THE CELL CYCLE OF SYMBIOTIC CHLORELLA

II. THE EFFECT OF CONTINUOUS DARKNESS

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SUMMARY

When green hydra were grown in continuous darkness the mean cell size of their symbiotic algae was smaller than when grown in light and numbers of algae per digestive cell were reduced. The former was due to a reduction in size at which the algae divided, and the latter to a loss of synchrony of algal mitosis with that of digestive cells after transfer to darkness. Eventually, algal mitosis regained synchrony with that of digestive cells. Division synchrony was not lost in reinfected hydra (with lower than normal numbers of algae per cell) transferred to darkness; this, and the observation that synchrony in normal animals transferred to darkness was regained when algal numbers per cell had fallen to a new, lower level, suggested that the initial inhibition of algal mitosis was due to competition for a limited supply of heterotrophically required metabolites.

When dark-grown hydra were returned to light there was no delay in algal division and a steady increase in the size of dividing cells, suggesting that the smaller division size in darkness was not due simply to the critical size for division being set at a lower value. In light, algal division size varied with frequency of host feeding, but this had less effect on algal division size in darkness.

It is suggested that the critical cell size that algae must attain before being able to complete the cell cycle is the same in light and darkness, but in light mitosis is restricted by some exogenous factor so that algae grow beyond the critical size without dividing. In darkness both algal cell growth and division are dependent on exogenously supplied metabolites, and cell growth rather than the division factor is limiting. The precise nature of the restriction on algal division remains unknown.

INTRODUCTION

In this paper, the effects of continuous darkness on cell growth and division of the symbiotic algae of green hydra are described. Growth in darkness considerably alters the relationship between the algal population and the host cells, although the precise reasons are unknown. Elucidation of the nature of these changes will test theories of host regulation of algal cell division and may provide further evidence for the way in which the cell cycle of the symbiotic algae is controlled within the host cell.

After hydra are transferred from light to continuous darkness, the mean number of algae per cell declines to a new stable level, which persists indefinitely (Pardy, 1974a,b; McAuley, 1981; Steele & Smith, 1981; Douglas & Smith, 1984). Pardy (1974b) suggested that algal division is inhibited in darkness because of competition for heterotrophically derived metabolites supplied by the host cell. Numbers of algae would fall because of the diluting effect of host cell division until a population size is

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reached where supply of metabolites is no longer limiting. Cook (1972) and Thorington & Margulis (1981) have shown that radioactively labelled substances introduced into the green hydra symbiosis via host feeding appear in algal metabolic pools, and experiments described by Douglas & Smith (1983) showed that in darkness green hydra with algae grew less well than those artificially rid of their algal symbionts (aposymbionts), presumably because in darkness algae competed with the host for essential metabolites.

Alternatively, Douglas & Smith (1984) suggested that division of algae in hydra may be controlled by host regulation of pH in the perialgal vacuole; low external pH diverts photosynthetically fixed carbon from cell growth to synthesis and release of maltose (Mews, 1980; Mews & Smith, 1982). Douglas & Smith (1984) suggested that when hydra are transferred to darkness the perialgal pH would decrease, possibly through inhibition of photosynthesis causing an increase in CO₂ levels, thus inhibiting algal division. However, there is no direct evidence to support either this or Pardy's theory, and in both cases inhibition of algal division was inferred from measurements of total numbers of algae in population of hydra. In this paper, the mitotic indices of algae and digestive cells were measured directly, after transfer of hydra to continuous darkness.

A second effect of growth of hydra in darkness is that the mean size of the algal cells declines (McAuley, 1980; Pardy, 1981; Douglas & Smith, 1984), although the cause and significance of this are not known. Pardy (1981) suggested that growth of the algae is arrested at a stage just after release from the mother cell, but even when a stable population size is reached in darkness, so that the algae are presumably dividing once more, the size of the algal cells does not increase. Here, measurement of the size of dividing algae in hydra grown in either diel photoperiod or continuous darkness suggested that the decline in algal cell size in darkness was due to division at a smaller size rather than inhibition of cell growth.

MATERIALS AND METHODS

Experimental organisms

Stock cultures of the European strain of *Hydra viridissima* were maintained in an illuminated incubator as previously described (McAuley, 1985). Hydra cultured in continuous darkness were maintained in the same incubator as stock cultures but in foil-wrapped dishes inside a light-proof box, and were exposed to ambient levels of light for less than a minute each day for feeding and cleaning.

Estimation of mitotic indices

Digestive cell and algal mitotic indices were measured as previously described (McAuley, 1982).

Measurement of algal cell size

Suspensions of algae were obtained by homogenizing gastric regions of five hydra (Pardy & Muscatine, 1973) in a glass microtissue homogenizer and were examined using ×1000 interference contrast microscopy. Diameters of 50 randomly selected algal cells were measured using an ocular micrometer and algal cell volumes were derived from these measurements by assuming the algae to be perfect spheres.
Estimation of numbers of algae per cell

Individual gastric regions were macerated on a glass slide by the technique of David (1973) and the resulting cell suspensions were examined using ×400 interference-contrast microscopy. Numbers of algae were counted in each of 30 digestive cells per slide.

Reinfection of aposymbiotic hydra

Suspensions of algae were produced by homogenizing large number of green hydra and separating algae from animal homogenate by centrifugation, finally resuspending in a dense slurry of about 10⁷ cells per ml. Aposymbiotic European hydra, derived from green hydra photobleached by the method of Pardy (1976) were injected with the algal slurry according to the method of McAuley & Smith (1982).

RESULTS

Effect of continuous darkness on algal and digestive cell division

Mitotic indices of algae and digestive cells were measured daily for 2 weeks in hydra transferred to continuous darkness or remaining in light (controls). At the beginning of the experiment 3-day starved hydra, in which digestive cell and algal mitosis are at low levels (McAuley, 1982), were fed and placed in darkness. Both algae and digestive cells showed a division response after this first feeding (Fig. 1A, B), but subsequently only the mitotic index of digestive cells increased after each feeding. Algal mitosis was not completely inhibited, but remained at a low level in comparison with peak values of that of digestive cells. In control hydra the mitotic indices of both algae and digestive cells increased after each feeding, confirming previous observations (McAuley, 1982).

After 2 weeks in darkness, algal mitosis began to increase after host feeding. The first division peak of the algae was delayed with respect to that of the digestive cells (day 13), but the second was similar to that of controls. Previous workers noted that numbers of algae per cell reached a new, lower, stable level in darkness within 2 or 3 weeks (Pardy, 1974a,b; McAuley, 1981; Douglas & Smith, 1984). The observation made here that division of algae and host digestive cells once more became synchronized after 2 weeks growth in darkness is consistent with the idea that algal division resumes once the new relationship is reached in darkness.

To test whether the loss of algal division synchrony with that of host cells was due to competition for a limited supply of heterotrophic metabolites, as suggested by Pardy (1974b), algal division was monitored in newly reinfected hydra transferred to darkness. In these hydra, numbers of algae per cell were comparable to those in hydra adapted to continuous darkness (cf. Fig. 5c), so that if loss of synchrony was simply due to competition, no change in the pattern of division would be expected.

Each day, using ×625 interference-contrast microscopy, numbers of algae were counted in 50 infected digestive cells in macerates of each of three gastric regions and the presence of dividing algae was noted. Controls were reinfected hydra maintained in light. The results (Fig. 2) showed that no loss of algal division synchrony was observed in reinfected hydra when transferred to darkness. Peak values of algal
mitosis declined in both dark-grown and control reinfected hydra as the experiment proceeded; a similar decline from initially high values of algal mitosis has been observed in reinfected European aposymbionts (McAuley & Smith, 1982). Numbers of algae per cell remained at a low level in dark-grown hydra but increased in those grown in light.

Fig. 1. Mitotic indices of algae (●-●) and digestive cells (○-○) in diel photoperiod (A), and after transfer to continuous darkness (B). Arrows indicate days when hydra were fed. Points are the amalgamated means of two experiments in which 1000 cells were scored each day for the presence or absence of division.
Cell cycle of symbiotic algae in darkness

![Graph showing changes in the number of algae per digestive cell and the mitotic index (%) over 8 days in darkness.](image)

- **Fig. 2**
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Size of algal cells in darkness

Daily measurement of algal cell size (Fig. 3) confirmed previous reports that it decreased in darkness (McAuley, 1980; Pardy, 1981; Douglas & Smith, 1984). Pardy (1981) suggested that this was due to arrest of growth of daughter cells after release from mother cells, but measurement of the size of dividing algae in darkness (Fig. 4) showed that the mean cell size of algae at division decreased after transfer to darkness. Measurement of sizes of dividing algae (mother cells) and autospores (daughter cells) in hydra kept for several weeks in continuous darkness confirmed that in darkness algae divided at a smaller size than in light (Table 1). While the size of dividing algae was significantly smaller in darkness \( (P > 0.001) \) the number of daughter cells produced per dividing cell was not \( (P < 0.10) \), so that daughter cell size was also significantly reduced \( (P > 0.001) \).

Growth of ‘stable’ populations of algae in darkness

Previous work has shown that the decline in numbers of algae per digestive cell in hydra transferred to darkness eventually ceases. Thereafter, numbers are maintained at a new stable level, lower than in hydra grown in light (Pardy, 1974a, b; McAuley, 1981; Steele & Smith 1981; Douglas & Smith, 1984). However, no attempts to characterize growth and division of algae in stable dark-grown populations have been made.

In the experimental conditions described here, it was found that about 2 weeks after transfer to darkness the mitotic index of the algae, previously at a low level, once more
Cell cycle of symbiotic algae in darkness

Fig. 4. Mean volume of dividing algae in light (○-○) or after transfer to continuous darkness (■-■). Points are amalgamated means of measurements from two experiments. Bars indicate standard errors of means.

Table 1. Size of dividing and daughter cells of Chlorella in light in darkness

<table>
<thead>
<tr>
<th></th>
<th>Mean cell volume (μm³)</th>
<th>No. of daughter cells/ dividing alga</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Dividing cells</td>
<td>Daughter cells</td>
</tr>
<tr>
<td>Light</td>
<td>175·54 ± 15·64 (100)</td>
<td>49·70 ± 0·72 (48)</td>
</tr>
<tr>
<td>Dark</td>
<td>83·02 ± 2·80 (100)</td>
<td>19·25 ± 0·60 (56)</td>
</tr>
</tbody>
</table>

Values are means ± standard error of mean. Numbers of measurements are given in parentheses. Diameters of algal cells were measured in duplicate homogenates of five gastric regions of 1-day starved hydra grown in diel photoperiod or in continuous darkness for at least 4 weeks. For the purpose of measurement of cell size, daughter cells were identified as outside the mother cell wall but still attached.

* No significant difference;  \( t = 0·498, \text{ d.f. } = 98. \)

increased each time the hydra were fed, in synchrony with digestive cell division (Fig. 1a). To investigate whether this was due to stabilization of the algal population, numbers of algae per cell, algal cell size, and division of algae and digestive cells, were measured in hydra during the fourth week of growth in darkness. The same parameters were measured in control hydra grown in light.

No change in either mean cell volume of algae or numbers of algae per digestive cell were observed in dark-grown hydra over the 5-day period of measurement (Fig. 4c,d). Both were lower than in controls. Algal and digestive cell mitotic indices increased in these 'stabilized' dark-grown hydra after each feeding, although peak values were consistently lower than in light (Fig. 4a,b). This may have been because
Fig. 5. A. Mitotic indices of algae (●-●) and digestive cells (○-○) of hydra grown in continuous darkness. B. As A, but from hydra grown in diel photoperiod. C. Number of algae per cell in gastric regions of hydra grown in diel photoperiod (○-○) or continuous darkness (●-●). D. Volume of algae from gastric regions of hydra grown in diel photoperiod (○-○) or in continuous darkness (●-●). Points are amalgamated means of duplicate experiments; bars indicate standard errors of means.

Fig. 6. A. Mitotic indices of algae from hydra previously kept in darkness, fed on day 0 and either transferred to diel photoperiod (○-○) or kept in continuous darkness (●-●). B. Volume of dividing algae from same hydra. (○-○) transferred to diel photoperiod; (●-●) kept in continuous darkness.
Cell cycle of symbiotic algae in darkness

![Graph A](image)

![Graph B](image)

Fig. 6
heterotrophically derived metabolites had to support both algal and animal cell growth and division in darkness (Douglas & Smith, 1983). Estimation of the population size of algae from daily measurements of mean algal cell volume and numbers of algae per cell showed that in darkness the volume of algae supported by digestive cells was approximately one-third of that in light.

**Effect of transfer from darkness to light and of feeding frequency on size of dividing algae**

When populations of algae have stabilized in darkness, algal mitosis regains synchrony with that of host cells but algal division size remains smaller than that in light. It is possible that there may be a critical size below which algal cells cannot initiate mitosis, and this may be set at a lower size in darkness than in light. To investigate this, mitotic indices of algae were measured after dark-grown hydra were fed and returned to light (Fig. 6). If division size was indeed set at a lower level in darkness then upon transfer to light a delay in algal mitosis might be expected, since algae would be smaller than the critical size for division in light and would first have to grow before being able to enter mitosis.

However, after feeding, the mitotic index of algae in hydra transferred from darkness to light showed a similar increase to that of control hydra normally maintained in diel photoperiod (Fig. 6A). Further, the mean size of dividing algae showed a gradual increase rather than a discontinuous jump from one critical size to another (Fig. 6B). Thus, in light, some factor other than the requirement to reach a critical size before being able to enter mitosis may control the timing of algal cell division.

Mitosis of symbiotic algae is stimulated by host feeding (McAuley, 1982, 1985). If algae do not divide at a critical cell size in light, increasing the frequency of host feeding (and so increasing the frequency of algal mitosis) would cause a decrease in algal cell size at division. This is because the time between each cell division would be decreased but the rate of cell growth (i.e. increase in cell volume per unit time) would remain constant. The effect of feeding hydra at different frequencies on the size of dividing algae in diel photoperiod and in continuous darkness is shown in Table 2. In diel photoperiod the size of dividing algae varied in direct proportion to the frequency at which their hosts were fed. However, in darkness there was no consistent variation in algal division size with frequency of host feeding.

Table 2. **Effect of feeding schedule on cell size of dividing algae (i.e. volume in μm³)**

<table>
<thead>
<tr>
<th></th>
<th>Each day (μm³ ± SEM)</th>
<th>Each 2 days (μm³ ± SEM)</th>
<th>Each 4 days (μm³ ± SEM)</th>
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<tbody>
<tr>
<td>Diel photoperiod</td>
<td>149.43 ± 2.80</td>
<td>169.56 ± 3.00*</td>
<td>185.96 ± 3.00*</td>
</tr>
<tr>
<td>Darkness</td>
<td>68.51 ± 1.76</td>
<td>73.66 ± 1.33**</td>
<td>68.64 ± 2.22</td>
</tr>
</tbody>
</table>

Values are derived from measurement of diameters of 50 dividing algae in replicate homogenates of five hydra, given as mean volumes (μm³) ± standard error of mean. Hydra were acclimatized to feeding regimes for 2 weeks, and measurements made 1 day after feeding.

*P > 0.001, ** > 0.05 that size differs significantly from size of algae in hydra fed each day.
DISCUSSION

In light, mitotic indices of symbiotic algae and digestive cells increase in parallel after hydra are fed (McAuley, 1982, 1985), but when hydra are transferred to darkness, feeding-associated cell division of algae was initially inhibited. Algal division resumed its normal pattern when numbers of algae per cell had fallen to a new stable level, and no inhibition was observed when reinfected hydra, with lower than normal numbers of algae per cell, were transferred to darkness. These observations support the hypothesis of Pardy (1974b) that in darkness symbiotic algae must compete for a limited supply of metabolites necessary for growth and division. The suggestion of Douglas & Smith (1984), that algal division is inhibited in darkness because perialgal vacuolar pH decreases, cannot explain why there is no inhibition in reinfected hydra, since change in vacuolar pH would affect algal division regardless of the number of algae per cell.

A number of workers have proposed that cells of microorganisms must attain a critical size before being able to complete the cell cycle (for review, see Fantes & Nurse, 1981). Although Donnan & John (1983) showed that mitosis of the unicellular alga *Chlamydomonas* is probably under a temporal rather than a size control, they suggested that at low growth rates (comparable to those of hydra algae) a size control could not be ruled out. Further, *Chlamydomonas* division differs from that of the hydra *Chlorella* in that cell size at division controls the number of daughter cells produced by *Chlamydomonas* (Craige & Cavalier-Smith, 1982; Donnan & John, 1983), while differently sized *Chlorella* dividing in darkness or light produced similar numbers of daughter cells. Separate size controls regulate entry into *S* phase and mitosis in the fission yeast *Schizosaccharomyces pombe* (Nurse & Thuriaux, 1977; Fantes & Nurse, 1977). In *Chlorella* only a single control point is possible, since *S* phase and mitosis alternate during cell division (twice in the case of symbiotic *Chlorella* to produce four daughter cells) with no intervening *G2* period (John et al. 1973).

The simple hypothesis, that algae divide at different sizes in light- and dark-grown hydra because the critical size necessary for cell division is set at a higher threshold in light, is not supported by experiments in which hydra were fed upon return to diel photoperiod, since the algae were able to divide immediately, at intermediate sizes. Further, workers growing free-living *Chlorella* heterotrophically in darkness have not noted a reduction in cell size compared to autotrophic cultures.

As an alternative explanation it is suggested that: (1) if there is a critical size for cell division of symbiotic algae it is the same in light and darkness; and (2) in light, algal cell division is restricted by requirement for some exogenous factor but cell growth is not (McAuley, 1985), so that although algae reach and grow beyond the critical size they are unable to divide. In darkness, however, both cell growth and cell division of algae are dependent upon metabolites derived from host digestion of prey, and those necessary for cell growth are more limiting than those necessary for cell division. Thus, algae tend to divide at the critical size, as cell growth rather than the division factor is restricting. In both cases the host cell may regulate supply of metabolites...
needed by the algae, and thus entrain algal division to its own cell cycle, so that populations of algae are maintained at a constant and optimum size.

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Cell cycle of symbiotic algae in darkness


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