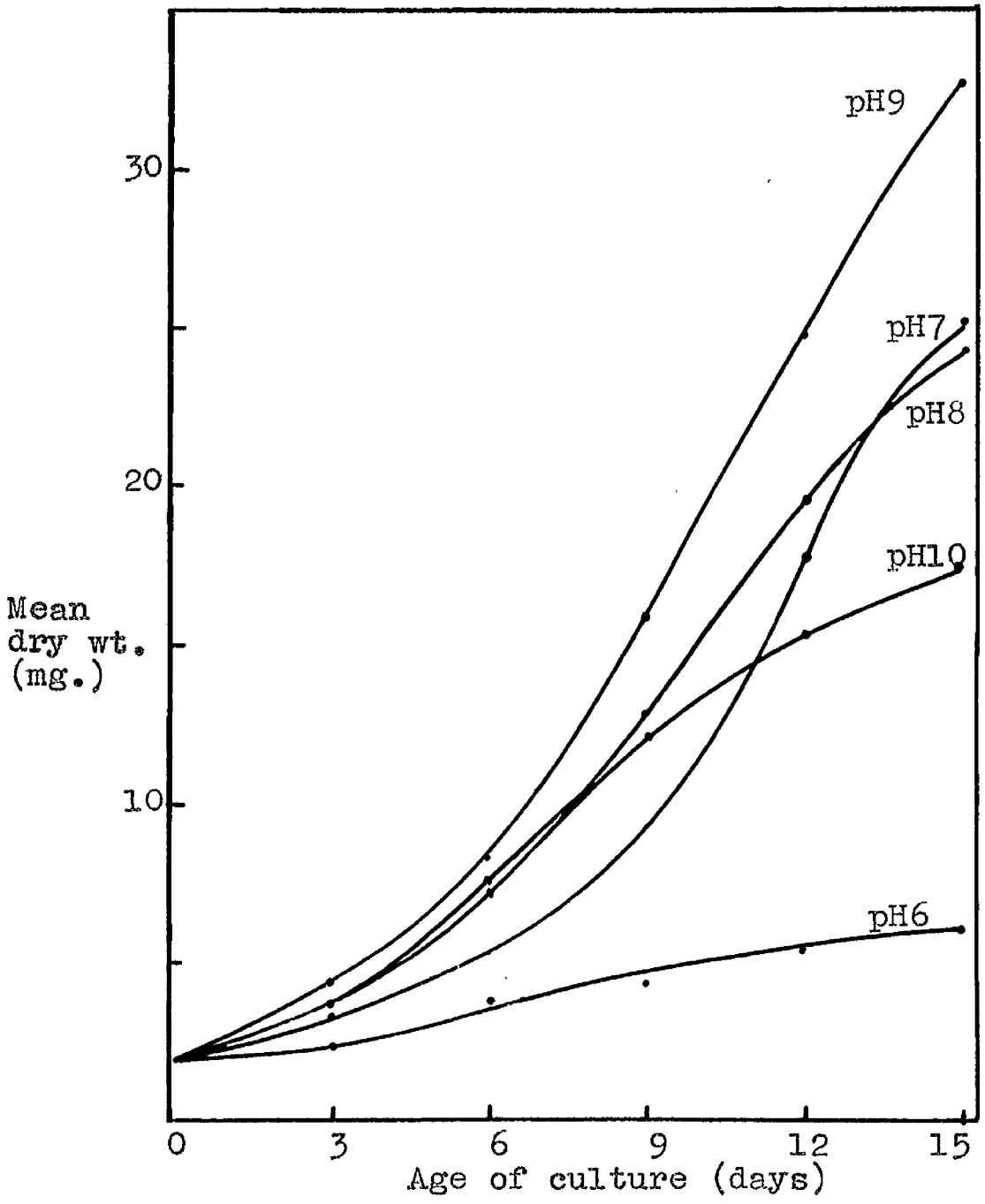
Difference between means necessary for significance at $P = 0.05$, at the 3, 6, 9, 12 and 15 day stages are 1.2, 3.0, 3.7, 4.2 and 4.6 mg. respectively.

TABLE 32

The pH limits of growth of Nostoc entophytum

	Density (x100) increase after 72 hours						
	Lower Limit				Upper Limit		
pH	Tube	Increase	Mean Increase	pH	Tube	Increase	Mean Increase
5.2	1	0	0	9.8	1	45	41
	2	-1			2	40	
	3	0			3	38	
	4	0			4	40	
5.4	1	1	0	10.0	1	24	21
	2	-2			2	19	
	3	0			3	20	
	4	0			4	22	
5.6	1	-1	0	10.2	1	14	9
	2	-1			2	10	
	3	1			3	0	
	4	0			4	12	
5.8	1	6	7	10.4	1	2	1
	2	8			2	0	
	3	7			3	3	
	4	9			4	-2	
6.0	1	14	16	10.6	1	5	1
	2	18			2	0	
	3	16			3	1	
	4	17			4	-2	
6.2	1	18	19	10.8	1	-7	-1
	2	20			2	0	
	3	20			3	0	
	4	18			4	2	

Figure 10



The effect of various pH levels on the growth of Nostoc entophytum in a nitrogen -free medium.

Throughout, there was no significant difference between growth at 7.0 and 8.0 although it was markedly better at the two latter levels than at 6.0. At pH 10.0 growth was rapid at first but fell off markedly during the later stages, becoming significantly less than at 7.0 by the fifteen day stage. No growth occurred at pH levels 5.0 and 11.0. Determinations showed that throughout, the pH levels did not vary outwith the ranges 4.9 - 5.1 (pH 5.0), 6.0 - 6.2 (pH 6.0), 6.9 - 7.2 (pH 7.0), 8.0 - 8.4 (pH 8.0), 8.7 - 9.2 (pH 9.0), 10.0 - 9.6 (pH 10.0), and 11.0 - 9.8 (pH 11.0).

The upper pH limit of growth

The results reproduced in Table 32 show that for this species the upper limit of growth is 10.2. At higher levels no growth occurred although at 10.4 the cultures remained healthy and green. Microscopic examination showed the presence of hormogonia at 10.2 while at 10.4 none were evident. Tests after seventy-two hours showed that no drift in pH had occurred. On examining the cultures macroscopically after a further period of seven days, it was evident that no growth had occurred above 10.2.

The lower pH limit of growth

In this experiment growth did not occur at pH 5.6 or lower, the cultures at these levels becoming colourless within twenty-four hours. At pH 5.8 growth although slow did occur, while marked growth was evident at 6.0 - 6.2. During the seventy-two hours of the experiment no pH drift occurred and

no new tubes showed growth after a further period of seven days.

OSCILLATORIA BREVIS

The optimum pH level for growth

The data obtained are presented in Table 33 and Fig.11 while Plate 11c shows typical cultures at the fifteen day stage. From the second harvest onwards growth at 9.0 was significantly better than at pH levels 5.0, 6.0, 7.0 and 11.0. There was no significant difference in growth at 8.0, 9.0 and 10.0 until the twelve day stage when growth at 10.0 had fallen off rather rapidly. No significant difference was noted between 8.0 and 9.0 until the last harvest when that at 9.0 was significantly better. There was no evidence of growth at pH levels 5.0 and 11.0. Throughout the experiment the pH levels did not vary outwith the following ranges, 5.0 - 5.3 (pH 5.0), 6.0 - 6.3 (pH 6.0), 7.0 - 7.4 (pH 7.0), 8.0 - 8.4 (pH 8.0), 8.8 - 9.2 (pH 9.0), 9.6 - 10.0 (pH 10.0) and 11.0 - 10.0 (pH 11.0).

The upper pH limit of growth

The results, reproduced in Table 34, show that for this species the upper pH limit is 10.2. At this level although growth occurred the filaments were more yellow in colour than at the lower levels. Above 10.2 the cultures rapidly became colourless. No drift in pH occurred throughout and no growth was observed in cultures above 10.2 even after a further seven days.

TABLE 33

The effect of various pH levels on the growth of Oscillatoria brevis

Days after inoculation / pH level	Mean dry weight of alga (mg.)*				
	3 Φ	6 Φ	9 Φ	12 Φ	15 Φ
5	1.5	1.3	1.7	1.5	1.5
6	2.3	4.0	5.5	6.7	7.7
7	3.2	5.5	8.8	10.3	11.8
8	4.3	8.2	10.8	14.3	17.2
9	4.3	9.0	12.7	16.3	19.0
10	4.3	8.7	11.0	12.5	14.0
11	1.5	1.5	1.7	1.7	1.7

Mean dry weight of inoculum = 1.5

* Each value represents the mean of the algal contents of three flasks.

Φ Difference between means necessary for significance at $P = 0.05$ at the 3, 6, 9, 12 and 15 day stages are 2.9, 3.3, 2.9, 3.0 and 1.8 mg. respectively.

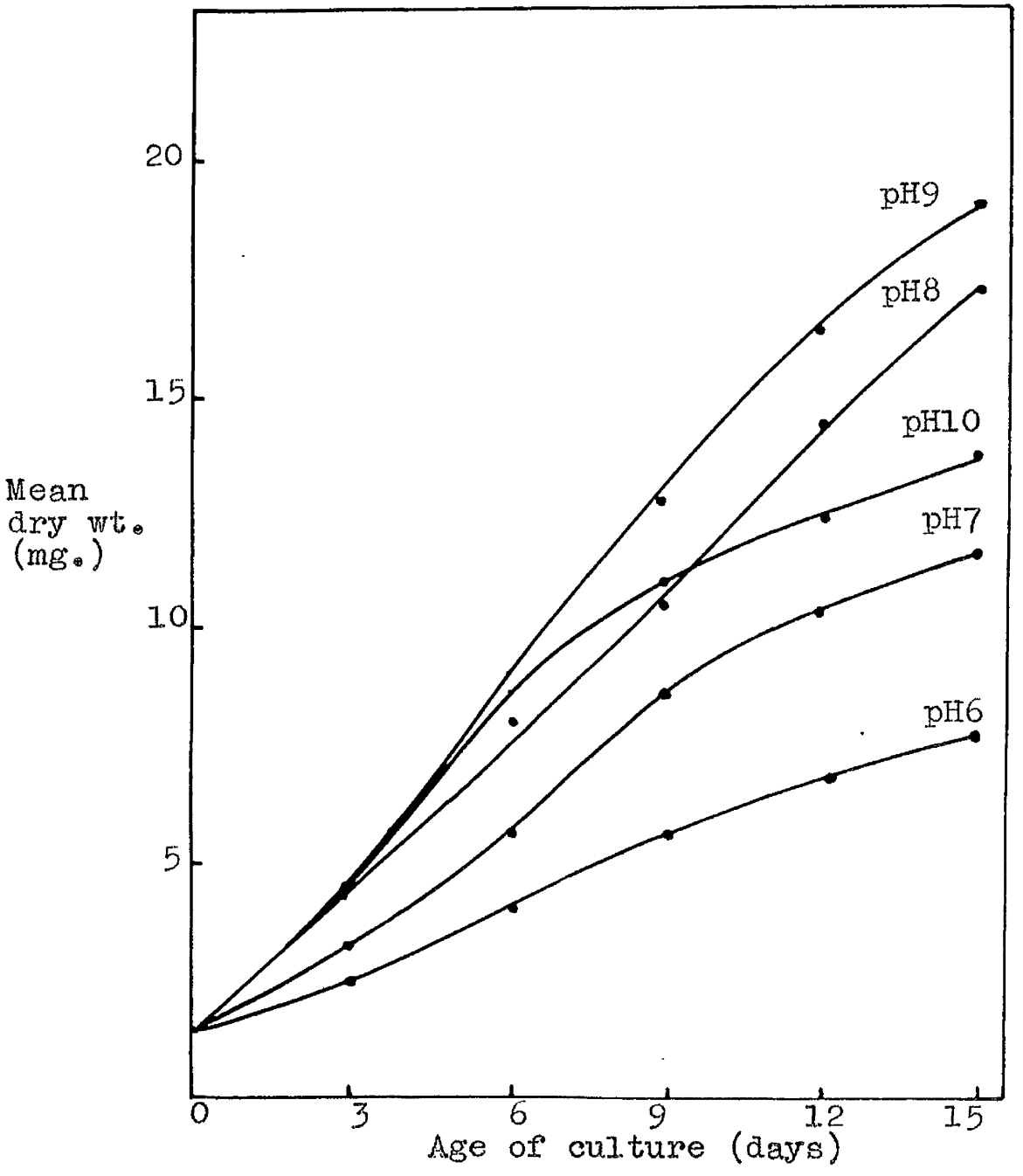
TABLE 34

The pH limits of growth of Oscillatoria brevis

Density (x100) increase after 72 hours

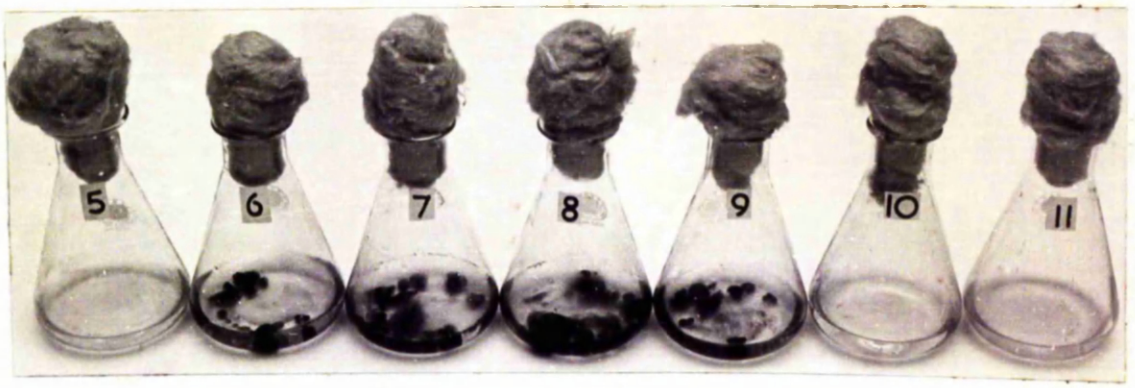
Lower Limit				Upper Limit			
pH	Tube	Increase	Mean Increase	pH	Tube	Increase	Mean Increase
5.2	1	-1	0	9.6	1	26	27
	2	1			2	28	
	3	2			3	30	
	4	-2			4	25	
5.4	1	0	0	9.8	1	25	25
	2	3			2	25	
	3	-1			3	28	
	4	-1			4	22	
5.6	1	4	3	10.0	1	17	15
	2	2			2	16	
	3	2			3	15	
	4	3			4	13	
5.8	1	9	9	10.2	1	8	6
	2	8			2	5	
	3	8			3	3	
	4	10			4	7	
6.0	1	10	12	10.4	1	-2	0
	2	12			2	3	
	3	10			3	2	
	4	14			4	-2	

Figure 11

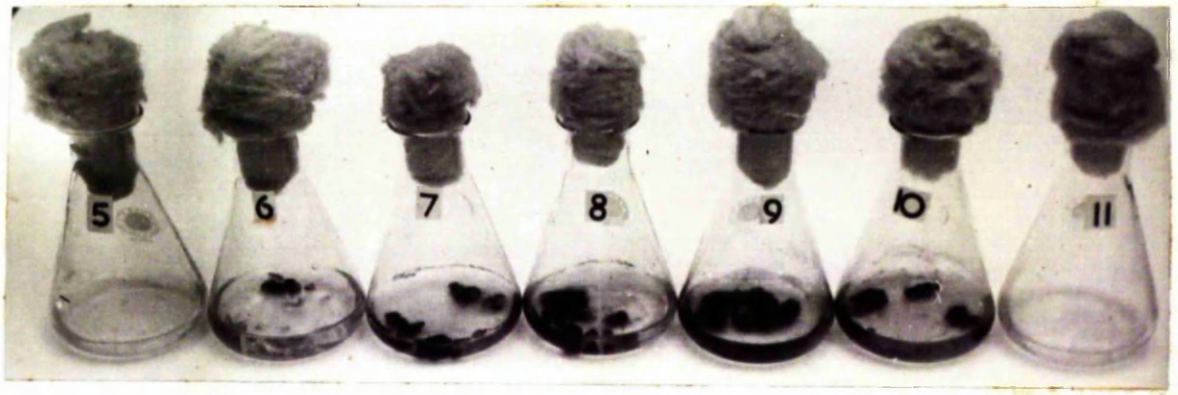


The effect of various pH levels on the growth of Oscillatoria brevis.

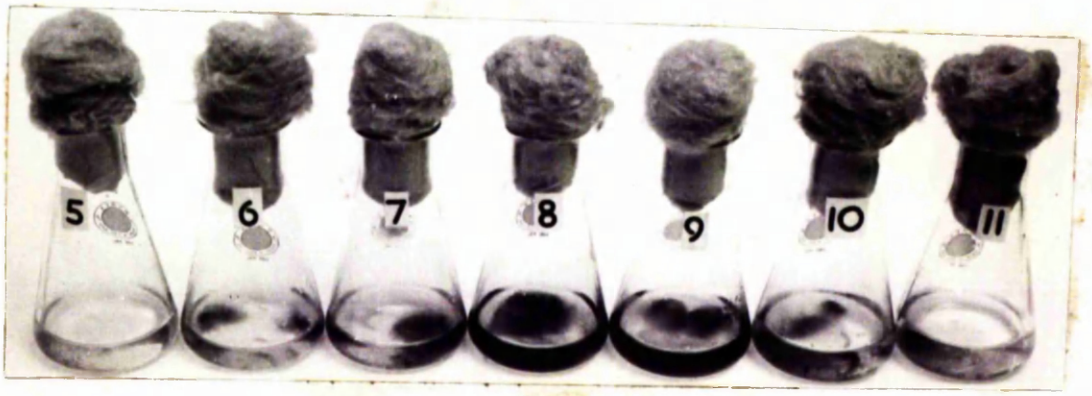
Plate 14



a) Calothrix



b) Nostoc



c) Oscillatoria

Typical fifteen day old cultures of Calothrix, Nostoc and Oscillatoria grown at different pH levels. Levels from left to right are, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. ($\times\frac{1}{4}$)

The lower pH limit of growth

The results reproduced in Table 34 show that this species closely resembles the two nitrogen-fixers in that the alga cannot grow below 5.6, the filaments becoming colourless within thirty-six hours. At 5.6 growth was slow but became more rapid with increase in pH. No drift in pH occurred and no growth was evident below 5.6 after a further period of seven days.

D I S C U S S I O N

In the salinity experiments, although artificial sea water media were employed it is unlikely that the effect would have been different had various dilutions and concentrations of natural sea water been used. In the first instance the species under consideration do not require organic substances present in natural sea water; secondly the constitution of the media was so designed that the salinity variations were due to differences in the overall concentrations of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Cl^{-} and SO_4^{2-}, the ions chiefly responsible for salinity variations in natural sea water. Droop (1958a) has shown that of the above ions, Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} chiefly influence algal growth, Cl^{-} and SO_4^{2-} being widely interchangeable. The proportions of Na, Mg and Ca relative to each other, were therefore maintained constant at ratios almost identical with those in natural sea water as can be seen from the following table:-

Ratio	Artificial sea water	Natural sea water (data from Macan and Worthington, 1951)
Ca/Mg	0.33	0.32
Na/Ca	25.16	25.52
Na/Mg	8.23	8.12

Owing to the necessity of using K_2HPO_4 as a non-nitrogenous buffer it was impossible to maintain the ratio of K^+ to the other ions constant throughout. The data obtained will nevertheless afford a worthwhile comparison with what is likely to occur in Nature.

Data on salinity ranges show that all species are capable of growth, and of nitrogen fixation in the case of Calothrix and Nostoc, over very wide salinity ranges. In Calothrix and Nostoc nitrogen fixation occurs over the ranges 1.21 - 53.27 and 1.21 - 26.83 parts per thousand respectively while Oscillatoria grew over the range 1.21 - 26.83. Although the lowest levels for growth were not determined the results indicate that Calothrix and Nostoc can grow and fix nitrogen at even lower salinities. Further experiments on Calothrix and Nostoc, in which the algae were inoculated into salinity levels of 26.83, 53.27 and 106.16 to which 0.01 gm. sodium nitrate per litre had been added, were carried out. These showed that growth occurred in the presence of nitrogen only at those levels which allowed growth in a nitrogen-free medium. These results show that the factor limiting growth of Calothrix and Nostoc in nitrogen-free medium at high salinities, is not inhibition of the nitrogen-fixing process.

The ability of these algae to withstand very high salinities is seen from the Nostoc and Oscillatoria experiments, where resumption of vigorous growth occurred

on the reduction of salinity from 53.27 to 10.62. Provasoli et al (1954) records a similar phenomenon with the flagellate Peridinium balticum; he found that at high salinities this species failed to divide although it remained alive. The present data imply that all three blue-green algae will be capable of survival in the supralittoral fringe even when large fluctuations in salinity occur.

The dry weight data show that optimum growth of Calothrix scopulorum occurred between 6.99 and 13.50. At 26.83 growth although significantly less than at 13.50 was still considerable and it is probable that the optimum salinity level for this strain of Calothrix scopulorum is nearer to 13.50 than 6.99.

In the case of Nostoc entophytum it is obvious that this species is very euryhaline and that a distinct optimum of 6.99 is reached only at the twenty day stage, for although growth at this level appeared better from the early stages, only after twenty days did it attain significance. At the earlier stages no difference in growth was noted over the range 1.21 - 6.99 and this species appears to have fresh water tendencies in its mineral requirements, a feature which accords with its frequency in brackish waters. At 26.83 growth, though slow at first, increased markedly by the twelve day stage, but always remained significantly less than at the lower levels. It is possible that this species takes a certain amount of time to adapt itself to high salinities but once it does so vigorous growth can occur.

Mean dry weight data for Oscillatoria brevis show that this species is intermediate between the two nitrogen-fixers in its salinity tolerance. Optimum growth occurs, from the sixteen day stage onwards, between 6.99 and 13.50 although growth at the 6.99 level is significantly greater than the others, except 13.50, by the twelve day stage. Compared with the two nitrogen-fixing species a marked feature is the poor growth which occurs at 1.21 suggesting that this level is very near the lowest salinity concentrations at which this strain can grow. At the other extreme, growth occurred at 53.12 during the first few days only; this level being near, or at, the uppermost salinity level for growth.

As the three organisms studied are all of upper littoral or supralittoral origin a comparison can be made with data obtained by Droop (1958a) on the effect of various concentrations of Na^{+}, Ca^{2+}, K^{+} and Mg^{2+} on the growth of the following supralittoral organisms: Helmiselmis virescens (a cryptomonad), Monochrysis lutheri (a chrysomonad), Phaeodactylum tricornatum and Nannochloris oculata (Chlorophyceae). From these data it can be calculated that Helmiselmis, Nannochloris and Monochrysis have optimum salinity levels near to 11, 11, and 4 parts per thousand, the overall salinity ranges of the three species being approximately 1 - 29, 1 - 29 and 2 - 58 parts per thousand respectively. The present results show that Calothrix scopulorum and Oscillatoria brevis resemble Helmiselmis

and Nannochloris in their optimum salinity levels for growth, while Nostoc entophytum resembles Monochrysis more closely in this respect. From the point of view of overall salinity tolerances Calothrix resembles Monochrysis in being extremely halotolerant while Oscillatoria and Nostoc resemble Helmiselmis and Nannochloris more closely. A notable feature is that the above blue-green algae appear capable of withstanding lower salinities than any of Dr. Droop's algae. Furthermore the results show that C. scopulorum is one of the most halotolerant algae so far isolated in pure culture.

The optimum total salinity concentrations for the present species are probably lower than that likely to be normally encountered in Nature. This phenomenon has been noted by other workers with different classes of organisms; for example Braarud (1951) and Braarud and Pappas (1951) showed the optimum salinity concentrations for several coastal species collected from waters of 25 - 30 parts per thousand to be 20 - 25 parts per thousand.

On comparing the results from an ecological point of view it is seen that the tolerances of these blue-green algae to pronounced variations in total salinity correspond with their ecological niche on the supralittoral fringe where wide variations in total salinity occur. In the case of Calothrix comparison can be made with the ecological data obtained by Ercegovic (1930) (Table 25) who estimated salinity by determining the chloride concentration and obtaining total

salinity by multiplying this result by the co-efficient of 1.811. As the culture solution employed in the present experiments consists almost entirely of chlorides, this method of estimating salinity can also be applied to the present culture medium. When this is done the salinity concentrations of 1.21, 2.03, 3.69, 6.99, 13.50, 26.83, 53.27 and 106.16 are equivalent to total salinities of 0.84, 1.67, 3.33, 6.66, 13.31, 26.62, 53.27 and 106.47 parts per thousand. From these data it can be said for comparative purposes that C. scopulorum is capable of growth and nitrogen fixation at salinity concentrations ranging from 0.84 to 53.24 parts per thousand. These data agree closely with those obtained in Nature by Ercegovic who observed growth of Calothrix at salinities of 4.33 - 37.6, but not in the upper littoral and supralittoral pools where salinity varied from 0 - 283 depending on factors such as rain and dessication. From the present data it would appear that inhibition of growth in these pools is due more to high salinity produced by evaporation than to low salinity produced by the influx of fresh water. This increase in salinity which no doubt occurs during the warm summer months may be one of the factors responsible for the disappearance then, of a large proportion of the supralittoral blue-green algae, and not solely dessication which has generally been accepted as the directly responsible factor.

From the results of the present experiments it can be

concluded that Nostoc entophytum, Calothrix scopulorum and Oscillatoria brevis are among the most euryhaline supralittoral species so far examined, and that Calothrix and Nostoc are capable of nitrogen fixation over very wide salinity ranges. Their euryhaline nature would appear to be one of the main factors responsible for their presence in the supralittoral fringe.

Turning to consider the data on the effect of various hydrogen ion concentrations on growth, it is seen that in addition to withstanding varying salinity concentrations the algae are capable of growth and nitrogen fixation (Calothrix and Nostoc) over wide pH ranges.

The optimum pH of 7.0 - 8.0 for Calothrix is rather similar to that for the fresh water species Nostoc muscorum, reported by Allison et al. (1937). It is of interest to note that while Calothrix grows better at a pH near to that of fresh water it is the most halotolerant of the three species examined. The optima of 8.0 - 9.0, and 9.0 for Oscillatoria and Nostoc respectively are close to (Oscillatoria) and slightly higher than (Nostoc) the pH of normal coastal water which is near to 8.2. The latter may vary considerably however in the supralittoral fringe depending on such factors as the amount of fresh water present, dessication and the rate of photosynthesis of the algae present.

It appears therefore that certain supralittoral blue-green algae have relatively high pH optima although species

with optima near to that of fresh water are present. It must however be noted that certain fresh water species have pH optima nearer to sea water than to fresh water (Zehnder and Gorham, 1960) and it is probable that the optimum pH levels for the present species do not greatly affect their distribution. Their distribution however may be affected by their tolerance to varying pH levels. A significant reduction in growth of all three algae, compared with that at 7.0 and 8.0 occurs at 6.0, the algae rapidly being killed at 5.0. The poor growth obtained at 6.0 agrees with most other data for fresh water species. At pH levels above the optimum, the algae grow vigorously during the earlier stages of growth but generally slow down as the cultures age. Too much stress however cannot be laid on this aspect as a tendency toward drop in pH occurred at these very high levels.

Data on the lower limits of growth show that Oscillatoria cannot grow below a pH level of approximately 5.6, while the two nitrogen-fixing species Calothrix and Nostoc cannot fix nitrogen below 5.6 and 5.8 respectively. These results are in close agreement with the findings of Allison et al.(1937) for Nostoc muscorum and differ sharply from those obtained by Walp and Schopbach (1942). It is evident therefore that although the optimum pH for growth of Nostoc and Oscillatoria is higher than that of Calothrix, the lower limits of all three species are very similar.

On comparing the upper limits of growth it is evident

that variation occurs depending on the species, being 10.2, 10.2 and 9.4 for Nostoc, Oscillatoria and Calothrix respectively. Comparison of the pH range for each species shows that Nostoc is capable of nitrogen fixation over the widest range.

The pH range for all three species is very wide and under the normal conditions of the supralittoral fringe the algae should be capable of withstanding any pH variations which are likely to occur. The fact that the optimum pH level varies for the three species suggests that the distribution of the algae on the shore cannot be correlated with optimum pH conditions, in fact it would appear that Calothrix scopulorum, the most abundant alga on the supralittoral fringe, is in natural sea water growing at a sub-optimal pH level.

S U M M A R Y

1) Experiments on the effect of various salinity concentrations on the growth of Calothrix scopulorum and Nostoc entophytum in nitrogen-free media have been carried out, while comparative data have been obtained for Oscillatoria brevis grown in the presence of combined nitrogen.

2) The results showed that all three species are very euryhaline in nature. Salinity optima occur in the region 6.99 - 13.50 parts per thousand for both Calothrix and Oscillatoria while the optimum for Nostoc is near to 6.99 parts per thousand.

3) The species are capable of survival at certain very high salinity levels although no multiplication occurs.

4) The inability of Calothrix and Nostoc to grow at very high salinity levels in the absence of combined nitrogen is due to factors other than inhibition of the nitrogen-fixing process.

5) The euryhaline nature of these algae accords with their position on the marine supralittoral fringe.

6) Experiments to determined the optimum, maximum and minimum pH levels for growth of Calothrix and Nostoc in nitrogen-free medium have been carried out. Similar experiments have been carried out in the case of Oscillatoria grown in the presence of combined nitrogen.

7) The results show that Calothrix, Nostoc and Oscillatoria have pH optima near to 8.6, 9.0 and 8.0 - 9.0, upper pH limits of 9.4, 10.2 and 10.2, and lower pH limits 5.6, 5.6 and 5.6 respectively.

8) The effect of hydrogen ion concentration is not thought to seriously affect the distribution of these algae on the supralittoral fringe.

P A R T IV

Studies on the nitrogenous composition of *Calothrix scopulorum*, *Nostoc entophytum* and *Oscillatoria brevis*

I N T R O D U C T I O N

The fact that Calothrix scopulorum and Nostoc entophytum are vigorous nitrogen fixers leads to speculation as to their nitrogenous composition. In the first instance it is thus desirable to determine the nature of the amino acids present, as these are the building blocks from which the cell proteins are derived. Determination of the amino acid composition of such algae is a necessary precursor of any future experiment on their nitrogen metabolism and in addition valuable data on the amino acid composition of marine blue-green algae will be recorded. Although data are available for fresh water Myxophyceae there appears to be no comparable information on the amino acid composition of marine species. Data on the composition of the above Calothrix and Nostoc species will afford a comparison with the non-nitrogen-fixing species Oscillatoria brevis and with nitrogen-fixing fresh water blue-green algae.

Furthermore the analyses of the amino acids present in marine blue-green algae may be of use in assessing the possible importance of the mass culturing of these species. Fogg (1958) suggested that many marine algae were suitable as feeding stuffs and perhaps as sources of valuable chemicals, but that no organism completely satisfactory for mass culture had yet been found.

The earliest recorded work on the amino acid composition of blue-green algae appears to be that of Mazur and Clarke (1938) who investigated the amino acid composition of two fresh water Phormidium species; only one, Phormidium valderianum Gom. was investigated in detail. In their experiments protein was extracted in hot 90 per cent formic acid, it being assumed that this fraction comprised all the protein components. After hydrolysis in 25 per cent sulphuric acid on a steam bath for three days aliquots of hydrolysates were then tested for the presence of various amino acids. The results (Table 35) showed the presence of fourteen amino acids, of which arginine, valine and proline contained the highest quantities of nitrogen. Cystine and lysine were reported as absent. The percentage total nitrogen recovered in the form of amino-nitrogen amounted to 46 per cent. In a further paper Mazur and Clarke (1942) reported that another fresh water blue-green alga Gleotrichia echimulata, like Phormidium, was devoid of cystine.

With the introduction of the techniques of chromatographic separation, the study of the amino acid composition of various organisms was very much facilitated. Watanabe (1951) by means of two-dimensional paper chromatograms qualitatively investigated the free acids present in four fresh water blue-green algae: Tolypothrix tenuis, Calothrix brevissima, Anabaenopsis sp. and Nostoc sp. The amino acids present in the free and combined state in Anabaena cylindrica were investigated qualitatively by Fowden (1951), using two

dimensional paper chromatography. Fifteen amino acids were detected in the free state, while eighteen were present in the bound form. Lysine, both as the free and combined form, was recorded for this species.

Quantitative analyses of the amino acid composition of the proteins of Anabaena cylindrica carried out by Fowden were quoted by Fogg (1953). In a further paper (Fowden, 1954), details of the procedure employed were given and the presence of cystine in A. cylindrica was also recorded. The results obtained are reproduced in Table 35. Fowden, after breaking up the cells in a cell-disintegrating apparatus extracted the protein in a solution of borax and ethanol-ether. The pH was adjusted to 4.5 with acetic acid and warmed to 70°C to flocculate the protein which was then centrifuged down and collected. The protein was hydrolysed, using a mixture of concentrated hydrochloric acid and glacial acetic acid containing stannous chloride, at 100°C for twenty-four hours. Amino acid estimations were carried out by a method of paper chromatography, while cystine and tryptophane were assayed by specific colorimetric procedures.

Column chromatography, carried out by Williams and Burris (1952), using either starch or an ion exchange resin, yielded a partial analysis of the total amino acid composition of the fresh water species Nostoc muscorum, Calothrix parietina and Microcystis (Diplocystis) aeruginosa. In these experiments the basic amino acids were not determined. However, Magee and

TABLE 35

The amino acid composition of several

The amino acid composition of several

fresh water blue-green algae

Amino Acid	Phormidium valderianum (Mazur and Clarke, 1938)	Anabaena cylindrica (Fowden, 1954)	Nostoc muscorum (Magee and Burris, 1954)
	*	*	ø
Alanine	5.2	6.0	6.8
Arginine	9.2	11.7	19.7
Aspartic acid	0.9	6.9	7.7
Cystine	0.0	-	1.1
Glutamic acid	4.4	5.6	6.2
Glycine	1.6	5.5	5.4
Histidine	3.8	2.5	2.6
Isoleucine	-	3.9	2.8
Leucine	2.1	6.2	4.6
Lysine	0.0	6.6	5.3
Methionine	2.0	1.2	0.4
Phenyl-alanine	1.1	2.9	2.4
Proline	7.0	5.0	2.5
Serine	-	2.4	4.2
Threonine	-	5.7	3.1
Tryptophane	0.2	1.0	-
Tyrosine	1.8	1.6	2.1
Valine	6.7	7.0	3.3
Amide	-	8.0	-
Hydrolysate ammonia	-	-	7.7
Total	46.0	89.7	88.9

* Amounts as percentage protein nitrogen present in each amino acid.

ø Amounts as percentage total cell nitrogen present in each amino acid.

Burris (1954) published a detailed account of the amino acid composition of Nostoc muscorum. In this study the nitrogenous substances were fractionated on a Dowex 50 column. Amino acids were analysed by the photometric ninhydrin method of Moore and Stein (1948). The results reproduced in Table 35 showed almost twice as much arginine as was recorded by earlier workers.

Work and Dewey (1953) reported the presence of a new amino acid from blue-green algae. This was α,ε-diaminopimelic acid which was present to the extent of 0.1 to 0.8 per cent of the dry weight of the blue-green algae, Anabaena cylindrica, Oscillatoria species, and Mastigocladus laminosus. Apart from the Myxophyceae, α,ε-diaminopimelic acid was reported by the above workers as occurring only in certain bacteria, although many other organisms were tested. Since then, minute quantities have been reported as present in Chlorella ellipsoidea by Fuziwara and Akabori (1954), and later confirmed by Hoare and Work (1957).

Smith (1954) reported analyses of the amino acid composition of certain marine Myxophyceae, but no detailed data were provided, either on the species used or on the amino acid composition. No further publications by the above author on this aspect have been traced.

Linko, Holm-Hansen, Bassham and Calvin (1957) isolated and identified citrulline from extracts of Nostoc muscorum and also recorded it as present in Anabaena. Prior to this Norris, Norris and Calvin (1955) had recorded an unknown spot on

chromatograms from Phormidium species, Nostoc muscorum, Nostoc species and Synechococcus cedrorum. This was later identified as citrulline.

From the above review it appears that, although data on the amino acid composition of blue-green algae are available for fresh water species and also for other marine algae (literature summarised by Lewis and Gonzalves, 1960), few data are available on marine Myxophyceae. To obtain such data it was decided to carry out the experiments to be described on the two nitrogen-fixing algae Calothrix scopulorum and Nostoc entophytum. For comparative purposes the non-nitrogen-fixing species Oscillatoria brevis was also included.

M E T H O D S

Culturing of the algae

In the present experiments the algae were grown aseptically from pure culture in 250 ml. Pyrex conical flasks stoppered with cotton wool. Each flask contained 100 ml. of medium. The medium, light intensity and temperature employed for the culture of each species were similar to those described in Part I of this Section. The algae were harvested after twenty days, washed three times in distilled water and then dried in vacuo.

Hydrolyses of the algae

This was normally carried out using 6N hydrochloric acid (constant boiling mixture) in sealed ampoules at 115°C for seventeen hours. Samples were also hydrolysed at 85°C for seventeen hours to detect the presence of any amino acids which might be destroyed by more severe hydrolysis. After hydrolysis the humin was removed by centrifugation and the supernatant evaporated to dryness in vacuo to remove the hydrochloric acid. The residues were then made up to a volume of 3 ml. with distilled water and duplicate 0.3 ml. samples taken and analysed for total nitrogen by the Kjeldahl procedure using a Markham still.

Paper partition chromatography

Preliminary qualitative investigations of the amino

acids present were carried out using two dimensional paper chromatography. Whatman No.1 chromatography paper was used throughout. The samples of hydrolysate applied to each chromatogram contained approximately 60 ugm of nitrogen. The solvent system employed in the first dimension was butanol/acetic acid/water (4:1:5 by volume), followed by water saturated phenol in the presence of ammonia vapour and potassium cyanide, in the second dimension. The amino acids were detected by dipping the chromatograms in a 0.2 per cent (w/v) solution of ninhydrin in acetone, the colour being developed at 90°C for five minutes.

Column chromatography

Fractionation of the nitrogenous substances in the hydrolysate of each alga was carried out on a sulphonated polystyrene resin column using the method of Moore and Stein (1954). The resin used in this instance was Zeokarb 225, which had a nominal degree of cross-linking of 5 per cent. 2.0 ml. fractions of the effluent were collected on an automatic fraction collector.

Analyses of 0.25 ml. samples from alternate fractions for amino nitrogen were carried out using the photometric ninhydrin method of Moore and Stein (1948) but employing the modified ninhydrin reagent of Cocking and Yemm (1954). Glycine was used as standard. Colour intensity was then plotted against effluent volume and the nature of each peak tentatively identified by comparison of their position of

elution with data obtained by Moore and Stein loc. cit. The nature of the substance or substances responsible for each peak was then confirmed using two dimensional paper chromatography as described above.

Because elution of the amino acids was carried out in a citrate buffer, it was necessary to remove the salt before the samples could be applied to paper. The electrolytic method of Stevens, Smith and Jepson (1954) proved unsatisfactory while high voltage electrophoresis (Smith, 1960) without prior removal of the salt was also unsatisfactory. The method which proved the most suitable was that of passage through ion exchange resins. The acidic and neutral amino acid-containing fractions were applied to a 0.9 x 1.2 column of Dowex 2 in the hydroxyl form, washed with 25 ml. of water and the amino acids then eluted with 1N acetic acid (Dreze, Bigwood and Moore, 1954). Desalting of the basic amino acids was carried out by using a 0.9 x 1.0 cm. column of Zeokarb 225 in the ammonium-form. After application of the sample, the column was washed with 0.01N acetic acid and the amino acids eluted with 0.05N ammonia.

From the data obtained the percentage of the total nitrogen, accounted for by each amino acid, was calculated.

DATA OBTAINED

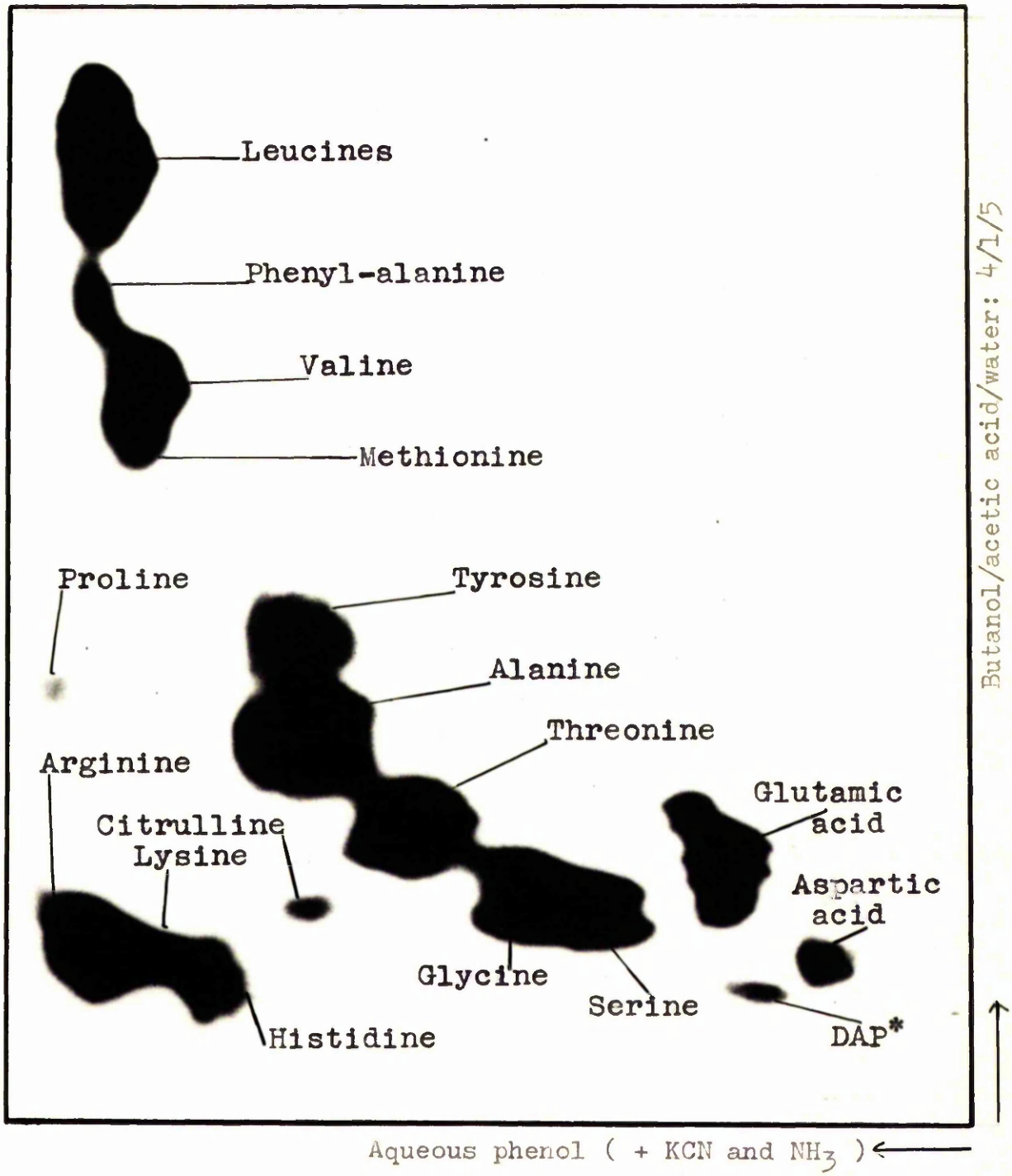
Nostoc entophytum

Paper chromatography revealed the presence of seventeen amino acids of which alanine, glutamic acid, valine, threonine, leucine and arginine were the most abundant. In addition to the common protein amino acids a spot suggestive of the presence of α,ε-diaminopimelic acid was detected, as can be seen from Plate 15. As cystine has similar R_F values in the solvent systems employed the presence of α,ε-diaminopimelic acid was confirmed using the peroxide treatment recommended by Work and Dewey (1953). Citrulline was detected on paper chromatograms of algae hydrolysed at 85°C. This was confirmed by spraying with p-aminobenzaldehyde reagent.

Data on quantitative analysis of the amino acids present are presented in Table 36. Column chromatography revealed a total of twenty-one amino acids, these representing 92.93 per cent of the total nitrogen present. Of those present arginine accounted for the highest percentage of total nitrogen (approximately 15 per cent) followed by alanine and glutamic acid, and almost equal proportions of lysine, valine and threonine.

In addition α,ε-diaminopimelic acid, cystine, cysteic acid and ornithine were detected. α,ε-diaminopimelic acid

PLATE 15



Two-dimensional paper chromatogram of an 85°C hydrolysate of Nostoc entophytum

*α,ε-diaminopimelic acid

accounted for 3.08 per cent of the total nitrogen while ornithine was present in appreciable quantity. No citrulline was detected. In addition to the above amino acids a substance (or substances) responsible for 5.82 of the total nitrogen was observed between the alanine and valine fractions. The nature of this substance was not determined.

Calothrix scopulorum

Paper chromatography of the algal hydrolysates revealed that the amino acids present were very similar to those of Nostoc but in addition there was present a fast running spot in the phenol direction, as can be seen from Plate 16. This spot was detected in all hydrolysates of C. scopulorum irrespective of whether the hydrolysis was carried out at 85°C or 115°C. α,ε-diaminopimelic acid was again detected as was citrulline in 85°C hydrolysates.

Quantitative data are presented in Table 36. In this experiment a total of twenty-two peaks were detected, of which twenty were identified as specific amino acids. The percentage nitrogen recovered in the form of amino acid nitrogen was 90.27 per cent. Very large quantities of nitrogen were again detected as arginine, followed by alanine and glutamic acid while the smallest proportions were present in cysteic acid and tyrosine. Distinct α,ε-diaminopimelic acid and ornithine peaks were detected. Two undetermined peaks were noted on fractionation, one between alanine and valine, and the other immediately after histidine.

TABLE 36

The amino acid composition of three marine blue-green algae

The amino acid composition of three marine blue-green algae

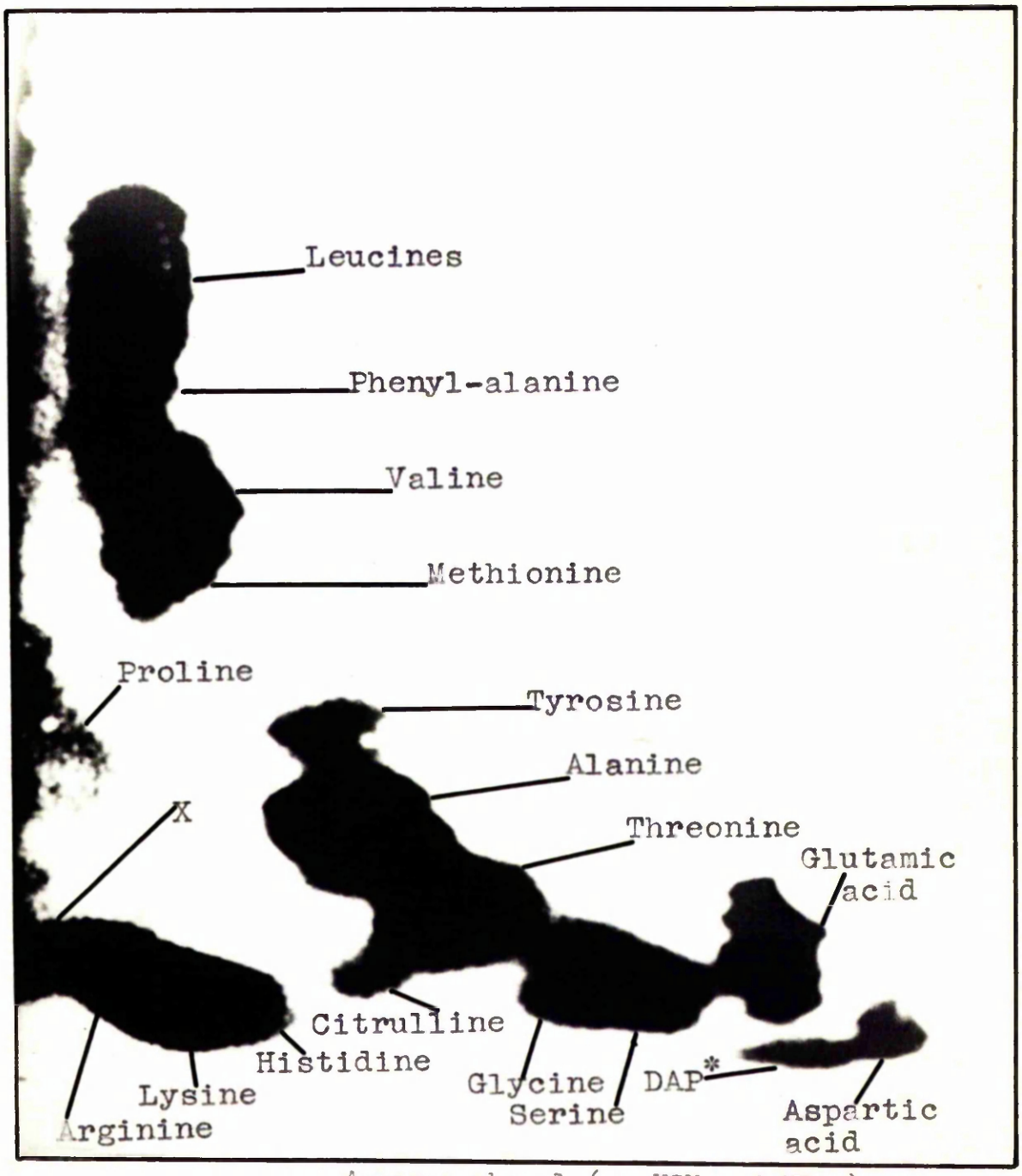
(expressed as nitrogen per cent of total hydrolysate nitrogen)

Amino acid	Nostoc entophytum	Calothrix scopulorum	Oscillatoria brevis
Alanine	8.06	7.11	8.14
Aspartic acid	5.01	4.38	4.62
Arginine	16.30	18.00	16.91
Cysteic acid	0.88	0.08	0.14
Cystine	0.62	1.29	0.67
α,ε-diamino-pimelic acid	3.08	2.62	2.48
Glutamic acid	7.13	7.26	7.58
Glycine	5.14	3.71	5.08
Histidine	3.09	2.68	2.65
Isoleucine	3.75	6.73	4.31
Leucine	4.19	4.71	3.37
Lysine	5.01	6.66	8.06
Methionine	1.29	1.31	0.94
Ornithine	5.17	2.27	4.43
Phenyl-alanine	3.04	2.62	2.92
Proline	3.36	2.98	2.31
Serine	2.83	1.91	2.00
Threonine	4.58	3.49	5.05
Tyrosine	1.72	1.51	1.49
Valine	4.96	5.45	7.35
Unknown I *	5.82	2.59	1.73
Unknown II Ø	0.00	1.91	0.00
Percentage recovery of total hydrolysate N	92.93	90.27	92.23

* Eluted between alanine and valine.

Ø Eluted between histidine and arginine.

PLATE 16



Two-dimensional paper chromatogram of an 85°C hydrolysate of Calothrix scopulorum

*α,ε-diaminopimelic acid

Oscillatoria brevis

Paper chromatography of the algal hydrolysates showed that this species was very similar in qualitative composition to the two nitrogen-fixing species. The common amino acids were all detected as were citrulline and α,ε-diaminopimelic acid.

Quantitative date (Table 36) show the presence of twenty-one amino acids which account for 92.13 per cent of the total nitrogen present. Highest quantities of amino acid-nitrogen were detected in arginine, with large quantities of alanine, glutamic acid and valine also present. Ornithine was again detected in appreciable quantity while the unknown peak between alanine and valine was present though only in small quantity.

D I S C U S S I O N

In all three experiments the amino acid nitrogen determined, amounted to over 90 per cent of the total present. The remaining 10 per cent was probably largely in the form of hydrolysate ammonia which was not estimated.

The qualitative results obtained are very similar for the three species, a total of twenty-one ninhydrin positive spots being detected in hydrolysates of Nostoc and Oscillatoria while twenty-two substances were recorded in Calothrix. All the amino acids normally found in plant hydrolysates were detected. Apart from these, α,ε-diaminopimelic acid was present in all three species, substantiating the suggestion that among the algae this substance is present in all the blue-green species. The presence of citrulline is also interesting in view of the fact that Linko et al. (1957) suggest that in the algae, relatively high concentrations of this amino acid are a peculiarity of the Myxophyceae. However, unlike α,ε-diaminopimelic acid citrulline is of more general distribution being recorded from mosses such as Funaria (Mansford and Raper, 1954) and higher plants such as alder (Miettinen and Virtanen, 1952) and melon (Wada, 1930).

Quantitative data show no pronounced variation among species or between nitrogen-fixing organisms and non-nitrogen-

fixing organisms. In each instance arginine accounts for the largest percentage of the total nitrogen followed by alanine, glutamic acid and lysine. Expressed in this manner, however, the results may tend to give an incorrect picture of the actual quantity of the amino acids in the hydrolysates for it must be remembered that while arginine possesses four nitrogen atoms per molecule and only one of these represents an α-amino group. Expressed in terms of α-amino nitrogen, therefore, the results show that alanine is the most abundant amino acid followed by glutamic acid and valine. The sulphur containing amino acids and tyrosine were detected in lowest concentrations. The traces of cysteic acid were probably derived from cystine during manipulation of the extract.

The results of the present study show a close comparison with those obtained by Magee and Burris (1934) for Nostoc muscorum and suggest that the blue-green algae in general have much higher concentrations of arginine than has been detected by some earlier workers, for example Fowden, (1954). While bearing a resemblance to fresh-water Myxophyceae, it can be seen on comparison with the data of Smith and Young (1955) that they also bear similarities to other groups of marine algae, in that generally aspartic acid, glutamic acid and arginine are present in largest quantities while tyrosine, serine, cystine and methionine are found in smallest quantity. These results differ markedly from those of Lewis and Gonzalves (1960) on other marine algae where generally proline and

methionine were detected in the highest quantities.

α,ε-diaminopimelic acid was observed as a marked peak between cystine and isoleucine in the quantitative experiments, and accounts for 3.08, 2.62 and 2.48 per cent of the total nitrogen in Nostoc, Calothrix and Oscillatoria respectively. Apart from Work and Dewey (1953) earlier workers give no indication of the presence of this substance although Watanabe (1951) records an unknown spot in the appropriate position in two dimensional chromatograms. Work and Dewey (loc. cit.) record it as present to the extent of 0.1 - 0.8 per cent of the dry weight of the three blue-green algae which they tested. Assuming the nitrogen contents of the present algae to be 5 per cent of the dry weight, the percentage of α,ε-diaminopimelic acid present, calculated on a dry weight basis would be 0.62, 0.52 and 0.46 per cent for Nostoc, Calothrix and Oscillatoria respectively. The presence of α,ε-diaminopimelic acid and its participation in lysine synthesis in certain organisms only, has been suggested by Vogel (1960) as being phylogenetically important and indicating a relationship between certain bacteria, the blue-green algae and certain green algae. In addition to α,ε-diaminopimelic acid it is possible that other amino acids, as yet unidentified may also shed light on evolutionary problems among the micro-organisms. Of particular interest in this connection is Unknown I, which occurs in all three species studied in the present experiments

The presence of ornithine in algae hydrolysed at 115°C for seventeen hours is of interest in view of the fact that this is the first time that this amino acid has been recorded as present in blue-green algae. This amino acid was not detected on the paper chromatograms, due to the fact that in the solvents used, its presence would be masked by the histidine. However, in the quantitative experiments a distinct ornithine peak was detected between phenyl-alanine and lysine. The detection of ornithine in the present experiments may be due to either or both of two factors. In the first instance ornithine may be present in marine blue-green algae although not detected in the fresh water species so far examined. If this is in fact the case marine blue-green algae are unlike other marine species where ornithine has been detected only in very small amounts, with the exception of Chondrus crispus where Smith and Young (1955) detected it in appreciable quantity. Although this is a possibility, an alternate and perhaps more likely explanation is that at least a large proportion of the ornithine detected has been formed by its production from citrulline on hydrolysis at 115°C. That this is probably the case is further strengthened by the fact that citrulline, although present on paper chromatograms of hydrolysates at 85°C was not detected on paper chromatograms or in the quantitative experiments using algae hydrolysed at 115°C.

The data on the amino acid composition of these blue-green algae lead to speculation as to their value as a source of food. From the point of view of the suitability of these algae the Essential Amino Acid Index (Oser, 1958) may give some indication of their nutritive value. This index allows comparison of the quantities of essential amino acids present in the algae to be made with those of egg protein. Since egg protein is as nearly completely utilised by most animals, including man, as any food protein it is given the standard value of 100.

The indices calculated for Calothrix, Nostoc and Oscillatoria are 84, 74 and 68 respectively. These compare favourably with a value of 62 for Chlorella quoted by Fisher, Little and Burlew (1953). It appears therefore, that, assuming tryptophane (which was not estimated in the present study) occurs in appreciable quantity, the blue-green algae are richer in the essential amino acids than is Chlorella, the alga on which most mass culture work has been carried out (Tamiya, 1957). No definite statement, however, on the suitability of these blue-green algae as protein sources can be made until biological tests on their digestibility are carried out, particularly when it is borne in mind that certain blue-green algae are known to be toxic to animals (Gorham, 1960)

S U M M A R Y

1) Amino acid analyses of the three marine supralittoral blue-green algae Calothrix scopulorum, Nostoc entophytum and Oscillatoria brevis have been carried out using methods of paper chromatography and column chromatography.

2) The results show that no marked differences occur between nitrogen-fixing and non-nitrogen-fixing species.

3) All the common amino acids have been detected as has α,ε-diaminopimelic acid, citrulline, ornithine and one (Nostoc and Oscillatoria) or two (Calothrix) undetermined ninhydrin positive substances.

4) The possible importance of blue-green algae as a source of essential amino acids has been briefly discussed.

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