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FLUCTUATION OF THE ADENYLATE ENERGY CHARGE IN SYNCHRONOUS *CHLORELLA RUBESCENS* CHOD. GROWN IN AUTO-, MIXO- AND HETEROTROPHIC CONDITIONS

BY

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ABSTRACT

The pool of adenine nucleotides (AN) and the adenylate energy charge (EC) were characterized during the cell cycle of the microalga *Chlorella rubescens* Chod. growing in auto-, mixo- and heterotrophic conditions. A synchronous autotrophic culture was established in a light: dark regime of 70: 24 h. The cells were growing actively during the light period and divided during the dark period. By transferring the culture in mixo- and heterotrophy, the cellular growth was considerably affected and the algae did not divide in the case of heterotrophy. In autotrophy, the pool of total AN remained remarkably constant throughout the life cycle, whereas the levels of individual AN varied significantly. Consequently, a fluctuation of the EC can be observed that is composed of three main peaks rising successively from the value of 0.72 to the values of 0.81 and 0.91 in the light, and to 0.82 in the dark. Despite the important changes in cellular growth and differentiation occurring in mixo- and heterotrophy, the adenylate system evolved basically as in autotrophy, showing the same fluctuation of the EC. Interestingly, this fluctuation was paralleled by a similar fluctuation in the ratio of chlorophyll *a*/*b*. These observations suggest that, during the cell cycle of the alga, the adenylate system and the energy state of the cell are submitted to a strong endogenous control and that the organization of the photosynthetic apparatus is apparently also affected by such a control.

Key-words: Energy charge, chlorophyll *a*/*b* ratio, synchronous *Chlorella rubescens* Chod.

Abbreviations: AN, adenine nucleotide(s); EC energy charge; PS, photosystem.

RÉSUMÉ

Le pool des nucléotides adényliques (AN) et la charge énergétique adénylique (CE) ont été caractérisés au cours du cycle de vie de la microalgue *Chlorella rubescens* Chod., en croissance auto-, mixo- et hétérotrophe. Une culture autotrophe synchrone a été établie dans un régime de lumière:obscurité de 70:24 h. Les cellules sont en croissance active pendant la période de lumière et se divisent pendant la période d'obscurité. En transférant la culture en mixo- et en hétérotrophie, la croissance cellulaire est considérablement modifiée et les algues ne se divisent plus dans le cas de l'hétérotrophie. En autotrophie, le pool des AN totaux reste remarquablement constant tout au long du cycle de vie, alors que le niveau de chaque nucléotide varie de façon significative. Il en résulte une fluctuation de la CE, composée de trois pics majeurs s'élevant d'une valeur originale de 0,72 et atteignant successivement les valeurs de 0,81 et de 0,91 à la lumière, et de 0,82 à l'obscurité. Malgré les changements importants de croissance et de différenciation observés en mixo- et en hétérotrophie, le système adénylique évolue essentiellement comme en autotrophie et présente une fluctuation similaire de la CE. De façon intéressante, le rapport des chlorophylles a/b subit une fluctuation parallèle. Ces observations suggèrent que, au cours du cycle cellulaire de l'algue, le système adénylique et l'état énergétique de la cellule sont soumis à un contrôle endogène puissant et que l'organisation de l'appareil photosynthétique est apparemment aussi touchée par ce contrôle.

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INTRODUCTION

The adenylate energy charge (EC) defined by Atkinson (1968) as the ratio of [ATP] + 0.5 [ADP] / ([ATP] + [ADP] + [AMP]) is considered to be a direct measure of the metabolic energy balance between anabolic and catabolic reactions and plays probably a significant role in metabolic regulation of prokaryotic and eukaryotic microorganisms (Chapman et al., 1971; Knowles, 1977). The changes in the adenine nucleotide content can be considered as a marker of the metabolic state of a given tissue or cell with a specific pool of enzymes and under given conditions, as was suggested by Pradet and Bomsel (1978). The level of ATP and the values of EC have been determined in *Chlorella* and other green algae (Syrett, 1958; St. John, 1970; Imafuku & Katoh, 1976; Kawada & Kanazawa, 1982) but there has been no work yet on the energy state during the life cycle of *Chlorella* in synchronized culture.

The purpose of this investigation was to characterize the adenylate EC during the cell cycle of *Chlorella rubescens* and to evaluate the impact of photosynthetic and respiratory metabolism on the energy state of the alga by modifying the environmental conditions. Therefore, the algae were synchronized in autotrophic conditions and were either maintened in these conditions or transferred to mixo- or heterotrophic conditions. The evolution of the ratio of chlorophyll *a*/*b* was also investigated in order to find an eventual relationship with the variations in EC, since it is known that the photosynthetic apparatus itself varies during the life cycle of microalgae (Sorokin & Krauss, 1961; Venediktov et al., 1981) and also that changes in the chlorophyll *a*/*b* ratio ressemble changes in EC (De Filippis et al., 1981a, b).

METHODS

Microalga and culture medium. The algal strain used was *Chlorella rubescens* Chod. B-24 (obtained from the Algal Collection, Department of Plant Biology of the University of Geneva, CH). The algae were cultivated axenically in Detmer-modified medium (Pringsheim, 1951; Sayegh and Greppin, 1971), supplemented with 0.5% (w/v) glucose in the preculture and in the mixotrophic and heterotrophic cultures.

Synchronisation of the algal cultures. Permanent synchronous growth was established according to the method of Pirson and Lorenzen (1966), in the conditions developped by Sayegh & Greppin (1973) and briefly described below. After a preculture of 2-3 weeks under continuous light at low intensity (2 to 3 klx) and constant temperature (25°C), the algae were inoculated in fresh medium (200 ml) in special flasks (Kuhl & Lorenzen, 1964). The flasks were then exposed successively to high intensity light (10 klx) during 5 days and to low intensity light (1 klx) during 2-3 days. The cultures were finally submitted to the optimal conditions for synchronous division, i.e. three to four cycles of 70 hours light (10 klx provided by Sylvania fluorescent lamps "daylight" 40

W) followed by 24 hours darkness (LD 70:24 h). After each cycle the cultures were diluted to 1000 cells/µl. The temperature was maintained at 29°C and agitation and aeration was provided by bubbling air enriched with 5% CO_2. In these conditions 98% of the cells were divided at the end of the last dark phase.

In our experiments, the synchronized cultures were either maintained in autotrophic conditions, or submitted to mixotrophic (0.5% glucose in the medium) and heterotrophic (0.5% glucose in the medium and light turned off) conditions.

Extraction and assay of adenylate nucleotides. Culture samples of 30 ml were harvested and the algae were collected by centrifugation at 12'000 x g for 10 min at 4°C (Beckman rotor JA-20). The algal pellets were resuspended in a minimal volume of culture medium (1-5 ml). The nucleotides were extracted from *Chlorella* cells by rapidly pipetting 1 volume (usually 1 ml) of cells (18,000 cells/µl) into 5 volumes of boiling ethanol in a 50 ml Erlenmeyer flask. After boiling for 1 minute, samples were blown dry and either stored at -20°C or reconstituted with 5 ml of water for analysis (St John, 1970). Preliminary determinations showed that this method of extraction caused no decomposition of added nucleotides.

The nucleotides were assayed according to Pradet (1967) in the conditions described by Bonzon et al. (1981). The concentrations of the adenine nucleotides were determined by comparison with standards treated in the same way as the samples. Possible inhibition of the test was evaluated by adding an internal standard (St John, 1970). The energy charge (EC) was calculated according to Atkinson (1968) as the ratio of [ATP] + 1/2 [ADP]/ ([ATP] + [ADP] + [AMP]).

Determinations of protein, chlorophyll and dry weight. For protein and chlorophyll determinations, the algae were first collected by centrifugation of 10-20 ml culture samples, as described above. Proteins were extracted after resuspending the algae pellet in 5 ml phosphate buffer pH 7 and disrupting the cells by ultrasonisation during 5 min. The proteins were precipitated with 5% TCA and the precipitate was dissolved in 1 ml 10% NaOH. The solution was completed to 20 ml with distilled water and an aliquot was used for the colorimetric determination of protein according to Lowry et al. (1951). Chlorophylls were extracted by grinding the cells exhaustively with a mortar and pestle in 80% acetone and estimated according to Arnon (1949). The dry weight of the algal mass was determinated by filtration of 10-20 ml of culture through a pre-weighed Whatman filter (type WCN, 1.2 µm). The filter with the algae was then dried at 70°C for 24 h, cooled at room temperature and weighed.

Determinations of the algal concentration and size. The number of cells was estimated by counting on a Zeiss-Thoma hemacytometer (Uehlinger & Linder, 1955). The cellular size was determined by measuring the cellular radian with a microscope equipped with a graduated ocular (Sayegh and Greppin, 1973).

Statistics. The experiments were repeated 6 times (6 consecutive cycles in the auto- and mixotrophic conditions, and 6 independent cycles in the heterotrophic condition). In each experiment, samples were assayed 3 times for adenylates, proteins, chlorophylls, dry weight and algal concentrations. The mean cellular radian was determined by measuring 20 cells.

The average level of nucleotides varied up to 18% between experiments. The pattern of the time courses of nucleotides levels was very stable from one experiment to the other and therefore typical representative results are shown in the figures. In each single run, the coefficient of variation on ATP measurements was 1.5-3%, that on ADP 3.5-6%, and that on AMP 4.5-7% (minimum and maximum values). The standards error for the energy charge was 2-3%, according to Friedrich & Mohr (1975). Values of the others parameters were determined as a mean of the 6 experiments with the following coefficients of variation (minimum and maximum values): that on proteins 6.0-8.7%, that on chlorophylls 3.9-5.2, that on dry weight 3.8-9.1%, that on algal concentration 3.0-5.4%, and that on algal diameter 4.0-8.5%.

RESULTS

Cell growth and division

The synchronized population of *Chlorella rubescens* was submitted to three different experimental conditions of culture: the cells were either maintained in autotrophy or transfered to mixo- or heterotrophy. The characteristics of growth and division under these conditions are illustrated in figure 1. In auto- and mixotrophy the cell cycle is relatively slow and is composed of a photophilic phase of 70 h followed by a nyctophilic phase of 24 h. In both conditions, the cell number remains constant during the period of light and increases during the period of darkness because of cell division and production of autospores, which is completed after 24 h (Fig. 1A). By that time, about 98% of the cells have divided. A mothercell yields usually ten daughter cell. Whereas the rate of cell division in autotrophy is maximal immediately after the onset of darkness, it increases progressively and is maximal only after 15 h of darkness in mixotrophy. Also, the number of autospores released in mixotrophy is slightly lower, eventhough the same amount of cells undergo division. In heterotrophy, the cell number remains constant and there is no cell division during the 94 h of culture.

The mean cellular diameter increases during the photoperiod in auto- and mixotrophy and then decreases rapidly because of cell division (Fig. 1B). Eventhough mixotrophic algae reach the largest cellular diameter before division and release a lower number of autospores (see above), the mean cellular diameter of the autospores at the end of the nyctoperiod is the same as in autotrophy. In heterotrophy, the cellular diameter increases also during the first 70 h of culture, although to a lower extent, and then decreases slightly during the last 24 h of culture.

The production of dry weight, protein and chlorophyll by the three types of culture is illustrated in figure 1B-E, successively. Autotrophic cells produce the lowest amount

of dry weight but the highest amount of protein and chlorophyll during the photoperiod. At hour 70, when the light is turned off, the decrease of the parameters corresponds to the production of daughter cells by auto- and mixotrophe algae. The heterotrophic culture is also characterized by a large increase of dry weight, almost as important as in mixotrophy followed by a decrease after 70 h of culture (Fig. 1C). The production of protein is however low and is reduced during the last 24 h of culture (Fig. 10). The level of chlorophyll remains fairly constant during the first 60 h, after which it raises slightly before decreasing to about half the value of time 0 (Fig. 1E).

In summary, the cell cycle of *Chlorella rubescens* cultivated in auto- and mixotrophy is characterized by a photophilic phase during which the cellular diameter, dry weight and protein and chlorophyll content increase, and by a nyctophilic phase during which cell division occurs simultaneously with a restructuration of the cellular dry weight and protein and chlorophyll content. The level of proteins is higher in autotrophic cells whereas the level of dry weight and the cell diameter are higher in mixotrophic cells. In heterotrophy, the algae do not divide and, even in complete darkness, the studied parameters, with the exception of chlorophyll, show a biphasic pattern, with a phase of increase during the first 70 h of culture and a phase of decrease during the last 24 h of culture.

Adenylates and energy charge

Variations of the cellular levels of the three adenine nucleotides (AN) and of the energy charge (EC) in the various conditions of culture are shown in figure 2. The cellular pool of total AN is quite similar in all three conditions and remains fairly constant throughout the auto- and mixotrophic cell cycles and the 94 h period of heterotrophic growth. The lowest value is observed in heterotrophy and the highest in autotrophy, whereas it fluctuates between these two values in mixotrophy. Contrarily to the other cellular components studied (see above) the algal cell maintains a constant pool of total AN during cellular growth and cell division. This means that the AN pool of young autospores is synthesized just before their release from the mother cells and corresponds to an eigth- to ten-fold amplification in a very short period of time.

The pool of total AN stays constant, but the levels of individual AN show important variations that are best understood by considering the values of the EC. Indeed, ATP is procured at the expense of ADP and AMP and, as a consequence, the EC varies in much the same way than the ATP level. During the cell cycle of autotrophic *Chlorella rubescens* a fluctuation of the EC with three peaks can be observed (Fig. 2A, I, II and III). The same peaks occur also when the algal cells are grown in mixotrophic and heterotrophic conditions, but with various proportions (Fig. 2B and C respectively). These 3 energetic events occur as follows: the first one during the period of adaptation to light (0-5 h), the second one during the last part of growth, just before cell division (35-60 h), and the third one in the middle of the period of division in darkness (80-90 h). The first peak is most evident in auto- and mixotrophy and reaches the value of 0.81 and 0.80, respectively, whereas in heterotrophy it reaches

only the value of 0.74. The highest peak is the second one in all conditions of growth and the highest value is reached in the autotrophic system (0.91) and the lowest in the heterotrophic system (0.84). This second peak is however wider in mixotrophy since the increase begins about 15 h earlier than in the two other conditions. As a consequence, values of EC are significantly higher during the period of 20 to 40 h. The third peak is more pronounced in auto- and mixotrophy, but the levels reached at time 85 h are the same in the three conditions. At the end of cell division, at time 94 h, the EC is back to values observed at the beginning of the auto and mixotrophic cycles (0.72 and 0.77 respectively). In heterotrophy, however, the EC stays at a relatively higher value (0.74 as opposed to 0.70 at the beginning) as cell division does not occur.

Nucleotide ratios have been postulated to control key enzymes in metabolic sequences (Atkinson, 1977) and, in this context, the ratio of ATP/AMP and of ATP/ADP were calculated and are represented in figure 3. Variations of the ATP/AMP ratios are coincident with the 3 energetic events mentioned above (Fig. 3, lower part). A rise of the ratio is particularly pronounced during mixotrophic growth, between 35 and 60 h. The same variations coincidental with the energetic events are also observed for the ATP/ADP ratio (Fig. 3, upper part). In this case, the fluctuation in mixotrophy is less pronounced than in autotrophy and the ratio tends to lower values than in auto- and heterotrophy. These observations are mostly related to the fact that mixotrophic cells possess a lower mean level of AMP and a higher mean level of ADP in comparison to auto- and heterotrophic cells.

There is apparently no direct correlation between the oscillations of the EC and the evolution of the growth parameters described in figure 1. In experiments with *Euglena*, De Filippis et al. (1981a, b) reported a change in EC that could be due in part to a disturbed development of the photosynthetic apparatus since a similar change in the ratio of chlorophyll *a*/*b* was observed. Furthermore, this ratio is known to undergo important fluctuations during the life cycle of *Chlorella* (Venediktov et al., 1981). On the basis of these observations, the evolution of the ratio of chlorophyll *a*/*b* in our experimental conditions was next investigated.

Chlorophyll a/b ratio

Changes of the ratio of chlorophyll *a*/*b* in our three conditions of culture are represented in figure 4. A fluctuation is clearly detectable in autotrophy. It corresponds to a decrease during the first 25 h of culture, followed by two peaks, one in the second half of the photoperiod and the other in the middle of the nyctoperiod. The first peak preceeds the second EC peak by 5 hours while the second one coincides with the third EC peak. These two peaks are also apparent in heterotrophy, but in this case the first one occurs 5 hours after the second EC peak while the second one coincides also with the third EC peak. Another small peak can be observed at the beginning of the period of culture. In mixotrophy, the chlorophyll *a*/*b* ratio rises during the first 25 h of culture and stays at this level with minor variations until it drops at the onset of darkness to lower values where it remains. It should be noted that, for comparison, the first rise of EC is very rapid and is immediately followed by the second fluctuation at time 15 h.

DISCUSSION

In this work we intended to find possible correlations between the adenylate energy state and the growth and differenciation events occuring during the life cycle of *Chlorella rubescens*. The transfer of a synchronous culture of *Chlorella* from autotrophic to mixo- and heterotrophic conditions induced significant alterations of growth and differentiation due to different cellular metabolisms, and we expected important related changes in the adenylate energy state that would help us to evaluate the importance of the energy metabolism during the life cycle of the algae. We found instead a persistence of the basic characteristics of AN and EC encountered in autotrophy: first, the total AN pool did not vary despite the important cellular growth and second, the three peaks of EC were maintained, although with various proportions. These observations suggest that the adenylate energy state is dependent on a strong endogenous control operating in various cellular metabolisms, but that it is also influenced by the environmental conditions, as will be discussed now.

Variations in the adenylate energy state in the light are most likely related to the energy input from photophosphorylation. Indeed, both chloroplastic and cytosolic EC are increased by active phosphorylation because, as soon as the reaction starts in the chloroplasts, a fast transfer of photosynthetic phosphorylation power to the cytosol has been described (Hampp et al., 1982). The higher values reached by the first peak of EC in auto- and mixotrophy could be explained by a stimulation of ATP production promoted by the onset of light. As a matter of fact, several authors have reported a rapid increase of ATP in the light, in chloroplasts and cytoplasm, followed by a slow decreases (Santarius & Heber, 1965; Holm-Hansen, 1970). At this early stage of algal culture (0-5 h), variations in the pattern of AN might depend mostly on photophosphorylation. After this first rise in EC the ratio decreases to the original level, probably because of the onset of energy-consuming reactions. The second peak of EC in the light period is also enhanced in auto- and mixotrophy and this effect can also be related to active photosynthesis. Studies of cell division in synchronized unicellular green algae have revealed that the photosynthetic activity of cells varies within the division cycle, with the greatest activity at the beginning of the cycle and the smallest before the release of autospores (Tamija et al., 1953; Sorokin & Krauss, 1961). Working with the same strain of *Chlorella rubescens*, Sayegh & Greppin (1973, 1975) have shown a transient increase in the photosynthetic capacity during the photoperiod that apparently coincides with the second peak of EC described in our work. This suggest again that the photochemical process is primarily responsible for maintaining the high ATP levels in the algal cells in the light. The high energy state in the light was discussed by Heber (1974) as the basis for photorepression of mitochondrial respiration. In this case, the highest degree of inhibition of respiration would occur between time 50-55 h. However, it should be reminded that the interaction between photosynthesis and respiration have not yet been clarified (Zelitch, 1971; Graham & Chapman, 1979; Kawada & Kanazawa, 1982), but we know that glycolysis and the tricarboxylic cycle can be inhibited by a

high phosphorylation potential of adenylates (Hoch et al., 1963; Atkinson, 1970; Turner & Turner, 1975; Holian et al., 1977). In the dark, when photosynthesis is turned off, the ATP is mainly produced by glycolysis and oxidative phosphorylation and the third peak of EC, although more apparent in auto- and mixotrophy, reaches the same level at time 85 h in the three conditions of culture.

In regard of the importance of photosynthesis in energy metabolism, the organization of the photosynthetic apparatus was next investigated by measuring the chlorophyll *a*/*b* ratio. Variations of this ratio have been correlated, together with the functional reaction centers PS I and PS II, to the process of formation of the photosynthetic apparatus (Nelle et al., 1975; Mell, 178). During the life cycle of *Chlorella* the ratio of chlorophyll *a*/*b* undergoes considerable variations (Venediktov et al., 1981; Kamada & Kanazawa, 1982) and, in *Euglena*, De Filippis et al. (1981a, b) have reported changes in the ratio similar to changes in the EC ratio. In our experimental system, the chlorophyll was present in low concentration when light was not limiting for growth (see also Beale & Appleman, 1971). Nevertheless, variations of the chlorophyll *a*/*b* were detected in all conditions and could be related, although not in strict coincidence, to the fluctuations of EC, except in the case of mixotrophy. It is nevertheless possible that an endogenous control somewhat related to the control of EC is also operating on the photosynthetic apparatus. Interestingly, when Sayegh and Greppin (1973, 1975) measured the photosynthetic capacity of the alga during the life cycle, they found an increase of the capacity in the nyctoperiod that is coincident with an increase in both radios, chlorophyll *a*/*b* and EC, presented here.

The variations of AN induced changes not only in the EC but also in the ratio of ATP/AMP and ATP/ADP. These ratios are particularly important since they have been postulated to control key enzymes of metabolic sequences and can be considered as regulatory parameters (see Pradet & Raymond, 1983). For example, a high ATP/AMP ratio inhibits phosphofructokinase (Atkinson, 1970) which is the most important element of control of glycolysis. In our system, a high ATP/AMP ratio was observed in mixotrophy between 35-60 h which might reflect an inhibition of glycolysis during this period of time. Also, the decrease in the ATP/ADP ratio between 55 and 70 h in the three conditions of culture could correspond to a rapid reversal of the inhibition of glycolysis, as proposed by Santarius & Heber (1965). The changes in AMP were opposite to the changes in ATP, which is consistent with an equilibrium maintained by adenylate kinase (Bomsel & Pradet, 1968).

Finally, the fluctuations in EC that were maintained in the three conditions of culture, with a period of 35 to 40 h, might be the expression of an internal temporal structure. Several works have already been dedicated to phenomena displaying circadian rhythmicity in algae, namely to photosynthetic capacity in various species (Driessche, 1966; Sweeney et al., 1967; Okada et al., 1978) and to chlorophyll *a*/*b* ratio (Sayegh & Greppin, 1973), nuclear division and the production of daughter cells (Chen & Lorenzen, 1986; Wu et al., 1986), and enzyme activities (Chen & Lorenzen, 1986) in synchronous *Chlorella*. This aspect needs further work to be demonstrated.

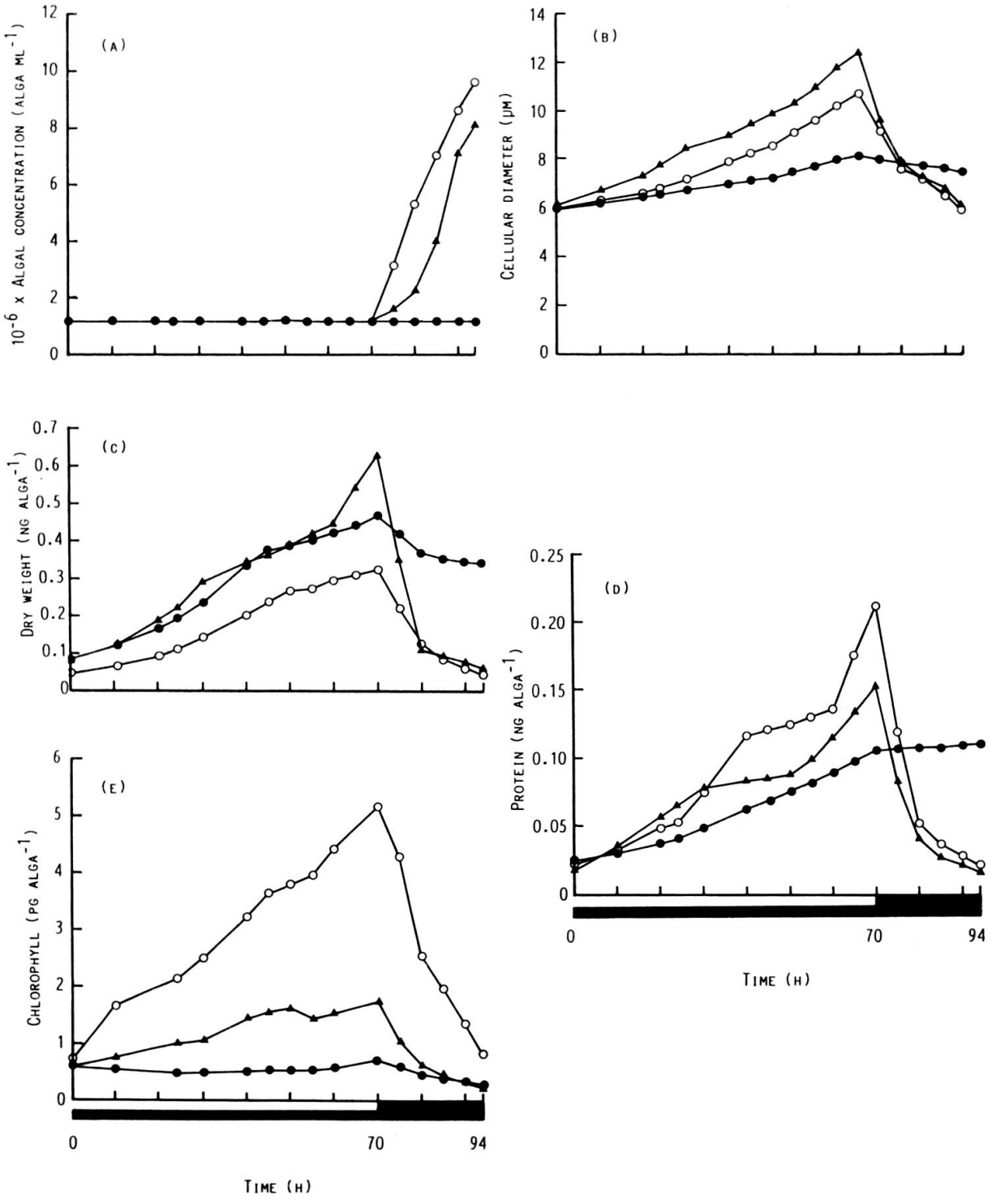

FIG. 1.

Variations of growth parameters of Chlorella rubescens. Algal concentration (A), diameter (B), dry weight (C), protein content (D) and chlorophyll content (E) were determined during synchronous growth in auto- (○), mixo- (▲) and heterotrophy (●). The auto- and mixotrophic cultures received a light: dark treatment of 72:24 h, and the heterotrophic culture was maintained in complete darkness for 94 h. Glucose (0.5%, w/v) was added to the medium of mixo- and heterotrophic cultures.

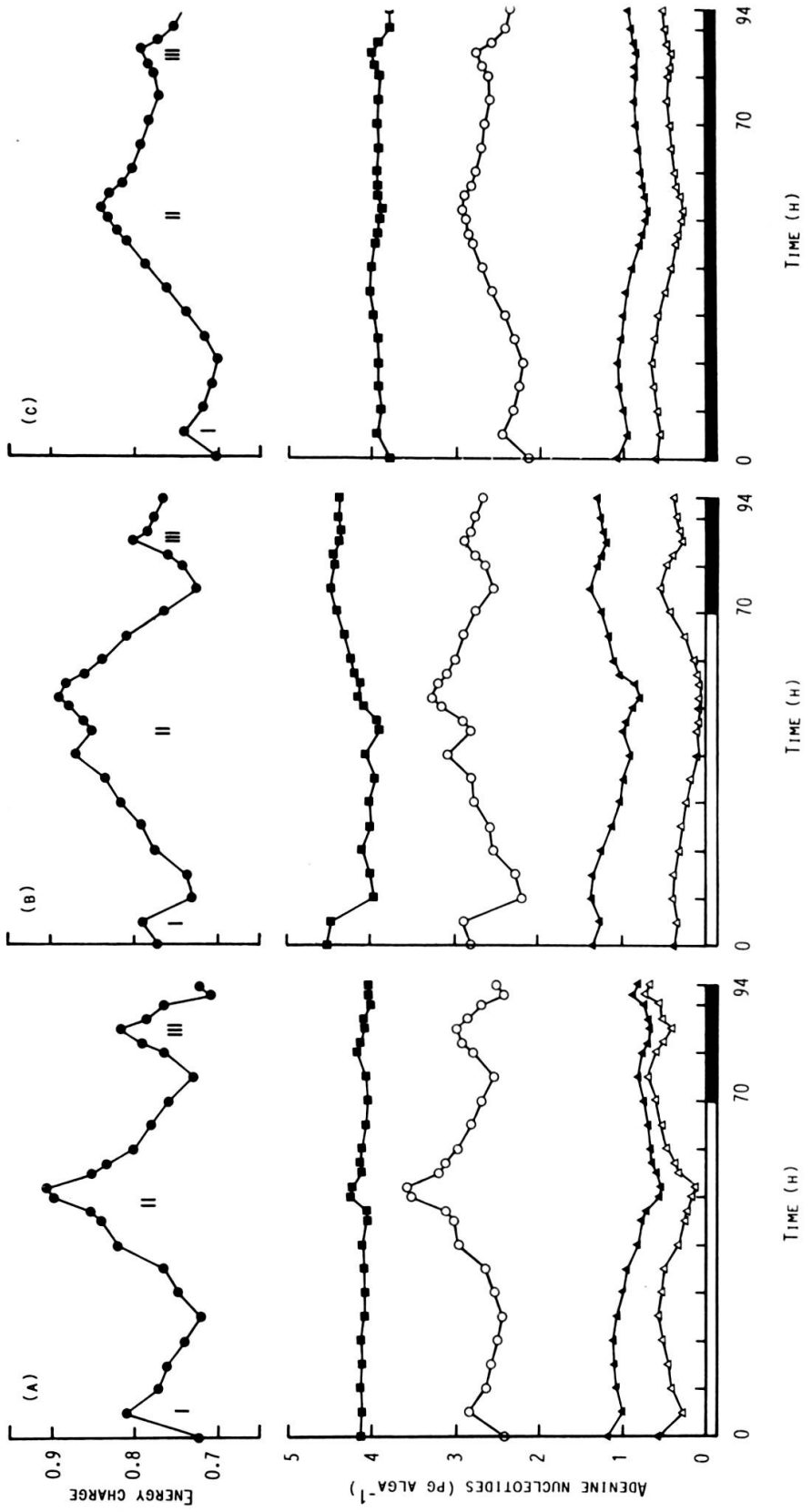

FIG. 2.

Fluctuations of the levels of adenylates and of energy charge. ATP (○), ADP (Δ), AMP (▲), ATP+ADP+AMP (■) and levels of energy charge (●) were determined in auto- (A), mixo- (B) and heterotrophy (C).

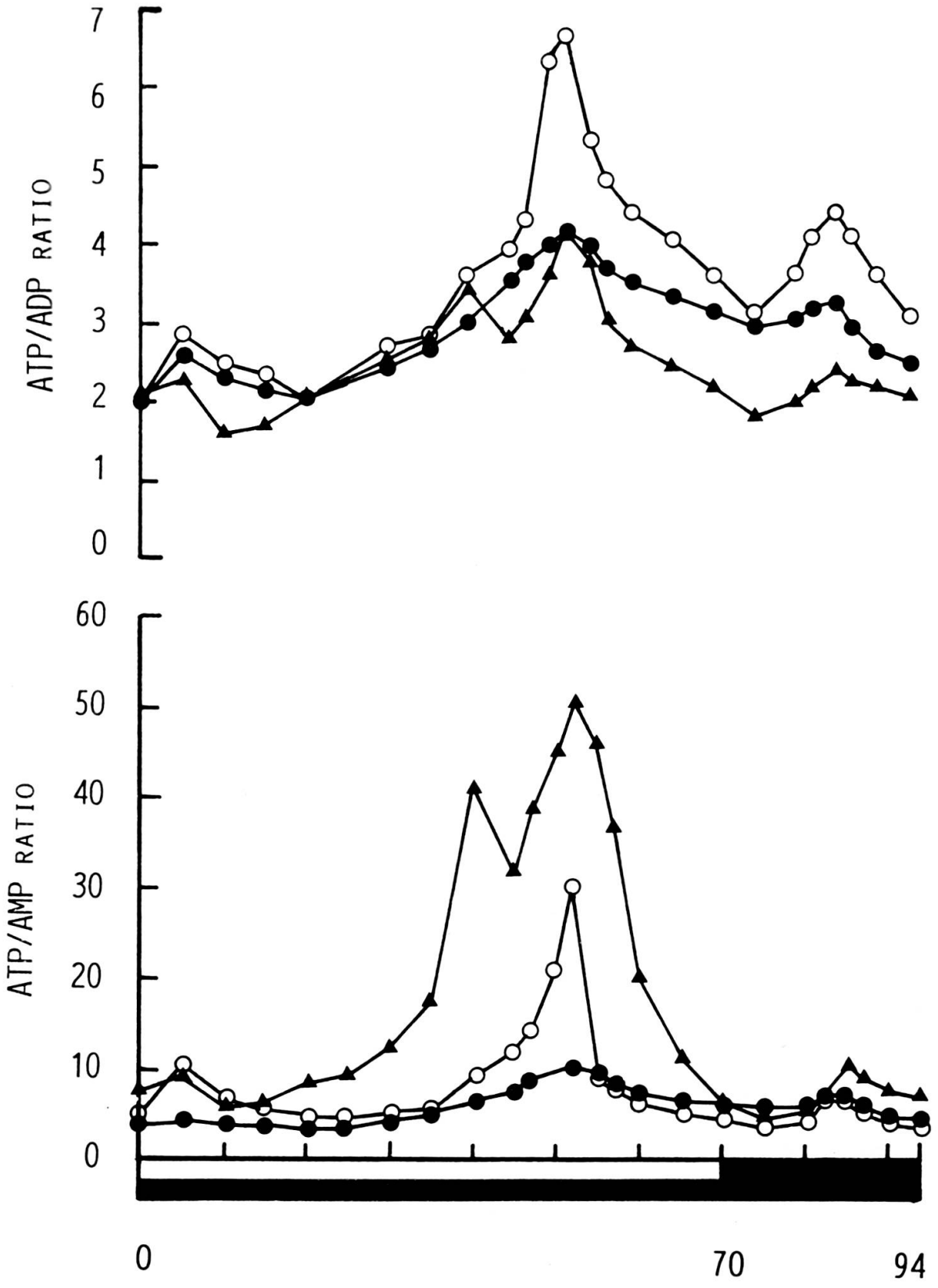

FIG. 3.

Fluctuations of adenylates ratios. ATP/AMP and ATP/ADP ratios were calculated from the values given in figure 2 for auto- (○), mixo- (▲) and heterotrophic (●) cells.

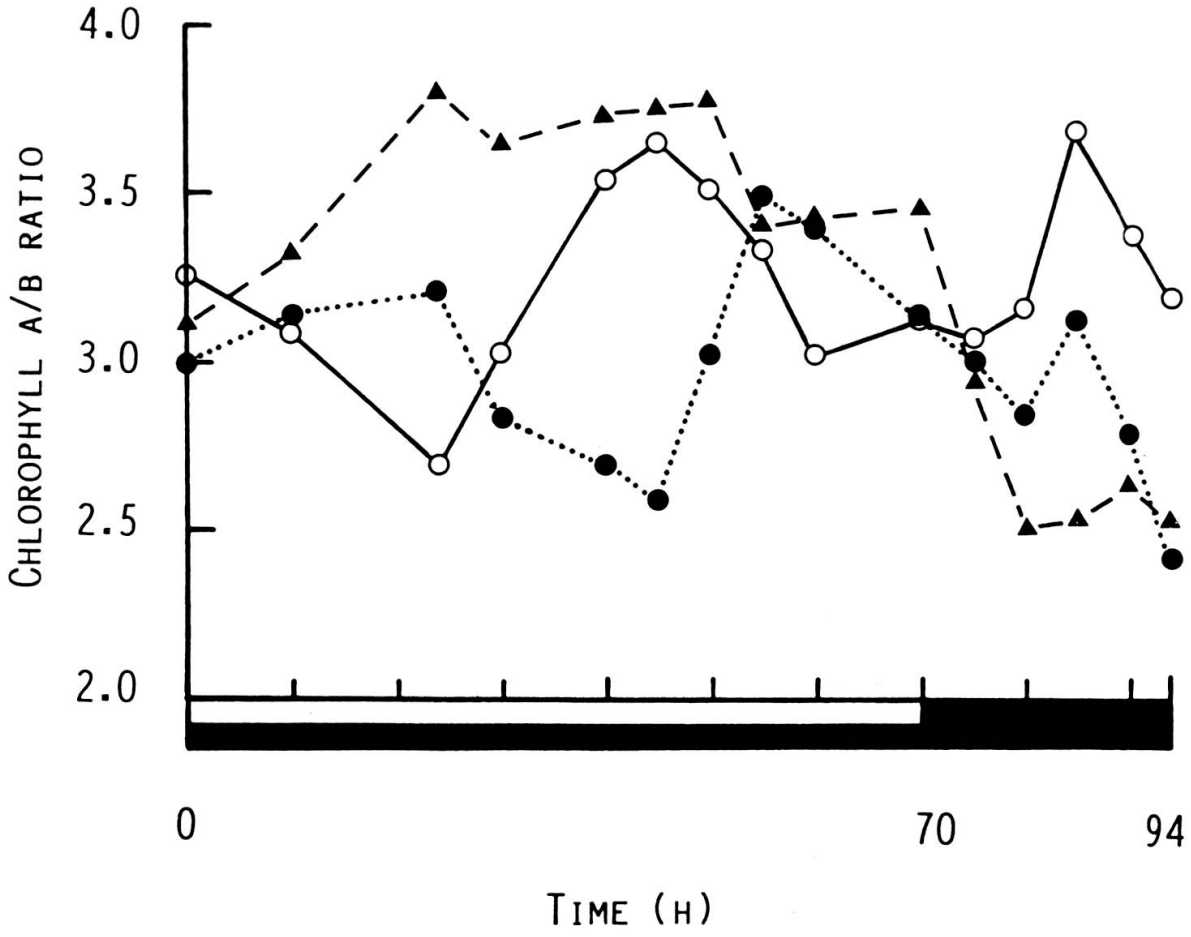

FIG. 4.

Fluctuation of chlorophyll a/b ratio. Values were determined in auto- (○), mixo- (▲) and heterotrophy (●).

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