Effects of Light Deprivation on RNA Synthesis, Accumulation of Guanosine 3'(2')-Diphosphate 5'-Diphosphate, and Protein Synthesis in Heat-Shocked *Synechococcus* sp. Strain PCC 6301, a Cyanobacterium

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The rate of total RNA synthesis, the extent of guanosine 3'(2')-diphosphate 5'-diphosphate (ppGpp) accumulation, and the pattern of protein synthesis were studied in light-deprived and heat-shocked *Synechococcus* sp. strain PCC 6301 cells. There was an inverse correlation between the rate of total RNA synthesis and the pool size of ppGpp, except immediately after a temperature shift up, when a parallel increase in the rate of RNA synthesis and accumulation of ppGpp was observed. The inverse correlation between RNA synthesis and ppGpp accumulation was more pronounced when cells were grown in the dark. Heat shock treatment (47°C) had an unexpected effect on ppGpp accumulation; there was a fairly stable level of ppGpp under heat shock conditions, which coincided with a stable steady-state rate of RNA synthesis even in the dark. We found that the pattern of dark-specific proteins was altered in response to heat shock. The transient synthesis of several dark-specific proteins was abolished by an elevated temperature (47°C) in the dark; moreover, the main heat shock proteins were synthesized even in the dark. This phenomenon might be of aid in the study of cyanobacterial gene expression.

Growing bacteria are strongly influenced by their nutritional, chemical, and physical environment, and they adjust their growth to alterations in these conditions in their habitat (18). Prokaryotic organisms respond to unfavorable alterations of these factors by a reduction of their growth rate and by a concomitant accumulation of guanosine 3'(2')-diphosphate 5'-diphosphate (ppGpp), the compound implicated as a key substance for the stringent control system (10, 16, 20, 22). The accumulation of ppGpp under shift down conditions is generally considered to be a signal for the curtailment of several metabolic processes. In heterotrophic bacteria, the intracellular level of ppGpp is inversely correlated with the rate of synthesis of stable RNA species, suggesting that the regulation of cell metabolism is directly or indirectly under the control of the effector molecule ppGpp (for reviews, see references 8, 12, 13, and 19). Recently it has been reported that a temperature shift up of *Escherichia coli* cells leads to a transient accumulation of ppGpp (10, 16, 20, 22), Ryals et al. (22), as well as Mackow and Chang (16), demonstrated that an inverse correlation exists between ppGpp level and the rate of total RNA synthesis in heat-shocked *E. coli*, which is consistent with this view.

In cyanobacteria, the mode of accumulation of ppGpp is comparable with that reported for heterotrophic bacteria (17). However, there are conflicting results for the correlation between nitrogen starvation and ppGpp accumulation (1, 2, 11, 17, 25) and the effect of light (hence, energy) deprivation, which does not seem to be comparable with the nutritional step-down of heterotrophic bacteria (3, 17, 25). In studies dealing with the light-dependent regulation of gene expression in *Synechococcus* sp., an obligate autotrophic cyanobacterium, the pleiotropic nature of regulatory responses has been emphasized (9, 23).

Indeed, under energy deprivation (dark) conditions, some specific polypeptides are synthesized (23). The heat shock phenomenon has recently been reported in *Synechococcus* sp. (6), and the response of the cells to elevated temperatures has been found to involve the reduction of growth rate and induction of a specific set of polypeptides, the so-called heat shock proteins. Since the reduction of growth during heat shock in *Synechococcus* and the nutritional or energy shift down in various cyanobacterial systems may have much in common with heterotrophic shift down systems, one would also expect a role for ppGpp in the temperature-induced regulation of stable RNA and protein synthesis in cyanobacteria.

Here we describe the effects of heat shock on the synthesis of RNA and the accumulation of ppGpp upon transfer of *Synechococcus* cultures from light to darkness. We relate these data to the pattern of proteins synthesized under heat shock (6) and in the dark (energy starvation [9]). Such studies are of interest not only in their own right, but from the viewpoint of light-dependent cyanobacterial gene expression as well.

**MATERIALS AND METHODS**

**Growth conditions.** *Synechococcus* sp. strain PCC 6301 (Anacystis nidulans ATCC 27144) was grown in the liquid medium of Allen (4) with jacketed glass culture vessels as described earlier (6).

**Heat shock conditions.** Exponentially growing *Synechococcus* cultures (0.500 to 0.700 A₅₇₀ units) were heat shocked as described earlier (6), with a minor modification, i.e., in the series of experiments, 10-ml jacketed glass culture vessels were used to study ppGpp accumulation and RNA synthesis at normal (39°C) and elevated (47°C) temperatures.

**Isotope-labeling conditions and analysis of RNA synthesis.**
The accumulation of RNA was monitored by measuring the incorporation of [3H]uracil into trichloroacetic acid (TCA)-
precipitable material. As exponential-phase culture of Synechococcus sp. was divided into two parts (time zero),
radioactive [5,6-3H]uracil at 185 kBq ml\(^{-1}\) (original concentration, 1.746 kBq mmol\(^{-1}\)) was added to both, and these
subcultures were grown at 39 or 47°C. The incorporation of radioactivity into cells was assessed by the removal of 0.2-ml
samples which were transferred into equal volumes of an ice-cold solution containing 0.4 mg of bovine serum albumin
and immediately mixed with 0.8 ml of cold 12.5% TCA solution. The precipitates were collected by centrifugation
and washed twice with cold 5% TCA solution and ethanol. The pellets were dried briefly and then hydrolyzed in 0.2 M
sodium hydroxide overnight at room temperature. After sodium hydroxide treatment, the samples were clarified by
centrifugation, and aliquots of supernatants were taken to measure their radioactivity in Bray solution. A Delta Searle
300 liquid scintillation spectrometer was used to measure radioactivity. To measure the rate of total RNA synthesis,
cyanobacterial cultures were labeled for several generations (usually overnight) with 5 kBq of [14C]uracil (13.32 GBq/ mmol; y emap, Prague, Czechoslovakia) per ml. The rate of total RNA synthesis was determined by pulsing 0.5-ml
samples with 1.2 MBq of [3H]uracil per ml for 10 min; labeling was stopped by withdrawing 0.2 ml of culture, adding this to an equal volume of bovine serum albumin solution, and immediately mixing the combination with cold
TCA as described above. The precipitates were treated as described for the accumulation of RNA. The rate of total
RNA synthesis was expressed as normalized values of [3H]uracil incorporated into alkali-soluble material per
10-min radioactive pulse. The ratio of incorporation of [3H]uracil to that of [14C]uracil at the end of the initial 39°C
incubation was defined as 1, and the rate of total RNA synthesis was calculated.

Measurement of guanosine tetrathosphate. The cellular nucleotide pool was labeled by the addition of carrier-free
\(^3\)P to the cultures (final radioactivity, 4.0 MBq ml\(^{-1}\)). Measurement of ppGpp and guanosine 3'-(2')-triphosphate
5'-diphosphate pools was done in formic acid extracts, which were chromatographed as described by Borbély et al.
(5) by using polyethyleneimine thin-layer chromatography plates (Macherey and Nagel Co., Dürren, Federal Republic of
Germany).

Polyacrylamide gel electrophoresis of *Synechococcus* proteins. Exponential-phase *Synechococcus* cultures (1-ml portions)
were labeled with 0.5 MBq of U-\(^{14}\)C-labeled protein hydrolysate for 1.5 h in the light and 3.0 h in the dark under both normal (39°C) and heat shock (47°C) conditions, unless
otherwise stated. After labeling, the samples were processed for 10 to 18% linear sodium dodecyl sulfate-polyacrylamide
gradient gel electrophoresis and autoradiography (6).

**RESULTS**

Effect of elevated temperature on RNA synthesis of *Synechococcus* sp. In *Synechococcus* cells, a shift in growth
temperature from 39 to 47°C resulted in an increased rate of accumulation of radioactive uracil for a 20-min period (Fig.
1). This initial increase was followed by a decrease in the rate of accumulation. A minimum for the uracil accumulation
was observed between 20 and 30 min after the temperature increase to 47°C. After this time, an increase in RNA
accumulation, as determined by the uptake of [3H]uracil, began and continued essentially at the same rate during the
remainder of the heat shock (Fig. 1). Since the mere accumulation of radioactive uracil under these conditions may
not be a true measure of RNA synthesis, we also measured the rate of total RNA synthesis in heat-treated cells by
[3H]uracil pulse-labeling of aliquots of *Synechococcus* cultures that had been continuously labeled with [\(^{14}\)C]uracil.
When the rate of total RNA synthesis was measured, an immediate increase in RNA synthesis was observed after the
shift to an elevated temperature (Fig. 2A). A subsequent gradual decrease was noted in the rate of total RNA synthesis
between 10 and 30 min. Subsequently, the rate of RNA synthesis gradually increased again and then reached a new
steady-state rate for the period of heat treatment. In cultures shifted back to the initial growth temperature (39°C), the rate
of incorporation of the radioactive RNA precursor decreased by some 30 to 40% for 60 min, after which the rate of total RNA synthesis returned to that characteristic of normal cells not stressed by heat treatment.

When the rate of total RNA synthesis of heat-shocked cells was measured in the dark, there was an immediate
decrease in the rate of RNA synthesis followed by a slight, transient increase (at about 40 min), and afterwards, a steady
state was maintained for the remainder of the heat shock period; this rate was notably lower than that observed under
light conditions (Fig. 2C).

To analyze more closely the effect of different down shifts (heat shock and darkness) on RNA synthesis, we examined
the consequences of different sequences of down shifts. Cells heat shocked in light responded to darkness under both
normal (39°C) and elevated (47°C) temperatures with a rapid decrease in the rate of total RNA synthesis (Fig. 2B). This
rate was slightly higher in cells maintained at 47°C than in those returned to the initial growth temperature (39°C); Fig.
2B). Cells heat shocked in the dark responded to illumination

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**FIG. 1.** Rate of RNA accumulation in heat-shocked *Synechococcus* cells. At time zero, an exponential-phase culture (A\(_{600}\) 0.120) was divided into two parts, and [3H]uracil (185 kBq ml\(^{-1}\)) was added to both cultures. The subcultures were incubated at normal (39°C, \(\bullet\)) and elevated (47°C, \(\bigstar\)) temperatures. Samples were pipetted into TCA, and uracil counts were determined in the TCA-insoluble fraction as described in Materials and Methods.
FIG. 2. Rate of total RNA synthesis in *Synechococcus* cells under various shock conditions. An exponential-phase culture continuously labeled with [*C*]uracil overnight was shifted from normal (30°C) to elevated (47°C) temperature in light and in the dark and pulse-labeled with [*H*]uracil for 10 min (1.2 MBq ml⁻¹). After each pulse, the alkali soluble radioactivity was measured as described in Materials and Methods. The ratio of incorporation of [*H*]uracil to [*C*]uracil at the end of the initial 39°C incubation was defined as 1, and the rate of total RNA synthesis was calculated.
with a rapid increase in RNA synthesis (Fig. 2C). Cells illuminated at 47°C displayed the more dramatic, immediate increase in RNA synthesis, followed by a gradual decrease which ultimately reached a new steady state of total RNA synthesis (Fig. 2C). Cells reincubated under the initial growth conditions showed a less pronounced initial response to reillumination, but one which seemed more evenly sustained over the time period. Cells which were maintained in the dark during and after heat shock and were shifted to the lower (growth) temperature did not synthesize RNA at the increased rate characteristic of similar, but illuminated, cells (Fig. 2D). Instead a second rapid decrease in synthesis, but of a more limited magnitude, typified the cells, which then established a moderate rate of RNA synthesis.

Darkness per se, rather than incubation temperature, was the imposed variable affecting the rapidity and magnitude of the cellular response vis-à-vis RNA synthesis when a treatment of heat plus illumination followed the initial 2-h stress period (cf. Fig. 2C and E). The kinetics of RNA synthesis in heat-shocked cells seemed to be similar, independent of the preceding fate of cells (Fig. 2A through D). Cells heat shocked in the dark respond to light shift up at the normal temperature with an initially rapid and then a gradual increase in the rate of RNA synthesis (Fig. 2C). However, the kinetics of RNA synthesis differed between the cultures heat shocked in light versus in the dark; the cells heat shocked in light responded to normal temperature and light first with a decrease and then an increase in the rate of RNA synthesis (Fig. 2A), while cells heat shocked in the dark responded to normal temperature and light only with an increase in the rate of RNA synthesis (Fig. 2C). These kinetics of RNA synthesis were the same as those seen when a culture grown in the dark at normal temperature was shifted to light (Fig. 2E). A decrease in temperature of a culture both heat shocked and kept in the dark resulted in a slight but significant decrease in the rate of total RNA synthesis. In addition, there was an immediate inhibition of rRNA synthesis in heat-shocked Synechococcus cells in the light, and the elevated temperature seemed to inhibit the light-dependent in vivo postmaturational cleavage of 23S rRNA (data not shown).

**ppGpp Accumulation after temperature upshifts under light or dark regimens.** In illuminated Synechococcus sp., after a temperature increase to 47°C, there was a rapid accumulation of ppGpp to about 6- to 10-fold over the basal level (Fig. 3). The accumulation of this nucleotide reached a peak after 10 min of heat shock in light, but this level was substantially lower than that noted in the dark (energy starvation) condi-
Effect of various separate and simultaneous treatments (heat shock and darkness) on the ppGpp level in *Synechococcus* cells. The cultures were shifted from normal temperature (39°C) and light conditions to the indicated conditions at time zero; 1 h later, the cultures were divided into two parts and shifted again. In the time zero samples of cultures, the basal level of ppGpp varied between 21 and 39 pmol per A₈₅₀ unit. The relative concentration of ppGpp was calculated as explained in the legend to Fig. 3.

FIG. 6. Effect of various separate and simultaneous treatments (heat shock and darkness) on the ppGpp level in *Synechococcus* cells. The cultures were shifted from normal temperature (39°C) and light conditions to the indicated conditions at time zero; 1 h later, the cultures were divided into two parts and shifted again. In the time zero samples of cultures, the basal level of ppGpp varied between 21 and 39 pmol per A₈₅₀ unit. The relative concentration of ppGpp was calculated as explained in the legend to Fig. 3.

Sections at the initial growth temperature (39°C). The decline of ppGpp level was fairly slow under either condition; cells subjected to a temperature increase maintained their increased ppGpp content at a rather stable level for at least 2 h, which was similar to the behavior of cells at 39°C in the dark (5, 17; Fig. 3). To study whether the increase in ppGpp level of heat-shocked cells was due to changes in synthesis or degradation or both, chloramphenicol (100 μg ml⁻¹) was added to the culture (7, 15, 23). After the addition of chloramphenicol, there was an immediate dramatic decrease in the pool size of ppGpp in heat-shocked *Synechococcus* sp. (Fig. 4), as would be expected if the effect of synthesis on the ppGpp level was more significant than was a change in the rate of degradation of ppGpp (7). The ppGpp level remained unexpectedly high for a longer period of time if light deprivation and heat stress regimens were simultaneous (Fig. 5). Under conditions of constant illumination, a shift up in temperature was followed by an increase in ppGpp level, and a subsequent return to the initial temperature resulted in an equally rapid decrease to a near-basal level of ppGpp (Fig. 6A). When the shift up was instead followed by darkness, there was another, even more significant increase in ppGpp levels under both normal (growth) and continued heat stress conditions (Fig. 6B).

When heat stress was applied concomitantly with dark incubation, the increase in ppGpp was 10-fold greater (Fig. 6C) than that seen under conditions of heat and illumination (Fig. 6A). The return of light brought about a rapid decrease
EFFECT OF DARKNESS ON THE PATTERN AND KINETICS OF THE SYNTHESIS OF HEAT SHOCK PROTEINS. To examine how darkness (energy deprivation) alters the response of *Synechococcus* sp. to heat shock treatment, the control (39°C) and heat-shocked (47°C) cultures were simultaneously put in the dark and pulse-labeled with 14C-labeled protein hydrolysate. In these cells, the same major heat shock polypeptides were synthesized as we have described elsewhere (6) for cells heat shocked in the light, albeit at a lower rate (Fig. 7). The specific pattern of polypeptide synthesis seen in cells in the dark at normal temperature was altered at the elevated temperature (Fig. 7); the synthesis of several dark-specific proteins with unknown functions was abolished in the dark, elevated-temperature conditions. The molecular masses of these polypeptides are indicated in Fig. 7. The disappearance of the 80.0-, 17.8-, 15.4- and most obviously, the 23.0-kilodalton (kDa) dark-specific proteins was characteristic.

To compare the kinetics of polypeptide synthesis in light-deprived cells (39°C) with cells both light deprived and heat shocked (47°C), samples were pulse-labeled for 30 min with 14C-labeled protein hydrolysate over a 2.5-h period. Some polypeptides (e.g., the 80.0-, 23.0-, 17.8-, and 15.4-kDa proteins; Fig. 8A) were synthesized transiently solely in the dark, while others (e.g., the 44.5-, 31.7-, and 26.2-kDa proteins; Fig. 8B) were synthesized transiently in the dark while heat stressed. On these two autoradiograms, the above-mentioned phenomenon, i.e., the lack of several dark-specific proteins in heat-shocked cells in the dark, is even more obvious (Fig. 8A and B). The results are summarized in Table 1.

**DISCUSSION**

In cyanobacteria, experimental perturbation of growth alters the rate of stable RNA accumulation (9, 17). Although few studies have been done on the phenomenon in cyanobacterial systems, it is clear that darkness (energy deprivation) may control stable RNA synthesis, as is known for heterotrophic systems in which availability of charged tRNA is altered by inhibitors or by nutritional step-down procedures (3, 9, 24, 25). Since we recently demonstrated that heat stress (elevated temperature [47°C]) reduces growth rate and protein synthesis in the cyanobacterium *Synechococcus* sp. (6), we expected changes in RNA synthesis and in the pool size of highly phosphorylated nucleotides to accompany this physiological stress as well. Indeed, after a temperature increase to 47°C, the RNA accumulation increased during the early period of stress treatment, subsequently decreased, and again increased near the end of the temperature shock period. The explanation of this oscillation in the rate of RNA accumulation is not known, but recent observations on *E. coli* (16, 21, 22) may be relevant. In *E. coli*, an inverse correlation exists between ppGpp content and the rate of total RNA synthesis during temperature shifts up and down except when the elevated temperature directly affects RNA chain elongation. To analyze more precisely the effect of elevated temperatures on RNA synthesis, we measured the rate of total RNA synthesis in *Synechococcus* sp. during transitions from a normal growth temperature (39°C) to an elevated one (47°C) and from light (photosynthesis) to the dark (energy deprivation). By measuring the rate of total RNA synthesis rather than only RNA accumulation, an immediate increase
in RNA synthesis was observed after temperature stress (shift up). A subsequent decrease in the rate of total RNA synthesis (at 10 to 30 min) preceded a gradual increase and a new steady-state rate of RNA synthesis during the latter period of heat stress. These alternations in the rate of total RNA synthesis were correlated with dramatic changes in ppGpp pool size. When the ppGpp level rose, cessation in RNA synthesis was observed. Under heat shock conditions, the higher level of ppGpp coincided with a lower rate of RNA synthesis. This phenomenon was even more apparent if the ppGpp level and the rate of total RNA synthesis were compared in the dark. The slight decrease in ppGpp content appeared to trigger a subsequent increase in the rate of total RNA synthesis; this seemed to be true not only for the heat shock period, but also for the recovery period, when the temperature was returned to normal (39°C), except for a lag period. Thus, this study extends to an obligate photosynthetic cyanobacterium the inverse correlation between ppGpp levels and the rate of RNA synthesis, a phenomenon which thus far had been known only for heat-stressed heterotrophic organisms (16, 21, 22). Under light, the primary difference observed was in the early period of heat shock, when the expansion of the ppGpp pool was accompanied by an increase in the rate of total RNA synthesis. The explanation of this phenomenon was not clear in our system. Although we have not measured the elongation rate of RNA chains in heat-shocked cyanobacterial cells, we see no reason to suppose that the elevated temperature has different effects on RNA chain elongation in Synechococcus sp. than in E. coli (21, 22). A similar intriguing irregularity existed between RNA synthesis and ppGpp pool size when heat-shocked cells were returned to normal (growth) temperature either in light or in the dark. Under these conditions of decreasing RNA synthesis, a lag period preceded the new steady-state rate of RNA synthesis. This irregular, concomitant oscillation of RNA synthesis and ppGpp pool size was seen only at the beginning of temperature shifts, when changes of temperature may have had an immediate and direct effect on RNA chain elongation rates.

For heterotrophic bacteria, the control of RNA and protein synthesis during transitions of growth rate is well understood in two systems, i.e., in situations in which alterations are due to restricted tRNA aminoaacylation and in systems in which changes are the consequence of manipulations of carbon or energy sources (19). However, unlike the situation in heterotrophs, in which the sources of carbon and energy are usually one and the same, for this cyanobacterium, the source of energy (light) differs from the source of carbon (bicarbonate); thus, the use of heat stress may be a valuable experimental approach for the analysis of potential differential effects of energy versus carbon starvation on RNA accumulation. In heterotrophic organisms, ppGpp plays a physiologically important role in the regulation of translation as well as of RNA synthesis (14, 18, 20).

Accordingly, this study is of interest, at least qualitatively, for the consequences of darkness on the cyanobacterial heat shock proteins. Singer and Doolittle (23) demonstrated that
the lack of light provokes specific changes in the pattern of protein synthesis in *Synechococcus* sp. Our more detailed analysis of this phenomenon at normal growth temperature thus extends and refines these observations by showing that there are indeed three classes of proteins that can be observed: (i) proteins transiently synthesized in the dark, (ii) polypeptides accumulated only in the dark, and (iii) proteins synthesized (but not necessarily at the same rate) both in light and in the dark. Nonetheless, we are left with the necessity of explaining what may be the function(s) of dark-specific proteins and now of the heat shock proteins in cyanobacteria. These proteins may provide an experimental system for a better understanding of dark-specific cyanobacterial gene expression. Indeed, we infer that the synthesis of heat-shock (stress) proteins in the dark indicate a significant role for these proteins in cell homeostasis. In cultures simultaneously light deprived and heat shocked (47°C), we observed (i) the main heat shock proteins, (ii) several transiently synthesized dark-specific proteins (of 44.5, 31.7, and 26.2 kDa), and (iii) polypeptides synthesized in the cells under each condition (light, dark, and heat shock in the dark). However, 80.0-, 23.0-, 17.8-, and 15.4-kDa proteins, which seem to be dark specific under normal growth conditions (39°C) (23), are abolished by heat. Thus, these proteins may have a specific role(s) in cells in the dark; the phenomenon needs further study in both obligate as well as facultative autotrophic cyanobacteria.

Our consideration of the results reported here leads us to the expectation that the high and more or less stable level of ppGpp in heat-shocked and light-deprived *Synechococcus* sp. may have pleiotropic effects on cyanobacterial metabolism.

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