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The Growth of *Chlorella* Cells in Culture

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Abstract

Some phenomena related to the growth of *Chlorella* were observed by a method of semi-synchronous culture with microscope under constant conditions of light (2,800 lux) and temperature (19.9±0.9C°).

The growth curves of individual cells were determined by measuring the variations of size distribution at twelve hour intervals for three weeks. Average growth rate during that period was 1.2 microns per day. The growth rates of the individual cell were getting slower as the culture aged. Life cycle of the individual cell showed three stages: (1) daughter cell, (2) early ripening cell, and (3) mother cell. The growth rate in each stage was approximately 4 : 1 : 3.

Introduction

The growth of living organisms involves two separate but interrelated processes: the growth of an individual organism and the growth of a population. Our understanding of the overall phenomenon would likely be better served if both aspects were observed in experimental work, as is common in the cases of larger animals or plants, but not for planktonic organisms. Most studies in phytoplankton have dealt with growth in bulk of the population, observing such things as total cell numbers and chlorophyll content in waters. Those methods do not permit analysis of individual growth which is the basic problem of growth study.

Direct observation of growth of individual algal cells ... e. g. continuous observation under a microscope ... is almost certain to bring about some disturbance of the natural growth pattern. In the present experiment, an indirect method for determining the growth rate of *Chlorella* cells was tried by measuring cell diameter with a method of semi-synchronous culture. Emphasis was placed upon the growth rate of individual cells through their life cycle.

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Material and Methods

The green alga *Chlorella pyrenoidosa* PRINGSHEIM, Woods Hole Collection, was used in the present observations. The composition of the culture medium was as follows: 1,000 ml distilled water, 150 mg $NaNO_3$, 10 mg $NaH_2PO_4 \cdot H_2O$, 3.03 mg $FeCl_3$, 6.97 mg EDTA, and 1.0 mg vitamin B_{12}. The stock cultures of algae were maintaintained on agar slants. The algae after about five months on the slants were transferred

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into the culture medium with a platinum rod. These old cells were in the stationary phase of population growth, and the individual cells showed very little variation in diameter. They developed almost synchronously for at least three weeks after innoculation into the new liquid medium.

A round flask (1,000 ml) was used for observing the growth of *Chlorella*. The medium (1,000 ml) was sterilized before innoculation, and aerated through an air breaker throughout the experimental period. Bacterial cells in the air supply were removed with Millipore air filter, but some bacteria might have been introduced accidentally into the medium by the method of sampling. The samplings were made at twelve hour intervals (10 am and 10 pm) for 38 days starting from the 7th day after innoculation. Samples of the cell populations collected in the foregoing manner were examined microscopically (950 magnifications). A hemocytometer was used for counting the cells and an ocular micrometer for measuring their diameter. The latter measurements were made along the long axis of these elliptical cells, since statistical analysis showed a significant difference between the long ($6.32\pm0.16\ \mu$) and the short diameter ($5.3\pm0.14\ \mu$). Fifty cells were measured in each sampling.

Dead cells were distinguished from living ones by observing the microscope with negative phase contrast lens. Living cells were remarkably bright while dead ones were half bright or dark circle as shown in Fig. 1.

The experiment was carried out in a constant temperature room at 19.9 ± 0.9°C. The light intensity at the level of culture was kept constant at 2,800 lux with flourescent lamps.

Results

1. Population growth

The total numbers of both living and dead cells in each sample are presented in Fig. 2 with mean cell diameters.

The number of living cells increased exponentially from the 7th day after innoculation, and it approached about 300×10^2 cells per ml around the 25th day. This number then decreased gradually to about 170×10^2 cells per ml by the end of the observations. The dead cells appeared suddenly from the 26th to the 30th day as shown in this figure. The number of dead cells then slightly increased to approximatly 80×10^2 cells per ml.

The mean cell diameters were getting smaller, showing some fluctuations, until the 25th day of the culture. The largest average diameter ($8.1\ \mu$) was measured on the 7th day when the first sampling was made. The smallest cells (mean diameter $5.0\ \mu$) were observed around the 25th day. The diameters very gradually increased for some days after the smallest size was found. The mean cell diameter then remained almost constant at the level of about $5.5\ \mu$.

On the whole, the phases might correspond to the exponential phase before the 25th day of the culture and the stationary phase after that.

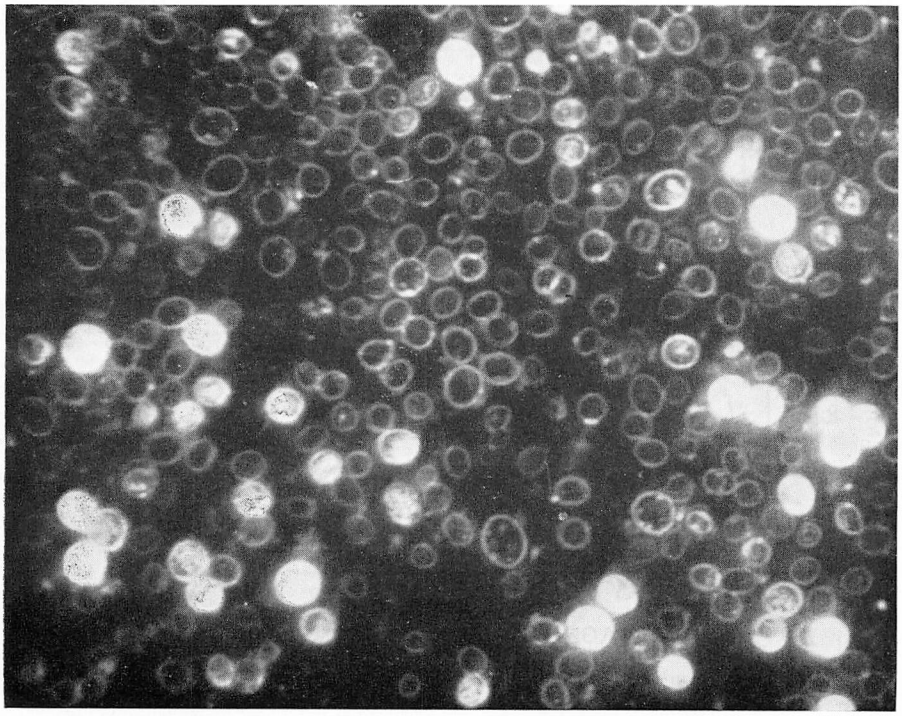

Fig. 1. Distinguish between living cells and dead ones. The potoghraph was taken with negative phase contrast microscope (Leiz). Living cells were remarkably bright while dead ones were dark circle.

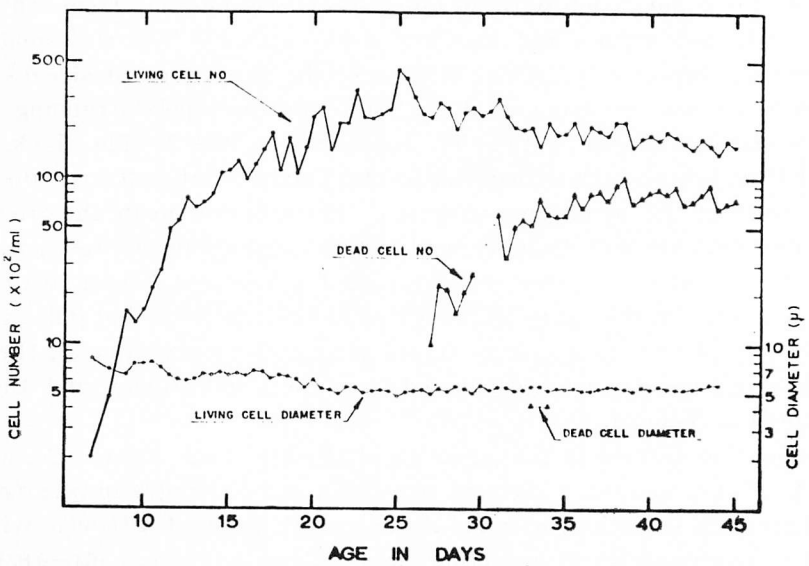

Fig. 2. Population growth of *Chlorella pyrenoidosa* in culture.

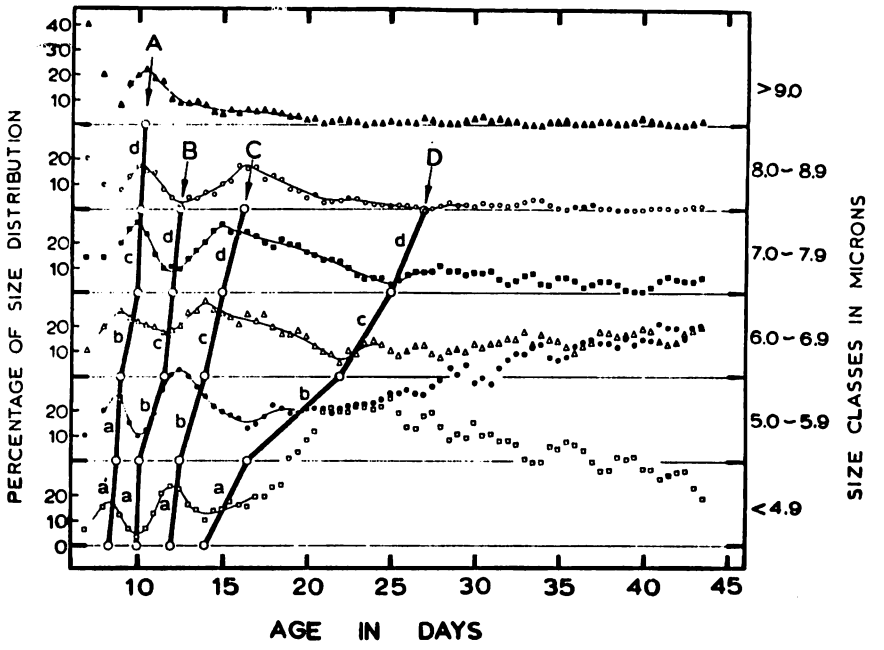

Fig. 3. Individual growth of *Chlorella pyrenoidosa* in culture.

2. Individual growth

The growth rates of the cells were determined indirectly by observing the cell diameters. The diameters ranged from about 3 to 10 or 11 μ, and this range was divided into six groups each with an interval of one micron, from 4.0 to 9.9 μ. The other cells below 3.9 μ and over 10.0 μ were included into the smallest and largest groups, respectively. The variation of the percentage of size distribution with each group was recalculated along X-axis, by three points running average, and the results are shown by the thin line curves in Fig. 3. The thick lines A, B, C, and D which are almost parallel to the Y-axis in this figure link up the limiting values of the percentage variation. These lines indicate the growth rates of the cells: dc/dt (c=cell diameter, and t=time). Most lines are comprised of four inflections, and these inflections are named *a*, *b*, *c*, and *d* from small to large diameter as shown in the figure. Line A shows five inflections, but inflection *a* and *a'* are probably of the same group. In order to analyze statistically, the growth rates in microns per day were calculated from these inflections, and the values are presented in Table 1.

The interesting feature is the inflection of these growth curves. None of the lines in this figure showed a sigmoid curve, but some changes in rate may be detected. From the inflections *a*, *c* and *d* the growth rates of *Chlorella* within the size ranges covered by these inflections were calculated to be 2.564 ± 0.831, 1.958 ± 0.709 and 1.554 ± 0.713 microns per day, respectively, none of which was significant-

ly different. However, growth rate of the cells within the range covered by inflection *b* was 0.929±0.146 microns per day, and its difference from the others was highly significant. Since the inflections *c* and *d* showed no significant difference in growth rates, they are probably of the same group. Therefore, inflections *a*, *b* and *c-d* may be considered to be the main stages of the life cycle of *Chlorella*. From these features, it may be concluded that the growth of the cells is indicated by a tangential curve.

On the other hand, lines A, B, C and D in Fig. 3 indicate that the growth rates were decreasing as the culture aged. The average rates were calculated to be approximately 3.0, 2.3, 1.1 and 0.4 microns per day from lines A to D, respectively. There was no difference in the growth rate between lines A and B, but these rates differed significantly from line C and highly significantly from line D. Lines A and B occurred from the 8th to 12th day of the culture, and this period fell within the exponential phase (cf. Fig. 2). The growth rate indicated by line D was the slowest, and during this period population growth was approaching the stationary phase (the 14th to 26th day). Line C was situated between exponential and stationary phases.

Table 1. Growth rates in microns per day of *Chlorella* cells, data from Fig, 3.

	Growth lines				Average	Significance
	A	B	C	D		
Inflection a	2.857	5.000	2.000	0.400	2.564±(0.831)	
b	1.000	0.667	0.667	0.182	0.629±(0.146)	a**
c	4.000	2.500	1.000	0.333	1.958±(0.709)	
d	4.000	1.000	0.714	0.500	1.554±(0.713)	
Average	2.964± (0.613)	2.292± (0.855)	1.095± (0.260)	0.354± (0.057)		
Significant			B*	B**		

3. Cell division

The number of daughter cells produced from a single mother cell ranged from 2 to 32. In general, 2 to 16 were formed; less frequently, 32 cells were formed by the division of one mother cell. The numbers corresponded with the growing phases of the population. Mother cells divided into 8 to 16 or 32 in the exponential phase and underwent 2 to 4 more cell divisions during the stationary phase. The number of cell divisions in the rapid growing phase was 4 to 8 times greater than in the stage when the population grew slowly.

The mother cells divided into certain multiples of two within the cell membrane. The membrane gradually became thinner and was ruptured by the growing daughter cells.

Discussion

These studies have yielded the first clues to the nature of individual growth rate ... i. e. change in cell diameter with change in time (*dc/dt*) ... in the life cycle of *Chlorella*. Growth rates of individual cells decreased as the population aged. The rates were faster in the exponential phase and slower in the stationary phase of population growth (cf. section 2). This might be caused by changing concentrations of nutrients in the medium (e. g. PRATT and FONG, 1940). At any age, however, the individual growth produced a tangential curve. It is well known that individual growth in large organisms shows a sigmoid curve. The difference between these curves could be caused by the reproductive mechanism. *Chlorella* is a single celled organism, and cell division is its method of reproduction. The cell division occurs within a mother cell, and the daughter cell may grow within the mother cell before it is released (cf. section 3).

The life cycle of *Chlorella* cells has been studied by some investigators using a method of complete synchronous culture (LORENZEN and RUPPEL, 1960; TAMIYA, 1957), and HASE (1962) reviewing those works stated that, LORENZEN and RUPPEL distinguished three successive developmental phases, I, II, and III, which seem to correspond, respectively, to *growth*, *early ripening*, and *late ripening* phases proposed by TAMIYA and his associates. However, there seems to have been no detailed description of growth rate in each stage. The tangential growth curve determined in the present study corresponds to the three stages in the life of *Chlorella* cells as observed by LORENZEN and RUPPEL using a different method. Further more, the growth rates during these stages I, II, and III were culculated to be 2.6, 0.6 and 1.9 (average of inflections *c* and *d*) microns per day, and the ratio was approximately 4 : 1 : 3, respectively. The slower rate of growth in stage II may be the result of metamorphosis ... i. e. a shift in physiological activities from daughter to mother cell. In this connection LORENZEN and RUPPEL (1960) reported that the greatest increase in DNA occurred in stage II.

MITCHISON (1957), using a single celled yeast (*Schizosaccharomyces pombe*) by a method of direct microscopic observation, found that growth curves for volume are sharply divided into two stages in a cell cycle: the 1st stage in which growth takes place and which take up 75 per cent of the generation time, and the 2nd stage or constant volume stage which preceds division. On the contrary, division of a *Chlorella* cell has an additional stage following the slow glowth stage during which accelarated growth accompanies the formation of daughter cells.

The number of autospores liberated per mother cell decreased as the culture aged. The larger nubemr of 8 to 32 divisions was observed when the population was in the exponential phase. The decrease in autospores liberated during the stationary phase might be caused by the changing concentrations of nutrients in the medium (HASE, 1962), since the nutrients are depleted as the population grows (PRATT and FONG, 1940).

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