CELL POPULATION KINETICS OF EXCISED ROOTS OF *PISUM SATIVUM*

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ABSTRACT

The cell population kinetics of excised, cultured pea roots was studied with the use of tritiated thymidine and colchicine to determine (1) the influence of excision, (2) the influence of sucrose concentration, (3) the average mitotic cycle duration, and (4) the duration of mitosis and the G_1, S, and G_2 periods of interphase.[1] The results indicate that the process of excision causes a drop in the frequency of mitotic figures when performed either at the beginning of the culture period or after 100 hours in culture. This initial decrease in frequency of cell division is independent of sucrose concentration, but the subsequent rise in frequency of division, after 12 hours in culture, is dependent upon sucrose concentration. Two per cent sucrose maintains the shortest mitotic cycle duration. The use of colchicine indicated an average cycle duration of 20 hours, whereas the use of tritiated thymidine produced an average cycle duration of 17 hours.

INTRODUCTION

The culture of plant organs and cells has given the experimenter a degree of control over the environmental conditions under which biological systems are maintained. The exercise of this control has provided answers to questions concerned with nutrition and the metabolism of various plant tissues (12, 19). Of immediate interest to cytologists concerned with cell reproduction is the development of an experimental system which can be manipulated and maintained *in vitro* for a period of time in excess of several complete mitotic cycles. Such a system would have ample time to adjust to culture conditions as well as provide time measurements of the mitotic cycle.

Although much work has been performed with excised, cultured roots, comparatively little is known concerning the time parameters of the mitotic cycle as described by Howard and Pelc (6). Of specific interest is the duration of DNA synthesis and the average mitotic cycle time, as well as the duration of mitosis. To obtain information on cell kinetic parameters, several forms of data

[1] *Glossary* (8)

S, Period of DNA synthesis
G_1, Postmitotic presynthetic period
G_2, Postsynthetic premitotic period
CT, Mitotic cycle ($T_m + T_{g1} + T_s + T_{g2}$) time
Tr, Number of cells in transition from proliferative to mature
F, Number of functional (mature) cells
T_m, Duration of mitosis
T_{g1}, Duration of G_1
T_s, Duration of S
T_{g2}, Duration of G_2
T_{tr}, Duration of maturation
T_f, Life time of a functional (mature) cell
N_{ct}, Number of cells in the mitotic cycle
N_t, Number of cells in the 2-mm terminal portion of the root ($Tr + F + N_{ct}$)
N_m, Number of cells in mitosis
I_m, N_m/N_t

are necessary. Among these are the average cycle duration as measured by two methods. One method utilizing tritiated thymidine was described by Quastler and Sherman (9) for mouse epidermal cells. This method has been used on plant cells also (21). The other method, which makes use of the cytological effects of colchicine, has been described by Van't Hof *et al.* (18). The use of two methods provides a check for possible radiation effects from tritiated thymidine and the mitotic delay from the use of colchicine.

The information obtained with the tritiated thymidine and colchicine methods and that of the mitotic index are also useful to estimate the time parameters of maturing and functional cells within the root. It was for these reasons that the present study was undertaken.

To obtain these objectives, three sets of experiments were performed. The first set was designed to determine the effect of excision and sucrose concentration on the minimum mitotic cycle duration. The second set was performed to find out the length of time required for acclimation of cell proliferation and tissue growth to the culture conditions. The third set of experiments involved the measurement of the mitotic cycle duration at a time when the cells had adjusted to the new environmental conditions.

MATERIALS AND METHODS

General procedures were as follows: Seeds of *Pisum sativum* (var. Alaska) were sterilized with Chlorox and aseptically germinated in vermiculite. Three days after germination, the terminal 1 cm was excised and placed in 125-ml Erlenmeyer flasks containing White's medium (19) supplemented with sucrose. Ten root tips were placed in a flask which contained 50 ml of culture medium. The flasks were shaken gently (about 60 cycle/min) for the duration of the experiment and samples were removed aseptically at designated time intervals. All experiments were performed at room temperature which varied between 23–25°C.

Experimental Set I

These experiments were designed to determine the effect of excision and sucrose concentration on the minimum mitotic cycle duration and the frequency of mitotic figures. The experiments were performed in the following manner. Just before excision, the seedlings were treated with sterile colchicine (3.76×10^{-4} M) for 30 minutes. Immediately following colchicine treatment the root tips were washed, excised, and the 1 cm segments placed in White's medium supplemented with 6, 3, 2, 1, 0.5, or 0.25 per cent sucrose. The flasks were shaken gently for the duration of the experiment, and samples were removed aseptically at designated time intervals. The root tips were prepared for cytological analysis and scored for the frequency of tetraploid and diploid mitotic figures.

Experimental Set II

The second set of experiments was performed to find out when the root meristems were no longer in a period of adjustment to their new environment and when cell population kinetics could be properly measured. This information was obtained by measuring the linear root growth and the frequency of mitotic figures in root meristems cultured for several days. The increase in length of the growing excised root was obtained by harvesting a single flask at designated time intervals and measuring the roots with a ruler having 1-mm divisions. After measurement, the roots were fixed in 3 parts ethanol and 1 part glacial acetic acid and used for determination of either the frequency of cells in mitosis or the number of cells per meristem. The number of cells in mitosis was determined by scoring 1,000 cells in squash preparations from three separate root tips stained by the Feulgen method.

The effect of excision on the mitotic frequency was measured in this second set of experiments at two points: initially when the 1 cm root segment was separated from the seedling, and after 100 hours in culture when 1 cm was removed for establishment of a subculture.

Experimental Set III

The third set of experiments involved the measurement of the average mitotic cycle duration, mitosis, and the interphase periods described by Howard and Pelc (6). These experiments took place after the roots were in culture for 72 hours.

The method utilizing tritiated thymidine (H^3-T) was described by Quastler and Sherman (9). H^3-T labels a block of cells in interphase, and the cycle time can be estimated from the rhythmic appearance and disappearance of the number of these labeled cells in mitosis with time. Generally, the mitotic cycle duration is taken to be the interval of time between two successive ascending portions of such a curve. In these experiments, the time interval between the 35 per cent intercepts was used in one experiment and the 40 per cent intercepts in another to estimate cycle duration.

H^3-T usually separates interphase into 3 periods (6). These are the DNA-synthesizing or S period, the pre-DNA–synthesizing or G_1 period, and the post-DNA–synthesizing or G_2 period. Following a pulse label, cells occupying the G_2 period appear in

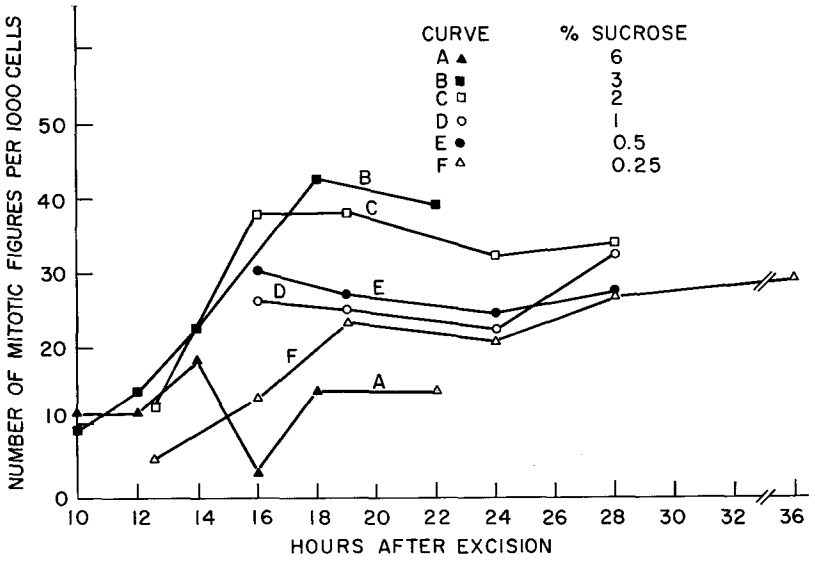

FIGURE 1 The influence of excision on the number of mitotic figures in the meristem of roots cultured in White's medium supplemented with different amounts of sucrose.

division first, the S period cells are next, and the G_1 period cells follow the S period cells. The 50 per cent intercepts of the rising and descending portions of the per cent-labeled mitotic figure *versus* time curve were used as estimates of the $T_{g_2} + T_m$ and T_s periods. T_{g_1} was calculated from the difference between the total cycle time and the sum of the T_{g_2}, T_m, T_s, and mitotic periods.

Radioautography

After being in culture for 72 hours, the roots were transferred to culture solutions containing 1 μc/ml. of tritiated thymidine (New England Nuclear, Boston, Specific activity 5.3 c/mM) and they remained in the radioactive solution for 30 minutes. They were then washed and returned to the nonradioactive medium.

All samples were fixed in a mixture of 3 parts ethanol and 1 part glacial acetic acid and stained by the Feulgen method. Squashes were made from the terminal portion of the roots using the technique of Conger and Fairchild (2). Radioautographs were made with Kodak NTB liquid emulsion. The emulsion was exposed for 12 days at 4°C after which the radioautographs were developed and prepared for microscopic examination.

Mitotic Cycle Time Measurements with Colchicine

The full details of the technique used have been published (18). The technique involves the production of tetraploid cells by treating the meristems with colchicine for short periods of time. The number of tetraploid cells produced during the treatment depends upon (1) the number of cells that entered metaphase while colchicine was effective, and (2) the concentration of colchicine used. The kinetochores of the chromosomes in the affected cells divide and enter interphase as usual. The next time these affected cells divide, they will be tetraploid and hence distinguishable from normal diploid cells. The period of time between the colchicine treatment and the appearance of the tetraploid cells in division is the mitotic cycle time.

Simultaneous Treatment with H^3-T and Colchicine

Simultaneous exposure of an asynchronous population of cells, such as that usually found in root meristems, to both H^3-T and colchicine produces the appearance of two successive waves of marked cells in mitosis: the first wave consists of H^3-T-labeled cells, and the second wave is composed of colchicine-produced tetraploid cells devoid off radioactive label (17).

RESULTS

Experimental Set I

While investigating some of the external factors required to maintain a dividing population of cells in the meristem of excised pea roots, Wilson *et al.* (20) showed that cell division almost ceased within

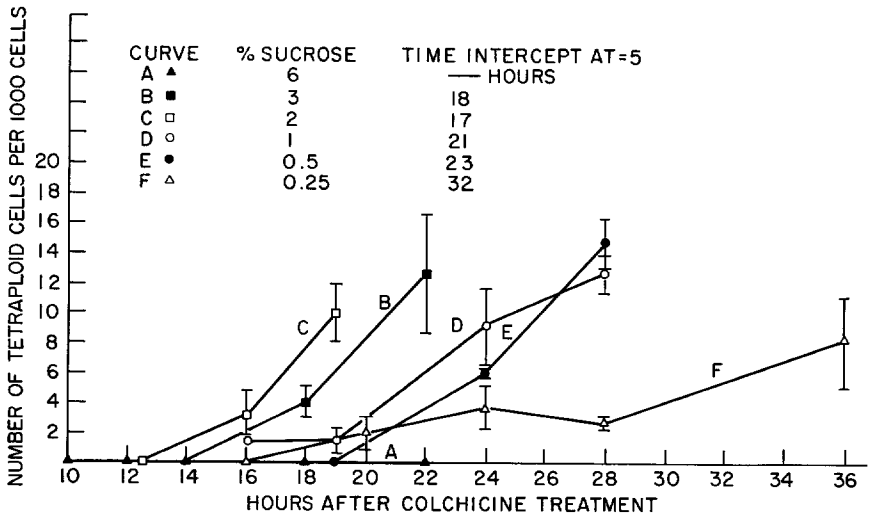

FIGURE 2 The appearance of colchicine-induced tetraploid mitotic figures in excised roots cultured in White's medium supplemented with different amounts of sucrose. Colchicine treatment preceded excision. Standard error of the mean is indicated.

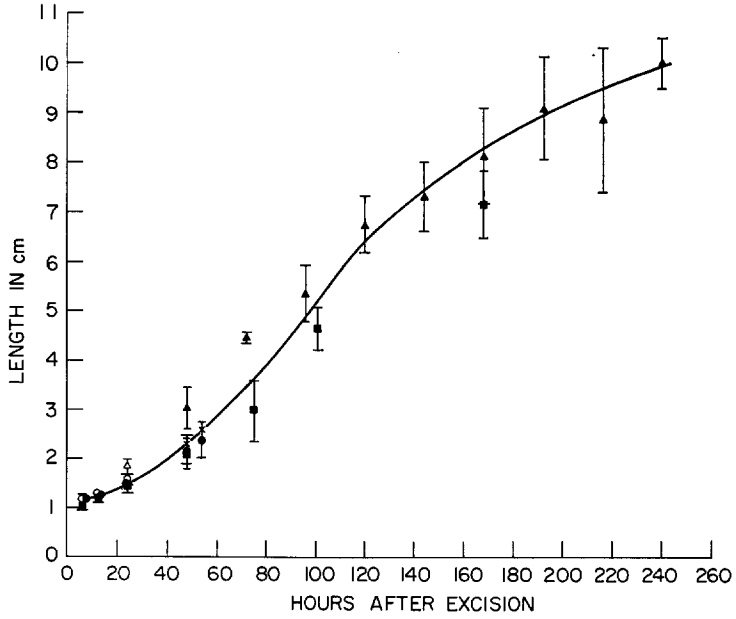

FIGURE 3 The increase in length of excised cultured roots in medium supplemented with 2 per cent sucrose. Each symbol represents a separate experiment, all performed with 2 per cent sucrose; 10 roots measured per point. Standard error of the mean is indicated.

12 hours after excision if a carbohydrate was not supplied. The information presented in Fig. 1 indicated that the reduction in the frequency of the mitotic figures, from about 70 just before excision to approximately 10 per 1,000 cells at 10 to 12 hours after excision, occurred in spite of the presence of sucrose and independent of sucrose concentration. The subsequent rise in number of division figures, however, was somewhat dependent upon sucrose concentration.

The appearance of tetraploid mitotic figures formed by colchicine treatment just before excision of the root suggested that 2 per cent sucrose maintained the shortest cycle duration (Fig. 2), closely followed by 3 per cent, and eventually, 1, 0.5, and 0.25 per cent, respectively.

Experimental Set II

From the results of the first set of experiments, it was decided to use a supplement of 2 per cent sucrose to White's medium for the second set of experiments. The changes in the frequency of dividing cells after excision indicated that the second set of experiments be performed for a duration in excess of 36 hours. Such an experiment would indicate when the root meristems were no longer in a period of adjustment to their new environment and when cell population kinetics could be properly measured. Fig. 3 shows the results of experiments in which linear growth was used as an indicator of culture conditions. The results are almost identical to those of Brown and Wrightman (1). From these data it is apparent that a slight lag in growth occurs immediately after excision. After approximately 20 hours in culture, linear growth begins and this continues for about 180 hours, at which time a tendency of decreased growth is suggested. The frequency of mitotic figures in these roots showed the effect of excision both initially and after 100 hours in culture when excision was performed for subculturing (Fig. 4). These data and those of Figs. 1 and 2 show that the first 24 hours after excision is a period of dramatic change in cell proliferation and not the proper time for measurements of mitotic cycle duration.

Experimental Set III

Results from the two preceding sets of experiments suggested that mitotic cycle measurements and determination of the interphase periods be conducted at some time between 60 and 160 hours. Therefore, the third series of experiments was initiated at 72 hours and continued to about 102 hours. Figs. 5 and 6, respectively, represent separate experiments in which cycle time was measured with either the colchicine or tritiated thymidine technique. Figs. 7 and 8 depict the results of a single experiment in which cells of the same meristem were marked simultaneously with tritiated thymidine and colchicine. The data obtained from

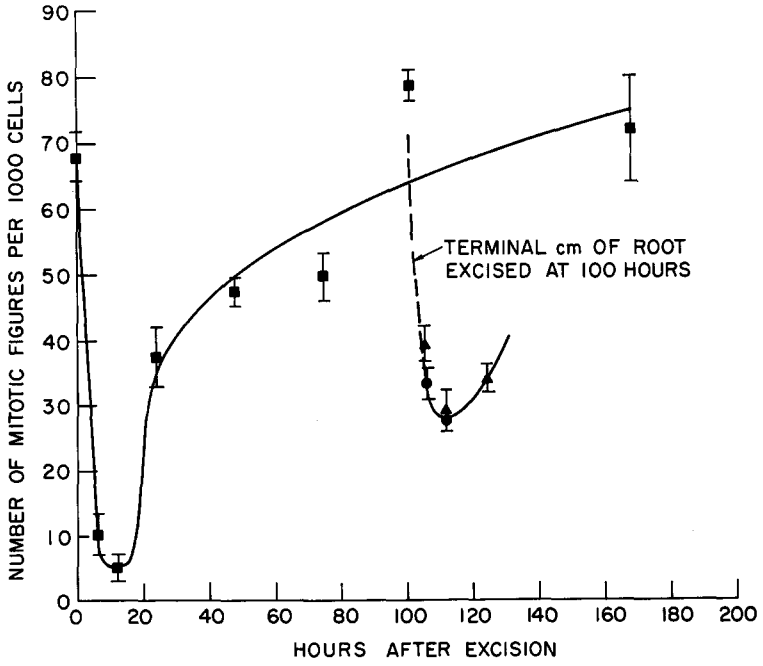

FIGURE 4 The influence of the initial excision and excision for subculture at 100 hours on the number of mitotic figures in the meristem of roots cultured in medium supplemented with 2 per cent sucrose. Standard error of the mean is indicated.

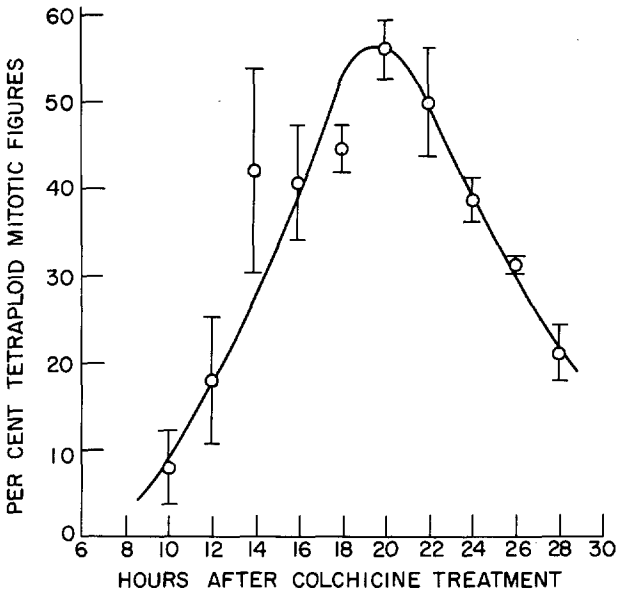

FIGURE 5 The per cent colchicine-induced tetraploid mitotic figures in roots cultured for 72 hours. Colchicine treatment time was 30 minutes. Standard error of the mean is indicated.

the curves in Figs. 5 through 8 are summarized in Table I.

The curves in Fig. 9 provide information necessary for the determination of the duration of mitosis. The number of labeled prophases at successive time intervals following a pulse label with H^3-T was determined and the curve extrapolated to the abscissa. The number of labeled metaphases at successive time intervals was also determined. The difference between the time intercepts for the prophase and metaphase cells is the minimum prophase duration. The relative proportion of prophase cells to all other mitotic figures was determined in 3 meristems. With these statistics, the duration of mitosis can be determined as follows:

$$\frac{\text{Duration of mitosis}}{\text{Duration of prophase}} = \frac{\text{Number of cells in mitosis}}{\text{Number of cells in prophase}}$$

The duration of mitosis and the total mitotic cycle time of roots were measured after 72 hours in culture. Extrapolation of the prophase curve causes it to intercept the abscissa at 0.7 hours. The metaphase curve indicates an intercept value of about 2 hours. The difference between the two intercept values is the duration of prophase. In this case, the value is 1.3 hours. Cytological examination of 3 slide preparations indicated that approximately 0.51 of the mitotic figures at 72 hours were prophases, thus

$$\frac{1.3}{T_m} = \frac{0.51}{1.0} \qquad T_m \sim 2.55 \text{ hours}$$

DISCUSSION

The response of excised *Pisum* roots to different sucrose concentrations was very similar to that of tomato roots. Street and McGregor (11) found that the rate of cell production in tomato roots grown in culture was greatest at 2.0 per cent sucrose. At concentrations greater or less than 2.0 per cent, cell production decreased. On the basis of the results obtained with *Pisum* roots (Fig. 2), the mitotic cycle duration of tomato roots also would be expected to be at a minimum at 2.0 per cent sucrose because of the inverse relationship between cycle duration and cell production.

Of interest is the observation that the frequency of mitotic figures at 12 hours after excision is at a common low, regardless of sucrose concentration (Fig. 1). Although the minimum at 12 hours is expected (20), the ineffectiveness of all concentrations to prevent this decrease corroborates the conclusions of Brown and Wrightman (1) that cell proliferation is very sensitive to events occurring at some distance from the meristem. Even after the roots have become acclimated to culture conditions, as indicated by the mitotic frequency and

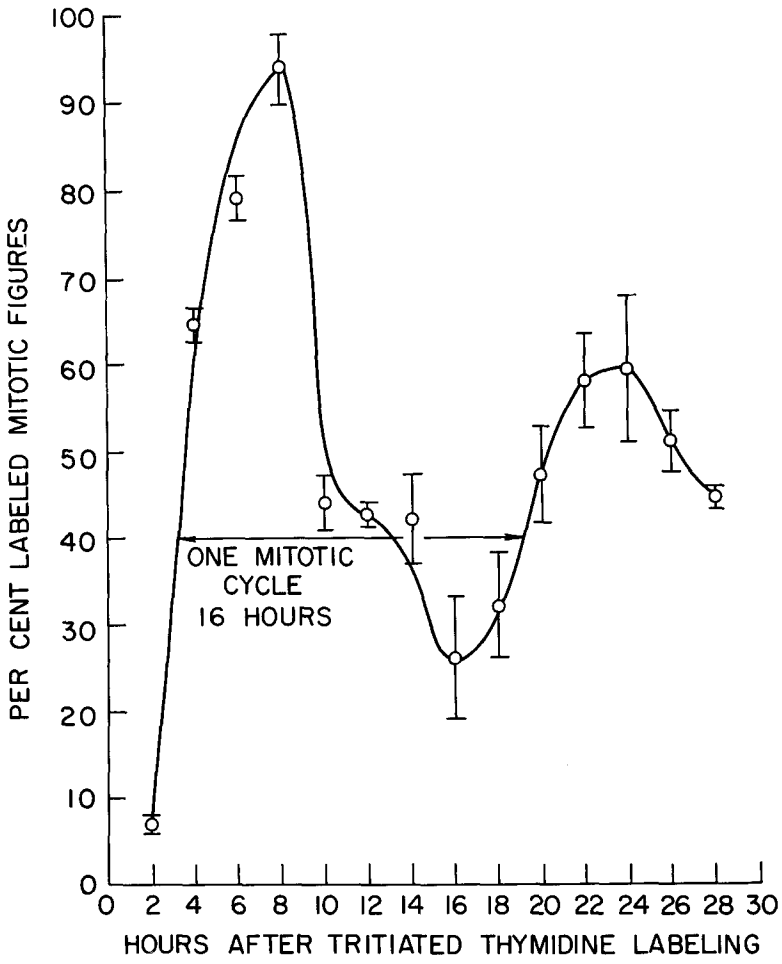

FIGURE 6 The per cent tritiated thymidine-labeled mitotic figures in roots cultured for 72 hours. Labeling time was 30 minutes. Standard error of the mean is indicated.

TABLE I

Duration of Interphase Periods (Hours) and Average Mitotic Cycle Duration of Excised Cultured Pea Roots

Excised roots	T_{g_1}	T_s*	T_{g_2}	T_m	CT
Tritiated thymidine (Fig. 8)	7.2	4.2	←6.5→		17.9
Tritiated thymidine (Fig. 6)	6.9	5.8	0.78	2.55	16.0
Colchicine (Fig. 5)					20.0
Colchicine (Fig. 7)					20.0

* The time interval between the 50 per cent intercepts of the rising and descending portions of the labeled mitotic figure curves minus 0.5 hour labeling period.

increase in length, they still display a sensitivity to excision when it takes place after 100 hours in culture (Fig. 4). The causal factors for this sensitivity remain unknown, even though various hypotheses have been presented (1, 12).

Although excision has an immediate but temporary effect on the frequency of mitotic figures, it causes little alteration in the total mitotic cycle duration. When a potential tetraploid population was formed by colchicine treatment before ex-

cision, the tetraploids began to appear in the subsequent division at about 16 hours (Fig. 2). The average cycle duration of cells in root meristems cultured for 72 hours, measured with either the colchicine or tritiated thymidine technique, ranged from 16.0 to 20.0 hours (Figs. 5 to 8). The insensitivity of cycle duration and the effect of excision on cell passage from interphase into mitosis suggest that cells are temporarily blocked in interphase (hence the decrease in the frequency of mitotic figures). Once the causal factors responsible for this block are overcome, the cells continue to proliferate with a cycle duration which is optimal for the culture conditions.

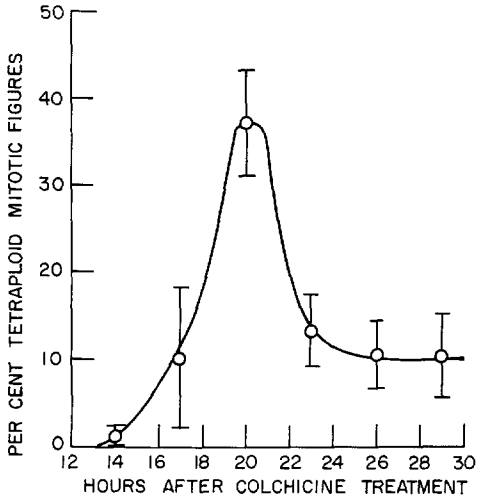

FIGURE 7 The per cent colchicine-induced tetraploid mitotic figures in roots cultured for 72 hours and simultaneously treated with tritiated thymidine and colchicine for 30 minutes. Standard error of the mean is indicated.

The information in Table I lists the durations of G_1, S, G_2, and mitosis (T_m) of excised roots after 72 hours in culture. The cycle duration of the tetraploid cells was longer than that reported for intact root meristem cells. In *Pisum*, at 22.5°C the tetraploids divided once every 12 hours on the average (18). On the other hand, the Ts of the culture meristem cells is almost the same as that of intact root cells maintained at 20°C (14). The similarity of S period durations is further support for the suggestion of Sisken and Kinosita (10) that the duration of DNA synthesis remains relatively constant while the other periods of interphase will vary under different environmental conditions. Although further comparisons of the interphase periods of intact and excised roots would be in-

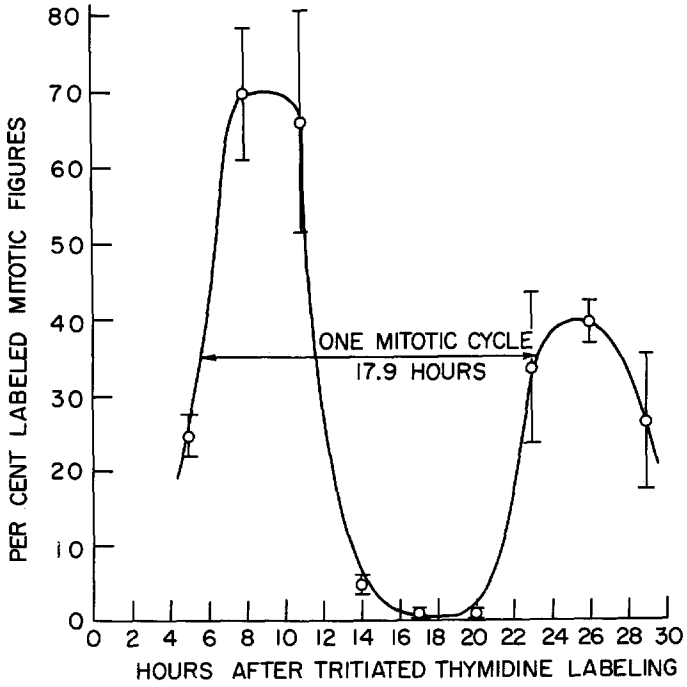

FIGURE 8 The per cent tritiated thymidine-labeled mitotic figures in the same roots from which the data in Fig. 7 were obtained. Standard error of the mean is indicated.

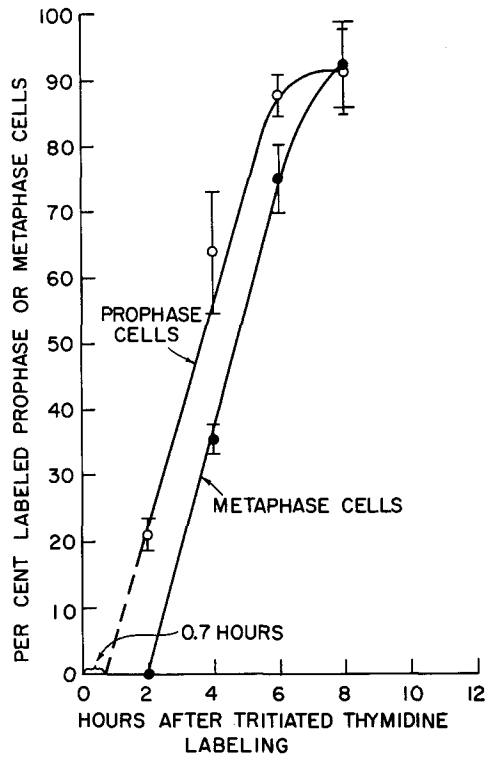

FIGURE 9 The appearance of tritiated thymidine-labeled prophase and metaphase cells in meristems of roots cultured for 72 hours. Standard error of the mean is indicated.

teresting, they cannot be made at present because of a difference in temperature and the amount of tritiated thymidine used in the two experiments.

The difference between the average mitotic cycle durations, when measured with tritiated thymidine and colchicine, may be expected in view of the suggestions of Das and Alfert (3) and the relationships described by Van't Hof and Sparrow (16) for several different plants. These investigators showed that the cycle duration is a function of the cellular DNA content. The additional 3 hours in cycle time may be the result of doubling the DNA content by the effects of colchicine, and much of the 3 hours may be spent in the *S* period (13).

It should be pointed out, however, that experiments with intact root meristems have yet to indicate a cycle duration, as measured with colchicine-induced tetraploids, which is longer than that measured by tritiated thymidine (14). Thus, it appears just as likely that the longer cycle duration of excised, cultured roots as measured by the colchicine method may be the result of an excessive delay which is not evident in intact roots. Although the cell population kinetics in tissues composed of heterogeneous cell populations are far from understood, it, nevertheless, does not seem unlikely for colchicine-induced tetraploid cells in a given species to have the same cycle duration as the diploid cells if all the chemical capacities of the cell are increased to the same degree as the DNA content.

In a root meristem the two daughter cells which result from cell division have, on the average, different proliferative potentials. One daughter cell will remain embryonic and eventually produce more cells, the other daughter cell will not remain proliferative but rather begin the transition from meristematic to maturity and differentiation. Evidence for this concept is derived from experiments in which the number of potential colchicine-induced tetraploid cells was compared to the number of actual tetraploid cells observed in division after the completion of one mitotic cycle (17). The comparison showed that nearly one-half of the potential tetraploid cells appeared in the first division after formation. The remaining half presumably were undergoing maturation. This maturing population of cells is not necessarily removed physically from the root apex immediately after the processes of maturation are initiated. The fact that continuous H^3-T labeling does not label all the cells in the meristem in cultured roots (15) indicates that the meristem contains a sizable population of cells which if not maturing are at least not synthesizing DNA at an appreciable rate.

On the basis of the observations just mentioned, the root meristem probably consists of at least three cell populations: one which is proliferative (N_{ct}), and two others which are non-proliferative (Tr and F). The mitotic index (I_m) is then

$$I_m = \frac{N_m}{N_{ct} + Tr + F}$$

The limits of I_m would be either zero or $\frac{N_{ct}}{N_{ct} + T_r + F}$, assuming complete synchrony of the proliferating cells. The root meristem, however, is generally an asynchronous system, and thus I_m under usual conditions would not approach either limit. The separation of a root from other plant parts is indeed an unusual situation for a root, and therefore I_m approached zero (Figs. 1 and 4). The rate at which I_m decreased was ampli-

fied by the fact that both the numerator and denominator of the above equation were changing.

When growth and cell division have adjusted to culture conditions (as it presumably has at 72 hours), some notion concerning the transition time for cells which are maturing can be obtained. In an asynchronous, steady-state cell population the number of cells in any stage or phase is proportional to the duration of that stage or phase (8). Thus,

$$I_m = \frac{T_m}{CT + T_{tr} + T_f}$$

and substituting the observed values obtained at 72 hours

$$0.055 = \frac{2.55}{20 + T_{tr} + T_f} \text{ or } \frac{2.55}{17 + T_{tr} + T_f}$$

For a 20-hour cycle, $T_{tr} + T_f$ is 26.3 hours; for a 17-hour cycle, $T_{tr} + T_f$ is 29.3 hours. If it is assumed that the number of cells in the meristem remains relatively constant for a time period equivalent to a complete mitotic cycle, the number of maturing and functional cells passing from the meristematic area to the zone of elongation should be approximately equivalent to the number of proliferating cells, and $T_{tr} + T_f$ would be equivalent to CT. The fact is, however, that not all transitional cells or functional cells leave the meristematic area. There are root cap cells and cells of the quiescent center which remain within the root meristem region. Cells such as these may account for the 6 to 12-hour discrepancy between the average mitotic cycle duration and $T_{tr} + T_f$. Added support for this explanation is derived from experiments in which the cultured roots were continuously labeled with H^3-T for 22 hours (15). Although these experiments exceeded in duration the average mitotic cycle time, the number of labeled cells observed at 22 hours was about 75 per cent. The remaining 25 per cent presumably were transitional and functional cells.

It was noted earlier that excision in some way interupts the emergence of cells from interphase to mitosis (Fig. 4). In view of this effect, it may be of interest to consider Gelfant's (4, 5) hypothesis that proliferative tissues may contain cell subpopulations which selectively arrest in either the G_1 or G_2 conditions when cell division is carried out at a much reduced rate. The root also contains cells which are distinctive because of their location, low rate of proliferation, and 2n DNA content. These cells also remain in G_1 for an unusually long time (7) and in this discussion are classified as either transitional or functional cells. In view of Gelfant's hypothesis, it would be of much interest to determine whether the decreased mitotic index after excision is the result of a selective accumulation of cells in a given interphase period. Completed experiments have indicated that such a study was possible and the results are presently being prepared for publication.

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