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Inhibition of Lytic Induction in Lysogenic Cyanophyces

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When the lysogenic strain SPI*cts*1 of the blue-green alga *Plectonema boryanum* carrying a temperature-sensitive mutation in the LPP2 prophage was heated at a nonpermissive temperature in the light, a lytic cycle occurred, with production of infectious viral particles. Inhibitors of transcription, translation, and photosynthetic functions interfered with this process and produced different effects when administered at different phases of the viral cycle. The presence of the inhibitors during the temperature shift did not allow a successful induction to take place; lysogens submitted to such a process produced a normal virus yield, however, when the drugs were removed and the temperature was shifted again. Incubation with the inhibitors during the early postinduction period reduced the virus yield; at later times, however, the inhibitory action rapidly declined. When cells were induced in the presence of chloramphenicol, incubated with actinomycin, and then grown in the dark, at either permissive or nonpermissive temperatures, virus multiplication was equally inhibited. These data indicate that: (i) provirus induction in lysogenic cyanophyces relies on the synthesis of early viral proteins; (ii) induction of mRNA is unstable and becomes rapidly inactivated when its translation is prevented; and (iii) inhibition of photosynthesis prevents the induction message from being expressed. It is suggested that the SPI*cts*1 prophage codes for a mutated repressor, which is reversibly inactivated at a nonpermissive temperature, and that the repressor must be inactivated at the same time that the message coded for by very early genes is translated, for a successful induction of the lytic cycle.

Discovery of lysogeny in *Plectonema boryanum* (2, 3, 25) has allowed the problem of virus-cell symbiosis in cells endowed with photosynthetic functions to be approached experimentally. The filamentous blue-green alga *P. boryanum*, which is the host of both the virulent LPP1 phages (22, 28) and the temperate LPP2 cyanoviruses (23, 29), is an ideal system for this sort of study. Its metabolic pathways are fairly well known, and the main relationships between virus development and metabolic functions have been established (1, 22).

The temperate cyanophage SPI*cts*1, which is the object of study in the present work, is a temperature-sensitive mutant of LPP2 that lysogenizes *Plectonema*. In contrast to the wild type, which has not been successfully induced so far, induction of SPI*cts*1 provirus can be obtained by heating the host cells to a nonpermissive temperature in the light (26, 27). Dependence of the induction process on photosynthetic reactions makes the LPP2-*Plectonema* system particularly interesting.

The aim of our work was to study the role played by transcription and translation on the lytic induction of SPI*cts*1. For this purpose, well-known inhibitors of these processes were used, such as actinomycin D and rifampin, which inhibit the RNA polymerase reaction, chloramphenicol and virginiamycin M (VM), which interfere with the reactions catalyzed by the peptidyltransferase and the elongation factor Tu (11, 14), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II (22), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupling agent (22). Virginiamycin, a streptomyces-produced antibiotic, contains two components, VM and virginiamycin S (VS), which have synergistic activity in different microorganisms (4, 10, 35, 36; C. Cocito, manuscript in preparation). These antibiotics have already been used to investigate the metabolism of macromolecules in bacteria (4, 6, 7, 8, 10) and algae (14, 15, 35, 36), as well as the development of virulent bacteriophages (5, 9) and cyanoviruses (11). In a recent work (14), it was shown that VM produced a transient block of protein formation in *P. boryanum*, whereas VS, which had no evident metabolic activity per se in this microorganism,

enhanced the inhibitory action of VM and made it permanent.

MATERIALS AND METHODS

Strains of algae and growth media. *P. boryanum* 594 (Gomot) from the University of Indiana Culture Collection (Bloomington, Ind.) was grown in modified CHU no. 10 mineral solution (28) either at 26°C or at 40°C, under continuous illumination by 10^4 ergs of incident white light per cm^2 per s. The lysogenic strain of this microorganism, P1 (SPI*cts*1), carrying a heat-inducible provirus, was isolated by infection of *Plectonema wt* with virus SPI*cts*1 (26, 27). This organism was grown at 26°C in light with the same medium.

Cyanoviruses. Phage SPI *wt* was an isolate of the temperate cyanophage LPP2, which formed turbid plaques on *P. boryanum* and *Phormidium luridum* (29). The temperature-sensitive mutant of this strain, SPI*cts*1, was obtained by chemical mutagenization of SPI-infected *Plectonema* (27). This mutant had a clear-plaque phenotype at 40°C and a turbid one at 26°C. Some experiments were carried out with SPIc1, a clear-plaque mutant of SPI *wt*. The virions of all these strains were counted by the plaque assay on lawns of *Plectonema* 594 *wt* after 3 days of incubation at 26°C in light.

Production and purification of viruses. Phages SPI *wt* and SPIc1 were multiplied by infecting exponential cultures of *Plectonema wt* at 26°C in the light. Multiplication of the *ts* mutants was obtained by heating exponential cultures of P1 (SPI*cts*1) at 40°C for 3 h, and the lytic cycle was terminated by addition of chloroform. After lysis and removal of residual cells and debris, viral particles were collected by ultracentrifugation, suspended in a mixture of 0.15 M NaCl and 0.1 M $MgCl_2$, purified by alternate cycles of low- and high-speed centrifugations, and concentrated by density gradient centrifugation (B. Boucau, unpublished data).

Induction of the lytic cycle. Vegetative multiplication of viral particles was obtained by shaking exponential cultures of the lysogenic strain P1 (SPI*cts*1) at 40°C in the light (10^4 ergs of white light per cm^2 per s) for variable lengths of time (10 to 60 min). As previously shown (26), heating and illumination had to be administered at the same time for induction to be produced. Thereafter, the multiplication cycle was allowed to occur at either 26 or 40°C, in the light and in the dark. The kinetics of virion formation under these four experimental conditions is traced in Fig. 1.

Antibiotics and inhibitors. Chloramphenicol (Synthomicetine) was purchased from Albic Chemical & Pharmaceutical Industries (Ramat Gan, Israel), actinomycin D was from Makor Chemicals (Jerusalem, Israel), and rifampin SV (Lepetit) was from Calbiochem. CCCP and DCMU were obtained from the du Pont de Nemours Experimental Station (Wilmington, Del.). Reference to the preparation of crystalline VM and VS and to the structure of these two compounds can be found in earlier publications (4, 11).

Radiochemical procedures. RNA synthesis was followed by adding [8-^{3}H]adenine to the growth medium, to a final concentration of 1 μCi/ml. Samples of labeled cells were collected by filtration, incubated for 14 h at 37°C with 0.5 M NaOH, transferred to an ice bath, neutralized with HCl, treated with 5% trichloroacetic acid, and filtered through glass fiber filters (GF/C22; Whatman, England), which were washed with trichloroacetic acid and ethanol-acetone (1:1), dried, and counted.

Incorporation of amino acids into polypeptides was traced by adding ^{14}C-labeled algal protein hydrolysate (0.1 μCi/ml) (Radiochemical Centre, Amersham, England) to culture medium supplemented with unlabeled amino acid mixture (1 μg/ml). Samples were precipitated with 5% trichloroacetic acid and filtered through glass fiber filters, which were washed with the trichloroacetic acid and ethanol mixture and counted. The kinetics of carbon dioxide photoassimilation was traced by growing cells in a modified CHU no. 10 medium containing 20 μmol of $NaHCO_3$ per ml (pH 9). It was previously shown that this medium supports a constant rate of carbon dioxide fixation in *Plectonema* over a 15-h period. Cells were labeled by addition of $NaH^{14}CO_3$ (Radiochemical Centre, Amersham, England) to a final specific activity of 0.03 μCi/μmol and were collected by filtration through glass fiber filters, which were washed with 1 mM NaOH and counted. Samples were counted in scintillation spectrometers, by using toluene containing 3 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-(5-phenyloxazolyl)benzene per liter as counting fluid.

RESULTS

Action of inhibitors on cell metabolism and virus yield in induced lysogens. In preliminary experiments, the three types of drugs used throughout the present work (inhibitors of RNA synthesis, protein formation, and photosynthesis) were tested for their capacity to

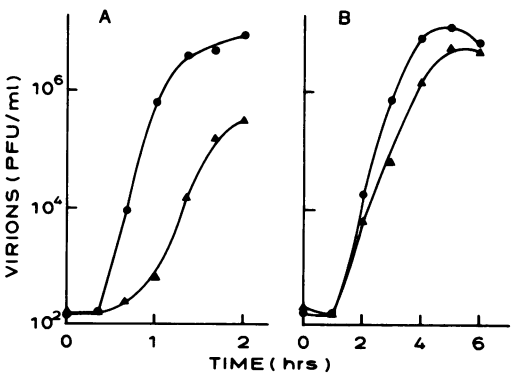

FIG. 1. *Kinetics of induced virion formation under different conditions. An exponential culture of lysogenic Plectonema SPIcts1 was induced (40°C, light, 30 min) and then split into four samples, which were incubated either at 40°C for 2 h (A) or at 26°C for 6 h (B), either in the light* (○) *or in the dark* (△), *for virus particle formation.*

block the multiplication of viral particles in induced lysogens. It was observed that: (i) all the inhibitors tested but VS inhibited virion production; (ii) although inactive per se, VS increased the inhibitory action of VM; (iii) all the active drugs produced a more evident effect when added before induction than after induction; (iv) inhibition by actinomycin, rifampin, DCMU, and CCCP was higher at 26°C in the dark than at 40°C in the light.

In addition, the inhibitors were checked for their capacity to block macromolecule formation and CO_2 photoassimilation in virus-infected cells, to exclude a possible aspecific effect. Indeed, as shown in Fig. 2, the synthesis of RNA in lysogenic *Plectonema* was halted by actinomycin and rifampin (Fig. 2A), and that of proteins was inhibited by chloramphenicol and VM (Fig. 2B). Moreover, CO_2 photoassimilation was blocked by DCMU and CCCP (Fig. 2C).

Inhibition of RNA formation during and after induction. Inhibition of the RNA polymerase reaction at different times in the lytic cycle might have different effects: the expression of some genes might be blocked irreversibly, whereas that of other genes might be reversibly blocked. If so, the use of transcriptional inhibitors would allow a distinction among the mRNA species that are made during prophage induction and vegetative virus development. To test this possibility, cultures of lysogenic cyanophyces were induced in the presence of either actinomycin or rifampin (controls were induced in the absence of antibiotics). Short periods of incubation with these inhibitors were also performed at randomly selected times during virus replication cycle. In all cases, antibiotic-treated cells were washed and transferred to fresh medium without inhibitors for 6 h, and the kinetics of virions formation was traced. To avoid photoinactivation of actinomycin and rifampin, cells were preincubated with the antibiotic for 15 min at 26°C in the dark, induced at 40°C in light, and then transferred to inhibitor-free medium.

No virions were produced when RNA synthesis was prevented during the induction period (Fig. 3A). Transcriptional inhibitors reduced the virus yield when added immediately after

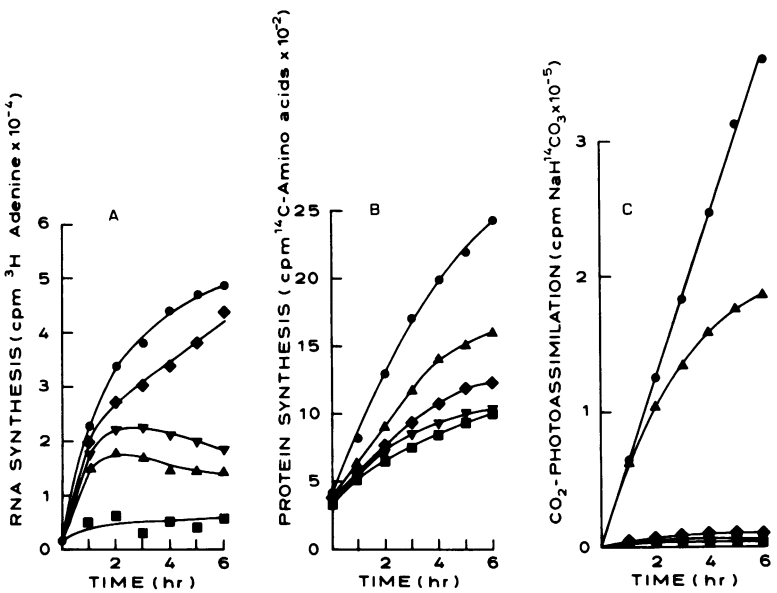

FIG. 2. *RNA and protein synthesis, and CO_2 photoassimilation in lysogenic Plectonema growing in the presence of different inhibitors. Cells were grown either in Na_2CO_3 medium without (A) or with (B) unlabeled amino acids (1 mg/ml), or in $NaHCO_3$ (20 μmol/ml) medium (C) without organic supplements. (A) Inhibitors and [³H]adenine (1 μCi/ml) were added at time 0, and trichloroacetic acid-insoluble KOH-hydrolyzable radioactivity incorporated into RNA was measured by scintillation counting. Inhibitors: none (○); actinomycin, 1 μg/ml (◇) and 10 μg/ml (▽); rifampin, 1 μg/ml (△) and 10 μg/ml (□). (B) Inhibitors and ¹⁴C-labeled amino acids (0.1 μCi/ml) were added at time 0, and the radioactivity incorporated into trichloroacetic acid-insoluble polypeptides was counted. Inhibitors: none (○); chloramphenicol, 1 μg/ml (◇) and 10 μg/ml (□); VM, 1 μg/ml (△) and 10 μg/ml (▽). (C) Inhibitors and $NaH^{14}CO_3$ (2 μCi/ml) were added at time 0, and cell samples were collected on glass fiber filters, which were washed with 1 mM NaOH and counted. Inhibitors: none (○); CCCP, 5 μg/ml (△) and 50 μg/ml (▽); DCMU 5 μg/ml (◇) and 50 μg/ml (□).*

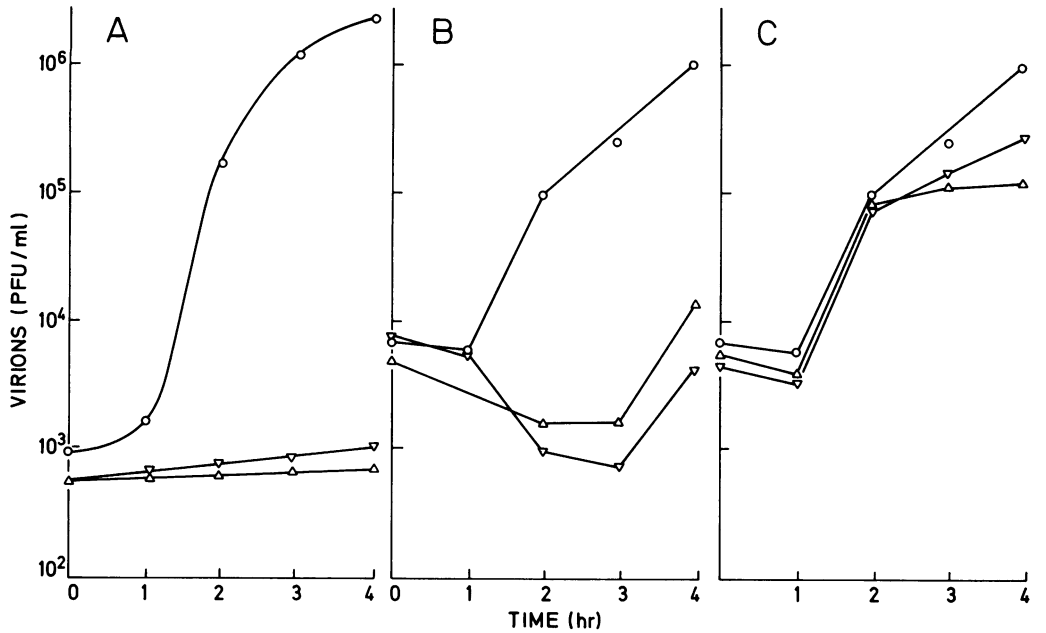

FIG. 3. *Action of transcriptional inhibitors. (A) Lysogens were induced (40°C, light, 10 min) in the absence* (○) *or in the presence of either rifampin* (▽) *or actinomycin D* (△) *(50 μg/ml), harvested, and transferred to antibiotic-free medium for virus multiplication. (B and C) Portions of cell cultures, which were induced in the absence of antibiotics, were incubated for 1 h with actinomycin (50 μg/ml, 26°C, dark) (control = no actinomycin) and grown further in antibiotic-free medium. (B) Control* (○) *and actinomycin during the 1st* (△) *or 2nd* (▽) *h after induction. (C) Control* (○) *and actinomycin during the 3rd* (△) *or 4th* (▽) *h after induction.*

induction (Fig. 3B), but they were ineffective at later times (Fig. 3C).

Inhibition of protein synthesis during different phases of the lytic cycle. Messages formed during different phases of the viral cycle might also be endowed with different metabolic stability. This possibility was tested by incubating lysogens with protein synthesis inhibitors either before, during, or after induction. Cells were then washed and transferred to fresh medium, and virions produced in the absence of inhibitors were counted.

Treatment with either VS or chloramphenicol during the period preceding induction did not reduce the virus yield (Fig. 4A). Although removal of VM by washing was more slow and less complete, the late increase in particle formation, occurring upon transfer of induced lysogens to antibiotic-free medium, pointed to a reversible effect (Fig. 4A). Instead, a complete inhibition of the viral cycle was produced when cells were preincubated with a mixture of the two virginiamycin components. Inhibition of protein synthesis during induction completely blocked subsequent virus development (Fig. 4B). In fact, almost no viral particles were formed when lysogens were induced in the presence of either chloramphenicol or VM, or a mixture of both virginiamycin components. Addition of either chloramphenicol or VM immediately after induction reduced virus yield: under such conditions, a complete halt of the lytic cycle was produced by a mixture of VM and VS (Fig. 4C). When time intervals of increasing length were allowed before the antibiotics were administered, the reduction of virus yield became progressively less evident, and finally no inhibition was observed (Fig. 5). It can be inferred, therefore, that the lytic cycle was irreversibly blocked by protein synthesis inhibitors only during the induction period; thereafter, the effect of the antibiotics progressively decreased and vanished in about 1 h. Inhibition by VM plus VS was irreversible at any time.

Action of photosynthesis inhibitors on the lytic cycle. Previous experiments have shown that illumination and active photosynthesis are both essential for a lytic cycle going to completion (26). Moreover, DCMU and CCCP, well-known inhibitors of photosynthetic functions, were more active when added before than when added after induction. Further study with these drugs showed that, after induction in the presence of either DCMU or CCCP, the subsequent lytic cycle in inhibitor-free medium yielded no virions (Fig. 6A). On the contrary, these sub-

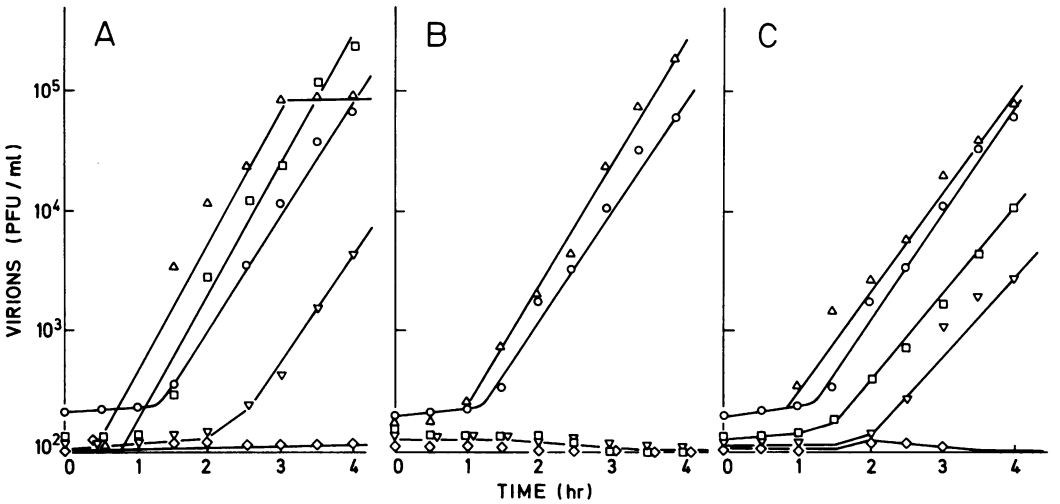

FIG. 4. *Effect of protein synthesis inhibitors on induction. Lysogenic cells were incubated with different inhibitors for 30 min, either before (A), during (B), or after (C) induction (40°C, light, 30 min), and then transferred to antibiotic-free medium for virus multiplication (26°C, light, 4 h). Inhibitors: none (control)* (○); *VS, 100 μg/ml* (△); *VM, 100 μg/ml* (▽); *VM + VS, 50 μg/ml* (◇); *chloramphenicol, 100 μg/ml* (□).

stances, when added during the lytic cycle, produced either a delay in virus particle formation or no effect at all (Fig. 6B). These results, in addition to data in Fig. 1, indicate that, among all the steps of the lytic cycle, the induction phase only has a stringent requirement for photosynthesis.

Multiple induction of inhibited lysogens. Since induction of lysogens in the presence of chloramphenicol was abortive (Fig. 4B), the question arose as to whether unsuccessfully induced cells retained their viability as well as the capacity of being subsequently induced. The possibility of complete and rapid removal of chloramphenicol (Fig. 4A and C) made an experimental approach to this problem feasible.

A culture of lysogenic *Plectonema* was divided into two portions, which were induced for 30 min at 40°C in the presence and in the absence of chloramphenicol. Induced lysogens were then collected, suspended in fresh medium, and further incubated at 25°C for 4 h, to follow the kinetics of particle formation. Two portions of the chloramphenicol-treated, heat-induced culture were induced again, in the presence and in the absence of the drug, and the virions produced in antibiotic-free medium were counted. This process was repeated four times, and the corresponding results show that unsuccessfully induced cells retained the capacity of being reinduced, yielding a normal crop of particles, as well as their susceptibility to chloramphenicol inhibition (Fig. 7).

Action of translational inhibitors on the lytic cycle at a nonpermissive temperature. Though lysogenic cells induced by heat plus light in the absence of protein synthesis remain viable and inducible when grown at 26°C in the light (Fig. 7), they might produce a lytic cycle at a nonpermissive temperature in the dark. This possibility was tested first by a three-step experiment in which cells were abortively induced (40°C, light, chloramphenicol), then incubated with actinomycin at 26°C, and finally grown without inhibitors in the dark at either permissive or nonpermissive temperatures; virion production was prevented (Table 1). Further control experiments, in which the actinomycin step was omitted, gave similar results. These results indicate that a block of protein synthesis during induction prevents a lytic cycle development at a nonpermissive temperature.

DISCUSSION

Induction of lysogens by a shift to a nonpermissive temperature (Fig. 1) can be explained by postulating that the temperature sensitivity of cyanophage SPI*cts*1 is due to a mutation of a regulatory viral gene, a situation encountered in other lysogenic systems (17, 24, 25): a repressor possessing an inactive configuration at a nonpermissive temperature is, thus, produced. Moreover, inactivation of the *cts*1 repressor by the temperature shift is a reversible process, since lysogens induced in the presence of chloramphenicol retain their viability and do not yield virions (Fig. 7).

As shown in Fig. 3, the presence of transcriptional inhibitors during induction completely

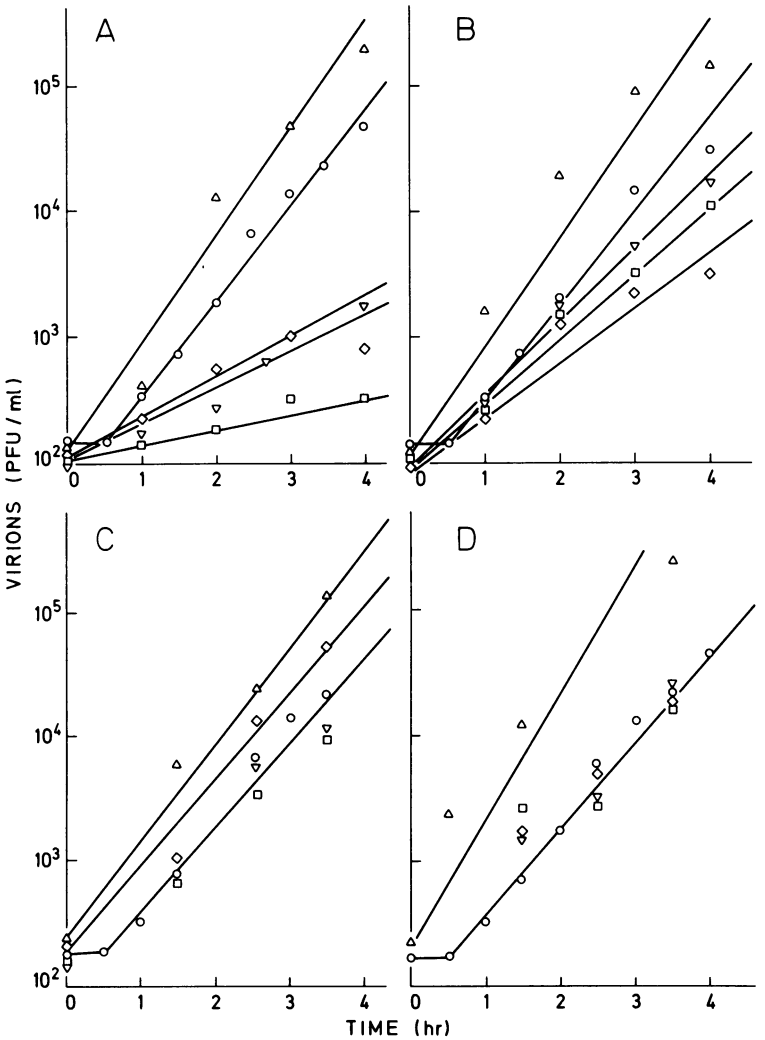

FIG. 5. *Inhibition of protein synthesis during different phases of the lytic cycle. Lysogens induced in the absence of inhibitor were incubated for 60 min with different antibiotics at different times in the viral cycle and then transferred to inhibitor-free nutrient for virus multiplication (26°C, light, 4 h). Time of antibiotic additions: 1 h (A), 2 h (B), 3 h (C), and 4 h (D) after induction.*

prevented subsequent virus multiplication in antibiotic-free medium. This indicates that the transcription of a very early message is compulsory for vegetative virus development. Similar conclusions were drawn for λ prophage induction (17–23, 32–34). Two alternative explanations for the block of lytic development produced by translational inhibitors during the induction of lysogenic cyanophyces (Fig. 4B) can be proposed: (i) the mRNA that is made during the induction step is very unstable and needs to be translated without delay, otherwise it is rapidly inactivated; (ii) the induction message is stable, but the protein that is coded for has no effect, in the presence of the repressor. The experiments reported in Table 1, in which induction mRNA was allowed to accumulate first (40°C, light, chloramphenicol) and then its translation (chloramphenicol removed) was subsequently allowed in the presence of an active (26°C) or inactive (40°C) repressor, were devised to differentiate between these two possibilities. The first hypothesis predicts that there is no difference whether translation of induction mRNA is allowed at 40°C or at 26°C (in both cases mRNA is degraded) and accounts equally well for the induction protein being a derepressor or a positive inducer. The second

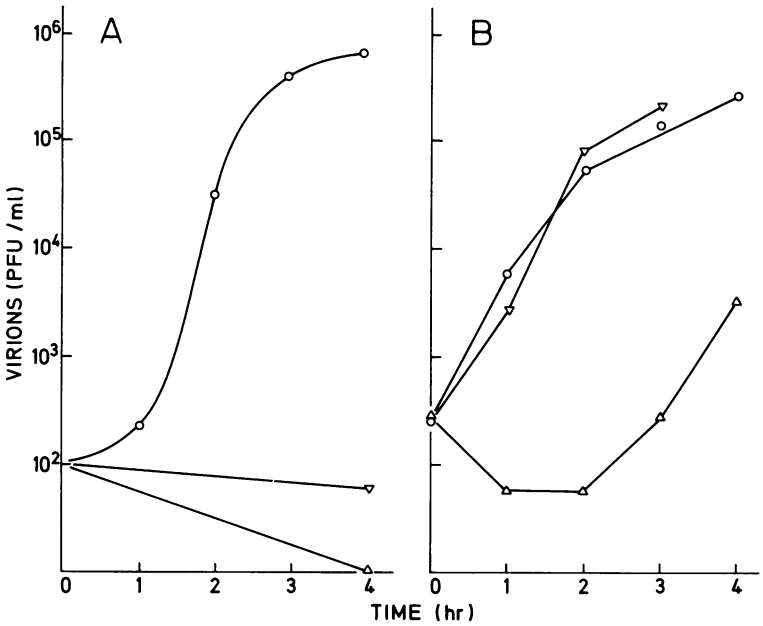

FIG. 6. *Action of inhibitors of photosynthesis and photophosphorylation. (A) Lysogens were induced (10 min, light, 40°C) in the absence (control, ○) or in the presence of either DCMU (▽) or CCCP (△) (50 μg/ml), harvested, and transferred to antibiotic-free medium for virus multiplication. (B) Lysogens were induced in the absence of inhibitors, incubated with either DCMU (▽) or CCCP (△) or nothing (○) during the 1st h after induction, and then transferred to inhibitor-free medium for virus multiplication (26°C, light, 4 h).*

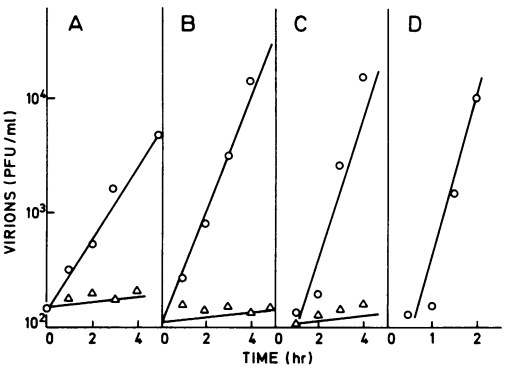

FIG. 7. *Multiple induction of inhibited lysogens. Lysogenic cells were induced (40°C, light, 30 min) in the absence (control, ○) and in the presence (△) of chloramphenicol (100 μg/ml). Cells were then harvested, transferred to antibiotic-free medium, and incubated at 26°C in light for 4 h to follow the lytic cycle (A). Portions of the culture previously induced in the presence of chloramphenicol were induced again, either in the presence (△) or in the absence (○) of the drug, and virions produced at 26°C were counted (B); this procedure was repeated twice more (C and D).*

hypothesis postulates that the product of the induction messenger translation is functional at 40°C but not at 26°C, excludes a derepressor

TABLE 1. *Lytic cycle at a nonpermissive temperature, after induction in the absence of protein synthesis*

Induction[a]	Lytic cycle[b]	Virus yield (PFU/ml)
No CMP	26°C, dark, 4 h	1.1×10^6
+ CMP		1.2×10^4
No CMP	40°C, dark, 2 h	1.4×10^6
+ CMP		4.0×10^3

[a] Lysogens were induced (40°C, light, 15 min) in the absence or in the presence of chloramphenicol (CMP, 100 μg/ml). In some experiments, induction in the presence of CMP was followed by additional incubation with actinomycin (10 μg/ml, 10 min, 26°C, light), with comparable results.

[b] Cells were washed, transferred to nutrient without antibiotics, and grown at two different temperatures in the dark.

function of the corresponding translation products, and predicts that the induction protein is a positive inducer that binds only to repressor-free DNA. Our experimental data (Table 1) support the first hypothesis. The additional possibility of a repressor that, at a nonpermissive temperature, loses the ability of binding to DNA while retaining that of linking other positive inducers is ruled out by the fact that virus

yielded is the same whether induced cells are maintained at 26°C or at 40°C (Fig. 1 and Table 1).

The lack of activity of VS (Fig. 4 and 5) was not surprising. In fact, this drug was found to block protein synthesis in uninfected (4) and phage-infected (5) bacteria, but not in eucaryotic (34) and procaryotic (12) algae. Moreover, this antibiotic does not affect the multiplication of virulent cyanoviruses (C. Cocito and M. Shilo, manuscript in preparation). Altogether, these data confirm the notion that the metabolic organizations for protein synthesis in bacteria and blue-green algae are different (14), and may also explain the different effects exerted by VS on the development of bacteriophages (5) and cyanophages (11). However, VS acted synergistically with VM in inhibiting the induced provirus development (Fig. 4). In agreement with this finding is a previous report that, although inactive per se, VS potentiates the VM-dependent inhibition of protein synthesis in *Plectonema* and prevents its reversal (14).

It has been reported (21) that induction of SPI*cts*1 is a light-dependent process and that DCMU, an inhibitor of photosystem II, reduces virus yield. In agreement with this finding are the data in Fig. 6 showing that, upon induction of lysogens in the presence of either DCMU or CCCP, no virus development takes place. Thereafter, these inhibitors have no action (DCMU) or less activity (CCCP). Thus, photosynthetic reactions are required only for induction and not for subsequent virus development. Such an inference is also supported by the kinetics of virion formation in light and in dark (Fig. 1). We have no explanation for the photosynthetic dependence of induction but suggest the possibility that, in lysogenic cyanophyces, genetic recombination and provirus excision require some coenzymes that are made by the photosynthetic apparatus. Since bacterial ligase is nicotinamide adenine dinucleotide dependent, the case of a nicotinamide adenine dinucleotide phosphate-dependent algal endonuclease is not inconceivable.

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