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Plant Tissue Culture and the Cell Cycle

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An extensive collection of methods and tissue culture systems exists for studying the plant cell cycle and its regulation. A number of recent observations and hypotheses involving asynchronous cell populations indicate that the transition probability model of cell proliferation developed with animal cells may also be applicable to higher plant cells. Repeated synchronous division may be induced by auxin addition to quiescent cells or starvation and regrowth of plant cell cultures. In a number of such cases cell division synchrony is associated with discontinuous biosynthetic events.

1 Introduction

It is now more than a quarter of a century since Howard and Pelc[1] proposed a model for the cell cycle of three obvious events (DNA replication, mitosis and cytokinesis) separated in time by synthetic "gaps" (Fig. 1a). Despite the fact that this pioneering work made use of a *plant* model system (*Vicia faba* root meristems), the "gaps" in the plant cell cycle remain more or less devoid of further direct information about critical events regulating the rate of cell proliferation.

The cycle of cell growth and division in plant meristems is a process at the very heart of plant growth and development, yet the number of scientific groups engaged in cell cycle studies has at any one time been disappointingly small. Furthermore, their work has largely relied upon the original labelling technique of Howard and Pelc, further developed by Quastler and Sherman[2], and has merely broadened the description of the cell cycle in terms of the original model to cover other tissues and species in various other conditions. Few new events have been observed and only a handful of groups have attempted to look at cycle *control*. Only very recently[3] has an alternative model for the cell cycle (the "transition probability" model[4]) been tested with plant cells.

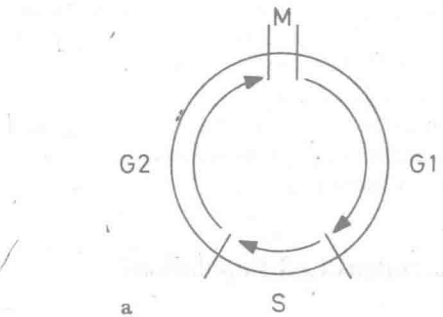
Paradoxically it appears that there is no shortage of potentially useful techniques and experimental systems for studying the plant cell cycle and the possibilities have been added to by recent developments in plant tissue culture. This article attempts to survey present methodology as applied to cell cycle studies in asynchronous and synchronous plant cell populations, in particular those involving cultured cells and tissues. A more detailed account of the biochemical information extracted using these methods may be found in recent reviews[5, 6, 7].

2 Experimental Systems

2.1 Cultured Meristems

The classical plant material for cell cycle studies is the primary root meristem, used, for example, in studies relating mean cycle time to nuclear DNA content in different species[8], or for examining the growth fraction and mean cycle times in physiologically different regions of the meristem[9, 10, 11]. Shoot apical meristems have also been the subject of several studies, for example, of alteration to mean cell cycle times during floral induction[12].

Some of the most imaginative work on the plant cell cycle uses meristem populations of cultured roots, as in the case of 1) the "principle control point" hypothesis developed by Van't Hof and coworkers using sucrose-starved sterile, primary root tips of *Pisum* and *Vicia*[13, 14, 15, 16], and 2) studies of the cycle progression of bi- and polynucleate cells induced by caffeine[18] or methyl-3, hydroxy-6, quinazolinedione-2-4 and deoxyguanosine[17, 74] in *Allium* root meristems. Although it is possible to block plant cell suspension cultures by sucrose starvation[19, 20] and caffeine[21] neither of these two approaches has yet been exploited with cell cultures, which have the advantages of being longer and more homogeneous than root tip meristems. Root meristem cells have been mainly used in an asynchronous state but also after induction of short-lived partial synchrony by e.g. caffeine[22] or 5-aminouracil[23] treatment.



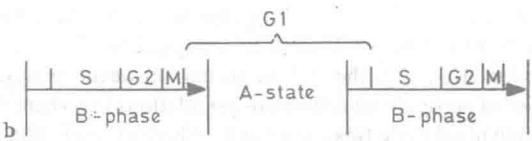


Fig. 1 Diagrams of models for the intermitotic period of dividing cells. (a) the cell cycle proposed by Howard and Pelc, 1953[1] with a determinate sequence of events: mitosis (M), DNA replication (S) and the "gaps", *G*1 and *G*2. (b) The intermitotic period according to Smith and Martin[4] in which the determinate sequences of the "cycle" are interrupted by an indeterminate "A-state"

2.2 Cultured Explants

Short-term cultures of slices from fleshy organs, for example, *Helianthus tuberosus* tuber[7, 24, 25, 26] and *Daucus carota* tap roots[27] have been widely used for studies of induction of cell division in quiescent tissues[25, 27], for cell cycle investigation[7, 24] and as a model system for cytodifferentiation[28, 29]. There are a number of advantages in the use of such explants: large amounts of tissue can be readily obtained and, in the case of the *Helianthus* explants, 90% of the cells in any explant may be induced initially to divide synchronously with further divisions showing a gradual loss of synchrony[30].

2.3 Cell Cultures

Freely-suspendable cell cultures from a wide range of species are now available which are an attractive system for cell cycle studies because of the ease of handling quantitatively on a large scale. They also have relatively short mean cycle times, are well mixed if not homogeneous and can be cultured continuously. Their main disadvantage lies in the degree of heterogeneity occurring in some cultures, particularly with respect to ploidy[31]. Cell cultures are furthermore not entirely stable: growth rate variation[32], changes in auxin dependence[33] and in DNA content per chromosome, even during the course of a single experiment[32], affect reproducibility. It is not at all clear unfortunately how such cell lines arise from the parent plant tissue but the process probably involves induction of cell division in normally quiescent tissue to give cell lines having apparently unlimited division potential. Whether or not meaningful studies of cell cycle *regulation* can be carried out with cells in such an

undefined, abnormal state is an important question. Nevertheless studies have been reported using cell lines of *Glycine max*[34,35,36], *Daucus carota*[37,38,39,40,41], *Haplopappus gracilis*[21,3], *Crepis capillarus*[42], *Acer pseudoplatanus*[43,44,45,46], *Althaea rosea*[47], *Nicotiana tabacum*[48,49,50,51,52], *Vinca rosea*[6] and *Datura innoxia*[53]. The majority of observations have been made with asynchronous populations (Sect. 3) but several methods have been recently reported which yield populations with varying degrees of synchrony (Sect. 4).

3 Asynchronous Cell Populations

The classical methods for cell cycle analysis which determine the frequency distribution at one moment in time of specifically marked cells in a population of known mean cell cycle time (see 3.1.1) or changes in the frecuency distribution of marked cells with time (see 3.1.2) were all designed for use with supposedly asynchronous cell populations e.g. the root meristem. However, precise calculations depend upon the use of *perfectly* asynchronous populations i.e. where the growth fraction is 1.0, where individual cycle times are equal, where all cycle phases are randomly represented at each moment in time etc. Cultured root meristems certainly do not conform to this definition and even in the supposedly more uniform cell culture populations the growth fraction is rarely 1.0[43,34,54], individual cell cycle times are not equal[38] and the age distribution of cells depends upon culture conditions, e.g. in an exponentially growing population there are always more young (early cycle) cells than old[55]. For these reasons the usefulness of the techniques outlined below relates directly to the type of cell population under study. Difficulties in application or interpretation of the methods have been discussed in several articles[55,56,57,58,59].

3.1 Analytical Techniques

3.1.1 Single-Sample Methods

These methods assume that in an asynchronous population the fraction of cells in a particular cycle phase at any given moment is equal to the fraction of the total cycle time which that phase occupies:

$$t = IT \tag{1}$$

where t is the phase duration, I the fraction of cells observed in that phase at a single point in time (the "index") and T the total cycle time. The proportionality is, however, altered by age distribution in the population as described by Cleaver[55]. Prior knowledge of the cycle time is required and a means of observing or marking the particular cycle events of interest.

3.1.1.1 Mitotic Index

Several thousand nuclei are examined for mitotic figures in cell squashes stained e.g. with Feulgen after hydrolysis, propionic-orcein, acid acetocarmine. The relative duration of mitosis or of constituent phases may be calculated [e.g.[37]]. Mitotic figures (particularly prophases in cell-culture cells) are often obscured by starch grains although choice of fixative can reduce this problem, e.g. use of 50% formic acid[31].

3.1.1.2 Labelling Indices

In a typical analysis of *Acer* cell cultures[43] thymidine (methyl-^{3}H, 5 Ci per mmol) was added at an activity of 0.05 μCi per ml culture (1×10^{-8} *M*) for 20 min. (This amount labels cells linearly for up to 10 h). The "flash" label was ended by fixation in 50% formic acid, the cells were Feulgen stained and squash preparations on microscope slides were autoradiographed using Kodak AR 10 stripping film. Several hundred nuclei were examined for label. The proportion of labelled cells (those cells replicating DNA at the time of the flash label) may be used to calculate the duration of S phase [e.g.[43, 8]]. Other specific labels could be used to estimate the duration of different synthetic phases, e.g. tritiated lysine or arginine to indicate histone synthesis[60, 61] (see Fig. 4).

3.1.1.3 Staining Indices

An alternative method for marking DNA-replicating cells, which may also be used for S-phase calculations (and might also be extendable to other synthetic events) involves a "flash" exposure of cells to BUdR to induce changes in the degree of nuclear staining with DNA-specific, fluorescing dyes e.g. the bis-benzimadazole dye 33258 Hoechst[62] or acridine orange[63]. The green fluorescence of DNA-bound acridine orange is suppressed as a result of prior BUdR incorporation. Light microscopic examination reveals the proportion of fluorescing cells. The RNA-specific red fluorescence is largely unaffected by BUdR. Use of flow cytometric systems has allowed automatic determination of cell fractions for animal cell cultures[63] and could presumably be extended to automatic sorting of cells in different phases or sorting cycling from non-cycling cells. This technique is mentioned here as an interesting possibility not yet applied to plant cells.

3.1.1.4 Nuclear Densitometry

Staining intensity of plant nuclei using Feulgen stain after strictly controlled fixation and acid hydrolysis is proportional to nuclear DNA content and may be conveniently measured with a scanning microspectrophotometer [e.g.[31, 15]]. The relative durations of *G*1 and *G*2 can be calculated from the frequency distribution of nuclear DNA content which normally separates into two peaks representing 2C and 4C nuclei (Fig. 2). In theory, *S*-phase nuclei can also be distinguished with values of DNA content intermediate between the 2C and 4C peaks. However, the spread of values around both main peaks usually precludes accurate estimates of *S*-phase duration. Mitotic index may usually be obtained from the same preparations. Whilst two peaks are resolved in populations with a single ploidy mode, cycling populations with two ploidy modes, e.g. mixed diploid and tetraploid, yield three peaks[31]. As the middle peak represents unknown proportions of 4C nuclei of the lower ploidy population and 2C nuclei of the higher ploidy population, no analysis is possible. Mixed ploidy populations could, however, be analysed if a small number of heterochromatic regions (chromocentres) were clearly visible in the interphase nuclei. The number of chromocentres per nucleus for any plant variety is constant and directly proportional to the number of chromosomes. Thus the middle peak of the frequency distribution curve might be analysed by simultaneously recording DNA content and chromocentre number of interphase nuclei[64]. The mean cell cycle times for both ploidy modes

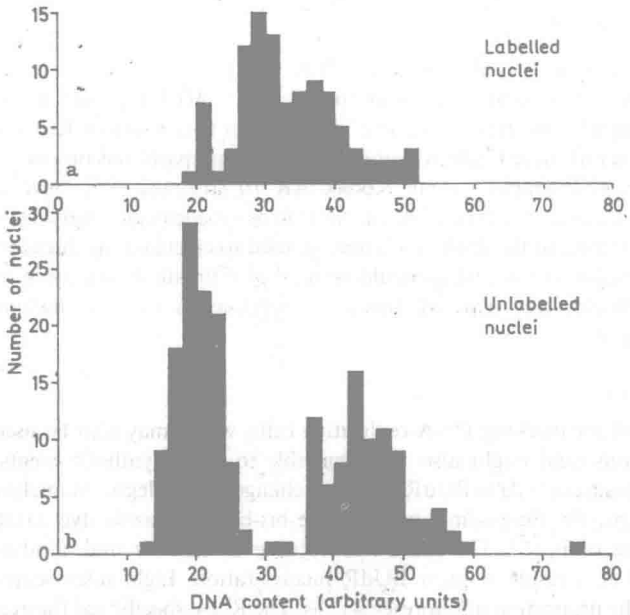


Fig. 2 The frequency distribution of nuclear DNA content in a logarithmically growing population of *Acer* cells after a flash label with ^{3}H-thymidine. Labelled nuclei (a) are from cells in S-phase at the moment of the flash label. Unlabelled cells (b) were either in *G*1 (left peak) or *G*2 (right peak). (From Gould et al.[43], with permission of the authors and Oxford University Press)

in a mixed population can be measured simultaneously using e.g. the "fraction of labelled metaphase" technique, colchicine or caffeine treatments (see 3.1.2). Diploid and tetraploid fractions of mixed populations have similar cycle times [e.g.[38,66]]. Unfortunately, not all plant varieties have chromocentres [e.g.[65]].

3.1.1.5 Autoradiography/Densitometry

A complete analysis of the relative durations of *G*1, *G*2, *S* and *M* can be carried out on a single sample by combining autoradiography of ^{3}H-thymidine flash-labelled cells with Feulgen densitometry on the same slide[43,67]. This technique clearly resolves the 2C and 4C cells as well as those cells in *S*-phase (Fig. 2). Accurate densitometry of *S*-phase cells is achieved by minimum use of ^{3}H-thymidine and by underexposing autoradiographs to give a minimum number of grains[43]. Again, the method is only directly applicable to populations of single ploidy mode.

3.1.2 Multiple-Sample Methods

Application of the single-sample methods described above requires an independent knowledge of the mean cell cycle time, which is usually determined as the population doubling time, td, during exponential growth in batch cell cultures. The specific

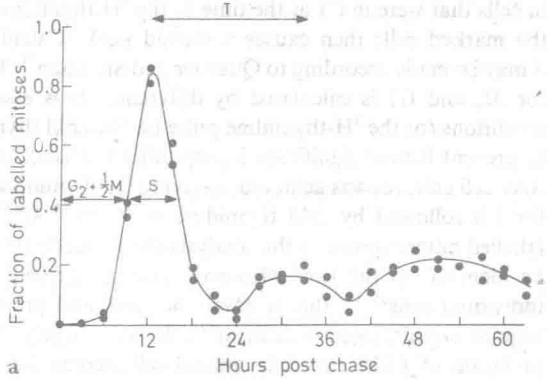
growth rate, μ, is obtained from a semilogarithmic plot of change in *cell number* with time, and td calculated from:

$$\text{td} = \frac{\log_e 2}{\mu} . \tag{2}$$

In most circumstances the population doubling time will not be equal to the mean of the individual cell generation times[55]. Such a procedure is not easily applicable to cells in a meristem and hence multi-sample methods were originally developed for following marked cells through one or more cycles, which allowed simultaneous calculation of phase durations and cell cycle times. In most of these multiple-sample analyses some cycle phases, in particular *G*1, are calculated by difference.

3.1.2.1 Fraction of Labelled Mitoses, FLM

This classical method, which has often been used for plant cell cycle studies *in vivo* [e.g.[1, 2, 68, 69, 9]] and *in vitro* [e.g.[70, 43, 34, 37]], entails marking a fraction of the cell population (those in *S*-phase) by a brief pulse of ^{3}H-thymidine, and determining the



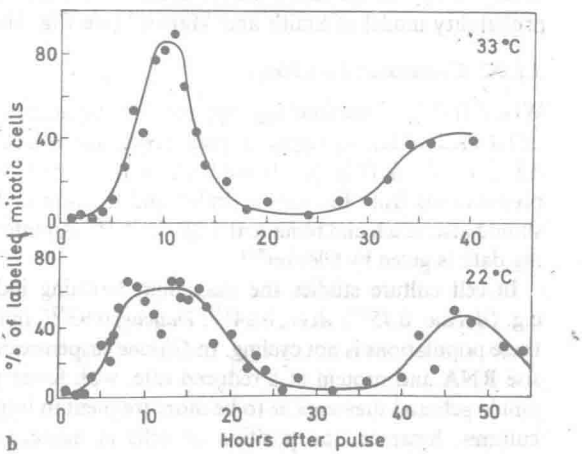


Fig. 3 (a) Changes in the fraction of labelled mitoses recorded at intervals after a ^{3}H-thymidine pulse/chase in an *Acer* cell culture. Cell cycle phases may be calculated as indicated. (From Gould et al.[43] with permission of the authors and Oxford University Press)
(b) Changes in the fraction of labelled mitoses at various times after a ^{3}H-thymidine pulse/chase in a cell culture of *Glycine max* at two temperatures. (From Chu and Lark[34] with permission of the authors)

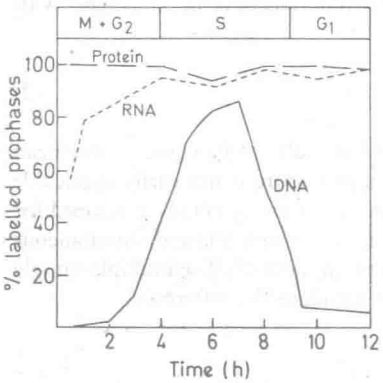


Fig. 4 Changes in the fraction of labelled prophases after exposure to ^{3}H-thymidine (DNA), ^{3}H-cytidine (RNA) or ^{3}H-leucine (protein) in meristems of primary *Pisum* roots. (After Van't Hof[76])

proportion of labelled mitotic figures which appear in a series of subsequent samples from the population (Fig. 3). The fraction of labelled mitoses rises to a peak as the marked cells reach mitosis. The labelled fraction then declines as mitosis occurs in cells that were in *G*1 at the time of the ^{3}H-thymidine pulse. The second mitoses of the marked cells then causes a second peak. Calculations of T, $G2 + 1/2M$ and S may be made according to Quastler and Sherman[2]. The mitotic index yields a value for M, and $G1$ is calculated by difference. It is essential to establish the correct conditions for the ^{3}H-thymidine pulse i.e. the cold thymidine chase must be adequate to prevent further significant incorporation of label. A satisfactory pulse/chase with *Acer* cell cultures was achieved by giving ^{3}H-thymidine (0.05 μCi per ml, 1×10^{-8} *M*) for 1 h followed by cold thymidine at 5×10^{-5} *M*[43]. In theory, the first peak of labelled mitoses given by this analysis should reach 100% and the second peak should be identical to the first. However, due to variability of cell-cycle times between individual cells[55, 38] this is never the case, and precise calculations from the data require sophisticated treatment[57]. It was largely dissatisfaction with the interpretation of FLM curves in animal cell studies that led Burns and Tannock[59] to develop a revised model for the cell cycle which was later expanded into the transition probability model of Smith and Martin[4] (see Fig. 1b and Sect. 3.3.1).

3.1.2.2 Continuous Labelling

When ^{3}H-Tdr is continuously supplied to an asynchronous cell population the fraction of labelled nuclei, beginning at a value equal to the labelling index, rises to 1.0 in a time equal to $T-S$ (Fig. 5). M and $G2 + 1/2M$ can be determined using the same preparations from the mitotic index and fraction of labelled metaphases (the latter should also reach and remain at 1.0)[9, 43, 34, 68]. An interpretation of continuous labelling data is given by Cleaver[55].

In cell culture studies the maximum labelling index was usually less than 1.0, e.g. *Glycine*, 0.75[34], *Acer*, 0.84[43], *Daucus*, 0.93[37], indicating that a small fraction of these populations is not cycling. In *Glycine* suspensions, the non-dividing cells synthesise RNA and protein at a reduced rate, with fewer protein fractions on polyacrylamide gels and they appear to be more frequent in larger cell aggregates[34]. In *Daucus* cultures, however, the position of cells in aggregates did not affect variation in individual cell cycle times[38].

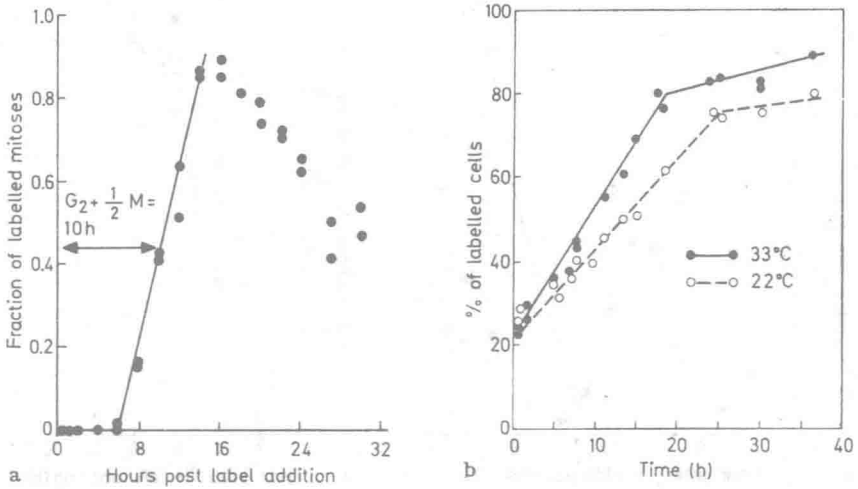


Fig. 5 Accumulation of labelled mitoses during continuous labelling with ^{3}H-thymidine in cell cultures of
(a) *Acer*: Gould et al.[43] and
(b) *Glycine max*: Chu and Lark[34].
(With permission of the authors and Oxford University Press)

There are other ways of "labelling" cells apart from using ^{3}H-thymidine, and it is clear, for example, that the above-mentioned BUdR acridine orange staining procedure could also be used either for FLM or continuous labelling analysis. Two effective methods for labelling cells by altering the configuration of a fraction of the nuclei are described in the last section.

3.1.2.3 Double Labelling

The progress of a ^{14}C-TdR flash-labelled fraction of an asynchronous population may be followed through the cycle by flash-labelling with ^{3}H-TdR at intervals and examining nuclei for double labelling. Cells which were in *S*-phase at the time of the ^{14}C-labelling will appear as ^{14}C/^{3}H double-labelled cells when they reach the next S-phase (Fig. 6a). This method was originally devised by Wimber and Quastler[71] and has recently been improved by the use of newly-available autoradiograph emulsions[12]. ^{14}C/^{3}H labelling can be distinguished by differences in 1) energy of emitted β particles (using layers of emulsion of different sensitivity); 2) distance travelled by β-particles, tritium β-particles travelling shorter distances (by focussing at different focal planes in the emulsion); 3) appearance of silver grain reaction (*tracks* of grains are produced by ^{14}C emissions). The data may be used to calculate other cycle phases[12].

3.1.2.4 Morphological Markers

— *Metaphase accumulation* induced by colchicine. The rate of accumulation of metaphases in a population exposed to colchicine (usually 0.05% w/v) has been used to determine the specific growth rate, μ, from which the mean cell cycle time

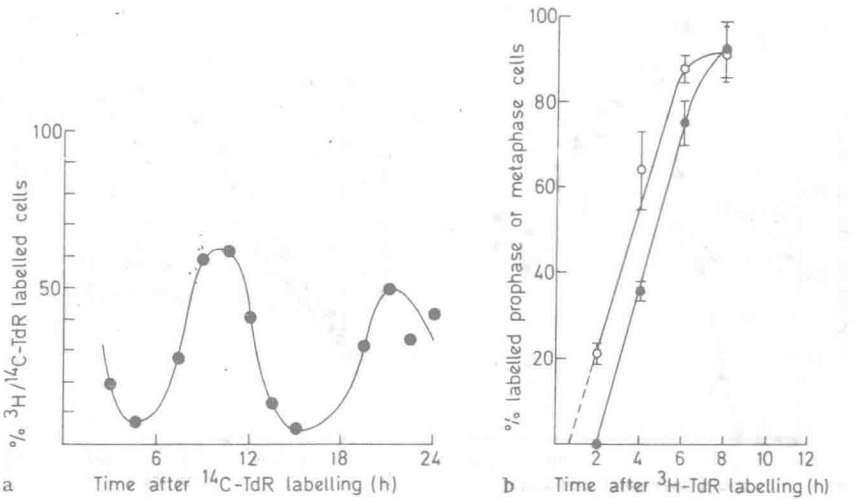


Fig. 6 (a) Change in the fraction of double-labelled cells observed in shoot apical meristems of *Silene coelirosa* by flash-labelling with ^{3}H-thymidine at intervals after the application of ^{14}C-thymidine. Plants were harvested on day 36 (after 7 long-day treatments) at the beginning of floral morphogenesis. (After Miller and Lyndon[121]) (b) Increase in fractions of labelled prophases (○) and metaphases (●) in meristem cells during continuous labelling of cultured *Pisum* roots with ^{3}H-thymidine. (After Van't Hof[68])

is calculated as shown in Eq. (2)[72, 9] (Fig. 7). Corrections can be applied for cells escaping into anaphase or interphase[72]. The assumption is made that the growth fraction is 1.0 and that the population is asynchronous and increasing exponentially. The latter assumptions are more readily confirmed for cell cultures than for root meristem cells. Nevertheless, much of the work with cultured root meristem populations which tries to determine cell-cycle times in different regions of the meristem, in an attempt to understand how differential rates of cell division influence the development of the meristem, has made use of this technique[9, 10, 11]. Some disagreement has been noted between cycle times derived by colchicine pulsing and those obtained from ^{3}H-TdR labelling analysis [e.g.[68]].

Bayliss[38] has made interesting use of continuous exposure to colchicine to estimate the variation in cell cycle time between cells within suspension cultures of *Daucus*. Two suspension culture lines, a diploid and a tetraploid, were treated with colchicine (0.05% w/v) and the frequency of cells of the initial ploidy level plotted against time (Fig. 8a). The first mitoses in presence of colchicine form double restitution nuclei (which would be scored as having the original ploidy), the second mitoses appear with doubled chromosome number. Thus in populations of cells with identical cycle times, the frequency of cells of the original ploidy would drop to zero instantaneously after one cycle interval in presence of colchicine. The form of the curve obtained (Fig. 8a) showed that individual cells in both cultures had different cycle times normally distributed about the culture means (51,2 h for the 2n culture, 46,1 h for the 4n culture). The range of cycle times (for 80% of the population) was 21,4—82,4 h (2n culture) and 14,6—77,5 h

(4n culture). Bayliss found also that such cycle-time variation severely hindered attempts to polyploidise diploid cultures using short-term colchicine exposure. Tetraploidy was not uniformly induced and the resulting mixoploid suspensions always reverted to diploid on further culture. This intra-population cycle variation has already been noted in the discussion of FLM data above (Sect. 3.1.2.1). The normal distribution of cycle times observed by Bayliss is

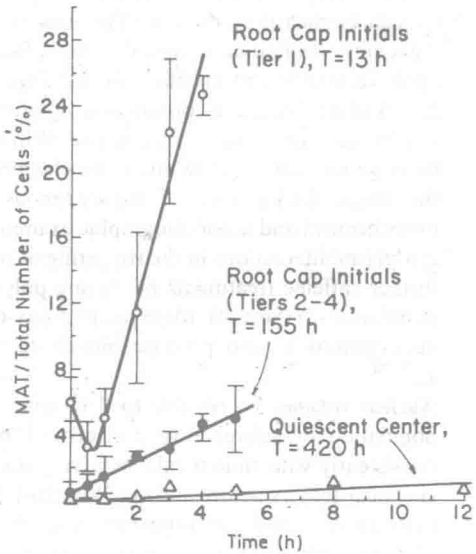


Fig. 7 Change with time in the proportion of cells at metaphase, anaphase and telophase (MAT) in the quiescent centre of *Zea mays* roots and at various distances (tiers) from the root cap junction after addition of colchicine (0.05%). T = the mean cycle time calculated from each curve. (From Phillips and Torrey[10] with permission of the authors and American Journal of Botany)

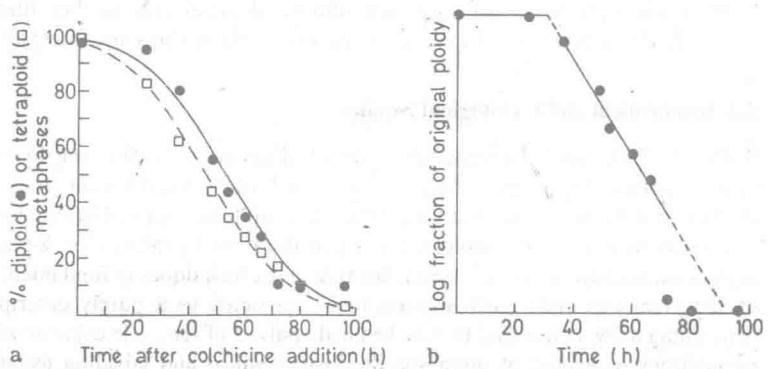


Fig. 8 (a) Change in frequency of diploid or tetraploid cells with time after addition of colchicine (0.05% w/v) to *Daucus carota* cell cultures. The gradual change to a tetraploid or octoploid population respectively indicated variation in individual cell cycle times. (From Bayliss[38] with permission of the author) (b) A semi-logarithmic plot of the data for the diploid cell culture shown in (a). For explanation see text

contrary to that predicted by the transition probability model of cell cycle control discussed in Sect. 3.3.1.

— *Binucleate cells*. Exposure of plant cells to caffeine halts progress through mitosis. During the recovery period after washing, fusion of Golgi vesicles and thus cell plate formation is inhibited[73]. The latter effect gives rise to a binucleate cell population, the size of which depends upon the duration of exposure. Charting the subsequent behaviour of this marked population as it proceeds towards the next mitosis can be used to estimate phase durations[18] (Fig. 18). With this method *G*1 can be measured directly. The unique and potentially useful feature of this "labelling" technique is that the dual effect of caffeine on 1) mitosis (which is easily reversible and produces no obvious effects on the subsequent cycle), and 2) cell plate formation, produces a significant population of cells which is both *synchronised* and easily recognizable. Whilst the size of the population may not be large enough to allow many biochemical events to be distinguished against the larger background of asynchronous cells, the situation is perfect for histochemical and autoradiographic examination of nucleus/nucleus and nucleus/cytoplasm interactions in the triggering of replication and mitosis, especially after further caffeine treatments producing polynucleate cells[18, 22] (see "synchronous populations") (Sect. 4). Methyl-3,hydroxy-6,quinazoline-dione-2-4 (MHQD) and deoxyguanosine also produce binucleate cells in meristems of *Allium sativum L.*[17, 74].

Nuclear volume. To be able to determine the cycle "position" of any cell in a population by reference to a clearly visible, structural marker which changes consistently with time would be a very useful tool for plotting changes in cytochemical activity before mitosis. Woodard, Rasch and Swift[75] reported reproducible changes in nuclear volume in *Vicia faba* root meristem cells which was used to plot stainable events in *G*1 and *G*2. However, the difficulties in measuring small changes in nuclear volume have limited further applications. Webster[100] recently found that nuclear volume distributions from proliferating and stationary phase meristem cells in *Pisum* roots are almost identical and further that a wide variability of nuclear volume occurs between cells of the same interphase age.

3.2 Biochemical and Cytological Studies

Within the limitations of interpretation already discussed, caused mainly by deficiences in the experimental systems used e.g. low growth fractions, the techniques described above and the innumerable variations may be confidently applied in situations where one *needs to know*, for example, the mean duration of mitosis or *S*-phase in an asynchronous population. However, the role of the techniques in fundamental studies of the plant cell cycle itself appears to be restricted to a purely descriptive one, concerning a few major events. Biochemical analysis of the cycle using asynchronous populations is dependent upon specific histochemical and labelling techniques for visualization and is thus largely confined to describing discontinuities in DNA, RNA and total protein synthesis. Studies of *control* of cell division have only been at the level of measuring cycle and phase times and arguing indirectly from conditions which affect the time intervals. To some extent these restrictions have been generated by the Howard and Pelc model of the cell cycle, and plant cell cycle studies will undoubtedly