Changes in Chemical Components and Energy Charge during Growth Cycle of *Chattonella antiqua*

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The changes in cellular content of adenosine triphosphate (ATP) in *Chattonella antiqua* (Hada) Ono (Raphidophyceae), a red tide microorganism, were determined during growth in laboratory culture using ESM-enriched seawater. Carbon, nitrogen, and phosphorus in the cells were also analyzed. The cellular content of ATP decreased within several hours of inoculation into fresh medium, then rapidly increased during the early exponential phase of growth to 20–30 pg ATP cell⁻¹. After the mid exponential phase ATP decreased and reached a constant level from the late exponential phase to the stationary phase at 5–10 pg ATP cell⁻¹. The decrease of ATP after inoculation seemed to be due to a lack of extracellular organic materials in the fresh medium. The addition of both low and high molecular fractions obtained by dialysis from early stationary phase cultures of *C. antiqua* increased the cellular ATP content in the early exponential phase. The energy charge decreased from 0.63 to about 0.5 during the lag phase and recovered during the exponential phase. The lag phase in *C. antiqua* culture is the period of adaptation to fresh medium possibly by the production of extracellular organic materials at the expense of cellular ATP.

For two decades physiological and biochemical studies of the growth of *Chattonella* spp. have been conducted to understand the cause of the outbreaks of *Chattonella* red tides. The nutritional requirements of *Chattonella* spp. have been examined by Iwasaki¹⁾ and Nakamura *et al.*²⁾ The biochemical mechanisms of iron absorption³⁾ and the role of vitamins^{4,5)} have also been investigated. Recently it was reported that the adenosine triphosphate (ATP) content of dinoflagellates changes remarkably throughout the life cycle.^{6,7)} Meksumpun *et al.*⁸⁾ also proposed that the amino acid contents in *C. antiqua* change markedly during the growth cycle.

Cellular contents of ATP are maintained at fairly uniform levels in various cells and tissues, indicating a very sensitive and efficient cellular mechanism for the control of ATP concentration.⁹⁾ However, in the case of *Scrippsiella trochoidea*,¹⁰⁾ the ATP content decreases from 4.0 pg ATP cell⁻¹ in the exponential phase to about 2.5 pg ATP cell⁻¹ in the resting phase. It is interesting that the ATP content in marine phytoplankton cells

may change with the phases of their growth cycles.

Chapman *et al.*¹⁰⁾ proposed that the physiological state of microorganisms could be expressed by the adenylate energy charge. The hypothesis that adenylate energy charge determines and defines the bioenergetic state of a living cell has been extended to phytoplankton.^{11–15)}

We investigated the changes in the total cellular contents of carbon (C), nitrogen (N), phosphorus (P), and adenylic nucleotide in *C. antiqua* (Hada) Ono throughout its growth cycle in batch culture. The influences of small and large molecular fractions obtained from dialysis of *C. antiqua* culture medium on the ATP content of *C. antiqua* were also examined.

Materials and Methods

Culture Conditions

The axenic culture strain of *C. antiqua* was routinely grown in 300-ml Erlenmeyer flasks containing 100 ml of autoclaved (120°C, 20 min) ESM-enriched seawater from the Seto Inland

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Sea (salinity $31 \pm 1\%$) without soil extract.¹⁰⁾ Illumination was provided by cool-white fluorescent lamps at an irradiance level of approximately $80 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 14:10 h LD cycle. The temperature was kept at $21 \pm 1^\circ\text{C}$. *C. antiqua* were grown in a 10-l glass carboy containing 9 liters of ESM-enriched seawater under the conditions described above. The samplings were performed 6 h after switching the fluorescent lamps on, except for the short-time experiments (48 hours long) which were carried out in two separate flasks. Cell number, cellular carbon, cellular nitrogen, cellular phosphorus, and cellular ATP and its related compounds were estimated. All samples were filtered through glass-filters (Whatman GF/C freed of organic matter by ignition at 450°C for 2 hours) and stored at -20°C till analysis, except for the samples used for cellular ATP and related compounds, which were extracted as described below.

Determination of ATP Content

A one ml subsample from cultures of *C. antiqua* grown was taken at appropriate intervals for five weeks and added to 10 ml of boiling TRIS-HCl buffer (0.025 M, pH 7.7). ATP extractions were done in triplicate. The extracted ATP was assayed using an SAI integration photometer, Model 2000 (Science Application, Inc.)¹⁷⁾

HPLC Analysis of ATP and Related Compounds

After the filtration had been completed on Whatman GF/C, the filters were immersed in boiling TRIS-HCl buffer (0.025 M, pH 7.7) at 100°C to inactivate all the enzymes. The extracted samples were analyzed by reverse phase HPLC. The HPLC system was composed of a Shimadzu LC-9A HPLC pump and a Shimadzu SPD-6A UV spectrometer detector, using a wavelength of 260 nm. The column was a Shimadzu shim-pack CLC ODS (M) analytical column (250×4.6 mm ID). The buffer used as eluent was 30 mM $\text{NH}_4\text{H}_2\text{PO}_4$ containing 1.5 mM PIC (tetrabutyl ammonium acetate) and 10% (v/v) methanol (pH 7.2).

The energy charge (EC) was calculated after Chapman *et al.*¹⁰⁾ as follows: $\text{EC} = (\text{ATP} + 1/2 \text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$.

Effect of Extracellular Low and High Molecular Weight Fractions from Culture Medium on ATP Content

One hundred ml of medium, in which *C. antiqua*

had been cultured for 30 days and reached a cell density of $15,000 \text{ cells ml}^{-1}$, was filtered and dialyzed against 1 liter of distilled water for 24 hours. The fractions inside and outside of the dialysis membrane (approx. cut range molecular weight is 12,000–14,000) were concentrated to 100 ml. Fifty ml of these solutions were added to 50 ml of fresh ESM medium. Ten ml of culture containing with cells at late exponential phase were inoculated into the flasks containing these media. The first sub-sampling was started 3 h after switching fluorescent lamps on. At appropriate intervals ATP and cell numbers were estimated.

Total Cellular Carbon, Nitrogen, and Phosphorus Analyses

The filtered samples were freeze dried. Carbon and nitrogen were measured by a Yanaco MT-3 CHN analyzer. Total cellular phosphorus was determined by the methods of Anderson *et al.*¹⁸⁾ and Strickland and Parsons.¹⁹⁾

Results

Changes in Cellular ATP During Growth

Chattonella antiqua grew in ESM-culture medium at a rate of 0.23 divisions/day during the first 14 days; during the early exponential phase it grew exponentially at a rate of 0.34 divisions/day. The changes in cellular ATP levels during growth are shown in Fig. 1. The cellular content of ATP was about $10\text{--}15 \text{ pg ATP cell}^{-1}$ during the first few days. The ATP pool of the cells was greatest during the early exponential phase of growth (25–

Fig. 1. Changes in cellular ATP during the growth of *Chattonella antiqua*.

Fig. 2. Changes in cellular ATP in *C. antiqua* under light and dark conditions.

30 pg ATP cell⁻¹). After that it decreased during the growth of the culture and seemed to reach a stationary level (5–10 pg ATP cell⁻¹) in the late exponential phase.

The ATP pool of cells incubated in the light for 3 hours decreased to 5 pg ATP cell⁻¹ (Fig. 2). After incubation in the light for 10 hours, cellular ATP increased sharply to 20 pg ATP cell⁻¹.

HPLC Analysis of ATP and Related Compounds and Energy Charge

The patterns of changes in ATP, ADP, and AMP levels are shown in Fig. 3A. The cellular contents of ATP and ADP in *C. antiqua* increased during the early exponential phase. The AMP content was less than that of ATP and ADP. The energy charge of *C. antiqua* calculated from the HPLC analytical data decreased from 0.63 to 0.53 after 2 days and then increased to 0.59 after 4 days (Fig. 3B). It seemed to become virtually stable from the early exponential phase to the late exponential phase of growth, remaining at about 0.6 for 2 weeks. During the late stationary phase, the energy charge declined slowly to a value of about 0.5.

Changes in Cellular Contents of Carbon, Nitrogen, and Phosphorus

The carbon content of *C. antiqua* varied between

Fig. 3A. Changes in the cellular contents of nucleotides during the growth of *C. antiqua*.

Fig. 3B. Changes in the energy charge (EC) of *C. antiqua* during growth.

Fig. 3C. Changes in cellular carbon, nitrogen, and phosphorus during the growth of *C. antiqua*.

1.7 and 2.9 ng cell⁻¹ throughout the growth of the culture (Fig. 3C). It increased remarkably during the early exponential phase of growth. Changes in cellular nitrogen followed the same general pattern as the changes in cellular carbon (Fig. 3C). After 3 days, the amount of nitrogen per cell had increased by about 40%, and thereafter it decreased markedly. An increase occurred again when cells reached the mid exponential phase of growth. Total phosphorus levels increased from 59 to 67 pg cell⁻¹ after the inoculation for two days. Then they decreased sharply until they

reached a nearly constant level during the stationary phase (Fig. 3C). The C/N ratio during the growth was between 7 and 10. This ratio decreased markedly during the lag and early exponential phases, after which it increased and then became more or less constant from the mid exponential phase to the end of the experiment. The changes in C/N and N/P ratios during the growth of *C. antiqua* are given in Table 1. The ratio of C and N to P increased from the lag phase to the mid exponential phase. Thereafter, this ratio increased when cells reached the stationary phase. The ratio of phosphorus to carbon was slightly low compared to that of the Redfield number (C: N: P=106: 16: 1).²⁰⁾ Holm-Hansen²¹⁾ proposed that algae ranging in size from 1 pg C/cell to 215000 pg C/cell had uniform levels of ATP relative to organic carbon (ATP=0.35% organic carbon). In this study, the C/ATP ratios in *C. antiqua* were nearly constant throughout the growth cycle. The mean value of ATP of *C. antiqua* during the growth was 0.22% organic carbon, with C.V. of 11.2%.

Effects of Extracellular Low and High Molecular Fractions on Cellular ATP Content

The ATP pools of cells in fresh ESM culture medium with the addition of 50 ml of low or high molecular weight fractions were remarkably higher than those of cells in control culture one day after inoculation (Fig. 4). ATP levels in cells exposed to the low molecular weight fraction increased greatly during the first day. The ATP pool of cells exposed to the high molecular weight fraction decreased after 3 hours, then increased markedly. After two days of inoculation, the cellular content of ATP in cells which were exposed to the low and high molecular weight fractions were higher than that of the control by about 80% and 100%, respectively.

Discussion

The result in Figure 2 shows that the cellular content of ATP decreased within 3 hours of inoculation into fresh medium and then increased during the light period of the first day. Upon transfer to the new medium from an old culture containing extracellular substances, *C. antiqua* needs to adapt to the fresh medium, thus the consumption of ATP and the production of extracellular substances may occur. A biochemical lag phase refers to this period when ATP is being consumed in the fresh medium. The addi-

Fig. 4. Changes in cellular ATP in *C. antiqua* as influenced by high and low molecular weight fractions in fresh ESM culture medium.

*High molecular weight fraction

**Low molecular weight fraction

tion of extracellular substances to fresh medium prevents the decrease of intracellular ATP to a certain extent. Corresponding with the increase of ATP that occurs about 48 hours after inoculation, cell division accelerates and growth enters the exponential phase. During the early exponential phase ATP is kept at a constant high level. Thereafter, cellular ATP gradually decreases and reaches a constant low concentration during the late exponential and stationary phases. Lirdwitayaprasit *et al.*^{6,7)} also reported that cellular contents of ATP of *Alexandrium catenella* and *Scrippsiella trochoidea* during the exponential phase were higher than those of the stationary phase when grown in the PO_4^{3-} -P-limited encystment medium.

In our study, ATP/cell measured by an ATP photometer was higher than that by the HPLC method. Although it is known that firefly luciferase is specific for ATP, it is likely that other molecules containing high energy phosphate bonds might cause some light emission. Holm-Hensen and Booth¹⁷⁾ reported that ADP, CTP, and ITP had some effect on light emission. This reason may contribute to the occurrence of such a high estimation of ATP in cell as measured by the ATP-photometer.

Energy charge (EC) is generally recognized as a metabolic regulatory parameter. Chapman *et al.*¹⁰⁾ found a correlation between EC and the growth state of *Escherichia coli*. active growth occurred only at EC values above 0.8. Cells were viable at values between 0.8 and 0.5, but died at values below 0.5. Growth cut off sharply as the EC declined past a narrow critical range.²²⁾ Recently, Salmeron *et al.*³³⁾ suggested that the EC

Table 1. Changes in cellular C, N, P contents and C/N/P ratio during growth of *Chattonella antiqua*.

Time (days)	C/cell (pM)	N/cell (pM)	P/cell (pM)	C:N	N:P	C:N:P
0	217	22.8	1.92	9.5	11.9	106:11.1:0.9
1	187	24.8	1.60	7.5	15.5	106:14.1:0.9
2	222	28.6	2.17	7.8	13.2	106:13.6:1.0
3	240	31.4	2.02	7.7	15.5	106:13.9:0.9
4	204	23.2	1.60	8.8	14.5	106:12.1:0.8
5	195	20.6	1.40	9.5	14.7	106:11.2:0.8
7	192	22.9	1.39	8.4	16.5	106:12.6:0.8
8	185	23.0	1.62	8.1	14.2	106:13.2:0.9
10	176	20.8	1.17	8.5	17.8	106:12.5:0.7
12	187	24.3	1.36	7.7	17.9	106:13.8:0.8
14	204	27.9	1.32	7.3	21.1	106:14.5:0.7
17	143	16.9	1.12	8.5	15.1	106:12.5:0.8
19	164	21.0	1.10	7.8	19.1	106:13.6:0.7
22	169	22.0	1.13	7.7	19.4	106:13.8:0.7
26	145	18.6	0.92	7.8	20.2	106:13.6:0.7

value of *Azotobacter chroococcum* was about 0.8 although cells were grown with a fixed nitrogen source or dinitrogen. Changes in light condition during night-sampling and extracting do not induce significant variations, in the adenylate nucleotide content or in EC values of microplankton.²⁴⁾ In our study, the EC value of *C. antiqua* decreased from 0.63 initially to 0.53 three days after inoculation, sharply increased to a stable value of about 0.6 during the exponential phase, and then decreased again when cells reached the stationary phase of growth. The decrease of the EC after inoculation into the fresh medium was probably due to the fact that cells were shocked by the new culture medium and subsampling on the first day. Riemann and Wium-Andersen¹²⁾ also reported that the EC in nitrogen-depleted *Periastrum duplex* cells decreased for 2 days after the addition of nitrogen. Presumably a "lag effect" was caused by the transfer of the extreme nitrogen-deficient algae to the new nutrient regime. The value of EC remained low for about 3 days. This result suggests that the ATP in the cell was used for cell adaptation during this period. Thereafter, ATP/cell increased markedly, probably because of the increase in cell activity. A decrease occurred again at the beginning of the stationary phase perhaps because of the decrease in metabolic activity, as reflected by the low levels of nitrogen and phosphorus in the cells. Reiman and Wium-Andersen²⁵⁾ reported that during the phosphorus deficiency (particulate C/P ~ 85) the EC values of *Selenastrum capricornutum*, *Scenedesmus acutus* and *Periastrum duplex* were low (mean 0.54).

Starvation by removal of all sources of phosphorus introduced a decline in the EC value and the level of photosynthetic activity of *Microcystis aeruginosa*.¹⁴⁾

The amount of cellular carbon in *C. antiqua* increased sharply during the lag and the early exponential phases of growth, probably reflecting the increase in photosynthetic rate and the accumulation of new products such as carbohydrate, protein and lipid necessary for cell division, which accelerated rapidly after this period. This result coincided with the definition of lag phase which was referred to a period of restoration of enzyme and substrate concentrations to the levels necessary for rapid growth.²⁶⁾ The amount of carbon per cell during the stationary phase was considerably lower than during the exponential phase, perhaps because of the low concentrations of nutrients in the culture medium and the reduction of cell activity.

During the lag phase and early exponential phases, the amount of nitrogen per cell increased by about 40% compared to its initial level. Moreover, the changes in cellular nitrogen followed the same general pattern as for cellular ATP. These results confirm that cells during these phases can increase their photosynthetic rate and accumulate the biochemical compounds necessary for cell division.

The level of cellular phosphorus in *C. antiqua* during the lag and the early exponential phases increased by more than 10% of its initial level. This level increased rapidly reaching a maximum just 2 or 3 days after the inoculation, presumably

reflecting a high rate of phosphorus absorption from the new culture medium for the production of ATP and other phosphorus compounds. The amount of phosphorus per cell of *C. antiqua* decreased about 30% between cells at the exponential and stationary phases. This is probably due to the low concentrations of nutrients in the culture medium and the reduction of cell activity. Cellular phosphorus of *Heterosigma akashiwo* increased during the growth processes and cells were able to continue their growth although the phosphorus in the culture medium was undetectable.^{27,28} Lirdwitayaprasit *et al.*⁷ also reported that cellular phosphorus of *Scrippsiella trochoidea* cultured in PO_4^{3-} -limited encystment medium decreased about 40% between vegetative cells at the exponential and stationary phases of growth.

The means of C/N ratio of *C. antiqua* during the exponential phase and the stationary phase were not so different. However, this ratio decreased remarkably during the early exponential phase in which cell division accelerated. A linear relationship between the C/N ratio and relative specific growth rate has been found in N-limited chemostats for phytoplankton.²⁹⁻³³ Davidson *et al.*³⁴ also reported that this relationship holds for non-steady-state growth in batch cultures.

Overall, these studies provide more detail about the changes in chemical components during the growth of *C. antiqua*. They show that after inoculation, the cells rapidly adjusted their biochemical conditions and accumulated chemical components for the preparation of cell division that increased rapidly after this period. Such a biochemical preparation in red tide organisms will also play an important role in the outbreak of red tides.

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