

EFFECT OF CELL POPULATION DENSITY ON G₂ ARREST IN *TETRAHYMENA*

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ABSTRACT

The ciliated protozoan, *Tetrahymena pyriformis* strain GL-C, has been used to study the effect of cell population density during starvation on the synchrony obtained after refeeding and on the number of cells arrested in the G₂ phase of the cell cycle. At high cell densities two peaks of division indices were observed after refeeding while only one was observed at low cell densities. Cell division began earlier in cultures starved at high cell densities. Most importantly, the proportion of cells in G₂ was considerably higher in populations starved at high cell densities. When tritiated thymidine was present during the refeeding period, radioautographs of cell samples at different times showed that the first cells to exhibit division furrows contained unlabeled nuclei. The first peak in the division index after refeeding was observed only at higher cell densities and is attributed to the cells arrested in G₂. These results suggest that *Tetrahymena* is an excellent organism to study the concept of resting stages in the cell cycle and their control.

Various strains of the ciliated protozoan, *Tetrahymena pyriformis*, may be synchronized by starvation for 24 h followed by refeeding (1). Upon refeeding, the cells synthesize DNA and then divide with some degree of synchrony. However, a small portion of the cells divide without synthesizing DNA (9, 11). Thus, the usefulness of this synchronization technique for analyzing the events leading to DNA synthesis could be improved if this population of cells, which is in the G₂ stage of the cell cycle, could be reduced or eliminated. This led us to study the effect of cell population density during the starvation period on the synchrony obtained and on the size of the G₂ population. The results indicate that the cell population density (hereafter simply referred to as cell density) during

the starvation period is important in controlling the percentage of cells arrested in G₂.

MATERIALS AND METHODS

T. pyriformis (strain GL-C) populations were synchronized by the starvation-refeed technique as outlined by Cameron and Jeter (1), except that the cells were starved at a number of different cell densities. Before refeeding, the cell samples were adjusted to approximately 20,000 cells per ml by centrifugation and resuspension in starvation buffer. Thus all populations were refeed at similar cell densities.

Drop cultures were followed to determine the increase in cell number (10). Cell densities were determined with a Coulter counter, model B (Coulter Electronics Inc., Hialeah, Flor.). Where indicated, solid hydroxyurea was added to give a concentration of 50 mM. Radioautogra-

phy was done as described elsewhere (1, 2, 11), using 10 $\mu\text{Ci/ml}$ (spec act 6.0 Ci/mmol) of tritiated thymidine ($^3\text{H}\text{T}$).

RESULTS

Fig. 1 shows the results of an experiment in which cells were starved for 24 h at a cell density of 100,000 cells per ml and then refed. Samples of the refed culture show no increase in cell number until after 240 min. There appears to be a two-step increase in cell number, separated by a plateau between 360–380 min. This plateau corresponds to a dramatic decrease or dip in the percentage of dividing cells (the "division index") which occurs between 350 and 370 min and is followed by a second peak of division activity at 390 min after refeeding. Thus, in cultures starved at high cell density, refeeding reveals that there are two dis-

tinct peaks of division activity as is evident from this figure and from other data shown below. A portion of this same culture was exposed to $^3\text{H}\text{T}$ immediately after refeeding, and samples were taken periodically for radioautography. The percentage of dividing cells containing a radioactive nucleus is shown at the bottom of Fig. 1. Clearly, the first cells to exhibit division furrows contain unlabeled nuclei, and most of the cells which divide during the first peak of division activity have an unlabeled nucleus, while most of the cells dividing during the second peak of division activity have a labeled nucleus. Clearly this shows two populations of cells—one which does not synthesize nuclear DNA before division and a second which does. Samples of this same 24-h-starved culture were exposed to $^3\text{H}\text{T}$ for 4–6 h and then samples prepared for radioautography. Of the several thou-

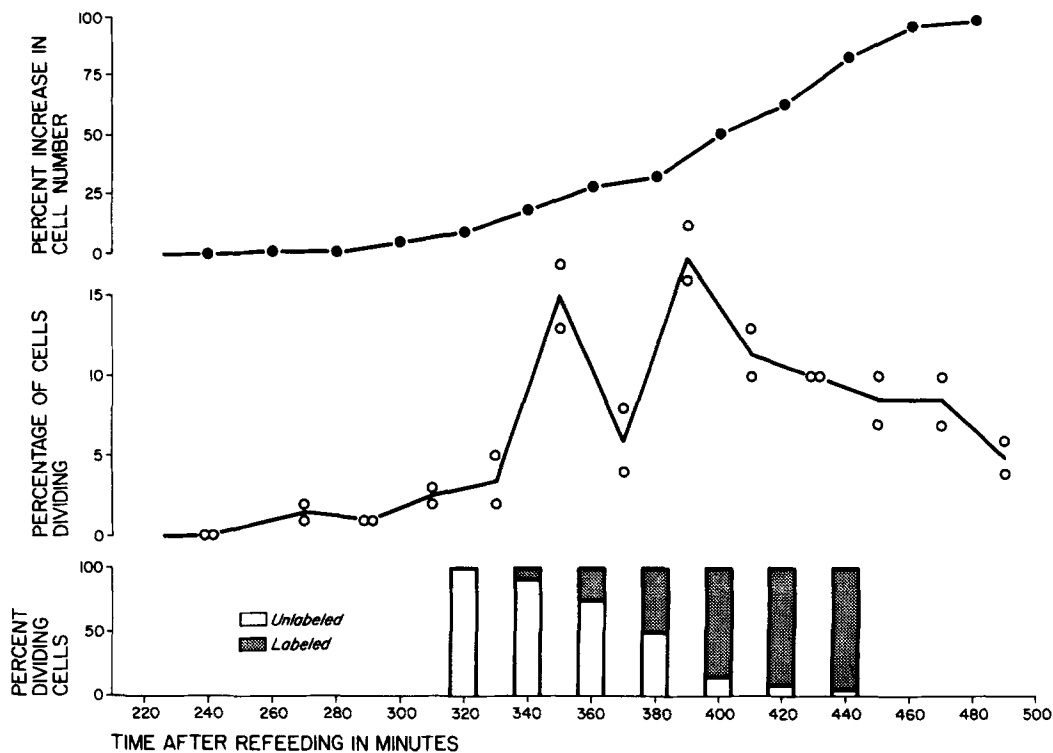


FIGURE 1 Time after refeeding of a 24-h-starved culture (initial starvation cell density of 100,000 cells per ml) plotted against: (a) (top of the figure) the percent increase in cell number, from drop cultures with 73 cells in the initial count (b) (middle of the figure) the percentage of cells dividing, when 200 cells per sample were scored for each point on the figure; and (c) (bottom of the figure) the percent of dividing cells showing $^3\text{H}\text{T}$ incorporation into the nucleus in radioautographs, when between 25–55 dividing cells were scored at each sample time.

sand cells examined in the radioautographs none showed indications of nuclear DNA synthesis.

At high and intermediate cell densities a plot of percentage of dividing cells vs. time after refeeding reveals two distinct peaks, while at the lowest cell concentration only one peak is present (Figs. 2 A, and 3 A). These data also show that in cultures starved at high cell densities an "early-dividing" and a "late-dividing" population are present. At low cell densities the early-dividing population is absent or very small.

A significant increase in cell numbers begins first in the culture starved at the highest cell density, followed by that starved at the intermediate cell density, and finally by the culture starved at the lowest cell density. The plots of percentage increase in cell numbers vs. time after refeeding again show the presence of an early-dividing and a late-dividing population in cultures starved at high and intermediate cell densities, showing that the early-dividing population is absent or very small in cultures starved at low cell concentrations. The increase in cell numbers occurs in two steps and the

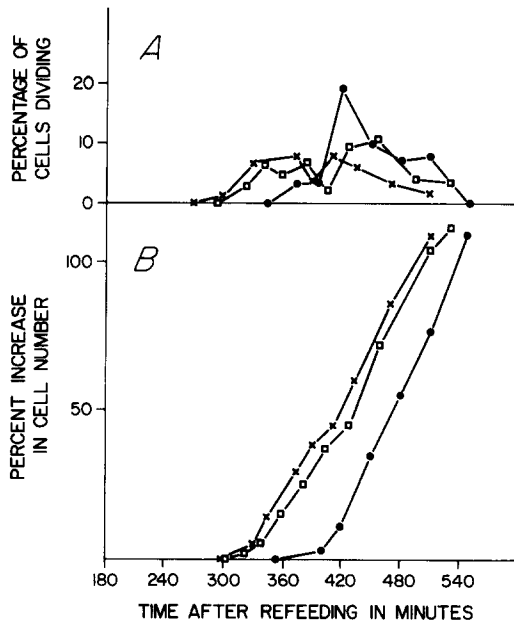


FIGURE 2 Effect of cell density during the 24-h starvation period upon the synchrony obtained after refeeding. The cultures contained at the beginning of the starvation period: (x—x), 200,000 cells/ml; (□—□), 60,000 cells/ml; (●—●), 3,500 cells/ml. (A) Percentage of cells dividing (showing division furrows). (B) Percentage increase in cell number.

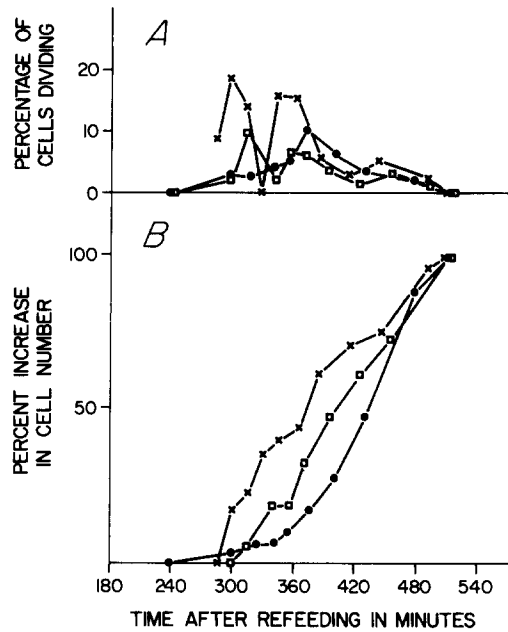


FIGURE 3 Effect of cell density during the 24 h starvation period upon the synchrony obtained after refeeding. The cultures contained at the beginning of the starvation period: (x—x), 215,000 cells/ml; (□—□), 72,000 cells/ml; (●—●), 3,700 cells/ml. (A) Percentage of cells dividing (showing division furrows). (B) Percentage increase in cell number.

size of the first step is dependent on the cell concentration during the starvation period.

Previous studies indicated that a G_2 population was present in starved-refed cells (9, 11). Perhaps the size of the early-dividing cell population depends upon the cell density during the starvation period. This was tested by refeeding cells in the presence of 50 mM hydroxyurea, which inhibits DNA synthesis in *Tetrahymena* (11). Cells dividing in the presence of hydroxyurea must have synthesized DNA before the addition of the drug and therefore must have been in G_2 . Radioautographs of cells exposed to hydroxyurea in the presence of [3H]T at the time of refeeding showed that no cells synthesize DNA, not even those cells that divide.

The number of cells dividing in the presence of 50 mM hydroxyurea is dependent on the cell density during the starvation period (Fig. 4). The highest G_2 numbers are observed in cultures starved at the highest cell densities, while the intermediate and lowest G_2 numbers are observed in cultures starved at intermediate and low cell

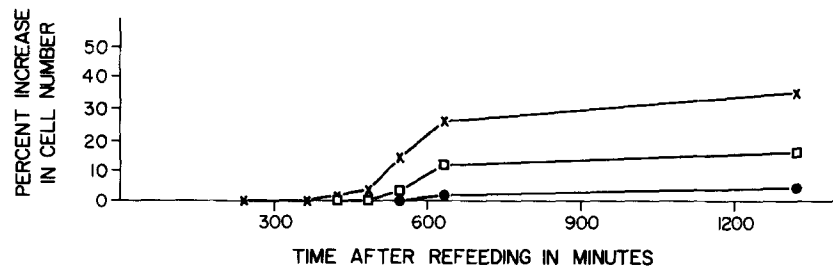


FIGURE 4 Effect of cell density during the 24-h starvation period upon the percentage of cells dividing in the presence of 50 mM hydroxyurea after refeeding. Cell densities are the same as in Fig. 2: (x—x), 200,000 cells/ml; (□—□), 60,000 cells/ml; (●—●), 3,500 cells/ml.

densities. However, the difference in the number of G_2 cells between the high and intermediate concentrations is not always as great as in Fig. 4. This suggests that there might be a saturation phenomenon such that increasing the cell density beyond a certain point will not bring about a corresponding increase in the number of G_2 cells. Indeed, when the results of a number of experiments are plotted, this is the case (Fig. 5).

A link between the early-dividing population and the G_2 population can be constructed. Since both populations are dependent in the same manner on the concentration of cells during the starvation period, this suggests that they are the same. Similarly, there appears to be a correlation between the height of the plateaus in graphs of percentage increase in cell numbers and in the numbers of cells dividing in the presence of hydroxyurea. Thus, the plateaus in Fig. 3 B occur at approximately 40, 18, and 6% while the corresponding G_2 numbers are 30, 26, and 6% as determined from the hydroxyurea data (Fig. 4). And finally, the absence of nuclear label in the first cells to divide also supports the contention that the early-dividing cell population and the G_2 population are the same.

DISCUSSION

The concept of two resting stages in the cell cycle has a relatively short history starting with and developed by the work on mouse ear epidermis by Gelfant (6-8). In a recent review (4) the cell cycle has been illustrated with a resting stage (R_1) in G_1 and another (R_2) in G_2 . This scheme implies that cells have the ability to arrest in both G_1 and G_2 . Van't Hof (12) believes that three populations exist in the root meristem of *Pisum*. One population arrests in G_1 and another in G_2 . A third population

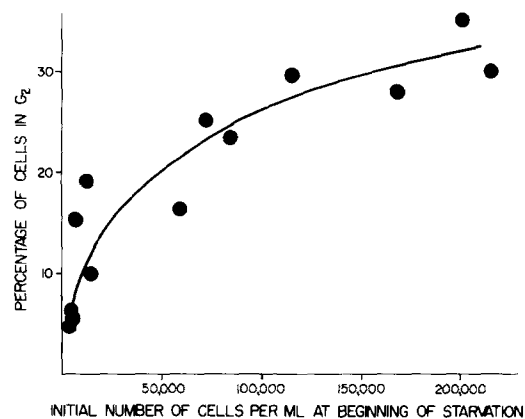


FIGURE 5 Effect of cell density during the starvation period upon the percentage of cells dividing in the presence of 50 mM hydroxyurea after refeeding (a measurement of the percentage of cells stalled in G_2).

arrests in either G_1 or G_2 , depending on the presence or absence of a G_2 factor (5).

The present study indicates that different percentages of *Tetrahymena* are arrested in either G_1 or G_2 , depending on the cell density at which the cells are starved. The observation that *Tetrahymena* stall in G_2 is supported by the work of B. B. McDonald (personal communication and footnote 1) on three different strains of *T. pyriformis* including the amiconucleate H and two of mating type II (variety 1, family B), II (Y), and II (O). The criterion for G_2 cells was the absence of labeled $^3\text{H-T}$ in nuclei of dividing cells after refeeding. Unpublished observations by M. Salamone and R. Pearlman (York University, Downsview, Ontario) of Feulgen cytophotometry measurements on starved *T. pyriformis* GL-C confirm that a percentage of 24-h-starved cells

¹ McDonald, B. B., Dickinson College, Carlisle, Pa. Manuscript in preparation.

have a G₂ content of DNA. We find no incorporation of [³H]T into the nucleus of 24-h-starved cells, which indicates that no 24-h-starved cells are in S phase and that no cells are passing from G₁ phase into S phase at this time. Because of these observations and because no dividing cells were seen before 240 min after refeeding, we conclude that the 24-h-starved cells must be arrested in the G₁ and the G₂ phases of the cell cycle.

Starved cells enter DNA synthesis and division after a lag (1), as opposed to immediately upon refeeding, which also supports the finding that starved cells are actually noncycling in G₁ and G₂ and not just passing through these stages slowly during the starvation period. If the cells were passing through these phases slowly, the number of cells entering S and division might be expected to increase immediately after the time of refeeding.

Three general types of hypotheses may be distinguished to explain how cell density, during the starvation period, alters the number of cells in G₂. The high G₂ number may be due to (a) some modification of the media brought about by the high cell density; (b) the release of a G₂-arresting factor by cells at high densities; or (c) the greater physical interaction between cells at high cell densities.

Hydroxyurea is an effective inhibitor of DNA synthesis in *Tetrahymena* as shown by the lack of [³H]T incorporation into the nuclei of starved and refeed cells (also see 2). However, the 50 mM dosage of hydroxyurea does delay somewhat cell division of the unlabeled G₂ cell population, which is probably due to the drug affecting protein synthesis (3).

The present study has practical ramifications in that the starvation-refeed technique of synchronization will yield the most homogenous population if the cells are starved at low cell densities; even then, a small G₂ population will be present.

In summary, *Tetrahymena* appears to be a promising organism for studying the concept of resting stages in the cell cycle. The present study

reveals one of the few systems in which the proportion of cells resting in G₁ and G₂ may be manipulated.

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