GROWTH AND CHEMICAL COMPOSITION OF THE BLUE-GREEN ALGA ANACYSTIS NIDULANS CULTURED AT HIGH LIGHT INTENSITIES*

J. C. GOEDHEER** and J. W. KLEINEN HAMMANS

Vakgroep Biofysica, Laboratorium voor Experimentele Fysica, Utrecht

SUMMARY

The influence of temperature, CO_2 availability, light intensity, and colour on growth of *Anacystis* at high (70–120 $W \cdot m^{-2}$) light intensities was investigated. In the first $2\frac{1}{2}$ days rapid formation of cells with a high nitrogen content was found. During this period the nitrogen content of the cells was about 10%. When the supply of bound nitrogen was exhausted, the over-all photosynthetic mechanism switched to production of nitrogen deficient products, probably carbohydrates. Only in the first period growth could be followed by measuring optical parameters, and a production of 0.5–1 $g \cdot l^{-1}$ dry weight per day could be obtained in single batch as well as in continuous cultures.

1. INTRODUCTION

KRATZ & MYERS (1955) studied nutrition and growth of several blue-green algae. They measured, especially with *Anacystis*, high rates of growth under favourable conditions. HALLDAL, HANG & FRENCH (1957) grew some species of blue-green algae in crossed gradients of light intensity and temperature. With three species of blue-green algae: *Anacystis*, *Anabaena*, and *Cyanidium*, a temperature of 40–45 °C supported good growth when the cells were grown on agar. Also ALLEN & ARNON (1955) and JÜTTNER et al. (1971) mentioned high growth rates with blue-green algae. Various aspects of growth, culture and nutrition of blue-green algae were reviewed by FOGG et al. (1973) and CARR & WHITTON (1973).

At high light intensities and high temperatures, photosynthesis may proceed at such high rates that its efficiency is primarily limited by the availability of carbon dioxide at the sites of chloroplasts or lamellae. As the ratio of the total lamellar surface to outer surface of the photosynthesizing organism is an important parameter in determining the diffusion of carbon dioxide (RABINOWITCH 1951), unicellular organisms may be expected to profit appreciably more from an increase in partial CO_2 pressure in the growth medium than leaves of

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higher plants. *Anacystis nidulans*, in which the thylakoids appear to be oriented parallel to the cell wall (ALLEN 1968, GANTT & CONTI 1969) seems a favourite organism in this respect.

In order to investigate the conditions under which a fast growth of *Anacystis* in appreciable quantities could be obtained, the influence of CO_2 supply, light intensity, spectral distribution of light and temperature was studied.

2. METHODS

Cells were cultured in flat bottles (200 cm^2 surface area and 5 cm thickness) in a medium as described by KRATZ & MYERS (1955), slightly modified. This medium contained 1.0 g KNO_3, 0.775 g NA_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.025 g $Ca(HO_3)_2 \cdot 4H_2O$, 0.165 g sodium citrate, 0.004 g $Fe_2(SO_4)_3 \cdot 6H_2O$ and 1 ml of a trace element solution in 1 l medium. The latter solution contained: H_3BO_3: 0.57 g, $MgSO_4 \cdot 2H_2O$: 0.2027 g, $ZnSO_4 \cdot 7H_2O$: 0.22 g, $(NH_4)_6M_7O_{24} \cdot 2H_2O$: 0.002 g, $CuSO_4 \cdot 5H_2O$: 0.008 g, $Co(NO_3)_2 \cdot 6H_2O$: 0.025 g per l. In some experiments KNO_3 was replaced by 0.6 g $(NH_4)_2SO_4$. The bottles were illuminated from both sides with light from fluorescent tubes (Philips TL 33, 70–120 W m^{-2} and flushed at a same rate (5 l.h^{-1}) either with air or with air enriched by 5% CO_2. The cultures were inoculated with cells obtained from a 3–5 days old stock culture grown in a shaker incubator at 27°C and at a light intensity of 10 W.m^{-2}. The phycocyanin/chlorophyll absorption ratio of the inoculate, used as a measure for the state of the cells (GOEDHEER 1976) was between 1.2 and 1.4. The density of the inoculation was such, that pigment absorption in the chlorophyll maximum at 680 nm after inoculation amounted to 0.01 in a 1 cm cuvette ($E_1^{680}{}_{cm} = 0.01$). Absorption spectra of the intact cells were recorded with a Shimadzu MPS 50 L spectrophotometer, which is suitable for scattering samples. Cells were also grown in a continuous culture apparatus, which consisted of a thermostated rectangular glass cuvette (200 × 40 × 5 cm) illuminated from both sides with a bank of fluorescent tubes (120 W.m^{-2}), and flushed by CO_2 enriched air. Pigment density was kept constant by addition of fresh culture medium and removal of cell suspension, either by adjustment of cell density with a photocell or by addition at a constant rate.

Growth of a culture can be measured in various ways. KRATZ & MYERS (1955) used optical density at 600 nm as a measure of growth. From their results they assumed that optical density is proportional with cell concentration. In our investigation growth was measured by determination of the dry weight of the cells produced in a fixed period of illumination. These dry weight values were correlated with absorption values of chlorophyll (680 nm), phycocyanin (630 nm) and light scattering at a wavelength at which no absorption occurs (800 nm). Estimation of growth in terms of cell numbers or cell size was not tried here.

Dry weight was determined on centrifuged samples (100 ml) after drying the cells at 55°C for two days. Drying for a prolonged period or drying over

vacuum yielded essentially the same results. Nitrogen and carbon contents were determined by the Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht.

The light intensity was measured with a Lux meter, which was calibrated vs. a thermopile. This occurred in parallel light to avoid effects caused by a different construction of these detectors. The light had passed a 5 cm water-filled glass cuvette to remove infrared and near ultraviolet radiation. Corrections for reflection losses were applied. With incandescent lamps a Calflex Av 700 reflection filter was added to remove light of a wavelength exceeding 700 nm. The samples were taken from the cultures after 2 days of growth at 40 °C and 100 $W \cdot m^{-2}$, and centrifuged for 15 min. They were diluted to an optical density of 0.16 at 680 nm.

Growth experiments were also performed with cells grown in light of incandescent lamps or in light of high pressure mercury lamps of the same energy in the wavelength region 350–700 nm (expressed in $W \cdot m^{-2}$).

3. RESULTS

An example of a short period growth experiment is given in *table 1*. As shown in this table (exp. A), increase in pigment content and light scattering as well as increase in dry weight was rapid during the first 48 hours of illumination if high light intensities from fluorescent tubes (equal to 70 $W \cdot m^{-2}$ in the spectral region 400–700 nm), high temperatures (35–40 °C) and good availability of CO_2

Table 1. Pigment absorption, light scattering and dry weight of cells of *Anacystis* cultures illuminated with fluorescent tubes (70 $W \cdot m^{-2}$) at 39 °C for $2\frac{1}{2}$ days. The cultures were flushed at a rate of 5 $l \cdot h^{-1}$ by air enriched with 5% CO_2 (A), or by plain air (B). After flushing 72 hours in the latter experiments plain air was replaced by CO_2 enriched air (B'). Pigment absorption and light scattering were measured in a 1 cm cuvette.

A					B			
illum. time h	chl. abs. 680 nm	phyc. abs. 630 nm	light scatt. 800 nm	dry weight $g \cdot l^{-1}$	illum. time h	chl. abs. 680 nm	phyc. abs. 630 nm	light scatt. 800 nm
0	0.001	0.012	0.003		0	0.010	0.012	0.003
6	0.013	0.017	0.004		6	0.015	0.015	0.004
12	0.041	0.056	0.021		12	0.017	0.014	0.005
18	0.133	0.234	0.055	0.10	24	0.026	0.021	0.013
24	0.33	0.53	0.14	0.22	72	0.205	0.130	0.120
30	0.69	1.10	0.24	0.47				
36	0.96	1.50	0.36		B'			
48	1.15	1.92	0.44	0.85	90	0.88	1.30	0.43
54	1.54	2.37	0.54		114	1.14	1.63	0.57
60	1.78	0.59	1.28					

(the cultures were flushed by air enriched with 5% CO_2 at a rate of 5 $l \cdot h^{-1}$) were applied. After inoculation a lag period of about 5 hours occurred before increase in chlorophyll content could be measured. After about 12 hours of illumination a doubling time of chlorophyll content of about 4 hours occurred. One and a half day after inoculation the absorption of the *in vivo* chlorophyll maximum (680 nm) had increased from 0.01 to 0.96 (1 cm light path). Between 12 and 48 hours after the start of the experiment there was an approximate proportionality between chlorophyll absorption (680 nm), phycocyanin absorption (630 nm), light scattering (800 nm) and dry weight of produced cells. Chlorophyll production stopped after about $2^1/_2$ days of illumination. The extinction value at 680 nm in our cultures (corrected for scattering) then amounted to $E_{1 cm}^{680} = 1.81 \pm 0.2$. The linear relationship between chlorophyll content and dry weight indicates that during the first days of illumination both parameters may be used as growth indicators.

Table 1 also shows the value of samples flushed at a same rate with plain air (exp. B), but in the same conditions of light and temperature. In these experiments chlorophyll increase was only 7% of that in CO_2 enriched air, while the phycocyanin/chlorophyll ratio decreased within 12 hours from 1.2 to 0.8 (cf. GOEDHEER 1976). When in the experiments B plain air was replaced by CO_2 enriched air and further conditions were kept the same, pigment absorption increased rapidly. After another $2^1/_2$ days a similar final chlorophyll content was reached as the one observed with experiments A. This suggests that the availability of CO_2 was a limiting factor for growth at the light intensities and temperatures used by us.

The availability of CO_2 to the cultures could also be improved when the rate of bubbling with plain air was increased and the contact with the culture medium improved by reduction of the size of the air bubbles. In culture flasks with built in sintered glass filter ("Kluyver flasks") the rate of chlorophyll formation in air-flushed flasks could be speeded up to about half that obtained with a control flask flushed with CO_2 enriched air at usual speed. Also the phycocyanin/chlorophyll ratio resembled that of the control. In these experiments a pH increase to 10.3 after 5 days of growth occurred. Therefore the pH was adjusted between 7.5 and 8.5 during growth.

In cultures containing potassium nitrate as the main nitrogen source and grown in air enriched with CO_2, the pH varied between 7.2 and 7.8 during growth. With cultures using ammonium sulfate as a nitrogen source the pH changed from 7.2 to 6.5 during 3 days of growth.

After $2^1/_2$ days of illumination, when chlorophyll formation had stopped, dry weight values continued to increase. In *fig. 1* the values of chlorophyll (680 nm), and phycocyanin (630 nm) absorption, light scattering (800 nm) and dry weight measured during 8 days of continuous illumination at high light intensities (100 W m^{-2}) are given. The cultures were kept at 39 °C and flushed with CO_2 enriched air. It follows from this figure that about 1 $g \cdot l^{-1}$ dry weight of cells per day was obtained during the first 48 hours. After 48 hours no more chlorophyll or phycocyanin appeared to be formed, and after this period
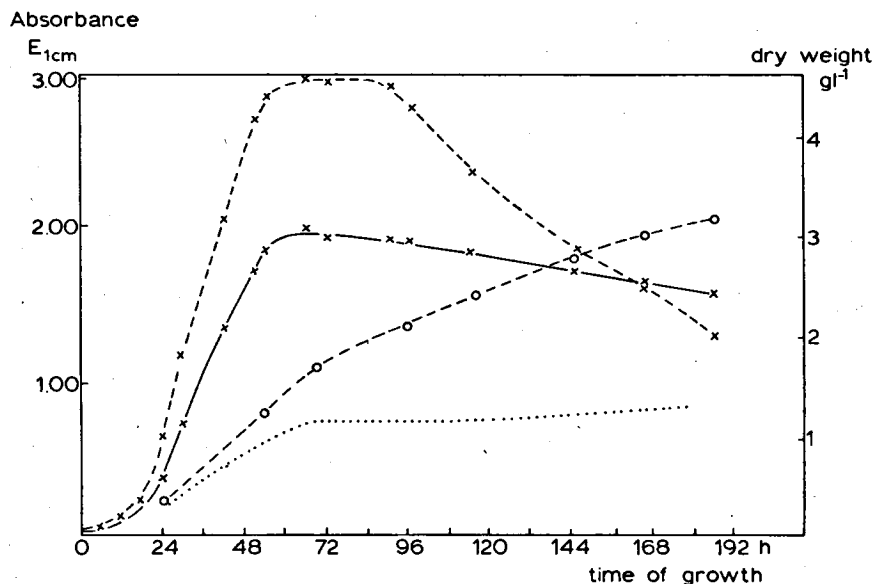
Absorbance

Fig. 1. Increase in pigment absorption, light scattering and dry weight of cells during eight days of growth of *Anacystis* cultures irradiated with light of high intensity ($100 \ W \cdot m^{-1}$).
——— chlorophyll absorption, measured at 680 nm ($E_{1 cm}^{680}$); – – – phycocyanin absorption, measured at 630 nm ($E_{1 cm}^{630}$); ... light scattering, measured at 800 nm; O - - - O dry weight of cells in $g \cdot l^{-1}$. Samples with values of extinction > 1.5 were diluted for measurements.

phycocyanin absorption decreased. Light scattering values were roughly proportional to those of chlorophyll absorption, although after prolonged growth scattering increased while the chlorophyll content decreased slightly.

If the temperature was over 45 °C the culture died and bleached. At a temperature under 30 °C little growth occurred at the light intensities used here and, although sufficient CO_2 was present, the absorption spectrum resembled that of cells grown in plain air.

Fig. 2 shows the increase in absorption ratio: 750 nm/680 nm. At the former wavelength the pigment complex P750, which is characteristic for *Anacystis* (GASSNER 1962, VAN BAALEN 1965, FISCHER & METZNER 1969) is located. The figure indicates that increase in P750 is observed when chlorophyll formation has stopped.

Illumination with incandescent lamps (intensity 50–80 $W \cdot m^{-2}$ between 400 and 700 nm) resulted in a somewhat higher rate of pigment formation than illumination with fluorescent tubes of the same light flux. Growth in light from high pressure mercury lamps on the other hand was 5 times less at the same flux than growth in light from fluorescent tubes.

Growth of *Anacystis* influences the ultraviolet absorption spectrum of the culture medium. Analysis of the u.v. spectrum of the constituents of the medium showed that the high absorption around 250 nm and a weak band at 300 nm
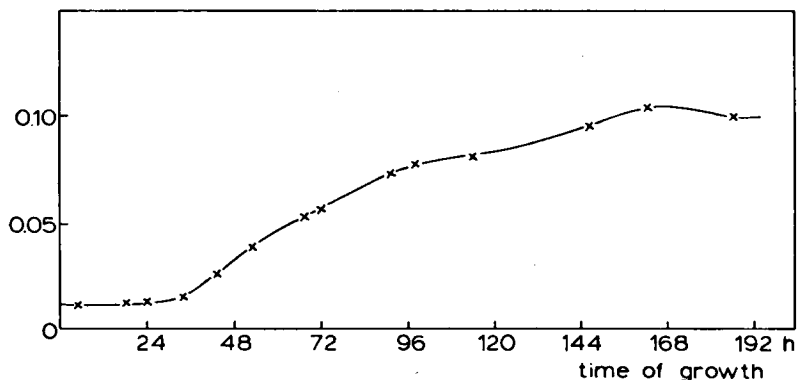
Ratio of absorbance

E_{750}/E_{680}

Fig. 2. Ratio of extinction 750/680 during growth at high light intensities, high temperatures and when flushed by air enriched with CO_2, of *Anacystis* cultures.

could be ascribed to nitrate ions. This absorption decreases as chlorophyll increases (*fig. 3*). In *fig. 4* the absorption spectra from 220–240 nm of a supernatant from *Anacystis* cultures are given at different stages of growth. Due to the high extinction values of nitrate ions below 240 nm, the solutions had to be ten fold diluted.

In *fig. 5a* the nitrogen content of the growth medium and of the cells is given. *Figs. 4* and *5a* show that after about two days of high intensity illumination, when no more new chlorophyll is produced, the absorption ascribed to the nitrate ion as well as the nitrogen content of the medium has been reduced to less than 10% of its original value. Apparently the source of bound nitrogen available to the culture has been nearly depleted after this period of growth. In *fig. 5b* dry weight values of cells and of the growth medium of the samples used in *fig. 5a* are given. At some points the carbon content was determined. As the dry weight values of the cells increase during prolonged illumination while, after $2\frac{1}{2}$ days, no more bound nitrogen is available, it could be expected that the nitrogen percentage of dry weight drops, unless the cells are able to fix atmospheric nitrogen. As shown in *table 2* a drop from 9.9% during the first days of illumination to 4% after 8 days of growth is measured.

Besides a decrease in absorption – ascribed to disappearance of the nitrate ions – an increase at other wavelengths in the ultraviolet part of the absorption spectrum of the growth medium was measured during prolonged growth. The absorption around 270 nm increases slowly during the first two days (*fig. 6*), but the rate of increase is markedly enhanced when chlorophyll accumulation has stopped and illumination proceeds. This results in the appearance of a maximum at 275 nm. After 6 days the absorption at 275 nm was approximately $E_{1\,cm}^{275} = 1.0$ in our conditions of growth. Also at other wavelengths an increase

$E^{225}_{0.1cm}$ E^{680}_{1cm}
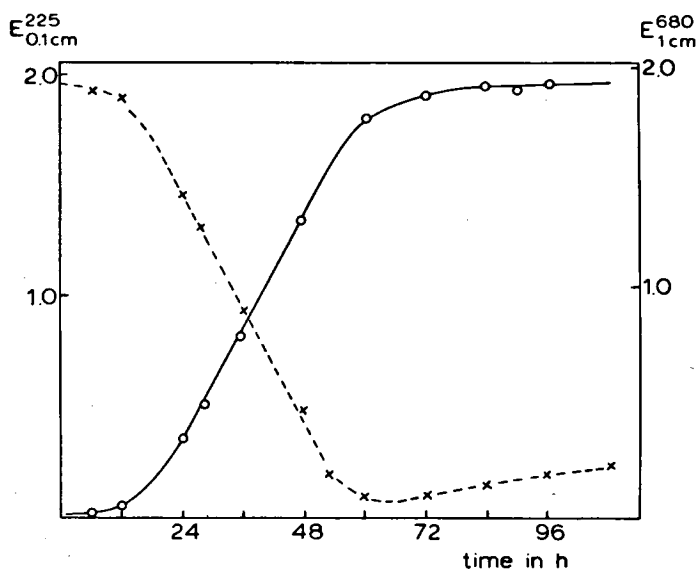

Fig. 3. Increase in chlorophyll extinction (680 nm) and decrease of nitrate ion extinction (at 225 nm) of an *Anacystis* culture grown at 100 W·m^{-2}. The extinction at 225 nm was measured with cell free growth medium.
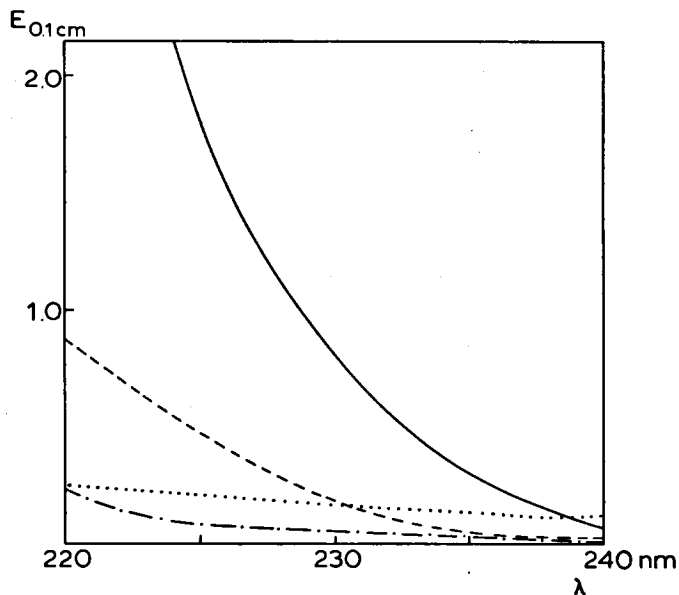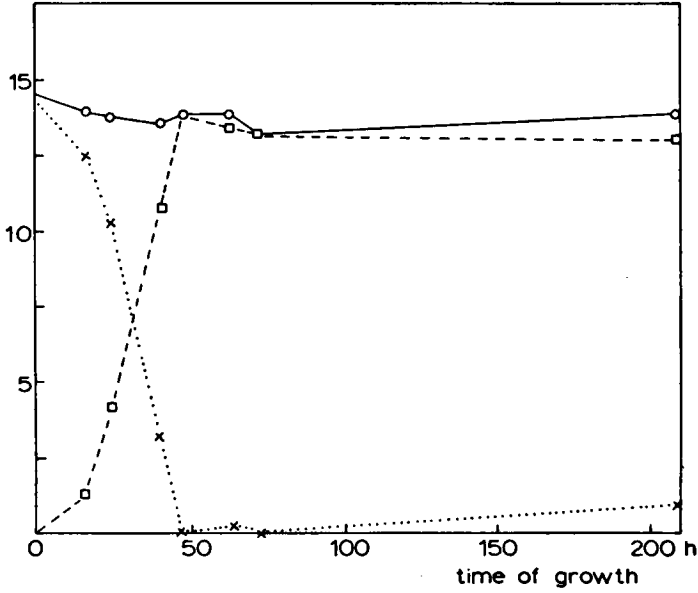
Absorbance

$E_{0.1cm}$

Fig. 4. Ultraviolet absorption spectra from 220 to 240 nm of the growth medium of *Anacystis* cultures. The spectra were measured —— immediately after inoculation; – – – after 2 days of growth; –.–. after 3 days of growth; ... after 6½ days of growth.
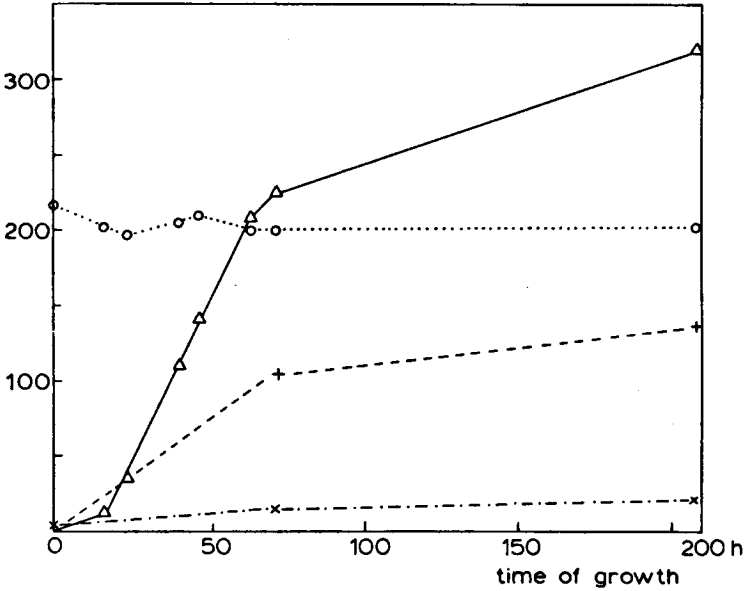
Fig. 5. a. Nitrogen content of 100 ml culture (——), separated cells (– – –), and supernatants
(.....) of *Anacystis* obtained after various periods of growth.
b. Dry weight of cells (——) and supernatant (. . .), and carbon content of cells (– – –) and
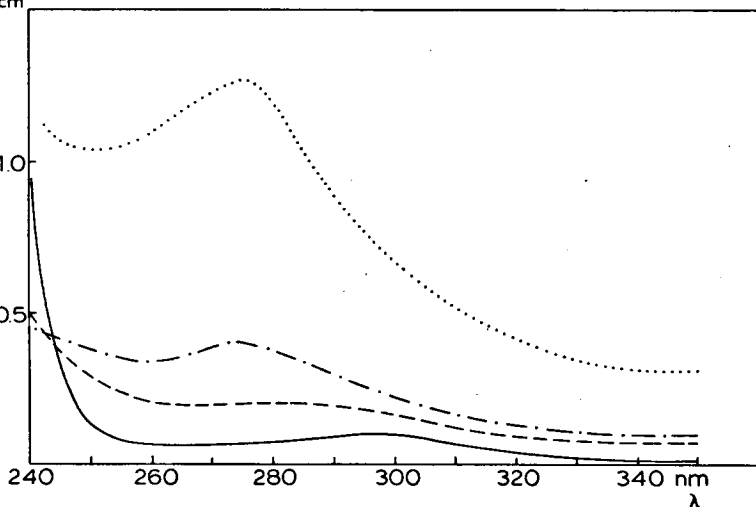supernatant (–.–.) of 100 ml culture of *Anacystis* obtained after various growth periods.

absorbance

Fig. 6. Ultraviolet absorption spectra from 240 to 350 nm of the growth medium of *Anacystis* cultures. The spectra were measured —— immediately after inoculation; – – – after 2 days of growth; –.–. after 3 days of growth; .... after 6½ days of growth.

in absorption occurs. At 225 nm, however, the absorption did not surpass 15% of that of the nitrate ion at the start of the experiment. The u.v. spectrum after 8 days of growth resembles somewhat that of the aromatic amino acid tyrosine, though the extinction values around 240 nm were found to be appreciably higher in the supernatant.

The nitrogen content of the supernatant increases slowly after 50 hours of illumination. About 6% of cellular nitrogen is found in the growth medium at

Table 2. Dry weight, nitrogen and carbon content of cells and growth medium from 100 ml samples of *Anacystis* cultures, taken after various periods of growth at 40 °C and 100 W·m^{-2}.

illum. time h	Cells total mg	N %	N mg	C %	C mg	Medium total mg	N %	N mg	C %	C mg
0	–	–	–	–	–	219.6	6.6	14.5	1.5	3.0
17	14.6	9.0	1.3			204.1	6.2	12.6		
24	37.2	9.8	3.6			198.7	5.1	10.2		
41	112.4	9.6	11.0			206.4	1.3	2.7		
47.5	144.2	9.6	13.8			210.4	–	–		
65	209.9	6.4	13.5			202.5	0.2	0.3		
71.5	226.4	5.8	13.2	44.8	101.4	200.1	–	–	7.0	13.9
211	319.4	4.1	13.0	42.6	136.0	204.0	0.4	0.9	11.7	21.9

the end of a growth period of 8 days, versus 16% of cellular carbon. In the nitrate medium 0.14 g l^{-1} of nitrogen is present as a nutrient. If, spectroscopically, nitrogen bound as nitrate was used up, cells with a dry weight of about 1.4 g were produced, which implies that the cells should have a nitrogen percentage of about 10%. This value fits in well with the measured one. From the values of the average nitrogen content of amino acids (about 16%) it may be estimated that the cells contain about 60% of protein during the first phase of growth, although part of the nitrogen will be bound in pigments and other cell constituents.

To check whether the high maximum rate of growth, measured in the single batch method (1 l bottles) could also be obtained in continuous cultures (25 l content), the daily production was measured during a 7 days period after the suspension had grown to a fixed pigment density. The production was found to be approximately equal to that obtained in the 1 l bottles.

A constant pigment density could, apart from adjustment with a photocell, also be obtained by continuous addition of fresh nutrient. Samples taken during 24 hours did not give any evidence of rhythmic processes.

4. DISCUSSION

Fast growth, and consequently efficient energy conservation at relatively high light intensities occurred in *Anacystis*, but only within a limited range of environmental conditions. Growth could only be adequately described by optical parameters (chlorophyll and phycocyanin content, light scattering) during the first days of illumination. In this paper no use is made of a specific growth rate defined by the equation: $\log_{10}(N/N_0) = kt$ (cf. KRATZ & MYERS 1955), in which N_0 and N are the cell numbers before and after a growth period: t. Although the number of cells seems proportional to the chlorophyll concentration during the first days of growth when ample nitrogen is present, k is a constant only in the light saturated state. Both growth determinations and measurements of photosynthetic carbon dioxide uptake indicate that with *Anacystis* at 40 °C the level of light saturation is appreciably above the level of illumination used here. As a consequence the specific growth rate is a direct function of light intensity. Hence also the doubling time of chlorophyll concentration is a similar function.

The uptake of nitrogen from the growth medium measured spectroscopically and by element analysis showed that, as soon as the nitrogen source in the medium is used up, no increase in pigment absorption occurs. A steady increase in dry weight however, is found. The relative nitrogen content of the algae per unit of weight dropped while the carbon content increased. Apparently the photosynthetic pattern changes from production of nitrogen containing compounds (primarily proteins and pigments) to nitrogen deficient compounds (primarily carbohydrates and fats). The dependence of the end products of the photosynthetic energy conservation chain on the growth conditions of the cells

appears to be a more general phenomenon with algae. *Chlorella*, essentially a protein synthesizing organism, can be forced into a predominantly carbohydrate metabolism (SPOER & MILNER 1949, MYERS 1970).

For fast growth at high light intensities the culture temperature should be between 35° and 41°C. At temperatures below 30°C little growth occurred, while at temperatures over 45°C the cultures died. The spectral distribution of light was found to be important for obtaining high growth rates. Light absorbed by carotenoids, especially xantophylls, is not transferred to either of the two photosystems in blue-green and red algae (GOEDHEER 1965). Therefore this light is probably not active in the photosynthetic process proper. This phenomenon may explain that the conversion efficiency in light of mercury lamps, which contains a high percentage of blue-violet light, absorbed partly by xantophylls, is low. The increase in growth rate, found when a yellow filter is placed between lamps and sample, might indicate that excess of blue light even inhibits growth.

Comparison of the ultraviolet absorption measurements and nitrogen content found by element analysis shows that decrease in nitrate absorption is a reliable measure of nitrogen consumption by the cells in our culturing conditions.

The changes in ultraviolet spectrum of the supernatant during prolonged growth indicate that ultraviolet radiation absorbing substances, possibly including aromatic peptides or amino acids, are excreted by the cells only after the nitrate of the medium has been used up. However, only about 6% of cellular nitrogen was excreted. FOGG (1952) gives values between 15 and 30% of total nitrogen present as extracellular nitrogen in polypeptides for *Anabaena cylindrica*; the lowest values were measured in the exponential phase of growth. Similar results were recorded for *Nostoc entophytum* (STEWART 1962), *Calotrix scopulorum* (JONES & STEWART 1969) and *Westielopsis prolifica* (PATTNAIK 1966), all heterocysteous blue-green algae, known to be able to fix atmospheric nitrogen. With *Anacystis*, no fixation of atmospheric nitrogen in our conditions of growth could be detected. It remains to be investigated whether the presence of nitrogen and carbon in the medium is due to lysis of dead cells or to excretion by living ones.

The marked increase in pH measured when the cells in the fast growth phase are flushed by air and grown with nitrate as a main nitrogen source can be expected if the overall equation of photosynthesis leads to production of protein instead of carbohydrates (cf. MYERS 1970). If the cells are flushed with air enriched by 5% CO_2 the increase in pH is much less as only a fraction CO_2 is taken up by the cells while the rest is used to buffer the suspension.

Our experiments suggest that about $1 g \cdot 1^{-2} \cdot day^{-1}$ dry weight of cell material, containing about 60% of protein, can be harvested with artificial illumination and CO_2 feeding, provided sufficient other nutrients are available. With an optical light path as used here of 5 cm, this corresponds to a yield of $50 g \cdot m^{-2}$ illuminated surface per day, resulting from continuous illumination. With an energy value of $5.5 kCal \cdot g^{-1}$, this is equivalent with a production of 275 $kCal \cdot m^{-2} \cdot day^{-1}$.

Solar radiation, averaged over day and night and winter and summer, does not exceed $260 \, W \cdot m^{-2}$ even in dry subtropic regions (CALVIN 1974). A multiple of this value, however, occurs during illumination in the midday hours. The decrease in photosynthetic rate and growth rate at very high intensities indicates that, although high production values of *Anacystis* in artificial light fields under optimum conditions of nitrogen can be obtained, this may not hold when the cells are grown in out-door conditions.

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