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Energetic and Metabolic Requirements for the Germination of Akinetes of the Cyanobacterium *Nostoc* PCC 7524

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Abstract. Although akinetes of *Nostoc* PCC 7524 lost little of their main photosynthetic pigments, phycocyanin and chlorophyll, with increasing age after the onset of sporulation, they lost at least 90% of their photosynthetic and respiratory capacities. Germination needed the supply of light throughout the process, though previous dark metabolism accelerated the following light process. In standard conditions, both respiratory and photosynthetic capacities increased markedly during the first 9–10 h, a time sufficient for the first doublets to appear, but when pigment contents had not yet changed. However, while respiratory capacity could be reacquired without de novo metabolism, resumption of photosynthetic capacity needed RNA and protein synthesis. The energetic requirement for germination was not efficiently fulfilled by cyclic photosynthesis on PSI alone or respiration alone. In the presence of both PSI and respiratory activities only 21% of the akinetes germinated, their endogenous carbon reserves thus being inadequate to support the process to completion. The addition of sucrose to such cultures permitted all of the akinetes to germinate, but at a very slow rate. Rapid and complete germination was only observed when both photosystems operated.

Key words: Cyanobacteria – Akinete – Germination – Respiration – Photosynthesis

Cyanobacterial akinetes, since they show little metabolic activity, resistance to several external factors, and have undergone morphological and macromolecular changes (Nichols and Carr 1978), are considered as the conservation state of certain species, through analogy with bacterial spores. They usually contain high amounts of cyanophycin (Miller and Lang 1968; Sutherland et al. 1979) and glycogen (Sutherland et al. 1979), respectively nitrogen and carbon reserve products, and have decreased their pigment contents (Fay 1969 a; Yamamoto 1972), CO_2 fixing capacity (Fay 1969 b) and photosynthetic activity (Fay 1969 b; Yamamoto 1976).

Akinetes, formed under unfavourable growth conditions, germinate and differentiate to the vegetative form when transferred to appropriate growth conditions. Though light always seems to be necessary for germination (Kaushik and Kumar 1970; Reddy et al. 1975; Yamamoto 1976; Braune 1979), contradictory arguments have been presented as to whether its action is through photosynthesis or another phytochromic process. (a) The most efficient activating light is in the red region of the spectrum (600–660 nm) (Reddy et al. 1975; Yamamoto 1976; Braune 1979), mostly absorbed by phycocyanin, the main photosynthesis collecting pigment to PSII centers. However, the akinete phycocyanin content has been described as being either reduced or the same as that of the vegetative cells. (b) Probable differences in the gas phase employed have led to contradictory results concerning the effect of the herbicide diuron (DCMU), an inhibitor to PSII and thus to CO_2 fixation, on germination. Though active on PSII, this inhibitor blocked either completely (Yamamoto 1976) or only slightly (Braune 1979) germination. An interpretation of this contradiction, which may be related to the need for oxygen also mentioned (Yamamoto 1976), could reside in the possibility for the akinetes to balance their pathways of energy production and carbon assimilation depending on available sources: reduction of CO_2 through non-cyclic phosphorylations on PSII + PSI in the presence of light; degradation and oxidation of glycogen or any available carbon source through cyclic phosphorylations on PSI alone in the presence of light and/or through respiration in the presence of O_2.

The work presented here aims at characterizing the development of photosynthetic and respiratory activities during germination of *Nostoc* PCC 7524 akinetes, and the need for these processes during this differentiation. The advantage of *Nostoc* 7524 is that it is very easy to obtain large amounts of pure akinete suspensions, which will germinate with excellent synchrony in 10 h (Chauvat and Joset-Espardellier 1981). This biological material also constitutes a convenient and original system to study the genesis and organization of photosynthetic membranes and activity. Most organisms, bleached or in resting conditions, lose an important proportion of their pigments in parallel to their photosynthetic capacity (Dubertret and Joliot 1974; Cahen et al. 1976; Akoyunoglou 1977; Evans et al. 1978; Grimme 1978; Diner and Wollman 1979; Dubertret 1981 b). In *Nostoc* 7524, modifications of the contents in the various pigments occur, depending on the age and conditions of obtention of the akinetes (Sutherland et al. 1979; Chauvat and Joset-Espardellier 1981), though nothing has been described concerning their photosynthetic activities.

In most cases, two phases can be determined during the greening process of etiolated or resting plant or algal cells. The first consists of reorganization of preexisting thylakoid constituents, including pigments, leading to active photo-

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Abbreviations: Chl, Chlorophyll; Phy, phycocyanin; DCMU, 3-(3,4-chlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; DPIP, 2,6-dichlorophenylindophenol

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synthetic units (Dubertret and Joliot 1974; Cahen et al. 1976; Evans et al. 1978). Active neosynthesis occurs only during the second phase. Light has been shown to be always necessary for chlorophyll synthesis (Ohad et al. 1978; Evans et al. 1978; Dubertret 1981 a), and may be required as an inducer for synthesis of other photosynthetic membrane constituents (Wild 1978; Dubertret 1981 b). The sequence of synthesis and integration into the membranes of the electron carriers and of the pigments varies with the organisms (Herron and Mauzerall 1972; Dubertret and Joliot 1974; Cahen et al. 1976; Akoyunoglou 1977; Wild 1978; Diner and Wollman 1979; Dubertret 1981 b).

Material and Methods

Strain Nostoc PCC 7524, (ATCC 29411), came from Dr. R. Y. Stanier's collection, at the Pasteur Institute, Paris (Rippka et al. 1979).

Growth Conditions. The growth medium was that of Herdman et al. (1973) modified by Astier (1976).

Vegetative growth from akinetes was obtained by diluting an akinete suspension at least 5-fold in new medium, and incubating at 34 °C, 3,700 lx, under a CO_2 enriched atmosphere in a rotatory incubator (standard conditions). Vegetative filaments grew to a cell concentration of 10^8/ml with a generation time of 7–8 h, and then started to differentiate akinetes.

Akinete suspensions were prepared from the above cultures after approximately 17 days of incubation, a duration sufficient to yield pure suspensions of isolated akinetes. These suspensions, which appeared brownish in color, were kept at room light and temperature conditions, under which they continued to differentiate slowly (modification of pigment contents, increase in size and germination lag) until they died, several months later. In the experiments described, they were never kept for more than 4 weeks. No differences were observed in the parameters measured during this time.

Germination kinetics were followed under a light microscope. The criterion for germination was the appearance of doublets, the first visible step towards cell division. Differential numerations were made on 5 to 10 microscope fields, amounting to about 10^3 total cells for each sample, to calculate the percent of germinating akinetes. Fluctuations were always lower than 5%.

Pigment contents were measured on whole cells, from absorption spectra performed on a Cary 14 spectrophotometer, and using the following formulae (Der Vartanian et al. 1981):

$$\text{Chl (mg/ml)} = 1.488 \times 10^{-2}\ A_{678} - 5.03 \times 10^{-4}\ A_{622}$$

$$\text{Phy (mg/ml)} = 0.170\ A_{622} - 3.69 \times 10^{-2}\ A_{678}.$$

Oxygen Exchanges. Respiratory and photosynthetic capacities were measured on whole cells, in a polarographic Clark electrode. A cell suspension (3 ml) containing $1-5 \times 10^7$ cells/ml was placed in the cuvette, and maintained at 34 °C. Respiration was measured as O_2 consumption in the dark, and photosynthesis as O_2 evolution (corrected for O_2 consumption) under saturating white light. The figures given represent either one typical experiment (in the figures) or 5 to 10 repeats of similar experiments (in the tables), each sample being measured once or twice only, since reproducibility was excellent.

PSI and PSII activities were measured on partially purified thylakoids. Cell suspensions, at concentrations no lower than 10^8 cells/ml, were broken through one passage in a French Press (91 kg/cm^2). After elimination of the unbroken cells (less than 5%) by centrifugation at 500 g for 5 min, the membranes were sedimented at 17,000 g for 1 h, at 0 °C, and resuspended in 10 mM Tricine buffer, pH 7.8, containing 10 mM NaCl, 10 mM $CaCl_2$, and 0.4 M sorbitol. PSI activity was measured by the Mehler reaction, on extracts containing 10–50 µg Chl/ml, in the following conditions: NH_4Cl 4 mM; NaN_3 0.1 mM; DCMU 0.01 mM, Na ascorbate 2 mM; DCPIP 20 mM; methylviologen 0.1 mM. Measures were performed at 34 °C, under saturating white light.

PSII activity was determined by the Hill reaction. Extracts containing 3–5 µg Chl/ml were mixed with 0.5 mM DPC and 0.2 mM DPIP, illuminated by saturating white light at 34 °C. Absorption changes were followed at 580 nm.

For each activity, 3 independent measures were performed for each sample.

Results

1. Kinetics of Germination and Outgrowth. When akinete suspensions (10^8 cells/ml) were diluted 5- to 30-fold in fresh mineral medium and incubated under standard conditions, the first doublets appeared after a lag of 7–8 h (Fig. 1). Within the range of dilution indicated above, the proportion of germinated akinetes then increased rapidly, reaching 10% at 10–13 h, and 60% at 15 h; 60% of the population appeared as 4-celled filaments after 25–30 h. The germination kinetics were consistently reproducible, within the limits of variation indicated in results, for all the parameters measured.

As already observed (Chauvat and Joset-Espardellier 1981), a very good synchrony of germination and of at least the first cell division made this material very convenient to study the sequence of events during germination. Since we were interested in the onset of germination, that is the events taking place before the first division, we considered only the period 0–9 h, at the end of which at most 10% of the akinetes were already seen as doublets.

Doublets appeared without increase in optical density at 580 nm. Since akinetes had about 8 times the volume of vegetative cells, the first generation daughter ones were still larger than normal.

2. Photosynthetic Constituents and Capacity in Resting Akinetes. The difference in these characteristics observed between vegetative cells and akinetes depended on the means of collecting and the age of the latter, and on the growth conditions for both types of cells. Very old (2 months or more) akinetes became brown, mainly because of an increase of their carotenoid content, while an increase in incident light during sporulation yielded green akinetes, with pigment contents very near to those of vegetative cells (data not presented). In our standard conditions, the akinetes had lost 10 to 12% of their phycocyanin and chlorophyll contents, compared to the vegetative state (Table 1). The constant ratio of the two main pigments suggested that the antennae to both photosynthetic

Table 1. Comparison of some characteristics of akinetes and vegetative cells. Akinetes were collected as described under material and methods. Vegetative cells were harvested during exponential growth. See methods for conditions of measures

	Akinetes	Vegetative cells
Phycocyanin [a]	4.60 ± 0.8 (7)	5.2 ± 0.3 (7)
Chlorophyll [a]	0.56 ± 0.05 (7)	0.64 ± 0.05 (7)
Respiratory capacity [b]	2.9×10^{-7} $\pm 1 \times 10^{-7}$ (12)	3.7×10^{-6} $\pm 1 \times 10^{-6}$ (3)
Photosynthetic capacity [b]	2.3×10^{-7} $\pm 0.4 \times 10^{-7}$ (12)	6×10^{-5} $\pm 1 \times 10^{-5}$ (3)

[a] in pg/cell; [b] in nmol O_2/min · cell
Figures in parentheses indicate the number of assays performed

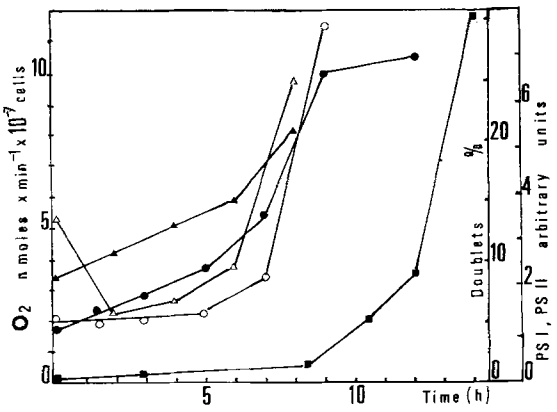

Fig. 1. Kinetics of germination of *Nostoc* PCC 7524 akinetes in standard conditions. Variation as a function of time of the proportion of doublets (in percent of the population, ■), the respiratory capacity (in nmol O_2 consumed/min/10^7 cells, ●), the photosynthetic capacity (in nmol O_2 evolved/min/10^7 cells, ○), PS I activity (Mehler reaction, ▲) and PS II activity (Hill reaction, △) in arbitrary units. See methods for the conditions of measures

reaction centers, PSII and PSI, were modified to similar extents. Both overall photosynthetic and respiratory capacities were decreased by factors larger than 10 (Table 1), compared to vegetative cells.

3. Evolution of Photosynthetic Constituents and Activities During Germination in Standard Conditions. Figure 1 shows a typical experiment, among at least 20 performed (PSI and PSII activities were measured only for two of them). The factors of increase of both photosynthetic and respiratory activities varied 6- (as in the experiment in Fig. 1) to 20-fold during the first 9 to 10 h of germination, thus almost reaching the final values in vegetative cells (Table 1). No significant increase in either phycocyanin or chlorophyll contents occured during this same period. Since no increase in cell mass, measured by turbidity, took place, these modifications could be explained either by a high turn-over (equivalent rates of synthesis and degradation) of, or by a reorganization of preexisting, constituents. The relative constancy in pigment concentrations would favour the second hypothesis, at least as far as photosynthesis is concerned. Average variations of PSI and PSII activities, measured on 2 experiments, indicated that their rates of increase were similar to that of overall photosynthetic activity measured as O_2 evolution (Fig. 1).

Table 2. Influence of the nature of the incubation medium on the efficiency of germination. The akinete suspension (10^8 cells/ml) was centrifuged, resuspended in distilled water at 5×10^8/ml. They were then diluted in the media described to yield a 13-fold dilution of the initial suspension (8×10^6 cells/ml). The supernatant is that obtained after centrifuging the initial akinete suspension

Medium	Germination	Respiratory capacity	Photosynthetic capacity
a Mineral medium	+	100	100
b H_2O	−	100	0
c Mineral medium (4 vol) + H_2O (1 vol)	+	100	100
d Mineral medium (4 vol) + supernatant from an old culture (1 vol)	+	100	100
e Supernatant from an old culture	−	100	60

Germination could not occur unless the akinete suspension was diluted at least 5-fold, and only in fresh growth medium. The increase in light intensity received per cell, resulting from the dilution of the suspension, was not the trigger for germination, since low light intensities (800 lx) promoted germination with the same kinetics as under normal conditions. Addition of new medium was necessary, since dilution by the same factor in distilled water (Table 2, b) or in supernatant from the akinete suspension (e) did not lead to germination. Varying the proportions of supernatant and fresh medium in the incubation flask (Table 2) suggested that no inhibitor of germination was present in the supernatant, since in condition d, its concentration per cell would have been higher than that in the initial (non-diluted) suspension.

Analysis of the activities of the energetic pathways of akinetes incubated under those various conditions (Table 2) indicated a correlation between the evolutions of photosynthetic capacity and germination. The different conditions had no effect on the development of respiratory capacity.

4. Implications of Respiratory and Photosynthetic Activities During Germination. Akinete suspensions placed in the dark, under otherwise standard conditions did not germinate, even after incubation periods up to 165 h. During the first 8 h, a small increase in both photosynthetic and respiratory capacities was observed. This increase then levelled off, having reached about 20 % of the values respectively obtained in the control at the same time (Fig. 2). Light thus appeared to be necessary for germination to go to completion. The question then was whether it was needed as an inducer for an early step of the process, or as an energy source, and then constantly necessary, or played both roles. This was tested by manipulating the light regime of the suspensions.

By submitting the akinetes to pulses of dark periods before illuminating them, a decrease in the lag period before the appearance of doublets was observed (Fig. 2 B). Thus some metabolism, usable for the early steps of the process, could take place in the absence of light. At least part of this metabolism represented the increase in photosynthetic and respiratory capacities described in Fig. 2 A. A limit appeared, though, since the latent period needed to obtain 50 % doublets in the light never decreased to less than 7 − 9 h, compared to about 14 in the control. This limit was reached after 5 − 7 h

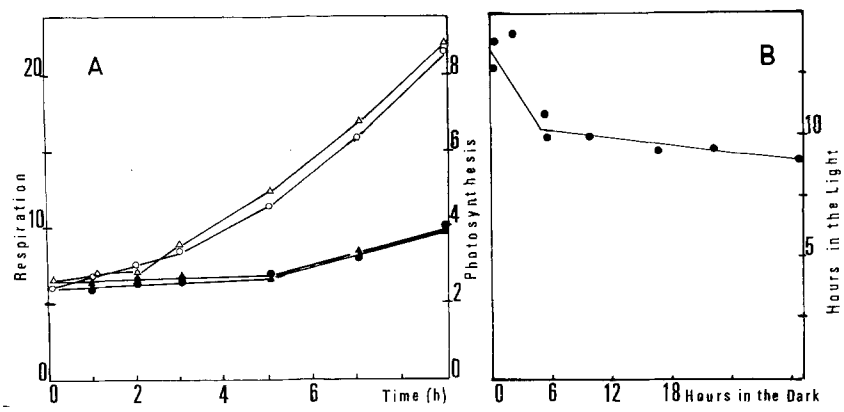

Fig. 2 A, B
Influence of incubation in the dark on efficiency and kinetics of germination. **A** The akinete suspension was either incubated in standard conditions (open symbols) or in the dark (closed symbols). Respiratory (circles) and photosynthetic (triangles) capacities, in nmol O_2 consumed and evolved, respectively/min · 10^7 cells, as a function of time. **B** The akinete suspension was incubated in the dark. After various durations, aliquots were transferred to the light. The appearance of doublets was followed. The time in the light necessary to obtain 50% doublets (ordinate) following dark preincubation (abscissa) was determined, and used as a criterion of germination

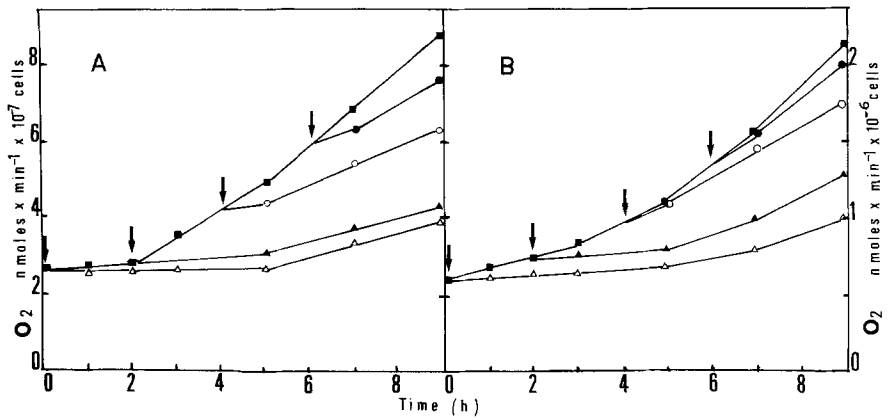

Fig. 3 A, B
Influence of previous light incubation on capacity of dark development of photosynthetic and respiratory activities of akinetes maintained otherwise in standard conditions. Aliquots of the light suspension (■) were transferred to the dark after 0 (△), 2 h (▲), 4 h (○) and 6 h (●). Photosynthetic **A** and respiratory **B** activities were measured as in methods

of dark incubation. The total length of time necessary in these conditions to reach 50% doublets (5–7 dark h + 7–9 light h), was very similar to that of the control, indicating that the dark metabolism was about as efficient as the light dependent one. These results further showed that: 1. the akinetes possessed at least enough endogenous energy to undergo part of their germinating process; 2. if a step inducible by light existed, it was not required, or needed not take place, before the 5th–7th h of differentiation.

The possible alternative roles of light were tested by allowing the akinetes to start germination in the presence of light, and then transferring them to the dark. The increases in photosynthetic and respiratory capacities were slowed down to similar extents after the transfer (Fig. 3). The average rate of residual development in the dark, measured until the 9th h of incubation, was grossly proportional to the duration of the preincubation in the light, as if the akinetes had accumulated energy and/or precursors of the photosynthetic and respiratory membranes during this period. Thus, at least until the 9th h, no light inducible step was necessary. Since about 10% of the cells had formed doublets by then, there probably existed no light inducible step during the whole process of germination. Thus light would only be necessary as an energy source.

The requirement for respiration during germination could not be assessed, since the two means of blocking this activity, known to be selective inhibitors in most organisms, i.e. dinitrophenol and KCN, proved here to be toxic to photosynthesis at concentrations which prevented very slightly the development of respiratory activity. No germination occured in these conditions, as could be expected from the previous results (Fig. 2 and Table 2).

Table 3. The influence of illumination and gas phase on germination

Culture conditions	Akinetes (%) germinated at			
	22 h	46 h	120 h	192 h
Light				
N_2/CO_2	100			
Air/CO_2	100			
$DCMU/N_2/CO_2$	0	4.3	4.0	4.0
$DCMU/N_2/CO_2$ + sucrose	0	4.0	4.5	4.1
$DCMU/air/CO_2$	0	20.7	21.3	21.0
$DCMU/air/CO_2$ + sucrose	8.6	28.5	76.0	100
Dark				
Air/CO_2	0	0	1.3	1.1
Air/CO_2 + sucrose	0	0	1.3	1.0

However, respiration alone did not permit germination: in the dark, under an atmosphere of air: CO_2 (95/5, v/v), akinete germination was not observed even after 192 h (Table 3) and was not stimulated by the addition of sucrose (0.5%, w/v), a utilisable carbon source for photoheterotrophic growth (Sutherland et al. 1979).

PSI activity alone did not support rapid germination (Table 3). In the presence of DCMU (10^{-5} M) and a gas phase of air: CO_2, no doublets were observed at 22 h, when all of the akinetes had germinated under normal conditions; 21% formed doublets by 46 h, but no further growth or germination occurred. When air was replaced by N_2, very few akinetes germinated (Table 3). The addition of sucrose permitted germination to occur at a slow rate in the presence,

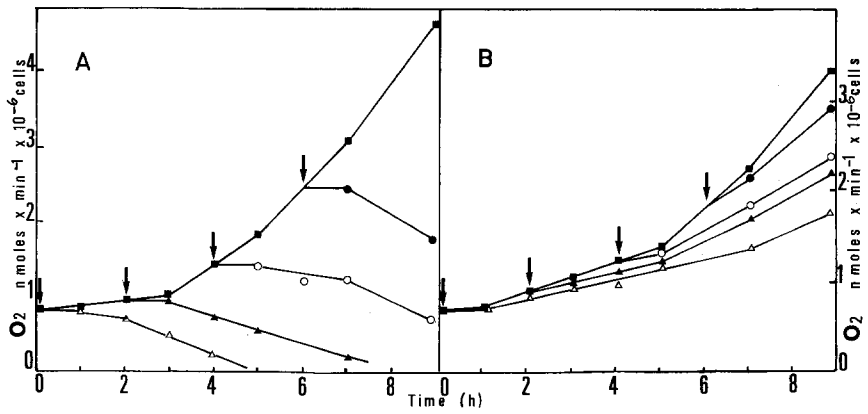

Fig. 4 A, B
Effect of the presence of Erythromycin on the development of photosynthetic **A** and respiratory **B** capacities of akinetes germinating in standard conditions. Erythromycin (1 µg/ml) was added at time 0 (△), or after 2 h (▲), 4 h (○) and 6 h (●) of incubation, in samples withdrawn from the control culture (■)

but not in the absence, of air (Table 3); after 192 h all of the akinetes had given rise to short filaments of mean length 3.3 cells. This rate of growth was similar to that of vegetative cells under the same conditions.

5. Need for de novo Synthesis During Germination. The 20% increase in photosynthetic and respiratory capacities occuring in the absence of light (Fig. 2 A), that is without an exogenous source of energy, may result from either the building up of efficient structures from preexisting proteic precursors, or the transcription of preexisting mRNAs, or may require de novo transcription and translation. Treatment of the akinetes, from time 0 of incubation, with either erythromycin (1 µg/ml) or rifampicin (10 µg/ml), at concentrations which prevented growth of the vegetative cells but were not bacteriocidal in the short exposures employed, resulted in complete inhibition of germination. The two antibiotics produced very similar degrees of inhibition. Development of respiratory and photosynthetic activities, once more, evolved differently. The inhibition of development of the respiratory capacity was very limited, after either treatment: 60% ± 10% of the oxygen consumption capacity of the control were observed in the treated suspension after 7 h (Fig. 4 B). The difference in rates increased afterwards, since only 40% appeared after 15 h. Thus, though limited, this development indicated that a fairly large amount of direct proteic precursors of the respiratory chain were present in the cells, and probably needed only to be integrated into efficient structures. This process would require little energy.

An opposite behavior was observed when photosynthetic activities were measured: treatment with either antibiotic immediately prevented any increase in O_2 emission capacity, and even ended in a loss of activity (5% left after 8 h, Fig. 4 A). This can be interpreted as the absence of a pool of at least some of the constituents of the thylakoid membranes in the akinetes, even at the mRNA level. The loss of activity might indicate a disintegration of the remaining active membranes, the cause of which could be a phototoxic effect or an unbalanced integration of some constituents into the preexisting membranes.

When the antibiotics were added after various periods of incubation in standard light conditions (Fig. 4 A and B), effects similar to those described above were observed: a partial inhibition of respiration development and a loss of photosynthetic activity. Both processes showed constant and similar evolutions with time, no matter when the antibiotics were added: 3 h of treatment limited the increase in respiratory activity to 60% of the control; 60% of the initial photosynthetic activity were lost after 4 h of treatment. Once more, an absence of correlation was observed between the development of respiratory capacity and that of germination and photosynthetic activity. Both RNA and protein syntheses appeared to be continuously needed to allow germination to proceed to completion.

Discussion and Conclusion

The increase in photosynthetic activity during the first 10 h of germination required de novo synthesis of RNA and protein, suggesting that the akinetes did not possess pools of stable mRNAs or of precursors of photosynthetic constituents. In contrast, respiratory activity could develop even when synthesis of RNA or protein was inhibited. The need for light is in agreement with the situation described for akinetes of several *Anabaena* species (Kaushik and Kumar 1970; Reddy et al. 1975; Braune 1979). Though a phytochromic effect of light, as has been discussed by Reddy et al. (1975) and Braune (1979), could not be ruled out for *Nostoc* akinetes, light was necessary as an energy supply through photosynthetic activity. No light inducible step was detected under the light regimes imposed. More refined determinations could be performed, though the biological significance of short pulses or small variations in intensities of light would need assessment.

Photosynthetic activity developed in the absence of correlated increases in pigment contents and optical density of the suspension. These observations might suggest a rearrangement of the pigments in the membranes, so as to build active photosynthetic units, and a proteolysis of some other constituents, such as cyanophycin, which is 8 times more abundant in akinetes than in vegetative cells (Sutherland et al. 1979).

It was not possible to determine whether respiration was necessary, though it was not sufficient, in our conditions, to promote germination in the dark. This pathway could develop in conditions where neither protein and RNA synthesis, nor photosynthetic function, could. However, light was necessary for its development. Although akinetes did not germinate in the dark even in the presence of oxygen and sucrose (a carbon source for photoheterotrophic growth), respiratory activity appeared to stimulate germination when PSI was operating in the absence of PSII since the proportion of germinated akinetes increased when oxygen was present. The differences observed under the gas phases of N_2:CO_2 and air:CO_2 may explain the contradictory results previously

described concerning the effect of DCMU on germination (Yamamoto 1976; Braune 1979), since DCMU was completely inhibitory only under anaerobic conditions.

Since little germination occurred in the absence of PSII activity, the glycogen content of the akinetes, although increased in comparison to that of vegetative cells (Sutherland et al. 1979), was not sufficient to support the process to completion. Although the addition of sucrose permitted all of the akinetes to germinate without PSII activity under aerobic conditions, the action of both photosystems was required for efficient and rapid germination.

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