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SYNCHRONIZED MAMMALIAN CELLS IN SUSPENSION CULTURE*

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High yields of synchronized mammalian cells in suspension culture have been obtained by reversible interference with DNA synthesis through introduction and removal of high concentrations (1 to 25 mM) of thymidine, deoxycytidine, deoxyguanosine or deoxyadenosine at two specified intervals in a double blocking procedure [Puck, Science (1964), in press; and Bootsma et al., Exptl. Cell Res. 33, 301 (1964)] originally suggested to us by Puck. On the basis of preliminary life cycle analyses in suspension culture [Puck, Sanders, and Petersen, Biophys. J. (1964), in press], Chinese hamster ovary cells were held in nucleoside blockade for a period equivalent to $G_2 + M + G_1$, released for a period slightly greater than S, and reblocked long enough for all cells in the culture to accumulate behind the second S block. Upon release, the resultant synchronous wave comprising 95 per cent of the cells was followed for four successive generations of synchronous division. Quality of synchrony in the first generation is sufficient for investigation

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of molecular events occurring sequentially in any of the major metabolic intervals, and yields are sufficiently high ($\sim 2 \times 10^8$ cells) to perform chemical manipulations on subcellular fractions isolated in specified phases of the life cycle. Subsequent generations exhibited a pattern of decay in synchrony in good agreement with theoretical predictions [Engelberg, Exptl. Cell Res. 23, 218 (1964)] based on the distribution of generation times within a population. Selected clones from the wild type population showed the same decay despite their improved genetic homogeneity. Release from blockade also could be readily accomplished by adding the corresponding purine or pyrimidine deoxynucleoside, except in the case of deoxycytidine blockade which was not reversible by addition of thymidine.

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ABSTRACT

High yields of synchronized mammalian cells in suspension culture have been obtained by reversible interference with DNA synthesis through introduction and removal of high concentrations (1 to 25 mM) of thymidine or deoxycytidine at two specified intervals. Appropriate spacing of blockade and release resulted in segregation of the entire population into a single phase of the life cycle. Upon release from the second deoxynucleoside blockade, a synchronous wave of cells comprising up to 100 percent of the population underwent mitosis within a period of approximately 4 hours. This degree of synchrony is sufficient to permit detailed investigations of molecular events occurring within any of the major areas of the cell life cycle.

Investigation of detailed biochemical events comprising the life cycle of mammalian cells necessitates the use of synchronized cultures in which particular stages can be selected and clearly defined. Attempts to synchronize large numbers of mammalian cells have resulted in a variety of methods based on the reversible inhibition of DNA synthesis. Klenow (1) originally reported that elevated concentrations of deoxyadenosine effectively inhibited DNA synthesis in Ascites tumor cells and that inhibition was released by addition of deoxyguanosine. Terasima and Tolmach (2) utilized deoxyadenosine inhibition to improve resolution obtained in HeLa cell cultures synchronized by rinsing weakly attached mitotic cells from the surface of a monolayer. Partial synchrony of HeLa cultures has been obtained by Rueckert and Mueller (3) by induction of a thymidine deficiency with amethopterin and subsequent release by addition of thymidine. More recently, Mueller has reported inhibition of DNA synthesis by deoxyguanosine reversible by addition of adenine (4), and Xeros (5) has used a single thymidine block released either by washing or addition of deoxycytidine. Bootsma et al. (6) and Puck (7) have synchronized several cell lines

grown as monolayers by a double blocking technique using excess thymidine. The present study was undertaken to improve the degree of synchrony attained by reversible intervention in DNA biosynthesis, taking advantage of precise knowledge of the duration of the major metabolic intervals in the life cycle of a diploid Chinese hamster ovary cell line grown in high yield in suspension culture. Life cycle analysis of both the wild type culture and derived clones was performed by the method recently devised by Puck (8) and modified for use with suspension cultures (9) using the conventional designations M, G₁, S, and G₂ to define the major metabolic divisions of the life cycle. Average intervals for each of the major phases of the life cycle of the wild type culture are summarized in Table 1. Using these values, the duration of inhibition of DNA synthesis was accurately controlled and release was suitably timed to optimize the degree of synchrony.

For all experiments, cells were grown in Puck's F-10 medium containing 10 percent calf and 5 percent fetal calf serum. The deoxynucleosides were dissolved in F-10 medium, adjusted to pH 7.0, and sterilized by membrane filtration prior to addition to the cultures.

In preliminary experiments, various concentrations of thymidine and deoxycytidine were employed to determine their

effect on DNA synthesis and subsequent mitosis. Random cultures, judged by a constant mitotic index, were labeled with 0.1 $\mu\text{c}/\text{ml}$ of tritiated thymidine (Schwartz, 3 c/mM) and arrested in metaphase by addition of 0.06 $\mu\text{g}/\text{ml}$ of colcemide (Ciba). Ten minutes after introduction of the label, amounts of the nucleoside were added to give final concentrations ranging from 1 to 25 mM and the mitotic index determined (9) on serial aliquots as a function of time after blockade. Labeled mitotic figures were detected autoradiographically.

Typical results summarized in Fig. 1 demonstrate that 25 mM thymidine effectively interrupted synthesis of DNA and prevented the appearance of labeled mitotic figures. However, cells which had completed DNA synthesis (non-labeled mitotic figures) at the time of blockade accumulated in metaphase colcemide arrest at the normal rate, indicating insensitivity of G_2 cells to high deoxynucleoside concentrations. Cells held in blockade for 18 hours exhibited a biphasic pattern of accumulation upon release. No mitoses appeared for 3 hours, then accumulated at the normal rate for 4 hours, followed by an increase in mitotic index at approximately 7 hours. These intervals correspond to G_2 and S, respectively, and indicate accumulation of a large number of cells at a point immediately prior to DNA synthesis. Similar results were obtained using 20 mM deoxycytidine as

the blocking agent. Lower concentrations of deoxynucleoside were also effective in arresting DNA synthesis but allowed a few labeled mitotic figures to leak through. Blockade in either case could be reversed by removal of the cells from the high deoxynucleoside concentration and resuspension in normal medium.

The appearance of labeled mitotic figures within approximately 3 hours after release indicates that deoxynucleoside blockade arrests synthesis in all cells actively engaged in making DNA regardless of the degree of completion. The remainder of the population proceeds to a point just before initiation of DNA replication before blockade takes place. This also appears to be the case in HeLa cells blocked by deoxyguanosine or amethopterin (4). Thus, it is apparent that synchrony achieved by a single block can have a resolution no greater than the duration of S (i.e., ca. 30 percent of the generation time). Improved resolution can be attained by segregating the entire population in a pre-S condition by a double blocking technique (6,7) in which block and release times are based on an accurate preliminary determination of the duration of the major metabolic intervals of a particular cell line. A random culture is held in deoxynucleoside blockade for a period equivalent to G_2 plus M plus G_1 (ca. 9 hours), during which S cells remain stationary and the

remainder of the population proceeds to a pre-S condition. After release from the first block, the culture is allowed to incubate in normal medium for a period greater than S (ca. 4 to 5 hours) but less than ($T_G - S$), and a second block is introduced. During incubation in normal medium, cells in S complete the remainder of their DNA synthesis while the pre-S cells synthesize an entire complement. The incubation time, however, is not sufficient for daughters of any previous S cell to re-enter S before the block is re-applied. Cells are then held in the second block for 9 hours, the entire population accumulates in a pre-S condition, and upon release enters S and the subsequent mitotic interval as a single synchronous wave. Results of such an experiment using a double thymidine blockade (25 mM) are shown in Fig. 2. Integration of the area under the curves for each generation indicates that in excess of 95 percent of the population participates in each mitotic wave. Volume of the culture at the time of the first mitotic peak was 900 ml containing 2.2×10^5 cells/ml, yielding approximately 2×10^8 synchronized cells. No limitation on volume of cultures handled in this way has yet been observed, and significant quantities of cells have been provided for investigations involving chemical manipulation of subcellular fractions at specified times during the life cycle.

In Chinese hamster cells, times for blockade and release which ensure quantitative survival of the culture have been found to be moderately critical. Prolonged blockades decrease survival rate and have proved to be as inadequate for production of tight synchrony as have shortened blockade and release times.

Although these data indicate resolution sufficient to provide a useful system for investigation of molecular events occurring in major divisions of the life cycle, decay of the synchrony is evident. For a time we entertained the idea that decay of synchrony might be due to genetic heterogeneity of the population. Individual clones were derived from the wild type culture and characterized with respect to generation time, modal chromosome number, and duration of major metabolic intervals. The homogeneous cells were then synchronized according to the technique described. Despite the fact that consistent generation times for the individual clones ranged from 10.5 to 18 hours, the decay of synchrony in the homogeneous populations proceeded at the same rate as in wild type cells. In virtually all of the test systems successfully synchronized to date, none appears to remain synchronous for more than a few generations. It appears, therefore, that capacity to desynchronize is a fundamental property of growing cells. As suggested by Puck, such a mechanism could be considered an asset to survival. A

population of cells in tight synchrony is subject to annihilation by a single insult to which cells in a particular phase of the life cycle are susceptible but to which cells in other phases are relatively immune. Reasons for decay of synchrony have been discussed at some length by Mazia (10), and the rate at which desynchronization occurs has been considered by Engelberg (11). Our results are in good agreement with Engelberg's predictions and provide a useful test system for investigation of mechanisms preventing prolonged synchrony.

The biochemical mechanism of deoxynucleoside inhibition of DNA synthesis has been the subject of numerous investigations which have generally confirmed the original observation of Reichard et al. (12) that phosphorylated deoxynucleosides inhibited the reductive conversion of ribonucleoside diphosphates to the corresponding deoxynucleoside diphosphate.

In this connection, we have observed the accumulation of thymidine triphosphate and deoxycytidine triphosphate, as well as of lesser amounts of the di- and monophosphates in cultures inhibited by the corresponding deoxynucleoside.

These data are in agreement with results reported by Overgaard-Hansen and Klenow (13), Munch-Petersen (14), and Xeros (5).

Inhibition of DNA synthesis by thymidine was reversible by addition of an equimolar amount of deoxycytidine, but deoxycytidine inhibition was not readily reversible by addition

of thymidine. However, more recent studies by Mueller (4) suggest that deoxyguanosine inhibits DNA synthesis at concentrations which do not affect production of the other deoxynucleosides. It does not appear, therefore, that the mechanism of deoxynucleoside inhibition is entirely settled, and our current studies are designed to investigate further details of inhibition and release (15,16).

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Fig. 1. Effect of 25 mM thymidine on DNA synthesis and mitosis in Chinese hamster ovary cells. Normal accumulation of mitotic figures in colcemide metaphase arrest is shown as a function of time for total mitotic figures (closed squares) and labeled mitotic figures (open squares) in the absence of inhibitory concentrations of thymidine. Accumulation of total mitotic figures (closed circles) and labeled mitotic figures (open circles) is shown in colcemide metaphase arrest after addition of 25 mM thymidine. Total mitotic figures following release from a single 18-hour 25-mM thymidine blockade (closed diamonds) is shown [0.06 $\mu\text{g/ml}$ colcemide added at time of release ($T = 0$)].

Fig. 2. Four generations of synchronous growth in a suspension culture of Chinese hamster ovary cells following release from double thymidine blockade.

Table 1. Generation time and duration of major metabolic intervals in a wild type culture of Chinese hamster ovary cells in vitro

Metabolic Interval	Time (hours)
Total Generation Time	12
M	0.4 - 0.6
G ₁	4.5
S	4.0
G ₂	3.0

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