LARC-6506

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SYNCHRONIZED MAMMALIAN CELLS. I. AN IMPROVED METHOD OF STUDY BY ELECTRONIC CELL COUNTING¹

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This paper reports some results of an improved method for the precise study of synchrony artificially induced in mammalian cell cultures. Limitations of previous methods (Xeros, 1962; Terasima and Tolmach, 1963; Bootsma et al., 1964; Puck, 1964; Marcus and Robbins, 1964) in terms of accuracy, available sample, time span covered, and experimental complexity are here avoided by the 1) use of suspension cultures rather than monolayers (Petersen and Anderson, 1964), and 2) use of electronic particle counting rather than microscopic determination of mitotic index. By virtue of their practically unlimited sample capacity, suspension cultures in addition to providing large enough samples for most

¹Work performed under the auspices of the U. S. Atomic Energy Commission.

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conventional analytical methods also permit frequent sampling over extended periods of time and can be adapted to automatic sampling systems, as well as to "chemostat" operation. The electronic particle counter (Kubitschek, 1958) is capable of an accuracy of a few tenths per cent because of the large numbers of cells which can be counted in times of less than a minute. Extended integral growth curves are obtained which can be differentiated to give growth rate curves equal or superior to those obtained by the more laborious mitotic index determinations. The methods used here have been in routine use for over a year and are in the process of being automated.

Methods

The method of induction of synchrony in suspension culture has been previously described (Petersen and Anderson, 1964). Briefly, Chinese hamster ovary (CHO) cells were grown in Puck's F-10 medium without calcium and containing 10% calf and 5% fetal calf serum and antibiotics. Deoxynucleosides (Calbiochem) used for blockade were dissolved in medium and sterilized by membrane filtration prior to addition to the cultures. Total cell concentrations were measured by electronic particle counting (Kubitschek, 1958), and for comparison mitotic indices were determined without colcemide block (Puck et al., 1964).

Results

Thymidine was found to be preferable to the other nucleosides as a blocking agent; no effect of concentration on quality of the block was noted over the concentration range 2 to 25 mM except for a suggestion of slight leakage during blockade with thymidine at the lower concentrations.

A comparison of particle count and mitotic index is shown in Fig. 1 for a typical experiment using a double block of 25 mM thymidine. The common logarithm of the total cell concentration is plotted against time in hours from the beginning of the experiment, and the magnitude of the statistical error ($1\sigma = 0.6\%$) is indicated. Integration of the mitotic index data for this plot involves an arbitrary constant related to the duration of the scored figure. The constant was chosen to normalize the two curves after second release (ca. 34 hours) and corresponded to 0.5 hour for the persistence of the recognizable mitotic pattern. An integral plot of this type is very convenient for monitoring a developing experiment in real time because of the ease of plotting and error estimation. Under conditions of good synchrony, the graph tends to be a succession of straight lines, more or less exponential growth alternating with zero growth between waves. Intersections of these lines provide reproducible fiducial marks. The electronic data were obtained in 50 seconds of counting time within a few minutes after sampling.

Fig. 1. Integral growth curve of wild type CHO cells. Synchrony induced by addition and removal of 25 mM thymidine. (O) Growth curve obtained by electronic cell counting; (Δ) normalized growth curve obtained by integration of mitotic index N_m (see text). Cells were diluted at the indicated intervals to maintain logarithmic growth at densities between 3×10^4 and 3×10^5 cells/ml.

Agreement between the two methods appears to be good, the principal discrepancy occurring about 42 hours following the first division wave after release from nucleoside blockade. At this time, the counting data indicate an essentially constant population, while the mitotic index data imply continued growth. Either a few per cent of erroneous positives among the scored cells or brief persistence of a few mitotic figures would account for this disagreement.

An important factor limiting future refinements of the method is the maximum attainable counting precision. The minimum possible error is set by counting statistics and has a value (1σ) equal to the square root of the total number of counts recorded (Evans, 1955). Thus, a coefficient of variation of 1% can be obtained from 10^4 and 0.3% from 10^5 cells in the counted sample volume (0.5 ml). However, considerable precision is lost in calculating division rates by differentiation of the primary data, so that 10^4 counts would give a precision of 10% on the average division rate over a period equal to 0.14 of the generation time (T_G). The number 10^5 counts would give the same precision over $0.06 T_G$, or alternatively, 5% over $0.12 T_G$. [One trades precision for time resolution, and optimum differentiation time is a function of the width of the synchronous wave.] These are minimum errors, and the experimental system must be analyzed to

determine how closely the actual total error can be made to approach the limiting value.

We have performed several such tests of the precision of our system. In one, the initial random growth (8 points) from the experiment of Fig. 1 was analyzed by a digital computer code which calculated the exponential function $[N = N_0 \exp(+\lambda t)]$, giving the best "least-squares" fit to the data in integral form. From the average total count of 24,000, a theoretical coefficient of variation of 0.65% was calculated. The experimental value given by the scatter about the computer curve was 0.87%, indicating a small additional error. In another test, using an automated sampling and counting system (Anderson, Van Dilla, and Petersen, to be published), samples were measured every 4 minutes over a 3-hour period of exponential growth, a 2-ml sample giving a total of 25,000 to 30,000 counts. These data were averaged in groups of 4 and division rates calculated for the resulting intervals of 16 minutes ($0.025 T_G$). The average rate was 0.062 ± 0.005 (σ_m) per hour. From the scatter of the individual rates about their mean, the coefficient of variation was found to be 23%, compared with the value of 25% derived from counting statistics. Over the somewhat limited duration of this experiment, it is concluded that this level of statistical precision can be reached by an automated system.

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