PIGMENT SYSTEMS IN ALGAE

ELECTRON TRANSPORT TO NITROGENASE IN *ANABAENA*

MARCEL DONZE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN
DE WISKUNDE EN NATUURWETENSCHAPPEN AAN
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DOOR

MARCEL DONZE

GEBOREN TE St. LAURENS
THANS GEMEENTE MIDDELBURG
IN 1943

PROMOTOR: PROF. DR. L.N.M. DUYSENS

CO-REFERENT: DR. J. AMESZ

CONTENTS

STELLINGEN

1. Het quantum rendement van de fotosynthese is onafhankelijk van het levensstadium van een cel.

Dit proefschrift

2. De waarnemingen die door Carr aangevoerd worden als verklaring voor de obligate fotoautotrofie van Anacystis nidulans, zouden eerder pleiten voor het tegendeel.

N.G.Carr, J. Gen. Microbiol.75 (1973), V-VI

3. De waarneming dat quantum rendement van fotoreductie en fotosynthese gelijk zijn betekent niet dat het zuurstof ontwikkelende mechanisme deelneemt aan de fotoreductie.

L. H. Grimme in: Proc. IInd Int. Congr. Photosynthesis Res. (1972) p. 2011.

4. Het onderwijs in de statistiek aan biologen en de toepassingen van statistiek door biologen nemen schrikbarende vormen aan.

5. De theorien van van Leeuwen over planten ecologie zijn overbodig.

C. G. van Leeuwen, Gorteria 2 (1965) 93-105

6. Het is jammer dat het wetenschappelijk Latijn uit de mode is. Het is vreemd wanneer het wetenschappelijk Chinees als schrijftaal ontwikkeld wordt.

C. van den Hoek et al. Verh. Kon. Ned. Akad. afd. Natuurk., tweede reeks, deel 61, No.2.

7. Synchrone celdeling kan een belangrijke factor zijn bij het ontstaan van waterbloei.

8. Het verband dat Fischer-Piette meent te vinden tussen het voorkomen van Himanthalia elongata en de isobath van 50 m is ver gezocht.

E. Fischer-Piette, J. Linn Soc. Zool. 40 (1936) 181-272.

9. Het pijproken is, evenals het roken van sigaren, en- zij het in mindere mate- het roken van sigaretten, in vele opzichten een maatschappelijk nuttige bezigheid.

23 mei 1973

M. Donze

INTRODUCTION

In photosynthesis the energy of light is converted into chemical free energy. In algae and higher plants the net result of the proces is the oxidation of water to oxygen, together with the reduction of carbondioxyde to organic compounds. The most important pigment which is present in all photosynthetic organisms that produce oxygen is chlorophyll a. Apart from chlorophyll a several other pigments may contribute the light they absorb to photosynthesis. Of these accessory pigments carotenes and xanthophylls are always present, while the presence of phycobilins, chlorophyll b and several other types of chlorophyll depends on the kind of organism.

Upon absorption of a quantum of light, a pigment molecule gets into an exited electronic singlet state. The exitation energy can be transferred rapidly among the different pigment molecules. By this proces almost all of the quanta absorbed by a plant are transferred to the lowest singlet state of chlorophyll a, which corresponds to an absorption band around 680 nm. There is evidence that the photochemical reactions obtain their energy from this exited state. In this band the exitation may follow a random walk over several hundreds of chlorophyll molecules. Only few chlorophyll molecules, called reaction center chlorophyll, are capable of photochemical reaction. If such a reaction center chlorophyll is excited an electron of the chlorophyll is transferred rapidly to a primary acceptor molecule. This reaction proceeds so fast as to make back transfer of the excitation energy to the bulk chlorophyll improbable. The hole in the reaction center chlorophyll can be filled by a reaction with the primary electron donor molecule. Reaction centers occur in a concentration of 1% to 0.2% of the chlorophyll concentration.

The oxidized and reduced sides of the reaction centers react with components of an electron transport chain. This finally leads to formation of the net products of photosynthesis.

Two photochemical reactions are required per electron transferred from water to carbondioxide. There is evidence that these reactions operate in series. The photoreaction which produces the strong reductant NADPH, needed to reduce carbondioxide is called system 1, the reaction which produces the strong oxidant, needed to oxidize water is system 2. The primary electron acceptor of system 2 reduces, via a chain of electron transport components, the primary electron donor of system 1. The arguments that have led to the model of the photosynthetic mechanism that was sketched above have been discussed by DUYSENS (1964).

The reaction centers of the two photosystems have different properties that can be studied by spectroscopic techniques. The structure of the reaction center of system 1 was discussed by KE (1973), and that of system 2 was discussed by van GORKOM and DONZE (1973).

The different pigments that are active in gathering light energy generally are distributed unequally over the two photosystems which makes their action spectra different from each other. With far red light system 1 can be excited exclusively. At all other wavelengths in the visible range of the spectrum both systems have a significant absorption, although the ratio of the amount of light absorbed may vary. The acessory pigments generally dominate in the action spectrum of system 2, while chlorophyll a, and especially its long-wavelength forms, dominates in system 1.

An increase in the fluorescence yield of the bulk chlorophyll of system 2 occurs if the acceptor becomes reduced, and no other quencher is present. In these investigations we mostly used this effect to study the behavior of system 2. No changes in fluorescence of system 1 have been identified at room temperature. To study this system the absorption decrease at 705 nm which occurs if the primary donor P700 of this system is oxidized was used most often.

In part 1 the role of the photosystems in electron transport to nitrogenase in the blue-green alga Anabaena cylindrica, and the localization of nitrogen fixation in special cells in this organism are investigated. In part 2 we report some results on the behaviour of photosynthesis in synchronous cultures of a green alga, Scenedesmus. A common theme in both parts is the hypothesis, that a large pool of reductant for the primary donor of photosystem 1 may accumulate. This reductant is generated in the cytoplasm, which occurs especially if the organisms are in a condition of high carbohydrate reserves. Part 3 comprises some experiments on fluorescence at room temperature and at liquid nitrogen temperature. It is shown that at liqiud nitrogen temperature light induced fluorescence changes from system 1 occur.

REFERENCES

DUYSENS, L. N. M.(1964) Prog. Biophys. Mol. Biol. 14,p. 1.

KE, B. (1973) Biochim. Biophys. Acta, 301, p.1-33.

van GORKOM, H. J. and M. DONZE (1973) Photochem. Photobiol. in the press.

PIGMENT SYSTEMS

AND ELECTRON TRANSPORT TO NITROGENASE

IN ANABAENA CYLINDRICA

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Localization of Nitrogen Fixation in *Anabaena*

THE function of heterocysts in blue-green algae has been controversial for some time; there are indications that these enlarged cells are the site of nitrogen fixation[1,2]. But non-heterocystous blue-green algae may fix nitrogen if grown under low oxygen tension[3], so that heterocysts are not essential for nitrogen fixation in these algae. According to a current hypothesis[4], in aerobic conditions nitrogen fixation is confined to heterocysts, while in anaerobic or semi-anaerobic conditions the vegetative cells fix nitrogen as well. We have evidence to support this view.

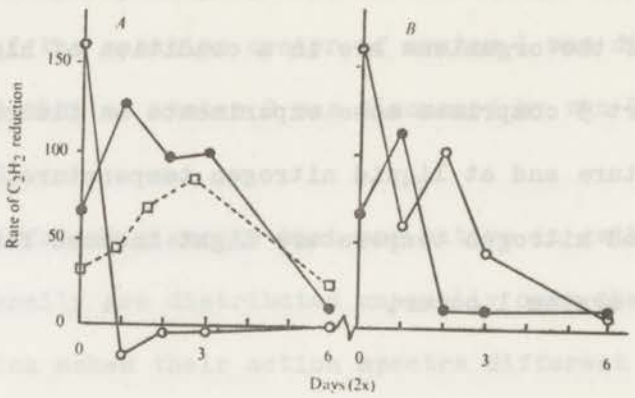

Fig. 1 Oxygen sensitivity of acetylene reduction after readmission of N_2 to an N-starved culture of *Anabaena*. A nitrogen-starved culture (grown under $H_2+5\%$ CO_2 for 5 days) was incubated at t=0 under air+5% CO_2 (*A*) and $N_2+5\%$ CO_2 (*B*). Ethylene production was measured gas chromatographically after 30 min incubation of samples under air+10% C_2H_2 (●), and under He+10% C_2H_2 (total). O_2 sensitive C_2H_2 reduction (○) is plotted as total minus O_2 resistant rate. Aerobic C_2H_2 reduction during the normal growth cycle of an aerobic culture (□) is also shown in *A*. Rate of C_2H_2 reduction was normalized on a chlorophyll basis

$$\left(\frac{\text{nmol } C_2H_2/\text{ml./30 min}}{\text{A(680 nm–740 nm)/cm}}\right)$$

and plotted against time after readmission of nitrogen.

One of the first obvious effects of nitrogen starvation in blue-green algae is the disappearance of phycobilin pigments, which normally constitute about 15% of the dry weight. The continuation of some growth and chlorophyll synthesis during starvation suggests[5] that phycobilins serve as a reserve source of nitrogen. We have studied the pattern of synthesis of one phycobilin, phycocyanin, after readmission of nitrogen to cultures starved of the element.

Cultures of *Anabaena cylindrica* (Cambridge Culture Collection No. 1403/2a) were grown in the medium of Allen and Arnon[6], and gassed with 5% carbon dioxide in air. Phycocyanin was observed by its autofluorescence and absorption under the microscope. Fluorescence was excited with a high pressure mercury arc from which the 546 nm line was isolated by a filter combination consisting of Balzers Calflex C and Corning CS 4–96 (2) and CS 3–368. Phycocyanin fluorescence was isolated with a Balzers B-40 632 nm and a Schott AL 638 interference filter, transmitting a band of 7 nm halfwidth at 632 nm. In cells of *Chlorella* no fluorescence could be seen in these conditions, so chlorophyll fluorescence is negligible at this wavelength. The absorption image was studied with the filter combination used for isolating the fluorescence. A phycobilin-free culture was obtained by bubbling a normal culture with $H_2 + 5\% CO_2$ for 6 days.

If nitrogen was readmitted aerobically (5% carbon dioxide in air) most series of vegetative cells situated between two heterocysts showed a gradient in phycocyanin fluorescence after 20 h. Intensity was greatest in the cells adjacent to heterocysts and least about halfway between the heterocysts. Normal mature heterocysts do not fluoresce. The most evident fluorescence gradients corresponded to gradients in phycocyanin absorption, so that the results are due to differences in phycocyanin concentration and not in fluorescence yield in different cells. If these gradients are caused by differences in the availability of nitrogen, this experiment shows that in an aerobically grown culture of *Anabaena*, N_2 is fixed exclusively or predominantly in the heterocysts.

If N_2 was readmitted anaerobically (5% CO_2 in N_2), the rate of phycocyanin synthesis was the same but gradients in phycocyanin concentration were rare and far less pronounced than in aerobic cultures. This indicates that in anaerobic conditions vegetative cells also fix nitrogen. It also seems that all cells reduce neotetrazolium in anaerobic cultures. After addition of ammonium phosphate, whether aerobic or anaerobic, no gradients of phycocyanin concentration develop during synthesis of the pigment.

An aerobic culture that was in the process of losing its phycocyanin due to molybdenum deficiency also exhibited concen-

tration gradients. Here heterocysts appeared also in the regions with low phycocyanin content, alternating with heterocysts whose neighbouring cells had a high concentration of phycocyanin. This pattern indicates that these were young heterocysts, differentiated after molybdenum had been exhausted in the medium, and so unable to synthesize nitrogenase. This experiment confirms that the gradients in phycocyanin concentration are due to nitrogen deficiency and that nitrogen is fixed chiefly by the heterocysts. It also seems that molybdenum does not migrate from one cell to another in significant amounts.

Another consequence of the hypothesis is that it should be possible to distinguish nitrogen fixation by vegetative cells and by heterocysts on the basis of their oxygen sensitivity. The reduction of acetylene by anaerobic cultures is strongly, and in part irreversibly, inhibited by oxygen. In aerobic cultures inhibition by oxygen, if any, is completely reversible. In Fig. 1 the oxygen sensitive and oxygen resistant parts of acetylene reduction by the aerobic (*A*) and anaerobic (*B*) nitrogen-starved cultures are plotted as a function of time after readmission of the gas. The dotted line in Fig. 1*A* represents the pattern of acetylene reducing activity during the growth of a normal aerobic culture, which roughly parallels the growth rate. On the first 2 days after nitrogen starvation there is an extra acetylene reduction, which is also resistant to oxygen in the anaerobic culture (Fig. 1*B*). If oxygen resistant nitrogen fixation is located in the heterocysts, this may account for the few and indistinct phycocyanin gradients seen in the anaerobic culture.

Other arguments have been published as evidence that the heterocyst is the site of nitrogen fixation[7]. As Smith and Evans[8] emphasized, the yield of acetylene reduction by isolated heterocysts was too low to permit any direct conclusions. Heterocysts reduce neotetrazolium to formazan crystals after removal of tetrazolium, and acetylene reduction was irreversibly inhibited in filaments in which the heterocysts contained formazan crystals. We found, however, that the formation of microscopically visible formazan crystals, but not the irreversible inhibition of acetylene reduction by neotetrazolium, can be prevented by 2 mM sodium azide during treatment with tetrazolium.

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[1] Fay, P., Stewart, W. D. P., Walsby, A. E., and Fogg, G. E., *Nature*, **220**, 810 (1968).
[2] Stewart, W. D. P., Fitzgerald, G. P., and Burris, R. H., *Arch. Mikrobiol.*, **62**, 336 (1968).
[3] Stewart, W. D. P., and Lex, M., *Arch. Mikrobiol.*, **73**, 250 (1970).
[4] Stewart, W. D. P., *Plant and Soil* (in the press).
[5] Allen, M. M., and Smith, A. J., *Arch. Mikrobiol.*, **69**, 114 (1969).
[6] Allen, M. B., and Arnon, D. I., *Plant Physiol.*, **30**, 366 (1955).
[7] Stewart, W. D. P., Haystead, A., and Pearson, H. W., *Nature*, **224**, 226 (1969).
[8] Smith, R. V., and Evans, M. C. W., *Nature*, **225**, 1253 (1970).

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BBA Report

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Absence of Photosystem 2 in heterocysts of the blue-green alga Anabaena

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SUMMARY

Heterocysts of the filamentous blue-green alga *Anabaena cylindrica* have a high concentration of the System 1 reaction center P700 and are able to photooxidize a cytochrome. They have a low yield of chlorophyll *a* fluorescence, do not show light-induced changes in fluorescence yield, show a very low intensity of delayed light emission and do not show Hill-reaction activity. It is concluded that heterocysts contain Photosystem 1 only.

Many filamentous blue-green algae form two kinds of cells: "normal" vegetative cells and heterocysts. Normally about 5% of the cells are differentiated into heterocysts; in Anabaena these enlarged cells occur singly and rather regularly spaced along the filament. They contain two cellular inclusions of about 1 μm; the function of these so-called polar bodies is unknown. Heterocysts are an interesting subject for the study of cell differentiation[1]. Under aerobic conditions heterocysts are believed to be the site of N_2 fixation[2,3]. Pigment composition and several other indirect arguments[2,4] suggested that heterocysts do contain System 1 but not System 2 of the photosynthetic mechanism.

Here we present direct evidence which supports this hypothesis. *Anabaena cylindrica* (Cambridge Culture Collection No. 1403/2a) was grown in the medium of Allen and Arnon[5] in 1 l Roux bottles. During growth the culture was gassed with 5% CO_2 in air, and stirred vigorously by a "vibro-Mischer" (Chemap AG, Switzerland) to prevent clumping of the filaments. Absorption spectra were recorded with a Cary 14 spectrophotometer equipped with a scattered light transmission accessory. Changes in intracellular light absorption were measured with a split-beam difference spectrophotometer[6]. The Hill reaction was measured with an Aminco–Chance dual wavelength spectrophotometer which was provided with a side-illumination for one of the cuvettes. Fluorescence spectra were recorded with the instrument used by Vredenberg and Slooten[7]. Light-induced changes in

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine.

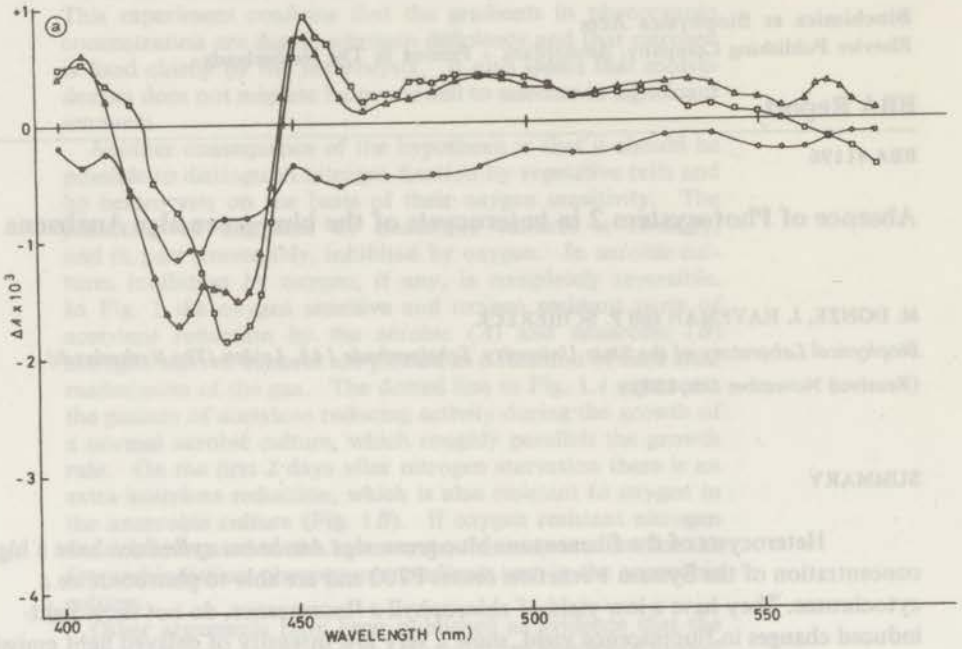
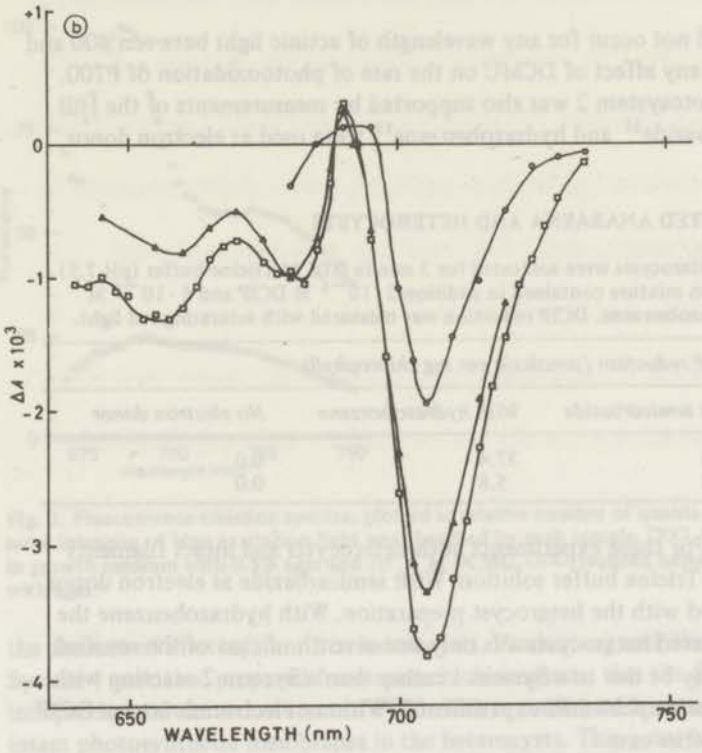


Fig. 1. Light *minus* dark absorption difference spectra. The absorbance of all samples at 680 nm, corrected for scattering was 0.30. (a) Saturating red actinic light (Schott RG 665-2 mm filter). (b) Saturating blue actinic light (Corning CS 4-96 and CS 5-61 filters). ○—○, intact filaments of Anabaena in growth medium with 0.5% agar to prevent movements of the organism. The initial rapid (2 sec) absorption change is plotted only. △—△, heterocysts isolated in growth medium with 0.25 M sucrose and 1 ‰ mercaptoethanol. Shortly before the measurement $2.5 \cdot 10^{-4}$ M ascorbate and 10^{-5} M DCIP were added to reduce P700 and cytochrome in the dark. □—□, heterocysts isolated in growth medium; ascorbate and DCIP added.

the fluorescence yield of chlorophyll were measured with an instrument analogous to the one used by Duysens and Sweers[8]. Fluorescence excitation light was filtered by a Corning CS 4-96 and a CS 5-61 filter, which transmit a broad band from about 400 to 500 nm. The emission was isolated with a Schott Al 683 interference filter together with a RG 5 cut-off filter. Delayed light from chlorophyll was measured with a Becquerel phosphoroscope at a chopping frequency of 570 Hz, with the same filter combinations as mentioned above. Chlorophyll fluorescence was also studied with a fluorescence microscope. A high-pressure mercury arc from which the blue lines were isolated with the same filter combination as mentioned above was used for excitation. The fluorescence image was observed through a Schott RG 695 cut-off filter. This filter transmitted only a negligible amount of phycocyanin fluorescence, as was checked by adding phycocyanin to the sample at such a concentration that the background fluorescence at 630 nm was much higher than that of the vegetative cells.

Heterocysts were isolated after destruction of the vegetative cells in a French press[9]. Cell debris was removed by filtration through a 2-μm Nuclepore filter (General Electric). Polar bodies were prepared by sonication of isolated heterocysts and separated from chlorophyll-containing membrane fragments by centrifugation in 25% fycoll (Pharmacia,



Sweden) solution. The polar bodies were identified under a polarization microscope by means of their optical anisotropy.

Light *minus* dark absorption difference spectra were measured for intact filaments and isolated heterocysts of Anabaena (Fig. 1). Illumination of isolated heterocysts caused an oxidation of the System 1 reaction center P700 with main bands at 705 nm and 437 nm and of a cytochrome with bands at about 420 nm and 560 nm. Isolated heterocysts do not reduce P700 and cytochrome in the dark, so an artificial reducing couple (ascorbate and DCIP) was added in our experiments. Cytochrome oxidation was observed only if the heterocysts were isolated in growth medium with sucrose and mercaptoethanol; washing in medium without these additions abolished this reaction. The time dependence of the light-induced absorption changes in intact filaments was rather complicated, only the first rapid (2 sec) change is plotted in the difference spectrum.

Assuming that the molar extinction of P700 at 705 nm is the same as that for chlorophyll *a in vivo*, we found a P700 concentration of 1 molecule to 90 chlorophyll molecules in heterocysts and in isolated polar bodies. The concentration in intact filaments was 1 to 170 chlorophylls. The same value was obtained by repeating the measurement in the presence of 10^{-6} M DCMU, 10^{-4} M ascorbate and 10^{-5} M DCIP.

These observations show that heterocysts contain an active Photosystem 1. The absence of Photosystem 2 is indicated by the high concentration of P700, which is about the same as found by Ogawa *et al.*[10] in System 1 particles isolated from *Anabaena variabilis*. This conclusion is also supported by the observations that in isolated heterocysts

photoreduction of P700 did not occur for any wavelength of actinic light between 400 and 750 nm and the absence of any effect of DCMU on the rate of photooxidation of P700.

The absence of Photosystem 2 was also supported by measurements of the Hill reaction (Table I). Semicarbazide[11] and hydrazobenzene[12] were used as electron donor,

TABLE I

HILL REACTION OF SONICATED ANABAENA AND HETEROCYSTS

Intact Anabaena and isolated heterocysts were sonicated for 3 min in 0.05 M Tricine buffer (pH 7.5) with 0.01 M $MgCl_2$. The reaction mixture contained in addition $2 \cdot 10^{-5}$ M DCIP and $5 \cdot 10^{-3}$ M semicarbazide or 10^{-4} M hydrazobenzene. DCIP reduction was measured with saturating red light.

	DCIP reduction (μmoles/h per mg chlorophyll)		
	With semicarbazide	*With hydrazobenzene*	*No electron donor*
Sonicated Anabaena	23.0	37.4	0.0
Sonicated heterocysts	0.0	5.8	0.0

DCIP as electron acceptor. For these experiments both heterocysts and intact filaments were sonicated for 3 min in Tricine buffer solution. With semicarbazide as electron donor no Hill reaction was observed with the heterocyst preparation. With hydrazobenzene the rate of reduction with sonicated heterocysts was only one-seventh of that of the sonified filaments. This (low) rate may be due to a System 1 rather than a System 2 reaction with hydrazobenzene (J. Haveman, unpublished experiments). Without electron donor no DCIP reduction was observed in either case.

Delayed light from chlorophyll has been shown to arise mainly in Photosystem 2[13] In agreement with this we found that delayed light from heterocysts was 0.2 ± 0.1% from the delayed light emitted by intact filaments for the same intensity of absorbed light.

In oxygen-evolving photosynthetic organisms the fluorescence yield of chlorophyll *a* belonging to System 2 is several times higher than the yield of System 1 chlorophyll *a*[14]. Changes in the fluorescence yield that occur upon illumination are generally ascribed to variations in the state of System 2[8]. Changes in the fluorescence of System 1 are considered small or nonexistent[7,14]

Fluorescence emission spectra of intact filaments and isolated heterocysts are shown in Fig. 2. The fluorescence from heterocysts was several times lower than from vegetative cells and especially the emission in the region 670–700 nm was strongly reduced. The low yield of chlorophyll fluorescence was not an artifact due to the isolation procedure. Observation of a preparation containing isolated heterocysts and intact filaments with a fluorescence microscope showed no observable difference between the fluorescence yield of isolated heterocysts and heterocysts in intact filaments.

Light-induced changes in the yield of chlorophyll fluorescence in intact filaments showed the normal pattern for blue-green algae as discussed by Duysens and Talens[15]. In isolated heterocysts no light-induced changes in the fluorescence yield exceeding 0.5% of the total fluorescence were observed. Addition of DCMU or reduction by dithionite did not affect the fluorescence yield, within the same error of measurement.

Both the low fluorescence yield and the absence of changes in fluorescence indicate

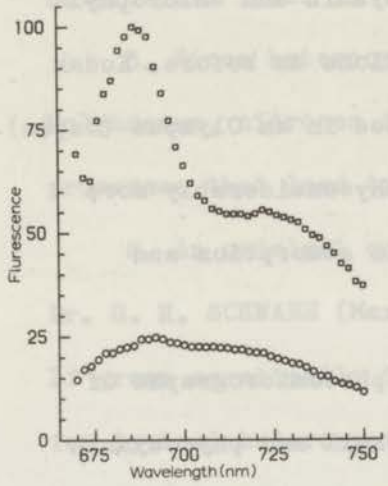


Fig. 2. Fluorescence emission spectra, plotted as relative number of quanta per wavelength interval. The same intensity of blue excitation light was absorbed by each sample. □□□, intact filaments of Anabaena in growth medium with 0.5% agar and 10^{-7} M DCMU. ○○○, isolated heterocysts in growth medium with agar.

the absence of Photosystem 2 in heterocysts. Vredenberg and Slooten[7] demonstrated with System 1 particles, prepared from spinach chloroplasts, that the fluorescence of System 1 is independent of the redox state of P700. This is confirmed by our experiments for the intact photosynthetic membranes in the heterocysts. This point has been discussed in detail by Duysens[14].

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REFERENCES

1 P. Fay and N.J. Lang, *Proc. R. Soc. London, Ser. B*, 178 (1971) 185.
2 P. Fay, W.D.P. Stewart, A.E. Walsby and G.E. Fogg, *Nature*, 220 (1968) 810.
3 W.D.P. Stewart and M. Lex, *Arch. Mikrobiol.*, 73 (1970) 250.
4 J. Thomas, *Nature*, 228 (1970) 181.
5 M.B. Allen and D.I. Arnon, *Physiol. Plant.*, 8 (1955) 652.
6 J. Amesz, Thesis, University of Leiden, 1964.
7 W.J. Vredenberg and L. Slooten, *Biochim. Biophys. Acta*, 143 (1967) 583.
8 L.N.M. Duysens and H.E. Sweers, in S. Miyachi, *Studies on Microalgae and Photosynthetic Bacteria*, Special Issue of *Plant Cell Physiol.*, Tokyo, 1963, p. 353.
9 P. Fay and A.E. Walsby, *Nature*, 209 (1966) 94.
10 T. Ogawa, L.P. Vernon and H.H. Mollenhauer, *Biochim. Biophys. Acta*, 172 (1969) 216.
11 T. Yamashita and W.J. Butler, *Plant Physiol.*, 44 (1969) 435.
12 J. Haveman and M. Donze, *Proc. 2nd Int. Congr. Photosynth. Res., Stresa, 1971*, in the press.
13 J.C. Goedheer, *Biochim. Biophys. Acta*, 64 (1962) 294.
14 L.N.M. Duysens, *Energy Conversion by the Photosynthetic Apparatus, Brookhaven Symp. Biol.*, 19 (1966) 71.
15 L.N.M. Duysens and A. Talens, in H. Metzner, *Progress in Photosynthesis Research*, Vol. II, Tübingen, 1969, p. 1073.

ADDENDUM TO THE PRECEDING PUBLICATIONS

A number of additional observations were made on pigment distribution and cell differentiation in Anabaena and Hapalosiphon, some of these are reported here. Photomicrographs of ligh absorption by the cells and of fluorescence of phycocyanin and chlorophyll were taken, using the same filter combinations as before. Kodak HIE 135-20 High speed Infrared Film was used in an Olympus (Japan) microscope camera. We found photomicrographyconsiderably more sensitive in detecting small differences in absorption and fluorescence than visual observation.

1. From a study of a large number of photomicrographs of the absorption and fluorescence of chlorophyll and phycocyanin the order of the changes in pigment composition during the differentiation of a vegetative cell into a heterocyst could be inferred. The first change we could observe was the disappearance of phycocyanin fluorescence and absorption. After this had happened the fluorescence yield of chlorophyll, as excited with blue light, started to fall off. So the breakdown of photosystem 2 probably is the second proces that can now be recognized in the differentiation. Only after the chlorophyll fluorescence had decreased formation of the thickened cell wall started.

2. Molybdemum is an essential element both in nitrogenase and in nitrate reductase. If a culture of Anabaena was starved for Molybdemum aerobically in the absence of combined nitrogen, the frequency of the heterocysts increased from about 4% to 15%. The newly formed heterocysts, when starvation became apparent, were smsller than the old ones, not exceeding the size of a vegetative cell. They were not associated with gradients in phycocyanin fluorescence, as reported earlier. No sporulation occurred under these conditions. Upon prolonged starvation the culture gradually died. However, if a

culture was starved for Molybdemum in the presence of nitrate, about 95% of the cells differentiated into heterocysts and spores. Heterocysts and spores were present in about equal number, generally alternating in the filaments. Upon prolonged starvation the heterocysts lysed in this experiment.

3. Since heterocyst formation occurred in the absence of Molybdemum, nitrogen fixation is not involved directly in the processes that lead to the differentiation.

4. An unialgal culture of Hapalosiphon sp. was a gift from Dr. G. H. SCHWABE (Max Planck Institut für Limnologie, Plön, Germany). If grown aerobically in the absence of combined nitrogen only very few typical heterocysts were observed in the filaments of this strain. However, if viewed under a fluorescence microscope at regular intervals in the filaments cells occurred which did not show phycocyanin fluorescence. They also had a low level of chlorophyll fluorescence as compared to most of the cells and from the absorption images it appeared that the concentration of phycocyanin was low or zero. Chlorophyll content was the same as in other cells. Otherwise these cells were under the microscope, in the normal, polarisation and phase contrast modes, indistinguishable from the normal cells. So these cells are with respect to the pigment content and fluorescence completely analogous to the heterocysts in Anabaena. Perhaps they are "physiologically" heterocysts, although the typical cell wall of heterocysts is lacking.

5. On p. 19 some microphotographs of Anabaena cylindrica are shown.

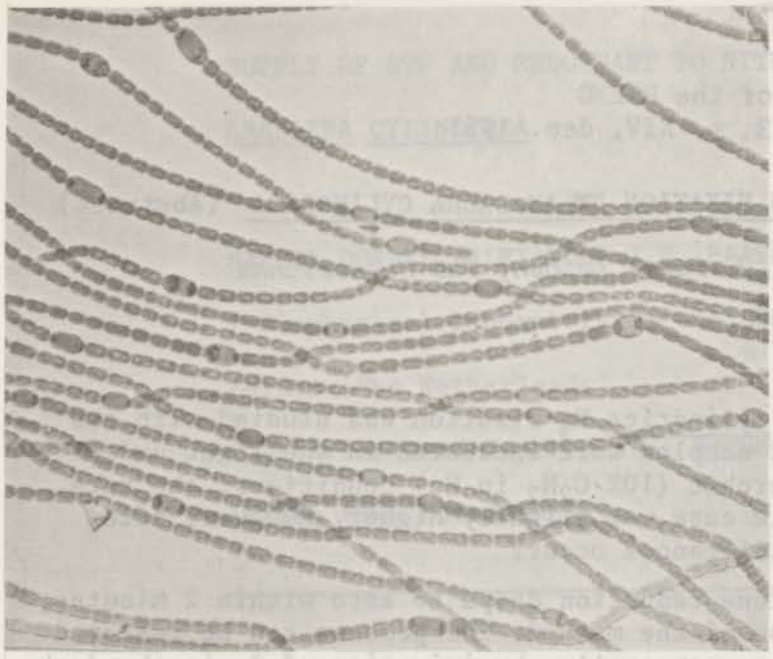
A. Absorption image with blue light. This picture shows absorption by chlorophyll. The heterocysts have about the same chlorophyll content as the vegetative cells have.

B. Absorption image with orange light, which is absorbed mainly by phycocyanin. The heterocycts have a considerably lower absorption, so they have a low content of phycocyanin.

C. Fluorescence image, excited with the green lines from a Mercury lamp and viewed through a filtercombination transmitting around 630 nm. This picture shows phycocyanin fluorescence. The heterocysts show extremely low or no fluorescence. In the rows of vegetative cells gradients in phycocyanin fluorescence can be seen.

D. Absorption image with blue light.

E. Fluorescence image, excited with the blue lines from a Mercury lamp and viewed through a filter combination with maximum transmission at 683 nm. This picture shows chlorophyll fluorescence. The heterocysts have a low level of fluorescence, as compared to the vegetative cells. Since the concentration of chlorophyll is not very different in the two kinds of cells, the heterocysts have a lower yield of fluorescence.



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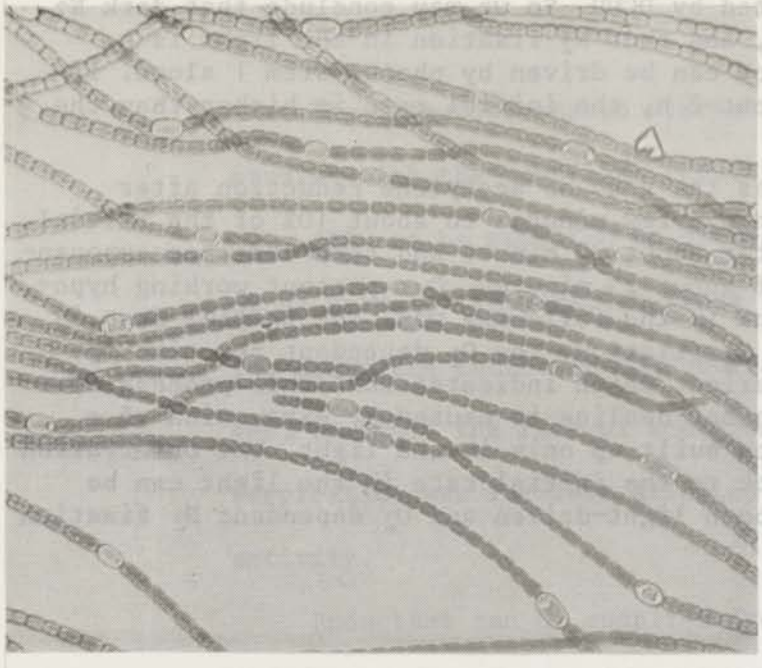

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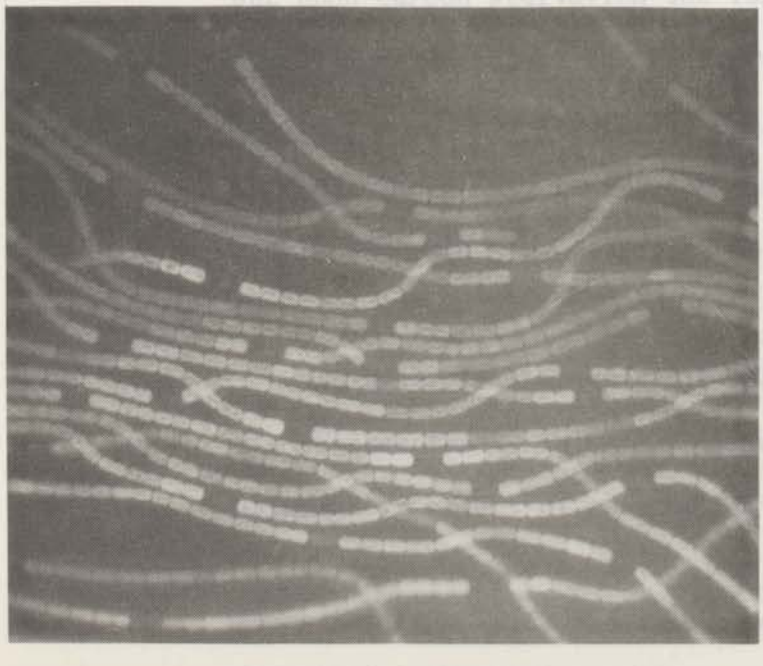

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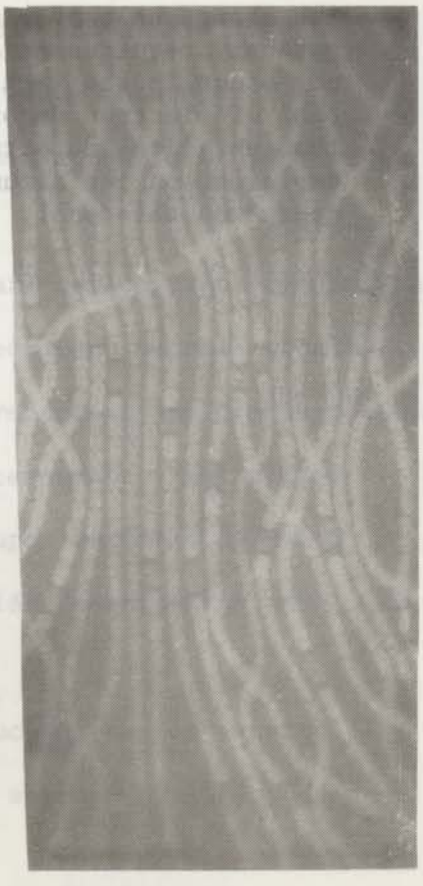

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LIGHT-DARK TRANSIENTS IN NITROGEN FIXATION BY ANABAENA CYLINDRICA (Abstract)

M. Donze, H.J. van Gorkom and A.J.P. Raat, Leiden, The Netherlands

1. In aerobically grown Anabaena cylindrica N_2 fixation was studied with the acetylene reduction technique. If samples were incubated in the light under aerobic (10% C_2H_2 in air) or anaerobic (10% C_2H_2 in He) conditions, the rate of C_2H_2 reduction in the anaerobic case was slightly higher. However, after switching off the light marked differences occur:

2. In the anaerobic sample acetylene reduction drops to zero within 2 minutes, which is about the time resolution of the method. The process can be restored either by readmitting the light or, temporally, by injection of O_2 in the dark. Restoration by light is not affected by DCMU. So we may conclude that dark N_2 fixation is strictly O_2 dependent, and that N_2 fixation in the light is a direct photosynthetic process which can be driven by photosystem I alone. If the light is switched on after about 2 h, the initial rate is higher than the steady state.

3. In aerobically incubated samples the rate of acetylene reduction after switching off the light drops within a few minutes to about 10% of the initial value; then rises to a maximum after about 20 minuts and then declines exponentially to zero with a half life of about 25 minutes. Our present working hypothesis accounts for these phenomena in the following way. The initial drop is the abrupt halt of photosynthetic N_2 fixation. The O_2 dependent reaction apparently requires an activation period, which indicates that this process does not occur in the light. The subsequent decline is caused by exhaustion of a pool of electron donor which can be built up only in the light. The observation that this decline extrapolates back to the initial rate in the light can be explained on the assumption that both light-driven and O_2 dependent N_2 fixation depend on this pool.

SUPPLY OF ATP AND REDUCTANT TO NITROGENASE IN THE BLUE-GREEN ALGAE ANABAENA CYLINDRICA

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SUMMARY

The effect of carbon starvation on nitrogenase activity was studied with the acetylene reduction technique in Anabaena cylindrica.

ATP can be supplied to nitrogenase either by photophosphorylation or by terminal respiration since either light or oxygen must be present for acetylene reduction to occur at a significant rate. ATP does not seem to limit the rate of acetylene reduction since the rate of respiration was far less affected by carbon starvation than nitrogenase activity.

Reductant can be supplied from intermediary carbohydrate metabolism, but upon carbon starvation the formation of reductant becomes progressively more dependent on light. Two types of reductant are distinguished that can move from vegetative cells to the heterocysts. They differ in redox potential, in rate of disappearance upon carbon starvation and in response time to light, to carbon dioxide assimilation and to DCMU.

In severely starved cells almost all reductant can be generated via photosystem 1. This is concluded from the strongDCMU independent stimulatory effect of reduced DPIP.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea;MFA, monofluoroacetate.

INTRODUCTION

From studies on cell free systems it is knwon that biological N_2 fixation requires ATP and reducing power. To incorporate nitrogen into organic compounds "carbon skeletons" are essential too, but since ammonium may be a product, these need not limit the rate of nitrogen uptake. The biochemistry of nitrogen fixation has been reviewed by BERGERSEN[1] and DALTON & MORTENSON[2], its physiology in blue-green algae by STEWART[3].

If nitrogenase activity is assayed by the acetylene reduction technique, the presumed need for carbon skeletons need not be taken into account, as pointed out by COX & FAY[4]. Acetylene reduction requires both ATP and reducing power[5].

In the blue-green alga Anabaena cylindrica acetylene reduction occurs both in the light and in the dark. Dark activity is strongly dependent on oxygen[6,7]. This shows that intermediary carbohydrate metabolism may provide reducing power and that terminal respiration is essential in the dark to provide ATP. This is consistent with the observation that under anaerobic incubation in the dark reduction occurs at a rate of about 5 % of an aerobic control[8]. This low rate may well correspond to the low yield in ATP of fermentative processes as compared to respiration. Also the observation that monofluoroacetate (MFA), a competitive inhibitor of aconitase in the Krebs cycle, inhibts acetylene reduction more strongly in the dark than in the light fits in well[9].

In the light the problem is more complex. Stimulation by light of nitrogen fixation and acetylene reduction has often been observed in blue-green algae. Under strictly anaerobic conditions photophosphorylation is the only likely source of ATP, while both C-metabolism and photosystem 1 of photosynthesis might provide electrons. Whether

photoreduction of nitrogenase occurs is controversial[9,10,11]. If it does, it has also to be decided whether photosystem 2 or C-metabolism functions as electron donor to system 1. In the presence of oxygen the contribution of respiration to ATP supply has to be evaluated too. The situation is further complicated by the inhibitory effect of oxygen on nitrogenase and the very high rate of photorespiration of which blue-green algae are capable[12].

The simplest approach to decide whether system 2 participates in electron transport to nitrogenase is to study the effect of its specific inhibitor DCMU. Older results did vary between no effect and about 50 % inhibition. Recently this problem was solved by LEX & STEWART[9], who demonstrated that in cells high in carbohydrate reserve DCMU did not affect acetylene reduction. Upon depletion of these reserves inhibition progressively appeared. The DCMU insensitive activity was strongly inhibited by MFA. In these cells low in carbohydrate, about 50 % of the reducing equivalents were supplied via system 2, the rest by dark metabolism. This is consistent with the action spectrum by FAY[13], which was measured with cells grown on gas with low carbon dioxide content. Involvement of the photosystem 2 pigment phycocyanin is apparent, especially if their spectrum is replotted in terms of quanta instead of energy. The absence of an Emerson enhancement effect[11] was observed in cells high in carbon content and thus fits in the picture.

Intermediary carbohydrate metabolism is, of course, a very wide term, but the actual pathways cannot at this time be specified. The effect of MFA indicate involvement of the Krebs cycle, and the stimulatory effect of pyruvate with concomitant carbon dioxide evolution[14] indicates that pyruvate decarboxylation may also play a role.

In this paper we present some experiments which were designed to allow a better distinction of some of the factors that were outlined above.

MATERIALS AND METHODS

Anabaena cylindrica (Cambridge Culture Collection No. 1403/2a) was grown as described earlier[15] in the medium of ALLEN & ARNON[16]. The cultures were gassed with air + 5 % CO_2 or with air from which CO_2 was removed. For the measurements of acetylene reduction 1 ml samples were incubated in 7.5 ml serum bottles, magnetically stirred and thermostated at 30° C. Samples could be illuminated with saturating white light from 100 W incandescent lamps. 0.8 ml C_2H_2 was injected in the serum bottles, and C_2H_4 production was followed by gas chromatography as described by AKKERMANS[17]. We reduced the column length to 90 cm and used a temperature of 90° C, thereby obtaining a retention time of less than 1 minute. The response time of our technique, including mixing and diffusion times was between 3 and 5 min, depending on which gasses were changed and on experimental care. The fastest response was obtained when the light was turned off or on in samples in which C_2H_2 was already in equilibrium. In these cases a constant rate of C_2H_4 production was sometimes observed within 2 minutes. C_2H_4 concentration was normalized on a chlorophyll basis and expressed as $\frac{\text{nmol } C_2H_4/\text{ml culture}}{A(680 \text{ nm} - 740 \text{ nm})/\text{cm}}$.

Respiration was measured in an oxygen polarograph fitted with a Clark-type electrode. DCMU was used in a concentration of 2.10^{-5} M and added with 1 % ethanol. DCIP was used at 10^{-5} M ascorbate at 10^{-4} M and NH_4Cl at 2.10^{-4} M.

Experiments were started with cultures three days after inoculation. Considerable variation in the absolute rate of acetylene reduction in the starting material was observed. The qualitative response to the variables used in this study was reproducible. Quantitative response to CO_2 starvation, expressed as percentage of the starting material, showed variations of about 20 %.

RESULTS

We call a culture high in carbohydrate reserve a high-C culture. In a high-C culture aerobic dark reduction of acetylene and anaerobic reduction of acetylene in the light occurred at about the same rate. DCMU does not affect the light process in a short-term experiment[9,18]. Apparently sufficient reductant is generated in the dark, so the dependence on light under anaerobic conditions is caused by a dependence on photophosphorylation only. This conclusion was also reached by BOTHE[10] on basis of inhibitor studies. The effect of injection of O_2 in the dark is shown in Fig. 1. The lag in the stimulation corresponds to the time-constant of our incubation technique, so the oxygen stimulation occurs within 5 minutes.

After starvation for CO_2 under aerobic conditions in the light during about 12 hours a high-C culture is converted to a low-C culture. In low-C cells the rate of anaerobic acetylene reduction in the light was similar to the rate before starvation. The aerobic rate in the dark however was halved by starvation. Since respiration in the dark was not significantly lower as compared to a high-C culture (on a chlorophyll basis), this lower rate of nitrogenase activity must be due to a limited supply of dark generated reductant.

We now realize that one of the induction phenomena we reported earlier[6] was observed in low-C cultures only. After switching off the light these showed a lag of about 10 minutes for aerobic acetylene reduction to reach its maximum rate (Fig. 2). Subsequently the rate drops exponentially to zero.

In agreement with our observations, LEX & STEWART[9] showed that in low-C cells the light dependent acetylene reduction is partially inhibited by DCMU. In our experiments the inhibition by DCMU after its addition occurred within the time-limit of our technique, which was 3 minutes in the best experiments.

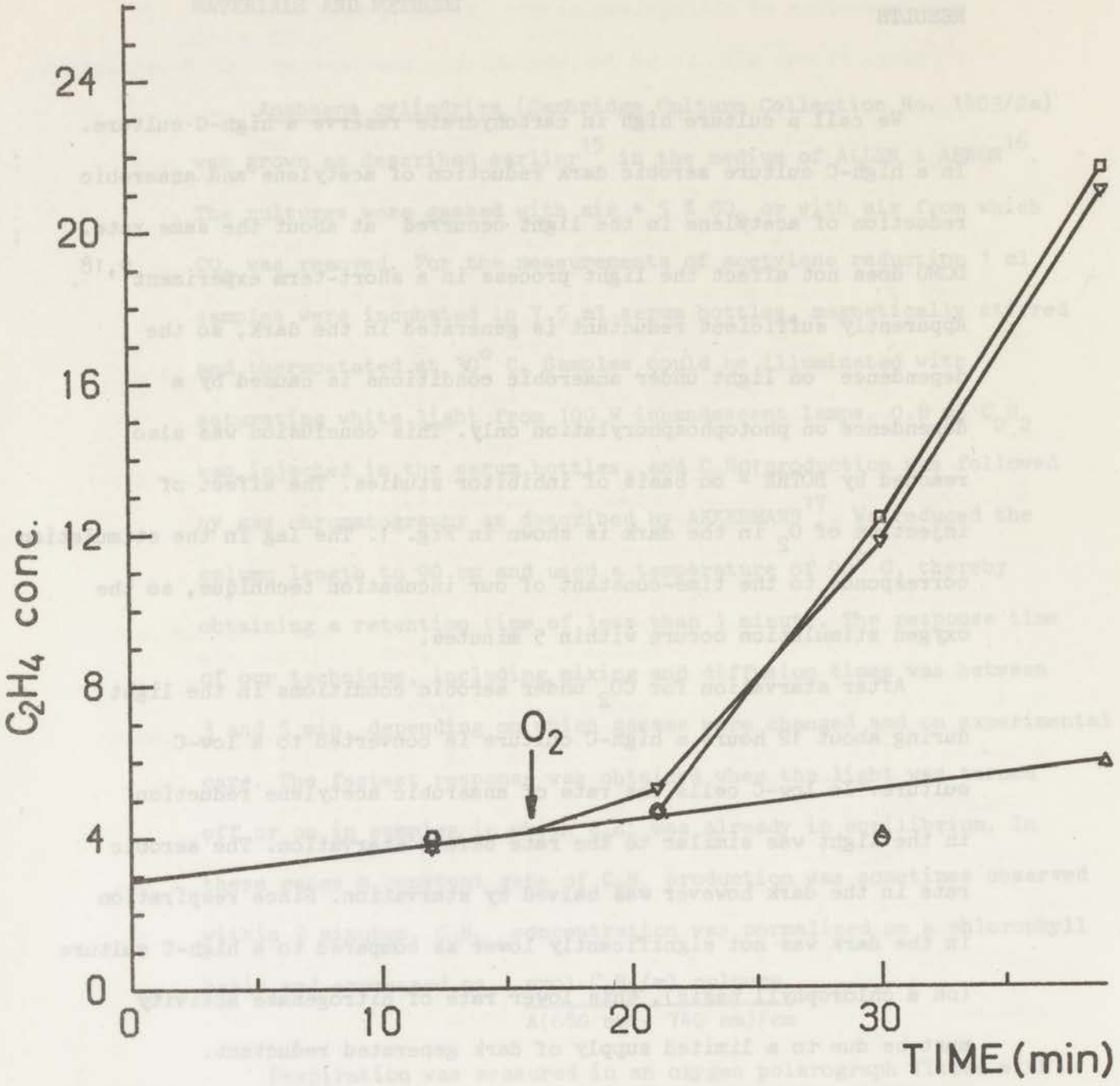


Fig. 1. Dependence of C_2H_2 reduction in the dark on O_2. Four samples from a high-C culture were incubated in the dark under N_2 + 10% C_2H_2. At the time indicated by the arrow 10% O_2 was injected in the gasphase of 2 smples (and). The rather low absolute rate of nitrogenase activity was due to fluctuations in maximal activity that occurred in our cultures. See also p. 8.

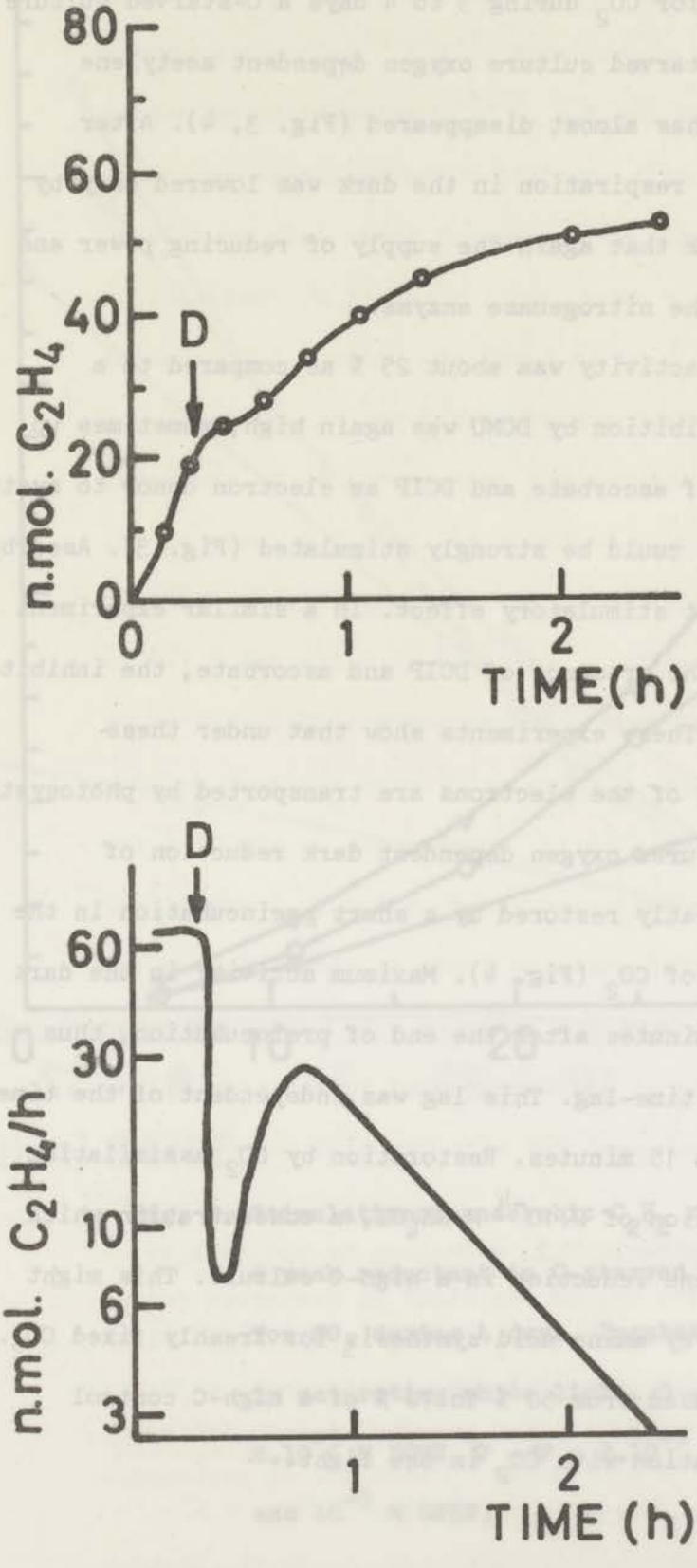


Fig. 2. Induction effect upon darkening a sample of low-C Anabaena. Incubation under air + 10% C_2H_2. At the time indicated by the arrow the smple was darkened. The lowere graph was obtained by graphical differentiation of the upper figure.

After starvation for CO_2 during 3 to 4 days a C-starved culture was obtained. In a C-starved culture oxygen dependent acetylene reduction in the dark has almost disappeared (Fig. 3, 4). After starvation for 3 days, respiration in the dark was lowered only by about 50 %, so we think that again the supply of reducing power and not ATP was limiting the nitrogenase enzyme.

In the light the activity was about 25 % as compared to a high-C culture and inhibition by DCMU was again high, sometimes up to 80 %. By addition of ascorbate and DCIP as electron donor to system 1, acetylene reduction could be strongly stimulated (Fig. 3). Ascorbate alone had only a slight stimulatory effect. In a similar experiment it was found that in the presence of DCIP and ascorbate, the inhibition by DCMU was relieved. These experiments show that under these conditions, almost all of the electrons are transported by photosystem 1.

In C-starved cultures oxygen dependent dark reduction of acetylene could be greatly restored by a short preincubation in the light in the presence of CO_2 (Fig. 4). Maximum activity in the dark was reached about 10 minutes after the end of preincubation, thus showing a significant time-lag. This lag was independent of the time of preincubation up to 15 minutes. Restoration by CO_2 assimilation was prevented by addition of 2.10^{-4} M NH_4Cl, a concentration which did not affect acetylene reduction in a high-C culture. This might be due to competition by amino acid synthesis for freshly fixed CO_2. Respiration was increased from 50 % to 70 % of a high-C control by 5 minutes preincubation with CO_2 in the light.

DISCUSSION

In the filaments of Anabaena differentiated cells, called heterocysts, occur singly and rather regularly spaced in a frequency of about 5 %. There is good circumstantial evidence that heterocysts

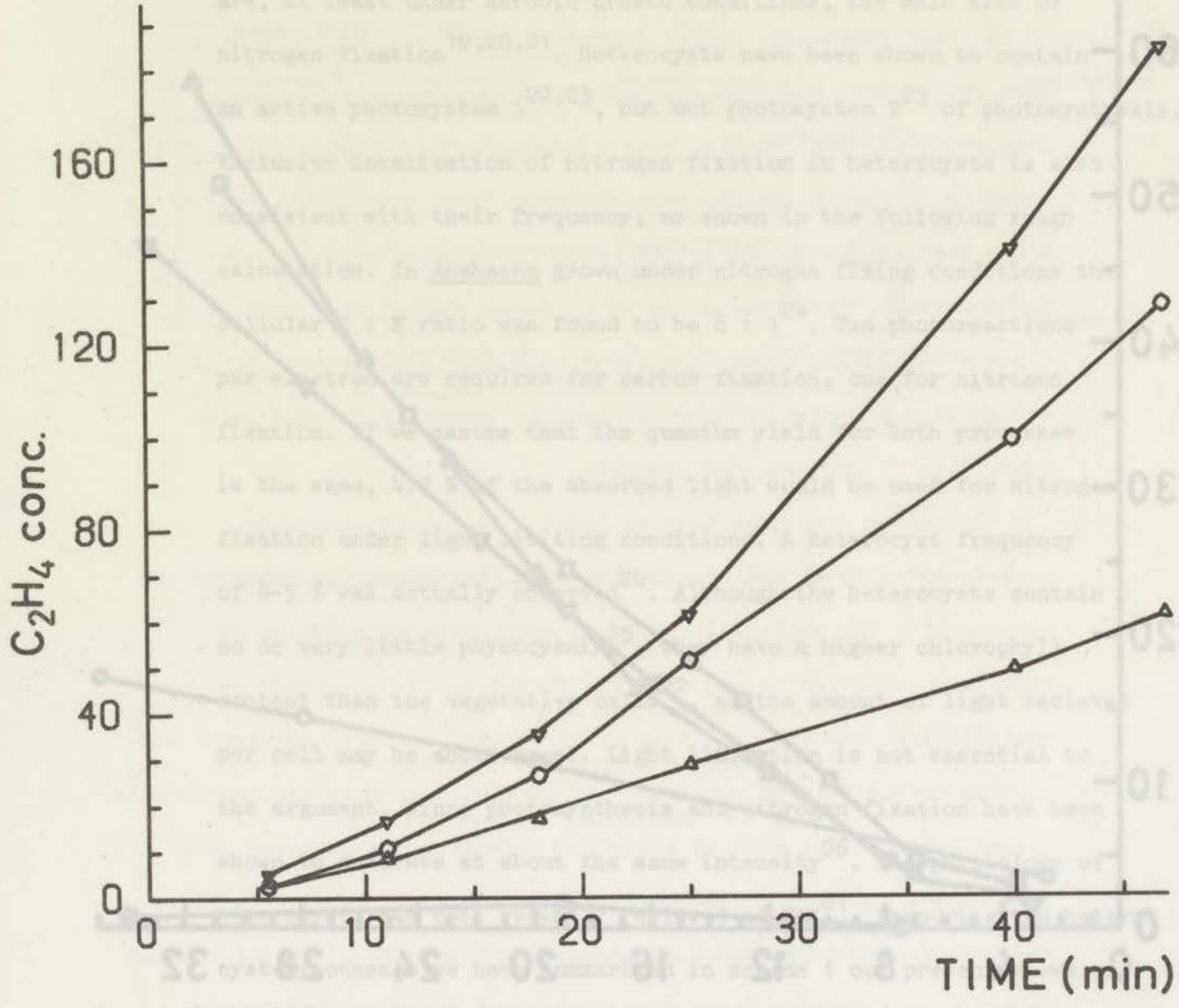


Fig. 3. Stimulationof anaerobic C_2H_2 reduction in the light by a weak reductant in C-starved cells. A culture was starved for CO_2 during 4 days. Incubation under He + 10% C_2H_2 in saturating white light. O - O : no additions. △ - △ : 2.10^{-5} M DCMU. ▽ - ▽ : 2.10^{-5} M DCMU, 10^{-4} M ascorbate and 10^{-5} M DPIP.

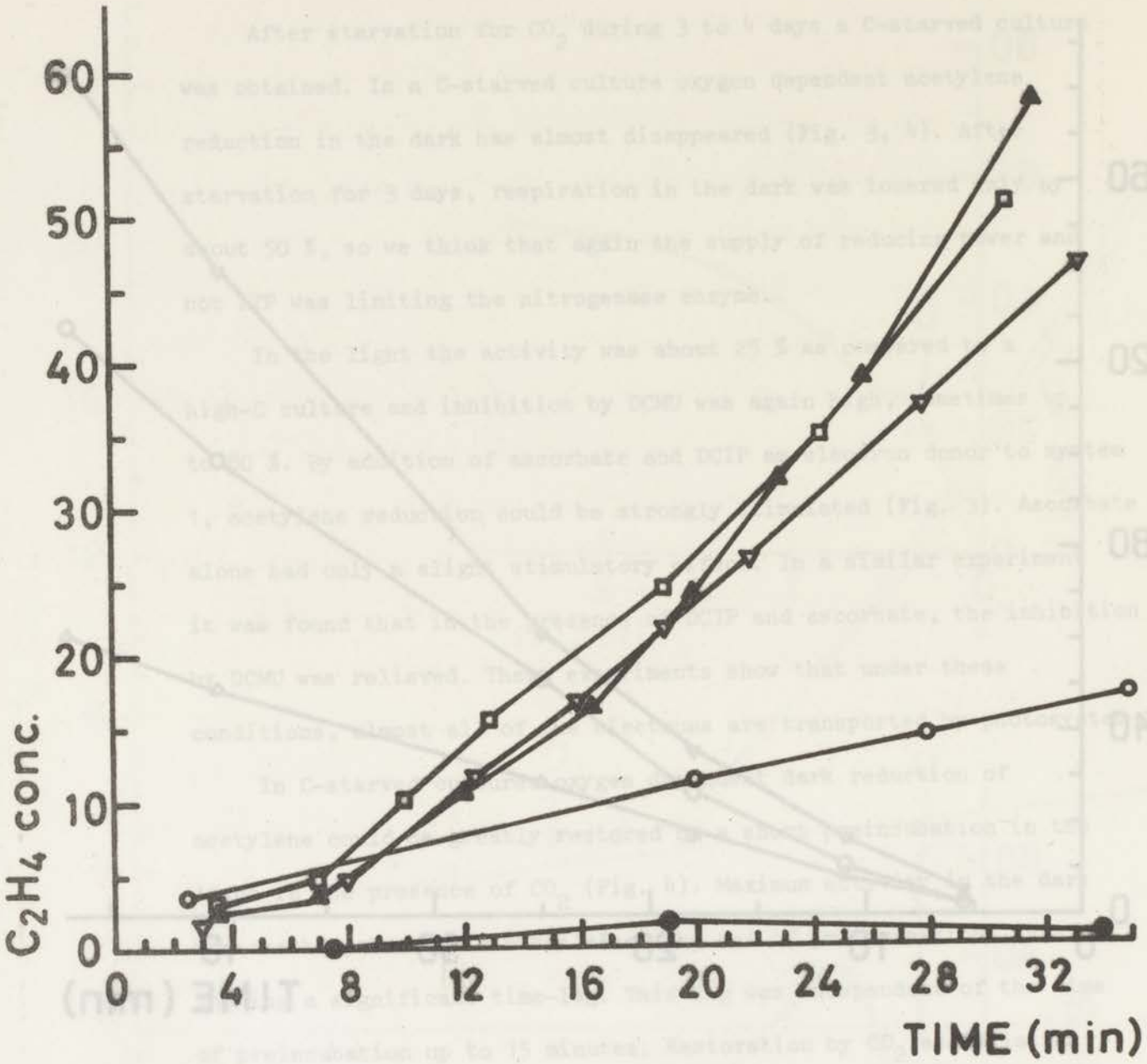


Fig. 4. Stimulation of aerobic C_2H_2 reduction in the dark by preincubation with CO_2 in the light in C-starved cells. A culture was starved for CO_2 during 3 days. C_2H_2 reduction was measured under air + 10% C_2H_2 in the dark. Before the measurements the samples were preincubated as follows.

● - ● : 30 minutes dark under N_2 with 0.1 ml carbonate-bicarbonate buffer of 1 M, pH 9.0

○ - ○ : 5 minutes preincubation under N_2 in the light

▲ - ▲ , ▽ - ▽ , □ - □ : 5, 10 and 20 minutes preincubation respectively in the light under N_2 with 0.1 ml carbonate-bicarbonate buffer.

are, at least under aerobic growth conditions, the main site of nitrogen fixation[19,20,21]. Heterocysts have been shown to contain an active photosystem 1[22,23], but not photosystem 2[23] of photosynthesis. Exclusive localization of nitrogen fixation in heterocysts is also consistent with their frequency, as shown in the following rough calculation. In Anabaena grown under nitrogen fixing conditions the cellular C : N ratio was found to be 8 : 1[24]. Two photoreactions per electron are required for carbon fixation, one for nitrogen fixation. If we assume that the quantum yield for both processes is the same, 4.7 % of the absorbed light would be used for nitrogen fixation under light limiting conditions. A heterocyst frequency of 4-5 % was actually observed[24]. Although the heterocysts contain no or very little phycocyanin[25], they have a higher chlorophyll content than the vegetative cells[22], so the amount of light recieved per cell may be about equal. Light limitation is not essential to the argument, since photosynthesis and nitrogen fixation have been shown to saturate at about the same intensity[26]. The physiology of heterocysts has been recently reviewed of FAY[27]. Favouring the heterocyst-hypothesis we have summarized in scheme 1 our present views on the different pathways that may lead to reduction and ATP supply of nitrogenase in Anabaena.

To simplify the discussion two types of reductant are defined. R_1 includes any compound that is capable of reducing nitrogenase and R_2 comprises all compounds that reduce the electron donor in photosystem 1.

In a high-C culture sufficient R_1 is generated in the dark to sustain nitrogenase activity. No dependence on light of the reductant could be detected[9,10]. ATP can be supplied by photophosphorylation in the light or by terminal respiration in thepresence of oxygen. Anaerobic dark processes can yield only insignificant amounts of ATP. During carbon starvation the content of the cells

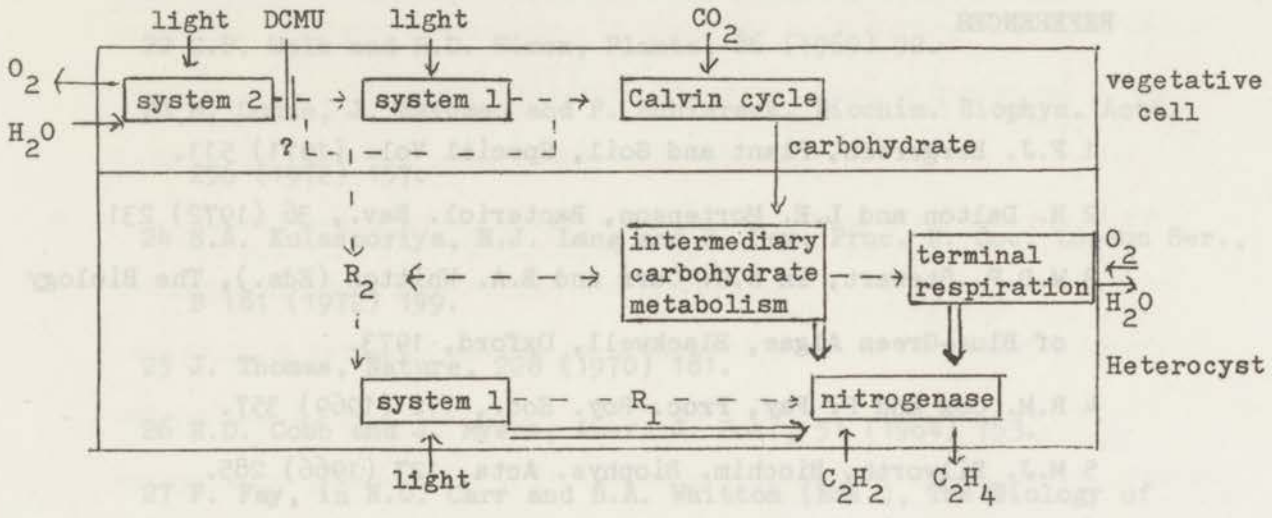
becomes progressively more oxidized. The capacity to form reductant in the dark decreases. Oxygen dependent acetylene reduction decays much faster than respiration, indicating the decrease of R_1. We interprete the pool in Fig. 2 as a measure of the amount of R_1. The capacity to form R_2 in the dark decreases also upon carbon starvation since light dependent acetylene reduction becomes dependent on photosystem 2. Dark generation of R_1 decays faster than that of R_2, since after carbon starvation for 3 to 4 days R_1 is absent while a significant amount of R_2 still occurs. In carbon starved cells inhibition by DCMU was not complete.

It can be concluded that at least 2 different compounds, corresponding to R_1 and R_2 can move from the vegetative cells to the heterocysts. The observations on carbon dioxide stimulation of acetylene reduction showed that the formation of R_1 depends on assimilation. The response time of 15 minutes in this experiment was similar to the rate of appearance of labeled carbon in the heterocysts[28]. A similar induction time of acetylene reduction was observed upon darkening a low-carbon culture in the presence of oxygen (Fig. 2). The photosystem 2 dependent nitrogenase activity in the light however showed a much shorter (less that 3 minutes) response time to light and DCMU. It did not depend on assimilation in experiments shorter than 30 minutes. This indicates that a rapidly diffusing electron carrier may play the role of R_2.

Finally we would like to remark that acetylene reduction experiments of the type described in this paper might prove useful in ecological research. Application in the field of these simple and rapid measurements could help to diagnose the physiological state of natural populations of blue-green algae.

ACKNOWLEDGEMENTS

Tank are due to Mr. H. Nienhuis for carrying out the oxygen measurements and to Professor Dr. A. Quispel for his interest during the work.



Scheme 1. Summary of pathways that may lead to reduction and supply of ATP to nitrogenase in Anabaena

- - - → electron or proton transport.

⟹ transport of ATP

other arrows as indicated.

REFERENCES

1 F.J. Bergersen, Plant and Soil, Special Vol. (1971) 511.

2 H. Dalton and L.E. Mortenson, Bacteriol. Rev., 36 (1972) 231.

3 W.D.P. Stewart, in N.G. Carr and B.A. Whitton (Eds.), The Biology of Blue-Green Algae, Blackwell, Oxford, 1973.

4 R.M. Cox and P. Fay, Proc. Roy. Soc., 172 (1969) 357.

5 M.J. Dilworth, Biochim. Biophys. Acta, 127 (1966) 285.

6 M. Donze, H.J. van Gorkom and A.J.P. Raat, J. Gen. Microbiol., 69 (1971)

7 C.P. Wolk, Ann. N. York Acad. Sc., 175 (1970) 641.

8 R.V. Smith and M.C.W. Evans, J. Bacteriol, 105 (1971) 913.

9 M. Lex and W.D.P. Stewart, Biochim. Biophys. Acta, 292 (1973) 436.

10 H. Bothe and E. Loos, Arch. Mikrobiol., 86 (1972) 241.

11 R.A. Lyne and W.D.P. Stewart, Planta, 109 (1973) 27.

12 M. Lex, W.B. Silvester and W.D.P. Stewart, Proc. R. Soc. London Ser., B 180 (1972) 87.

13 P. Fay, Biochim. Biophys. Acta, 206 (1970) 353.

14 R.M. Cox and P. Fay, Arch. Mikrobiol., 58 (1967) 357.

15 H. Hoogenhout and J. Amesz, Arch. Mikrobiol., 50 (1965) 10.

16 M.B. Allen and D.I. Arnon, Plant Physiol., 30 (1955) 366.

17 A.D.L. Akkermans, Nitrogen fixation and nodulation of Alnus and Hippophae under natural conditions., Ph.D. Thesis, University of Leiden, DSW, Dordrecht, 1971.

18 W.D.P. Stewart and H.W. Pearson, Proc. R. Soc. London Ser., B 175 (1970) 293.

19 P. Fay, W.D.P. Stewart, A.E. Walsby and G.E. Fogg, Nature, 220 (1968) 810.

20 H.J. van Gorkom and M. Donze, Nature, 234 (1971) 231.

21 C.P. Wolk and E. Wojciuch, Planta, 97 (1971) 126.

22 C.P. Wolk and R.D. Simon, Planta, 86 (1969) 92.

23 M. Donze, J. Haveman and P. Schiereck, Biochim. Biophys. Acta, 256 (1972) 157.

24 S.A. Kulasooriya, N.J. Lang and P. Fay, Proc. R. Soc. London Ser., B 181 (1972) 199.

25 J. Thomas, Nature, 228 (1970) 181.

26 H.D. Cobb and J. Myers, Amer. J. Bot., 51 (1964) 753.

27 P. Fay, in N.G. Carr and B.A. Whitton (Eds.), The Biology of Blue-Green Algae, Blackwell, Oxford, 1973.

28 C.P. Wolk, J. Bacteriol., 96 (1968) 2138.

PART 2

STUDIES ON PHOTOSYNTHESIS IN SYNCHRONOUS CULTURES OF SCENEDESMUS

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SUMMARY

1. A new type of culture apparatus for photosynthesic microorganism was developed that is especially useful if small amounts of concentrated suspensions are desired. High cell densities can be obtained due to a small diameter and to a dialysis system. A new turbidostatic control unit employing flash light from a light-emitting diode was designed.
2. Some cytological and physiological observations on synchronous cultures of Scenedesmus grown in this apparatus were made. Synchrony was obtained by submitting the cultures to a light-dark cycle.
3. The arrangement of daughter cells after division was found to be quite variable, which does not accord with the importance given to it in systematics.
4. In cultures grown in relatively low light intensity the quantum yield of photosynthesis was found to be high and independent of the life cycle stage of the cells, except sometimes for a short induction period upon onset of illumination.
5. In cultures grown at high light intensity the quantum yield increased in the beginning of the light period to its maximal value, which was the same as found in low-light cultures, and than dropped again.
6. Sometimes the quantum yield was lower during the first 15 minutes of weak illumination. This phenomenon depended on the life cycle stage

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Abbreviations: DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

under our culture conditions. It occurred only during the dark-period, the lowering of quantum yield was strongest during the stage in the life cycle when cell walls are synthesized.

7. Measurements of P_{700} kinetics and of changes of the yield of chlorophyll fluorescence indicated that this induction process was caused by a variable amount of reductant leaking from carbohydrate metabolism to P_{700}. So this reductant competes with the electron acceptor of photosystem 2 in reducing P_{700}, thus lowering the rate of electron transport by system 2.

8. The concentrations of P_{700} and of cytochrome f were, on a chlorophyll basis, independent of the life cycle stage.

1. INTRODUCTION

If a culture of unicellular organism can be synchronized, that is, if all cells can be made to divide simultaneously, it is reasonable to expect that the whole culture behaves approximately as a single cell during its life-cycle. In a synchronous culture variations in metabolism that are associated with the life cycle can be investigated. Many aspects of research in this direction have been discussed in the book by PADILLA and CAMERON (eds)[1]. In studies employing synchronous cultures of algae, a regime of alternating light and dark periods was most often used as a means of obtaining synchronous division. This field of research has been recently reviewed by LORENZEN[2].

The main pitfall in work on synchronous cultures is due to the technique of synchronization itself, since all techniques use a periodic disturbance of some kind to keep the cells in pace. This problem has been discussed by JAMES[5].

From the point of view of photosynthesis research. the main question with respect to the cell cycle is, whether the composition

and functioning of the photosynthetic apparatus depend on the life cycle stage. The literature on these aspects has been discussed by SENGER[3] and WANG[4]. The problem which has attracted most interest was, whether the efficiency of light utilization depends on the life cycle stage. Varying results have been reported[3,4].

We investigated some aspects of photosynthesis in cultures of the unicellular green alga Scenedesmus that were synchronized by a light-dark regime, using different intensities of light during the light period. Measurements were made on oxygen exchange, absorption changes of cytochrome f and P700 and prompt and delayed fluorescence of chlorophyll.

2. MATERIALS AND METHODS

Scenedesmus sp., clone D3 (Gaffron) Cambridge Culture Collection no. 276/6a was grown in an inorganic medium of the following composition. Per liter: KNO_3: 0.81 g, NaCl: 0.47 g, Na_2HPO_4: 0.14 g, $NaH_2PO_4 . 2H_2O$: 4.1 g, $CaCl_2 . 6H_2O$: 22 mg, $MnCl_2 . 4H_2O$: 0.1 mg, $MgSO_4 . 7H_2O$: 0.25 g, $ZnSO_4 . 7H_2O$: 0.05 mg, Versene: 0.02 g.

The automatic culture apparatuses to be described in the next section, were thermostated at 30° C and illuminated at 0.31 mW/cm^2 by 20W/33 Philips fluorescent lamps. The cultures were gassed with 5 % CO_2 in air. Light intensity was measured with an Si photocell and averaged over the surface of the culture apparatus. The Si cell was calibrated against an YSI-Kettering Model 65 radiometer (Yellow Springs Instrument Co., Ohio, U.S.A.) in light of the same fluorescent lamps. Synchrony of cell division was obtained by applying a light-dark regime of 14 hours light, 10 hours dark. The beginning of the light period was taken as time zero.

Some experiments were carried out with batch cultures grown as

described earlier[6] at 25° C, 0.12 mW/cm^2 light intensity from similar lamps on air with 5 % CO_2 or on air from which CO_2 was removed. The culture vessel was modified from ref. 6.(Fig. 1) The tapering end, as compared to a spherical, strongly reduces the tendency for algae to grow on the glass near the air inlet. The constriction in the gas inlet prevents backflow of the algal suspension into the tube. Insertion of a piece of silicon tubing in the gas supply reduced accidental breakage by a factor of about 10. Except for the constriction, these modifications also reduced the work in making the vessel.

The concentration of cells was determined with a counting chamber (Thoma ruling), either directly or from photomicrographs. A volume containing about 150 large cells or 1200 small cells was counted each time.

Cell volume was calculated, assuming that the shape of the cells is a prolate spheroid, from microscopic measurements of the length and width of the cells. About 10 cells were averaged each time. Packed cell volume (PCV) was calculated from average cell volume and cell concentration. We could not determine PCV directly by centrifugation, since in the Tromsdorff-type maematocrit tubes at hand too many cells did stick to the wall of the tube, even at top speed of our table centrifuge.

Chlorophyll was estimated by the method of MacKinney[7].

Cell production in the automatic apparatus could be followed by feeding the outflow into a fraction collector.Absorption spectra were recorded with a Cary-14 spectrophotometer equipped with a scattered light transmission accessory.

Photomicrographs were taken with a Leitz "Aristophot" automatic photomicroscope. A blue filter (Schott BG 12, 2 mm) was used to enhance contrast of the chloroplasts[8].

Oxygen exchange was measured by means of a Clark-type microelectrode (Yellow Spring Instrument Co.) in a magnetically stirred reaction

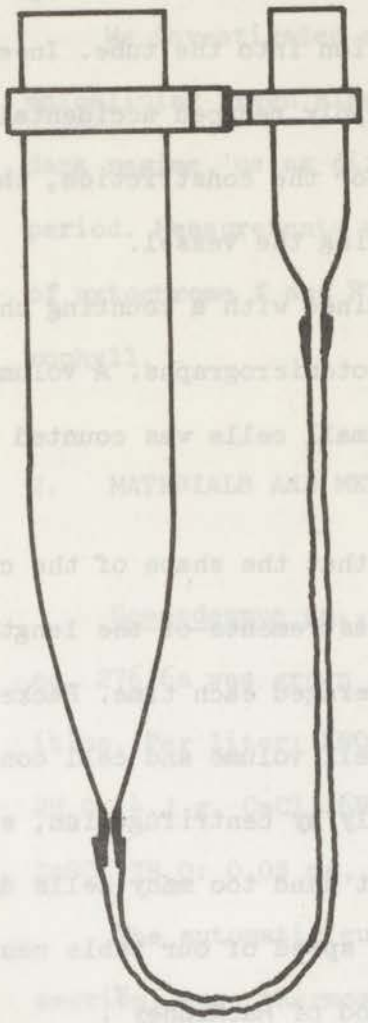


Fig. 1. Culture vessel for batch cultures. 1/3 x its actual size. the vessel and the holder of the cotton plug are fixed together by means of two "TERRY" clamps

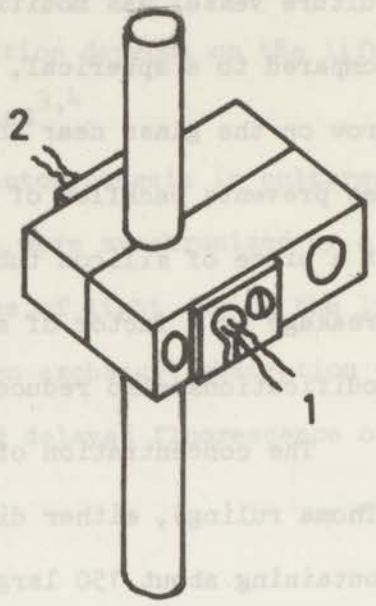


Fig. 3c. Optical control unit. Mounting of the LED (1) and photodiode (2) in a block of black perspex that is fitted round the tube of the culture vessel shown in fig. 2. (p. 41).

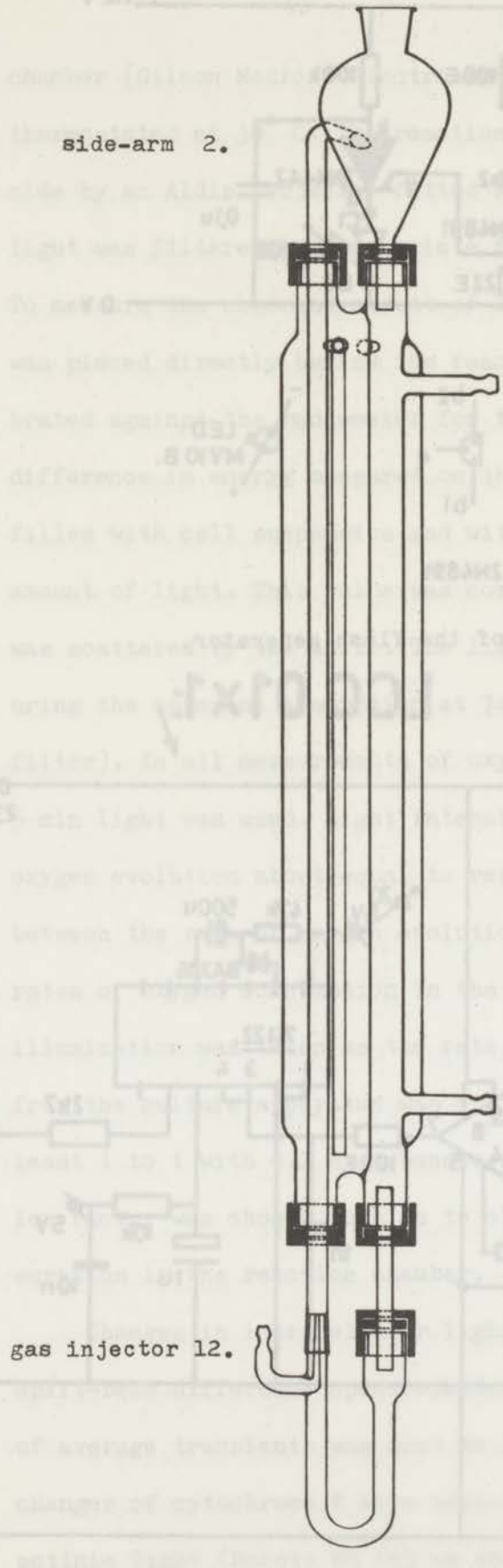


Fig. 2. Drawing of the culture apparatus, 0.38 x its actual size.

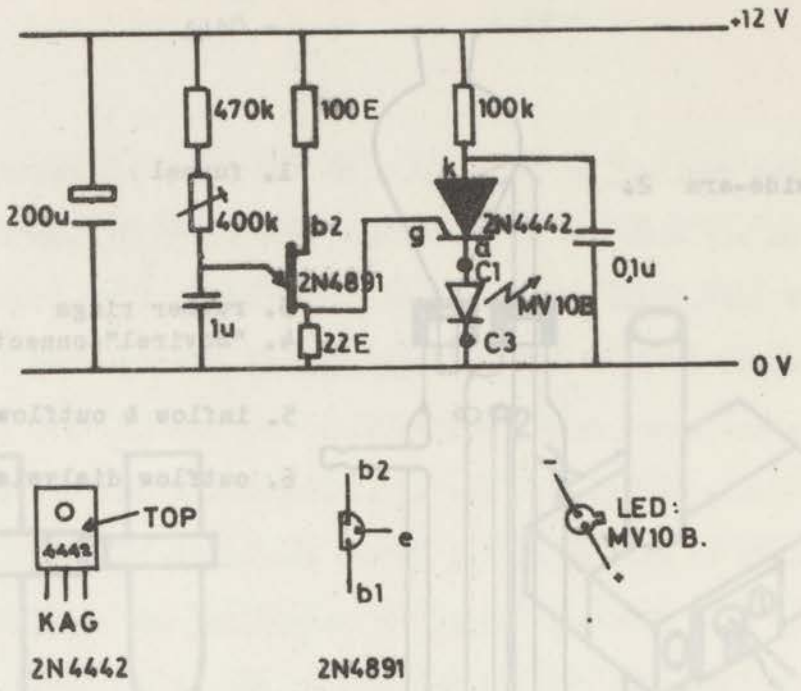
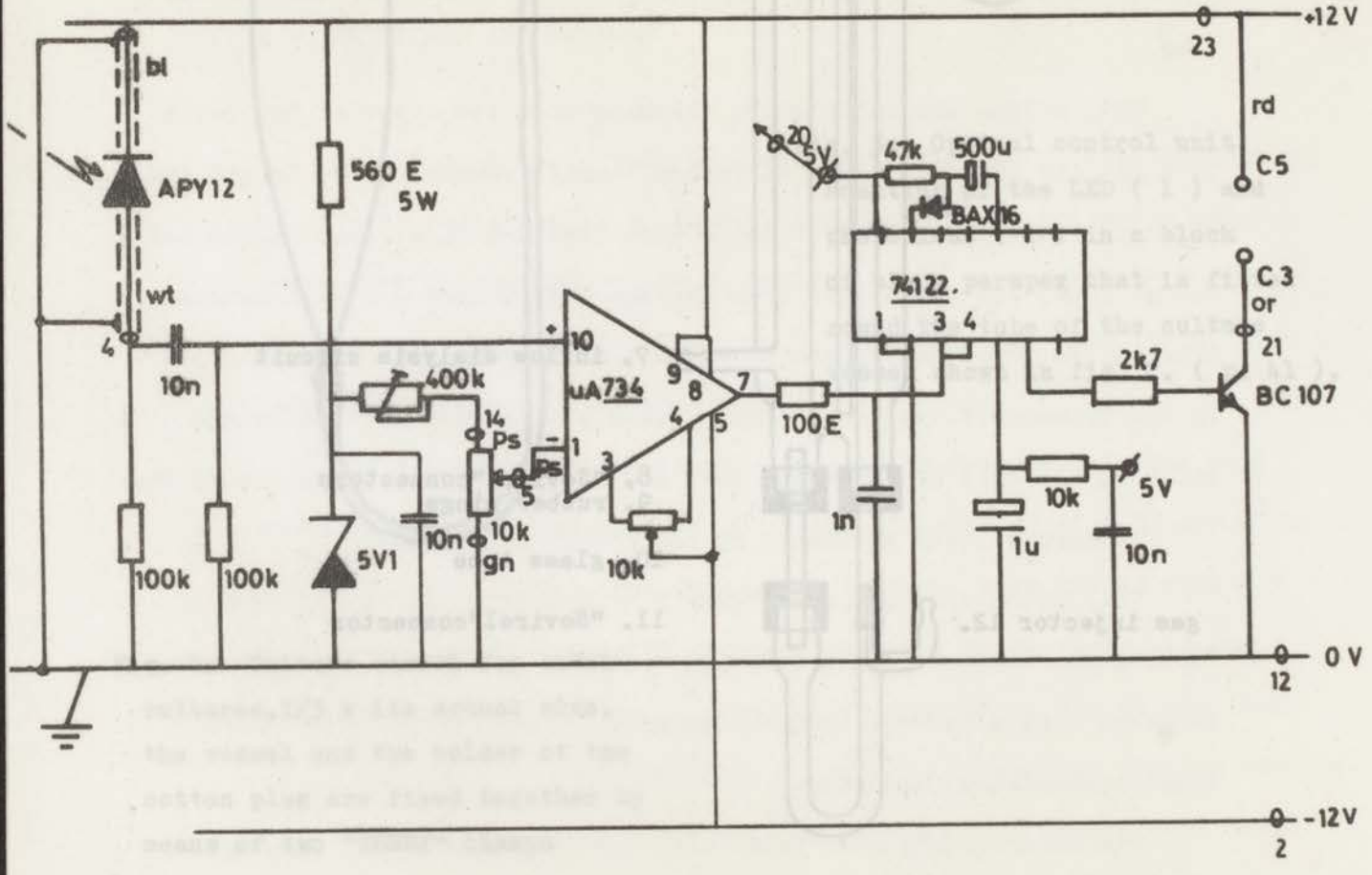


Fig. 3a. Circuit of the flash generator.

LCC 01x1:

CONTROL CIRCUIT:



OPM: -0- is connecterpunt 4.
4
: de LED en de FOTODIODE zijn met kabels aangesloten

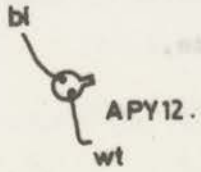


Fig. 3b. Circuit of the comparator. Fig. 3b is shown on p.40.

chamber (Gilson Medical Electronics, France, ref. OX 70_) which was thermostated at 30° C. The reaction chamber was illuminated from one side by an Aldis projector fitted with a 500 W incandescent lamp. The light was filtered by appropriate filters as indicated in the text. To measure the absorbed amount of light, a Si photocell of 42 mm diameter was placed directly behind the reaction chamber. The Si cell was calibrated against the radiometer for the filter combinations we used. The difference in energy measured on the Si cell between a reaction chamber filled with cell suspension and with water was taken as the absorbed amount of light. This value was corrected for the amount of light that was scattered by the cells. The light scattering was determined by measuring the apparent absorption at 740 nm (Balzers B40 740 nm interference filter). In all measurements of oxygen exchange a cycle of 5 min dark, 5 min light was used. Light intensity was chosen such as to cause a net oxygen evolution about equal to respiration in the dark. The difference between the rate of oxygen evolution in the light and the average of the rates of oxygen consumption in the dark periods before and after the illumination was taken as the rate of photosynthesis. The cell suspension from the culture apparatus was collected in the dark. It was diluted at least 1 to 1 with 0.2 M carbonate-bicarbonate buffer, pH 9.0. The dilution factor was chosen such as to obtain between 50 % and 20 % light absorption in the reaction chamber.

Changes in intracellular light absorption were measured with a split-beam difference spectrophotometer[9]. In some experiments a computer of average transients was used to increase the sensitivity. Absorption changes of cytochrome f were measured at 421 nm, using saturating red actinic light (Schott RG 665 nm absorption filter), P700 was measured at 705 nm using saturating blue actinic light (Corning CS 4-96 and Schott BG 12 absorption filters). DCMU was added with 1 % ethanol to a concentration of 5.10^{-6} M.

"Prompt" and "delayed" fluorescence from chlorophyll a were measur-

ed in the following way. Three light beams from 250 W 24 V iodine tungsten lamps were used in these experiments. The excitation or measuring beam was very weak; it was filtered through a Balzers B40 419 nm interference filter and a Corning CS 4-96 absorption filter. The two actinic beams were chopped in phase by means of rotating discs. A chopper in front of the photomultiplier was out of phase with these. Light from the actinic beams, or fluorescence caused by these beams was intercepted by the chopper in front of the photomultiplier; only light from the measuring beam was admitted by this chopper. The kinetics of fluorescence observed in this way were the same as those observed, if continuous actinic light of the same average intensity was used (L.N.M. Duysens, unpublished observations). The measuring beam was made sufficiently intense so that delayed light from the actinic beams was negligible. The chopping frequency was 300 Hz.

3. DESIGN OF THE CULTURE APPARATUS

An apparatus for the continuous culture of photosynthetic microorganisms was developed especially to fit the requirements of spectroscopic investigators working with intact cells. These requirements are the following:

1. A relatively low production is sufficient, as compared to the amounts of cell material needed in biochemical investigations.

2. It is desirable to operate the culture apparatus at high cell densities (at least 2-3 volume percent) to eliminate the need of centrifugation and storage of cell material before experiments.

Other requirements are:

3. Ease of maintenance and long-term reliable operation.

4. It should be possible to operate it as a chemostatic culture, a turbidostatic culture and a "pigment static" culture.

To obtain high cell densities it is essential to use a thin layer of culture liquid[10], otherwise the growth rate drops due to mutual shading of the cells. This was obtained by circulating the culture in a narrow tube (internal diameter 6 mm). The apparatus is shown in Fig. 2. The liquid was kept in motion by an air lift (Fig. 2-12). The stream of gas bubbles and liquid that moves upward flows into a funnel (1) through the side arm 2. The side arm was mounted tangentially to the funnel to cause a vortex. This vortex ensures that also in the funnel the layer of liquid is thin. The side arm was tilted about 15° from the horizontal. Otherwise splashing occurred and algae would start growing on the glass near the liquid-gas interface, eventually these cells would fall back into the culture causing severe clumping. Passing down the funnel gas bubbles separated from the liquid, which was essential to the operation of the optical control system. To be able to investigate whether variations in chemical composition of the growth medium, due to the activity of the organisms, affect anny process under investigation, two apparatus were built. One consisted entirely of glass, the other (shown in Fig. 2) of similar dimensions, was equipped with a dialysis system. In the dialysis circuit the culture was dialyzed against a surplus of growth medium. The dialysis system would remove any toxic products of low molecular weight from the organisms and replace any component from the medium if exhausted by the organisms. It has been shown with a number of heterotrophic organisms that much higher cell concentrations can be obtained in a dialysis culture[11], as compared to a closed vessel.

A piece of dialysis tubing of about 7 mm diameter can be fitted around the narrow end of the funnel (1) and a glass tube (10) to complete the culture circuit. The membrane could be fixed by tightening the rings of silicon rubber (3 and 9) with the screw caps (Sovirel, France). The dialysis tube could be spanned by moving the lower glass tube (10) in the second "Sovirel" connector (10).

The funnel was closed on top by a silicon stopper which contained 4 tubes. A wide one leading to a cotton plug for escape of the gas; a stainless steel capillary for medium supply; a steel capillary for sampling; and a steel capillary for keeping the liquid level constant. This last capillary was connected to a peristaltic pump which was continuously pumping. The medium supply tube and the supply tube of the dialysis circuit contained membrane filters of 0.1 micron pore width to eliminate the risk of infection when the medium bottles were changed. Medium was supplied via a peristaltic pump which was switched on and off by the optical control system. The flow rate of the culture liquid was 12 cm per sec. This high rate was obtained through the special design of the gas inlet of the air lift. The gas was injected through a capillary in the middle of the tube. So the kinetic energy of the gas stream (injected at 0.3 atmospheres) contributed to the upward movement of the liquid. The more important factor probably was the reduction of friction in this design. The capillary injects small bubbles of about 3 mm diameter. These seem to bounce against the glass wall of the tube and move quickly upward in a helicoidal path. Other injection methods in a tube of 6 mm diameter caused big 6 mm diameter lens-shaped bubbles, that moved slowly upwards. In this case a capillary film of liquid separated the gass bubbles from the glass. This film could be seen to move quickly downward by adding some small particles to the culture. This causes much friction and so slows down the action of the air lift. A problem with capillary gas inlet systems is, that they may be blocked by crystal growth from the medium. Occasionally this occurred in a prototype of our apparatus which had a stainless steel capillary. With the glass capillary this effect was never observed, during periods of continuous operation of up to two months.

The time during which a culture apparatus could be kept in operation was set by the moment at which the cells started to grow on the glass

or on the dialysis membrane. With Scenedesmus this occurred suddenly, within one or two days, through the whole culture apparatus. At low cell densities (up to an absorbance at 680 nm of 0.1/mm) this occurred in the glass apparatus after 4 to 6 weeks, in the dialysis apparatus after 6 to 8 weeks. So we believe this effect is due to a slow accumulation of extracellular products. At the low cell densities used in this investigation, this was the only significant difference in behaviour of the two types of apparatus.

The optical control unit employed a light emiiting diode (LED) as a light source. The LED was mounted in a small block of black perspex (Fig. 3c). The perspex blox was mounted around the culture below the lowest "Sovirel" connector (Fig. 2-11) of the culture apparatus. A photodiode was placed at the other side of the culture tube in the perspex block. The LED was fed by a pulse generator (Fig. 3a) which caused flashes of 0.5 microsec. at maximal LED current in a frequency of 1 per sec. The amplitude of the flashes as measured by the photodiode is a measure of the absorption of the culture. The LED we used hat its maximum emission at 670 nm and a half band width of 40 nm. The current from the photodiode was fed into a comparator (Fig. 3b). If the light intensity from the LED, as seen be the photodiode, was lower than the level determined by the potentiometer of the comparator, no pulses occurred. After 5 pulsed had been missing, a monostable multivibrator would switch a relais. The relais switched on the peristaltic pump of the medium supply.

The main advantage of this system was, that due to the very short flashes combined with suitable filtering it did not see the light that illuminated the culture. So it was not necessary to blacken a significant part of the culture volume. The system reacted to a combination absorption by chlorophyll and scattering by the algae. It kept "absorption", as measured with the Cary spectrophotometer at 680 nm, constant within 6 %.

4. MICROSCOPICAL OBSERVATIONS

The number of daughter cells, cell size at any given stage of the cycle and the time of cell division of Scenedesmus in synchronous culture were found to depend strongly on the amount of light that is absorbed by a cell during the light period.

At the lowest intensity at which complete synchrony in a 14 h light 8 h dark cycle was obtained, all cells divided in four daughter cells around the 22th hour. Upon increase of the light intensity division occurred progressively earlier, and cell size increased. This process developed until the majority of cells divided around the 16th hour. At this intensity, however, a small percentage divided in eight daughter cells around the 22th hour. If light intensity was increased further, an increasing percentage of the cells divided in eight daughter cells. These also increased in size and divided earlier upon increase in light intensity. When the light intensity was increased further, all cells finally divided in eight around the 16th hour. A still further increase did not cause an earlier division. A synchronous culture grown under these conditions we call a light saturated culture; a synchronous culture grown at lower intensity is a light limited culture. Similar effects of light intensity on the time of division and number of daughter cells were observed by WANKA[12] in Chlorella.

During the light period the average volume of the cells increased exponentially (Fig. 4), except perhaps during the first two hours.

The amount of light absorbed by a cell during a light period does not only depend on the average light intensity in the culture, but also on the initial amount of chlorophyll per cell. The amount of chlorophyll per cell, cell volume and dry weight were found to be in first approximation proportional to each other. This agrees

with our incidental observations on cell volume and chlorophyll content. Since the size of daughter cells depended upon the previous history of a culture and especially on the number of daughter cells, at least one cycle was needed for adaptation of a particular culture to a change in light intensity. We found that it generally takes four to five days before a stable situation is reached after changing the light intensity. The variations during the adaptation period were particularly prominent when a transition from division in four to division in eight daughter cells, or vice versa, was involved. In one case we even obtained an oscillating culture in which the majority of cells divided alternatively in four and eight. This occurred after lowering the intensity in a fully synchronous culture which divided in eight small daughter cells. At the lower intensity during the subsequent light period these small cells presumably did not obtain enough light to be able to divide in eight cells again, but they produced four large daughters, containing large chloroplasts. The next cycle these large cells were capable of obtaining enough food to permit division in eight small daughter cells. This alternation lasted for six days, when we interrupted the experiment.

The above conclusions are based on many incidental observations on the behaviour of our cultures during our search for physiological effects occurring in photosynthesis. So the documentation is rather scattered, and more systematic experiments in this direction are desirable. In most experiments we varied the light intensity in the culture by varying the absorption (by means of the optical control unit), thus so varying mutual shading of the cells. The results agreed with a few control experiments in which the light intensity was varied and absorption kept constant. This indicated that variations in the chemical environment did not play an important role.

Als the glass apparatus and the dialysis apparatus gave very similar results. Chemical variations would be expected to be damped in the dialysis apparatus. The dialysis system allowed linear growth up to cell concentrations of at least 10 % of the culture volume (M. Donze, unpublished results), while in a glass apparatus growth stopped around 3 %. This indicated that the system was effective, but the effects were negligible at the lower cell densities used in this investigation. Maximum concentration used was 0.16 % corresponding to an absorbancy at 680 nm of 0.1/mm.

Two effects in the absorption spectrum during a life cycle were noted (Fig. 5). During the period of cell wall synthesis, which in this particular culture took place between the 16th and the 20th hour the absorption increased at all wavelengths due to an increase in scattering. This can be concluded, since at 730 nm,where absorption by pigments in negligible, the same increase in absorption of the sample occurred. During release of the daughter cells the absorption of the pigments increased without a marked change in scattering. Since, as was found by extraction, no pigments were synthesized during this period, this effect is probably due to a decrease in the flattening effect[13], due to cell division.

Young cells have a single chloroplast with the shape of an oval dish. During growth the chloroplast gradually expands, lining the cell wall. Finally the chloroplast has an urn-like shape, leaving a very small hole at one of the poles of the cell. In Fig. 6 a few cells are still in this condition, the others are in the first stage of chloroplast division. At this stage in the life cycle, and at no other, the central protoplasm can be seen to be in a vigorous motion. This was especially prominent if observed between crossed polars under a polarization microscope. A few optically anisotropic inclusions that are generally present in the protoplasm at this

stage move and rotate quickly around. In Fig. 7 a number of cells in the process of building the cell walls for the daughter cells is shown; in Fig. 8 the release of daughter cells is in full progress. The photographs were taken during one cycle of a light-limited culture in which over 90 % of the cells divided in eight.

The arrangement of daughter cells was quite variable in our cultures (Fig. 9). Eight daughter cells may be lined up in one straight row if viewed from the side. Viewed from top this row has a more or less pronounced S shape. Another characteristic arrangement is one in which the cells alternate in a row of eight which is straight if seen from top (Fig. 9c). Two rows of four, where the cells adhere at their poles is another possibility. The tree-like arrangement of Fig. 9d which was rather common might be an intermediate between the former two possibilities. Whether this variability of arrangement is peculiar to this strain, or of a more widespread occurrence in the genus Scenedesmus we do not know. So possibly the importance attached to arrangement as a distinctive character in taxonomy[14,15] is exaggerated.

A few times we had the impression that division in two or sixteen daughter cells occurred. We cannot be sure of this since we did not have the means to follow a single cell, and the frequency of these possibilities was too low to be detectable by counting. Moreover artefacts in observations like these are likely to arise due to the strong tendency of cells of Scenedesmus to adhere to each other.

5. MEASUREMENTS OF OXYGEN EXCHANGE

Except for a short induction period, no significant changes in the quantum requirement of photosynthesis during the life cycle

of Scenedesmus were observed in light limited synchronous cultures Fig. 10). Only in cultures grown above light saturation (as defined in section 4) a periodic lowering of the quantum yield of about 20 % was observed. This lowering roughly followed the time course as found by other investigators[3,4]. We did not study these conditions in more detail.

During the first few illuminations in the polarograph sometimes a higher quantum requirement was found, which after some time declined to its normal value. This induction process was observed only during the dark period of the culture cycle. The maximal effect occurred always in the period during which the cell walls of the daughter cells were synthesized. Whether this induction process occurred and how strong it was depended on the light intensity in the culture. It was maximal under two circumstances: in light limited cultures, in which the majority of cells divided in four but about 10 % in eight; and in cultures grown at about light saturation. This suggested that this temporal inhibition of photosynthesis might be associated with an excess of carbohydrates that might be present in these two types of cells. At intermediate light intensities in the culture this process often was not measurable. The normal low quantum requirement was between 9 and 10 quanta per oxygen molecule. In red and blue light (filtered by a Schott Al 430 nm and AL 662 nm interference filter, respectively) the quantum requirement was the same within 5 %. Samples from exponentially growing batch cultures gave the same value.

The behaviour of respiration in the dark showed considerable variations with the life cycle stage in our cultures (Fig. 11). It also showed large induction phenomena upon illumination. If respiration had a high rate, it generally was lowered after weak illumination. The pattern in the dependence of respiration on life

cycle stage was very similar to that observed by WANG[4]. We did not analyse respiration in more detail.

SPECTROSCOPIC MEASUREMENTS

The total concentrations of cytochrome f and the reaction centrum pigment of photosystem 1, P700 were determined at various phases of the same cycle. On a chlorophyll basis these concentrations did not vary more than 5 %, which was the accuracy of the measurements[7]. However, the chlorophyll content per cell may vary by a factor 8. We may conclude that the reaction centers of system 1 are formed at the same rate as that of chlorophyll synthesis. In a qualitative way it was observed that at the 18th hour P700 and cytochrome required a considerably higher intensity of actinic light before saturation of their oxidation level could be reached. Since DCMU was present in these samples, this effect could not be due to reductant accumulated by photosystem 2. This suggested an accumulation of reductant for P700 from the cytoplasm. A similar hypothesis has been used by HEALEY[16] to account for photoevolution of H_2 in the presence of DCMU working with Chlamydomonas, and by LEX and STEWART[17] as a possible pathway of reductant to nitrogenase in the blue-green alga Anabaena.

This hypothesis was investigated in more detail by measuring fluorescence and delayed fluorescence changes. A light induced increase in fluorescence in generally ascribed to reduction of the primary electron acceptor of photosystem 2, delayed fluorescence to back reactions between Q and the primary donor Z of this system. A theoretical discussion of these processes had been given by VAN GORKOM and DONZE[18]. Measurements of these phenomena made it possible to use the antagonistic effect of system 1 light and system 2

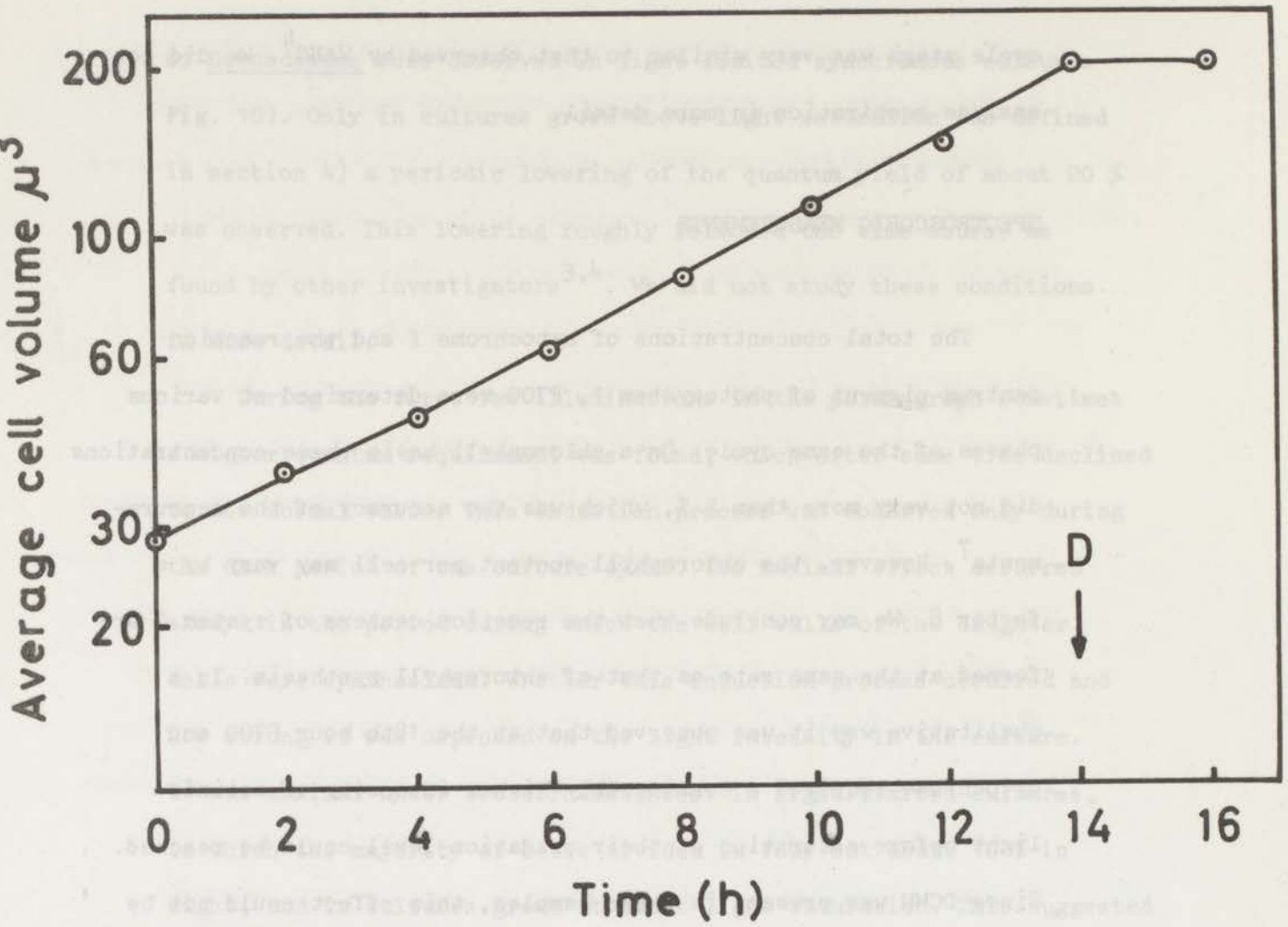


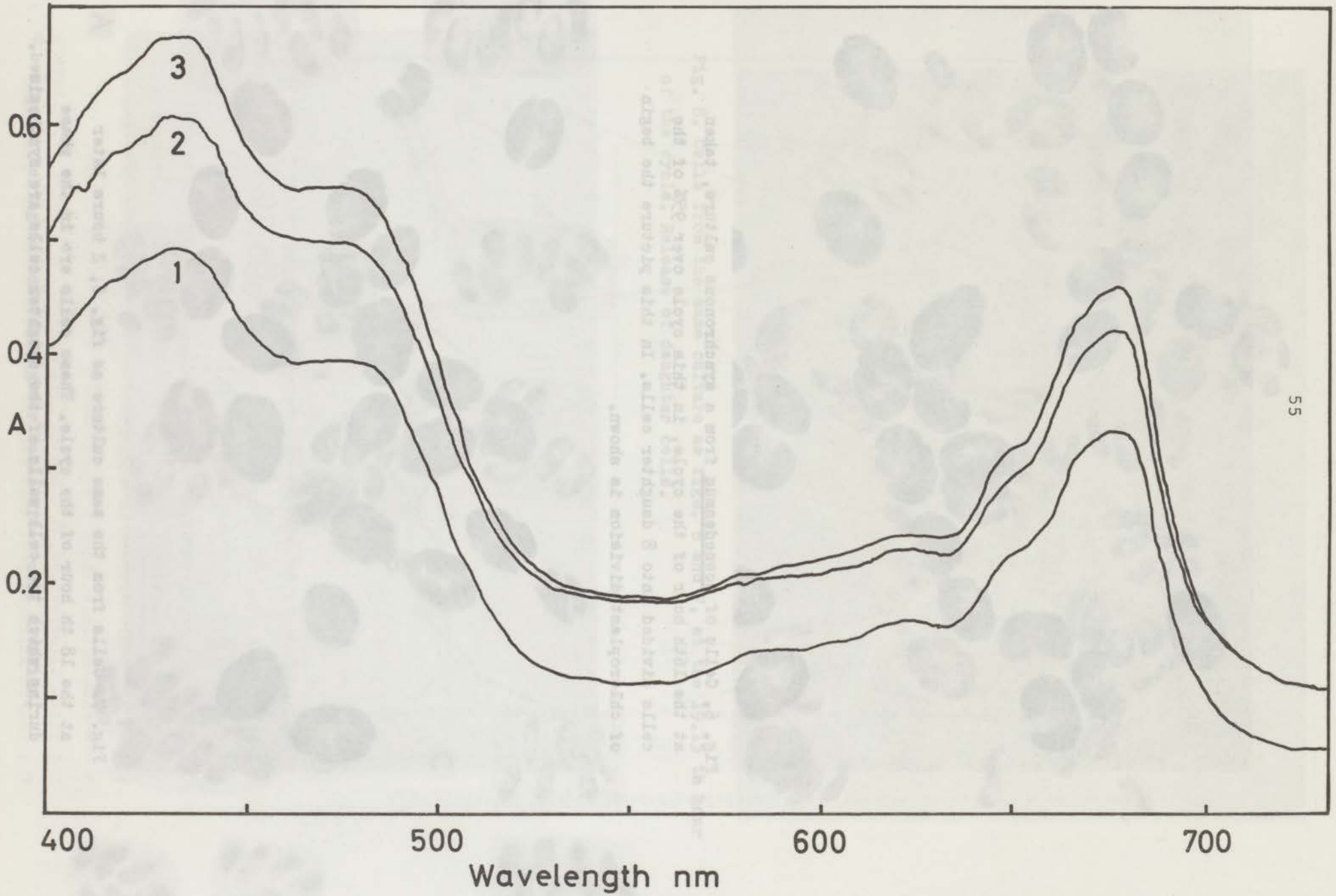
Fig. 4. Exponential increase of the average cell volume during the light period in a synchronous culture of Scenedesmus. These data are from a culture which was grown under identical conditions of illumination as the cultures on which figs. 5,6,7,8 and 9 are based. 90% to 95% of the cells divided in 8 daughter cells.

Fig. 5. Absorption spectra at several stages in the cycle of a synchronous culture of Scenedesmus.

curve 1 : The 16th hour of the cycle

curve 2 : The 18th hour of the cycle. The cell walls of the daughter cells has been formed, which caused scattering to increase. See also Figs. 6 and 7.

curve 3 : The 21th hour of the cycle. Cell division has occurred and the absorption bands of the pigments are sharpened, due to decrease of the flattening effect.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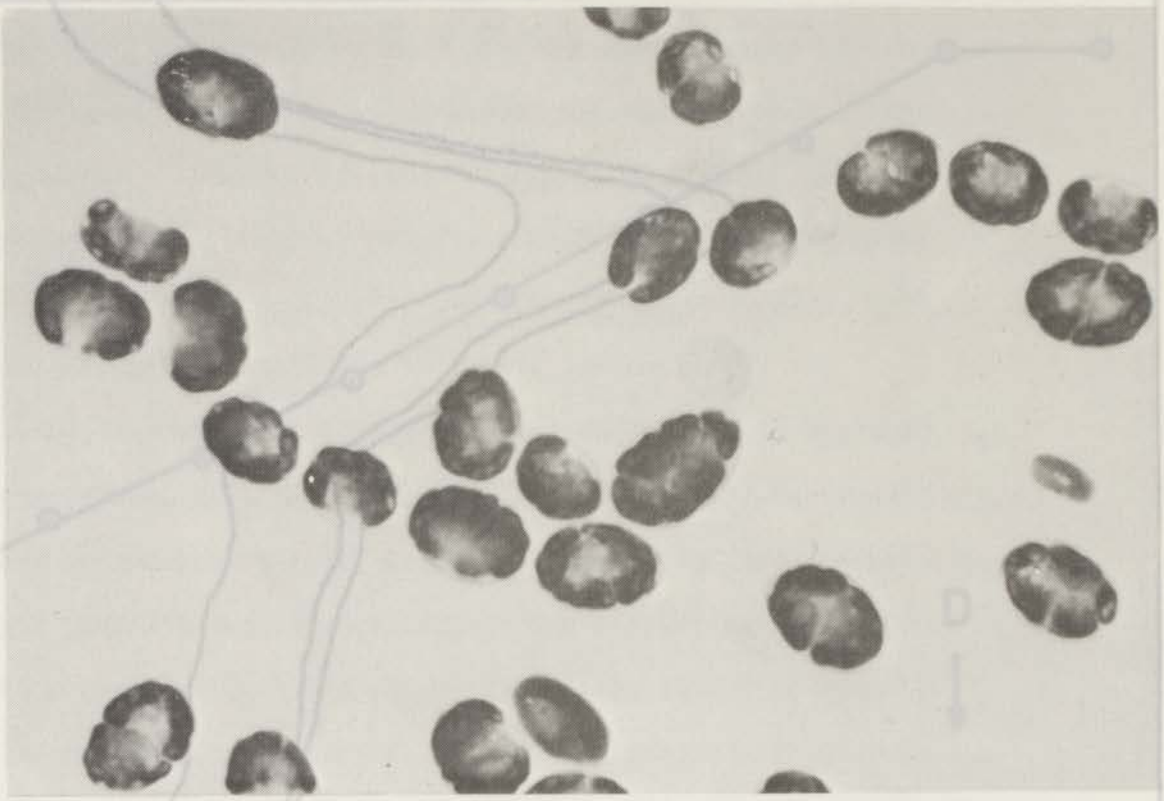


Fig. 6. Cells of Scenedesmus from a synchronous culture, taken at the 16th hour of the cycle, in this cycle over 95% of the cells divided into 8 daughter cells. In this picture the begin of chloroplast division is shown.

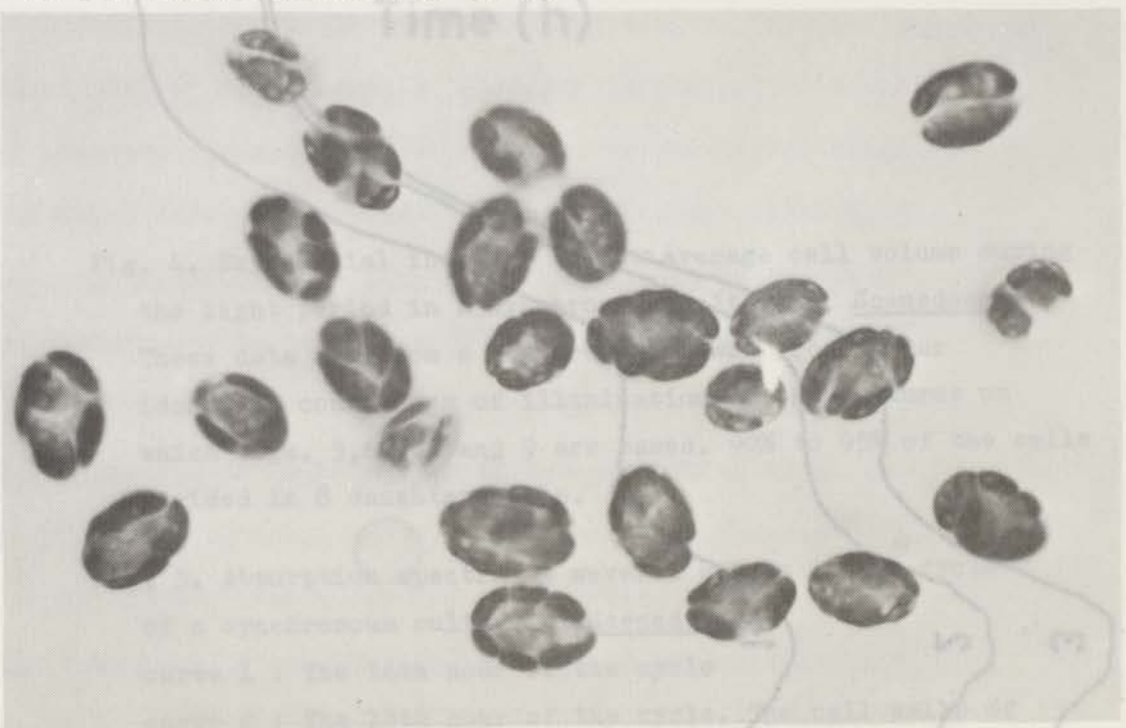


Fig. 7. Cells from the same culture as fig. 6, 2 hours later at the 18 th hour of the cycle. These cells are in the phase during which the cell walls of the daughter cells are synthesised.

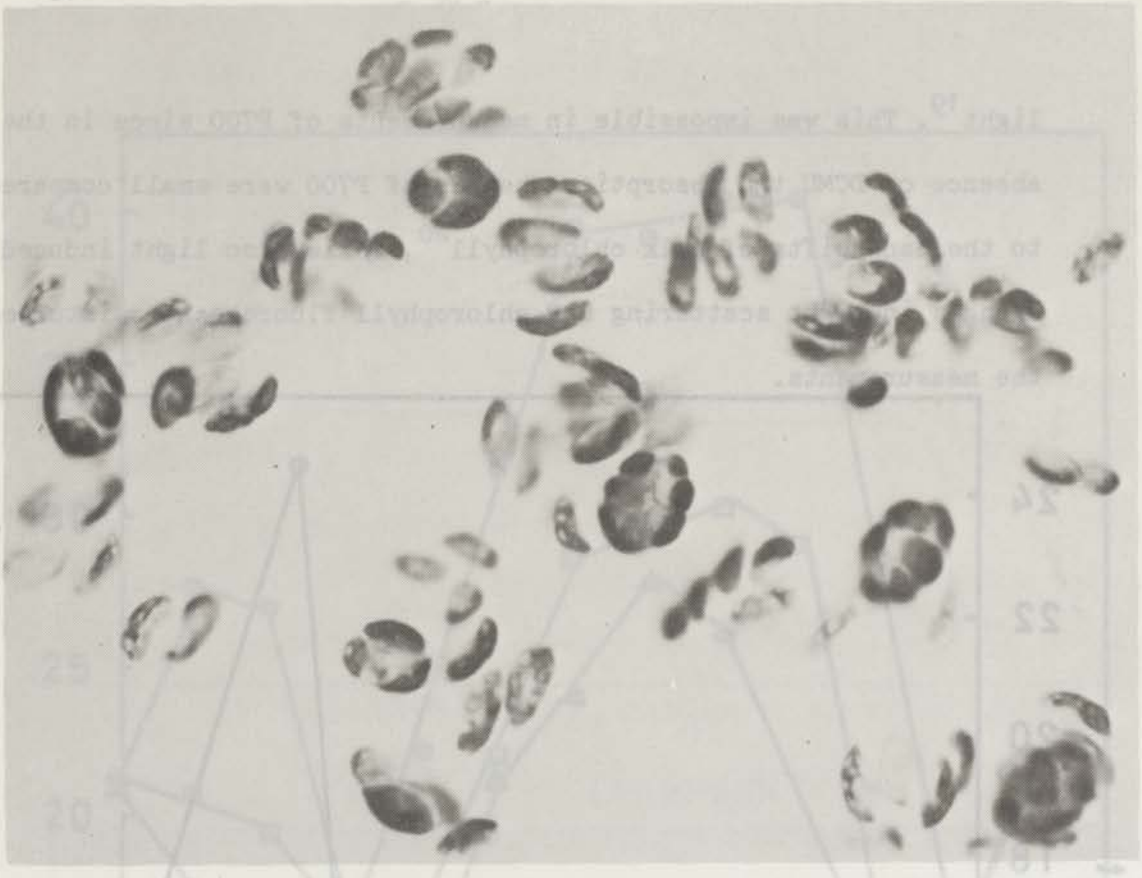


Fig. 8. Cells from the same culture as figs. 6 and 7, at the 19.15 th hour of the cycle. Release of daughter cells.

Fig. 9. Configuration of daughter cells after cell division.

light[19]. This was impossible in measurements of P700 since in the absence of DCMU the absorption changes of P700 were small compared to the bandshifts of bulk chlorophyll[20], while also light induced changes in light scattering and chlorophyll fluorescence disturbed the measurements.

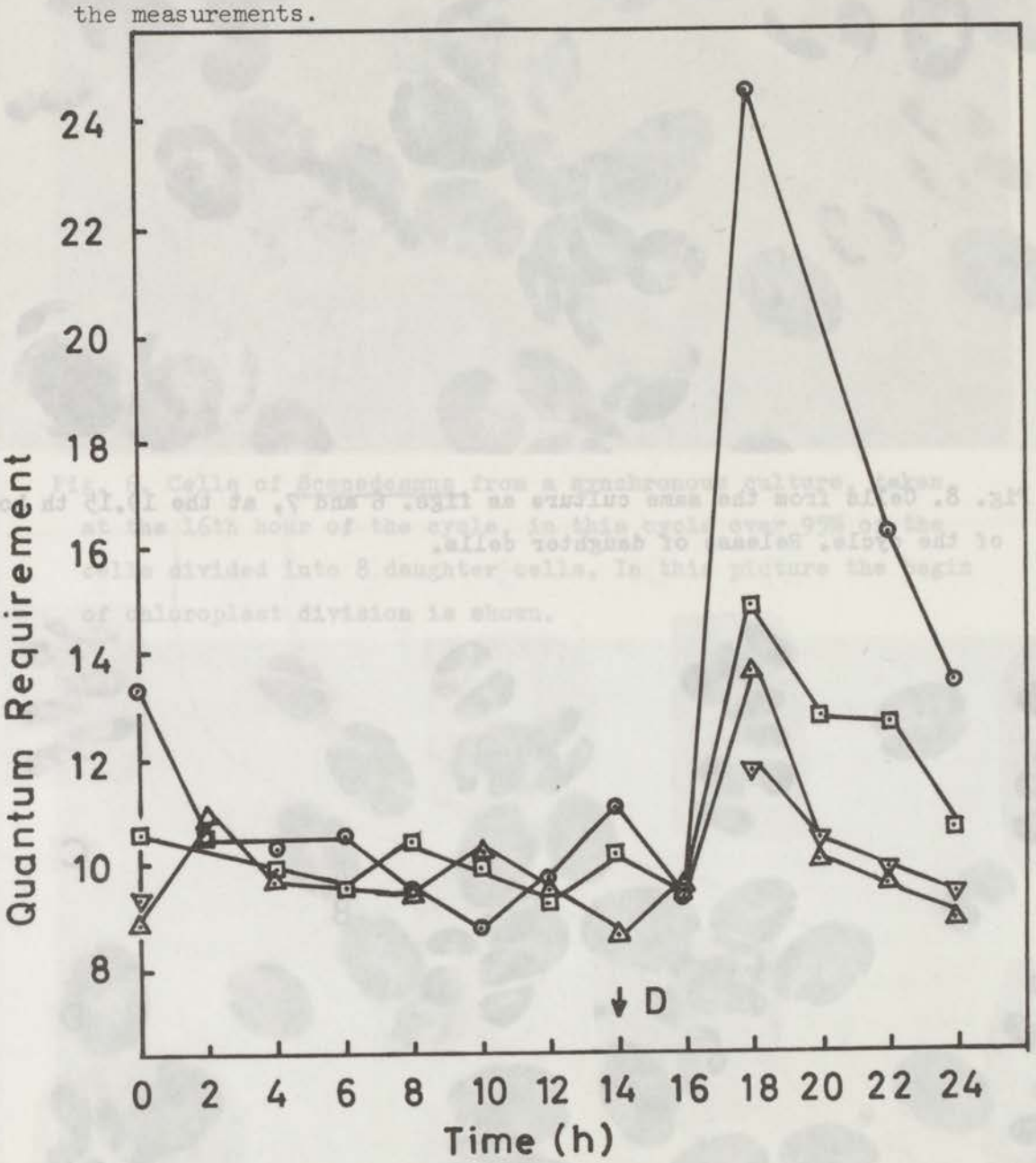


Fig. 10. Quantum requirement of photosynthetic oxygen evolution during a cycle of Scenedesmus in synchronous culture. In this culture over 95% of the cells divided in 4 daughter cells between the 20th and 22th hour. Blue actinic light (Schott Al 435 interference filter. o : first illumination, □ : second illumination, △ : third illumination ▽ : fourth illumination. This culture showed a pronounced induction effect.

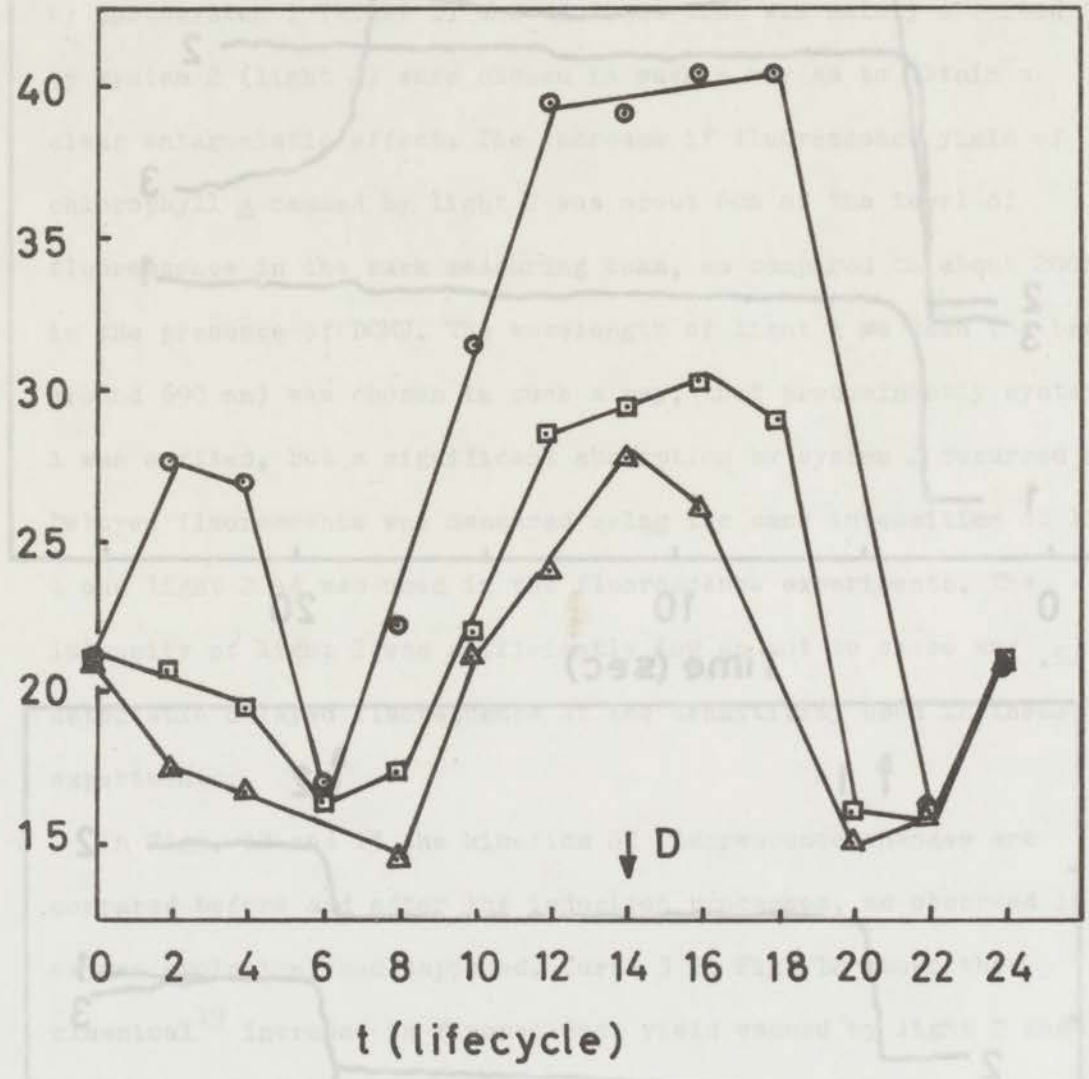


Fig. 11. Rate of oxygen uptake in the dark during a cycle of Scenedesmus in synchronous culture. These measurements were carried out with the same samples as shown in fig. 10.

o : rate of oxygen uptake before the first illumination.

□ : after the first illumination.

△ : after the second illumination.

Oxygen uptake is expressed in units of 2.10^{-2} nmol/sec.ml.

The extinction of the samples at (680 nm - 740 nm) was 0.18 ± 0.01 /cm.

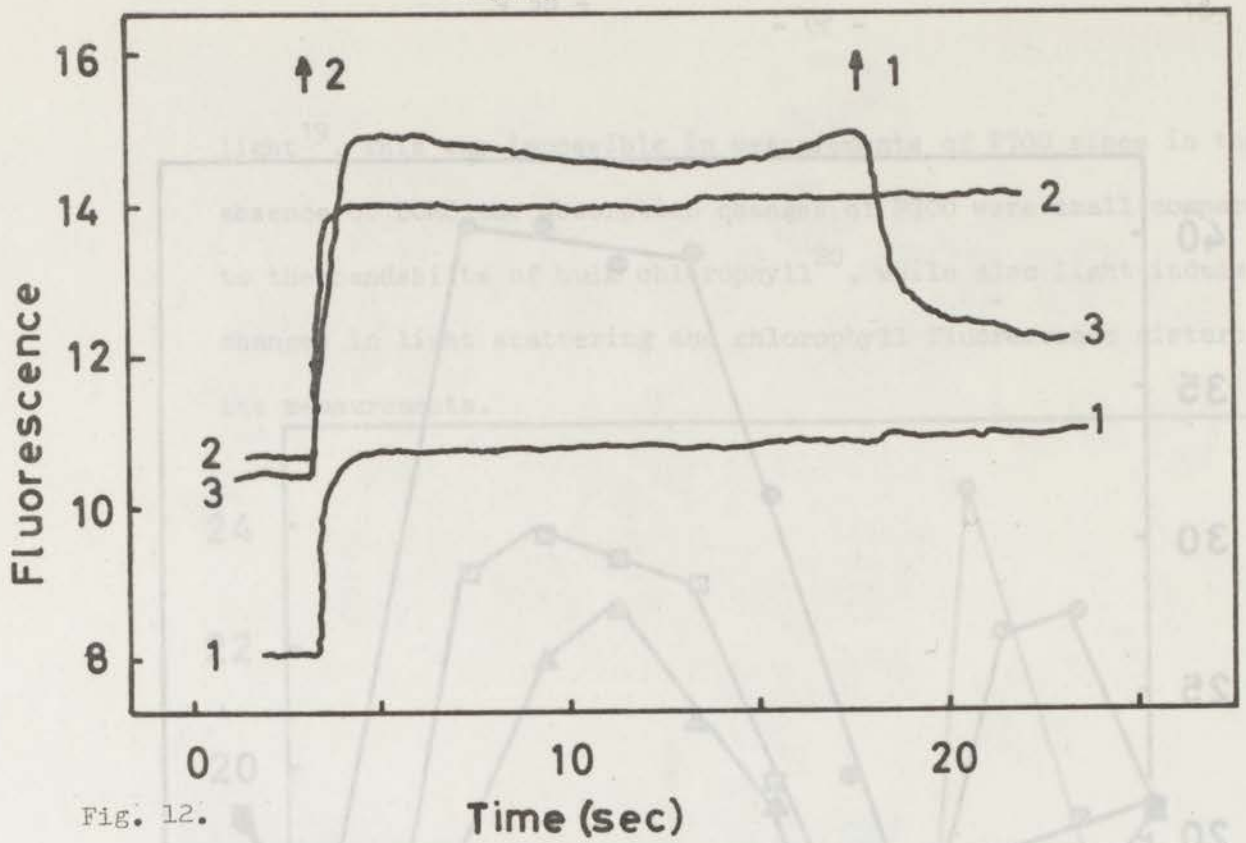


Fig. 12.

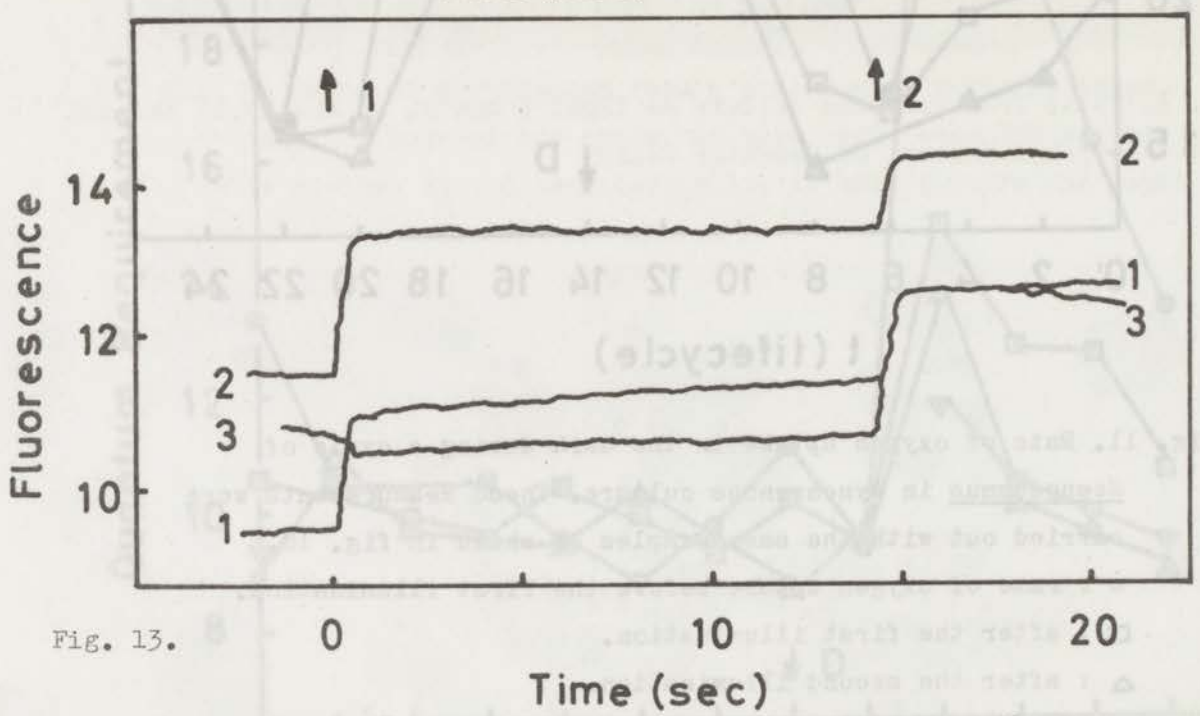


Fig. 13.

Fig. 12. Kinetics of fluorescence changes in a sample from the same culture as used for Fig. 10, at the 22th hour. Light 1 and light 2 were switched on as indicated by the arrows. Curve 1: sample taken directly from the culture aparatus and diluted with carbonate-bicarbonate buffer in the dark. Curve 2: the same after illumination for 2 minutes with light 2 and a dark period of 14 sec. Curve 3: the same sample after completion of the induction process in oxygen evolution as measured in a polarograph, and a short dark period.

Fig. 13. Legend see fig. 12. The sequence in which light 1 and light 2 are admitted was interchanged.

Relatively weak intensities of light that was mainly absorbed by photosystem 1 (light 1) and of light that was mainly absorbed by system 2 (light 2) were chosen in such a way as to obtain a clear antagonistic effect. The increase if fluorescence yield of chlorophyll a caused by light 2 was about 60% of the level of fluorescence in the waek measuring beam, as compared to about 200% in the presence of DCMU. The wavelength of light 1 we used (a band around 690 nm) was chosen in such a way, that predominantly system 1 was excited, but a significant absorption by system 2 occurred also. Delayed fluorescence was measured using the same intensities of light 1 and light 2 as was used in the fluorescence experiments. The intensity of light 1 was sufficiently low as not to cause any detectable delayed fluorescence at the sensitivity used in these experiments.

In Figs. 12 and 13 the kinetics of fluorescence changes are compared before and after the induction processes, as observed in oxygen evolution, had happened. Curve 3 in Fig. 12 shows the classical[19] increase in fluorescence yield caused by light 2 and its decrease induced by light 1. The increase is interpreted as reflecting the reduction of the primary electron acceptor Q in photosystem 2; the decrease as its oxidation via system 1. This antagonistic effect of light 1 in the presence of light 2 was absent before the induction of a high quantum yield in oxygen evolution had occurred. This indicates, that before this had happened, system 1 was inefficient in oxidizing Q. The hypothesis that, under these conditions, a pool of reductant is present which competes effectively with the electron transport chain from Q in the reduction of P700, accounts well for this effect in fluorescence kinetics.

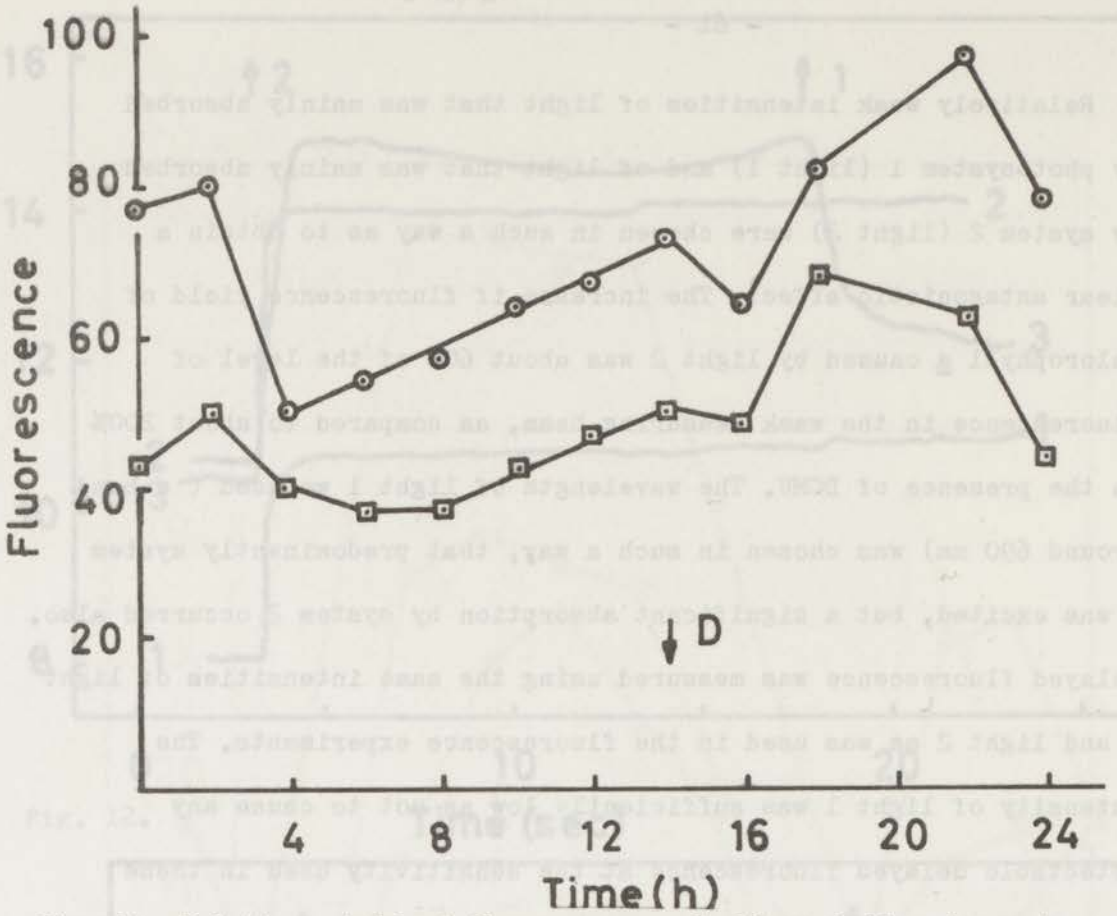


Fig. 14. Relative yield of fluorescence of chlorophyll a in weak measuring light (Fo), as measured in the same samples that were used for the experiments of Fig. 10, after the induction processes in oxygen evolution had occurred o-o, and the increase in fluorescence upon switching on light 2 □-□.

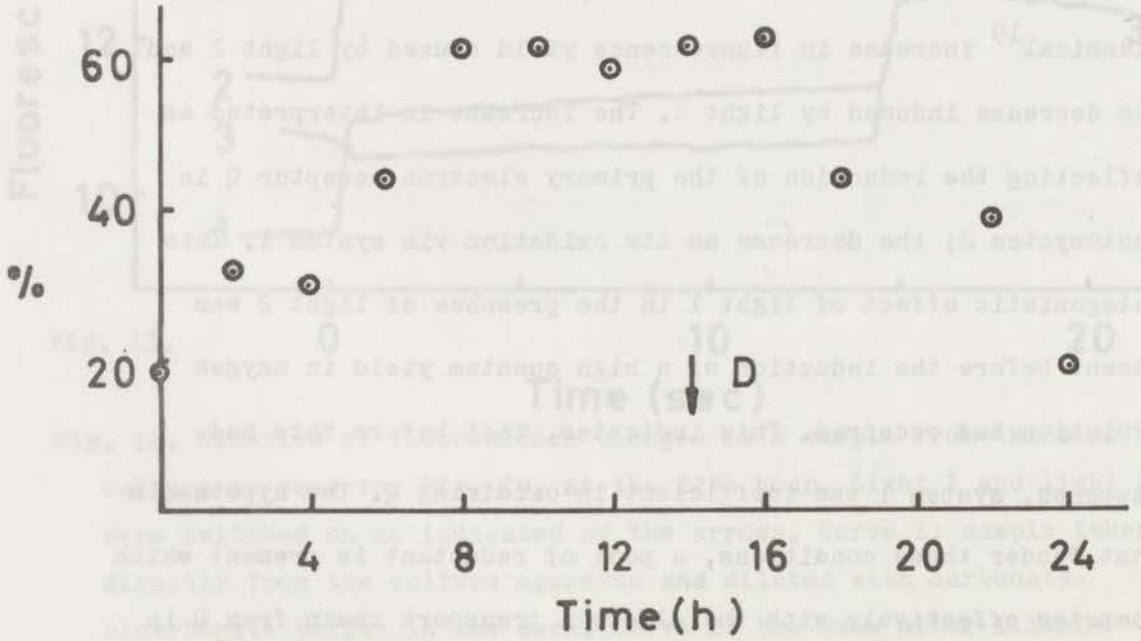


Fig. 15. Decrease in fluorescence caused by system 1 light, divided by the increase from Fo in system 2 light, expressed as a percentage. Fluorescence levels were measured 10 sec. after light 2 and after light 2 and light 1 were switched on. This percentage is a measure of the efficiency of light 1 in oxidizing the electron acceptor Q in system 2. Same samples as used for Fig. 14.

Fig. 13 shows that in a sample taken directly from the culture, light 1 increased the fluorescence. This also fits in our explanation, since as was mentionned above, the light 1 also excited system 2 to some extent.

These samples that were taken directly from the culture aparatus also showed slow changes in the fluorescence yield in weak measuring light upon illumination, and in the amplidude of the fluorescence change caused by light. This appears from comparison of curves 1 and 2 in Figs. 12 and 13. We did not analyse this effect in more detail.

After the measurements of oxygen exchange shown in Figs. 10 and 11, the same samples were used for measurements of prompt and delayed fluorescence. So after they had been taken from the culture aparatus, these samples had been illuminated in the polarograph for 15 or 20 minutes. In this way we were sure that the induction processes in oxygen evolution were over, and its quantum yield approximately constant and at its maximum.

The yield of fluorescence in the waek measuring beam (Fo) showed a variation by a factor of almost 2 during the cycle (Fig. 14). Upon switching on light 1 which, in the presence of light 2 gave a significant decrease in fluorescence, no decrease exceeding 1% in Fo was observed. So changes in the redox level of Q in the dark probably were not the cause of these variations in Fo. The increase in fluorescence upon switching on light 2 showed variations that followed a similar, though not identical, pattern as Fo did. This indicates that the variations in Fo are mainly due to variations in the pigment system that affect the fluorescence yield of the light harvesting chlorophyll in photo-system 2. These changes in Fo apparently did not affect the efficiency

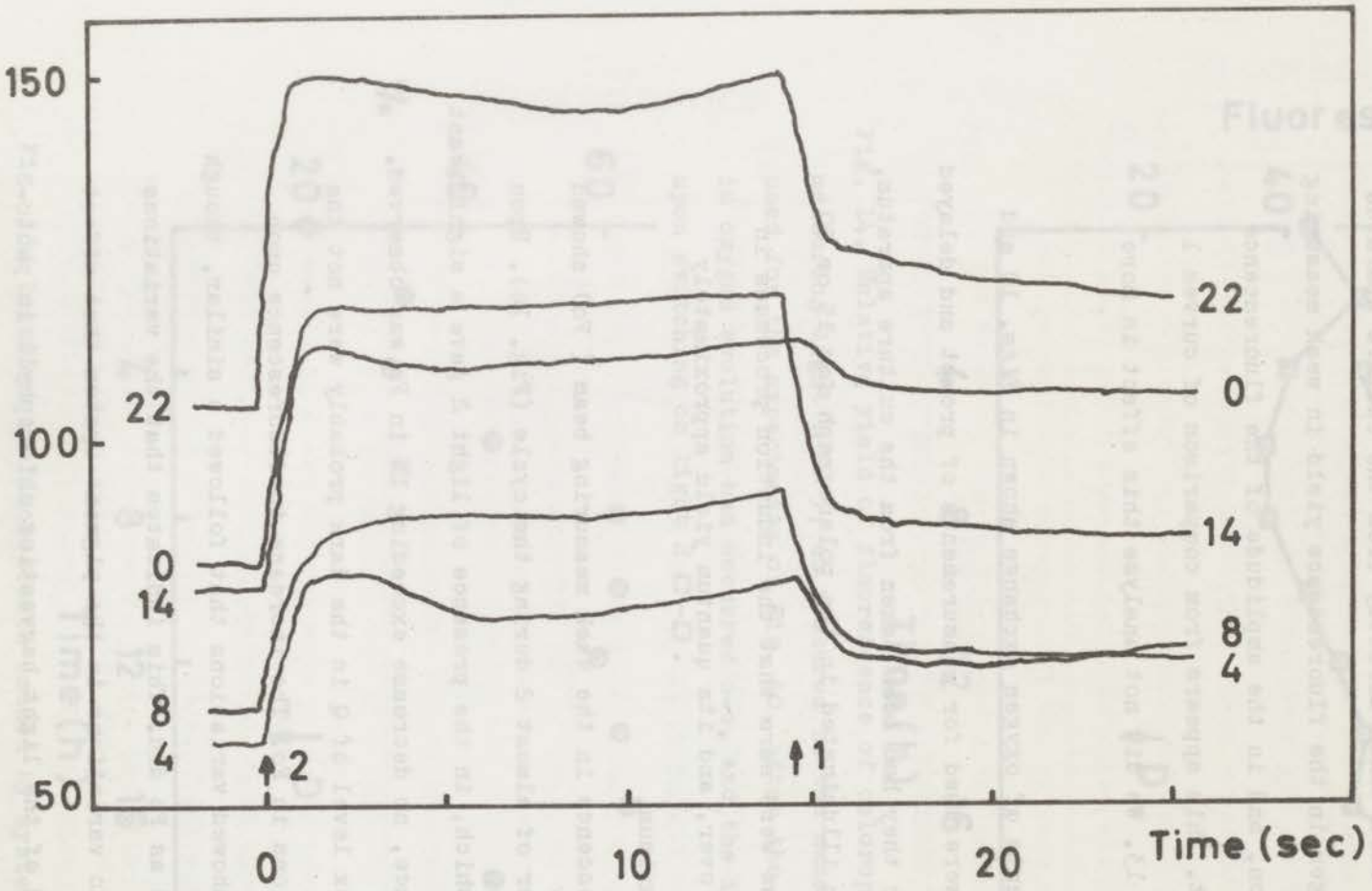
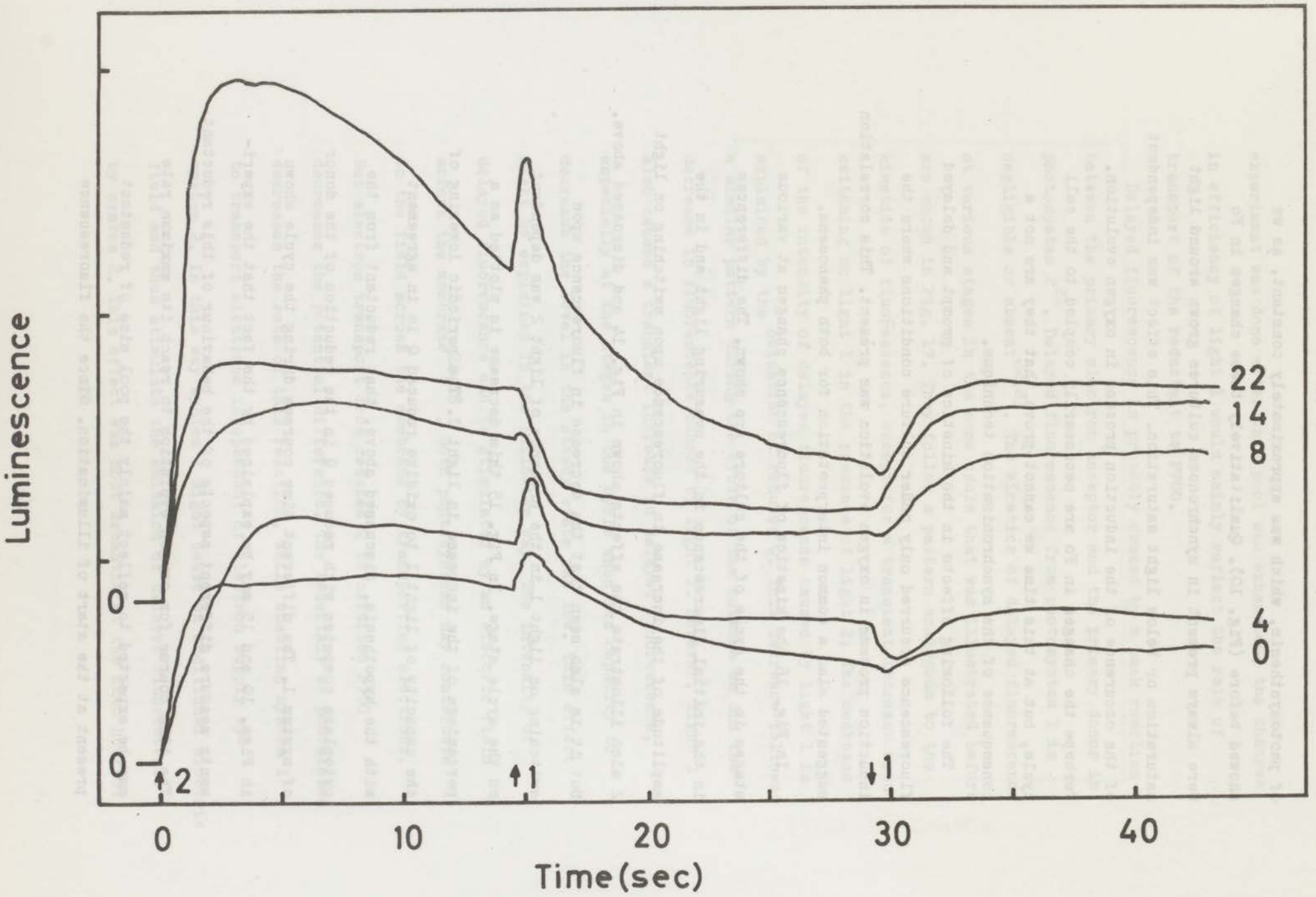


Fig. 16. Kinetics of light induced changes in the fluorescence yield of chlorophyll a at various stages during the same cycle as shown in Fig. 10. Light 1 and light 2 were switched on and off at the moments indicated by the arrows. The measurements were made on the same samples as used in the measurements of oxygen exchange (Fig. 10), after the induction processes were complete. The time in the light- dark cycle of the culture is indicated at the curves in hours.

Fig. 17. (p. 65) Kinetics of delayed fluorescence of chlorophyll a, measured on the same samples as used for Fig. 16, using identical intensities of light 1 and 2, only the very weak beam used for excitation of fluorescence in Fig. 16 was turned off. Legend see Fig. 16.


Luminescence
0
0
↑2
↑1
↓1
0
10
20
30
40
Time (sec)
22
14
8
4
0

of photosynthesis, which was approximately constant, as we showed before (Fig. 10). Qualitatively these changes in Fo were always present in synchronous cultures grown around light saturation or below light saturation. This effect was independent of the occurence of the induction process in oxygen evolution. Perhaps the changes in Fo are necessarily coupled to the cell cycle, but at this time we cannot prove that they are not a consequence of the synchronization technique.

The following effects in the kinetics of prompt and delayed fluorescence occurred only under culture conditions where the induction process in oxygen evolution was present. This correlation suggested also a common interpretation for both phenomena.

In Fig. 16 the kinetics of fluorescence changes at various stages in the cycle of the culture are shown. The differences in the initial fluorescence in the measuring light and in the amplitude of the increase in fluorescence upon switching on light 2 also illustrate the effects shown in Fig. 14 and discussed above. But it is also seen that the decrease in fluorescence upon switching on light 1 in the presence of light 2 was dependent on the cycle stage. In Fig. 15 this decrease is plotted as a percentage of the increase in light 2. The periodic lowering of the capacity of light 1 to oxidize reduced Q is in agreement with the hypothesis, discussed above, that reductant from the cytoplasm competes with reduced Q in the reduction of the donor of system 1. The different time courses during the cycle shown in Figs. 10 and 15 may be explained by the fact that the experiments measure different aspects of the behaviour of this reductant. The time course for oxygen evolution to reach its maximum rate may be expected to reflect mainly the pool size of reductant present at the start of illumination. Since the fluorescence

experiment was done after this pool was exhausted, the changes in efficiency of light 1 would mainly reflect the rate of transport of the reductant to P700.

Delayed fluorescence is probably caused by a back reaction between the primary electron acceptor and the primary donor in photosystem 2[21]. Delayed fluorescence from photosystem 1 is negligible or absent[22,23]. The kinetics of delayed fluorescence at various stages in the same cycle that was illustrated before are shown in Fig. 17. They follow a pattern analogous to the kinetics of fluorescence, except for a transient increase upon switching on light 1 in the presence of light 2. The decrease of the intensity of delayed fluorescence caused by light 1 is explained by the oxidation of reduced Q. This decrease followed a similar pattern during the cycle of the culture as did the decrease in fluorescence caused by light 1. However, light 1 also caused a transient increase in delayed fluorescence, especially at the stages in the cycle when its capacity to decrease the level in the steady state was low. This effect might be explained in the following way. The intensity of delayed fluorescence can be stimulated by an electric field across the membrane[24,18]. Both photosystems may contribute to the field across the membrane by transporting electrons. But electron transport from reduced Q to oxidized P700 decreases the potential difference. The reductant we postulated decreases the rate of this reaction, while enabling system 1 to transport electrons independent of electron supply by system 2. In this way system 1 might cause an additional electric field and thus stimulate the emission of delayed fluorescence by system 2. This effect was most dramatic at the 22th hour of

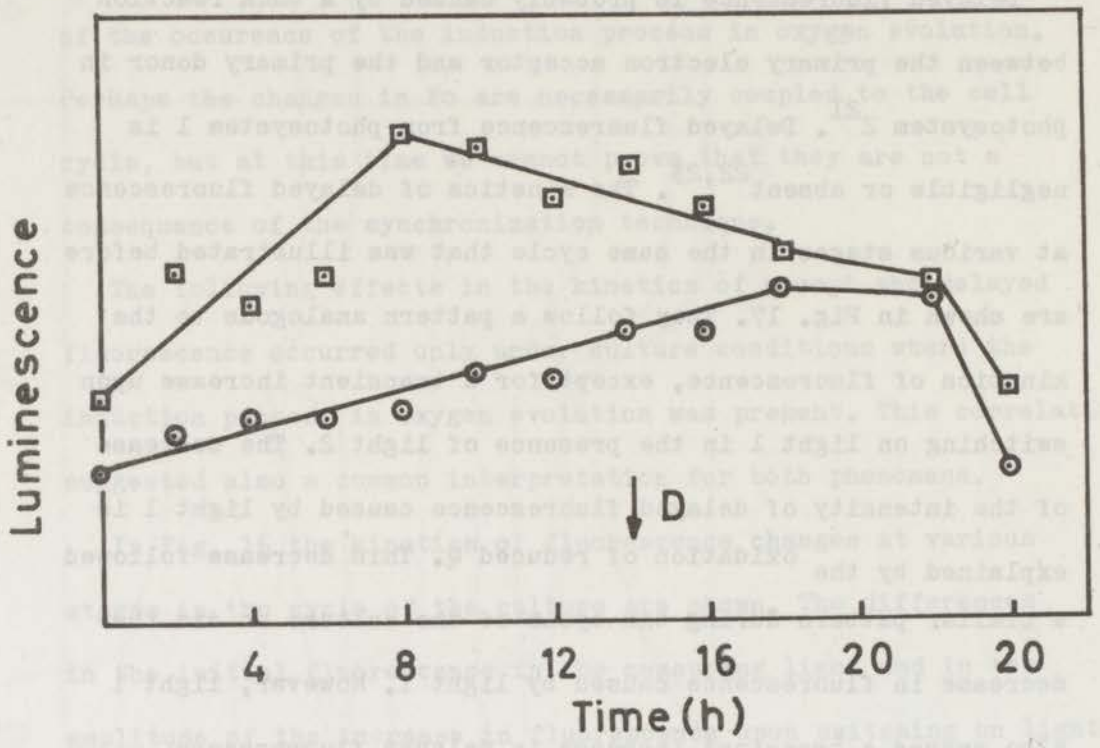


Fig. 18. Steady state level of delayed fluorescence during the cycle of a synchronous culture of Scenedesmus in light 2 (o). □ : 5.10^{-6} M DCMU added. Same samples as used in Fig. 10.

cycle of the culture. In this sample light 1 decreased fluorescence, but induced a marked transient increase in delayed fluorescence. So we have to assume that in this case the effect of the membrane potential overcompensates the decreasing effect of the disappearance of reduced Q.

In Fig. 18 the intensity of delayed fluorescence in light 2 after its steady state was reached is plotted, as function of stage in the cycle of the culture. In tha absence of DCMU a steady increase occurred up to the 18th hour. This may reflect the increase in fluorescence yield of chlorophyll, as discussed before (Fig. 14). DCMU increased the intensity of delayed light, presumably since reduced Q, one of the substrates in the reactions which lead to the emission of delayed fluorescence, accumulates in the presence of the inhibitor. We have then to assume that the increase in Q^- overcompensates a decrease in oxidize donor in system 2, which is the other substrate in the emission of delayed fluorescence. The stimulatory effect of DCMU decreased strongly during the dark period of the culture. This can be explained in a similar way by the accumulation of reductant.

The half life time of the decrease in fluorescence yield in the measuring light upon switching off light 2 in the presence of DCMU was 0.16 ± 0.02 sec. This time constant was independent of the stage in the cycle, indicating that the reductant did not react with reduced Q nor with the donor side of system 2. There is good evidence that this decrease in fluorescence is due to a back reaction between Q and the donor side of system 2[25].

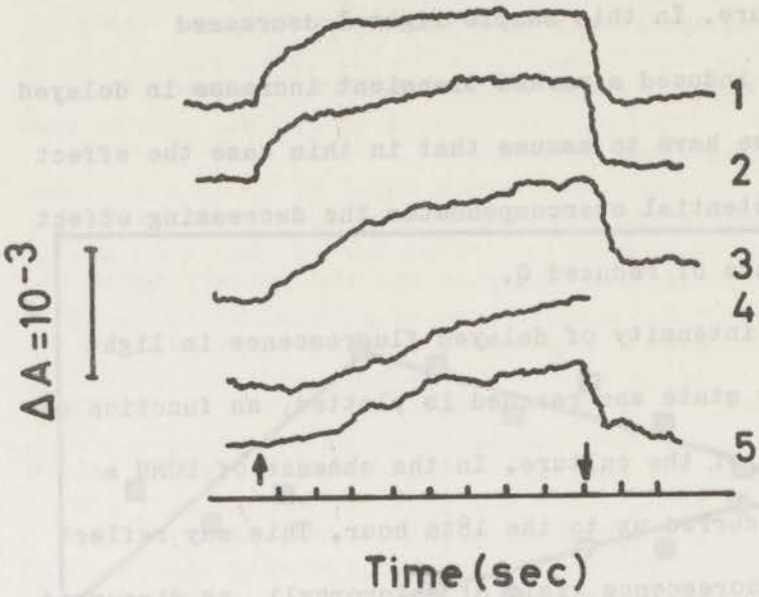


Fig. 19. Absorption changes at 700 nm in a batch culture which was starved for CO_2 during 2 days. Actinic light of 430 nm was switched on and off as indicated by the arrows. Curves 1 and 2 : no additions. Curve 1 was measured after 10 sec darkness, curve 2 after 2 minutes darkness. Curves 3, 4, and 5: the same sample after addition of 10^{-3} M glucose. Curve 3 after 5 sec darkness, curve 4 after 20 sec darkness and curve 5 after 2 minutes darkness. 5.10^{-6} M DCMU in all samples.

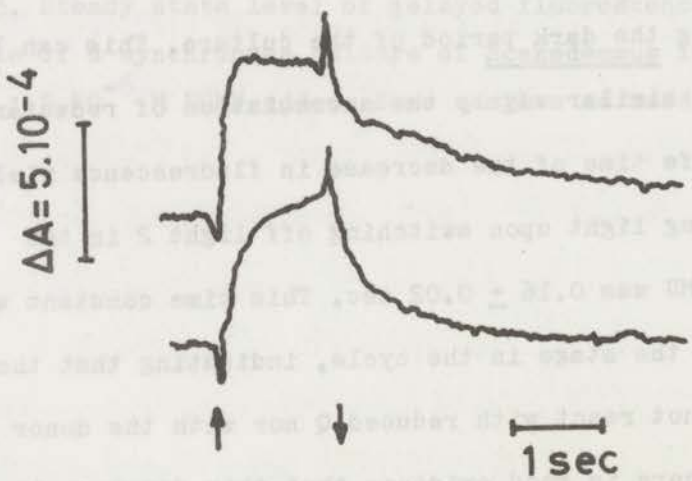


Fig. 20. Absorption changes at 700 nm in an exponentially growing batch culture. Strong actinic light (a broad band between 400 and 500 nm) was switched on and off as indicated by the arrows. The transient upon switching on and off the light is an artefact due to chlorophyll fluorescence. Upper curve: no additions, Lower curve: 10^{-3} M KCN added. 5.10^{-6} M DCMU in all samples.

A separate series of experiments was carried out on batch cultures of Scenedesmus to strengthen the conclusions discussed above. A batch culture was starved for carbondioxide in the light during 2 days to exhaust the internal carbohydrate reserve. The kinetics of P700 oxidation by light are shown in Fig. 19 in a sample from this culture. Generation of reductant by system 2 was blocked by DCMU. Using blue actinic light of an intensity just sufficient to saturate the amplitude of the absoption change at 700 nm, the shape of the induction cuve was almost independent of the dark time between measurements. Only after a dark time of 5 minutes ore more, when also the weak measuring beam was turned off, a lowering of the initial rate of photo-oxidation of P700 was observer during the first actinic illumination. After addition of glucose to this sample, the rate of photooxidation of P700 was retarded, an effect which increased with increasing dark time between the measurements. This effect indicates that reductant formed from glucose reacts with oxidized P700. Glucose itself does not reduce P700, as was checked with photosystem 1 particles from Anabaena. Accumulation of a pool of reductant for P700 was also noted by VREDENBERG and AMESZ[26] and AMESZ[26] in several algae that had not been starved for carbondioxide.

The following experiments were done with exponentially growing non-starved batch cultures of Scenedesmus. KCN, at a concentration of 10^{-3} M completely inhibited respiration, while the quantum yield of oxygen evolution was slightly lowered. In the presence of DCMU the rate of dark reduction of P700 was strongly increased, as shown in Fig. 20. KCN

inhibits terminal respiration, and so reductant normally consumed by oxygen may be expected to accumulate in the presence of KCN. The inhibition of oxygen evolution by KCN may be due to competition of this reductant with system 2.

The experiments discussed in this section show that slow spectroscopic measurements on the photosynthetic mechanism, using low light intensities, do not only detect the electron transport components of photosynthesis. Diffusable redox compounds from the cyctplasm can also be studied with these techniques.

6. DISCUSSION

We have shown that in cultures of Scenedesmus, synchronized by a regime of light and dark periods, at light intensities at or below light saturation of the growth rate, the quantum yield of photosynthesis is essentially independent of the life cycle stage of the cells. Our conclusion does not agree with the conclusion of SENGER and BISHOP[26] and of several other workers, using different organisms[3,4]. Aconstant quantum yield during the life cycle of Porphyridium sp. was observed by GENSE et al.[27]. In the above mentionned investigations where a periodicity in quantum yield was found this normally consisted in a lowering of the efficiency of photosynthesis at the end of the light period of the culture cycle, and a gradual increase in the beginning of the light period. Also the maximum rate of photosynthesis varied in a similar manner. These changes in the quantum yield were observed by us only if the cultures were grown at light intensities higher than necessary to saturate the growth rate. Under such conditions other processes than light absorption may limit the growth

rate, and the average rate of carbondioxide fixation would be expected to become independent of the light intensity in the culture. We now assume that these variations in the quantum yield and maximum rate of photosynthesis are caused by an adaptation process to high light intensity. This assumption implies that this adaptation process does depend on the life cycle stage of the cells. Otherwise a constant quantum yield would be observed which would become lower upon increase of the light intensity. This hypothesis is also consistent with the observations by SOROKIN[28]. In this investigation cells corresponding to different stages in the life cycle were separated by differential centrifugation form a culture grown under continuous illumination. These cells showed similar variation in quantum yield as did cells from a culture synchronized by a light-dark regime. We can be sure that the cultures used by SENGER[3] were grown far above light saturation, since he observed that halving the light intensity did not reduce the average growth rate. Even at the maximum intensities used in our work this halving of the light intensity would have significantly lowered the average number of daughter cells, and thus the growth rate of the culture. In the cultures grown above light saturation SENGER and BISHOP[26] found that the quantum yield of photoreduction with hydrogen as electron donor was constant during the life cycle. This reaction is driven by system 1 only. The Hill reaction with p.benzoquinone, which is driven by system 2 only, showed similar variations in quantum yield during the cycle as oxygen evolution did.[29] So most probably the quantum yield of photosystem 2 is lowered

in Scenedesmus if the light intensity is higher than that required for the maximum growth rate.

The mechanism by which this decrease of the efficiency of system 2 happens is not known. A possible mechanism would be photodestruction of part of the reaction centers of system 2. This would also account for the lowering of the maximum rate of photosynthesis, and is consistent with the measurements of the Hill reaction. Another interpretation could be based on the hypothesis discussed before, that cell contents become progressively reduced if more carbohydrate reserves are accumulated in the cells. This reduction could explain an apparent lowering of the quantum yield of photosynthesis in two ways. Firstly a sufficient amount of reductant, reacting with oxidized P700, could be present to account for a lowering of the quantum yield of oxygen evolution in a similar way as in our proposed mechanism for the induction process in oxygen evolution that was discussed before. Secondly cell contents could become sufficiently reduced to keep part of the reaction centers of system 2 in the reduced state. This reaction can occur under anaerobic conditions[30].

ACKNOWLEDGEMENTS

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Thanks are due to Mr. P. J. M. Vreeburg for his help in the desing and for actually making the culture aparatusses, to Mr. J. B. Ruben who designed the optical control unit, and to Miss W. van der Plas for technical assistence.

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REFERENCES

1. PADILLA, G. M. and I. L. CAMERON (Eds) The Cell Cycle Academic Press, London 1969.
2. LORENZEN, H. in: P. HALLDAL (Ed.) Photobiology of Microorganisms. Wiley-Interscience, London 1970 pp. 187-213.
3. SENGER, H. Planta 90 (1970) 243-266.
4. WANG, T. Thesis, University of Rochester, New York 1969.
5. JAMES, T. W. In: E. ZEUTHEN (Ed.) Synchrony in Cell Division and Growth. Interscience, New York 1964 pp.323-349.
6. HOOGENHOUT, H. and J. AMESZ, Arch. f. Mikrobiol. 50 (1965) 10-24.
7. MACKINNEY, G. J. Biol. Chem. 140 (1941) 315-322.
8. FRIEDMANN, I. Phycologia 6 (1966) 29-37.
9. AMESZ, J. Thesis, University of Leiden 1964.
10. MYERS, J.,J. N. PHILLIPS and J. R. GRAHAM, Plant Physiol. 26 (1951) 539-548.
11. GERHARDT, P. and J. S. SCHULTZ, J. Ferm. Technol., 44 (1966) 349-356.
12. WANKA, F., Arch. Microbiol. 34 (1959) 161-188.
13. DUYSENS, L. N. M., Biochim. Biophys. Acta, 19 (1956) 1-12.
14. CHDAT, Rev. Hydrologie, 3 (1926)
15. UHERKOVICH, G. Die Scenedesmus Arten Ungarns. Akad. Kindo, Budapest 1966.
16. HEALEY, F. P., Plant Physiol. 45 (1969) 153-159.
17. LEX, M. and W. D. P. STEWART, Biochim. Biophys. Acta 292 (1973) 436-443.
18. GORKOM, H. J. van, and M. DONZE, Photochem. Photobiol. in the press.
19. DUYSENS, L. N. M. and H. E. SWEERS, in S. MIYACHI (Ed.) Studies on Microalgae and Photosynthetic Bacteria, Special Issue of Plant Cell Physiol, Tokyo 1963, p.353-372.
20. AMESZ, J. and J. W. M. VISSER, Biochim. Biophys. Acta, 234 (1971) 62-69.

21. BENNOUN, P., Biochim. Biophys. Acta 216 (1970) 357.

22. GOEDHEER, J. C., Biochim. Biophys. Acta 64 (1962) 294.

23. DONZE, M., J. HAVEMAN and P. SCHIERECK, Biochim. Biophys. Acta 256 (1972) 157-161.

24. BARBER, J. and G. P. B. KRAAN, Biochim. Biophys. Acta 197 (1970) 49-59.

25. BENNOUN, P., Biochim. Biophys. Acta 216 (1970) 357.

26. SENGER, H. and N. I. BISHOP, Nature 214 (1967) 140-142.

27. GENSE, M. T., E. GUERIN-DUMARTRET , J. C. LECLERC and S. MIHARA, Phycologia 8 (1969) 135-141.

28. SOROKIN, C., Biochim. Biophys. Acta 94 (1965) 42-52,

29. SENGER, H., Planta 92 (1970) 327-346.

30. LAVOREL, J. in: H. Metzner (Ed.) Progress in Photosynthesis Research, Tübingen 1969,pp 883-898.

Progress in Photosynthesis Research, Vol. II, 991–995 (1969)

Forms of Reaction Center II as Deduced from Low Temperature Fluorescence Changes

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Upon cooling to 77°K the fluorescence yield of chlorophyll in intact bluegreen algae increases about tenfold. Also the form of the emission spectrum changes. At room temperature it shows one major band at 685 nm; at low temperature three bands or humps are visible, generally called F 685, F 696 and F 720. It will be assumed that these bands correspond to three different types of chlorophyll a, which are also present but not manifest at room temperature.

From excitation spectra of chloroplasts and algae and from the emission spectra of chloroplast particles it was concluded that the two short wavelength emission peaks arise mainly from System II chlorophyll and that the long wavelength band F 720 belongs to System I (for a review see GOVINDJEE et al. [1]). VREDENBERG and SLOOTEN [2] concluded that System II also contributes to F 720, as follows also from our experiments.

At room temperature the cells show fluorescence changes, generally ascribed to the variable redox state of the reaction center Q of System II. From experiments on the kinetics of these fluorescence changes DUYSENS and SWEERS [3] concluded that, in addition to the non-quenching reduced state QH and the oxidized quenching state Q, there exists a second quenching state Q′ of this reaction center. DUYSENS and TALENS [4] concluded that Q′ is slowly formed in a dark reaction from the state QH or Q and slowly removed by a reaction driven by System I. After a period of darkness and especially after prolonged illumination with weak System II light, Q′ predominates; in increasing intensities of System I light the steady-state shows an increasing concentration of Q. In both cases the fluorescence yield is low. A high fluorescence yield is only obtained as a transitory phenomenon after switching on System II light in a state with a high concentration of Q. Poisoning with DCMU blocks the oxidation of QH by System I, but does not affect the reactions of Q′.

After freezing to 77°K in the dark the system still shows a light-induced fluorescence rise [5]. This paper contains a more detailed study of this reaction in the blue-green alga *Schizothrix calcicola*. In this species the reaction at 77°K is irreversible, and of approximately first order kinetics.

Methods

The sample is contained in a 1 mm perspex cuvette. It can be preilluminated with light of different wavelengths and intensities. After 3 minutes it is cooled in the dark by moving it into a brass cuvette holder cooled by liquid nitrogen. For details on the fluorescence measuring apparatus see DUYSENS and TALENS [4]. Essentially, the relative fluorescence yield is recorded as a function of time. Fluorescence is isolated with Schott AL interference filters having their maximum transmission at 683, 696 and 727 nm.

Results and Conclusions

1. *F 685 and F 696*

The initial and final fluorescence yields f_o and $f\infty$ and thus the total change in fluorescence yield $f\infty - f_o = \Delta f$ appear to depend on preillumination (see fig. 1). This makes it possible in principle to correlate measurements at low temperature with the properties of the photosynthetic system at normal temperature. At all three wavelengths of measurement, with green and with blue excitation, f_o and Δf are lowest after preillumination with very weak System II light. Both f_o and Δf increase with increasing intensities of System I light as pretreatment. The relative reaction rate, defined as the initial rate divided by Δf, is for green exciting light the same within 5% in the three emission bands, indicating that only one reaction occurs. The measurements for blue exciting light were less precise.

If we assume that the increase in fluorescence yield at 77° K is due to the reduction of Q to QH, and that for $f\infty$ all Q is converted into QH, it follows from the dependence of $f\infty$ on preillumination that in addition to Q a second quenching form exists, which does not react at low temperature. Our data are consistent with the assumption that this is the Q′ state of the reaction center of System II.

Fig. 1 suggests that after different preilluminations we find a unique and linear relationship between f_o and Δf for the emission at 696 nm. This was confirmed in a number of other experiments, and – in a smaller number of experiments – for the emission at 683 nm. Such a relationship can be explained as follows: We may write for f_o after a certain pretreatment

$$f_o = f_c + K_1 [Q'_1] + K_2 [Q_1] + K_3 [QH_1]$$

where $[Q_1']$, $[Q_1]$ and $[QH_1]$ are the initial concentrations of the different states of the reaction centers, f_c is a constant background fluorescence and the K's are the relative fluorescence yields per unit concentration of the different states of the reaction center. Further we assume that

$$[Q_1'] + [Q_1] + [QH_1] = [Q_o].$$

Q_0 is total concentration of the reaction center II. The assumptions that for $f\infty$ all Q present is converted into QH and that at low temperature Q′ is not converted by light give

$$\Delta f = (K_3 - K_2)\,[Q_1].$$

To obtain a unique and linear dependence of Δf on f_0 one more linear relation between [Q′], [Q] and [QH] must hold in these experiments. Such a linear relation would obtain if a relatively rapid chemical equilibrium was established in the time between preillumination and the moment in which a sufficient low temperature is reached to stop this reaction. Since we know that the reactions in which Q′ takes part are too slow for this, this might be an equilibrium reaction of Q, QH and a relatively large pool AH and A of approximately constant oxidation-reduction level. The simplest assumption is the special case that $[QH_1] = 0$. Then it follows from the dependence of f_0 on preillumination that Q′ is a more effective quencher than Q is.

2. *F 720*

The F 720 band behaves different from the other two bands. Here f_0 is almost independent of preillumination, while Δf shows a strong dependence (see fig. 1). The relative change $\Delta f/f_0$ in fluorescence yield at 727 nm is smaller in blue excitation light than in

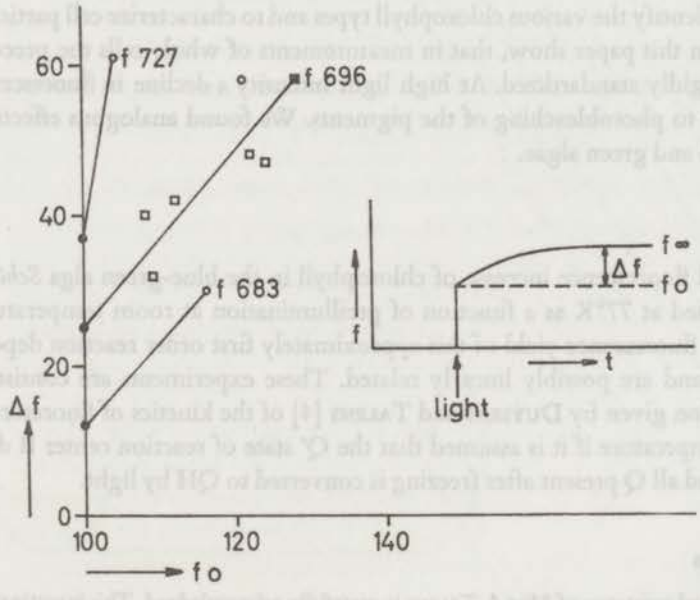

Fig. 1: Fluorescence changes of *Schizothrix calcicola* at 77°K as function of preillumination at room temperature. The sample is excited by a broad green band, fluorescence is isolated with interference filters. The amplitude of the change in fluorescence yield, Δf, is plotted against the initial relative fluorescence yield f_0. The initial fluorescence yield after a pretreatment of 3 min darkness is set at 100 for each emission wavelength.
● = 3 min darkness;
○ = 3 min strong blue preillumination;
□ = intermediate intensities of blue and green light;
■ = strong far-red preillumination.

green excitation light. It is concluded that the variable fluorescence at 727 nm is excited by or *via* the System II pigment complex. This is consistent with the result that the relative initial reaction rates are about the same at all three wavelengths. So we have to assume that part of the 727 fluorescence arises in System II chlorophyll, or that the fluorescence *changes* at this wavelength are due to spill-over from System II to System I chlorophyll. However, the smallness of the variation in f_0 at 727 nm requires an additional explanation. Such an explanation might be that after preillumination, even with green light, an appreciable fraction of reaction center II remains in the state Q and that this fraction and not Q′ occurs in the neighbourhood of the pigment emitting at 727 nm. The long wavelength emission then is relatively more influenced by variations in [Q] and [QH] than in [Q′].

3. *Effect of DCMU*

Addition of DCMU before freezing increases f_0 and $f\infty$ by only about 20%, without a significant effect on the preillumination effect and the relative rate and amplitude of the low temperature reaction. This is also consistent with the interpretation of room temperature experiments [4].

4. *Emission spectra*

Fluorescence emission spectra (of $f\infty$) at liquid nitrogen temperature are commonly used in attempts to identify the various chlorophyll types and to characterize cell particles. The effects studied in this paper show, that in measurements of whole cells the preconditions should be rigidly standardized. At high light intensity a decline in fluorescence occurs at 77°K, due to photobleaching of the pigments. We found analogous effects in several species of red and green algae.

Summary

The light-induced fluorescence increase of chlorophyll in the blue-green alga *Schizothrix calcicola* is studied at 77°K as a function of preillumination at room temperature. The initial and final fluorescence yield of this approximately first order reaction depend on preillumination and are possibly linearly related. These experiments are consistent with the interpretation given by Duysens and Talens [4] of the kinetics of fluorescence changes at room temperature if it is assumed that the Q′ state of reaction center II does not react at 77°K, and all Q present after freezing is converted to QH by light.

Acknowledgments

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References

[1] GOVINDJEE, G. PAPAGEORGIOU, and E. RABINOWITCH in: G. G. GUILBAULT (Ed.), "Fluorescence; Theory, Instrumentation, and Practice", pp. 511–565 (1967).

[2] VREDENBERG, W. J., and L. SLOOTEN: Biochim. Biophys. Acta **143**, 583–594 (1967).

[3] DUYSENS, L. N. M., and H. E. SWEERS in: "Microalgae and Photosynthetic Bacteria", pp. 353–372, Special Issue of Plant and Cell Physiol., Tokyo 1963.

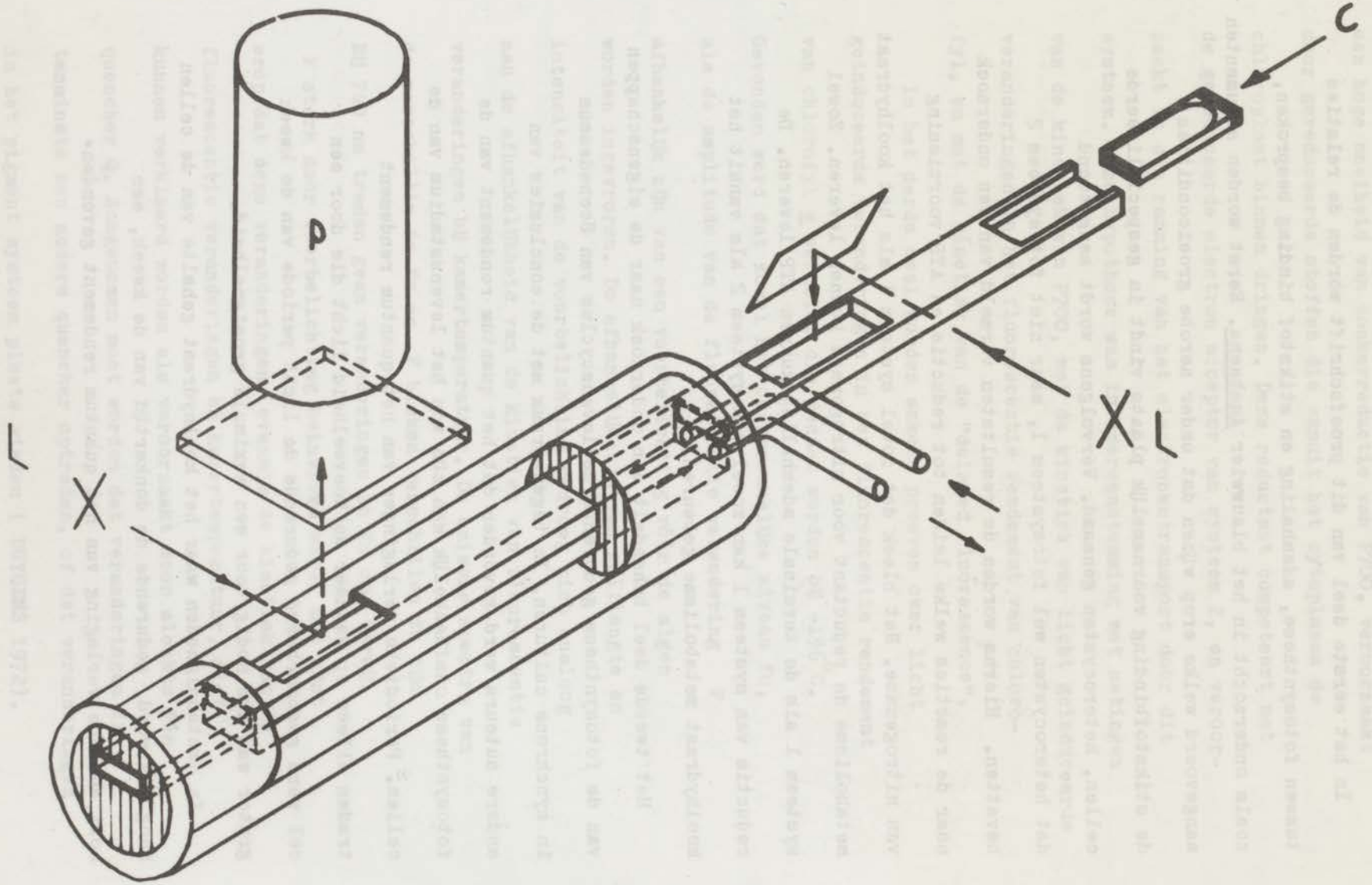
[4] DUYSENS, L. N. M., and A. TALENS in H. METZNER (Ed.): "Progress in Photosynthesis Research", Vol. II, 1073–1081, Tübingen 1969.

[5] KOK, B. in: "Photosynthetic Mechanisms of Green Plants", Natl. Acad. Sci. – Natl. Res. Council, No. 1145, pp. 45–54, Washington, D. C., 1953.

A SAMPLE HOLDER FOR MEASUREMENTS OF FLUORESCENCE AT THE TEMPERATURE OF LIQUID NITROGEN.

A sample holder for measurements of fluorescence at liquid nitrogen temperature was designed (p. 83). It allows a rapid change of samples. This was especially convenient in studies of light induced changes in the fluorescence yield of chlorophyll in photosynthetic organisms, since these reactions were irreversible.

The sample holder consists essentially of a tunnel. Perspex cuvettes can be pushed forward through this tunnel. The tunnel consists of two parts, milled out of brass. The first part of the tunnel is kept at room temperature. It contains a window which, by means of a mirror and suitable optics, permits a preillumination of samples before they are frozen. The second part of the tunnel is kept at liquid nitrogen temperature, by flowing the liquid through a channel which was milled below the tunnel. The cold part is isolated by means of a dewar vessel, open at both ends. This part contains three cuvettes. The last cuvette lies beneath a window, the fluorescence measurements are made on this sample. After the measurements a new sample can be pushed into the tunnel, which will push forward all cuvettes one place. So this arrangement makes it possible to do an experiment on one sample, while at the same time two other samples are being cooled in the dark part of the cold tunnel and a fourth sample is being preilluminated at room temperature.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XL

SAMENVATTING

In het eerste deel van dit proefschrift worden de relaties tussen fotosynthese, ademhaling en stikstof binding besproken, zoals onderzocht in het blauwwier Anabaena. Eerst worden argumenten aangevoerd welke erop wijzen dat onder aerobe groeicondities de stikstofbinding voornamelijk plaats vindt in gespecializeerde cellen, heterocysten genaamd. Vervolgens wordt aangetoond dat heterocysten wel fotosysteem 1, maar niet fotosysteem 2 bevatten. Hierna worden de resultaten vermeld van een onderzoek naar de reacties welke leiden tot reductie en ATP voorziening van nitrogenase. Het bleek dat zowel systeem 1 als het koolhydraat metabolisme de reductant voor nitrogenase kunnen leveren. Zowel systeem 1 als de terminale ademhaling kunnen ATP leveren. De reductie van systeem 1 kan zowel via systeem 2 als vanuit het koolhydraat metabolisme gebeuren.

Het tweede deel behandelt een onderzoek naar de eigenschappen van de fotosynthese gedurende de levenscyclus van Scenedesmus in synchrone culturen. In tegenspraak met de conclusies van andere auteurs werd gevonden dat het quantum rendement van de fotosynthese onafhankelijk kan zijn van het levensstadium van de cellen. Periodieke verlagingen van het quantum rendement traden alleen op wanneer de hoeveelheid licht die door een cel werd geabsorbeerd gedurende de licht periode van de kweek groter was dan nodig voor een maximale groeisnelheid.

In omstandigheden waar het koolhydraat gehalte van de cellen hoog was werd, gedurende de donkertijd van de kweek, een kort durende verlaging van het quantum rendement gevonden.

Dit inductie proces kan worden verklaard als het gevolg van een hoge snelheid van donkerreductie van P700, veroorzaakt door gereduceerde stoffen die vanuit het cytoplasma de chloroplast binnen dringen. Deze reductant competeert met de gereduceerde electron acceptor van systeem 2, en veroorzaakt zo een remming van het electronentransport door dit systeem. Deze hypothese was in overeenstemming met metingen van de kinetiek van P700, met de kinetiek van licht geinduceerde veranderingen in het fluorescentie rendement van chlorofyl, en met de kinetiek van de "delayed fluorescence".

In het derde deel worden enkele proeven over licht geinduceerde veranderingen in het fluorescentie rendement van chlorofyl a vermeld, die gedaan werden bij -196°C. Gevonden werd dat zowel het oorspronkelijke niveau Fo, als de amplitude van de fluorescentie verandering F afhankelijk zijn van een voorbelichting vóór de algen worden ingevroren. De afhankelijkheid van golflengte en intensiteit van de voorbelichting gedroeg zich analoog aan de afhankelijkheid van de kinetiek van fluorescentie veranderingen bij kamertemperatuur. De emissie spectra van de verschillin in Fo en F bleken verschillen te zijn. Bij 740 nm traden geen veranderingen in Fo op, terwijl F sterk door voorbelichting beinvloed werd. Dit wijst erop dat deze veranderingen, evenals de kinetiek van de fluorescentie veranderingen bij kamertemperatuur, niet kunnen verklaard worden als veroorzaakt door slechts één quencher Q. Aangenomen moet worden dat veranderingen in tenminste een andere quencher optreden, of dat veranderingen in het pigment systeem plaats vinden (DUYSENS 1972).

DUYSENS, L. N. M., Biophysical J., 12 (1972) 858-863.

CURRICULUM VITAE

Na het behalen van het einddiploma HBS - B aan de Rijks HBS te Vlissingen begon ik in september 1960 met de studie aan de Rijksuniversiteit te Leiden. Het candidaatsexamen in de natuurkunde met bijvakken wiskunde en scheikunde werd in 1964 afgelegd. De studie werd daarna voortgezet aan het Rijksherbarium waar, in het kader van het Leidse onderzoek aan de Ria de Arosa, onder leiding van dr. C. van den Hoek de vegetaties van zeewieren in deze baai in NW-Spanje werden onderzocht. Het tweede onderwerp voor mijn doctoraal studie verrichtte ik op het Biofysisch laboratorium, waar onder leiding van Prof. Dr. L.N.M. Duysens fluorescentie veranderingen van chlorofyl bij lage temperaturen werden onderzocht. Tevens werd in die tijd een begin gemaakt met het onderzoek naar de groei van fotosynthetiserende micro-organismen. Het doctoraal examen in de biologie werd cum laude op 13 december 1966 afgelegd.

Hierna begon ik met het onderzoek, zoals vermeld in dit proefschrift.

De volgende betrekkingen werden door mij bekleed:

Candidaats-assistent namens Z.W.O. bij het onderzoek aan de Ria de Arosa onder leiding van Prof. Dr. A.J. Pannekoek van 1 jan. - 30 juni 1965 en van 1 jan. - 30 juni 1966.

Candidaats-assistent namens Z.W.O. sinds 1 juni 1966; na 13 december 1967 wetenschappelijk medewerker aan het Biofysisch Laboratorium tot 15 juli 1967. Op dezelfde datum trad ik in dienst van de Rijksuniversiteit Leiden als wetenschappelijk medewerker aan hetzelfde instituut.