Effect of Cyanophage Infection on CO₂ Photoassimilation in *Plectonema boryanum*

DALIA GINZBURG, ETANA PADAN, AND MOSHE SHILO

Department of Microbiological Chemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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Cyanophage infection effects a rapid and complete cessation of CO₂ photoassimilation in *Plectonema* cells. From the amount of infected cells lysed, it was established that this phenomenon cannot be ascribed to lysis of the host cells either from within or from without. The possibility that the effect is due to nitrogen starvation, induced secondarily by cyanophage multiplication, was ruled out when it was found that nitrogen supplementation did not influence the inhibition. It is suggested that the arrest of CO₂ photoassimilation is an integral part of the cyanophage infection cycle in *P. boryanum*. This idea is supported by the nondependence of the cyanophage-induced inhibition on the input multiplicity, by the light requirement for the inhibition, and by the fact that infected *Plectonema* cells with inhibited CO₂ photoassimilation support normal multiplication of the cyanophage. The pattern of light requirement for this viral inhibition further supports this suggestion.

The study of the behavior of photosynthetic apparatus under viral infection is complicated by a number of experimental difficulties (11). This is owing to the fact that until now such study has been restricted to tissues of higher plants.

Only recently, a new group of viruses which attack *Cyanophyceae* was isolated (9). Since these agents have characteristics of bacteriophages (7) and their blue-green algal hosts can be cultivated like bacteria, the cyanophage-alga system can be manipulated conveniently. Such a system allows for homogeneity of host population and of infection. Furthermore, the resemblance of the photosynthetic apparatus of blue-green algae to that of higher plants makes the alga a very promising model for studies of photosynthesis under conditions of viral attack.

On finding that the cyanophage attaches to the photosynthetic lamellae of the blue-green host cell (7, 12), it seemed that there might be an interrelationship between the virus and the photosynthetic apparatus of the host.

Recently, Wu and Shugarman (14) investigated photosynthetic O₂ evolution and respiration in cyanophage-infected *Plectonema* cells. They concluded that the rate of O₂ evolution did not change until the infected cells had ruptured, but that the rate of respiration after infection increased considerably.

In our studies of the effect of viral attack on the photosynthesis of *Plectonema*, we chose to study

the CO₂ fixation which is drastically affected in the infected cells. Detailed descriptions of this cyanophage-induced inhibition are given in this paper.

MATERIALS AND METHODS

Growth of alga and cyanophage. Cyanophage GIII, isolated and characterized by Padan et al. (7), and *Plectonema boryanum* 594 (Gomot) from the University of Indiana Culture Collection (Bloomington, Ind.) were used.

The alga was grown in the modified Chu No. 10 broth described by Safferman (10). Methods for cultivation and enumeration of *Plectonema* cells and the virus, as well as for concentration and purification of the virus, have been described (7). The number of infected *Plectonema* cells during the experiment (Table 2) was obtained as described by Luftig et al. (5). Trichomes were counted in a hemacytometer.

In certain experiments, the medium was enriched with a nitrogen source by the addition of 0.16 g of NaNO₃/liter.

CO₂ fixation by *Plectonema* cells. Cells of *Plectonema* in the logarithmic phase of growth were washed twice in the growth medium that contained 20 μmoles of NaHCO₃/ml (instead of Na₂CO₃) and titrated to a final pH of 9. This amount of bicarbonate supported a constant rate of CO₂ fixation in *Plectonema* over a 15-hr period. The cells were resuspended in this medium to give a final concentration of 1.5 × 10⁷ cells/ml. A 40-ml amount of the washed cell suspension was incubated with agitation in 100-ml stoppered flasks at 26 C, under constant illumination with an incident light intensity of 500 ft-c. After 15 min

of preincubation, $\text{NaH}^{14}\text{CO}_3$ (Radiochemical Centre, Amersham, Buckinghamshire, England) was added to each reaction mixture to give a final specific activity of $0.03 \mu\text{C}/\mu\text{mole}$.

At different time intervals, samples were removed and drawn through HA membrane filters (Millipore Corp., Bedford, Mass.; average pore size, 0.45μ). The cells retained on the filters were washed twice with cold water. The membrane filters were glued onto aluminum planchets and heat-dried for 5 min under a tungsten lamp. Samples were counted in a gas-flow, end-window planchet counter (model D-47, Nuclear-Chicago Corp., Des Plaines, Ill.). Self-absorption was negligible, and the counts were corrected for background radiation. Counting efficiency was approximately 25%.

For infection, 1 ml of dilute suspension of the cyanophage in TSM buffer (7) was added to the reaction mixture 30 min after the addition of the labeled bicarbonate to give the desired input multiplicity (IM) with respect to algal cells, unless otherwise stated; 1 ml of buffer was added to the uninfected controls.

Light was eliminated from the reaction conditions by wrapping the flasks in aluminum foil.

One-step growth experiment. A growth study of GIII, patterned after the one-step growth experiment of Ellis and Delbruck, was performed as described by Adams (1). *Plectonema* cells were prepared for the experiment and incubated as above. After 15 min of preincubation, they were infected with virus at IM of 1 plaque-forming unit per trichome. After 1 hr, on the assumption that this period is sufficient to give complete adsorption (4), samples were withdrawn and diluted to 10^{-4} and 10^{-5} of the original cell density in the bicarbonate medium to give a final volume of 40 ml each. After further incubation, samples were withdrawn from these growth tubes and assayed for plaque-forming units.

Radioactive labeling of *Plectonema* cells. Radioactively labeled *Plectonema* cells were obtained by adding 0.5 mc of $\text{NaH}^{14}\text{CO}_3$ (Amersham) to 100 ml of culture in the logarithmic phase. After 3 hr of incubation in the given growth conditions, a specific activity of $10 \mu\text{C}/\text{mg}$ of cell protein was obtained. The labeled algal cells were harvested and washed in 1 liter of unlabeled bicarbonate medium.

Measurement of leakage of intracellular components from infected *Plectonema* cells. The radioactively labeled algal cells were suspended in medium and incubated as for study of CO_2 fixation. After 15 min of preincubation, cyanophage was introduced at an IM of 10. At the indicated times (in Table 3), 1-ml samples were withdrawn; each sample was diluted 1:5 in water and filtered through Millipore membrane filters (HA 0.45μ) which had been washed with 5 ml of water. Radioactively labeled cells were retained on the filters, and the filtrate contained labeled intracellular materials released from the cells. The amount of isotope in the filtrates was measured in a liquid scintillation counter (model 314, Ex-2, Packard Instrument Co., Inc., La Grange, Ill.), as described by Bachrach and Eilon (2).

RESULTS

One-step growth experiment. The growth pattern of GIII, the locally isolated cyanophage strain, in the medium enriched with bicarbonate is shown in Fig. 1. The latent stage lasted for 7 hr; the rise period continued for another 6 hr. The average burst size was about 100 viruses per infected algal cell.

This growth pattern is similar to that of LPP-1 (4). In addition, the previously reported data (7) and the evidence that both viruses are inactivated by the same antibody (R. Haselkorn, *personal communication*) indicate that the viruses are very similar. Therefore, the local strain will be designated LPP-1G, as suggested to us by R. S. Safferman.

Effect of cyanophage infection on CO_2 photoassimilation in *Plectonema* cells. Noninfected *Plectonema* cells in logarithmic phase showed a constant rate of CO_2 fixation over a period of at least 7 hr (Fig. 2). This activity of the algal cells is strictly light-dependent, since no fixation was observed when light was excluded from the incubation conditions.

When such algal cells were infected with cyanophage at input multiplicities of 5 and 10, a drastic decrease leading to complete cessation of the CO_2 photoassimilation was observed at 2 to 2.5 hr after infection (Fig. 2 and 4a). Since at least 85% of the phage particles were adsorbed within 0.5 hr of infection (4), the cyanophage-induced inhibition must start 1.5 to 2 hr after completion of adsorption and long before the visible

FIG. 1. Growth curve of cyanophage LPP-1G (GIII).

FIG. 2. Rate of CO₂ photoassimilation in *Plectonema* populations at different IM. *Plectonema* populations were infected with given IM after 30 min of incubation. Solid lines show observed rate of CO₂ fixation; broken lines represent expected rate of CO₂ fixation for the given IM. The expected rate was obtained by calculating the fraction of the population infected according to Poisson's law and assuming that every single phage infection causes complete cessation of CO₂ fixation of the cell.

clearing of the culture and the release of phage particles from the infected cells, as detected by the one-step growth experiment.

In cells infected with smaller input multiplicities (less than 2), the onset of inhibition occurred at about the same time after infection as with higher multiplicities, while the rate of CO₂ uptake in the cell suspensions varied. The variously inhibited rates of photoassimilation could be due to different proportions of infected cells in *Plectonema* populations infected with different input multiplicities. Assuming that the infective phage particles are distributed at random over the cells in *Plectonema* trichomes, the expected fraction of infected cells was estimated from the IM according to Poisson's Law (13). [As *Plectonema* is constructed of short and long trichomes, it is conceivable that terminal cells with larger surface areas have a greater probability of infection than the inner cells. Assuming that the shape of *Plectonema* cells is cylindrical, which seems to give the most generous estimate, and that the cell length (on the average, 2 μ) is roughly twice its width, the expected number of phages adsorbed on a terminal cell is only one-sixteenth more than

on an inner cell. Furthermore, assuming that one-fourth of the cells are terminal (also an overestimate) the fraction of infected cells obtained is, at the most, $1 - e^{-m(\frac{1}{4} + \frac{1}{4}\exp[-\frac{1}{4}m])}$ instead of $1 - e^{-m}$ (13). This gives, for an IM of 1, a relative error of 0.02. This degree of error allowed us to adopt the uncorrected standard formula in our calculations.] Furthermore, assuming that even a single viral particle is sufficient to cause complete cessation of CO₂ fixation in an infected algal cell, the rate of CO₂ uptake in the attacked cultures could be predicted. The expected and observed rates of CO₂ uptake in *Plectonema* cells infected with multiplicities of 1, 5, and 10 are shown in Fig. 2. There is a persistent agreement between the expected and observed values of inhibition of CO₂ fixation in the infected algae. Thus, it is concluded that neither the onset nor the magnitude of inhibition per infected cell is dependent upon the IM.

The number of viruses produced during several experiments was detected by plaque-forming unit assay of samples withdrawn at the beginning of each reaction and after 15 hr (at which time the initial burst of infected cells was expected to be complete). Viral propagation was observed in every experiment (Table 1). On the basis of the established burst size of 100 viral particles per infected cell and the estimated fraction of attacked host cells, the expected increase in the number of viruses was calculated for each IM (Table 1). A good correlation was found between the expected and observed values for the extent of viral propagation. It could thus be concluded that infected *Plectonema* cells, in which CO₂ fixation was completely blocked, nevertheless supported multiplication of the cyanophage.

Uninfected *Plectonema* populations retained their original filamentous structure and cell numbers throughout the experimental period (Table 2). On the other hand, microscopic observation of infected *Plectonema* filaments showed that they had fragmented once or twice by the time of the onset of the CO₂ photoassimilation inhibition. This fragmentation seems to involve the lysis of no more than 1 cell out of 10 (in estimates made by microscopic inspection), and thus is below the range of discrimination in the hemacytometer enumeration technique (Table 2).

The possibility of lysis of the infected cells (IM of 10) during the latent period could be determined with greater sensitivity by the amount of leakage of labeled intracellular components from labeled, infected algal cells (Table 3). It is evident that even when CO₂ photoassimilation ceased, the magnitude of leakage induced by infection did not exceed 13 to 15%. Uninfected, labeled *Plectonema* populations show a certain

TABLE 1. *Cyanophage multiplication in Plectonema boryanum with different input multiplicities*^a

Expt no.	IM (PFU/cell) ^b	Input (PFU/ml) (A)	Yield (PFU/ml) (B)	Observed factor of increase (B/A)	Expected factor of increase
1	10	3.0×10^8	9.7×10^8	3.2	10.0
	5	1.5×10^8	2.4×10^9	16.0	20.0
2	5	1.5×10^8	2.7×10^9	18.0	20.0
3	1	2.5×10^7	1.5×10^9	60.0	63.0

^a Infected cell suspensions were sampled immediately after infection and after 15 hr. Expected factors of increase were calculated on the basis of the found burst size (Fig. 1) and the number of infected cells as calculated by Poisson's law.

^b IM = input multiplicity; PFU = plaque-forming units.

TABLE 2. *Number of cells and trichomes in infected and noninfected Plectonema suspension*^a

Incubation time (hr)	Noninfected		Infected	
	No. of cells (5×10^4 /ml)	No. of trichomes (10^4 /ml)	No. of cells (5×10^4 /ml)	No. of trichomes (10^4 /ml)
0	300	90	290	90
2	290	95	280	60
4	300	100	325	241
6	310	98	300	227

^a Cells were infected with an input multiplicity of 10 at 0 hr. Cells and trichomes were enumerated microscopically in a hemacytometer. The values given are means of three different experiments.

TABLE 3. *Leakage of labeled intracellular components from infected and noninfected Plectonema cells*^a

Incubation time (hr)	Leakage of labeled cell material from algal cells (at total lysis = 100)		Leakage due to cyanophage infection (B/A)
	Noninfected host (A)	Infected host (B)	
	%	%	%
0	1.2	1.0	-0.2
1	2.9	2.6	-0.3
1.5	3.3	4.0	0.7
2	3.3	5.1	1.4
3	4.6	13.0	8.4
4	7.2	22.7	15.5
5	16.8	29.8	13.0
24	17.4	100	

^a Cells were infected with an input multiplicity of 10 at 0 hr.

amount of progressive release of labeled cell components, which could possibly reflect release of the ¹⁴C in decarboxylation reactions and the like.

It has been shown (15) that virus multiplication in plants can have a secondary effect on the Hill reaction and photosynthetic phosphorylation in isolated chloroplasts, by inducing a state of nitrogen stress at the cellular level. By using a similar experimental approach to test the possible role of nitrogen stress on cyanophage-induced inhibition, *Plectonema* cells were grown before infection in a medium enriched with NaNO₃. Such cells, when infected, showed the same pattern of inhibition as was observed with cells grown without supplementary nitrogen. Moreover, the addition of nitrogen to reaction mixtures containing cells grown with and without nitrogen supplement did not change the outcome of the inhibition. Thus, the cyanophage-induced inhibition cannot be ascribed to nitrogen deprivation.

Light requirement of cyanophage-induced inhibition of CO₂ photoassimilation in infected Plectonema cells. As light is the sole energy source of *Plectonema boryanum*, an obligate autotroph, its requirement for the virus-induced inhibition was studied. Thus, light was eliminated for different periods during the incubation of infected *Plectonema* cells.

It was first necessary to determine the extent to which dark periods influence the rate of CO₂ photoassimilation in uninfected algal suspensions. It is apparent from Fig. 3 that 2.5 hr or less of darkness, after 1 hr of linear CO₂ photoassimilation, did not influence the rate of photoassimilation of uninfected cells. Immediately after light restoration, the initial rate of CO₂ fixation was regained and maintained for at least another 4 hr.

The effect of dark periods on the cyanophage-induced inhibition of CO₂ photoassimilation is presented in Fig. 3, 4, and 5. The IM used throughout was 10. In one experiment (see Fig. 3), the reaction mixture was illuminated for 0.5 hr after infection, during which time most of the cyanophage particles should have adsorbed (4). This reaction mixture was then incubated for 2.5 hr in the dark and then reilluminated. At the same time, inhibition was complete in the continuously illuminated mixture. Because of the dark period, the onset of the cyanophage-induced inhibition was delayed and expressed later. Light, therefore, is required for the viral inhibition.

By excluding light for different time periods and at different stages, the duration of light needed for the cyanophage-induced inhibition and the stage at which light is required were established. The possibility that darkness directly

FIG. 3. Effect of dark periods on the rate of CO₂ photoassimilation of infected and noninfected *Plectonema* cells. Cells were infected with IM of 10 after 30 min of incubation. Broken lines show rate in non-infected cells; complete lines represent rate in infected cells. Dark periods commenced 1 hr after beginning of incubation ($\downarrow$), and were terminated at the indicated times ($\uparrow$).

modifies the cyanophage-induced inhibition was also examined.

When light was excluded 2 hr before infection (Fig. 4b), the time of the onset of inhibition and its magnitude did not differ from that of the continuously illuminated infected reaction mixture (Fig. 4a).

When light was omitted immediately after infection (Fig. 4c) for 0.5 hr, the viral inhibition appeared at the same time as in the continuously illuminated reaction mixture (Fig. 4a). Thus, light is not required for adsorption. This conclusion was corroborated directly by determining the adsorption rate of cyanophage in the dark (unpublished data).

When light was interrupted for longer periods (1 to 2.5 hr) immediately after infection (Fig. 4d-g), the general pattern of the cyanophage-

FIG. 4. Effect of dark periods, immediately before and after infection, on the onset and rate of cyanophage-induced inhibition of CO₂ photoassimilation in *Plectonema* cells. Infection with IM of 10 at 0 hr. Shaded areas represent dark periods.

induced inhibition did not change, but its onset was delayed. The delays observed agree well with the values obtained by subtracting adsorption time (0.5 hr) from the corresponding dark periods. Apparently, the light requirement for the cyanophage-induced inhibition starts immediately after adsorption is complete.

Light was interrupted at different times after adsorption (Fig. 5c-e) for up to 2 hr, at which time the cyanophage-induced inhibition was evident in the continuously illuminated reaction mixture (Fig. 5a). Similarly, as with previous results (Fig. 4d-g), the magnitude of inhibition did not change, but its onset was delayed for periods corresponding exactly in time to those of the dark periods. On the other hand, the introduction of dark periods after the onset of inhibition (Fig. 5g-j) did not cause any modification in the rate and magnitude of the inhibition as compared with the continuously illuminated reaction mixture (Fig. 5f).

Thus, it was established that after adsorption a fixed period of illumination (1.5 to 2 hr) is required for cyanophage-induced inhibition to be effective. This illumination period need not necessarily be continuous nor need it be applied immediately after adsorption.

When dark periods were introduced 2 hr be-

FIG. 5. Effect of 1-hr dark periods, introduced after infection, on the onset and rate of cyanophage-induced inhibition of CO_2 photoassimilation in *Plectonema* cells. Infection with IM of 10 at 0 hr. Shaded areas represent dark periods.

fore infection (Fig. 4b), for 2 to 2.5 hr immediately after infection (Fig. 4f, g), and 1 hr after adsorption (Fig. 5d, e), the rate of the cyanophage-induced inhibition was increased on the whole. This could be ascribed to synchronization induced in the algal cells, or in the virus-host system, by excluding light before or after infection.

DISCUSSION

The present study shows that the cyanophage LPP-1G induces a rapid and complete cessation of CO_2 photoassimilation in infected *Plectonema* cells. As the cyanophage has characteristics of a bacteriophage (7), one of the following two circumstances might account for this cyanophage-induced inhibition. (i) Lysis of infected *Plectonema* cells induced by the cyanophage, either from within or without, would evidently involve, secondarily, destruction of all cellular activities. (ii)

Multiplication of the cyanophage could have a secondary effect on different enzymatic reactions of the host (15), or could directly induce inhibition of CO_2 fixation as an integral part of its propagation cycle.

With regard to the first alternative, we found that the cyanophage-induced inhibition of CO_2 photoassimilation starts long before cell burst is apparent, either by direct enumeration or by indirect determination of leakage of prelabeled cellular constituents. Thus, the explanation of cyanophage-induced inhibition by lysis from within or from without is untenable.

On the other hand, the small amount of lysis of infected cells ($\leq 15\%$) observed directly and indirectly during the latent period is interesting and deserves further investigation. It is of the same order of magnitude as that detected by Puck et al. (8) in bacteria immediately after phage infection. These authors concluded that this may reflect a reversible increase in the permeability of the host cells induced by the virus invasion.

With regard to the second alternative, we found that the cyanophage-induced effect starts and is completed during the first half of the latent period. Neither the onset nor the magnitude per infected cell of the cyanophage-induced inhibition is dependent on the IM. Furthermore, infected algal cells with completely blocked CO_2 fixation support, nevertheless, cyanophage multiplication. All these facts suggest that the cyanophage-induced inhibition of CO_2 photoassimilation is connected directly or indirectly with the early phase of the infection process of the cyanophage. The light requirement for this viral inhibition is consonant with this idea.

The notion that multiplication of the virus can have a secondary effect on different enzymatic reactions of the host was suggested by Zaitlin and Jagendorf (15) in studies of tobacco mosaic virus. They showed that nitrogen stress, rather than direct viral induction, was the actual cause of inhibition of various photosynthetic reactions in the infected plants. As our study shows that nitrogen supplementation does not influence cyanophage-induced inhibition in the infected *Plectonema* cells, this possible explanation can be withdrawn in the case of cyanophage-induced inhibition.

Our observations show that a fixed period of illumination (1.5 to 2 hr) is required after adsorption for cyanophage-induced inhibition. Thus, it is suggested that, after viral attachment, a process that depends directly or indirectly on light is induced by the cyanophage. This process continues for 1.5 to 2 hr and leads to the observed cessation of CO_2 photoassimilation in the host cells. Dark periods before or during the light-dependent process do not intrinsically change the cyano-

phage-induced inhibition but do delay its onset. After the light-dependent process is accomplished, dark periods no longer influence inhibition of CO₂ fixation.

A recent study of the mechanisms of lysis in *Escherichia coli* infected with a mutant strain of T4 lacking active lysozyme (6) showed that the bacteriophage induces cessation of oxygen uptake at the end of the latent period. In the wild type, such an interruption seems to trigger off lysozyme lysis of the cells, although lysozyme was already present 8 min after infection. Thus, it appears that the energy metabolism of the host cells may be more directly controlled by the phage than was previously believed.

These results suggest the possibility that the cyanophage-induced inhibition of CO₂ photoassimilation may cause lysis of the algal host as well. There is a possibility that in algae packed with lamellae (7) such lysis may be delayed.

On the other hand, the LPP-1G-induced cessation of CO₂ photoassimilation in *P. boryanum* could reflect a unique interaction between this agent and the metabolism of its obligate autotrophic host.

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