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A circadian rhythm of gametangium formation in *Pseudobryopsis* sp. (Chlorophyta, Codiales)*

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Coenocytic gametophytes of a macroscopic green alga, *Pseudobryopsis* sp., were grown under various environmental conditions to determine the factors controlling synchronous gametangium formation. The process of gametogenesis is controlled by a circadian rhythm which is maintained even under constant conditions, with continuous light or darkness. In the formation of gametangia by plants controlled by the rhythm of an L/D cycle, formation is periodic and synchronous, not only in individual plants, but also in a population of plants, even if individual plants had been given induction treatments at different times. Both light-dark cycle and temperature change act as entraining stimuli (Zeitgebers) for the endogenous rhythm. However, the former is more effective. The effects of the circadian rhythm of synchronous gametangium formation gradually decline in plants exposed to continuous light for a long period (more than 60 h) and to continuous darkness of more than 36 h, but in the former case, one dark break of more than 4 h recovers the rhythm. The process of gametogenesis itself may help entrainment of the circadian rhythm. A 4 h dark break affects a free running circadian rhythm and shifts specific phases of the rhythm. DCMU inhibits the process of gametogenesis but has no effect on the circadian rhythm. These results demonstrate that an endogenous circadian rhythm controls the timing of gametangium formation in the organism studied.

Key Index Words: *circadian rhythm*; *gametangium formation*; *entraining stimulus (Zeitgeber)*; Pseudobryopsis.

Periodicity or the rhythm of reproductive activity has been studied in many algal species. Among these studies, BÜHNEMANN (1955) demonstrated that in the fresh water alga, *Oedogonium cardiacum*, sporulation shows a regular diel periodicity under a constant condition of light and temperature, and it is controlled by an endogenous circadian rhythm. Furthermore, RUDDAT (1960) investigated the circadian rhythm of sporulation of the same species with various entraining stimuli and demonstrated that a transition of dark-to-light and a sudden temperature change may also act as an entraining stimulus for endogenous rhythms. These studies of *Oedogonium* have contributed much to our understanding of endogenous circadian rhythms in algae.

Among marine macroscopic algae, several species have displayed periodicity of reproductive activity controlled by endogenous rhythms. For example, the lunar rhythm of egg-release in *Dictyota* has been fully investigated (BÜNNING and MÜLLER 1961, MÜLLER 1962, VIELHABEN 1963) and also gamete formation and release in *Derbesia* has been reported to be controlled by an

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endogenous rhythm at 4-5 day intervals (PAGE and SWEENEY 1968). However, with a few exceptions, including those mentioned above, most data are not clear regarding the demonstration of endogenous rhythms because of the incomplete presentation of laboratory conditions or difficulty in defining the natural conditions. These problems prevent unequivocal information as to the factors controlling reproductive activity. It seems, therefore, that there is no conclusive evidence regarding a circadian rhythm of reproductive activity in marine macroscopic algae.

The aim of the present study is to obtain positive evidence of gametogenesis controlled by an endogenous circadian rhythm in the marine coenocytic alga, *Pseudobryopsis* sp. and also to test responses to factors of light, dark, temperature, etc. as entraining stimuli for an endogenous rhythm in this alga.

Materials and Methods

Gametophytic plants of *Pseudobryopsis* sp. were collected at Ushuku (28°28′N; 129°43′E), Amami-Oshima, Japan in 1977 by Dr. S. ENOMOTO. and were maintained as axenic unialgal stock cultures grown in an artificial medium ASP_{12} (PROVASOLI 1963) under 22°C and 14 : 10 h L : D cycle.

Monosiphonous sterile plants about 2 months old were selected and isolated from the stock cultures and single upright axes (ca. 10-30 mm in length) without any lateral ramelli were cut into pieces of 4-5 mm in length. Fifteen fragments were grown unialgally (but not axenically) as a preculture in a petri-dish (65×80 mm) containing 150 m*l* ASP_{12} medium. They developed normally into mature gametophytes (15-20 mm in length) within 3 or 4 weeks.

For pre-cultures, the following growth conditions were employed for temperature-photoperiod regimes: 22°C, 14 : 10 h L : D cycle (14L/10D); 22°C, 10 : 14 h L : D (10L/14D); 22°C, 17.5 : 12.5 h L : D (17.5L/12.5D); 22°C, continuous light (L/L); 26°C for 14 h and 22°C for 10 h in continuous light (14TH/10TL-L/L). Illumination was provided by Toshiba "cool white" fluorescent lamps (ca. 3000-4000 lux).

For periodicity experiments, the basal parts of two sterile (reproductively immature) plants grown under each pre-cultural condition for 3 or 4 weeks, were cut off and transferred into screw-cap test tubes (18× 135 mm) containing 10 m*l* of a modification of ASP_{12} medium (a modification containing nine-tenths dilution of NaCl, $MgSO_4$, $MgCl_2$, KCl, $CaCl_2$, Na_2SiO_3, and Tris, and one-thirtieth concentration of Na_2glyceroPO_4 and K_3PO_4). The test tubes were placed in a water bath (50×28×26 cm) made of transparent plastic (acrylic resin) boards containing about 25 *l* tap water. The water in the bath was stirred and kept at 26±0.1°C using a portable thermo-regulator (T-80, Tokyo Rikakikai Co. Ltd.). Culture test tubes were incubated in the water bath with lateral lights mounted behind a plastic board outside the bath wall. Continuous illumination was provided by Toshiba "cool white" fluorescent lamps (20 W×2) and a constant light intensity of about 6000 lux was maintained at the surface of the test tubes in the water.

In general, gametogenesis of this plant could be induced by cutting the sterile plant, changing the medium, and transferring plants to a higher temperature. Consequently, these treatments will be called an "induction treatment" or "inducing" gametangium formation in this paper.

For observation of the process of gametangium formation, a plant grown in the test tube was transferred to a glass slide, mounted in several drops of the medium and quickly observed under a microscope (×100). After observation the plant was put back in the test tube. Observations were done at intervals and four stages in the process of gametangium formation were recorded in alphabetical order (Fig. 1). At the initiation of gametangium formation (stage A) there was no difference in color between contents of the gametangium and its mother plant (Fig. 1A). Then the gametangium (stage B) increased in size and showed an increased

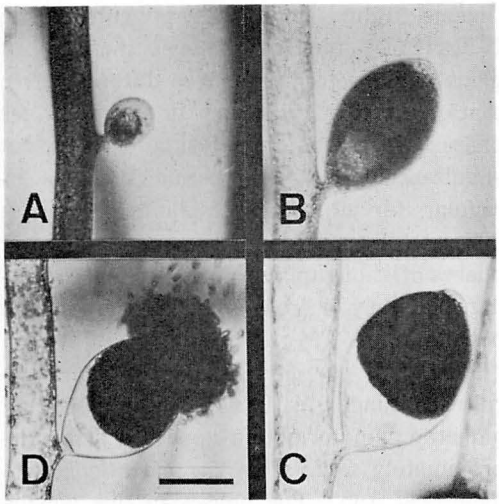

Fig. 1. The process of gametangium formation, which progresses synchronously in a plant, is divided into four stages. A. initial stage; B. further development of gametangium; C. mature gametangium; D. liberation of gametes. Scale: 50 μm.

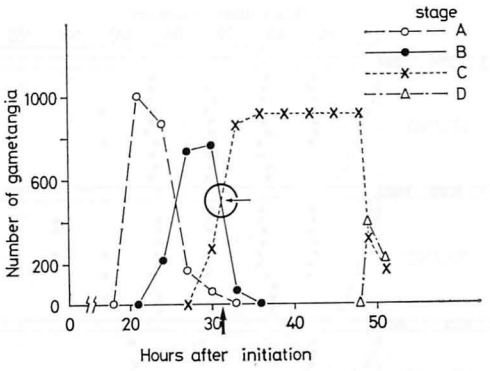

Fig. 2. Time course of gametangium formation. Point of intersection of decreasing line B and increasing line C (large open circle), i.e., the horizontal co-ordinate of the intersection (arrow), is regarded as the time of the formation of a series of gametangia. The time of initiation is the beginning of a continuous light period.

intensity of color (Fig. 1B). It developed into a reproductively mature gametangium (stage C) which formed a distinct papilla at its distal end, with the contents massed toward its upper part (Fig. 1C). Finally, gamete liberation (stage D) occurred (Fig. 1D).

To determine the rhythm of gametangium formation, the number of stage B and C gametangia were counted as an index. Plants which attained reproductive maturity under 14L/10D were observed to produce different stages of gametangia at the time of transition from the dark to the light period. This gametangium formation was synchronous and periodic and continued for more than two weeks. One ramullus on the mature plant bore a few gametangia and produced gametangia repeatedly and periodically. Those gametangia produced on each ramullus at the same time developed into the same stage. Two plants pre-cultured in 14L/10D were given an induction treatment and placed in an L/L condition, and the number of gametangia at each stage (stage A, B, C and D of Fig. 1) was counted and plotted at 3 h intervals (Fig. 2). Among the four stages, stage C was most easily distinguished from the others because of a protoplasmic accumulation at the apex of the gametangium which separated it completely from its stalk as mentioned above. Consequently, the phase change from stage B to C could be exactly determined. As shown in Figure 2, the two lines plotting the numbers of gametangia of both stages B and C intersected nearly at 31 h (between 30 and 33 h). At this time there were as many stage C gametangia as stage B. Therefore, the phase of the rhythm for gametangium formation was given by counting the numbers of stages B and C at 3 h intervals in the present experiment.

To observe the effects of an inhibitor of rhythmical gametangium formation, DCMU was added to a modified medium at various concentrations and finally a concentration of 10^{-6} M was employed in this experiment. Plants were given induction treatments and cultured in the medium with 10^{-6} M DCMU for certain periods. Then DCMU was removed by washing them 3 times in fresh medium.

Results

Free-running rhythm of gametangium

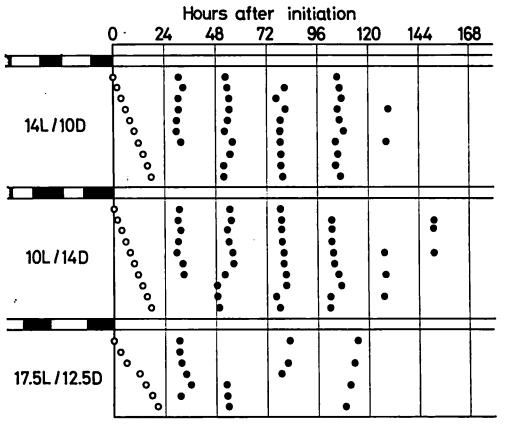

Fig. 3. Endogenous rhythm of gametangium formation of plants grown under 3 different light and dark cycles in pre-culture. Open and dark bar, ▬=light and dark period in pre-cultures; open bar, ▭=continuous light period in experimental cultures or pre-culture: ○=a group, at the time of induction treatments: ●=phase (time) of the formation of series of gametangia. These symbols are used in the following figures unless otherwise mentioned. The time of initiation is the beginning of a continuous light period.

formation in plants pre-cultured under 3 different light-dark cycles: Plants pre-cultured under conditions of 14L/10D, 10L/14D and 17.5L/12.5D, respectively were given induction treatments at 2 h intervals and transferred into test tubes incubated in a water bath with an L/L regime.

Figure 3 shows the relationship between the time of induction and the time of gametangium formation in populations of plants grown in 3 different pre-culture conditions. In the population of plants pre-cultured under 14L/10D or 10L/14D, the plants induced before 12 h after initiation exhibited the first phase at about the same time (30-33 h). The second phase occurred at 52-56 h in these populations. The third and subsequent phases also occurred approximately at 76-81 h and 100-104 h, respectively. As mentioned above, the phases of synchronous gametangium formation in the populations of plants induced at different times appeared rhythmically at about 24 h intervals under a constant condition of continuous light. In plants pre-cultured in 17.5L/12.5D, the rhythmical manner of gametangium formation was different from that of the former group. In this case, the phases of gametangium formation were not simultaneous and their periodicity was irregular for all groups of plants induced at different times. In some groups the different phases of gametangium formation were lacking, but they were partly rhythmic as a population.

Timing by one dark break: In plants pre-cultured under L/L, the first phase of gametangium formation appeared after approximately 26 h from the induction treatment (Fig. 4A). The phases in this population were not synchronous, but in some groups of plants induced at different times, the successive phases of gametangium formation occurred periodically at about 24 h intervals. This suggests that the induction treatment itself is an entraining stimulus

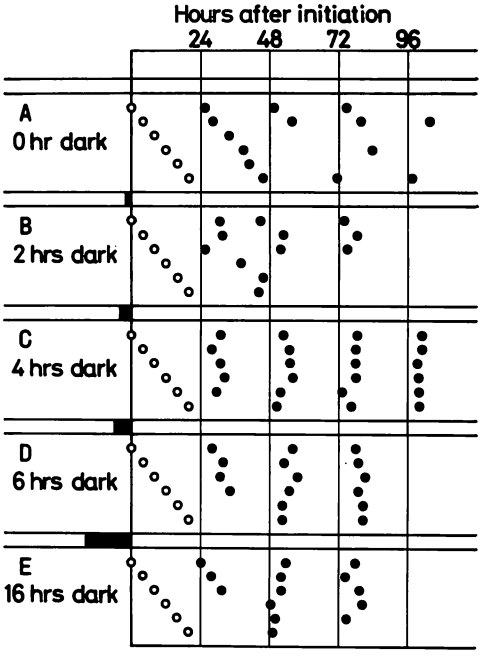

Fig. 4. Synchronous circadian rhythm in populations with one dark break during continuous light. The population of A, B, C, D and E were given one dark break (part of dark bar) for 0, 2, 4, 6 and 16 h respectively. The time of initiation is the end of a dark period.

for the circadian rhythm. In plants grown in L/L, however, the phases were changed by giving one dark break. Figure 4B shows the results for plants given a 2 h dark period. In this population, each phase of the groups induced at 0, 4 and 8 h after initiation appeared at about the same time, but were not simultaneous. In the population of plants given a 4 h dark period, however, each phase of the group induced at the times from 0 to 20 h appeared simultaneously (Fig. 4C). Also the populations of plants given 6 and 16 h dark periods showed the same results as plants given 4 h darkness (Fig. 4D-E). These results were quite similar to those obtained for plants pre-cultured in 14L/10D and 10L/14D. The phases of gametangium formation appeared at about the same time regardless of the different length of the dark break. This suggests that one continuous dark period of at least 4 h may recover the synchronous rhythm and periodic gametangium formation in a population of plants which has lost synchronization and periodicity under constant light. Thus the dark break is an entraining stimulus for the circadian rhythm of gametogenesis in this alga.

Synchronous gametangium formation by temperature cycle: Plants grown under conditions of 14TH/10TL-L/L (pre-cultural condition) for 14 days were transferred into L/L with a constant high temperature (26 °C, experimental condition) with induction treatments at 3 h intervals. The first phase of gametangium formation in the groups of plants given induction treatments from 0 till 12 h appeared at about the same time (36-40 h), but was delayed in other groups after 15 h, and appeared 31-33 h later (Fig. 5A). In some groups (those induced at 9 and 18 h), a diel periodicity could be observed.

In the population of plants exposed to the TH/TL cycle, the phases of gametangium formation appeared synchronously, whereas the control plants did not show any synchronous phase (cf. Fig. 4A). From this, it can be concluded that a temperature cycle given as a pre-cultural condition induces

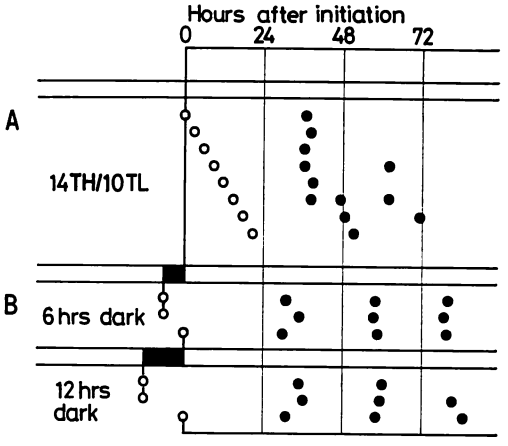

Fig. 5. Effect of temperature and one dark period on synchronous gametangium formation. 5A. Synchronization by temperature change, during the first phase in population grown in 14 TH/10 TL with continuous light in pre-culture. 26°C constant condition in experimental culture after hour 0. 5B. Synchronization by induction treatments with one dark break (part of dark bar). The time of initiation is the end of a dark period.

synchronous gametangium formation to some degree, regardless of the time of induction.

Although it only confirmed the results shown in Figures 4 C-E, the following experiment was attempted to demonstrate the relationship between one dark break and induction treatments as entraining stimuli.

Plants pre-cultured under an L/L condition were given a single dark break for 6 h or 12 h and also given the induction treatment before or after the dark period followed by the continuous light period. In the populations of plants given induction treatments before the dark break of 6 h or 12 h, the phases of gametangium formation coincided approximately at both darkperiodo and appeared rhythmically at about 24 h intervals (Fig. 5B). There was no significant difference in either the length of the dark period or the time of induction. In the populations of plants induced after the dark break (6 h or 12 h), the occurrence of phases also nearly corresponded with that of the former (before the dark break). So, induction treatments produced no significant difference when given before or after the dark period. This sug-

gests that a single dark break is more effective than induction treatment as an entraining stimulus (Zeitgeber) for synchronous and periodic gametangium formation in this alga.

Disappearance of synchronous rhythms in gametangium formation: Plants grown under 14L/10D were transferred into a constant condition of continuous light (L/L) or darkness (D/D) to remove any exogenous rhythmic signal. After incubation under both conditions, two plants from each culture were taken out at 3 h intervals and given induction treatments.

In the population of plants exposed to continuous light for 57 h, the phases of gametangium formation were the same as those of plants exposed to the light for shorter periods (Fig. 6A). However, when plants were exposed to continuous light for more than 57 h, the phases appeared less simultaneously and gradually disappeared. On the other hand, in the population of plants exposed to continuous darkness for 33 h or less, the plants maintained their synchronous phases of gametangium formation. In plants exposed to the dark for more than 36 h, the phases did not appear simultaneously (Fig. 6B).

The endogenous rhythms of a plant acquired during the L/D cycle were lost after 60 h when the plant was transferred to L/L or after 36 h in D/D. However, the circadian rhythm remained until at least the fifth phase even in L/L when plants attained reproductive maturity (cf. Fig. 3). It thus appears possible that this rhythm is entrained whenever the plant initiates gametogenesis by a circadian rhythm.

Phase-shift of a circadian rhythm by a single dark break: One dark period of more than 4 h is an entraining stimulus of the circadian rhythm controlling the phases of gametangium formation in this alga.

It was observed how the rhythmical phases of gametangium formation were influenced by a free-running circadian rhythm when exposed to a 4 h dark period at different times during an L/L condition. Plants grown in 14L/10D were given the induction treatment in the light period and were transferred into the L/L condition. Then they were exposed to a 4 h dark period at 2 h intervals from 0 h till 48 h (Fig. 7). In this population, the phases of gametangium formation were disturbed in the groups of

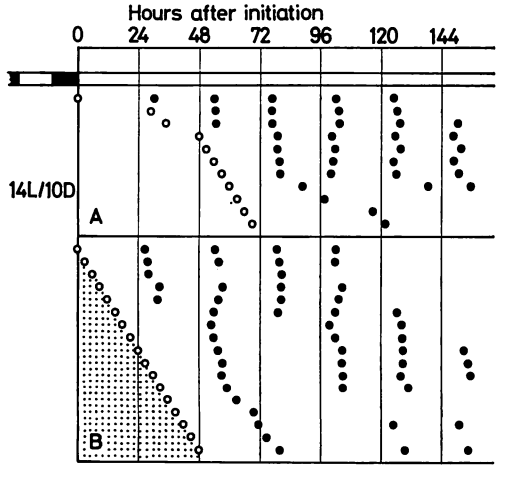

Fig. 6. Duration of endogenous rhythm under continuous light or dark conditions in plants. Dots in Fig. 6B show darkness after the last dark period in pre-culture. The time of initiation is the end of the last dark period in pre-culture.

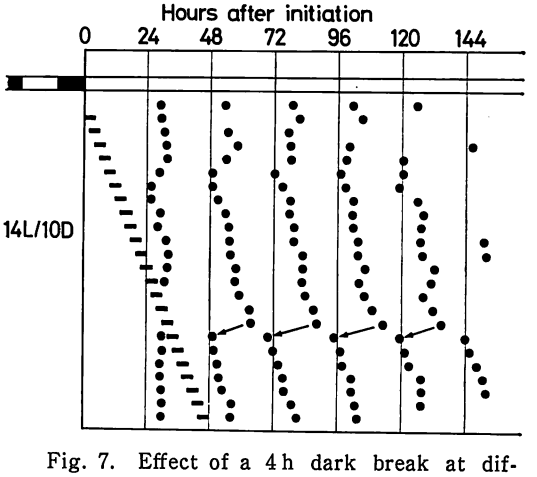

Fig. 7. Effect of a 4 h dark break at different times on the free running circadian rhythm. Short horizontal bar = 4 h dark break given to each group at 2 h intervals; Arrows = reverse of the phases from delay to advance after the second phases. The time of initiation is the end of the last dark period in pre-culture.

plants given a dark break at different times, particularly between the second phases and successive ones. In plants given the dark break between 8 h and 12 h, the phases were accelerated and in plants exposed to the dark between 14 h and 24 h the occurrence of phases was not greatly disturbed. However, in plants exposed to the dark between 26 h and 30 h the phases were gradually delayed, but were suddenly regained by almost one phase (about 24 h) in plants exposed to the darkness after 32 h.

As shown in Figure 7, an inverse sigmoid pattern of phase-shift is observed in the groups of plants given a dark period between 8 h and 30 h, and this continues periodically at about 24 h intervals for 144 h.

Effects of DCMU on gametangium formation: Although DCMU is a potent inhibitor blocking O_2 evolution during photosynthesis, it also suppressed gametangium formation at a concentration of 10^{-6} M in an experiment. Figure 8 shows the relationship between DCMU treatments and phases of gametangium formation in a population of plants grown in 14L/10D. DCMU at 10^{-6} M was added to the medium in each group of cultures for a specific period and then was removed. After removal of DCMU, in plants given the induction treatment gametangium formation appeared again. Each phase in the groups treated by DCMU appeared almost simultaneously. Although DCMU treatments of short and long duration completely inhibited gametangium formation, neither the phase nor the period of the rhythm was injured by its application.

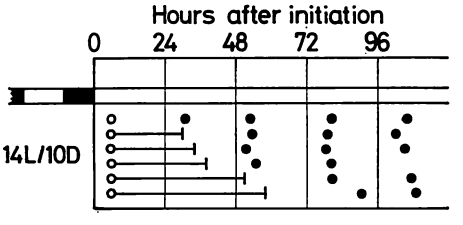

Fig. 8. Effect of DCMU on the phases of gametangium formation. ——=period of plants exposed to DCMU at 10^{-6}M after induction treatment. The time of initiation is the beginning of a continuous light period.

These results suggest that only the "hand" (meaning the phenomenon controlled by a circadian rhythm) of a basic oscillator is affected by DCMU treatment, but not the oscillator itself.

Discussion

Processes of reproductive activity occur in many organisms depending on periodic changes of environmental conditions. However, if reproductive activity in those organisms is maintained by periodical rhythms under constant conditions, it should be determined whether the process is controlled by a certain endogenous rhythm. In macroscopic algae, endogenous rhythms controlling their reproductive activities have been reported in *Dictyota*, as a lunar rhythm of egg-production, in *Derbesia*, as a 4-5 day rhythm of gametogenesis, and in *Oedogonium*, as a circadian rhythm of asexual reproduction. As mentioned above, each species has a periodic and endogenous rhythm which may be species specific.

In the present study, it is confirmed that gametangium formation of *Pseudobryopsis* sp. is controlled by an endogenous circadian rhythm and the rhythm is maintained even under constant conditions. For example, a population of plants pre-cultured under a long day or short day condition, and given induction treatments at different times, shows control of gametangium formation by a diel rhythm even under continuous light. However, another population of plants grown in 17.5L/12.5D cycle (30 h photoperiod) shows a 24 h period of gametangium formation under the L/L condition, but not 30 h periodicity. These findings suggest that gametangium formation of *Pseudobryopsis* sp. is controlled by an endogenous circadian rhythm under constant conditions and that it also occurs independently of a shift of the light-dark cycle.

PAGE and KINGSBURY (1968) have demonstrated that sudden temperature change and medium renewal act as entraining stimuli for endogenous rhythms in gametogenesis

of *Derbesia*. In this study, we also employed induction treatments, such as cutting the plant, changing the medium and transferring thalli to higher temperatures for gametangium formation. Such induction treatments are to act as entraining stimuli. These induction treatments were given to plants in the same population at different times at specified intervals and it was observed whether periodic and synchronous gametangium formation occurred in the population or not. Further, we attempted to equalize the effects of environmental factors by including induction treatments for an endogenous rhythm. From these experiments, it is demonstrated that an endogenous rhythm is affected by light and dark conditions or temperatures. The induction treatment seems to act as an entraining stimulus and also as a coupling of a basic oscillator to the "hand" (gametangium formation) in plants grown under L/L conditions (cf. Fig. 4A), whereas it may act only as the coupling in plants grown under L/D conditions (cf. Fig. 3) or given one dark break in an L/L (cf. Fig. 4 C-E). In some case, however, it seems that the basic oscillator is entrained again by induction treatments because phases at 24 h intervals appear in groups of plants (at 18 h in Fig. 5A, 60 h in Fig. 6A, and 42 h and 48 h in Fig. 6B) given the treatments. Therefore, the effect of induction treatments may be variable according to the amplitude of the basic oscillator, with an increase and a decrease at respective time, especially as it induces an entrainment of the basic oscillator when the amplitude decreases.

In a leaf of *Vicia fava* grown under a 11L/13D cycle, the movement and change in nuclear size of the epidermal cells are controlled by a circadian rhythm even when it is transferred into an L/L condition, but no rhythm appears when the plant is cultured under the L/L condition from the beginning (WASSERMANN 1959). In *Pseudobryopsis* sp. the population of plants pre-cultured in an L/D cycle shows synchrony of gametangium formation when transferred to an L/L condition and the phases of formation correspond to the endogenous rhythm. However, there is no synchrony of formation in the populations of plants pre-cultured under the L/L condition. So our results agree with the data on *Vicia fava* described by WASSERMANN.

It appears that one factor which controls the phases of endogenous rhythm in a population of plants is the length of the dark period inserted during L/L conditions. According to WASSERMANN (1959), in *Phaseolus multiflorus* grown under L/L conditions, the occurrence of leaf movement controlled by a circadian rhythm requires at least a 9 h dark period. In a population of *Pseudobryopsis* sp. plants grown under L/L conditions, synchrony of gametangium formation reappears after a single dark break of more than 4 h. These results indicate that one dark break acts as an entraining stimulus for an endogenous circadian rhythm.

WASSERMANN (1959) has considered that in *Vicia fava* grown under L/L conditions, the rhythmical phenomena of epidermal cells become free from the control of endogenous rhythms. In *Pseudobryopsis* sp. pre-cultured under L/L conditions, gametangium formation is not controlled by endogenous rhythms. Endogenous rhythms in populations of plants exposed to L/L conditions are lost on and after the fourth phase of gametangium formation and, in populations transferred to D/D conditions, endogenous rhythms are lost on and after the third phase. However, gametangium formation in plants controlled by endogenous rhythms appears synchronously and periodically untily at least the sixth phase (for about 150 h) through the mechanism of circadian rhythm.

According to OKADA *et al.* (1978), in *Bryopsis maxima* a diel rhythm of photosynthesis was disturbed at the period when gametes are discharged from gametangia, but the rhythm reappeared with a larger amplitude after this period. Similarly, it is expected that an endogenous rhythm for gametangium formation in *Pseudobryopsis* sp. is amplified at every period of gamete liberation and is maintained synchronously for

many phases of gametangium formation in the same population of plants.

In zoospore production of *Oedogonium cardiacum*, a sudden temperature change acts as an entraining stimulus for an endogenous circadian rhythm (RUDDAT 1960). According to RUDDAT, in this circadian rhythm of zoospore production a strong fluctuation of phase occurs when thalli are given a low temperature during the primary light period rather than during the primary dark period. He has considered that there are two phases in the circadian rhythm; one, a high-tension period, reacts to a low temperature, and another, a low-tension period, does not easily respond to such a stimulus. These phases may alternate with each other. In *Pseudobryopsis* sp. the high-tension period for the circadian rhythm is the primary light period. There is a strong fluctuation of phases caused by one dark break of more than 4 h. The low-tension period is the primary dark period, which does not significantly change the phase of gametangium formation. The present alga appears to have a similar sensitivity to temperature changes as *Oedogonium*.

In general, the gametes of most marine algae do not survive for a long period after liberation from the mother plant, if they can not develop parthenogenetically. Therefore, the population of a species growing in the same habitat must be able to discharge gametes of both sexes synchronously by endogenous rhythms which are entrained by the same environmental factors. However, there are many difficulties in understanding the endogenous rhythms of reproductive activities in these algae, because of our lack of precise data, especially concerning the relationship between the rhythmic phenomena of morphology and the physiological or biochemical changes which occur during reproductive processes, including gametogenesis. The present investigation attempts to contribute to our understanding of endogenous rhythms and the controls of gametogenesis for algal species.

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奥田一雄・舘脇正和: *Pseudobryopsis* sp.（緑藻類ミル目）の配偶子嚢形成の概日リズム

海産多核緑藻 *Pseudobryopsis* sp. を種々の環境条件下で培養し，配偶子形成が概日リズムの制御によって同調的にしかも周期的に起こることを明らかにした。配偶子形成を誘導する条件，明暗交代および温度変化は，この内在リズムの同調因子として作用する。配偶子形成の概日リズムは60時間以上の連続明期下，または36時間以上の連続暗期下で同調性を失ってくるが，前者の場合4時間以上の暗中断によって回復する。自由継続する概日リズムは，4時間の暗中断によって位相変移（前進または後退）を起こす時間帯を有する。DCMU は配偶子形成の過程を阻害するが，内在リズム自体には影響を与えない。（051 室蘭市母恋南町 1-13，北海道大学理学部附属海藻研究施設）