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THE CELL CYCLE OF SYMBIOTIC *CHLORELLA*

IV. DNA CONTENT OF ALGAE SLOWLY INCREASES DURING HOST STARVATION OF GREEN HYDRA

P. J. McAULEY

Department of Plant Sciences, University of Oxford, Agricultural Science Building, Parks Road, Oxford OX1 3PF, UK

AND L. MUSCATINE

Department of Biology, University of California, Los Angeles, 405 Hilgard Avenue, Los Angeles, CA 90024, USA

SUMMARY

The distribution of DNA content of symbiotic *Chlorella* algae freshly isolated from green hydra was compared with that of cultured *Chlorella* of the NC64A strain, using flow cytometry. In non-logarithmic cultures of NC64A most cells had accumulated in G_1 phase, while in logarithmic cultures a peak containing cells in S phase and mitosis could be distinguished from the larger G_1 peak. However, symbiotic algae showed a single broad peak in which there was no clear distinction between G_1 and S phase/mitosis. When hydra were starved for a prolonged period, inhibiting host cell and algal division, the DNA content of the symbiotic algae slowly increased, and the number of daughter cells produced after a single feeding increased with the length of the preceding period of starvation. This suggests that symbiotic algae are able to cycle slowly through S phase, but unless the host is fed they cannot traverse into mitosis and complete the cell division cycle. No significant difference in cell size was found between algae producing either four or eight daughter cells after 1-day- or 22-day-starved hydra were fed, suggesting that algal cell size did not determine the number of daughter cells produced. Instead, this may be dependent upon the length of time the cell had spent in S phase prior to receiving the, as yet unknown, stimulus to enter into mitosis.

INTRODUCTION

The cell division cycle of free-living strains of *Chlorella* has been extensively characterized using synchronized cultures (Tamiya, 1963; John *et al.* 1973). When a cell divides to produce, for example, four daughter cells, there are two separate rounds of DNA synthesis each directly followed by mitosis with no intervening G_2 period. The first round of mitosis produces two daughter cell nuclei, the second four. This basic pattern can be extended, depending upon mother cell size (Donnan *et al.* 1985), to multiply further the number of daughter cell nuclei. Cytokinesis immediately follows the final round of mitosis, yielding discrete autospores, which grow and rupture the enveloping mother cell wall.

In *Chlorella* symbiotic within digestive cells of green hydra, the cell division cycle is controlled at some unknown point by the host cell. When the host cell divides, a

Key words: *Chlorella*, green hydra, symbiosis, cell cycle, flow cytometry.

proportion of the symbiotic *Chlorella* algae also divide, but otherwise division of algae is inhibited (McAuley, 1981, 1982, 1985*a*). This relationship between host cell and algal mitosis is part of the control mechanism whereby the population of symbiotic algae within a hydra is maintained at a constant level in constant conditions, since growth rates of algae and host cells are closely similar (Pardy, 1974). During host starvation, both algal as well as digestive cell division ceases, so that algae do not overgrow their host (McAuley, 1982, 1985*a*; Douglas & Smith, 1984).

Initiation of cell division in symbiotic *Chlorella* appears to depend upon supply of exogenous 'division factor' ultimately derived from host heterotrophic nutrition (McAuley, 1985*a*), although the nature of the factor and precisely where it operates in the *Chlorella* cell cycle are unknown. Furthermore, it is not known if all the algae eventually complete the cell cycle or whether a proportion are non-proliferating. During host starvation, it is possible that the majority of the symbiotic *Chlorella* cells are resting in a quiescent stage analogous to the G_0 stage of non-cycling mammalian or yeast cells (Lajtha, 1963; Tobey, 1973; Dethlefsen, 1980; Iida & Yahara, 1984), as algal mitosis falls to a very low level and increase in algal cell size and protein content is very slow (McAuley, 1985*a*). When hydra are fed after prolonged starvation, algal but not digestive cell mitosis is delayed (McAuley, 1985*a*), suggesting the need for a recovery period before the normal cell cycle is resumed.

In this paper, the DNA content of populations of symbiotic *Chlorella* was measured using flow cytometry. In starved hydra, the non-dividing *Chlorella* slowly accumulated DNA, suggesting that although they are able to enter and slowly proceed through *S* phase, nuclear division was inhibited.

MATERIALS AND METHODS

Maintenance of organisms

Stock cultures of the European strain of green hydra were maintained in an illuminated incubator in constant light, as described previously (McAuley, 1985*a*). *Chlorella* of the NC64A strain, derived from *Paramecium bursaria* (Muscatine *et al.* 1967) and *Chlorella vulgaris* 211/1e, obtained from the Cambridge Culture Centre of Algae and Protozoa, were cultured axenically in the same conditions as hydra, in flasks containing 30 ml Kessler's nutrient solution (Kessler *et al.* 1963) adjusted to pH 6·8.

Isolation, fixation and DNA-staining of algae

Algae isolated from hydra by the sodium dodecyl sulphate-washing technique, which removes contaminating animal material (McAuley, 1986), or cultured algae, were suspended in 1:1 (v/v), phosphate-buffered saline, containing 0·2% (w/v) sodium azide (PBSa)/absolute ethanol, and were fixed in darkness for 45 min at 4°C. Fixed algae were washed twice with PBSa, resuspended at a final concentration of 10^6 cells ml^{-1} and stored until needed, in darkness at 4°C.

Samples (1 ml) were photobleached by exposure to a light intensity of $6{\cdot}9\times10^3$ μEinsteins $m^2 s^{-1}$ for at least 12 h at room temperature (Yentsch *et al.* 1983), treated with RNase (type 1A from bovine pancreas, Sigma Chemical Co., St Louis, Miss.) for 30 min at 37°C, and resuspended in 2 ml propidium iodide (25 μg ml^{-1} in hypotonic citrate) to stain cell DNA.

Flow cytometry

The fluorescence emission of individual, DNA-stained algal cells was measured in a Coulter EPICSV flow cytometer (Coulter Electronics, Hileah, Florida) after excitation by a 500 mW argon ion laser at 514 nm. Fluorescence of the propidium iodide–DNA complex was measured between 540 nm and 570 nm using selective filters, and recorded on floppy diskettes. Between 20 000 and 30 000 cells were analysed in each sample.

Chemical analysis of DNA content

Pellets of washed symbiotic or cultured algae (2×10^7 to 5×10^7 cells) were extracted twice for 30 min at room temperature with 2% perchloric acid/50% ethanol and twice for 10 min at 60°C with 3:1 (v/v), ethanol/ether to remove acid and lipid-soluble impurities (Wanka, 1962). Nucleic acids were extracted from the residue by heating for 6 h at 45°C in 0·5 N perchloric acid (Wanka & Geraedts, 1972). The DNA content of the extract was determined by the method of Burton (1956) using calf thymus DNA (Sigma Chemical Co. Ltd) as a standard.

Mitotic index and cell size measurements

Mitotic indices were determined by counting the number of dividing algae per 1000 cells using ×1000 Nomarski optics. Cell diameter was measured at the same magnification using an ocular scale; cell volumes were calculated assuming the algae to be perfect spheres.

RESULTS

DNA content of cultured and symbiotic algae

The mean DNA content of algae isolated from 24-h starved European hydra was compared with that of algae from 2-week-old asynchronous cultures of NC64A and 211/1e *Chlorella*. These cultures were no longer in logarithmic growth and their mitotic indices were similar to those of the symbiotic European algae (Table 1). Despite this, the mean DNA content of European algae was about three times higher than that of the cultured algae.

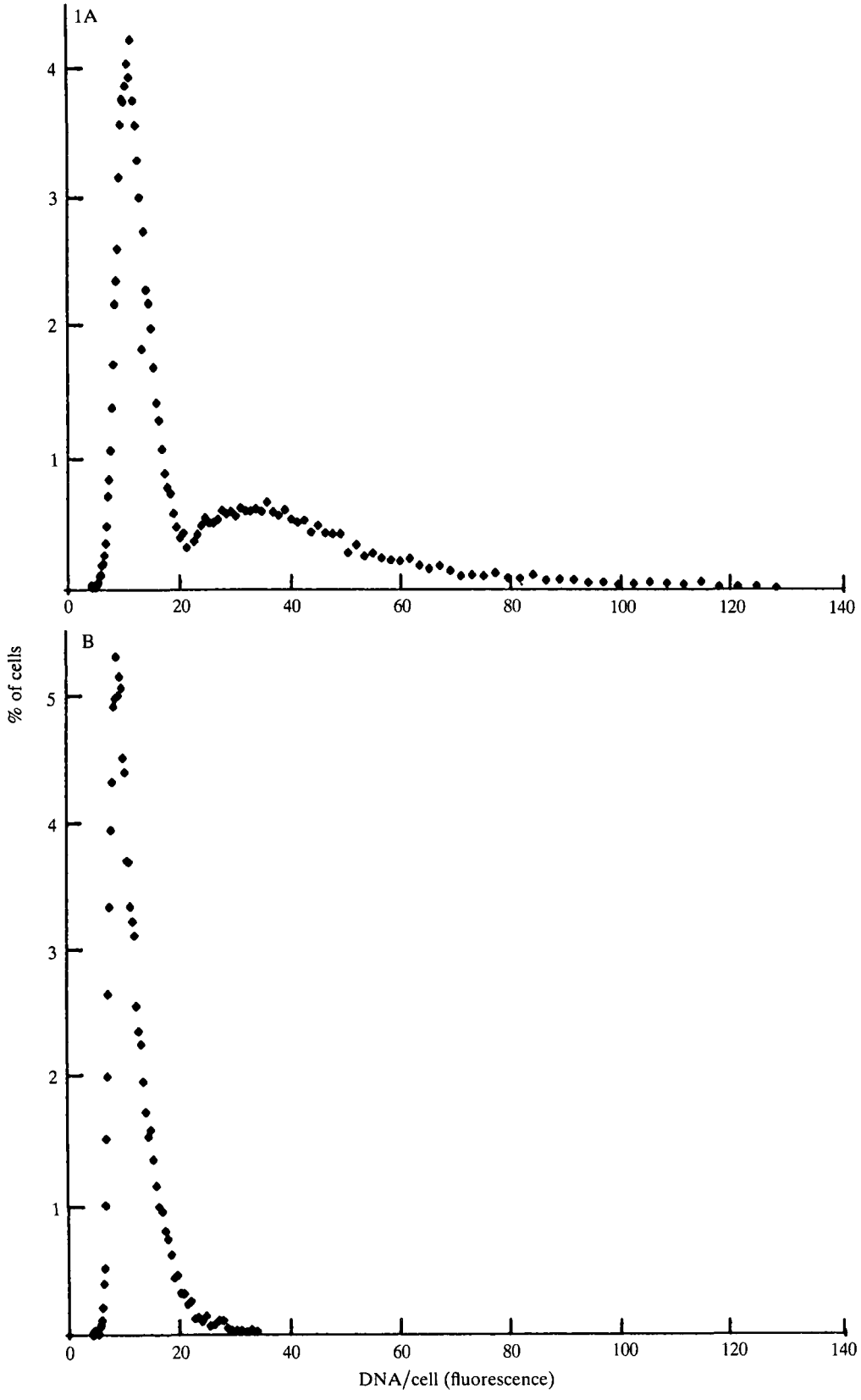
Flow cytometry

The distributions of DNA content of logarithmic (1-week-old) and non-logarithmic (2-week-old) populations of cultured NC64A algae were measured and compared with that of symbiotic European algae (Fig. 1). In the asynchronous logarithmically growing population of NC64A algae it was possible to distinguish a major and minor peak, the former presumably containing cells in G_1 and the latter

Table 1. *DNA content of symbiotic and cultured algae*

	DNA per cell ($g\times10^{-13}$)	Mitotic index (%)
Freshly isolated European algae	4·72 ± 0·46 (8)	1·8
Cultured NC64A	1·69 ± 0·16 (8)	1·6
Cultured 211/1e	1·53 ± 0·14 (12)	1·2

The amount of DNA in pellets of algal cells was determined as described in Materials and Methods. Values are means ± S.E.M. Number of replicate samples given in parenthesis.


1A
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DNA/cell (fluorescence)

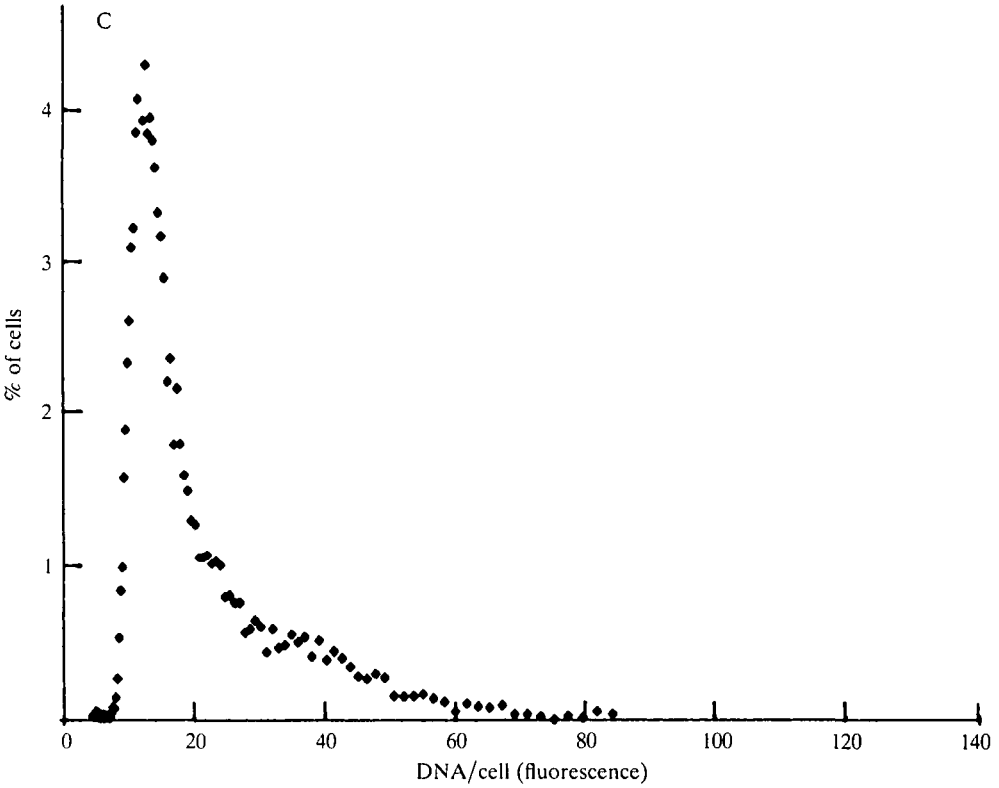
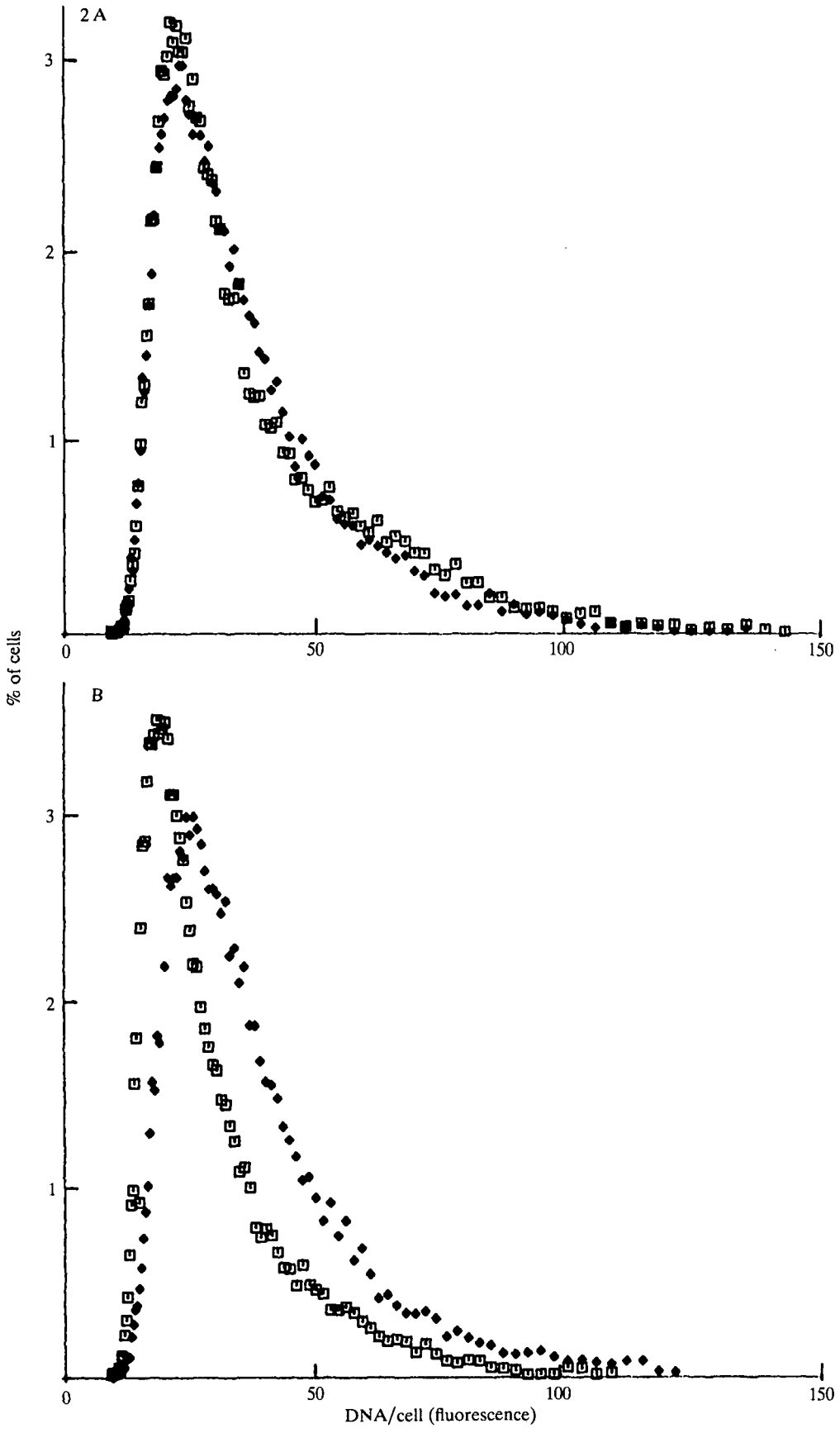


Fig. 1. Histograms of DNA content of algal cells measured as fluorescence of the propidium iodide–DNA complex. A. Logarithmic asynchronous NC64A. B. Non-logarithmic NC64A. C. Symbiotic algae freshly isolated from 1-day starved green hydra.

cells in *S* phase and mitosis. The minor peak was absent from the non-logarithmic population of NC64A. In contrast, the DNA content of European algae showed a single peak with a right-hand tail, with no clear distinction between G_1 and *S* phase/mitosis. This resembled distributions of DNA content in populations of mammalian cells whose cell cycle had been inhibited in *S* phase (Fried *et al.* 1983). If a large proportion of algae in 24-h starved hydra were in *S* phase (and therefore presumably committed to cell division), this is not reflected in measurements of mitotic indices, which are consistently less than 3% (McAuley, 1982, 1985*a*,*b*; Table 1, this paper).

This anomalous distribution was investigated further by measuring distribution of DNA content in algae isolated from long-term starved hydra. While host feeding stimulates algal division in hydra, host starvation causes cessation of both host cell and symbiont mitosis (McAuley, 1982, 1985*a*). During prolonged starvation, a shift in the DNA distribution towards the G_1 peak would be expected if algal cell cycle transit through *S* phase and mitosis were dependent upon host feeding.

Algae were isolated from 8-, 15- and 22-day starved hydra, and distribution of DNA content was compared with that of 1-day starved controls (Fig. 2). Algae in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150
DNA/cell (fluorescence)

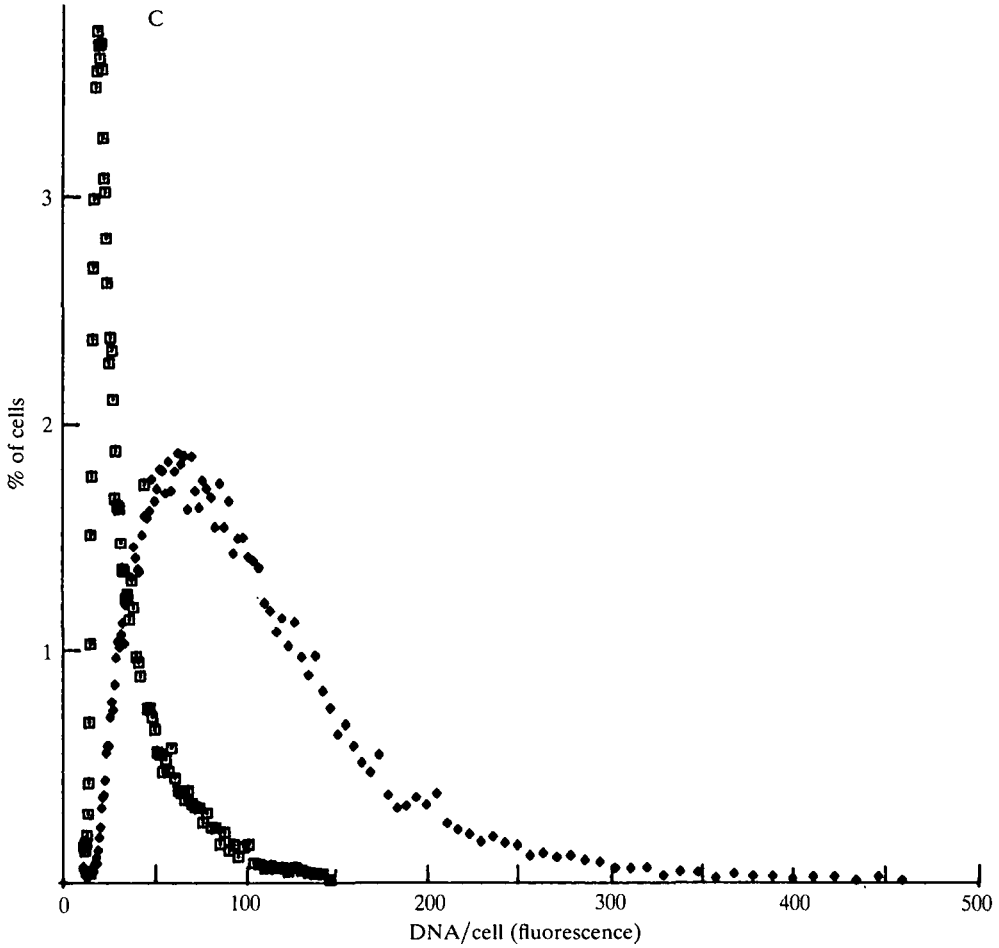

Fig. 2. Histograms of DNA content of symbiotic algae from hydra starved for different periods of time (◆) compared to 1-day starved controls measured at the same time (⊡). A. 8-day starved; B, 15-day starved; C, 22-day starved. Note that machine settings differed from those in Fig. 1 and varied slightly between the three pairs of measurements.

8-day starved hydra showed no difference in DNA content from 1-day starved controls; it is possible that some small amount of cell division was taking place, since numbers of algae per cell increase in European hydra during the first 10 days of starvation (Douglas & Smith, 1984; McAuley, 1985*a*). After 15 days of starvation the DNA distribution had moved slightly to the right to controls, rather than a single G_1 peak becoming apparent as in non-logarithmic cultured NC64A. After 22 days of starvation the peak value of DNA content was more than three times that of controls, and the distribution had developed a very broad right-hand tail. Previous work has shown that the symbiotic algae remain uninucleate when green hydra are starved (McAuley, 1985*a*). Thus, mitosis and cytokinesis are inhibited, but DNA synthesis is not, so that after a prolonged period of host starvation algal cell nuclei contain several times the amount of DNA at G_1.

Table 2. *Mean number of daughter cells produced by dividing algae 48 h after feeding starved hydra*

Days starved	Mean number of daughter cells	
	Starved	Controls*
8	4·52 ± 0·14 (13)	4·32 ± 0·11 (8)
15	4·76 ± 0·16 (19)	4·24 ± 0·10 (6)
22	5·28 ± 0·19 (32)	4·28 ± 0·10 (7)

Values are means ± S.E.M. of 100 observations from duplicate experiments. Values in parenthesis are numbers of cells dividing to produce more than four daughter cells.
* 3-day starved.

Effect of length of host starvation on algal division after a single feeding

Since non-dividing algae in starved hydra continued to replicate DNA, it might be expected that when algal division is stimulated by host feeding the number of daughter cells produced would be proportional to the length of time the algal host was starved. This was tested by feeding hydra 8, 15 and 22 days after beginning starvation, and examining the number of autospores produced by dividing algae. Controls in each case were 3-day starved hydra fed at the same time; algal division was examined 48 h after feeding since there is a delay in the algal division response after prolonged starvation (McAuley, 1985*a*).

The results, given in Table 2, show that the mean number of daughter cells produced per dividing alga increased as host starvation proceeded. Algal cell size also increases during host starvation (Douglas & Smith, 1984; McAuley, 1985*a*), and this may cause the increased number of daughter cells observed here, since in free-living *Chlorella* cell size at division determines the number of daughter cells produced (Donnan *et al.* 1985). However, comparison of the cell volume of mother cells producing four and eight autospores after feeding 3-day and 22-day starved hydra showed that this was not the case (Table 3).

A chi^2 test showed that when 22-day starved algae divided, a significantly greater proportion produced eight autospores than 3-day starved controls (chi = 19·91, d.f. = 1, $P < 0·001$). While there was a significant difference in size of 3-day and 22-day starved dividing algae ($P < 0·001$), in both populations there was no significant difference in size between algae producing four or eight autospores ($P > 0·10$). Although algae producing eight autospores tended to be larger, the distribution in size overlapped that of algae producing four autospores.

Table 3. *Size of algae dividing 48 h after feeding 3-day or 22-day starved hydra*

	Volume of dividing cell (μm^3)	
	4 autospores	8 autospores
3-day starved	157·8 ± 2·7	176·2 ± 10·9
22-day starved	184·6 ± 3·1	194·7 ± 7·8

Values are means ± S.E.M., from duplicate experiments.

Furthermore, 22-day starved algae producing four autospores were significantly larger than those in the 3-day starved population ($P < 0{\cdot}001$), which would not be the case if size alone determined the number of autospores produced.

DISCUSSION

Chlorella algae symbiotic with green hydra did not arrest in G_1 when cell division was blocked by host starvation, but appeared to cycle slowly through S phase. No distinct S/mitosis peak could be distinguished in algae from 1-day starved hosts, and if host starvation was prolonged, algal DNA content, as shown by flow cytometry, increased. In the first paper of this series it was shown that during prolonged host starvation the percentage of algae with multiple nuclei fell almost to zero (McAuley, 1985*a*). Thus, algae were able to synthesize DNA but were unable to initiate mitosis. In contrast, an S phase/mitosis peak was distinguishable from a larger G_1 peak in logarithmically growing cultures of free-living *Chlorella*, and in older cultures, in which mitosis was virtually arrested by exhaustion of nutrients in the medium, almost all of the algae accumulated in G_1.

To the best of our knowledge these results are the first to show that plant cells in which mitosis is blocked can progress slowly through S phase rather than arresting in G_1. Growth of flax in suboptimal conditions causes a number of heritable changes, including change in nuclear DNA content due to differences in number of repeated DNA sequences (Evans, 1968; Cullis, 1973; Timmins & Ingle, 1973); but the maximum increase in DNA content is only 10%, considerably less than that observed in symbiotic *Chlorella* during host starvation. That the number of daughter cells produced by *Chlorella* after host feeding was related to the length of the preceding period of starvation suggests that increase in DNA content was due to normal, albeit slow, replication of the entire genome rather than stress-induced amplification of selected portions. Studies of various mammalian cell lines have shown that in some cases a proportion of cells arrest in S phase (having an intermediate DNA content but failing to incorporate DNA precursors) when deprived of nutrients or subjected to crowding (Bhuyan *et al.* 1977; Nicolini *et al.* 1982; Drewinko *et al.* 1984). Dipasquale & McGuire (1977) found that treatment with dibutyryl cyclic AMP prevented cell division of cultivated Cloudman melanoma cells but not cell growth or DNA synthesis, while Clausen *et al.* (1983, 1984) identified subpopulations of slowly cycling cells in S and G_2 phase in mouse epidermis. While a number of workers have identified important restrictions controlling entry of unicellular algae into S phase and mitosis within the G_1 period (Donnan & John, 1983; Craigie & Cavalier-Smith, 1982; McAteer *et al.* 1985; Donnan *et al.* 1985), restrictions controlling exit of cells from S phase into mitosis are not inconceivable; for instance, deprivation of essential nutrients after G_1 traverse has been completed. The presence of an unidentified thermolabile component in the food of green hydra, which stimulates algal mitosis (McAuley, 1985*a*), suggests such a type of restriction.

It is not known if symbiotic *Chlorella* slowly and continuously synthesize DNA during host starvation or whether synthesis proceeds in bursts, so that there are subpopulations containing two, four, eight, etc. times the G_1 amount of DNA that are obscured by those cells traversing from one value to the next. Neither is it known if all cells in long-term *S* phase are able to divide when the host is fed, or if division is limited by competition for 'division factor'. However, the results described here are consistent with the model of symbiotic algal regulation in which mitosis but not cell growth is restricted (McAuley, 1985*a*). Thus, after feeding, algae divide at a larger size in 22-day starved than in 3-day starved hosts (Table 3), but the number of daughter cells produced is not dependent upon the size of the mother cell. This agrees with previous results, which showed that although algae in dark-grown hydra divided at about half the size of those in light, there was no difference in the number of daughter cells produced (McAuley, 1985*b*). In free-living *Chlorella*, the number of daughter cells formed depends upon cell size at initiation of division (Donnan *et al.* 1985). In symbiotic *Chlorella*, the number of daughter cells produced was correlated with the length of time the host had been starved and hence, presumably, the increase in mother cell DNA content (Table 2), rather than mother cell size. Residence within green hydra digestive cells appears to cause fundamental alterations in the cell cycle of the symbiotic *Chlorella*. Flow cytometry provides a powerful tool with which to analyse these changes.

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