EVIDENCE FROM THYMIDINE-^{3}H-LABELED MERISTEMS OF *VICIA FABA* OF TWO CELL POPULATIONS

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ABSTRACT

Treatments with tritiated thymidine (TdR-^{3}H) have revealed the existence of two populations of mitotically active cells in meristems of lateral roots of *Vicia faba*. A rapidly dividing population, with a cycle time of 14 hr, constitutes about half the cells in the meristem. A second population of cells, with a cycle time in excess of 30 hr, is also present. Estimates of the relative size of this slowly dividing population are more difficult to make, but we calculate that this population includes 27–43% of meristem cells. The remaining fraction of the meristem is made up of cells that divide rarely or not at all. Since, at all times, both populations contribute to the mitotic index, the curve of the percentage of labeled mitoses that can be determined after a pulse label with TdR-^{3}H differs from the curve expected of an ideal population in an important way: the peak value of the curve of the percentage of labeled mitoses is always less than 100%, usually between 75 and 80%. This heterogeneity within a meristem must be borne in mind in terms of the response of meristems to disruptive treatments, the mechanisms controlling mitotic cycle duration, and the spatial organization of a heterogeneous population in an organ that shows polarized growth.

INTRODUCTION

Two of the most useful contributions to the study of cell cycle kinetics are the mitotic cycle model of Howard and Pelc (13) and the pulse-labeling technique of Quastler and Sherman (18). The pulse-labeling technique has been used successfully in studies of cell kinetics in root apical meristems, notably by Wimber (24, 25), Howard and Dewey (12), and Van't Hof (20–22). The root meristem, however, is not always a homogeneous population of cells (1, 14). In roots of *Vicia faba* in particular, there are cells that divide very rarely (1, 19), and there is variation in the duration of the mitotic cycle in cells that are undergoing division (1,12). Rasch, Rasch, and Woodard (19) measured nuclear volume in meristematic cells of *V. faba* and found evidence for two discrete populations of cells. They also showed, by long treatments with tritiated thymidine (TdR-^{3}H), up to 96 hr, that there were discrete populations of cells. We have treated lateral roots of *V. faba* with TdR-^{3}H for 1 hr, and from the curve of the percentage of labeled mitoses we have made an estimate of the degree of heterogeneity in mitotic populations of the lateral root meristems. The sizes and cycle times of the different subpopulations contributing to the heterogeneity have been determined and will be reported here.

MATERIAL AND METHODS

The method of seed germination and growing *V. faba* seedlings has been described (5). The beans used in the present experiments were grown at 21–22°C.

Measurements of the duration of the mitotic cycle and its component phases were made by the pulse-labeling method of Quastler and Sherman (18). The phases of the mitotic cycle are (13): G_1, presynthetic interphase; S, period of DNA synthesis; G_2, postsynthetic interphase; and mitosis. Following a brief exposure to tritiated thymidine (TdR-^{3}H), a specific precursor for DNA, the first cells to enter mitosis after treatment are unlabeled; they were in G_2 at the time of treatment and did not undergo S in the presence of labeled precursor. These cells are followed into mitosis by cells that were in S during treatment and hence they are labeled. As these cells pass out of mitosis, they are followed by a second group of unlabeled cells, the ones that were in G_1 during treatment.

The duration of the mitotic cycle can be estimated from the rhythmic appearance and disappearance of labeled mitoses. In the present experiments, the intercepts used in the timing determinations were the values at 50% of the maximum frequency of labeled mitoses. Mitotic cycle duration was taken as the interval between the 50% intercepts of two successive ascending portions of the curve. The interval between the time of exposure of the roots to TdR-^{3}H and the time when the 50% intercept is reached on the first ascending portion of the curve of the percentage of labeled mitoses represents the duration of $G_2 + \frac{\text{mitosis}}{2}$. This interval is the sum of the mean duration of G^2 and of half the mean duration of mitosis for the population of cells contributing to the curve of the percentage of labeled mitoses. The duration of S is obtained by subtracting labeling time from the interval between the 50% intercepts of the first ascending and descending portions of the curve of the labeled mitoses, and G_1 is then calculated by subtracting the sum of the values for S, G_2, and mitosis from the total cycle time.

The values presented for the durations of cell cycles and the component phases have been calculated directly from the curve of the percentage of labeled mitoses curve and, in the case of the duration of mitosis, from cycle time and mitotic index. Values were calculated to one decimal place.

Mitotic indices were fairly constant in control and treated roots over the period of the experiment. Mean mitotic index was used to calculate duration of mitosis, and the value obtained agrees with previous estimates (9, 12).

Roots were treated with TdR-^{3}H (1 μc/ml; specific activity 3 c/mmole) for 1 hr. Incorporation of tritiated thymidine into DNA begins within 5 min, and no further incorporation occurs 10 min after removing roots from precursor (7). It is assumed, therefore, that the 1-hr exposure can be regarded as a genuine 1-hr pulse.

Lateral roots were fixed, every 3 hr after exposure to TdR-^{3}H, in acetic acid–alcohol (1/3, v/v). They were hydrolyzed for 8 min in 1 N HCl at 60°C and stained with the Feulgen reaction. Meristems were prepared as squashes (4). The slides were coated with Kodak NTB2 liquid emulsion and exposed, at 2°C, for 2 wk. Some preparations were given longer exposures as a check on possible false negatives, i.e. labeled cells that failed to produce grains in a 2-wk exposure because they had incorporated little TdR-^{3}H. We found no increase in the frequency of labeled cells. Thus, false negatives can be making little contribution to the data obtained from roots exposed for 2 wk. The radioautographs were developed and permanent preparations were made.

Mitotic indices were determined by scoring 1,000 cells from each of at least three roots. The frequencies of labeled mitotic figures were determined by scoring 100 mitoses from each root; again, at least three roots were scored from each fixation.

RESULTS

The mitotic index remained fairly constant over the duration of the experiment. The range of values was 8.8–11.5%; the mean mitotic index was 9.7% (Table I). The frequencies of cells in the different stages of mitosis also remained constant.

Mitotic Cycle Measurements

From the curves for the percentage of labeled prophases, metaphases, and total mitoses (Fig. 1), the duration of the mitotic cycle, C, can be meas-

TABLE I

Mitotic Index (MI) and Frequencies of Cells, per 1,000 Cells Scored, in the Various Stages of Mitosis

Time (t) was measured, in hours, from the end of treatment with TdR-^{3}H. P = prophase; M = metaphase; A = anaphase; and T = telophase.

t	P	M	A	T	MI
3	55	19	10	11	9.5 ± 1.9
6	55	19	15	12	10.1 ± 1.1
9	55	21	11	15	10.1 ± 1.2
12	56	16	10	13	9.5 ± 1.9
15	48	16	12	19	8.8 ± 0.2
18	50	20	10	9	8.8 ± 0.6
21	60	23	16	16	11.5 ± 1.0
24	48	19	14	19	9.1 ± 1.1
27	52	20	14	12	9.7 ± 0.1

ured directly. The estimates from these three curves are similar:

Fig. 1 *a*	Prophase curve	C = 14.4 hr
Fig. 1 *b*	Metaphase "	C = 13.6
Fig. 1 *c*	Mitoses "	C = 14.0
	Mean	C = 14.0

The durations of G_2 and mitosis are calculated as follows:

Fig. 1 *a* $G_2 + P/2 = 3.8$ hr

Fig. 1 *b* $G_2 + P + M/2 = 4.5$

$$\therefore \frac{P + M}{2} = 0.7$$

$$\therefore P + M = 1.4$$

The mean P:M ratio (P and M values from Table I) is 2.77:1.

$$P/M = 2.77$$

$$\therefore 3.77\,M = 1.4 \text{ hr}$$

$$\therefore M = 0.4 \quad (0.37)$$

$$\therefore P = 1.0 \quad (1.03)$$

$$\therefore G_2 = 3.3$$

From Fig. 1. *c* $G_2 + \frac{\text{mitosis}}{2} = 4.3$

$$\therefore \text{mitosis} = 2 \text{ hr}$$

The duration of the S period, allowing for a labeling time of 1 hr (see Material and Methods), is calculated as follows:

From Fig. 1 *a* S = 5.6 hr

" Fig. 1 *b* S = 6.6

" Fig. 1 *c* S = 6.4

Mean S = 6.2

$G_1 = C - (G_2 + S + \text{Mitosis})$

$= 14 - (3.3 + 6.2 + 2.0)$

$= 2.5$ hr

It must be stressed that these values apply to the majority, but not to all of the dividing cells. From these estimates, however, the sizes of the populations with different cycle times have been calculated.

If all dividing cells in a meristem have the same cycle time, C, then over a period equal to C all cells will have divided once. Thus, the area under the mitotic index curve taken over this period C will represent the total number of cells that contribute to mitotic cycles (23). Similarly, following a pulse label with TdR-^{3}H, the area under the curve of the labeled mitosis index over the same time period will be proportional to the number of cells in S at any one time. If we assume that the number of cells in any one phase is proportional to the duration of that phase, then the duration of S, relative to the duration of the cycle,

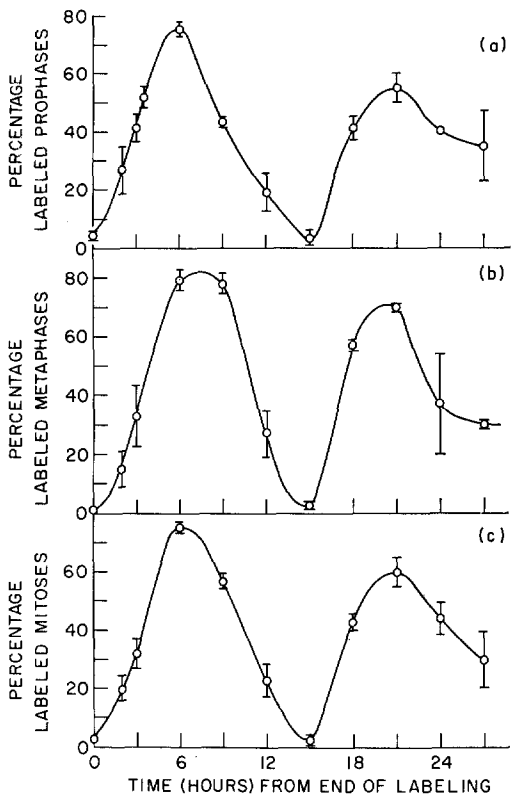

FIGURE 1 Per cent of labeled prophases, metaphases, and mitoses in lateral roots of *V. faba* treated with TdR-^{3}H for 1 hr and fixed up to 27 hr later.

can be calculated by comparing the areas under these two curves.

The mitotic index and the labeled mitosis index (i.e. number of labeled mitoses per 100 cells) have been plotted against time (Fig. 2). The time period chosen is 0–14 hr, i.e. the measured duration of the mitotic cycle. A comparison of the areas under these curves gives the relationship:

$$\frac{S + t^1}{C} = 0.39$$

where t^1 = duration of exposure to TdR-^{3}H. Direct measurements of the durations of $S + t^1$ and C, however, yield values of 7.2 and 14 hr, respectively, and hence

$$\frac{S + t^1}{C} = 0.51.$$

Thus, the actual value of 0.39 obtained by com-

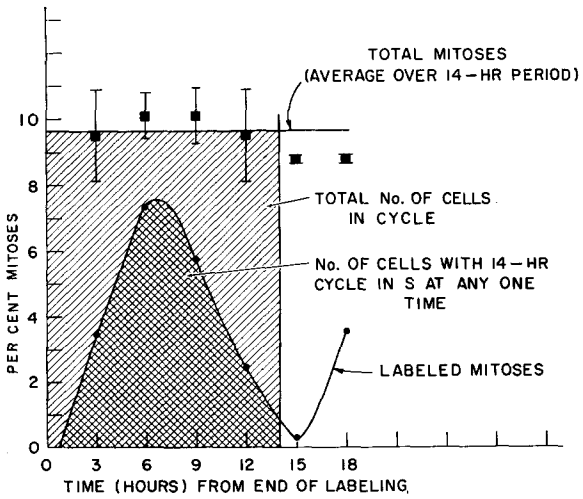

FIGURE 2 Per cent of cells in mitosis and per cent of cells in mitosis that are labeled in lateral roots of *V. faba* treated with TdR-^{3}H for 1 hr and fixed up to 18 hr later.

paring the areas under the curves is only 75% of the expected value based on direct measurement. The labeled cells that pass through mitosis in the 14-hr period we are considering constitute the population whose cycle time is estimated following exposure to TdR-^{3}H; since the value for this population of labeled cells (0.39) is lower than the value for all cells (0.51), the difference between these two values must result from the presence in mitosis, in the 14 hr period we are considering, of cells which have a cycle time considerably longer than 14 hr. Furthermore, this population of cells contributes 25% of cells seen in mitosis at any one time; the other 75% of cells in mitosis represents the population with a mean cycle time of 14 hr. An estimate of the size of this latter population, of rapidly dividing cells, can be found as follows.

It is possible to derive a relationship involving the proportions of cells that are actually proliferating.

$$MI = tm/C \times p$$

where MI = mitotic index, tm = duration of mitosis, C = cycle duration, p = proportion of proliferating cells.

It would be more accurate, however, from the estimates above, to consider the dividing cells in the meristem as comprising two populations: those with a shorter cycle time (A hours) and those with a longer cycle time (B hours). Hence:

$$MI = \frac{tm^1}{A}\cdot p^1 + \frac{tm^{11}}{B}\cdot p^{11}$$

where superscript 1 refers to the population with cycle time A hours and superscript 11 refers to the population with cycle time B hours, or

$$0.097 = \frac{2}{14}\cdot p^1 + \frac{tm^{11}}{B}\cdot p^{11} \qquad (1)$$

Now, since the population with a cycle time of 14 hr contributes 75% of the cells in mitosis, while the population with cycle time B hours contributes 25% of these cells

$$\therefore \frac{2}{14}\cdot p^1 = 3\left(\frac{tm^{11}}{B}\cdot p^{11}\right) \qquad (2)$$

Substituting in equation (1),

$$0.097 = \frac{2}{14}\cdot p^1 + \frac{1}{3}\left(\frac{2}{14}\cdot p^1\right)$$

$$\therefore p^1 = 0.51.$$

Hence from equation (1)

$$\frac{tm^{11}}{B}\cdot p^{11} = 0.024.$$

The assumption has been made in these calculations that the duration of mitosis in the slowly dividing population is also 2 hr (see below). i.e.

$$\frac{2}{B}\cdot p^{11} = 0.024 \qquad (3)$$

Now the mean cycle times ($\bar{C}$) for all dividing cells in the meristem is given by the relationship

$$\bar{C} = Ap^{I} + Bp^{II}$$

or

$$\bar{C} = 14 \times 0.51 + Bp^{II} \quad (4)$$

Another relationship involving $\bar{C}$ can be defined:

$$\bar{C} = \frac{1}{R} \cdot p,$$

where R = rate of entry of cells into division, p = proportion of cells that are proliferating.

We now have three equations, (3), (4), and (5), involving four unknowns: $\bar{C}$, B, p^{II}, and R. However, a value for R can be obtained experimentally by a determination of the rate of accumulation of metaphases following colchicine treatment (6, 8, 16). For this purpose, beans were immersed in 0.025% colchicine for up to 3 hr. Lateral roots were fixed at ½-hr intervals.

The rate of accumulation of metaphases will be an accurate indicator of the rate of entry of cells into mitosis only if two conditions are satisfied. First, the rate of passage of cells into and out of prophase must be constant, i.e. unchanged by colchicine, and secondly, cells must not be leaving metaphase.

Roots that had been in colchicine for only 0.5 hr still had cells in anaphase in high frequency, but after 2 hr or more restitution nuclei were present in sufficiently high frequency to show that significant numbers of cells were leaving c-metaphase and returning to an interphase condition. Roots fixed after 1 and 1.5 hr in colchicine lacked anaphases and restitution nuclei, while their prophase frequency had remained constant. From each of the first four half-hourly fixations (i.e. 0.5–2 hr), four roots were scored, 1,000 cells were counted per root. After 1 and 1.5 hr, the metaphase fequencies per 1,000 cells were 29 and 50, respectively. In this half-hour period, therefore, the frequency of metaphases per 1,000 cells increased by 21.

$\therefore$ Rate of entry per hour, of cells into mitosis $(R) = 0.042$

$\therefore$ in equation (5) $\bar{C} = \frac{1}{0.042}(0.51 + p^{II})$

i.e.

$$\bar{C} = 24(0.51 + p^{II}) \quad (6)$$

When we solve for equations (3), (4), and (5), $B = 36$, $p^{II} = 0.43$, $\bar{C} = 23$.

These values are based on the assumption that $tm^{II} = 2$ hr. If, however, the duration of mitosis is twice as long as it is in the rapidly dividing population of cells then equation (3) becomes

$$\frac{4}{B} \cdot p^{II} = 0.024 \quad (3a)$$

When we solve again, $B = 45$, $p^{II} = 0.27$, $\bar{C} = 19$.

It must be stressed that the above values are only approximations; nevertheless these values permit a description of the meristems of lateral roots of *V. faba* in terms of mitotic activity. Roughly 51% of all cells in the meristem has a cycle duration of about 14 hr; the rest of the dividing cells have a much longer mean cycle time. The size and mean cycle time of this second population cannot be estimated with any degree of accuracy since the duration of mitosis in this population is not known. However, it is unlikely that mitosis lasts less than 2 or more than 4 hr; assuming that these limits are valid, one can assign limits to the other parameters. Thus, 27–43% of all cells in the meristem, the slowly dividing cells, have a mean cycle time of between 36 and 45 hr. The mean cycle time for all dividing cells in the meristem is between 19 and 23 hr. The remaining cells in the meristem, 6–22% of all cells, do not divide at all.

It has not been possible to measure directly the cycle duration of the more slowly dividing cells, but their existence can be demonstrated experimentally. After treatment of roots with 0.025% colchicine for 3 hr, anaphase is inhibited for a further 27 hr. Those cells, therefore, which enter a second mitosis in this interval are tetraploid, and hence are distinguishable from the cells entering their first mitosis following colchicine treatment. From 18 to 27 hr following colchicine and TdR-^{3}H treatment, both labeled and unlabeled diploid cells are present in fairly high frequency, e.g. at 24 and 27 hr after treatment, 33% and 20%, respectively, of all labeled metaphases are diploid. The presence of labeled diploids at these times shows that such cells must have taken up to 27 hr to progress from S to mitosis; this observation is clearly consistent with the idea of a population of cells with a mean cycle duration of more than 30 hr.

DISCUSSION

The duration of a mitotic cycle, 14 hr, in lateral root meristems of *V. faba* has been measured by the method involving determination of the percentage of labeled mitoses. The durations of the different phases of the mitotic cycle are: G_1 = 2–2½ hr; S = 6–6½ hr; G_2 = 3–3½ hr, and mitosis = 2 hr. Two further determinations of the time parameters of the mitotic cycle in untreated *V. faba* lateral roots have yielded almost identical values (unpublished results). Although the value of 14 hr is lower than previous estimates (9, 11, 13), the differences can be explained on technical grounds. Gray and Scholes (11) and Howard and Pelc (13) reported values of about 25 hr for whole meristems. Their determinations, however, would include two populations of cells described earlier (Results) that would increase mean cycle time, i.e. (*a*) the cells that are in the meristem but do not divide, and (*b*) the cells with a long cycle time. The mean cycle time of all cells in the meristems will be much longer than the 14 hr determined for the rapidly dividing population described here. That inherent variation in cycle times occurs in meristems is, indeed, indicated by Gray and Scholes (11); they showed that the mean cycle time for the first 3 mm of the root tip was 25 hr, but that for the apical 1.5 mm the cycle time fell to 19 hr.

This variation, however, does not account for the difference in values obtained by Evans and Scott (9) and Howard and Dewey (12) and in the present experiment. These other workers, using the same technique of labeling mitoses, obtained cycle durations of 19 and 18 hr, respectively. However, they used primary roots of *V. faba*, not lateral roots, and the difference in results almost certainly reflects the differences in the two types of meristem. Furthermore, the difference in temperature at which the experiments were carried out (21–22°C, cf. 19°C) may also account in part for the difference in cycle time; both Murín (17) and Wimber (25) have shown that in the temperature range 10°–30°C the duration of the mitotic cycle is temperature dependent.

It is clear, therefore, that at least three factors must be considered in any measurement of mitotic cycles: (*a*) temperature, (*b*) the type of meristem used, and (*c*) the technique used, which determines whether the mean or the mode cycle time is measured.

With regard to this latter point, the present results indicate that approximately one-half of the cells in the meristem has a cycle duration of 14 hr, and that the method involving determination of the percentage of labeled mitoses measures the cycle duration of this population. Also included in the root meristem are cells whose average cycle duration is very much longer, and cells which do not divide at all. Murín (17), Howard and Dewey (12), and Rasch et al. (19) also point out that, in primary roots of *Vicia faba*, although a majority of cells comprise the rapidly dividing population, there are cells with a much longer cycle time and cells which do not divide at all. Rasch et al. (19) have shown that there are nondividing cells distributed throughout the meristem. From their study of nuclear volume, they concluded (19) that the cells showed a bimodal or skewed distribution. The results presented here indicate that the same distribution is found with regard to cycle times. In other roots there is much greater homogeneity of cycle times, and PLM[1] curves may approximate to 100% at their peak (24). Heterogeneous populations of cells also have been described in systems that can be stimulated to initiate mitosis (10).

Such variation in the duration of the mitotic cycle confirms that the apical meristem of *V. faba* is not a completely homogeneous population of dividing cells. Furthermore, it has been shown (1–3) that the different mitotic cycle times are a property of cells in specific parts of a meristem. Mattingly (15) reached the same conclusion from a study of the synchronization of division induced by 5-aminouracil. The mitotically inactive cells presumably include cells of the quiescent center (1) as well as some cells that have differentiated while in the meristematic region. Although no attempt was made in the present study to identify the position of the different populations in the meristem, the quantitative estimates of the degree of heterogeneity with respect to mitotic activity does contribute to our understanding of the functional organization of the meristem. The differences in mitotic cycle times existing within a meristem and the locali-

[1] Percentage labeled mitosis.

zation of different cycle times in recognizable tissue-type histogens provide evidence, if not of differentiation, then of closely controlled programming of the columns of cells in the meristem.

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