REGULATION OF INITIATION OF DNA SYNTHESIS IN CHINESE HAMSTER CELLS

I. Production of Stable, Reversible G_1-Arrested Populations in Suspension Culture

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ABSTRACT

Suspension cultures of Chinese hamster cells (line CHO) were grown to stationary phase (approximately 8–9 × 10^5 cells/ml) in F-10 medium. Cells remained viable (95%) for at least 80 hr in stationary phase, and essentially all of the cells were in G_1. Upon resuspension or dilution with fresh medium, the cells were induced to resume traverse of the life cycle in in synchrony, and the patterns of DNA synthesis and division were similar to those observed in cultures prepared by mitotic selection. Immediately after dilution, the rates of synthesis of RNA and protein increased threefold. This system provides a simple technique for production of large quantities of highly synchronized cells and may ultimately provide information on the biochemical mechanisms regulating cell-cycle traverse.

INTRODUCTION

It is generally assumed that, on the basis of proliferative capacity in vivo, there are three general classes of mammalian cells. One type, exemplified by the lining epithelium of the crypts of the small intestine, continuously traverses the life cycle and divides. Thus, preparations of this class always contain cells in all phases of the life cycle: in the pre-DNA synthetic G_1 period, the DNA synthetic S period, the premitotic G_2 period, and the mitotic or M period. Another class, which Baserga designates as "non-dividing cells" (1, 2), contains differentiated cells incapable of initiating DNA synthesis and division. Examples of this class are erythrocytes and polymorphonuclear leukocytes. The third class, under normal conditions, exists within the animal in a state of G_1 arrest (or G_0, according to Patt and Quastler [3]). When appropriately stimulated, these cells can be induced to synthesize DNA and to divide. Examples of this class are liver cells, which resume cell-cycle traverse following partial hepatectomy, and cells in mouse salivary glands stimulated to divide by injection with isoproterenol. In vivo systems of this nature have been exploited to great advantage to yield information on growth regulation in animal cells (see Gorski and Notides, 4; Baserga et al., 5; Cooper, 6; Lieberman, 7; Bucher et al., 8; and Allfrey, 9).

The principal disadvantage of in vivo systems is that multiple sampling of events in a single animal is difficult or impossible, necessitating the use of many animals in each investigation with the attendant risk of variation among individuals in the population. It would be advantageous to obtain a system which could be stimulated to undergo the transition from G_1 arrest to cell-cycle traverse in

vitro under conditions in which a large homogeneous pool of cells could be sampled repetitively for multiple analysis of biochemical properties. One system of this nature currently in use consists of lymphocytes cultivated in vitro and stimulated to divide by administration of phytohemagglutinin and other chemical agents (reviewed by Oppenheim, 10). This report describes a second in vitro system for production of cells in a state of G_1 arrest. Under appropriate conditions of growth in suspension culture, the entire population of Chinese hamster cells can be made to stop in the G_1 phase. On subsequent dilution with fresh medium, these cells synthesize DNA and divide synchronously.

MATERIALS AND METHODS

Chinese hamster cells (line CHO) were maintained free of PPLO in suspension culture in F-10 medium (11), supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. The line employed was hypodiploid with a modal chromosome number of 21.

Cell concentrations were determined by counting aliquots of cell suspension diluted with isotonic saline in the electronic cell counter described in detail elsewhere (12). The magnitude of the statistical error (σ) was 0.6%. Reproducibility of results indicated that a precision of better than 1% was obtained.

Radioautographs of cells labeled with ^{3}H compounds were prepared by the method of Puck and Steffan (13) except that the cells were stained after development. The resultant radioautographs had a very low background. Only those cells with five or more grains were considered to be labeled, and 500 cells were scored for each determination.

Volume spectra were determined with the spectrometer described in detail elsewhere (14). Under the conditions employed (14), a statistical precision of $\pm 2\%$ in a theoretical electronic volume resolution of approximately 3% at peak amplitude was obtained.

The distribution of DNA contents of cells in exponential and stationary phases was determined with the high-speed microfluorometer described elsewhere (15), utilizing auramine-O as the DNA stain. Synthesis of RNA and protein was determined by measuring the incorporation of uridine-^{3}H and leucine-^{3}H into trichloroacetic acid-precipitable material as described previously (16).

Thymidine-methyl-^{3}H (6 Ci/mmole), uridine-5-^{3}H (4 Ci/mmole), and L-leucine-4,5-^{3}H (6 Ci/mmole) were all purchased from Schwarz BioResearch, Orangeburg, New York. Uridine-6-^{3}H (10 Ci/mmole) was purchased from the New England Nuclear Corporation, Boston, Massachusetts.

RESULTS

Formation of G_1-Arrested Populations

When suspension cultures of CHO cells were set up in freshly prepared medium and stirred vigorously, the cells grew exponentially (doubling time 16 hr) from a concentration of 1–2 $\times$ 10^5 cells/ml to approximately 6.5 $\times$ 10^5 cells/ml. Over the ensuing 24–36 hr, the cell number increased by about 30–40% and remained constant thereafter at an approximate concentration of 8–9 $\times$ 10^5 cells/ml (Fig. 1). More than 95% of cells remained viable during the stationary phase for at least 80 hr (corresponding to t = 140 hr in Fig. 1), as indicated by their resistance to digestion by trypsin and exclusion of trypan blue. Furthermore, as will be shown in a later section, essentially all cells—even those in cultures in a prolonged stationary state—could be induced to divide upon appropriate dilution.

The volume spectra of cultures undergoing transition from exponential to stationary phase were determined, and the coefficient of variation of the stationary phase cells remained equivalent or increased slightly over that obtained in exponential populations (30%). In contrast, the coefficient of variation of newly born CHO cells prepared by mitotic selection was 15% (17). Therefore, even though cells are accumulating in G_1 from a biochemical standpoint (as will be shown in a later section), the distribution of cell volumes in stationary phase cultures exhibits a much greater degree of heterogeneity than that observed for G_1 populations prepared by mitotic selection, indicating that cell volume and biochemical "age" are very loosely coupled. In this regard, Fox and Pardee (18) have very recently demonstrated that cells of different sizes initiated DNA synthesis at similar times after mitosis in cultures of CHO cells prepared by mitotic selection.

So as to measure the fraction of cells synthesizing nucleic acids during the transition period, aliquots of cell suspension were pulse-labeled with thymidine-^{3}H or uridine-^{3}H, and the fraction of cells incorporating these isotopes into DNA and RNA, respectively, was determined radioautographically (Fig. 2). Nearly all cells continued to synthesize RNA throughout the entire period, but the fraction synthesizing DNA gradually decreased during the transition period until the population was depleted of cells in S upon entering the stationary phase. Thus, a redistribution of cells occurred during

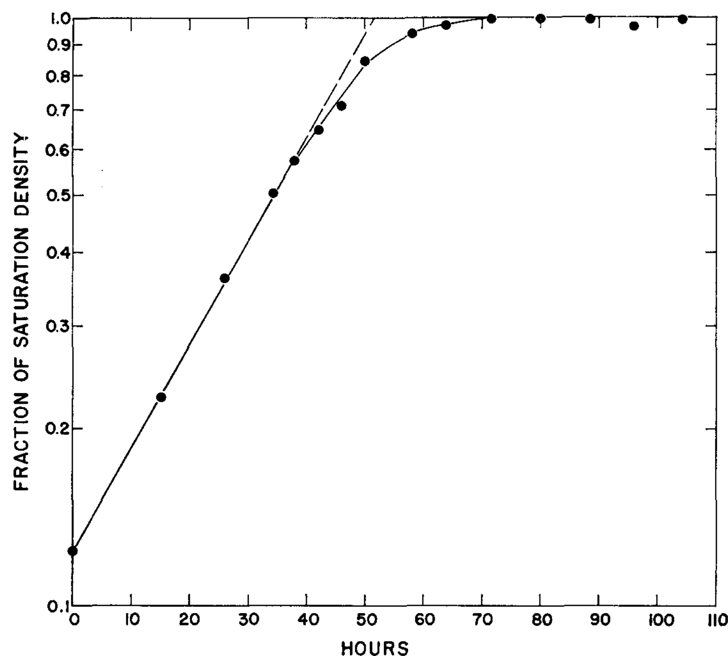

FIGURE 1 Pattern of growth of CHO cells in F-10 medium in suspension culture. The culture was set up in fresh medium and stirred vigorously throughout the experiment. The saturation density for this culture was 9×10^5 cells/ml. In repeated experiments in which suspension cultures were allowed to grow to stationary phase, the saturation density concentrations were usually in the range of 8–9 $\times 10^5$ cells/ml.

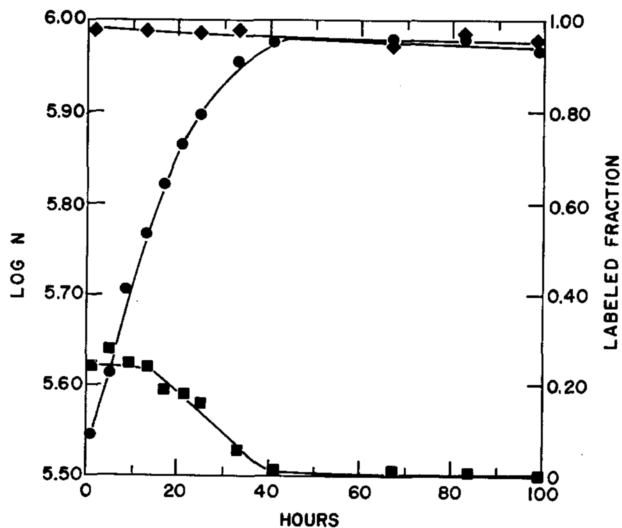

FIGURE 2 Fraction of cells incorporating thymidine-^3H into DNA or uridine-^3H into RNA in suspension cultures of CHO cells growing to stationary phase. So as to determine the labeled fractions, aliquots of 5 ml of cell suspension were added to 5 μCi of either thymidine-^3H or uridine-^3H, and were incubated for 15 min at 37°C in a shaker water bath. Samples were then fixed, and the labeled fractions were determined by radioautography: —●—●—, cell concentrations; —■—■—, fraction synthesizing DNA; —◆—◆—, fraction synthesizing RNA.

transition to stationary phase to the extent that no cells were found in the DNA synthetic phase of the cell cycle.

The rates of RNA and protein synthesis during the transition period were determined through incorporation rate measurements of uridine-^3H and leucine-^3H, respectively, as cells attained the stationary phase (Fig. 3). It is assumed that the rates of precursor incorporation accurately reflect the rate of synthesis of macromolecules during the

transition to high-density cultures, although the exact quantitative relationships between the processes have not been firmly established. That is, one cannot rule out alterations in transport mechanisms, pool sizes, or rates of equilibration and turnover which could occur during transition to stationary phase. Synthetic rates of RNA and protein were reduced in stationary-phase cultures to values approximately 30% of those found in exponentially growing cultures.

Resumption of Cell-Cycle Traverse

Cells maintained 48–80 hr in stationary phase were stimulated by either resuspension or dilution with fresh medium; in both types of experiment the fresh medium contained thymidine-^{3}H, and the label was present continuously at all stages thereafter. The fraction of cells synthesizing DNA (incorporating thymidine-^{3}H determined by radioautography) and cell number were determined at intervals thereafter (Fig. 4). It is readily apparent that synthesis of DNA precedes cell division, indicating that the stationary-phase cells were arrested in G_1. (Confirmatory evidence for arrest of stationary-phase cells in G_1 is provided by measurements of the distribution of DNA contents with a high-speed microfluorometer [15]; more than 95% of the stationary-phase cells possessed the DNA content expected for G_1 cells.) Since 4 hr elapsed after dilution before the first cells in Fig. 4 began to synthesize DNA, it is suggested that the G_1-arrested population is located at least 4 hr from the G_1/S boundary. The time of appearance and rate of increase in the fraction of DNA-synthesizing cells are very similar to the pattern obtained with cells synchronized by the mitotic selection technique (19, 20). It is known that the initial population prepared by the latter method consists of cells occupying 1% of the life cycle at the M/G_1 boundary (14). Further indication that the culture has been partially synchronized by the technique of growth to high density is provided by the pattern of cell division in Fig. 4 which, once again, resembles the second round of division observed in cultures prepared by mitotic selection (19, 20). Note also that approximately 90% of the stimulated cells in Fig. 4 synthesized DNA and divided. As expected from the microfluorometric and radioautographic data, there was no evidence for early-dividing cells or cells synthesizing DNA in the very early stages after dilution, confirming our conclusion that cultures maintained in stationary phase

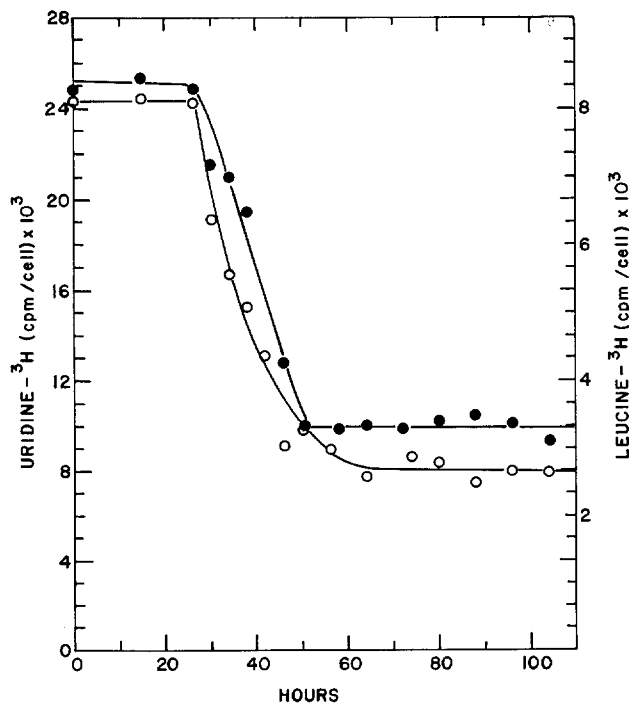

FIGURE 3 Rate of incorporation of uridine-^{3}H or leucine-^{3}H into suspension cultures of CHO cells growing to stationary phase. Aliquots of 5 ml of cell suspension were added to either 10 μCi of uridine-5-^{3}H or leucine-^{3}H, and were incubated for 15 min at 37°C in a shaker water bath. Samples were precipitated with trichloroacetic acid at 0°C by the method described elsewhere (13). The cell concentration data for this figure are those given in Fig. 2: —○—○—, rate of incorporation of uridine-^{3}H; —●—●—, rate of incorporation of leucine-^{3}H.

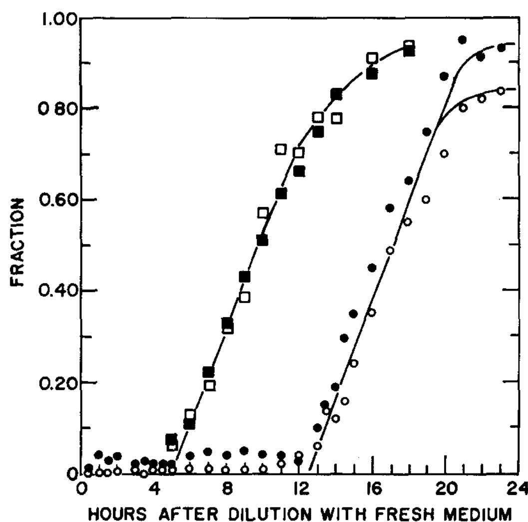

FIGURE 4 DNA synthesis and cell division in suspension cultures of CHO cells maintained in stationary phase for 48 hr, then resuspended or diluted with fresh medium at $t = 0$. Cells from the stationary-phase culture were resuspended (open figures) or diluted (solid figures) to a concentration of approximately 2×10^5 cells/ml in fresh medium containing 0.1 μCi/ml thymidine-^{3}H. At intervals thereafter, aliquots were removed, fixed, and prepared for radioautography: —□—□—, fraction of cells incorporating thymidine-^{3}H into DNA; —○—○—, cell concentrations.

for 24 hr or more were essentially devoid of cells in S, G_2, or M. However, when cultures maintained in stationary phase for less than 24 hr were resuspended in fresh medium, a small fraction (about 5%) of the population divided during the interval 4–7 hr after resuspension—well in advance of the synchronous population which commenced dividing at 12 hr. These early-dividing cells apparently represent a fraction of cells which completed synthesis of DNA but were not able to complete G_2 during the transition and stationary phases. After 24 hr in stationary phase, these cells cannot be stimulated to divide again by fresh medium. It is not yet known whether or not this fraction represents the 5% trypan blue–permeable fraction observed in cultures maintained in stationary phase for long periods.

In order to determine the patterns of macromolecular synthesis during the postdilution period, the rates of incorporation of uridine-^{3}H and leucine-^{3}H into RNA and protein were determined at intervals after dilution of a culture in stationary phase for 72 hr (Fig. 5). After seeding at a lower density in fresh medium, there was an immediate threefold increase in rate of synthesis of protein and RNA, with a constant rate thereafter. Synthetic rates of protein and RNA following dilution were approximately 85% of those observed in the exponential culture from which the stationary culture described in Fig. 5 was derived. These results indicate that the cells in stationary phase are capable of attaining a new biochemical state without delay.

DISCUSSION

The technique of growth to stationary phase and subsequent dilution of CHO cells provides an easy and inexpensive means for providing large quantities of highly synchronized cells. Whether or not this technique is generally applicable to other cell lines remains to be seen. At least one cell line differs in this response to growth to high density in suspension culture. Ward and Plagemann (21), in studies with Novikoff rat hepatoma cells, noted that the rates of synthesis of DNA, RNA, and protein in stationary phase were less than 10% of the corresponding rates during exponential growth and that the viable cell fraction dropped precipitously within 20 hr in stationary phase. Furthermore, upon dilution of stationary-phase cultures, the pattern of cell division was that of the exponentially growing population. Thus, the Novikoff hepatoma cells were not synchronized by this technique, were less viable, and were inhibited to a greater extent in synthesis of macromolecules during stationary phase than were CHO cells. We have no explanation for these differences at the present time, since we do not know the mechanism by which CHO cells accumulate in G_1 during transition to stationary phase. Preliminary experiments have suggested that cell redistribution results from a depletion of medium components rather than from serum factors or diffusible toxic substances. Studies into the nature of the depletion are continuing.

If the incorporation rates of uridine-^{3}H and leucine-^{3}H do accurately reflect the rates of synthesis of RNA and protein, there are several puzzling aspects of the data in synthetic rates following dilution with fresh medium of a stationary-phase culture. Synthetic rates do not return to the level of synthesis in the exponential culture, although the cells can divide at least three times (unpublished data) and presumably many more times if the diluted stationary-phase cultures are then

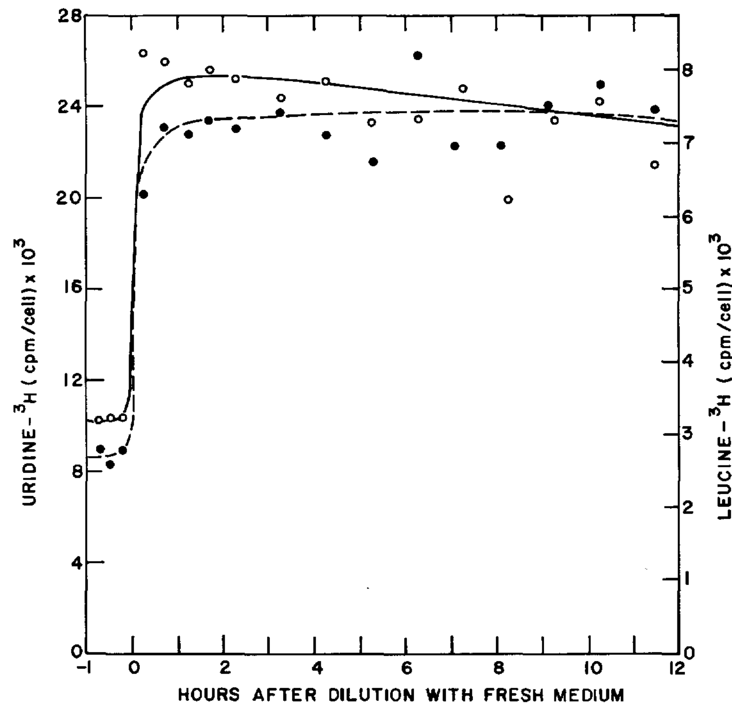

FIGURE 5 Rate of incorporation of uridine-^{3}H or leucine-^{3}H into a suspension culture of CHO cells prepared by diluting stationary-phase cells into fresh medium at $t = 0$. At intervals thereafter, aliquots of 5 ml of cell suspension were added to 10 μCi of either uridine-6-^{3}H or leucine-^{3}H and were incubated at 37°C for 15 min in a shaker water bath. Samples were then precipitated with cold trichloroacetic acid by the method described elsewhere (16): —○—○—, rate of incorporation of uridine-^{3}H; —●—●—, rate of incorporation of leucine-^{3}H.

maintained as standard suspension cultures with frequent dilution. Unlike CHO cell cultures prepared by mitotic selection in which the rates of synthesis of RNA and protein show a twofold or a greater increase across interphase (20), the rates of macromolecule synthesis in diluted stationary-phase cultures of CHO cells remain constant throughout interphase. This observation perhaps suggests that either part of the biochemical operations required for cell-cycle traverse have been completed during stationary phase or that the metabolic and catabolic capabilities of recovering stationary-phase cells are very different from those of the exponential-phase cell. Regarding this latter point, Warren and Glick (22) have noted that the rate of turnover of surface membrane and cell particulate material is much greater in nondividing than in dividing cultures of mouse L cells. Similar studies with the CHO cell are required before the biochemical significance of the incorporation data is known. It is strongly suggested that the period immediately following dilution of stationary-phase cultures is a transitional one of unbalanced growth, whereas both the exponential and stationary phases may represent two different states of biochemical balance. Perhaps the pre- and postdilution synthetic patterns represent biochemical operations predominantly concerned with maintenance of G_1 and cell-cycle traverse, respectively. Transition periods between the two balanced states may be extremely interesting from a biochemical standpoint.

Transition from a non-DNA synthetic state to one of active synthesis and immediate increase in synthetic rates of protein and RNA upon dilution of stationary-phase suspension cultures of CHO cells are at least superficially similar to the results obtained when cells in G_1 arrest (or G_0) in vivo are stimulated to initiate DNA synthesis and to divide. Extensive studies will be required before the degree of analogy between in vitro and in vivo systems can be established. In any event, reversal of the stationary phase in suspension cultures of CHO cells may ultimately provide information on the biochemical mechanisms which regulate initiation of DNA synthesis and cell-cycle traverse.

The authors wish to thank Drs. M. A. Van Dilla and P. F. Mullaney and Mr. T. T. Trujillo for providing the volume and microfluorometric determinations and also Drs. E. C. Anderson and D. F. Petersen for helpful comments during preparation of the manuscript. The excellent technical assistance of Mrs. Evelyn Campbell, Mrs. Phyllis Sanders, and Mrs. Susan Carpenter is gratefully acknolwedged.

K. D. Ley is the recipient of a National Institutes of Health Postdoctoral Fellowship (1-F02-CA43809-01) from the National Cancer Institute.

This work was performed under the auspices of the United States Atomic Energy Commission.

Received for publication 23 December 1969, and in revised form 28 January 1970.

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