REGULATION OF INITIATION OF DNA SYNTHESIS IN CHINESE HAMSTER CELLS

II. Induction of DNA Synthesis and Cell Division by Isoleucine and Glutamine in G_1-Arrested Cells in Suspension Culture

K. D. LEY and R. A. TOBEY

From the Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544

ABSTRACT

Suspension cultures of Chinese hamster cells (line CHO), which had stopped dividing and were arrested in G_1 following growth to high cell concentrations in F-10 medium, could be induced to reinitiate DNA synthesis and to divide in synchrony upon addition of the appropriate amounts of isoleucine and glutamine. Both amino acids were required to initiate resumption of cell-cycle traverse. Deficiencies in other amino acids contained in F-10 medium did not result in accumulation of cells in G_1, indicating a specific action produced by limiting quantities of isoleucine and glutamine. In the presence of sufficient glutamine, approximately 2×10^{-6} M isoleucine was required for all cells to initiate DNA synthesis in a population initially containing 1.5×10^5 cells/ml. Under similar conditions, about 4×10^{-6} M isoleucine was required for all G_1-arrested cells to progress through cell division. In contrast, 1×10^{-4} M glutamine was necessary for maximum initiation of DNA synthesis in G_1 cells, along with sufficient isoleucine. A technique for rapid production of G_1-arrested cells is described in which cells from an exponentially growing population placed in F-10 medium deficient in both isoleucine and glutamine or isoleucine alone accumulated in G_1 after 30 hr.

INTRODUCTION

An in vitro method for producing large quantities of cells arrested in the G_1 portion of the cell cycle was established in this Laboratory and has been previously described (1). By this method, suspension cultures of Chinese hamster cells (line CHO) are grown to stationary phase in F-10 medium, and, upon subsequent addition of fresh medium, cells resume traverse of their life cycle in synchrony. Experimental evidence that stationary-phase cells are arrested in G_1 was provided by (*a*) radioautographic studies which showed that these cells did not incorporate tritiated thymidine into DNA, (*b*) microfluorometric measurements which verified that the arrested cells contained the G_1 complement of DNA, and (*c*) data which indicated that induction of cell division in these cells was *always* preceded by synthesis of DNA. Because all cells in stationary phase are arrested in G_1 and the entire population can be stimulated to divide merely by altering the nutritional state, this system provides a means for studying factors inducing synthesis of DNA in mammalian cells. The present

paper reports that isoleucine and glutamine are the medium components responsible for inducing reinitiation of DNA synthesis and cell division.

MATERIALS AND METHODS

Cells

Chinese hamster cell line CHO (2) was cultured in suspension as a monodispersed population in spinner flasks, and cell concentrations were determined with an electronic cell counter as described in a previous paper (1). No pleuropneumonia-like organisms (PPLO) were detected in cells routinely tested for contamination by the method of Chanock et al. (3).

The fraction of cells incorporating thymidine-methyl-^{3}H (6 Ci/mmole, Schwarz BioResearch Inc., Orangeburg, N. Y.) was measured by iadioautography and scored by the procedures described elsewhere (1).

Media

CHO cells were propagated in F-10 medium supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. The original F-10 formula (4) has been modified in this laboratory by omitting stock solutions D ($FeSO_4$, $CuSO_4$, and $ZnSO_4$) and E ($CaCl_2$) and sodium pyruvate. The F-10 medium prepared without isoleucine and glutamine contained 10% calf and 5% fetal calf sera which were dialyzed against Earle's balanced salt solution at a 1:10 volume for 6 days at 3°C with changes of salt solution every other day. Stock solutions of 100 × L-isoleucine and L-glutamine (A grade, Calbiochem, Los Angeles, Calif.) were prepared by dissolving isoleucine (0.26 g/liter) and glutamine (14.62 g/liter) in triple-distilled water.

RESULTS

Initiation of Cell Division by Isoleucine and Glutamine

Before attempting to elucidate the factors in fresh complete medium that are responsible for resumption of cell-cycle traverse in G_1-arrested cells (1), it was necessary first to determine whether these factors were present in the serum or in the F-10 medium. Medium from stationary-phase cultures which would no longer support culture growth is referred to as "spent" medium. The influence of fresh F-10 medium or fresh serum on synchronous cell division was determined by resuspending G_1-arrested cells in either (*a*) F-10 medium containing 15% fresh serum, (*b*) 60% serum-free F-10 plus 40% spent medium, (*c*) spent medium plus 15% fresh serum, or (*d*) spent medium with 15% dialyzed serum. As seen from the data presented in Fig. 1 *A*, cells in F-10 plus fresh serum began dividing at 12 hr, and synchrony in cell division was evident from the doubling time of 8 hr *versus* about 17 hr during exponential growth. The time of appearance and rate of increase for dividing cells are very similar to those observed for the second round of division in cultures prepared by mitotic selection (5, 6). Cells resuspended in 60% serum-free F-10 also began dividing in synchrony at 12 hr (Fig. 1 *B*). In spent medium plus fresh serum, cells began dividing at 12 hr but grew much more slowly than random cultures (Fig. 1 *C*). Moreover, serum that was dialyzed as described in the Materials and Methods section did not stimulate the cells to divide in spent medium (Fig. 1 *D*). These results indicated that a component of F-10 medium was responsible for synchronous recycling of G_1-arrested cells independent of addition of fresh serum.

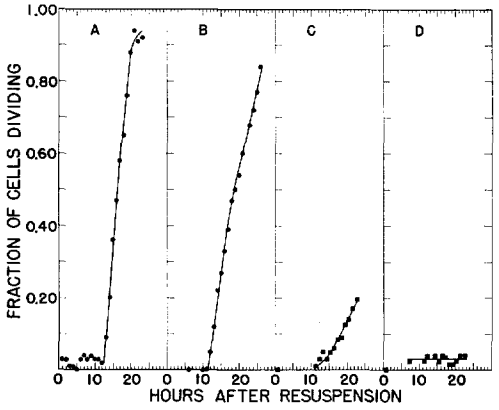

FIGURE 1 Patterns of cell division after resuspension of stationary-phase cells at approximately 1.5×10^5 cells/ml at t = 0 in (*A*) F-10 medium plus 15% calf serum, (*B*) 40% spent medium plus 60% serum-free F-10, (*C*) spent medium plus 15% fresh serum, and (*D*) spent medium plus 15% dialyzed serum.

The factors in F-10 medium that induced cell division in the G_1-arrested cells were determined by seeding stationary-phase cells in spent medium and adding serum-free F-10 media from which various specific ingredients had been omitted. By this process, it was found that cells placed in fresh F-10 medium lacking isoleucine or glutamine were unable to initiate cell division. Confirmatory evidence that medium from stationary-phase cultures was deficient in one of these two amino acids came

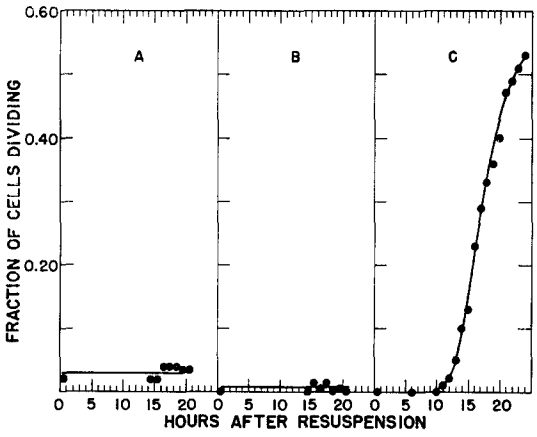

FIGURE 2 Patterns of cell division after resuspension of stationary-phase cells at approximately 1.5×10^5 cells/ml at t = 0 in (*A*) spent medium plus 1×10^{-4} M glutamine, (*B*) spent medium plus 2×10^{-5} M isoleucine, and (*C*) spent medium plus both isoleucine and glutamine at the above concentrations.

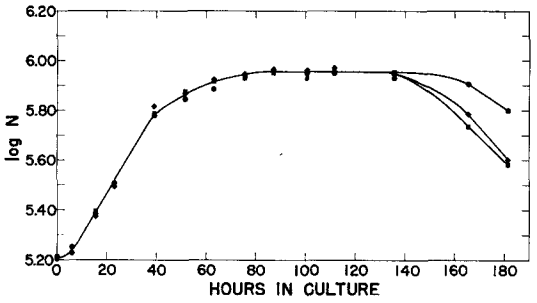

FIGURE 3 Patterns of cell division following resuspension of cells from an exponentially growing culture in medium containing two to four times the concentration of isoleucine and glutamine normally present in F-10 medium: (●) 2 × isoleucine and glutamine, (◆) 3 × isoleucine and glutamine, and (■) 4 × isoleucine and glutamine.

from amino acid analyses which revealed that the spent medium was completely devoid of isoleucine. Additional proof that isoleucine and glutamine were the sole nutrients required for resumption of cell-cycle traverse was provided when addition of isoleucine and glutamine to cells in spent medium brought about synchronous cell division without addition of fresh serum. Also note in Fig. 2 that neither isoleucine nor glutamine alone stimulated cell division. Since addition of concentrated stock solutions (100 ×) to reestablish normal concentrations of isoleucine and glutamine (2×10^{-5} M and 1×10^{-3} M, respectively) produced negligible dilution (~2%) of the spent medium, it is also apparent that cessation of cell division in stationary-phase cultures is not the result of accumulated inhibitory substances in the medium.

Effects of Increased Levels of Isoleucine and Glutamine on Stationary-Phase Cultures

On the basis of experimental evidence that isoleucine and glutamine become limiting to CHO cells, the effects of added isoleucine and glutamine on production and stability of stationary-phase cells were measured. As seen from Fig. 3, addition of two to four times the normal concentrations of isoleucine and glutamine to F-10 medium during growth to stationary phase did not increase the growth rate of these cultures. However, these cultures reached cell concentrations in stationary phase that were usually 15–20% higher than the cell concentrations in cultures in regular F-10 medium (1). The rapidity with which these cells began to die after 60 hr in stationary phase was directly proportional to the amount of isoleucine and glutamine added. The decrease in cell number (t = 181 hr in Fig. 3) was accompanied by an increase in percentage of cells permeable to trypan blue (57%, 79%, and 89% for 2 ×, 3 ×, and 4 × amino acids, respectively). In contrast, cells grown in F-10 medium containing the normal amount of isoleucine and glutamine remained viable in stationary phase for periods in excess of 80 hr (1). When cultures grown in elevated concentrations of isoleucine and glutamine were resuspended in fresh medium before the cells began to die, cell division patterns were highly variable, but highly synchronous cell division was not observed. These results suggested that quantities of isoleucine and glutamine in excess of the standard F-10 formulation allow the cells to grow to higher concentrations but that, in the process, other nutrients may become limiting and cells die from starvation.

Rapid Production of G_1-Arrested Cells in Medium Deficient in Isoleucine and Glutamine

Since growth of CHO cells to high cell densities leads to establishment of stationary-phase cultures and concurrently to a reduction in amount of available isoleucine and glutamine after 80 or more hr, the ability of F-10 medium without isoleucine

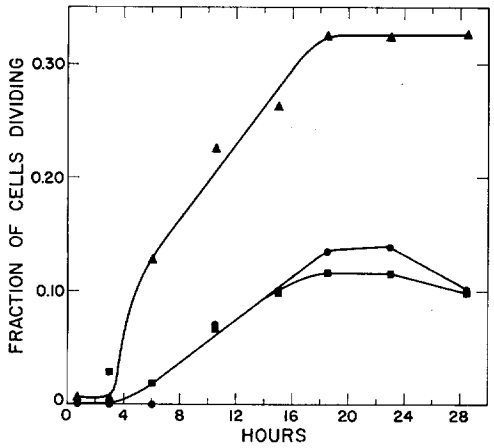

FIGURE 4 Patterns of cell division in exponentially growing CHO cells after resuspension at t = 0 in F-10 medium containing dialyzed serum and deficient in (●) isoleucine and glutamine, (■) glutamine, or (▲) isoleucine. Initial cell concentrations were approximately 1.5×10^5 cells/ml.

and glutamine or without isoleucine alone to allow accumulation of cells in G_1 was tested. Dialyzed calf serum was used for these cultures because the complete serum contained sufficient isoleucine and glutamine to allow the cells to divide several times. The patterns of division for cells from an exponential culture after seeding into (*a*) F-10 without isoleucine and glutamine, (*b*) F-10 without glutamine, and (*c*) F-10 lacking isoleucine are shown in Fig. 4. About 12–14% of cells in cultures deficient in both isoleucine and glutamine or glutamine alone had divided at 18.5 hr. Cells which were supplied with glutamine but no isoleucine reached stationary phase after approximately 33% of the cells had divided. These cells remained viable for at least 5 days and could be induced to divide in synchrony. Cells arrested in these deficient media could be induced to divide in synchrony as early as 30 hr after seeding in the deficient medium either by resuspension in complete F-10 or by addition of isoleucine and glutamine (Fig. 5). Furthermore, incorporation of tritiated thymidine into DNA preceded cell division (Fig. 5), as was the case in G_1-arrested cells produced by growth to stationary phase (1).

To ensure that the lack of sufficient isoleucine and glutamine resulted in specific biochemical effects on initiation of DNA synthesis and cell division rather than simple amino acid starvation, CHO cells were placed in F-10 medium which was singly deficient in other amino acids (valine, leucine, arginine, histidine, or methionine). The

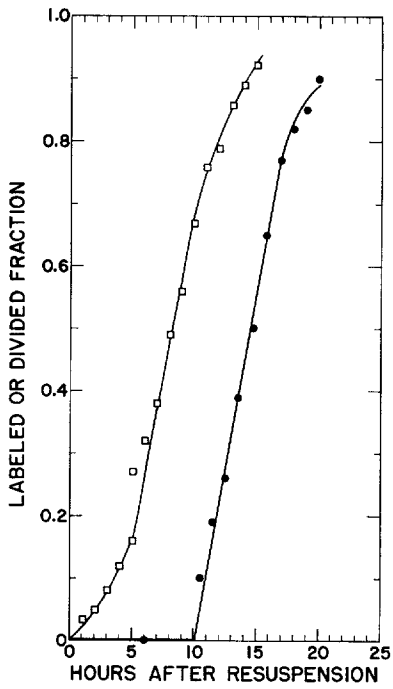

FIGURE 5 Patterns of DNA synthesis and cell division obtained by addition of isoleucine and glutamine (at t = 0) to a culture of cells previously maintained for 30 hr in F-10 medium containing dialyzed serum and deficient in isoleucine and glutamine. Initial cell concentration was approximately 1.5×10^5 cells/ml, and the fraction of cells dividing was determined by the increase in cell number. Tritiated thymidine (0.05 μCi/ml) was added at t = 0, and at intervals thereafter portions of cells were removed, fixed, and prepared for radioautography: (□—□) fraction of cells incorporating thymidine-^{3}H into DNA, and (●—●) fraction of cells dividing.

doubling time increased to about 56 hr immediately after resuspension in the various deficient media, and this slow rate of growth continued until, by 90 hr, cell division had ceased in all cultures. Upon resuspension in fresh complete medium at this time, cultures began dividing after a lag period of 7 hr or less, and the rate of cell division was the same as that obtained in nonsynchronized cultures.

Relative Amounts of Isoleucine and Glutamine Required to Reinitiate Cell-Cycle Traverse

To obtain an estimate of the amount of isoleucine or glutamine necessary for initiation of DNA

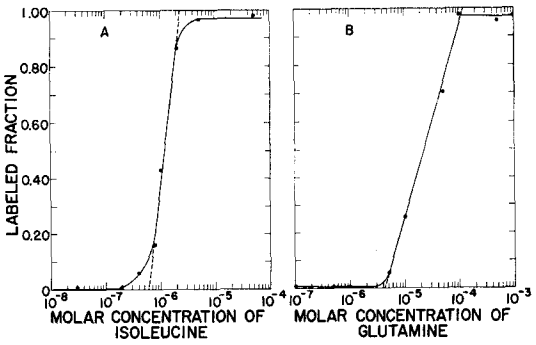

FIGURE 6 Fraction of cells incorporating thymidine-^{3}H into DNA as a function of varying concentrations of (*A*) isoleucine or (*B*) glutamine. Cells in G_1 prepared by mitotic selection were resuspended at t = 0 (about 1.5×10^5 cells/ml) in F-10 medium containing dialyzed serum and either twice the usual concentration of glutamine and different amounts of isoleucine (*A*) or twice the usual concentration of isoleucine and different amounts of glutamine (*B*). Tritiated thymidine was added to the cultures at t = 0 to a final concentration of 0.05 μCi/ml, and samples were collected at t = 16 hr. Samples were fixed, and the labeled fraction was determined by radioautography (1). Corrections were made for increases in cell number during the 16 hr labeling period where necessary.

synthesis, cells in G_1 prepared by the mitotic selection technique [i.e., collected at the M/G_1 boundary without use of mitotic inhibitors and, therefore, minimally perturbed biochemically (5, 6)] were placed in F-10 medium supplemented with dialyzed calf serum but singly deficient in either isoleucine or glutamine. Then different quantities of isoleucine were added back to the cultures deficient in isoleucine. Similarly, cultures were set up in F-10 medium containing varying quantities of glutamine. Tritiated thymidine was added to all cultures, and the fraction of cells labeled after 16 hr was determined radioautographically. The fraction of cells incorporating thymidine-^{3}H is plotted in Fig. 6 as a function of concentration of either isoleucine (*A*) or glutamine (*B*) added to the medium. Under these conditions, approximately 2×10^{-6} M isoleucine was required to stimulate DNA synthesis in all cells. It was possible to decrease the fraction of cells initiating DNA synthesis from essentially 100 to 0% merely by decreasing the concentration of isoleucine 4-fold (i.e., from 2×10^{-6} M to 6×10^{-7} M). The concentration of glutamine required to allow DNA synthesis in all cells was about 4×10^{-5} M. The fraction of cells synthesizing DNA increased from 0 to 100% by increasing the glutamine concentration over a 25-fold range of 4×10^{-6} M to 1×10^{-4} M. A similar experiment was performed to determine the amount of isoleucine required for cell division. Cultures of stationary-phase cells were resuspended in portions of F-10 medium supplemented with dialyzed serum and different quantities of isoleucine. In Fig. 7 is shown the fraction of cells dividing within 28 hr after resuspension as a function of isoleucine concentration. The isoleucine requirement for dividing cells corresponded to a concentration of about 4×10^{-6} M isoleucine, which compares well with the 2×10^{-6} M isoleucine required to initiate DNA synthesis in the same number of cells (Fig. 6). At lower concentrations, the dividing fraction was reduced and the rate of division was also decreased.

DISCUSSION

Evidence that isoleucine and glutamine are not only essential for cell growth but also can be

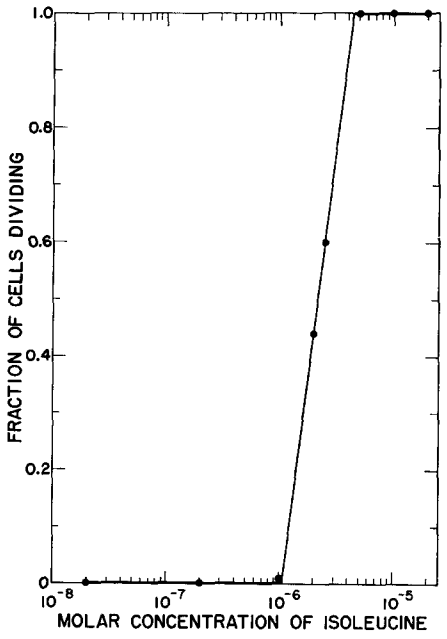

FIGURE 7 Fraction of cells dividing as a function of isoleucine concentration. Cells from a stationary-phase culture were resuspended in aliquots of F-10 medium containing dialyzed serum and different quantities of isoleucine. Initial cell concentration was about 1.5×10^5 cells/ml. Cell number was determined at t = 28 hr, at which time no further increases in cell number were observed for cultures given limited amounts of isoleucine.

growth-limiting has been reported for other types of cells. Griffiths and Pirt (7) found that, if suspension cultures of mouse cell strain LS were grown in medium containing excess glutamine, isoleucine and leucine then became the growth-limiting nutrients. Mohberg and Johnson (8) showed that isoleucine, glutamine, and leucine were the only amino acids depleted from the medium after growth of mouse L-cells had ceased in monolayer cultures. It is not known if refeeding of these amino acids would have stimulated synchronous cell division in the monolayers. Littlefield (9) has reported partial synchronization of L-cells after diluting stationary-phase suspension cultures with fresh medium. His evidence for partial synchronization was based primarily on the rate of DNA synthesis per volume cell suspension and not on serial cell counts. The medium components responsible for these observations were not determined. Further experiments with different types of cells will be required to establish whether or not G_1-arrest arising from deficiencies in available isoleucine and glutamine is a general phenomenon. Failure of other cell lines to be synchronized by growth to stationary phase may reflect imbalances in nutrients or contamination with PPLO.

It is evident from the results presented here that isoleucine and glutamine are required to induce cell division in high-density cultures that have been maintained in stationary phase or in cells that have been purposely deprived of isoleucine and glutamine. Evidence that stationary-phase cells are trapped in the G_1 portion of the cell cycle has been provided by studies of tritiated thymidine incorporation by radioautography and DNA content by microfluorometry (1). Evidence that synchronized cells produced when isoleucine and glutamine are deleted from the medium (Fig. 4) are also in a state of G_1-arrest is consistent with the experimental results obtained for cells in stationary-phase cultures [i.e., initiation of DNA synthesis, as measured by tritiated-thymidine incorporation, preceded cell division (Fig. 5)]. It follows hen that addition of isoleucine and glutamine fulfilled some specific requirement(s) that allowed the G_1-arrested cells to initiate DNA synthesis before cell division could occur. The 33% increase in cell number observed after exponentially growing cells were resuspended in medium deficient in isoleucine (Fig. 4) corresponds to the number of cells in S, G_2, and M at time of resuspension. The fraction of CHO cells in these portions of the cell cycle in asynchronous cultures with a doubling time of 17 hr may be calculated if one assumes that the cells are exponentially distributed throughout the cell cycle and that increases in culture doubling time arise from increases in duration of G_1 (10). This value is about 35% and compares well with the 33% increase measured. When cells from an exponentially growing culture were placed in F-10 medium deficient in glutamine or glutamine and isoleucine, the resultant increase in cell number was only 12–14% (Fig. 4)—the approximate number of cells in G_2 and M. These results suggest that, in the absence of isoleucine, cells in S, G_2, and M continue traverse of the cell cycle and accumulate in G_1, whereas in glutamine-deficient medium most cells synthesizing DNA at the time of transfer to deficient medium never divide. A specific requirement for glutamine during S would be consistent with the known role of glutamine in the *de novo* synthesis of purines and pyrimidines. Further studies will be required to determine whether these nondividing cells die or remain in S or G_2 for long periods of time in glutamine-deficient medium. The mechanism by which isoleucine and glutamine induce DNA synthesis in cells arrested in G_1 is not known. Whether or not isoleucine plays a role in regulation of cell proliferation in vivo is also not clear at this time. Research at this Laboratory is currently directed toward attempting to answer these fundamental questions.

The authors wish to acknowledge the excellent technical assistance given by Mrs. Evelyn Campbell, Mrs. Phyllis Sanders, and Mrs. Susan Carpenter. We also thank Dr. George Shepherd and Mrs. Carol Roberts for the amino acid analyses and Dr. D. F. Petersen for his helpful comments during preparation of the manuscript.

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

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

Received for publication 1 April 1970, and in revised form 19 May 1970.

REFERENCES

1. Tobey, R. A., and K. D. Ley. 1970. Regulation of initiation of DNA synthesis in Chinese hamster cells. I. Production of stable, reversible G_1-arrested populations in suspension culture. *J. Cell Biol.* **46**:151.
2. Tjio, J. H., and T. T. Puck. 1958. Genetics of

somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. *J. Exp. Med.* **108**:259.

3. CHANOCK, R. M., L. HAYFLICK, and M. F. BARILE. 1962. Growth on artificial medium of an agent associated with atypical pneumoniae and its identification as a PPLO. *Proc. Nat. Acad. Sci. U. S. A.* **48**:41.
4. HAM, R. G. 1963. An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp. Cell Res.* **29**:515.
5. TOBEY, R. A., E. C. ANDERSON, and D. F. PETERSEN. 1967. Properties of mitotic cells prepared by mechanically shaking monolayer cultures of Chinese hamster cells. *J. Cell. Physiol.* **70**:63.
6. PETERSEN, D. F., E. C. ANDERSON, and R. A. TOBEY. 1968. Mitotic cells as a source of synchronized cultures. *In* Methods in Cell Physiology III. D. M. Prescott, editor. Academic Press Inc., New York. 347.
7. GRIFFITHS, J. B., and S. J. PIRT. 1967. The uptake of amino acids by mouse cells (strain LS) during growth in batch culture and chemostat culture: The influence of cell growth rate. *Proc. Roy. Soc. Ser. B.* **168**:421.
8. MOHBERG, J., and M. J. JOHNSON. 1963. Amino acid utilization by 929-L fibroblasts in chemically defined media. *J. Nat. Cancer Inst.* **31**:611.
9. LITTLEFIELD, J. W. 1962. DNA synthesis in partially synchronized L cells. *Exp. Cell Res.* **26**:318.
10. TOBEY, R. A., E. C. ANDERSON, and D. F. PETERSEN. 1967. The effect of thymidine on the duration of G_1 in Chinese hamster cells. *J. Cell Biol.* **35**:53.