STUDIES ON NITROGEN FIXATION BY BLUE-GREEN ALGAE

I. NITROGEN FIXATION BY *ANABAENA CYLINDRICA* LEMM.

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(With Two Text-figures)

Recent work, particularly that of De (1939), has shown that some blue-green algae are capable of utilizing the free nitrogen of the atmosphere in the synthesis of cell material. Much further work will be necessary, however, before the importance of nitrogen fixation by these organisms in nature can be estimated, our knowledge both of the distribution of the property within the group of the Myxophyceae and of the physiology of the process being very vague.

The aim of the present paper is to present proof of nitrogen fixation by a species of *Anabaena* which has not hitherto been examined from this point of view. Later it is hoped to publish the results of investigations on some physiological aspects of the fixation process in the same species.

1. ISOLATION OF THE ALGA

(*a*) *Source and identity of material*

The alga upon which this work has been carried out was isolated by Mr S. P. Chu of the Botany Department, Queen Mary College, London, from a small garden pond in Surrey. De's solution (De, 1939) was found to be a suitable culture medium.

This alga has been identified as *Anabaena cylindrica* Lemm. (Geitler, 1932). It differs from the original description of this species in that the curious mucilaginous envelopes, described by Lemmermann, surrounding the heterocysts have not been observed.

(*b*) *Isolation of the alga in pure culture*

Isolation in pure culture is a necessary preliminary to the study of nitrogen fixation in a given organism. Since it is possible that different physiological races of a species of alga may exist, it is desirable that the cultures used should have originated from a single individual. Such unialgal cultures of *Anabaena cylindrica* were obtained by isolating an individual filament with a sterile capillary pipette under the low power of the microscope and inoculating it into sterile culture medium. Cultures obtained by this method were, however, still contaminated with bacteria.

The difficulty of obtaining cultures of Myxophyceae free from bacteria has been stressed elsewhere (De, 1939). Various methods have been tried in the course of the present investigation, including repeated subculture on silica gel and irradiation with ultra-violet light. Bacteria-free cultures were eventually obtained by treatment of the alga with dilute chlorine water. A small portion of algal material was immersed in chlorine water of a concentration of 25 p.p.m. for 2 min., washed in sterile water and plated out. Exhaustive examination failed to show the presence of contaminating organisms in this culture or in subcultures derived from it. All the cultures used in the subsequent work were grown from this culture. It has not so far been found possible to obtain bacteria-free cultures of any other species of blue-green alga by this method. Its success in this particular instance is thought to be due to the absence of spore-forming bacteria in the original material.

(*c*) *Tests of purity of the cultures*

The most reliable method of demonstrating the absence of bacteria in a given culture is by means of special culture media. De (1938) has pointed out two possible sources of error that must be borne in mind when using this method:

(1) The culture may include bacteria incapable of growing in the media used.

(2) Some bacteria may remain embedded in the mucilage of the alga, and, failing to pass out into the medium, will not produce visible contamination.

In order to minimize the first source of error a range of media suitable for a wide variety of physiological types of bacteria was used. The standard method of preparing the inoculum for a bacteriological examination of an algal culture was to shake a sample of the material vigorously for 5 min. with sterile glass beads and about 10 ml. of De's medium in a sterile tube closed with a sterile rubber stopper. Drops of this suspension were then used to inoculate the test cultures. Tests have been made with the following media:

(1) Caseinate agar (Taylor, 1940).

(2) Lemco-peptone agar.[1]

(3) Sucrose-nitrate-soil-extract agar (De, 1939).

(4) Albuminate agar (Waksman, 1922).

(5) Sucrose-nitrate-soil-extract liquid medium (De, 1939).

(6) Litmus milk.

(7) Dextrose peptone broth (1 % each of dextrose and "Difco" proteose peptone).

(8) Bacto-tryptone broth ('Difco', 1 % solution).

(9) 2 % mannite in tap water + 0·02 % potassium monohydrogen phosphate (a medium specially suited for *Azotobacter*).

Incubation was in the dark at a temperature of 25 or 30° C. for a period of 3 weeks. In the case of bacto-tryptone broth both aerobic and anaerobic cultures were set up. Test cultures were always prepared in triplicate.

[1] The composition of this medium, for which I am indebted to Miss J. Meiklejohn, Rothamsted, is as follows: agar, 15 g.; 'Lemco' meat extract, 3 g.; peptone (bacteriological, B.D.H.), 10 g.; sodium chloride, 5 g.; distilled water, 1 l. After neutralization to phenolphthalein it is sterilized at 1 atm. for 15 min.

In case the second source of error was operative, another method of preparing the inoculum was also used. A test-tube, 7·5 × 2·5 cm., containing some kieselguhr and about 10 ml. of water was sterilized. Some algal material was introduced into this and ground up by means of a second sterile test-tube, 15 × 1·75 cm., used as a pestle. Sterile cotton-wool was bound round the middle of the second tube in order to close the mouth of the first tube and so reduce risk of contamination during the process. It was sometimes necessary to subculture cultures prepared with this inoculum since the turbidity produced by the kieselguhr rendered the recognition of contamination impossible.

Bacterial contamination as indicated by turbidity in the liquid cultures or growth of colonies on solid media was absent in all cases. The media used showed themselves when tested to be perfectly capable of supporting bacterial growth. Direct microscopical examination of smears of algal material stained with carbol-thionin blue also supported the view that the cultures were bacteria-free.

2. METHODS

(*a*) *Culture methods*

(1) *The medium.* Growth of the purified alga in De's modification of Benecke's solution (De, 1939) being unsatisfactory, experiments were made to find a modification giving better results. It was found that vigorous growth could be obtained by adding ferric chloride in greater amount, the optimum being about 0·4 p.p.m. of iron. Since molybdenum has been found to be necessary for nitrogen fixation by *Azotobacter* (Burk, 1934) a trace of this element was added, though no significant improvement of growth was observed as a result. The composition of this modified medium is as follows:

Potassium monohydrogen orthophosphate ...	0·2 g.
Magnesium sulphate heptahydrate	0·2 g.
Calcium chloride decahydrate	0·2 g.
Ferric chloride anhydrous	1·2 mg.
Potassium molybdate (K_2MoO_4)	0·25 mg.
'Pyrex' redistilled water	1 l.

The chemicals used were of analytical quality. The medium was sterilized at 1 atm. for 15 min. in the autoclave and allowed to stand at least 48 hr. before inoculation. Its *p*H was 7·3.

(2) *Culture vessels.* 'Pyrex' conical flasks of 250 ml. capacity, plugged with cotton-wool, were used throughout this work. The flasks were cleaned with chromic acid, steamed and rinsed with 'Pyrex' distilled water before use.

(3) *Culture chamber.* The culture chamber used was the larger of the two described by De (1939). Owing to the heating effect of the lamps (which was reduced as far as possible by the interpolation of two sheets of $\frac{1}{4}$ in. plate glass between the lamps and the cultures), it was found impossible to maintain a constant temperature of below 30° C. by means of a Hearson capsule. Since a constant

temperature was not essential for this portion of the work, no attempt was made to regulate the temperature except that it was not allowed to drop below 21° C. The temperature remained at 21° C. for most of the time, rising to about 27° C. during illumination. Illumination was for a period of about 8 hr. daily.

(4) *Stock cultures.* These were maintained on De soil extract agar slopes in test-tubes. Before using a culture for preparing inoculum for a series of experiments it was thoroughly examined for contaminants by inoculating, from a suspension prepared by shaking with glass beads, into media, 1, 2, 5, 7 and 8 listed on p. 79.

(5) *Inoculation.* A portion of material from a young stock culture was shaken with sterile medium in a sterile flask closed with a tightly fitting sterile rubber stopper for 15 min. The heavier material was allowed to settle, and after decantation portions of 0·25 ml. of the suspension were used as inoculum. The suspension prepared in this manner was found to be sufficiently uniform for all ordinary purposes.

(*b*) *Chemical methods*

When the contents of a culture flask were to be analysed, the alga was first detached from the sides of the flask by means of a rubber-tipped glass rod, then the alga and medium were separated by centrifuging (at 2500 r.p.m.). The alga was washed once with distilled water and the washings added to the medium. The medium was concentrated for analysis by evaporation in vacuo at *c.* 75° C. in Pyrex distilling flasks.

Total nitrogen was estimated by the micro-Kjeldahl method described by Pregl (1930). Nitrate and nitrite nitrogen was estimated by the micro-Kjeldahl method after reduction to ammonia by reduced iron in acid solution, a parallel blank determination, in which the iron was omitted, being made to allow for ammonia already present or produced by hydrolysis. Where nitrite or nitrate were present total nitrogen was also determined after reduction. It was thought that, during reduction in this manner, nitrites might perhaps react with amino acids present to produce gaseous nitrogen and hence cause an error in the estimation, but experiments showed that the method was perfectly reliable when nitrites and amino acids were present together. At all stages of the analyses and preparation for analysis, blank determinations were carried out and the results, which were usually small, subtracted from the results of the actual estimations.

3. THE NITROGEN-FIXING CAPACITY OF THE ALGA

Any experiment designed to show nitrogen fixation by a given organism must be carried out with the following two points in mind:

(1) The organism must be in absolutely pure culture. Even if a contaminant is present which is known to be otherwise incapable of fixing nitrogen, the possibility cannot be precluded that it may fix nitrogen in the presence of the organism which is being examined.

(2) It must be absolutely certain that any increase in fixed nitrogen which takes place in a culture is due to the uptake of free nitrogen. Ideally, a manometric

method, showing decrease in free nitrogen accompanying increase in fixed nitrogen should be used, but this is frequently inconvenient. A method showing increase in combined nitrogen is satisfactory provided that precautions are taken to exclude the possibilities that combined nitrogen may be supplied from an unsuspected source, e.g. the atmosphere, or that the apparent increase may be due to some defect in the method of estimation.

Since pure bacteria-free material of *Anabaena cylindrica* shows vigorous growth in a medium free from combined nitrogen, it is probable that it is able to fix nitrogen. It is possible, however, that nitrogen is being absorbed in the form of ammonia or oxides of nitrogen from the atmosphere. In order to show that this was not so in the case of the algae studied by him, De (1938) carried out an experiment in which air was bubbled through sulphuric acid to remove ammonia, and potassium bicarbonate to remove oxides of nitrogen, before passing over the cultures. In the course of the present work it has been found that gas washing bottles of the type used by De are quite inefficient for this purpose, and, in the absence of proof to the contrary, it must be assumed that all the combined nitrogen present in the air which passed over the cultures was not removed. De's contention (1939) that the increase in combined nitrogen observed in his cultures could not be due to absorption of ammonia or oxides of nitrogen from the atmosphere since uninoculated control flasks, exposed side by side with the cultures, did not show any increase of nitrogen, cannot be regarded as valid since a growing alga would form a very much more efficient absorbing system for such substances than the medium alone. It is unlikely that the comparatively large increases in combined nitrogen found by De in his cultures were actually due to absorption of fixed nitrogen from the atmosphere, but this defect in his methods introduces an element of uncertainty which is undesirable in an investigation of this kind.

An experiment similar to that of De, but using improved apparatus, has therefore been carried out with *Anabaena cylindrica*. Before passing over the cultures, air was purified from traces of combined nitrogen by passage through 1 % sodium bicarbonate solution (Fig. 1 *B*) and 25 % sulphuric acid (*D*), the wash bottles being of a type fitted with fritted glass bubblers. It was necessary to have the reagent with the lower vapour pressure (the sulphuric acid) nearer to the culture vessels since otherwise the cotton wool filter *E* became wet and allowed the cultures to become contaminated. The four culture flasks (*F*, *G*, etc.), one of which was left uninoculated as a control, were connected in series in the culture chamber. This part of the apparatus was sterilized and assembled under aseptic conditions and the air passing through it was sterilized by the sterile cotton wool filter *E*. A second sterile filter (*H*) and wash bottle containing sulphuric acid (*J*) were included respectively to prevent contamination and to absorb ammonia in the event of any sucking back. *A*, *C* and *I* were safety flasks. All connexions were sealed with paraffin wax. Air was drawn slowly (approximately 3 l. per hour) and continuously through the apparatus by means of a filter pump.

The efficiency of the gas-washing system was tested as follows. In each of two culture flasks was placed 100 ml. of *N*/50 sulphuric acid (to absorb ammonia) and

in each of two others 100 ml. of $N/50$ sodium hydroxide (to absorb oxides of nitrogen). Air was drawn through the wash bottles and then through these flasks for a period of 10 days, the rate of flow being somewhat more rapid than that employed for aerating cultures. At the end of the experimental period the contents of the flasks were found to show no increase in combined nitrogen compared with controls analysed at the beginning of the experiment. This demonstrates that ammonia and oxides of nitrogen were effectively removed from the air passing over the cultures.

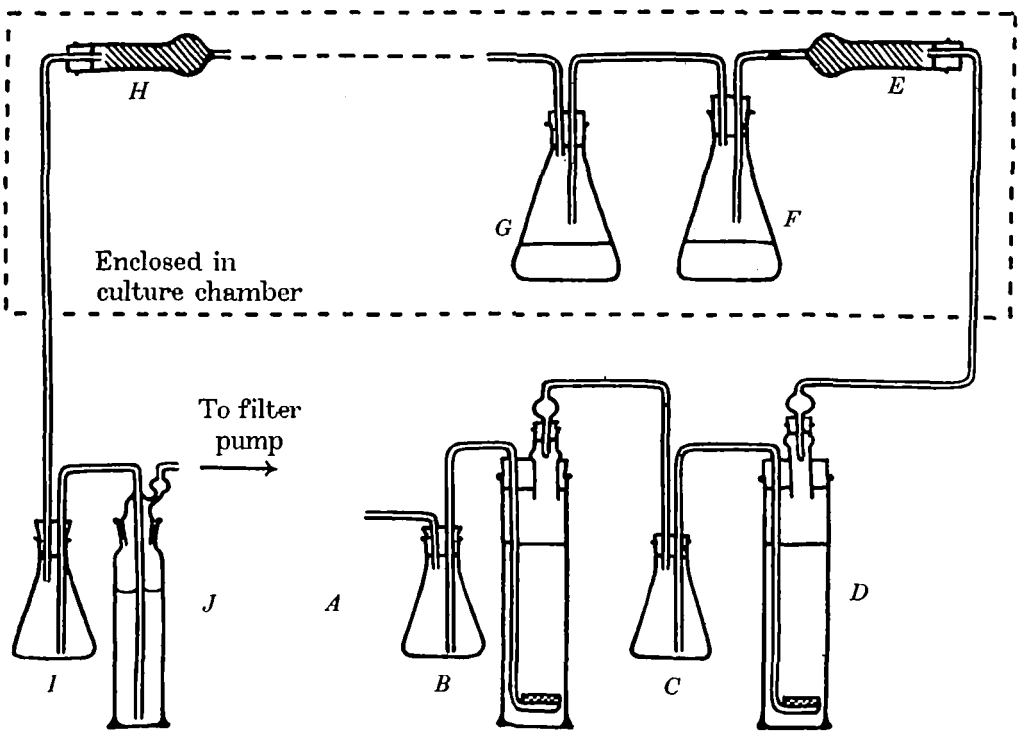

Fig. 1. Diagram of the aeration apparatus.

The cultures were inoculated from a pure bacteria-free stock culture. In order to ensure that the cultures had not become contaminated during the course of the experiment a small amount of material from each of the flasks was examined bacteriologically at the end of the experimental period by the same means as those used for the examination of stock cultures. No contamination by fungi or bacteria was found.

The results of micro-Kjeldahl analyses of the cultures are given in Table 1.

This table shows clearly that free nitrogen has been fixed by the alga. The small amount of nitrogen found in the control flask, the medium in which was completely free from combined nitrogen at the beginning of the experiment, may perhaps be due to ammonia derived from the culture flasks. It is to be noted that

a large proportion of the nitrogen fixed appears in a soluble form in the medium. The nature of this excreted nitrogen is being investigated.

Table 1. *Nitrogen fixation by* Anabaena cylindrica *in aerated culture after* 50 *days. Alga showing no sign of senescence.* 100 *ml. nitrogen-free medium per flask. Nitrogen in mg.*

	Alga	Medium	Total
Control	—	0·019	0·019
1	1·403	0·093	1·496
2	1·163	0·073	1·236
3	1·878	0·133	2·011

Table 2. *The changes produced by* Anabaena cylindrica *in a medium containing potassium nitrate.* 75 *ml. medium per flask. Nitrogen in mg.*

Age of culture	N in alga	Total N	Deviation of total N from control	Nitrate N in medium	Total N in medium	Other N in medium (by difference)
Control	—	1·243	—	1·243	1·243	—
	—	1·371	—	1·371	1·371	—
Means	—	1·307	—	1·307	1·307	—
20 days	0·173	1·328	+0·014	1·114	1·155	0·041
	0·193	1·359	+0·052	1·167	1·166	0·000
	0·212	1·392	+0·085	1·134	1·180	0·046
Means	0·193	1·360	+0·050	1·138	1·167	0·030
30 days	0·332	1·324	+0·017	0·861	0·992	0·131
	0·326	1·261	−0·046	0·908	0·935	0·027
	0·369	1·373	+0·066	0·932	1·004	0·072
Means	0·342	1·319	+0·012	0·900	0·977	0·077
40 days	0·488	1·126	−0·181	0·533	0·638	0·108
	0·685	1·278	−0·029	0·503	0·593	0·093
	0·450	—	—	0·521	—	—
Means	0·541	1·202	−0·105	0·519	0·615	0·100
50 days	1·384	1·621	+0·314	0·160	0·237	0·177
	1·161	1·593	+0·286	0·189	0·432	0·243
	1·368	1·629	+0·322	0·006	0·261	0·255
Means	1·304	1·614	+0·307	0·085	0·310	0·225

4. INHIBITION OF NITROGEN FIXATION BY COMBINED NITROGEN

As in other nitrogen-fixing organisms, nitrogen fixation by *Anabaena cylindrica* does not take place in the presence of a sufficient amount of readily available combined nitrogen. The changes taking place in cultures supplied with nitrate have been followed up experimentally. Cultures of *Anabaena* were set up in a medium containing nitrate and samples of three, chosen at random, analysed at intervals. The nitrogen in the alga, the total nitrogen in the medium and the amount of nitrate remaining were determined. The results are given in Table 2 and Fig. 2 and may be summarized as follows:

(1) As the nitrogen in the alga increases there is a corresponding decrease in nitrate nitrogen.

(2) Extracellular nitrogen other than nitrate increases as the cultures grow older, amounting to about 14 % of the total nitrogen after 50 days.

(3) Total nitrogen remains roughly constant until the nitrate is nearly exhausted, thus showing that nitrogen fixation does not take place in the presence of this form of combined nitrogen. Total nitrogen decreases slightly between 20 and 40 days,

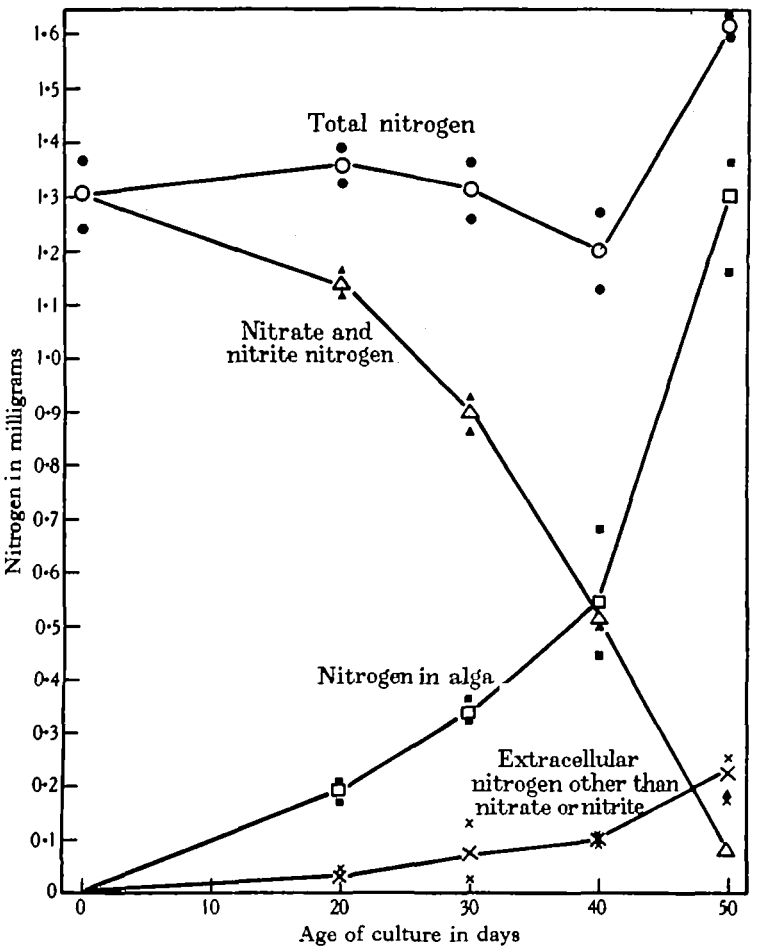

Fig. 2. The changes occurring in cultures of *Anabaena cylindrica* in a medium containing nitrate.

but the decrease is not statistically significant. If this decrease is real, it may be due to denitrification or conversion of fixed nitrogen to a form which is missed by the Kjeldahl method. Nitrogen fixation takes place from 40 to 50 days, by which time the nitrogen in the medium has dropped to about 4 p.p.m.

A similar experiment in which ammonium chloride was used as the source of nitrogen gave comparable results.

5. CONCLUSION

The work described above shows clearly that *Anabaena cylindrica* is able to fix nitrogen. Four species of *Anabaena* have now been shown to possess this capacity (De, 1939). It is only in this genus and in species of *Nostoc* (for references see De, 1939) that reliable evidence is available of the capacity of assimilating nitrogen in the elemental form. *Nostoc* and *Anabaena* are, however, comparatively easy to work with under laboratory conditions, while other blue-green algae are more difficult to obtain free from contaminants. Further work may possibly show that the property is more widely distributed in the Myxophyceae than would appear at present.

Anabaena cylindrica was isolated from an aquatic habitat. This alga has not been reported as occurring in any quantity in fresh waters and it probably plays no very important part as a fixer of nitrogen. Other species of *Anabaena*, however, occur in great abundance in certain types of fresh water and may be of considerable importance in this respect. Hutchinson (1941) has reported nitrogen fixation by a planktonic *Anabaena* which, however, was not free from bacteria. Further critical work on this and similar species is necessary.

Even if an alga has been shown to fix nitrogen it is quite possible that the concentration of available combined nitrogen in a given habitat will be sufficient to inhibit the process.

6. SUMMARY

1. *Anabaena cylindrica* Lemm. has been obtained in pure unialgal bacteria-free culture.

2. Due precautions having been taken against contamination by other organisms and error due to absorption of fixed nitrogen from the atmosphere, this alga has been shown to possess the capacity of fixing nitrogen.

3. Nitrogen fixation does not take place in the presence of a sufficient quantity of readily available combined nitrogen.

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