The Effects of Counting Threshold and Emulsion Exposure Duration on the Percent-labeled Mitosis Curve and Their Implications for Cell Cycle Analysis

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SUMMARY

Percent-labeled mitosis curves were obtained at different counting thresholds and emulsion exposure durations in Sarcoma 180 cells grown in vitro and pulse labeled with tritiated thymidine. The shape of the percent-labeled mitosis curve was found to be dependent on counting threshold and on emulsion exposure duration. At low counting thresholds and prolonged emulsion exposure durations more mitoses were labeled than at higher thresholds and at shorter emulsion exposure times. The results could not be attributed to emulsion background noise.

The data suggest that the onset and termination of DNA synthesis do not occur abruptly in individual cells as they progress through the cycle; the rate of DNA synthesis and corresponding nuclear tritium uptake rise gradually to a maximum, especially in cells with long cell cycle times. When conditions are such that there is increased detection of low levels of nuclear tritium uptake, the apparent duration of S phase is increased.

It is suggested that the identification of distinctive patterns of relative labeling intensity as a function of age within the cycle may prove to be more useful than attempts to measure cell cycle phase durations in the kinetic characterization of growing cell populations.

INTRODUCTION

The kinetic behavior of mammalian cell populations is commonly studied by radioautographic methods using tritiated thymidine as a radiotracer for active DNA synthesis. Ordinarily in such studies, cells whose grain counts exceed some predetermined background counting threshold are considered to have been in a state of active DNA synthesis (S phase) at the time of exposure to tritiated thymidine, while cells whose grain counts fall below this threshold are considered to have been in a kinetic phase (G1, G2, or G0) that is totally devoid of DNA synthesis. Obviously, the validity of measurements of cell cycle phase durations by radioautographic methods hinges on the sharpness of the distinction between S phase and non-S phase, i.e., it depends on the sharpness of the distinction between labeled and unlabeled cells. It might be expected that if DNA synthesis and its detection were all-or-none phenomena, then radioautographic measurements would, for the most part, be independent of threshold levels or of factors that determine overall labeling intensity. Conventional interpretations of PLM curves, labeling indices, and related radioautographic data, based on either graphic methods (10, 18) or computer-implemented analyses (1, 15, 16), presuppose that this is indeed the case.

If, on the other hand, the onset and termination of DNA synthesis in individual cells progressing through the cycle is not abrupt, it might be expected that the observed durations of the cycle phases would be dependent on background threshold and on conditions which affect radioautographic labeling intensity.

In this paper, we wish to examine the effects of background threshold and emulsion exposure duration on the shape of the PLM curve and consider the implications of these effects for cell cycle analysis.

MATERIALS AND METHODS

All studies were carried out in Sarcoma 180 (Foley Strain CCRF11, supplied by American Type Culture Collection, Rockville, Md.) grown in vitro in Medium 199 (Flow Laboratories, Rockville, Md.) containing Hanks' basic salt solution, 5% fetal bovine serum, 2 mM glutamine per ml, 100 units penicillin per ml, and 100 μg streptomycin per ml. Cultures were grown at 37° in an atmosphere containing 5% CO2. Cells were grown in monolayer in 250-ml plastic flasks (Falcon Plastics, Oxnard, Calif.) containing 10 ml of medium, at an initial concentration of 1 x 10⁴ cells per ml. Medium was changed on Days 2 and 4, and cells were transplanted on Day 6 or 7.

Cell counts for growth curves included free-floating cells in the supernatant medium. For cell counting purposes, cells were classified as viable on the basis of trypan blue dye exclusion.

Radioautographic Studies. Two-day-old cultures were incubated with tritiated thymidine (specific activity, 2 Ci/mM; New England Nuclear Corp., Boston, Mass.) at a final concentration of 0.05 μCi/ml for 30 min, washed 4 times in fresh Hanks' medium containing 10⁻⁸ M cold thymidine, and resuspended in fresh medium containing 10⁻⁸ M

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1 The abbreviation used is: PLM, percent-labeled mitosis.
cold thymidine. Mitoses were collected by the method of Terasima and Tolmach (17). Cells from 3 flasks were pooled for each time point. Slides were prepared by a modification of the method of Hungerford (9). Cells were centrifuged at 300 x g for 10 min, incubated in 0.075 M KCl at 37° for 15 min, fixed and washed 3 times in cold methanol: acetic acid (3:1), spread on cleaned glass slides, flame dried, and stained with acetic orcein.

Radioautographs were prepared by dipping in Kodak NTB2 emulsion diluted with an equal part of distilled water. Slides were air dried, exposed to H2O2 vapor for 3 hr (2), sealed in light tight boxes containing Drierite, and stored in the dark at room temperature for 2 to 16 days. The slides were air dried, exposed to H2O2 vapor for 3 hr (2), sealed in light tight boxes containing Drierite, and stored in the dark at room temperature for 2 to 16 days. The radioautographs were developed in Kodak D19 developer at 15° for 4 min.

Slides for PLM curve studies were prepared at 2-hr intervals; 200 mitoses were counted at each time point.

The area of each counted cell was estimated with the aid of an eyepiece reticle. Background values were obtained over 3 adjacent areas of comparable size. Cell grain count values were corrected by subtraction of the average local background value for each cell.

RESULTS

Growth Characteristics. Generally, cell growth tends to exhibit considerable variability on the 1st day of transplantation (Day 0). Log phase growth ordinarily extends from Days 1 to 3. Population doubling time during mid-log phase is about 18 hr. Retardation of growth becomes apparent on Day 4, and stationary growth follows from Day 5 or 6 onward, when cell concentrations of 8 x 10⁵ to 1 x 10⁶ cells per ml are attained.

Growth curves show no observable toxic effects of thymidine in the replenishment medium at concentrations of 10⁻³ M.

Background Grain Counts. Background grain counts were low, with a mean of about 1 grain per cell and an S.D. of 1. Background grain count distributions were similar from slide to slide; no significant increase in background was discernible over a range of exposure times extending from 2 to 16 days (Chart 1).

The PLM Curve as a Function of Threshold and Emulsion Exposure Duration. The effects of threshold and emulsion exposure duration are shown in Chart 2.

In general, labeling intensity at each time point increases with increasing emulsion exposure duration. With the exception of the 2-hr point, time points at which mitoses appear unlabeled at short emulsion exposure times exhibit an increasing percentage of labeled mitotic cells with increasing emulsion exposure duration. It is readily apparent that there is no single PLM curve whose shape uniquely reflects the proliferative characteristics of the population; there is a large family of PLM curves whose shapes are affected strongly by counting threshold and emulsion exposure duration.

Maximum labeling intensity occurs early in the 1st labeled mitotic wave. The most heavily labeled cell class is poorly represented in the 2nd labeled mitotic wave, while the grain count class whose lower and upper limits are approximately one-half lower and upper limits, respectively, of the most heavily labeled class, is comparably well represented in the 2nd wave. This is attributable to the halving of nuclear tritium content at telophase. This relation appears to hold for more lightly labeled cell classes and between the 2nd and 3rd waves as well, but it is less clearly demonstrated. Cell tritium content halving effects are evident at all emulsion exposure times but are most clearly demonstrated in data obtained at longer exposure times.

The Labeling Index as a Function of Time. Tritiated thymidine pulse characteristics were studied in a separate experiment. Interphase cells were obtained by trypsinization at 0.5, 1, 2, 4, and 6 hr following exposure to tritiated thymidine. Labeling conditions and the washing procedure were identical to those used for mitotic collection, as described under “Materials and Methods,” except that the incubation in hypotonic KCl was omitted. Five hundred interphase cells were counted at each time point.

The thresholded labeling index as a function of time after 4 days of emulsion exposure with and without the addition of cold thymidine is shown in Chart 3. A and B, respectively. It is apparent that values for the labeling index are dependent on counting threshold as well as on the time of collection. The rise of the fraction of interphase cells with > 50 and > 100 grains per cell at 2 hr suggests that extensive washing and the cold thymidine chase did not reduce intracellular tritiated thymidine concentrations sufficiently to prevent significant continuing incorporation in cells with brisk DNA synthesis rates.

The fall in the lightly labeled fractions between 1 and 4 hr in the presence of cold thymidine (Chart 3A) can be attributed to dilution of the interphase population by daughter cells derived from lightly labeled and unlabeled late cycle (G2) cells. By 4 hr, nearly all dividing cells were heavily labeled, the interphase population was being enriched with labeled daughter cells, and the falling trend in the labeling index was reversed.

Graphs of mean grain count per cell as a function of time with and without the addition of cold thymidine are shown in Chart 3C. This parameter provides a better estimate of accrual of label to the population with time than the thresholded labeling index. It would appear that with the addition of cold thymidine effective pulse duration was about 2 hr, but because of the confounding effects of tritium content halving at mitosis, and the absence of data at 3 hr, it is possible that true pulse duration may have been somewhat longer.

DISCUSSION

Background threshold and emulsion exposure duration play an important role in determining the shape of the PLM curve. Mitoses that might have been unlabeled or very lightly labeled at short emulsion exposure durations appear progressively more heavily labeled as emulsion exposure duration is increased.
This effect cannot be attributed to an increase in background grain count with increasing emulsion exposure duration. A local background correction was applied to each mitotic cell represented in the PLM curves shown in Chart 2. Since mean background values were of the order of 1 grain per cell, with an S.D. of approximately 1, the magnitude of this correction was small. Furthermore, there was no significant increase in background grain count with increasing emulsion exposure duration.

The Reconstruction of Population Age Structure and DNA Synthesis Characteristics. Several studies have suggested that the rate of DNA synthesis is not constant but peaks in mid-S phase (11) or late S phase (5, 6, 13). The present study suggests that DNA synthesis rate varies with cycle age, with the highest rates of synthesis occurring about 4 hr prior to mitosis, as evidenced by the occurrence of maximum mitotic labeling intensity 4 hr following pulse exposure.

Our results can be explained on the basis of a reduction in the radioautographic threshold for detection of incorporated tritium when emulsion exposure duration is increased and/or when low background counting thresholds are chosen. With increased detection of low-level labeling in cells that were synthesizing DNA at low rates at the time of exposure, the apparent duration of S phase increases, as shown schematically in Chart 4. This would suggest that the apparent discreteness of the cell cycle phases proposed by Howard and Pelc (8) is due at least in part to threshold effects inherent in the radioautographic method and that the G1 and G2 periods should properly be treated as periods within the cycle during which DNA synthesis rates and corresponding tritium uptakes were too low for radioautographic detection.

As suggested by the thresholded labeling index data (Chart 3), the effective tritium pulse extended over a period of about 2 hr, despite extensive washing and the addition of a cold thymidine chase at 0.5 hr. Similar results were obtained in vitro by Cleaver and Holford (4) in mouse L-cells. There results indicate that extracellular concentrations of tritiated thymidine following pulse exposure may not accurately reflect intracellular pulse duration. This may have some bearing on the estimation of pulse duration in
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vivo, suggesting that rapid falloff of blood levels of tritiated thymidine may not reflect pulse characteristics at the tissue level. Staroscik et al. (14) have shown that the bulk of tritiated thymidine incorporation occurs in vivo within the 1st hr of tritiated thymidine administration. Chang and Looney (3) could not demonstrate continued tritiated thymidine incorporation beyond 1 hr in vivo by radioautographic methods, but with liquid scintillation counting techniques, they did show continued incorporation for up to 3 hr in their system.

In rapidly growing cell populations (mean cell cycle time: 8 to 12 hr) and in the presence of a pulse as long as 1 to 2 hr, it might be expected that tritiated thymidine uptake in cells with rising DNA synthesis rates will exceed that of cells with initially identical but falling DNA synthesis rates and that relative tritium uptake in cells with low and falling rates (late G2) will be particularly low. It is not surprising, then, that at the 2-hr time point in the PLM curve (Chart 2) there is no significant rise in labeling intensity with increasing emulsion exposure duration. This would suggest that the increase in height and labeling intensity of the 1st trough as emulsion exposure duration is increased is due in large part to increased detection of low uptakes early in long cycles (long G1), although the effects of desynchronization and growth retardation on the late portion (G2) of daughter cell cycles may also play a role.

Some of the more detailed effects of intracycle variation in DNA synthesis rate on the PLM curve at different thresholds and different exposures are summarized schematically in Chart 5. A distribution of subpopulations with different cell cycle times is presumed to have been present at the time of exposure to tritiated thymidine. Since the amount of DNA to be synthesized between divisions is constant, regardless of cell cycle time duration, the areas under the DNA synthesis rate curves are assumed to be constant; thus, the presence of prolonged low-level synthesis early in long cycles is associated with reductions in subsequent peak rates. Relative uptakes of tritiated thymidine are

![Chart 2](image)

Chart 2. The PLM curve as a function of counting threshold and emulsion exposure duration. For discussion, see text.

![Chart 3](image)

Chart 3. A, the labeling index as a function of counting threshold and as a function of time following the onset of tritiated thymidine pulse with $1 \times 10^{-4}$ M cold thymidine added to the washing medium and the final medium at 0.5 hr; B, same as A but without added cold thymidine; C, interphase cell labeling intensity, expressed as mean grains per cell as a function of time following the onset of tritiated thymidine exposure. For discussion, see text.
presumed to reflect DNA synthesis rate, with some differences due to nonideality of the pulse, as noted above. The differences in late cycle (G2) uptake between the parent and daughter cycles shown in Chart 5 are intended to represent the effects of desynchronization and, perhaps, retardation in the growth rate of some of the more rapidly proliferating cells.

We assume that the most heavily labeled mitotic cell class represents those cells with the shortest cell cycle times that were synthesizing DNA at peak rates at the time of pulse exposure. We estimate from the position and height of the daughter cell peak (Chart 2 and 5) that cells with cycle times of the order of 7 to 9 hr at the time of pulse exposure account for 5 to 15% of the population.

The failure of the trough to disappear completely at 10 hr, even after prolonged emulsion exposure, suggests that the 9- to 11-hr cell cycle time class is well represented in the cell cycle time distribution. Judging from the depth of the trough at low thresholds after 16 days of exposure (Chart 2), we would estimate that perhaps 20 to 40% of the population had cell cycle times in the range of 9 to 11 hr.

From the magnitude of the increase in lightly labeled cells between 12 and 16 hr at increased emulsion exposure duration, which we attribute largely to the uncovering of light labeling early in long cycles, and from the slurring of the 2nd wave with the occurrence of peak labeling at about 18 hr, we would estimate that some 40 to 60% of the population had cell cycle times in the range of 11 to 17 hr.

**Comparison with Conventional Methods of Analysis.** The population under study is relatively homogeneous in comparison with slowly growing in vivo populations in man, for example. And yet, even when data regarding threshold levels and radioautographic labeling intensity at different exposure levels are utilized in the analysis, the reconstruction of population age structure and DNA synthesis characteristics remains fairly crude. This is in sharp contrast with conventional methods of PLM curve analysis that purport to characterize cycle time and cycle phase duration distributions from single PLM curves. The difference lies primarily in the fact that when a gradual change in the rate of DNA synthesis in individual cells as they progress through the cycle is considered to be an important feature of cell growth behavior, the amount of kinetic information required for the analysis of population age structure is greatly increased.

Conventional methods of PLM curve analysis have bypassed this aspect of cell growth behavior and its attendant problems by simply treating the onset and termination of DNA synthesis in each cell as if they were abrupt events. The phases of the cycle could then be treated as sharply defined entities, whose durations could further be assumed to be independent of one another. This treatment implies that the shape of the PLM curve and the values of kinetic parameters derived from it are independent of threshold levels and radioautographic labeling intensity. In fact, conventional PLM curve analyses are ordinarily carried out without reference to such variables as background threshold, tritiated thymidine dosage and pulse characteristics, and emulsion exposure duration.

It is clear from the present study that there is no single PLM curve from which truly representative cycle time and phase durations and their distributions can be extracted. It might be expected that at higher counting thresholds, and/or at shorter emulsion exposure times, S phase durations computed by conventional methods would tend to be

Chart 4. Schematic representation of the dependence of the observable duration of S phase on tritiated thymidine detection threshold, when the onset and termination of DNA synthesis are not abrupt. At high detection thresholds (Threshold 1, as when emulsion exposure duration is short and/or background counting threshold is high), the apparent duration of S phase is shorter than at low detection thresholds (Threshold 2, as when emulsion exposure duration is prolonged and/or when counting threshold is low). The apparent durations of the G phases of the cycles are correspondingly longer at higher thresholds. \( T_s \), and \( T_{s-1} \), apparent S phase durations at Thresholds 1 and 2, respectively; \( T_c \), cell cycle time.

Chart 5. A schematic reconstruction of the patterns of DNA synthesis rate throughout the cycle for subpopulations with different cell cycle times (left) and their contribution to observable PLM curve behavior (right). Additional PLM curve data are found in Chart 2. Peak tritium uptakes occur in cells with short cell cycle times 4 hr prior to mitosis. The frequency of this subclass is low. Cell cycle time can be estimated to be 7 to 9 hr, judging from the position of the daughter cell peak. The cell subclass which contributes most to the preservation of the trough at long emulsion exposure times has cell cycle times in the 9- to 11-hr range. In cells whose cycle times range from 11 to 17 hr, tritium uptake is low early in the cycle. At long emulsion exposure times, the detection of this low level labeling is increased, and a larger proportion of mitoses emerging 12 to 16 hr following pulse appear labeled. For additional discussion, see text.
Threshold Effects on PLM Curves

We are aware of one published study (7) in which PLM curves obtained in vivo in a rat leukemia were analyzed by the automated method of Steel and Hanes (15) at several different counting thresholds. Emulsion exposure duration was long (80 days), and it is clear from reported peak mean mitotic grain count values of approximately 120 grains per cell that radioautographic labeling intensity was quite high. For the PLM curve obtained at the >4-grain threshold, “there was only a slight dip in the curve before a second rise, and the computer could give no reasonable estimate of cell cycle parameters” (7). The PLM curve obtained at a threshold of >19 grains per cell exhibited a deeper trough and a lower 2nd wave than that obtained at a threshold of >9 grains per cell; computed mean S phase duration was shorter at the higher threshold, and computed mean G1 and G2 durations were correspondingly longer.

Intensity of DNA Synthesis versus Duration. A computer model had been developed by one of us that not only accounts for variability in cell cycle time but also takes into account variations in tritiated thymidine uptake throughout the cycle and the statistics of radioautographic detection (Ref. 12; S. E. Shackney, manuscripts in preparation). Our experience with this model suggests that the variability in apparent cycle phase durations due to threshold effects and differences in radioautographic labeling intensity may be quite pronounced, especially in heterogeneous cell populations. Even if the variability in observed cycle phase durations were minimized by the adoption of a set of standard conditions for data collection and processing, the intensity of DNA synthesis, and quite possibly the effectiveness of cycle-stage-specific chemotherapeutic agents, could vary over a wide range throughout the observed duration of S phase. Thus, the identification of patterns of intensity of synthesis in relation to the cell cycle may ultimately prove to be more useful in describing population kinetics than attempts to characterize apparent phase durations. Our modeling studies have suggested that kinetic data contain differential features that should permit the assignment of 1 of 3 predominant general patterns of DNA synthesis to a given population. The 1st pattern, which we have called type I, is one in which slowly proliferating cells synthesize DNA at low rates throughout much of the cell cycle. In the type I pattern, DNA synthesis is restricted to the terminal portion of the cycle. This pattern can be described in conventional terms by long G1, G2, and constant S + G2 phases. A pattern of DNA synthesis intermediate between the type I and type II patterns can also be identified. The details of this approach to kinetic analysis are given elsewhere (S. E. Shackney, manuscripts in preparation). The advantages of this method are that it is especially well suited for the characterization of heterogeneous cell populations, and it is not seriously affected by moderate variations in radioautographic exposure.

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