

# CONTROL OF CELL PROGRESSION THROUGH THE MITOTIC CYCLE BY CARBOHYDRATE PROVISION

## I. Regulation of Cell Division in Excised Plant Tissue\*

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### ABSTRACT

A stationary phase in the root meristem of excised pea roots was established by prolonged carbohydrate deprivation in sterile culture medium. When the stationary phase had been established, cells that had collected in the  $G_1$  period of the mitotic cycle were induced to enter the S stage by subjection to relatively short intervals of carbohydrate provision (sucrose spurts). Progression and cycle location of the  $G_1$  cells induced to enter S were measured with tritiated thymidine and radioautography. The results indicated that the number of  $G_1$  cells induced to enter S increased directly with the spurt duration and that cells could be positioned and retained in the S and/or  $G_2$  periods by varying the duration of the spurt. The data support the hypothesis that S and maybe M stages have a relatively larger dependence on carbohydrate availability, and presumably a greater energy requirement, than  $G_1$  and  $G_2$ .

### INTRODUCTION

In an earlier paper, cell population kinetics and tissue growth were described in excised roots of *Pisum* (12). It was found that separation of the root tip from the seedling produced a drop in the mitotic index that lasted for approximately 12 hr, and that the subsequent rise in cell division was dependent on sucrose concentration. The optimum sucrose concentration, defined as that which produced the shortest mitotic cycle duration, was 2%. These results suggested that cell division and the mitotic cycle could be controlled by sucrose concentration. In a second paper, it was further demonstrated that mitosis and DNA synthesis could also be controlled by deprivation or availability

of sucrose in the culture medium (14). If the excised root tips were cultured in medium without sucrose for a minimum of 24 hr and up to 72 hr, the meristem would have few, if any, mitotic figures, and cells capable of incorporating tritiated thymidine ( $T\text{-}^3\text{H}$ ). Further experimentation indicated that the interphase cells in the meristem were in either the  $G_1$  (presynthetic) or the  $G_2$  (postsynthetic) periods of the mitotic cycle. The actual distribution was approximately 90% in  $G_1$  and 10% in  $G_2$ . A root tip meristem with this distribution is referred to as being in a stationary phase (15).

The work to be described in the present paper was carried out to determine whether cell progression through the mitotic cycle could be experimentally controlled. To be more specific, can

\* This paper is dedicated to the memory of Prof. G. B. Wilson who introduced the author to the fascinating subject of cell division.

a cell population be placed preferentially in  $G_1$ , S, or  $G_2$  and retained in a given period for a certain length of time before being induced to complete a mitotic cycle and continue proliferation?

## MATERIALS AND METHODS

### General

Seeds of *Pisum sativum* (var. Alaska) were sterilized by a 5 min immersion in Clorox. Germination was effected in sterile, moist vermiculite in 3 days at 22–23°C. The terminal 1 cm of the primary root tip was excised and suspended in 50 ml of White's medium (16) that contained no sucrose. Erlenmeyer flasks, 125 ml, were used as culture vessels and each vessel contained 10–12 root tips. In all experiments the flasks were maintained in constant motion by a reciprocating shaker reduced to the lowest speed. The temperature remained at 23°C for the duration of the experiment.

### Transfer of Cultured Root Tips

To change from the nondividing, nonDNA synthesizing stationary phase to the actively dividing, DNA synthesizing proliferative phase, we transferred the root tip from culture medium without sucrose to medium with sucrose. This transfer was performed by pouring the contents of the culture flask through a sterile gauze filter. The root tips were then removed from the gauze filter and resuspended in White's medium that contained 2% sucrose.

### Measurement of DNA Synthesis and Radioautography

Tritiated thymidine ( $T\text{-}^3\text{H}$ ) obtained from Schwarz Bio Research Inc. (Orangeburg, N. Y.), specific activity 6.0 c/mmole, was used for measurement of DNA synthesis and marking of cells of the S (DNA synthesis) period of the mitotic cycle. Cells of the root tip meristem were labeled by transfer to medium that contained the radioactive isotope. The presence of cells in S was determined by radioautography in the following manner. After aseptic removal of sample root tips from the culture medium, they were fixed in a mixture of three parts ethanol and one part glacial acetic acid. Next they were stained by the Feulgen method that consisted of a 15 min hydrolysis with 1 N HCl at 60°C and staining with Schiff's reagent. Squash preparations were obtained from the terminal 1–1.5 mm portion of the root tip according to the method of Conger and Fairchild (5). Radioautographs were prepared with Kodak NTB liquid emulsion and a 2 day exposure at 4°C. Because excised root tips have a high  $T\text{-}^3\text{H}$  incorporation rate, only a short exposure period is required to produce satisfactory radioautographs

(13). After development the preparations were often stained with toluidine blue (6) as a way of easing microscopic analysis.

### Experimental Design

#### PART I

A pictorial representation of the experimental design is shown in Fig. 1. These experiments were designed first for accumulation of cells in  $G_1$  and  $G_2$  by the establishment of 48 hr stationary phase; second, as a determination of whether a 6 or 12 hr provision of 2% sucrose would produce sufficient energy to cause  $G_1$  cells to proceed into S; and, third, for determination of whether the cells, once in S, would remain in this state until further provision of carbohydrate. Figure 1 *a* represents the control group. Root tips of this group remained in the stationary phase for 72 hr and then were transferred to medium with 2% sucrose and  $T\text{-}^3\text{H}$ . Fig. 1 *b* represents root tips that were in the stationary phase 48 hr, in medium with sucrose for 6 hr, in the stationary phase for 18 hr, and then transferred to medium with sucrose plus  $T\text{-}^3\text{H}$ . Figure 1 *c* represents root tips that were handled in the same manner as in Fig. 1 *b* except that they experienced a 12 hr spurt with sucrose followed by 12 hr in the stationary phase before being transferred to medium with sucrose plus  $T\text{-}^3\text{H}$ .

#### PART II

We designed the second part of the experiment as shown in Fig. 2 to determine whether  $G_1$  cells were going into S during the 6 or 12 hr of sucrose provision and to determine the time when these cells entered mitosis. Fig. 2 *a* represents the control group. Root tips of this group spent 72 hr in the stationary phase, and  $T\text{-}^3\text{H}$  was made available for 12 hr (from 48 to 60 hr) during this period. After the 72 hr stationary phase, the root tips were transferred to medium that contained sucrose. Figure 2 *b* represents root tips that were in the stationary phase 48 hr, incubated in sucrose medium with  $T\text{-}^3\text{H}$  for 6 hr, returned to the stationary phase for 18 hr and then transferred to medium with sucrose at 72 hr. Fig. 2 *c* represents root tips that were in the stationary phase 48 hr, incubated for 12 hr with sucrose and  $T\text{-}^3\text{H}$ , returned to the stationary phase for 12 hr and then transferred to medium with sucrose.

### Sampling and Cytological Analysis

72 hr after excision, when all groups were transferred to medium with sucrose (Figs. 1 and 2), four to five roots were aseptically removed at 4, 8, 12, and 16 hr after the transfer at 72 hr. For measurement of the entrance of  $G_1$  cells into S ( $G_1 \rightarrow S$ ), the

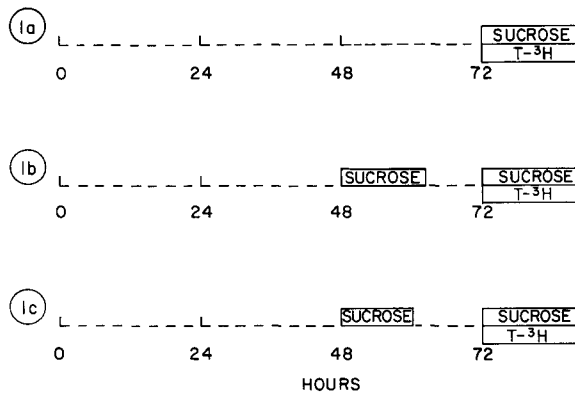


FIGURE 1 Diagrams depicting experimental design, part I. Root tips were excised at 0 hr. The dashed line indicates time spent in medium without sucrose (stationary phase); the solid line indicates time spent in medium with sucrose. At 72 hr the root tips were transferred to medium with sucrose and tritiated thymidine and sampling was initiated.

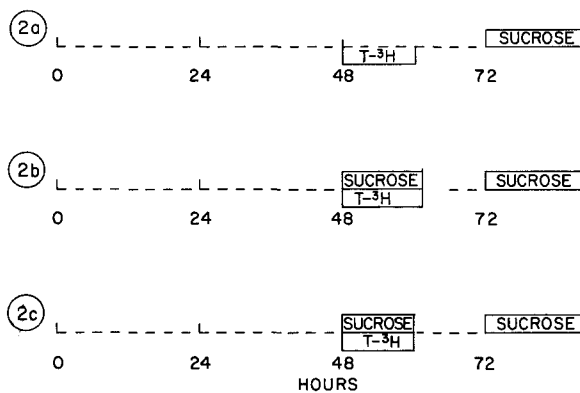


FIGURE 2 Diagrams depicting experimental design, part II. Root tips were excised at 0 hr. The dashed line indicates time spent in medium without sucrose (stationary phase); solid line indicates time spent in medium with sucrose. The periods at which tritiated thymidine labeling was performed are also indicated. At 72 hr the root tips were transferred to medium with sucrose and sampling was begun.

percentage of T-<sup>3</sup>H-labeled interphase cells was obtained by scoring more than 400 interphase cells per meristem. The appearance of G<sub>1</sub> cells in mitosis (M) (G<sub>1</sub> → S → G<sub>2</sub> → M) was measured as the per cent T-<sup>3</sup>H-labeled mitotic figures observed in each meristem scored. The entrance of G<sub>2</sub> cells into mitosis (G<sub>2</sub> → M) was measured by obtaining the percentage of dividing cells observed in 1,000 cells in each of the meristems scored.

## RESULTS

### *Experimental Design*

#### PART I

The increase in the number of T-<sup>3</sup>H-labeled interphase cells as a function of time in medium with carbohydrate in roots that were provided sucrose for 0, 6, and 12 hr during the stationary phase is shown in Fig. 3 *a*. It clearly indicates that the 6 and 12 hr sucrose provision increased the number of cells incorporating T-<sup>3</sup>H and affected the rate at which the increase occurred. The lowest rate was that of the control which was not provided

any sucrose for 72 hr. Once carbohydrate was provided, and even after it was present for 16 hr, only 6% of the interphase cells was labeled compared to 25 and 32% for the roots provided sucrose for 6 or 12 hr, respectively. The influence of carbohydrate on the number of G<sub>1</sub> cells induced to enter S varied directly with the duration of incubation (Fig. 4). The data include results from the present and other experiments in order to demonstrate the point. The curve in Fig. 4 represents the percentage of labeled interphase cells at 8 hr (0 hr is the time at which the stationary phase was terminated) expressed as a function of the duration of sucrose incubation at the 48th hr of the stationary phase. The 8-hr percentages were chosen because it was felt that this was sufficient time for T-<sup>3</sup>H to be incorporated by the G<sub>1</sub> cells induced to enter S and insufficient time for former G<sub>2</sub> cells to initiate DNA synthesis. The data suggest that cells given sucrose for relatively short periods during the stationary phase did progress into S. If this did occur, then the appearance of T-<sup>3</sup>H-labeled former G<sub>1</sub> cells in mitosis should be in the following order: cells

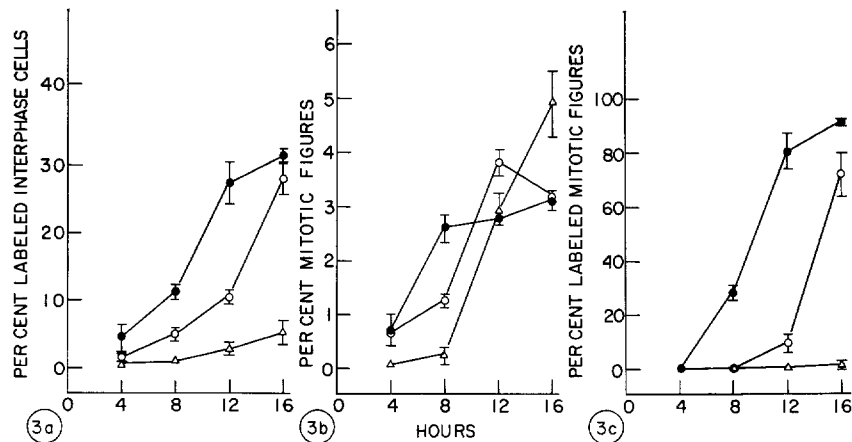


FIGURE 3 Results of part I experiments. *a*, measurement of  $G_1 \rightarrow S$  changes; *b*, measurement of  $G_2 \rightarrow M$ ; and *c* measurement of  $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$  progression after transfer to medium with sucrose and tritiated thymidine at 72 hr (0 hr in Fig. 3). Triangles, no sucrose spurt; open circles, a 6 hr sucrose spurt; solid circles, a 12 hr spurt of sucrose at 48 hr. Standard error of the mean is indicated.

provided sucrose for 12 hr, first; those provided sucrose for 6 hr, second; and those not provided sucrose, last. Figure 3 *c* shows that the appearance of  $G_1$  cells in mitosis followed the order predicted.

The influence of sucrose spurts during the stationary phase on  $G_2 \rightarrow M$  is least apparent (Fig. 3 *b*). In fact, for a full understanding of the  $G_2 \rightarrow M$  curves (Fig. 3 *b*), the  $G_1 \rightarrow M$  curves of Fig. 3 *c* must be jointly consulted. For instance, almost no labeled mitotic figures were observed in the control root tips (Fig. 3 *c*), whereas many division figures were actually observed (Fig. 3 *b*). These unlabeled dividing cells originated from  $G_2$ . Thus to determine whether the sucrose spurt reduced the number of cells arrested in  $G_2$  by inducing them to proceed from  $G_2 \rightarrow M \rightarrow G_1$ , one should consider only the unlabeled cells. Experiments of part I design, however, are incapable of distinguishing between  $G_1$  cells induced to enter  $G_2$  by the sucrose spurt and cells arrested in  $G_2$  during the stationary phase. Little can be said, therefore, about the influence of the sucrose spurt on  $G_2$  cells other than that they are not retained in  $M$  in large numbers after the spurt. If retention in  $M$  did occur, a higher frequency of mitotic figures would be expected at 4 hr (Fig. 3 *b*).

## PART II

Before the results of this portion of the experiment are described, it would be well to become familiarized again with the design (Fig. 2 *a-c*). As

seen in Fig. 2, this experiment should mark the  $G_1$  cells that enter  $S$  during the spurt because  $T\text{-}^3\text{H}$  was provided simultaneously with the sucrose; no  $T\text{-}^3\text{H}$ , however, was provided at 72 hr when the root tips were transferred to medium with sucrose and samples were taken. In effect, part II design experiments constitute the labeling of a small population of  $G_1$  cells as they enter  $S$ . The size of the population induced to enter  $S$  by the sucrose spurt can be estimated by the percentage of labeled interphase cells, a value that should remain relatively constant within a given mitotic cycle. Fig. 5 *a* expresses the per cent labeled interphase cells in root tip meristems that were incubated for 12 hr with  $T\text{-}^3\text{H}$  (control), 12 hr with 2% sucrose plus  $T\text{-}^3\text{H}$ , and 6 hr with 2% sucrose plus  $T\text{-}^3\text{H}$ . The curves are flat as predicted, since a population size is measured and the height of the curves increased with increased duration of the sucrose spurt. Root tips that were provided  $T\text{-}^3\text{H}$  and no sucrose for 12 hr had 1–2%  $T\text{-}^3\text{H}$ -labeled interphase cells; those that were provided sucrose and  $T\text{-}^3\text{H}$  for 6 hr had 3–6%; and, those that had sucrose and  $T\text{-}^3\text{H}$  for 12 hr had 8–15% labeled interphase cells. If labeled interphase cells did proceed from  $G_1 \rightarrow S$  and if the extent of their progression in  $S$  was related to the duration of the sucrose spurt, then the appearance of  $T\text{-}^3\text{H}$ -labeled cells in mitosis would be in the following order: the 12-hr incubated cells would appear first; the 6-hr cells, second; and the cells that were provided no

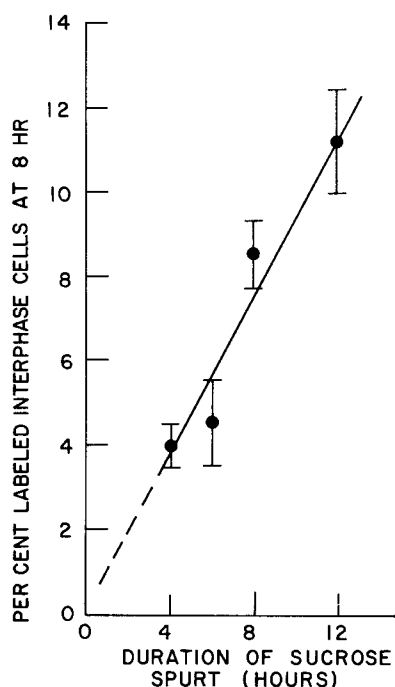


FIGURE 4 The per cent labeled interphase cells observed at 8 hr expressed as a function of the duration of the sucrose spurt. All results are of part I experiments (see Fig. 1). Standard error of the mean is indicated.

sucrose, last. Figure 5 *c* shows that the order of appearance in mitosis followed the expected sequence.

The combined data in Fig. 5 *b* and *c* are capable of revealing something about the influence of the sucrose spurt on  $G_2 \rightarrow M$ . Again, as in Fig. 3 *c*, Fig. 5 *c* shows few mitotic figures at 4 hr and thus few, if any, cells were retained in M after the sucrose spurts. In general, the data of Fig. 5 *b* indicated that sucrose provision during the stationary phase caused  $G_2$  cells to enter division. This effect is particularly evident in roots incubated with sucrose for 6 hr. Included in the early dividing group, however, were labeled cells (Fig. 5 *c*); this suggests that 12 hr of sucrose provided enough energy to drive cells from  $G_1$  through S and into  $G_2$  and 6 hr of sucrose appeared to drive  $G_1$  cells well into S. The absence of sucrose provided little or no drive and, therefore, very few of the mitotic figures were  $T\text{-}^3\text{H}$ -labeled even at 16 hr (Fig. 5 *a* and *c*).

## DISCUSSION

### *Control of Cell Progression and Cell Distribution Patterns*

The question posed for these experiments as set forth in the Introduction concerned the possibility of controlling experimentally the position of cells in a given period of the mitotic cycle and determining whether the cells can be retained in a given position for an extended period of time. The motivation for such experimentation was derived from two sources. First, if cells could not be retained in a given period of the cycle, some insight would be gained about cell populations that are subjected to metabolic variation, as for example, during hibernation or dormancy. Second, if the cells could be controlled and retained in the cycle, a new path would be opened for obtaining information about various mechanisms that are peculiar to a given mitotic cycle period and to proliferative cells that are constituents of growing tissue.

The diagrams shown in Fig. 6 will be helpful in discussing the experimental results. If Fig. 6 *a* approaches the cell distribution in root tips cultured for 72 hr in medium without sucrose, then on transfer to medium with sucrose plus  $T\text{-}^3\text{H}$  and with subsequent sampling, the expected results would be: (*a*) a slow but steady increase in labeled interphase cells with time, (*b*) after a delay, an abrupt increase in the percentage of mitotic figures as  $G_2$  cells enter M, and (*c*) the absence of any  $T\text{-}^3\text{H}$ -labeled mitotic figures until the later sampling times. Inspection of the curves in Fig. 3 *a-c* shows that they support the proposed cell distribution pattern in Fig. 6 *a*.

In cases where the stationary phase was interrupted by 6 or 12 hr incubation in medium with sucrose, the cell distribution patterns would be as described in Fig. 6 *b* and *c*, respectively. With transfer to medium with sucrose and  $T\text{-}^3\text{H}$ , the expected results of the 6- and 12-hr incubation groups would be similar except that the 6 hr group should exhibit a lower and a delayed response when compared to the 12 hr group. The expected results would be: (*a*) an obvious increase in  $G_1 \rightarrow S$  as revealed by  $T\text{-}^3\text{H}$ -labeled interphase cells, (*b*) the degree of increase should follow the order  $0 < 6 < 12$ -hr sucrose incubation groups, (*c*) cell division should appear in the early samples, and (*d*) there should be  $T\text{-}^3\text{H}$ -labeled mitotic figures in the early samples. The curves in Fig. 3 *a-c* are in full agreement with these expectations, except

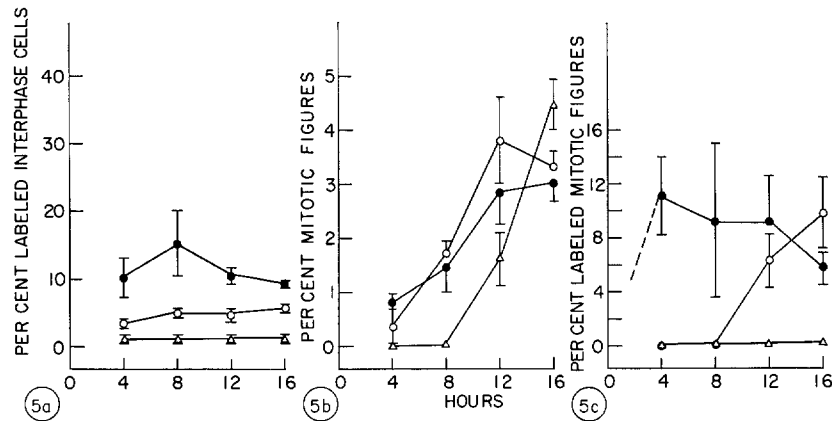


FIGURE 5 Results of part II experiments. *a*, measurement of  $G_1 \rightarrow S$  changes; *b*, measurement of  $G_2 \rightarrow M$ ; and *c*, measurement of  $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$  progression after transfer to medium with sucrose at 72 hr (0 hr in Fig. 5). Triangles, no sucrose spurt but did incubate for 12 hr with tritiated thymidine; open circles, a 6 hr spurt with sucrose and tritiated thymidine; closed circles, a 12 hr spurt with sucrose and tritiated thymidine at 48 hr. Standard error of the mean is indicated.

for the percentage of cells in division in the early samples of the 6 hr incubation group.

The relative number of cells participating in the  $G_1 \rightarrow S$  transition during interruption of the stationary phase by carbohydrate provision was determined by incubation in medium with sucrose and  $T\text{-}^3\text{H}$ . Since  $T\text{-}^3\text{H}$  was not provided after the transfer at 72 hr (Fig. 2 *a-c*), the cells involved in the  $G_1 \rightarrow S$  transition constituted a marked population of a particular size. If the proposed distribution patterns are correct (Fig. 6 *d-f*), then (*a*) the per cent labeled interphase cells would increase as the duration of the sucrose spurt increased, (*b*) labeled cells would appear in division in order with the 12 hr incubation group first, the 6 hr group second, and the 0 hr group last, and (*c*) the unlabeled  $G_2$  cells in the sucrose-incubated root tips would divide before those of root tips that were not provided sucrose. The curves in Fig. 5 are in agreement with these expected results and thus support the distribution patterns proposed. The reversed relationship between the 6- and 12-hr incubation groups with regard to the appearance of  $G_2$  cells in M (Fig. 5 *b*) is also in agreement with expected results if it is assumed that some of the  $G_2$  cells were provided sufficient energy during the 12 hr incubation period to carry out mitosis and enter  $G_1$ . The result would be a reduced number of arrested  $G_2$  cells. Therefore, after the root tips were transferred to sucrose me-

dium at 72 hr, fewer  $G_2$  cells would divide in the 12 hr group than in the root tips that were incubated for 6 hr.

#### *Activity Level of Cells in the Mitotic Cycle*

The present experiments confirm earlier observations that indicated that an asynchronous population of proliferating cells will collect in the  $G_1$  and  $G_2$  periods of the mitotic cycle if deprived of carbohydrate (14). Because oxygen consumption of carbohydrate-deprived cells declines as the length of the stationary phase is extended (15), it is reasonable to state that preferential cell collection in  $G_1$  and  $G_2$  is associated with a reduction in carbohydrate metabolism. This appears to be the case whether metabolism is reduced experimentally as in the present work or naturally with the establishment of a dormant (3, 4, 7, 10) or a mitotically inactive (9) state. The question raised by these observations is, what is peculiar about S and M that makes them the least probable conditions for a cell with low metabolic level? If one assumes that the matter of available energy is involved, this question has two possible answers. One possibility is that the energy requirements of  $G_1$  and  $G_2$  are greater than those of S or M. If this were true, cells caught in S or M during reduced metabolism would have a "downhill" path relative to either  $G_1$  or  $G_2$  cells, and accumulation would

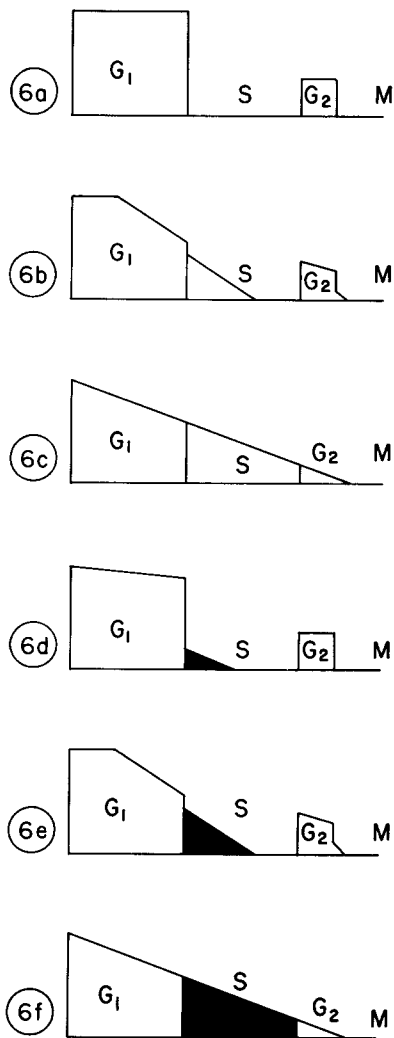


FIGURE 6 Schematic representation of postulated cell distributions in the mitotic cycle of stationary phase root tips that received no sucrose spurt, *a*, a 6-hr spurt *b*, and a 12 hr spurt of sucrose, *c*, during the 72 hr stationary phase. *d-f* represent cell distributions of tritiated thymidine-labeled interphase cells: after no sucrose spurt and a 12 hr labeling period, *d*; after a 6 hr spurt with sucrose and simultaneous labeling with tritiated thymidine, *e*; and after a 12 hr sucrose spurt and tritiated thymidine labeling, *f*. The shaded portions in *d-f* represent tritiated thymidine-labeled interphase cells.

occur in either  $G_1$  or  $G_2$ . However, since most cells appear to collect in  $G_1$  (14), the energy requirements of  $G_1$  may be greater than those of  $G_2$ . If

one takes this factor into consideration, the relative probabilities that a cell will remain in any given period would be  $G_1 > G_2 > (S \text{ or } M)$ . The second possibility is that S and M or the initial events of these periods have greater energy requirements than  $G_1$  or  $G_2$ . Once a cell reaches the  $G_1$  or  $G_2$  state, it is unable to generate enough energy to enter S or M. Again, because cells collect preferentially in  $G_1$ , the relative probabilities that a cell would remain in a given period would be  $G_1 > G_2 > (S \text{ or } M)$ . The experimental evidence presented in the present work tends to support the second possibility, particularly with respect to S. A 6 hr sucrose interruption of the stationary phase increased the percentage of  $T\text{-}^3\text{H}$ -labeled interphase cells by a factor of approximately 2.5 (Fig. 5 *a*). These cells apparently had sufficient energy to initiate DNA synthesis as indicated by  $T\text{-}^3\text{H}$  incorporation but not enough energy to carry them through S and into  $G_2$ , as suggested by the fact that the labeled cells did not divide with the unlabeled  $G_2$  cells (hr 4 and 8, Fig. 5 *b*) but did divide at 12 and 16 hr (Fig. 5 *c*). A 12 hr sucrose interruption of the stationary phase did supply sufficient energy for the  $G_1$  cells to synthesize DNA and enter  $G_2$  (Fig. 5 *a-c*). Thus, at 72 hr while still in  $G_2$  and *again* provided sucrose, the former  $G_1$  cells divided at a time when  $G_2$  cells normally divide, from 0 to 12 hr after termination of the stationary phase (Fig. 5 *c*; reference 14).

If one assumes that oxygen requirement is equivalent to carbohydrate requirement, the present experiments and those performed with meiotic (8) and mitotic (1, 2, 11, 17) cells of plants offer increasing evidence that cell progression through the mitotic cycle is characterized not only by marked fluctuations in macromolecular synthesis, but also by changes in energy requirements. The general pattern at the moment appears to be that the events of S and perhaps those of M require relatively more energy than those of  $G_1$  and  $G_2$ .

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