Plant Physiol. (1986) 80, 918–925
0032-0889/86/80/0918/08/$01.00/0

Effects of Environmental Stresses on the Cell Cycle of Two Marine Phytoplankton Species[1]

Received for publication October 5, 1985 and in revised form December 10, 1985

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ABSTRACT

Cell cycle phase durations of cultures of *Hymenomonas carterae* Braarud and Fagerl, a coccolithophore, and *Thalassiosira weissflogii* Grun., a centric diatom, in temperature-, light- or nitrogen-limited balanced growth were determined using flow cytometry. Suboptimal temperature caused increases in the duration of all phases of the cell cycle (though not equally) in both species, and the increased generation time of nitrogen-limited cells of both species was due almost wholly to expansion of G_1 phase. In *H. carterae* light limitation caused only G_1 phase to expand, but in *T. weissflogii* both G_2 + M and G_1 were affected. These results are discussed in relation to cell division phasing patterns of these two species and to models of phytoplankton growth. Simultaneous measurements of protein and DNA on individual cells indicated that under all conditions, the protein content of cells in G_1 was a constant proportion of that of G_2 + M cells. Simultaneous measurements of RNA and protein on each cell indicated that the amounts of these two cell constituents were always tightly correlated. Under conditions of nitrogen limitation both protein and RNA per cell decreased to less than one-third of the levels found in nonlimited cells. This indicates, at least for nitrogen-replete cells, that neither protein nor RNA levels are likely to act as the trigger for cell cycle progression. Strict control by cell size is also unlikely since mean cell volume decreased as growth rates were limited by light and nitrogen supply, but increased with decreasing temperature.

In many cell types, a point (called restriction point, transition point, or 'start') has been identified, beyond which the cell is committed to go through the DNA cycle to mitosis and cytokinesis (28). The determining factor for the restriction point has been suggested (10) to be size-related (critical cell mass or protein content) or to be a function of a specific protein (21). The time required for a cell to fulfill the critical condition can be variable, and adverse environmental conditions often result in an increase in the duration of G_1 phase of the cell cycle (23). The durations of the S and G_2 phases can also vary, but generally not by as much as G_1 (12).

Our interest in the interaction between the growth cycle and the DNA-division cycle began in the context of attempts to understand observed cell division patterns of phytoplankton populations growing in periodic environments. It is well known that cell division in unicellular algae can be entrained to periodic supplies of light (2) or limiting nutrients (17, 20). The simplest interpretation of this observation is that the physiological processing of the limiting substance has a 'preferred' timing in the cell division cycle, so that cells whose cycles are out of phase with the periodic supply are forced to 'wait' for the supply before progressing to the next stage of the cycle. This is, in a sense, an expression of the block point hypothesis of Spudich and Sager (24), who were able to explain the cell division patterns of *Chlamydomonas* growing on L/D[4] cycles by hypothesizing an arrest and transition point in the cell cycle which bound a light-requiring segment in G_1.

We have been investigating for some time the unusual cell division phasing patterns of marine diatoms, some of which show multiple division peaks during each L/D cycle (3, 4, 19). It is clear that these cells process their light supply differently from species such as coccolithophorids and dinoflagellates which exhibit strict phasing to L/D cycles (17). Since it is likely that these differences can be explained in terms of constraints on cell cycle progression, we have begun to examine these species in this context.

There are two logical approaches to identifying stages of the cell cycle which contain block points or disproportional dependencies on a given growth factor. One is to deprive completely the cells of the factor being examined, and determine at what cell cycle stage the cells arrest when population growth ceases. The results of studies using this approach are reported elsewhere (26), and are consistent with the conclusions of this paper. A second approach, which we have employed here, is to look at the expansion of various cell cycle stages in response to a suboptimal supply of a growth regulating substance. If the average growth rate of an exponentially growing culture is known, the duration of each cell cycle stage can be calculated from the distribution of DNA/cell measured using flow cytometry (23). When the growth rate is reduced by suboptimal environmental conditions (such as reduced light energy), one or more of the cell cycle stages must be extended. The relative expansion of each stage with increasing generation time reflects the sensitivity of that stage to a particular environmental variable; those stages which expand preferentially are likely to have transition points which are dependent upon the variable in question. Similarly, the phase angle of the cell cycle alignment to a periodic supply of the variable should reflect the position of this transition point. In addition to measuring the response of the DNA-cycle to growth-limiting substances, we have also measured several cell growth parameters (volume,

[1] Supported in part by National Science Foundation grants OCE-8211525, OCE-8118475, OCE-8121270, OCE-8316616, CEE-8211525, by grant N00014-83-K-0661 from the Office of Naval Research, and by the Doherty Professorship of Ocean Utilization awarded to S. W. Chisholm.

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[4] Abbreviations: L/D, light/dark; PI, propidium iodide; FITC, fluorescein isothiocyanate.

protein, and RNA per cell) in an attempt to identify the link between the DNA-division cycle and the growth cycle of the cell.

MATERIALS AND METHODS

Culture Conditions. *Hymenomonas carterae* Braarud and Fagerl (clone Cocco II) and *Thalassiosira weissflogii* Grun. (clone Actin) were obtained from R. Guillard (Bigelow Laboratories, Boothbay Harbor, ME) and were grown in f/2 enriched seawater medium (13) with ammonium as the nitrogen source. The sea water was obtained from ESL, Woods Hole Oceanographic Institution. Nitrogen-limited cultures were grown in 1-L chemostat vessels at 21°C in continuous light (cool-white fluorescent lamps, 100 μE m^{-2} s^{-1}). The cultures were stirred with Teflon-coated stirring bars and bubbled with sterile-filtered, moisturized air. The medium for the chemostat cultures was made up with twice the standard amount of Si and with 50 μM ammonium. It was prepared from seawater which had been previously stripped of N by addition of N-limited phytoplankton and all f/2 nutrients except N. Dilution rates were varied by changing the rate of medium addition, and cell concentration and cell volume distributions were measured daily using a Coulter Counter (model Z_F with P-128 size distribution analyzer). The cultures were allowed to reach steady state and remain there for at least 4 days before sampling for flow cytometric analyses.

Light- and temperature-limited cultures were maintained in 1- or 2-L Erlenmeyer flasks, which were mixed daily and/or before sampling by swirling. The f/2 medium with 200 μM ammonium as the N source was used for these experiments. Illumination was continuous at 100 μE m^{-2} s^{-1} for the temperature-limited cultures and from 10 to 70 μE m^{-2} s^{-1} for the light-limited cultures. Temperature was maintained at 21°C for the light-limited cultures and from 13 to 23°C for the temperature-limited cultures. Cell division rate was analyzed by monitoring changes in cell concentration (with the Coulter Counter) and the cultures were diluted with new medium to maintain exponential growth for at least six generations before sampling for flow cytometry. Transfers and sampling were done when cell concentrations were at 25% or less of the asymptotic cell concentration for each condition to avoid possible effects of approach to stationary phase.

With the goal of linearizing the data, the growth rates of the populations are expressed as either the specific growth rate, μ,

$$\mu = 1/t \ (\ln N_t/N_o) \quad (1)$$

or as doubling time, t_D,

$$t_D = \ln 2/\mu \quad (2)$$

depending upon the characteristics of the growth-dependent parameter being analyzed.

Sampling for Flow Cytometric Analysis. In preparation for fixation and staining, several replicate samples containing 10^6 cells each were concentrated by centrifugation in 50-ml tubes at 2000*g* for 10 min. All but 0.5 ml of the supernatant was removed by aspiration and the pellet was resuspended. The cell suspension was then injected through a hypodermic needle (24 gauge) into 10 ml of ice cold absolute methanol to fix the cells and remove Chl (18). The samples are stable in this form at 4°C and can be stored for up to a year before analysis.

Staining and Analysis. Cells were stained for DNA and protein with PI and FITC, using a modification of the method of Crissman and Steinkamp (5). Cells (10^6) were removed from methanol by centrifugation at 2000*g* for 10 min. The pellet was resuspended in 1.5 ml PBS and spun down at 14,000*g* for 3 min; the cells were then resuspended in PBS. PI (4 μg/ml), FITC (5 μg/ml), and RNase A (0.25 mg/ml = 1,250 units; Worthington), which eliminates interference by double-stranded RNA, were then added and the sample allowed to incubate at room temperature (20°C) for 1 h. The samples were then refrigerated at 4°C and analyzed within 4 h.

Cells were stained for RNA and protein by a modification of the same method (26): RNase was replaced by DNase (40 μg/ml = 470 units; Worthington) and the cells were stained in filtered seawater rather than PBS.

Flow Cytometric Analyses. The stained samples were analyzed using an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). A 488 nm laser line (500 mW) was used to excite PI fluorescence (greater than 630 nm), and FITC fluorescence (515–560 nm), which were measured simultaneously on each cell. Each sample represents measurements of 2 to 5 × 10^4 cells. The data were stored on floppy disks in two ways: as single-parameter histograms of 256-channel resolution, and as two parameter sets of correlated data, of 64 × 64 channel resolution. Typical output from the flow cytometer (from cultures in unlimited exponential growth) is illustrated in Figure 1. Note that the distributions of protein (A, F) and RNA (B, G) per cell are unimodal and skewed toward smaller values, while DNA (C, H) has a bimodal distribution. The correlated histograms of RNA *versus* protein (D, I) reveal a tight correlation between these two parameters while those of DNA *versus* protein (E, J) are clearly resolvable into G_1 and G_2 + M populations (note that cells in G_2 + M have higher protein contents than G_1 cells). We must point out that these fluorescence measurements have not been independently calibrated against chemical measurements of DNA, RNA, or protein; this is not a serious problem in the case of DNA because of its 'quantized' nature, but precludes the assignment of absolute values to our results for RNA and protein levels.

Data Analysis. The flow cytometric data was transferred to an IBM 9000 computer, where subsequent data analyses were carried out. Single-parameter DNA distributions (Fig. 2, A, C) were analyzed by a modification of the method of Dean and Jett (8). Their original method fits Gaussian distributions to the G_1 and G_2 + M peaks, and a quadratic equation to S phase; an alternative is to approximate S phase by a series of broadened rectangles (P. Dean, personal communication), or as a broadened rectangle extending from the center of the G_1 peak to the center of the G_2 + M peak, as we have adopted here. The values of the parameters (coefficient of variation and positions of peak, height of S, and number of cells in G_1, S, and G_2 + M) is determined by a nonlinear fitting routine supplied by P. Dean.

The DNA histogram analysis method used was chosen because it is relatively insensitive to skewness in the G_1 and G_2 + M peaks. In many (but not all) samples, the G_1 peak was indeed skewed toward larger values of DNA/cell (26). When the histograms were analyzed by the other, less rigidly constrained methods, a better fit to the data could be obtained, but the analysis often resulted in large and variable values for the proportion of the population in early S phase. The results were also quite sensitive to sample-to-sample differences in peak resolution. Having established that the conclusions of this study are independent of the DNA histogram analysis method used, we have chosen to present the results of the simplest fitting routine, which yielded the greatest internal consistency in the data. A detailed discussion of the possible causes of the skewness in the G_1 peak appears elsewhere (26).

The durations of the cell cycle stages, $T(x)$, were calculated from the proportions of the population in each stage, $P(x)$, and the doubling time of the population in balanced growth, t_D, using the equations of Slater *et al.* (23):

$$T(G_1) = -(t_D/\ln 2) \ln (1 - [P(G_1)/2])$$
$$T(G_2) = (t_D/\ln 2) \ln (1 + [P(G_2)])$$
$$T(S) = (t_D/\ln 2) \ln (1 + 3P(S)/[1 + P(G_2)])$$

Dual-parameter correlated histograms (of DNA *versus* protein and RNA *versus* protein) were analyzed using programs devel-

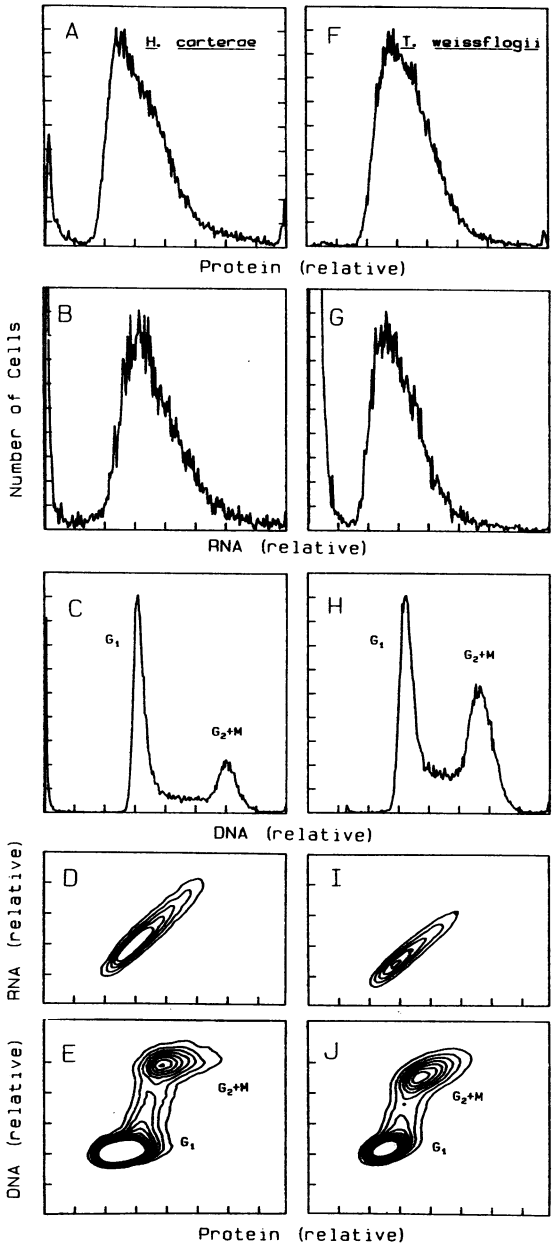

FIG. 1. Flow cytometric analysis of protein, RNA and DNA contents of *H. carterae* (A–E) and *T. weissflogii* (F–J) cells in unlimited exponential growth. At least 2.5×10^4 cells were analyzed. A and F, Distribution of relative protein/cell (FITC fluorescence); B and G, distribution of relative RNA/cell (PI fluorescence of DNase-treated cells); C and H, distribution of relative DNA/cell (PI fluorescence of RNase-treated cells); D and I, 2-parameter contour plot of RNA *versus* protein. Contour intervals were from 20 to 100 cells in steps of 20; E and J, 2-parameter contour plot of relative DNA *versus* protein. Contour intervals are from 10 to 100 cells in steps of 10.

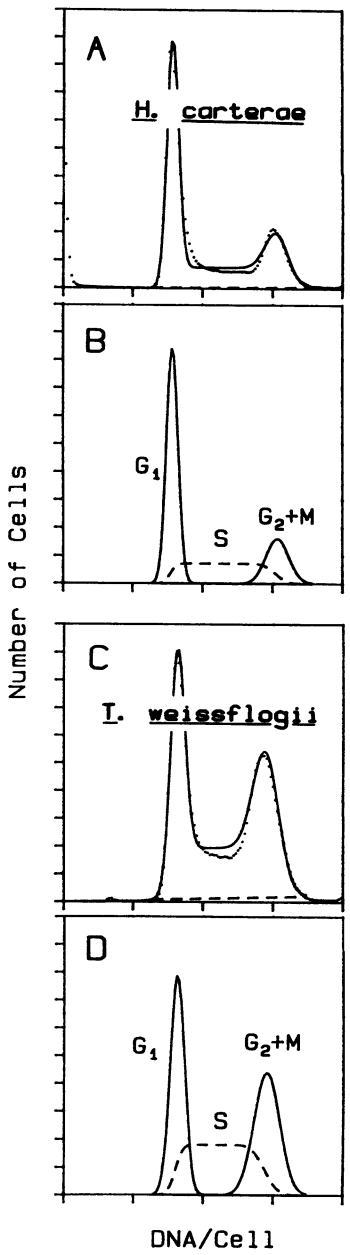

FIG. 2. Distribution of cell cycle stages during exponential growth under optimal conditions for *H. carterae* (A, B), and *T. weissflogii* (C, D). (A, C), Raw data (····) and the curves fitted to them (——); (B, D), deconvoluted cell cycle stages.

Table I. *Cell Cycle Durations under Optimal Growth Conditions*

Species	t_D	Percent of Cell Cycle Spent in			Duration of		
		G_1	S	$G_2 + M$	G_1	S	$G_2 + M$
	h					*h*	
H. carterae	14	42	34	24	5.8	4.8	3.4
T. weissflogii	8	26	33	41	2.1	2.6	3.3

oped in this laboratory for the IBM CS 9000 computer. Analyses include enumeration and projection of single-parameter histograms from selected windows in the 2-parameter data, simple statistics on these (mean, mode, CV, correlation coefficients), and contour plotting of the correlated histograms.

RESULTS

Hymenomonas carterae. The maximum growth rate attained by *H. carterae* in our experiments was 1.2 d^{-1} (corresponding to a population doubling time [t_D] of 14 h). Examination of the raw DNA distribution (Fig. 2A) gives the immediate impression that most *H. carterae* cells in unlimited exponential growth are in the early part of their DNA cycle. Analysis of the histogram (Fig. 2B; Table I) confirms this impression: *H. carterae* cells spend the largest portion of their cell cycles in G_1 phase (42%) with 34 and

24% in S and G_2 + M, respectively. The mode of protein content of the G_1 subpopulation was 0.67 that of the G_2 + M subpopulation.

When growth was limited by low temperature, all DNA cycle phases in *H. carterae* were lengthened, though not equally (Fig. 3A). As total cycle time increased, the length of G_1 phase increased slightly faster than the other phases (39% of total increase). The mean cell volume of *H. carterae* decreased as the temperature-controlled growth rate increased (37%/d^{-1}) (Fig. 4) but the mode of the protein and RNA values per cell did not change significantly as a function of temperature and the ratio RNA/protein remained nearly constant (Fig. 5) (slope not significantly different from 0, $P < 0.10$). The mode of protein content of the G_1 subpopulation remained a constant fraction (mean ± 95% confidence limits = 0.66 ± 0.03) of that of the G_2 + M cells.

Limitation of growth rate by light caused only minor expansion of S and G_2 + M phases in *H. carterae* (Fig. 3B); nearly all the increased cycle time (82%) was spent in G_1. In contrast to the temperature-limited case, mean cell volume increased with increasing growth rate (58%/d^{-1}) (Fig. 4). Also, it appears that RNA per cell and the RNA/protein ratio in *H. carterae* increased slightly with light-controlled growth rate, but there was too much noise in the protein data for this regression to be significant (Fig. 5B). The ratio of protein in the G_1 cells to that in G_2 + M cells again was relatively constant, at 0.63 ± 0.02.

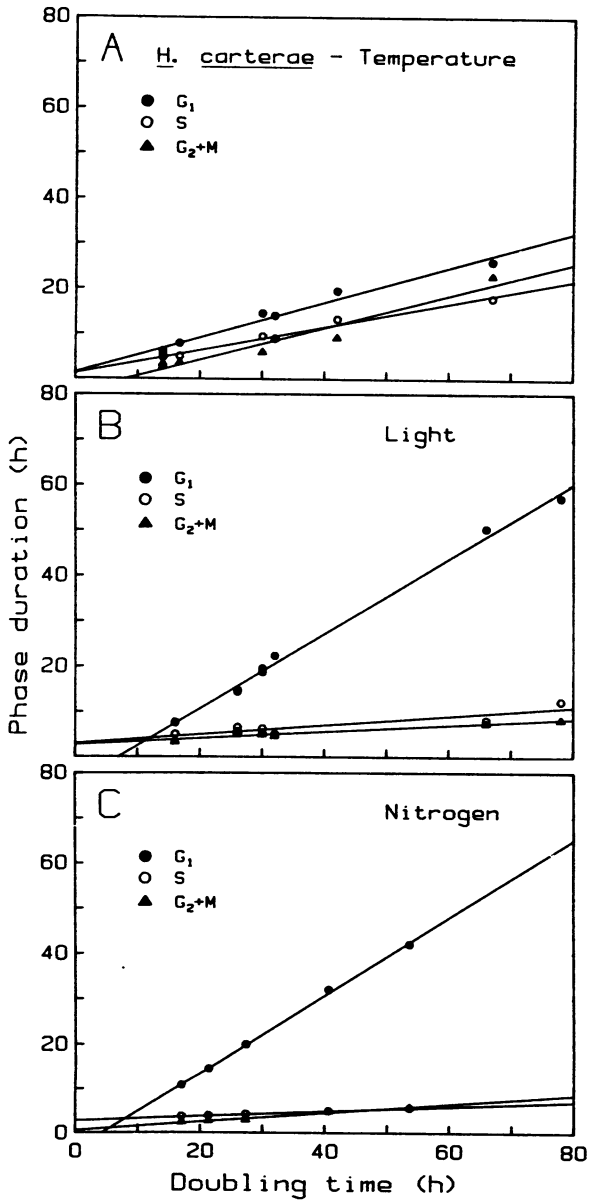

FIG. 3. Durations of cell cycle stages in *H. carterae* as functions of the population doubling time (t_D) controlled by temperature (A), light (B), and ammonium limitation (C). Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$).

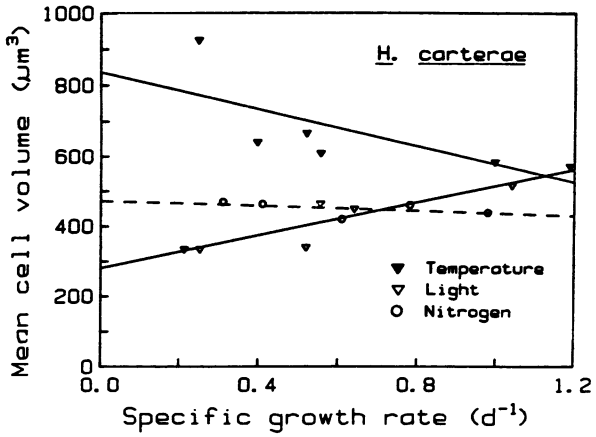

FIG. 4. Mean cell volume (as measured with a Coulter Counter) for *H. carterae* as a function of the specific growth rate (μ) controlled by temperature, light, and ammonium. Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$). Slopes of dashed lines were not significantly greater than zero.

Limitation of growth rate by N deficiency caused only minor expansion of S and G_2 + M phases; about 85% of the increased cycle time was spent in G_1 (Fig. 3C). Mean cell volume was virtually unaffected by increasing degrees of N-limitation (Fig. 4) but the cell volume of these N-limited cells was about 30% lower than for nonlimited cells. The mode of protein and RNA values increased dramatically with division rate (Fig. 5C), such that the protein value at the slowest growth rate was about 40% that of N-replete cells. The RNA values dropped to about 20% of the N-replete value, so that the ratio of RNA/protein *increased* with increasing division rate. The ratio of protein in G_1 cells to that in the G_2 + M cells was, as before, relatively constant, at 0.65 ± 0.02. Thus, none of these modes of growth limitation caused significant differences in this ratio.

Thalassiosira weissflogii. The maximum growth rate of *T. weissflogii* in our experiments was 2.0 d^{-1} (population doubling time of 8 h). In contrast to *H. carterae*, the largest portion of *T. weissflogii*'s cell cycle was spent in G_2 + M (41%), with 33% in S and only 26% in G_1 (Fig. 2B; Table I). The ratio of protein contents of G_1 to G_2 + M cells was slightly higher than that of *H. carterae* at 0.75.

The cell cycle response of *T. weissflogii* to limitation by suboptimal temperature was similar to that of *H. carterae* in that all phases of the cycle were expanded, but in *T. weissflogii* the length of G_2 + M increased slightly more than the others (44% of the total; Fig. 6A). The mean cell volume of *T. weissflogii* decreased as temperature-controlled growth rate increased, although to a lesser extent (13%/d^{-1}) than in *H. carterae* (Fig. 7). In contrast to the situation in *H. carterae*, protein mode values increased with temperature-controlled growth (Fig. 8A), but the RNA/protein ratio did not change. The ratio of protein contents of G_1 and G_2 + M cells was constant over the range of growth rates but higher (0.73 ± 0.02) than in *H. carterae*.

The effects of N limitation on the cell cycle of *T. weissflogii* were similar to those noted for *H. carterae* (Fig. 6C). The decrease in mean cell volume compared to nutrient-replete cells (about

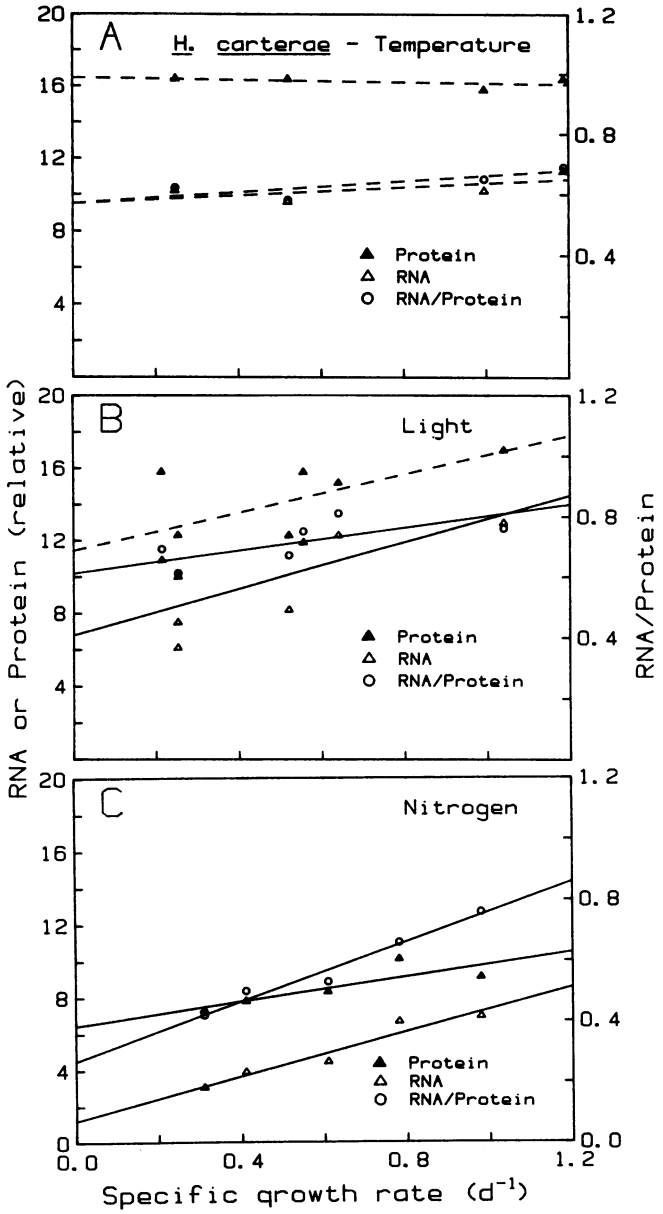

FIG. 5. Mode of the protein and RNA distributions (as determined by flow cytometry) for *H. carterae* as functions of the specific growth rate (μ) controlled by temperature (A), light (B), and ammonium limitation (C). Units of protein and RNA are relative. Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$). Slopes of dashed lines were not significantly greater than zero.

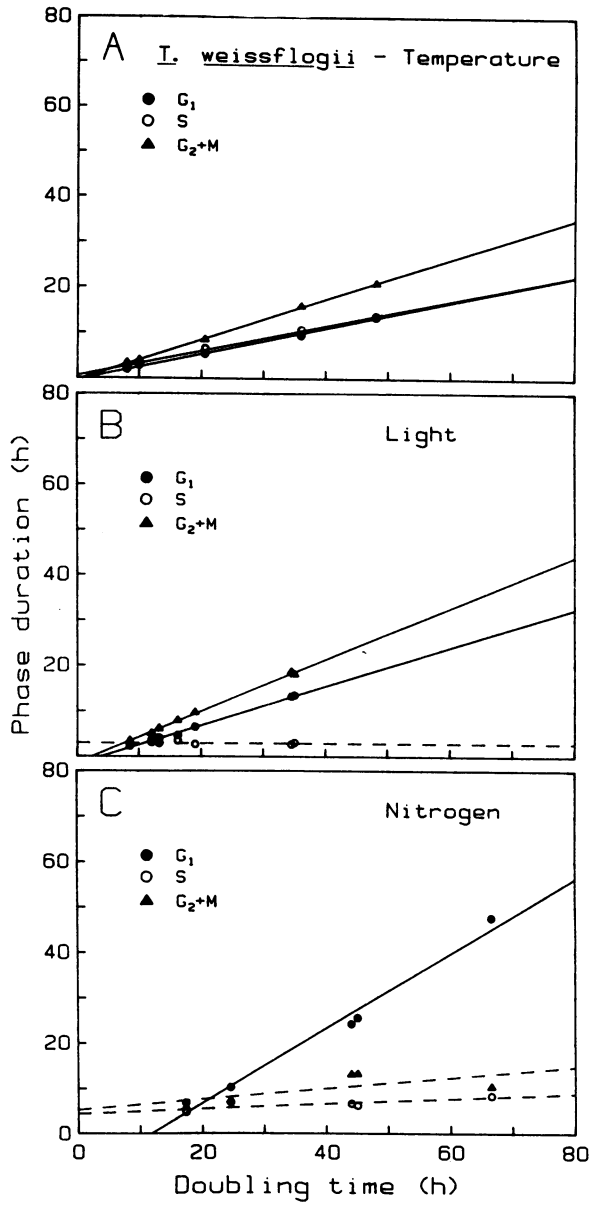

FIG. 6. Durations of cell cycle stages in *T. weissflogii* as a function of the population doubling time (t_D) controlled by temperature (A), light (B), and ammonium limitation (C). Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$). Slopes of dashed lines were not significantly greater than zero.

15%) was somewhat less striking than in *H. carterae* (Fig. 7), but the decline in protein/cell was more dramatic, decreasing by 70% (Fig. 8C). The ratio of protein in G_1 cells to that in G_2 + M cells was again invariant with growth rate but slightly higher (0.71 ± 0.01) than in *H. carterae*.

Limitation of the growth rate of *T. weissflogii* by light revealed a major difference between the two species. In *H. carterae*, only the duration of G_1 phase of the cell cycle was expanded by light limitation, whereas in *T. weissflogii* the G_2 + M phase was expanded by the largest amount (57%). G_1 accounted for the rest of the increased cycle time (Fig. 6B). In terms of cell growth parameters, the responses of *T. weissflogii* were rather similar to those of *H. carterae*, although the change in mean cell volume was less striking (12%/d^{-1}) (Fig. 7). The ratio of protein in G_1 to that in G_2 + M cells was again higher than *H. carterae* (0.71 ± 0.01) but still constant. The RNA/protein ratio did not change significantly with light-controlled growth rate, but the RNA values in light-limited cultures were lower than those in temperature-limited ones, so the mean RNA/protein was lower for the light-limited cells (Fig. 8B) ($P < 0.05$, *t* test).

Observations of *T. weissflogii* cells made using time-lapse video microscopy indicated that there was about a 2 h lag between cytokinesis and cell separation during rapid growth (D Vaulot, SW Chisholm, unpublished data). Since unseparated postmitotic cells might 'physiologically' be in G_1 phase, yet are counted as G_2 + M cells by the flow cytometer, it is possible that the duration of G_2 + M phase in nonlimited *T. weissflogii* cells has been overestimated and that of G_1 phase underestimated. It is also possible that some of the increased duration of G_2 + M we observed using flow cytometry could actually be due to G_1

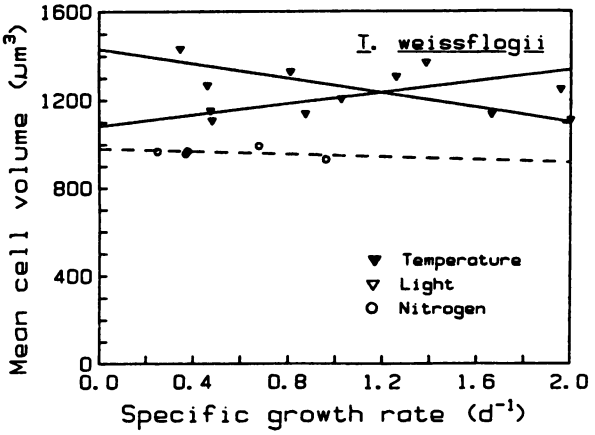

FIG. 7. Mean cell volume (as measured by Coulter Counter) for *T. weissflogii* as a function of the specific growth rate (μ) controlled by temperature, light and ammonium. Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$). Slopes of dashed lines were not significantly greater than zero.

phase expansion. To evaluate the latter possibility, we made direct counts of unseparated postmitotic cells with epifluorescence microscopy in those samples which exhibited large increases in the duration of G_2 + M. Eight hundred cells were counted from each sample and the duration of the postmitotic stage calculated by the same method as for the flow cytometry data. The duration of the postmitotic stage in the most severely light-limited culture was similar to that in the unlimited culture (2.6 h *versus* 2.8 h), which indicates that the influence of phase expansion in unseparated G_1 cells in the light-limited cultures was small.

We can also place an upper limit on the extent to which unseparated G_1 cells 'contaminate' the G_2 + M peak, and thereby influence the apparent trends of cell cycle phase expansion, by examining the data for N-limited cells. The expansion of G_2 + M as growth rate is slowed was only about 15% that of G_1 expansion, so (making the reasonable assumption that the duration of G_2 + M does not decrease as generation time increases) the contribution of G_1 cells to the measured G_2 + M peak must be less than 15%.

In contrast to the above cases, the duration of the postmitotic doublet stage in *T. weissflogii* in the most severely temperature-limited culture was increased to 9.5 h, *i.e.* by more than 3-fold. Thus, low temperature caused expansion of both G_2 + M and postmitotic stages in this species. Similar measurements of *H. carterae* cultures revealed that low temperature (the only environmental stress tested which affected the duration of G_2 + M phase in this species) also caused the duration of the postmitotic stage to increase relative to that in nonlimited growth (from 1.4–7.2 h).

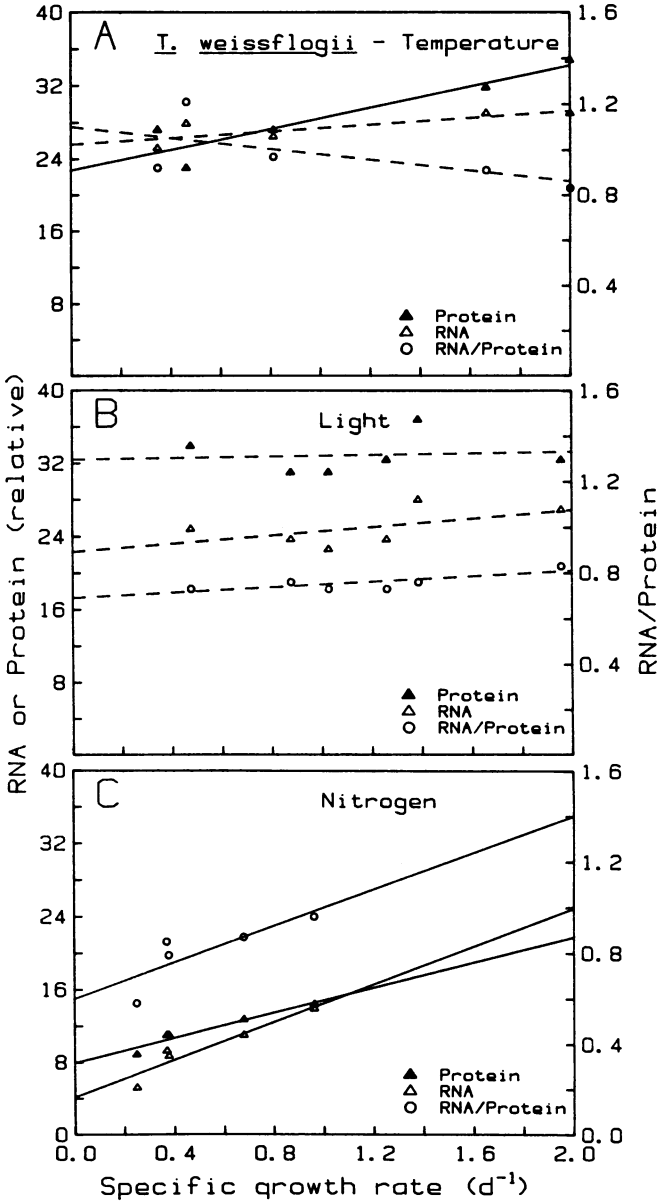

FIG. 8. Mode of the protein and RNA distributions (as determined by flow cytometry) for *T. weissflogii* as functions of the specific growth rate (μ) controlled by temperature (A), light (B), and ammonium limitation (C). Units of protein and RNA are relative. Solid lines are least-squares linear regressions whose slopes were significantly greater than zero ($P < 0.10$). Slopes of dashed lines were not significantly greater than zero.

DISCUSSION

DNA Cycle. The expansion of a cell cycle phase when temperature is lowered presumably is due to decreased enzymic reaction rates. Large increases in a particular phase duration might then indicate a preponderance of reactions with large Q_{10} values in that phase. Alternatively, if the cell cycle is controlled by a unique 'trigger' constituent, the Q_{10} of a single critical reaction might determine the temperature dependence. The extent to which each cell cycle phase expands when the total cell cycle time is increased by suboptimal temperature varies considerably among species, suggesting different modes of regulation in different organisms. In mammalian cell systems, there are reports of equal expansion of each phase, and of preferential expansion of G_1 phase (28). In yeasts, it has generally been found that all the phases expand as temperature is lowered, but that S expands less than the others (25). This was the response to suboptimal temperatures of both the phytoplankton species we examined.

The almost exclusive lengthening of G_1 phase of the cell cycle by N-limitation in both *H. carterae* and *T. weissflogii* implies that this phase is more N-dependent than the others. This is consistent with earlier findings that N-depleted cells of these species are arrested in G_1 phase (18), and that cell division in cultures in continuous light can be entrained to pulses of limiting ammonium (17). The preferential lengthening of G_1 we observed here was similar to that in N-limited chemostats of the dinoflagellate *Amphidinium carteri* (RJ Olson, SW Chisholm, unpub-

lished data), cultured higher plant cells (11) and the fission yeast *Schizosaccharomyces pombe* (16), and in batch cultures of the budding yeast *Saccharomyces cerevisiae* growing on different N sources (15).

The effects of light limitation on the durations of cell cycle phases are consistent with the findings of Vaulot (26) that *H. carterae* arrests in darkness only in G_1 while *T. weissflogii* can arrest in either G_1 or G_2 + M (but not in S). The expansion of G_1 phase in light-limited *H. carterae* may be related to the expansion of the light-dependent stage described for *Chlamydomonas* (24) and *Thalassiosira pseudonana* (14). The additional expansion of G_2 + M phase in *T. weissflogii* implies that in this species there is a light-dependent segment of the cell cycle in G_2 + M as well.

This striking difference between the species' responses to light limitation helps us interpret their cell division patterns on L/D cycles: *H. carterae* entrains strictly to L/D cycles with division occurring primarily during the dark period, while *T. weissflogii* entrains poorly, with division occurring throughout the L/D cycle, and preferentially during the light period (3). Vaulot (26) hypothesized that the division patterns of *T. weissflogii*, if regulated by a cell cycle block point, must originate at least in part from a light dependent transition point occurring late in the cell cycle. This is consistent with the hypothesis that there exists a light-dependent cell cycle segment in G_2 + M, and intuition suggests that the complex division patterns expressed by L/D entrained diatom populations have their origins in this unique requirement. Finally, we note that *A. carteri*, whose cell division pattern in L/D cycles resembled that of *H. carterae*, also showed expansion of only G_1 phase when grown in continuous limiting light (RJ Olson, SW Chisholm, unpublished data).

Cell Growth Parameters. The increased volume of cells growing slowly due to suboptimal temperatures is consistent with observations of other species of phytoplankton (27), as is the increase in volume with increasing light-controlled growth rate (22). The decrease in volume that occurred between N-replete and N-limited conditions (about 30% for *H. carterae* and 15% for *T. weissflogii*) was not magnified by increasing degrees of N-limitation. This lack of variation in cell volume with varying growth rate has been observed for other species of marine phytoplankton grown in N-limited chemostats (1).

Changes in the average cell volume of populations in balanced growth must represent differential adaptation of cell growth and division rates. Since volume increases under temperature limitation, but decreases under light and N limitation, it is clear that low temperature affects the rate of cell division more than it does cell growth *per se*, while light and N limitation cause growth rate to be slowed more than division rate. The latter situation is undoubtedly linked to the primary importance of light and N in biosynthetic processes, although it is apparent from the cell volume trends that light and N supply must regulate cell growth in different ways.

The main point to be drawn from the RNA and protein data is the difference between N and the other limiting factors. The large decrease in both parameters as the cells are more N-stressed (which was not mirrored in cell volume) is to be expected because of the high N content of both RNA and protein molecules. Another indication of the difference between light and N in regulating growth is the observation that protein and RNA values in severely light-limited cells did not decrease in parallel with cell volume and in fact *increased* in *T. weissflogii*.

The decrease in RNA/protein ratio with increasing severity of N-limitation, brought about by the faster decline in RNA, suggests that RNA is more 'dispensable' to the cells. Since the bulk of RNA is ribosomal RNA, this may reflect the possibility that at low growth rates sufficient protein can be synthesized from a reduced amount of rRNA.

Links between Growth and DNA-Division Cycles. Identification of the critical factor for initiation of cell division would greatly facilitate modeling of the cell cycle. We can evaluate some possibilities as to this factor from this data set, since cell size, protein, and RNA content have all been suggested as possible controllers of the cell cycle. One of the most striking results of this work is the remarkable constancy of the ratio of protein contents of G_1 and G_2 + M cells (0.65 ± 0.02 and 0.71 ± 0.02 for *H. carterae* and *T. weissflogii*, respectively), over a wide range of protein levels and growth rates regulated by temperature, light, and N-limitation. This constancy is similar to that observed in the yeast *S. cerevisiae* (for the ratio of protein/cell at initiation of S and M phases) (25) and suggests that the DNA cycle is regulated by a link to cell growth at least two points in the cycle.

Regulation is not necessarily by protein, however; RNA content was highly correlated with protein in all the samples we analyzed ($r^2 \geq 0.81$), and has been proposed to regulate both yeast (15) and mammalian (7) cell cycles. A similar high degree of correlation between protein and RNA contents of individual cells has been reported in a mammalian system (6). In addition, the existence of a specific trigger protein that regulates cell cycle progression is also consistent with these observations. In fact, neither total protein nor total RNA are likely to be the primary means of regulation, since they can vary so much under conditions of N-limitation. At the lowest N-limited growth rates, the mean cell protein and RNA were less than half those of nonlimited cells; DNA synthesis must therefore have been initiated at cell protein and RNA levels less than those of newly born nutrient-replete cells. Thus, for instance, a light-limited cell would have at birth already attained the 'critical' levels of protein and RNA; clearly another mode of cell cycle reguation must be operating in this case. Such multiple levels of regulation have been described for systems such as the yeast *S. pombe* (16), in which a 'cryptic' size control has been found to operate only when the cells are grown under N-limitation (and are thus much smaller than normal cells). Under nutrient replete conditions all cells are larger than the critical size and the cell cycle is under the control of a 'timer' initiated at the time of mitosis of the mother cell (S phase begins at a constant time after mitosis rather than after a transition point in G_1). Both size and timer control of the cell cycle has been reported for *Chlamydomonas* (9). This may be analogous to the situation we have observed with N-limitation in the phytoplankton. The opposing trends we observed in the mean cell volume of cells whose growth had been limited by temperature and the other stresses are also not consistent with strict size control of the cell cycle.

Acknowledgments—We thank Sheila Frankel for technical assistance and Jane Marsh for typing the manuscript.

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