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STUDIES ON NITROGEN-FIXING BLUE-GREEN ALGAE. I. GROWTH AND NITROGEN FIXATION BY ANABAENA CYLINDRICA LEMM.[1]

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Many members of the blue-green algae (Myxophyceae) possess the ability to fix molecular nitrogen. This property, combined with their photosynthetic habit of life, places these algae among the most completely autotrophic living organisms. Organisms with these properties are obviously desirable objects for investigations of photosynthesis, nitrogen fixation, and the possible relationships between these two synthetic processes. However, the blue-green algae are commonly regarded as slow-growing forms which are difficult to maintain in culture. Although there are a few reports of algae of this group which grow at a rapid rate with combined nitrogen [Emerson and Lewis (13), Kratz and Myers[3] (18)], the yields commonly reported for nitrogen-fixing blue-greens vary from 0.3 to 2.0 gm dry weight of cell material per liter of culture medium in periods of time varying from 20 to 60 days (4, 14, 24). Even in those cases where rapid growth has been reported, it has not been shown that high cell yields can be obtained in a short time. For example, the Chroococcus grown by Emerson and Lewis (13) produced 60 mg dry wt per liter of culture in 5 to 7 days. By contrast, the cultures of Chlorella and Scenedesmus commonly used in laboratory studies give yields of 5 to 10 gm dry wt per liter in 5 to 6 days (6, 7, 11). The small yield obtained with the Myxophyceae and the reputed difficulty of growing these algae has prevented them from being used in studies of photosynthetic nitrogen fixation (19).

Since dense growths of blue-green algae can appear in a short time under natural conditions, it appeared possible that many of the low yields for these organisms reported in the literature were the result of unfavorable physical or nutritional conditions for growth. The importance of favorable physical conditions for good results in the culture of Myxophyceae has previously been pointed out (1). Several unsolved problems in the nutrition of blue-green algae were also apparent. Was calcium required for growth with combined nitrogen or was it only needed for nitrogen fixation (4)? Could calcium be replaced by strontium (4; cf. 27)? Could molybdenum be replaced by vanadium (10)? Were there other inorganic nutrients required by these algae?

As a result of investigation and clarification of these points we have now found that under favorable conditions the growth of *Anabaena cylindrica* Lemm., a filamentous, nitrogen-fixing member of the Myxophyceae, is such that 7 to 8 gm dry wt of cells per liter of culture medium can be obtained in 6 days (3), an increase of approximately 200 fold over the yield previously reported for this and similar organisms (4, 14, 29).

The high yields obtained for *Anabaena cylindrica* (and for other blue-green algae not discussed here) have resulted not from the attainment of higher growth rate constants (K) than those previously reported by other investigators but rather from the maintenance of exponential growth rates at high cell densities. The purpose of this paper is to discuss the nutrient requirements and the physical conditions of the environment under which the vigorous growth of nitrogen-fixing blue-green algae was obtained.

METHODS

The alga used in these studies was Fogg's strain of *Anabaena cylindrica* Lemm. (14). It was found possible to obtain a reasonably uniform suspension of filaments by growing the culture with continuous shaking (see below). In most cases, uniform samples for inoculation of fresh cultures could be obtained by

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[3] In a paper which appeared after this manuscript was submitted for publication, these authors (Amer. Jour. Bot. 42: 282–297. 1955) report for *Anacystis nidulans* a growth constant (K) of 3.55 at cell density of ca 0.4 gm dry wt per liter of culture.

pipetting aliquots from a mature culture; 0.1 to 1.0 ml samples, depending on the density of the culture, were used for inoculation. If lumpy cultures were obtained, a uniform suspension of filaments for use as inoculum was prepared by filtering a portion of the culture through a thin mat of glass wool.

Cultures were grown in Roux bottles, on an illuminated shaker, at ca 25° C, with 5 % CO_2 in air passing over the liquid; 250 ml of medium was used in each bottle; the illuminated area was 220 cm^2. The apparatus is described elsewhere (6, 7). Unless otherwise described, the following nutrient solution was used: 0.001 M $MgSO_4$, 0.0005 M $CaCl_2$, 0.004 M NaCl, 0.002 M K_2HPO_4, and a micronutrient supplement based on the A4 and B7 solutions (9) which supplied the following final concentrations of metals: Fe [as the EDTA complex (7)] 4 ppm Mn (as $MnSO_4 \cdot 4\,H_2O$) 0.5 ppm, Mo (as MoO_3) 0.1 ppm, Zn (as $ZnSO_4 \cdot 4\,H_2O$) 0.05 ppm, Cu (as $CuSO_4 \cdot 5\,H_2O$) 0.02 ppm, B (as H_3BO_3) 0.50 ppm, V (as NH_4VO_3) 0.01 ppm, Co (as $Co(NO_3)_2 \cdot 6\,H_2O$) 0.01 ppm, Ni (as $NiSO_4 \cdot 6\,H_2O$) 0.01 ppm, Cr (as $Cr_2(SO_4)_3K_2SO_4 \cdot 24\,H_2O$) 0.01 ppm, W (as $Na_2WO_4 \cdot 2\,H_2O$) 0.01 ppm, Ti (as $TiO(C_2O_4)_x \cdot y\,H_2O$) 0.01 ppm. The micronutrient supplement included several elements not so far shown to be essential for plant growth to ensure that growth was not being limited by an unknown element. The phosphate was autoclaved separately and added to the medium after cooling, to prevent precipitation. Potassium nitrate, 0.02 M, was added for experiments in which growth with combined nitrogen was measured.

All media were prepared with glass-distilled water. For experiments on Mo and V requirements, the salts used were purified by treatment with $CaCO_3$ and CuS, as described elsewhere (7, 17). The salts used in Ca and Sr experiments were C.P. grade purified by three recrystallizations from hot water. For other experiments, C.P. salts were used without further purification. The methods of cleaning glassware and other experimental details are given elsewhere (7, 17).

The dry weight of the algal cells was used as a measure of growth. Since the medium used contained only ca 0.6 gm dry wt of salts per liter, growth was determined by drying 30 to 50 ml aliquots of the culture, evaporating to dryness, weighing, and correcting for the weight of salts in the medium. Growth figures obtained in this way are slightly low, since algae contain 5 to 10 % ash (20), but the error is small for an organism which obtains both its C and its N from the atmosphere.

For measurements of nitrogen in the cells and in the medium, the algae were removed by centrifuga-

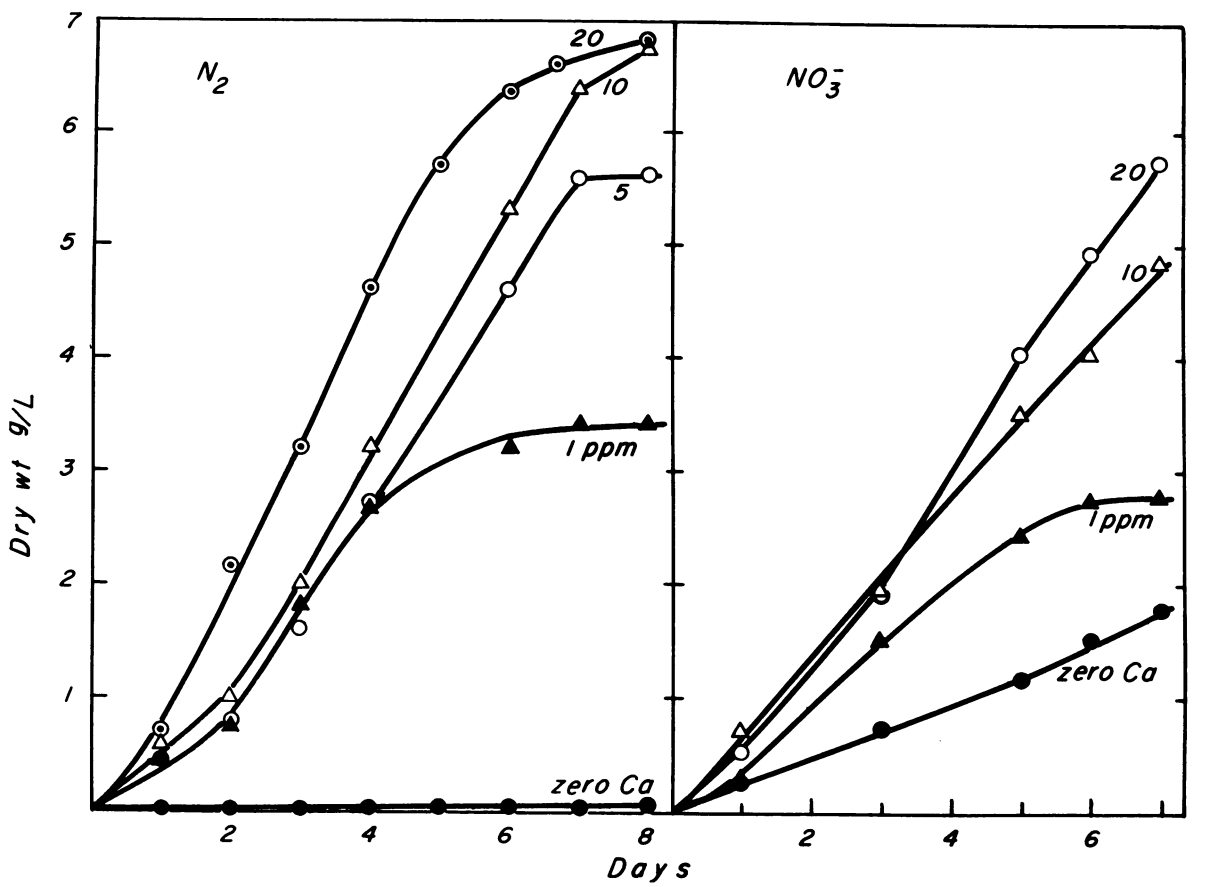

FIG. 1. The calcium requirement of *Anabaena cylindrica*. Light intensity 7500 lux. Calcium concentrations (ppm) are shown on the curves.

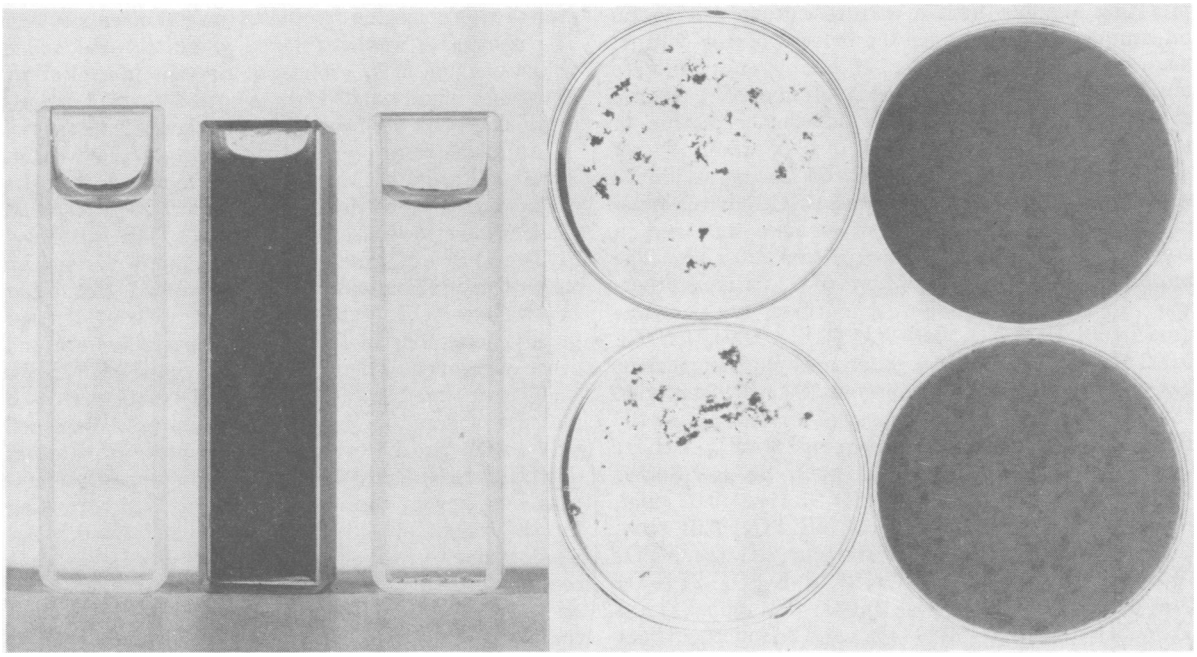

FIG. 2 (*left*). Effect of Ca and Sr on growth of Anabaena. Seven-day cultures. Light intensity 7500 lux. Left, no Ca or Sr added; center, 20 ppm Ca added; right, 20 ppm Sr added.

FIG. 3 (*right*). Effect of Mo and V on growth of Anabaena. Cultures on right received 0.01 ppm Mo and 0.01 ppm V, those on left received 0.01 ppm V. Light intensity 7500 lux.

tion and the nitrogen in the cell mass and the supernatant liquid determined by the microkjeldahl method (12).

RESULTS

NUTRITION: Little systematic work has heretofore been done on the nutritional requirements of Myxophyceae [cf. (1) for a review of the literature]. The proportions of the major nutrients used in the present study were based, in the main, on those which have been found to give satisfactory growth of higher plants (8) and of green algae (6, 7). Since these quantities of nutrients were also favorable for growth of the blue-green algae, the effects of varying the proportions of the major nutrients have not been extensively investigated here.

The importance of an adequate supply of nitrogen, molecular or combined, in obtaining good yields should, however, be emphasized. Since healthy algae contain 5 to 7 % N, calculations show that 0.02 to 0.04 M nitrate is required to support a population of 6 to 8 gm dry wt/l when the organisms are growing with combined nitrogen. When the algae are growing with molecular nitrogen, the yield obtained may be limited by the rate of diffusion of nitrogen to the cells unless provision is made by agitation of the culture, growth in thin layers of liquid, or by a combination of both measures, for free access of air to the organisms. This point will be discussed more extensively below.

Two elements, Ca and Mo, were of especial interest, since it has been reported that, although both are needed for growth of Myxophyceae, Ca could be replaced by Sr (4) and Mo by V (10). Since no other cases are known as yet in which an essential element for plant growth can be completely replaced by another (5), an examination of the requirements of Anabaena for these nutrients was made.

Calcium was shown to be required for the growth of Myxophyceae by early investigators of the physiology of these algae (23). In spite of one report that Ca is not needed (16), based on cultures in which the total growth was small, later work has generally substantiated the early results (1). However, Allison et al (4) claimed that Ca was needed by *Nostoc muscorum* only when this alga was growing with molecular nitrogen, not when nitrate was the nitrogen source.

As shown in figure 1, Ca is needed by Anabaena whether it is growing with molecular or with combined nitrogen. At least 20 ppm Ca was needed for optimum growth. Anabaena thus resembles the higher plants in requiring calcium in relatively large amounts, in contrast to the green algae, which can grow with micronutrient quantities of this element (22, 25, 27). The slight growth in the absence of added Ca with nitrate as N source may reflect the presence of a small amount of Ca contamination in the KNO_3, in spite of recrystallization of this salt.

Figure 2 shows that calcium canont be replaced by strontium for the growth of *Anabaena cylindrica*. The evidence for calcium as an irreplaceable essential element (5) for the growth of blue-green algae was obtained with purified nutrient salts under conditions

favoring vigorous growth and high cell density. Such conditions are desirable for demonstrating the nutrient requirements of plants.

Similarly, as shown in figure 3, the requirement of Anabaena for molybdenum was confirmed, and it was shown that V cannot replace Mo. Examination of the experimental results published by Bortels (10) shows that the evidence for the replaceability of Mo by V was not as definite as it has been accepted to be. Bortels studied 18 cultures of various Myxophyceae. The growth of 13 of these was stimulated by Mo, but not by V, two of them were stimulated by both Mo and V, and three did not respond to the addition of either of these elements. The two cultures which responded to both Mo and V gave yields of 0.48 and 0.28 gm dry wt/l respectively, in the absence of added Mo or V. Addition of Mo raised the yields to 0.98 and 0.74 gm/l, while addition of V resulted in 0.65 and 0.57 gm/l. In the present study, growth of Anabaena without Mo or V resulted in a yield of 0.28 gm/l, addition of V resulted in a yield of 0.36 gm/l, and addition of Mo raised the yield to 5.8 gm/l.

Supply of Nitrogen and Carbon Dioxide: Since Anabaena growing with molecular nitrogen obtains both its C and its N from the gas phase above the culture, it is clearly necessary to provide for free access to these gases to the cells. In our experiments this was accomplished by passing a mixture of 5 % CO_2 in air over the liquid surface and shaking the cultures to ensure mixing of the gas with the liquid. Aeration was further favored by growing the cultures in thin layers of nutrient medium in Roux bottles, which provide a large liquid-gas interface. Under these conditions, growth with molecular nitrogen was as fast as growth with nitrate, as shown in figure 4.

Shaking, while desirable for obtaining uniform cultures, is not strictly necessary for good growth when a large liquid-gas interface is available. For example, in one experiment a shaken culture yielded 7.1 gm/l of dry Anabaena cells, whereas a parallel culture, treated in the same manner except that it was not shaken, gave a yield of 6.9 gm dry wt/l. The shaken culture, however, was uniformly blue-green, whereas the unshaken one, which had grown only at the gas-liquid interface, was partly old and bleached out while other portions were still growing. Thus it appears desirable to shake cultures which are to be used for experimental purposes in the laboratory, but provision of a large surface exposed to air, without agitation, might be satisfactory if it were desired to establish mass cultures of nitrogen-fixing blue-green algae. It seems likely that the improvement of growth of filamentous blue-green algae previously noted on introduction of a solid substratum such as glass wool into the culture medium (1) resulted from the more disperse growth of the algae under these conditions, with consequent better access of gases to the cells.

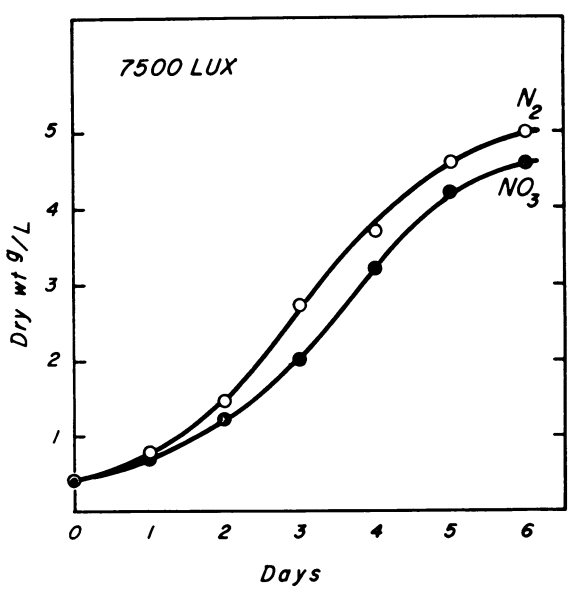

Fig. 4. Growth of Anabaena with molecular and combined nitrogen.

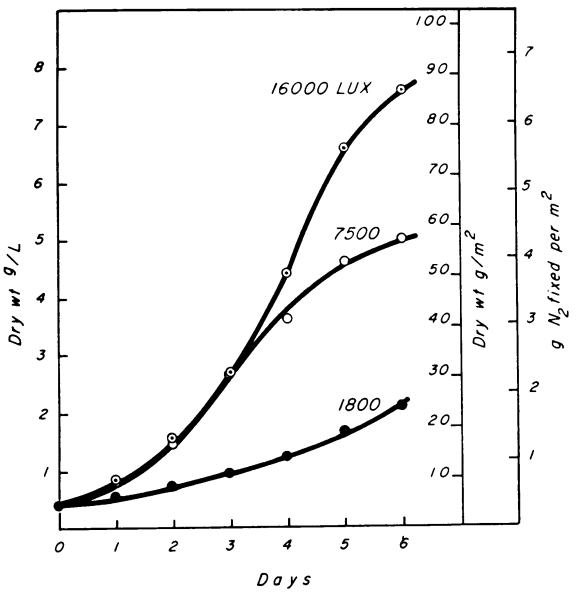

Fig. 5. Effect of light intensity on growth of Anabaena. N_2 as nitrogen source.

Intensity and Duration of Light: It has been common practice in the past to grow blue-green algae at rather low light intensities, from 300 to 3000 lux. Some workers have claimed that these algae are injured by high light intensities (4). Our experiments have shown, however, that under good conditions of mineral nutrition and with an adequate supply of nitrogen and CO_2 Anabaena can grow well at high light intensity, and that the yield of cells increases with increasing light intensity up to at least 16,000 lux, as illustrated in figure 5.

The question whether algae, under controlled laboratory conditions, grow better when given a dark period each day, or whether maximum growth per unit time is obtained when the cells are continuously illuminated has never been fully resolved. Earlier

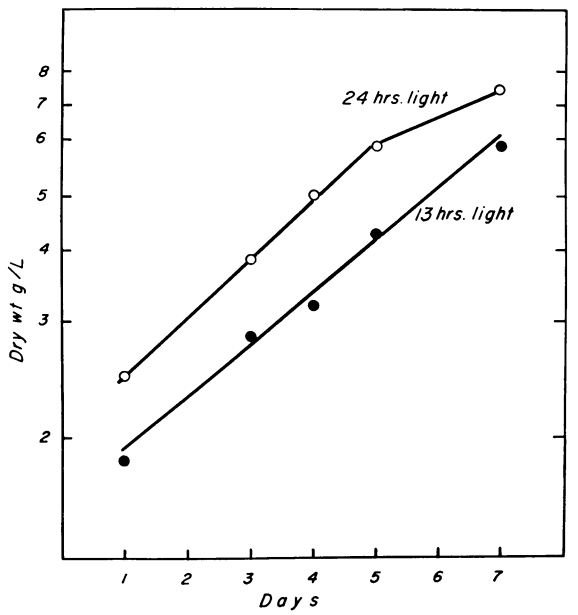

FIG. 6. Comparison of growth of Anabaena in continuous and intermittent light. Light intensity 16,000 lux. N_2 as nitrogen source. The upper curve shows growth in continuous light, the lower, growth with 13 hrs light and 11 hrs darkness each day. The ordinate is a logarithmic scale.

workers tended to give their cultures a dark period; Allison et al (4), for example, reported slightly better growth of *Nostoc muscorum* with 18 than with 24 hours of light each day. Recent practice, however, has been to grow cultures of algae under continuous illumination (21). Our experiments with Anabaena have shown that the rate of growth, under the conditions tested, was essentially the same in cultures receiving 11 hrs of darkness and 13 hrs of light as in those receiving 24 hrs of light each day, as is illustrated in figure 6. The cultures in continuous light had a shorter lag period, but once active growth had started there was no effect of the dark period on growth. Similar results have been previously obtained for Chlorella (2), suggesting that this behavior may be found in many algae. Similar results have been obtained by other workers; Tamiya et al (26) observed no retardation of growth of *Chlorella ellipsoidea* on interposition of a 12-hour dark period each day as long as the cells were growing logarithmically. It follows from these results that the yields of algae which might be expected under natural conditions with alternating day and night periods need not be very different from those obtained in continuous light in the laboratory.

DISCUSSION

It is evident from the growth figures reported in this paper that there is no longer any need to consider the common nitrogen-fixing Myxophyceae as slow-growing algae. Further emphasis of this point is provided by figure 7, where the growth of *Anabaena cylindrica* is compared with that of the green alga Scenedesmus (strain D_3). Although the growth of Scenedesmus shown here is higher than most figures previously published, the yield of Anabaena, while somewhat lower, is comparable to that of the green alga. The maximum daily increment of Anabaena growth was 2.0 gm/l, or 26 gm dry wt/m^2 of illuminated surface. The corresponding figures for Scenedesmus were 2.8 gm/l, or 36 gm/m^2.

These high yields have not been obtained by any increase in the growth rate constant of the alga, but rather by the maintenance of high rates of growth in dense cultures. The growth rate constant is an expression of the potentialities of an organism. Suitable conditions for the realization of these potentialities must be provided if high yields are to be obtained. Thus Fogg (14) reported a growth rate constant of 0.70 to 0.75 for this strain of *Anabaena cylindrica*, which agrees well with values which we have obtained, but his cultures required 60 days to reach a density of 1.7 gm dry wt/l. Fogg obtained exponential growth of the alga only in cultures so thin that the only measure of growth which could be used was microscopic observation of increases in filament length. In our cultures, on the other hand, exponential growth continued up to approximately 6 gm dry wt/l (fig 6). The growth rate constant of an

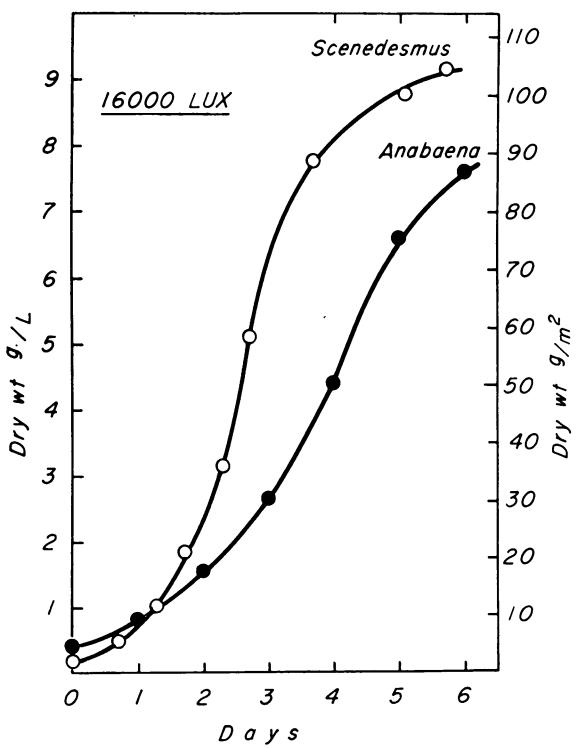

FIG. 7. Comparison of growth of Scenedesmus and Anabaena. Medium and experimental conditions for Anabaena as described in Methods; Scenedesmus was grown as described elsewhere (6, 7).

alga may be of less importance for obtaining high cell yields than the provision of physical and nutritional conditions which permit exponential growth at high cell densities.

Several workers have shown that blue-green algae excrete a portion of the nitrogen which they fix into the medium in the form of amino acids and peptides (15, 28). According to Fogg (15), as much as 30 to 40 % of the nitrogen fixed by *Anabaena cylindrica* appears in the medium in soluble form. Under our conditions, however, most of the nitrogen remained in the cells, no more than 7 to 8 % appearing in the medium. Fogg concluded (15) that the excretion of nitrogenous substances by Myxophyceae is favored by deficiencies of micronutrient elements, especially iron. The influence of nutrition and other conditions of growth on the excretion of nitrogen compounds by Anabaena is being studied further.

Summary

The capacity for growth of blue-green algae with molecular nitrogen has been found to be of the same order of magnitude as the growth of vigorously growing green algae with nitrate nitrogen.

An investigation of the nutrient requirements of the blue-green alga *Anabaena cylindrica* Lemm. disclosed that calcium was essential for growth whether nitrate or molecular nitrogen was used. Previous reports had suggested that calcium was required with molecular nitrogen only.

The specificity of two elements previously reported to be replaceable in the growth of blue-green algae was established. Calcium was not replaceable by strontium nor molybdenum by vanadium.

Growth of Anabaena increased with increasing light intensity up to 16,000 lux. An intensity of 16,000 lux for 13 hrs each day permitted as rapid growth as 24 hrs of illumination.

Under the conditions described, a yield of Anabaena of 7 to 8 gm dry wt/l of culture solution in 5 to 6 days was obtained with either nitrate or molecular nitrogen.

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CONVERSION OF CARBON-14-LABELED UREA INTO AMINO ACIDS IN LEAVES[1,2]

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It has become increasingly evident that the foliar application of urea to many different plants represents an extremely valuable nutritive technique. The urea is absorbed, as such, by the leaf cells and is then presumably hydrolyzed by cellular urease to carbon dioxide and ammonia. Unfortunately, our knowledge of urea metabolism in plants, based on actual experimental evidence, is very small. Especially lacking is information on the effect of urea nutrition on cellular nitrogen metabolism. In this connection it should be pointed out that evidence for the universal occurrence of even the presumed first step in the metabolism of urea, its hydrolysis to carbon dioxide and ammonia, is not at all clear. For example, Walker (6) has reported that Chlorella cells grown with urea as the sole nitrogen source contain no detectable urease. In spite of this they assimilate urea readily and grow, with a concurrent increase in their levels of cellular arginine and argino-succinic acid. Likewise, Williams and Sharma (8) have reported that urease essentially disappears from seedlings of *Citrullus vulgaris* within nine days of germination.

In contrast, Hinsvark et al (2) have concluded from isotopic studies that the leaves of a number of higher plants hydrolyze urea readily. It seems, therefore, that further knowledge of the metabolic fate of urea in plant cells is needed. This problem has recently been investigated by Boynton et al (1) who have reported that treatment of apple leaves with urea results in increased levels of total, amino, and amide nitrogen. The present communication reports the results of a study carried out on the metabolism of urea-C^{14} in leaves of bean plants.

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[2] Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.
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EXPERIMENTAL MATERIAL AND METHODS

The experimental material (*Phaseolus vulgaris*) was grown in the manner described by Rogers (5). Thirty grams of young leaves were detached from the plants and mechanically agitated under weak (50 fc) white light at 23° C for six hours while completely submerged in 200 ml of 0.01 M urea-C^{14} having a total activity of 1 mc. In a parallel experiment, 30 gm of young leaves were incubated with 0.01 M $NaHC^{14}O_3$ with a total activity of 1 mc in a solution containing 0.01 M NH_4Cl. The incubation was stopped by quickly freezing the leaves in a mixture of solid carbon dioxide and acetone. Out of a total of 2,220,000,000 cts/min in each incubation solution, the leaves absorbed a total of 15,300,000 cts/min from the urea-C^{14} solution and 28,500,000 cts/min from the $NaHC^{14}O_3$ solution. The leaves were ground in a Waring blendor with 100 ml of water, filtered, and the heavy cellular particles sedimented at 18,000 × g. The remaining clear supernatant was treated with two volumes of acetone to precipitate protein. The protein was sedimented by centrifugation in a Servall refrigerated centrifuge. The protein-free supernatant was reduced to a small volume in a low temperature rotating evaporator. The protein obtained by acetone treatment was combined with that of the sedimented cellular particles and the total protein hydrolyzed in a sealed tube at 150° C with 20 % HCl. To the amino acids obtained from both the cellular extracts and the protein hydrolysates, a mixture of 17 carrier amino acids was added. The amino acids were separated either on a Dowex-50 column or on a combination of ion exchange columns by the method of Hirs, Moore and Stein (3) with the aid of an automatic fraction collector (Autonomos Instruments, Columbus, Ohio). The two ml fractions were evaporated to dryness on copper planchets, and the radioactivity of each determined with a Nuclear-Chicago G-M tube