A METHOD FOR COMPARING EFFECTS OF DIFFERENT SYNCHRONIZING PROTOCOLS ON MAMMALIAN CELL CYCLE TRAVERSE

The Traverse Perturbation Index

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ABSTRACT

After treatment of Chinese hamster cells (line CHO) with various protocols for synchrony induction, the subsequent ability of cells to traverse the cell cycle (i e., to perform an essential cell cycle process) has been determined by measurement of the DNA distribution pattern among cells in large populations with the Los Alamos flow microfluorometer In the cultures prepared by the various synchronizing techniques the vast majority of cells traversed the cell cycle in a normal fashion; however, in all cultures examined there remained small subpopulations which, though remaining viable for several days, could not carry out normal traverse. After reversible inhibition of DNA synthesis by means of a double-thymidine blockade, approximately 17% of the cells were unable to complete genome replication. After reversal of G_1 arrest resulting from cultivation of cells in isoleucine-deficient medium, 12 4% of the cells commenced synthesis of DNA but were unable to complete the S phase. Cells prepared by mitotic selection yielded a subpopulation (5 5% of the total cells) with a G_1 DNA content which remained viable but noncycling for at least 5 days. We propose a term "traverse perturbation index" which is defined as the fraction of cells converted to a noncycle-traversing state as the result of experimental manipulation. A knowledge of the perturbation index will allow direct comparison of effects on cell cycle traverse of various synchrony-induction protocols

INTRODUCTION

Techniques which accumulate or select cultured mammalian cells in short, specific segments of the cell cycle are extremely useful in studies of periodic biochemical events and regulatory mechanisms controlling macromolecular biosynthesis However, many of the physical or chemical techniques presently available for preparing synchronized populations may cause a state of unbalanced growth (see review by Petersen et al , 1969) and may even prevent part of the population from carrying out subsequent cell cycle operations. Obviously, the less perturbation induced, the more useful the synchronizing technique in preparing cells for definitive biochemical studies. A problem arises in attempting to compare effects of synchronizing protocols on subsequent cell cycle traverse. Standard biochemical measurements of macromolecular biosynthetic capacities yield population averages which provide no information concerning the properties of individual cells in the culture A

technique is required that will provide a population distribution measurement of a basic cycle-specific process, yielding information on capacities of individual cells in the population.

In this report we have examined the capacity of populations to synthesize DNA subsequent to synchronization by measuring the DNA contents of large numbers of cells with the Los Alamos flow microfluorometer (FMF) Three main classes of synchrony-induction techniques have been examined and compared for effects upon completion of genome replication: (*a*) reversible suppression of DNA synthesis with thymidine; (*b*) removal of an essential component from the culture medium, and (*c*) separation of cells in a specific phase of the cell cycle by physical means, represented by mitotic selection. Results indicate differential effects upon cell cycle processes induced by these methods and allow us to define a new term, the perturbation index, relating to relative inhibitory effects upon cycle traverse induced by various synchronizing protocols

MATERIALS AND METHODS

A line of Chinese hamster cells (line CHO obtained from Dr T T. Puck) was maintained free of pleuropneumonia-like organisms (PPLO) in F10 Medium, supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. In all studies described herein, the cells were maintained in suspension culture. In certain studies, cultures were prepared in a reversible state of G_1 arrest by growth in isoleucine-deficient medium (Tobey and Ley, 1971, Enger and Tobey, 1972). In other experiments, cells were prepared by mitotic selection (Tobey et al., 1967, Petersen et al., 1968). Cultures were also accumulated near the G_1/S boundary by means of double-thymidine blockade involving treatment of an exponential culture with 20 mM thymidine for 8 hr, followed by resuspension in normal F10 Medium for 8 hr, at which time thymidine to 20 mM was added for an additional 8 hr period; reversal was accomplished by resuspending the cells in regular F10 Medium

The following protocol was employed for dispersing, fixing, and staining cells for analysis with the FMF. Cells were washed in saline G solution (Merchant et al , 1960) lacking magnesium and calcium (i e., saline GM), then incubated for 20 min at 37°C in 0.5 mM EDTA and 0 1 mg/ml crystalline trypsin in saline GM. Soybean trypsin inhibitor, DNase I, and bovine serum albumin in saline G were added to final concentrations of 0 1, 0 005, and 1 mg/ml, respectively. After chilling in an ice bath the cells were resuspended in cold saline G, and an equal volume of cold saline G containing 20% formalin was then added. For optimum results, cells were fixed in the cold at least 18 hr before staining. After fixation, the cells were washed twice with distilled water, then hydrolyzed for 20 min in 4 N HCl at room temperature. The cells were then washed with distilled water and stained by a modification of the acriflavine-Feulgen procedure of Culling and Vassar (1961), employing a 0.03% acriflavine solution. Finally, cells were washed three times in an acid-alcohol solution (1 ml concentrated HCl in 100 ml of 70% ethanol) and resuspended in distilled water for analysis in the FMF.

RESULTS

The Los Alamos FMF has been described in detail elsewhere (Van Dilla et al , 1969; Kraemer et al , 1972). Basically, cells to be examined are washed, dispersed, and fixed, and are then stained with the fluorescent Feulgen procedure employing acriflavine (see above) The amount of dye bound is proportional to amount of DNA present in the cells (Kraemer et al., 1972), and FMF yields a distribution pattern which provides the DNA contents of individual cells in the population. An example of the type of data obtained with the FMF instrument is shown in Fig 1 Four separate cultures of known composition in regard to position in the cell cycle were examined Because of the long duration of G_1 in exponentially growing cells under our cultivation conditions (9 hr G_1, 4 hr S, 3 5 hr G_2 + M), the major peak represents cells with the G_1 DNA content, while at twice the mode of the G_1 DNA peak is a mode representing cells with the DNA content of cells in G_2 + M. S-phase cells with varying degrees of completion of DNA replication are distributed between the peaks representing G_1 and G_2 + M DNA contents. Essentially all the cells in a culture maintained for 30 hr in isoleucine-deficient medium possess the G_1 DNA content as expected from biochemical measurements of DNA mass and standard cell cycle analysis techniques (Enger and Tobey, 1972). Cells subjected to the double-thymidine procedure described in the Materials and Methods section accumulate in the S phase Galavazi and Bootsma (1966), Studzinski and Lambert (1969), and Bostock et al. (1971) have shown by other techniques that cells enter the S phase and complete replication of part of their genome. Finally, a population of cells prepared by mitotic selection containing 99% of the cells in mitosis exhibited the DNA content expected of a nearly pure M population. The above data provide evidence of the reliability of

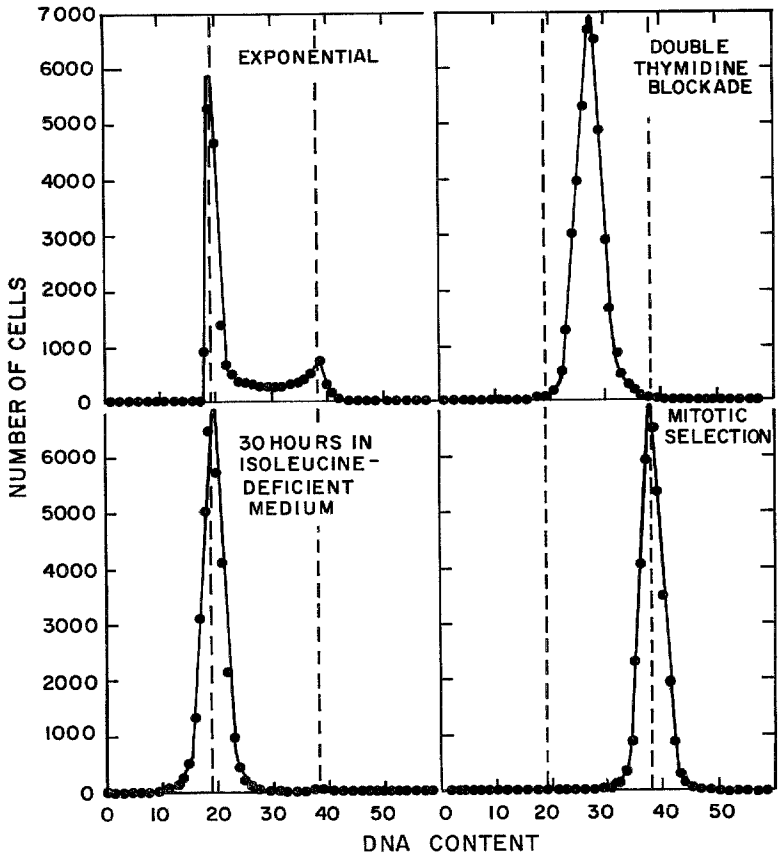

FIGURE 1 DNA distribution patterns in various types of cultures of CHO cells examined with the Los Alamos FMF. Cells were examined directly from an exponentially growing culture, from one maintained for 30 hr in F10 Medium from which the isoleucine component was omitted (Tobey and Ley, 1971), from a culture receiving thymidine to 20 mM for 8 hr followed by resuspension in fresh medium for 8 hr at which time thymidine was added for an additional 8 hr period, and finally from a culture prepared by mitotic selection (Tobey et al., 1967). Broken lines represent the modes for cells with G_1 DNA content (scale value of 19) and for cells with the DNA content of cells in G_2 + M (scale value of 38). The number of cells examined for each culture was: exponential, 20,000; isoleucine-mediated G_1, 34,000; double-thymidine blockade, 42,000; and mitotic selection, 45,000.

the FMF and also serve to indicate the quality of synchronization (in regard to DNA content) of the techniques employed in this study.

After reversal of double-thymidine blockade (FMF pattern at time of reversal in Fig. 2 A), the bulk of the population resumed traverse of the cell cycle, completed genome replication, and ultimately divided However, it was repeatedly observed that, when such cultures were examined at 6 hr after washing out the thymidine (i e , at a time immediately before any increase in cell number), a sizable fraction of the population remained in the early S phase, clearly distinct from the major, rapidly traversing portion of the population (Fig. 2 B)

In other experiments not shown, Colcemid (Ciba Pharmaceutical Co., Summit, N. J) was added to 0 2 μg/ml to these cultures to prevent traversing cells from completing mitosis, dividing, returning through the cell cycle, and obscuring the fate of nontraversing cells in S. There was no increase in DNA content for the fraction in S over the ensuing 20 hr, indicating that they had either stopped traverse altogether or were traversing S at an imperceptible rate after removal of thymidine. The nontraversing fraction in Fig 2 B represents approximately 17% of the total population. As shown in Table I, as the number of blockades is increased, there is an increase in number of cells in the non-

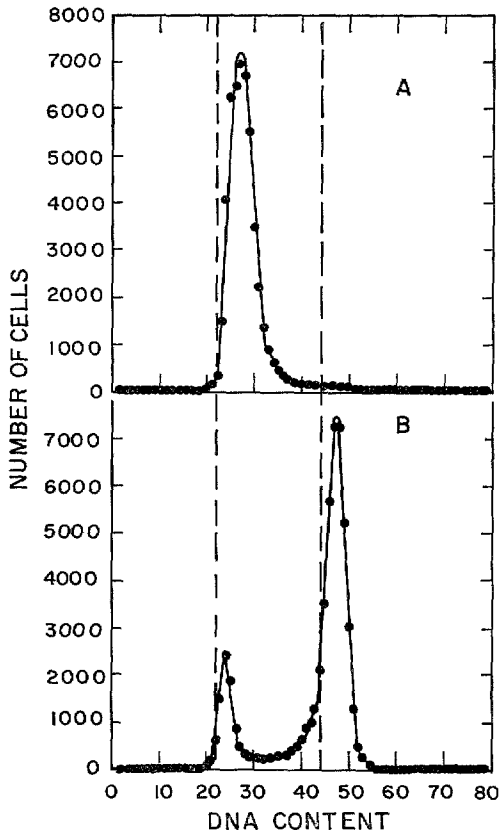

FIGURE 2 DNA distribution patterns showing nontraversing fraction of cells after reversal of the double-thymidine blockade synchronizing technique Broken lines represent values for G_1 and G_2 + M DNA calculated from controls Cells were prepared via the double-thymidine blockade technique described in the Materials and Methods section. FMF patterns in the culture at time of removal of the second thymidine blockade (DNA pattern shown in A) and at 6 hr later, immediately before the first increase in cell number (shown in B). The numbers of cells examined in (A) and (B) were 51,000 and 52,000, respectively.

traversing fraction after blockade reversal Thus, cultivation in high concentrations of thymidine induces a sizable fraction of the population to remain in S, unable to complete genome replication.

CHO cells may be reversibly arrested in G_1 by cultivation in isoleucine-deficient (i e , limiting quantities of isoleucine) F10 Medium. Such cells do not enter a state of gross biochemical imbalance, even though biosynthetic capacities for major classes of macromolecules (except DNA) remain at high levels (Enger and Tobey, 1972). By merely adding back isoleucine to the medium cells resume

TABLE I

Effect of Multiple-Thymidine Blockade on the Nontraversing Fraction of Cells after Reversal of Blockade

Blockade number	Nontraversing fraction after blockade reversal	Increase in nontraversing fraction relative to value after 2 thymidine blockades
		%
2	0 169	—
3	0.190	12
4	0.217	28
5	0.228	35

Cells were treated with thymidine (to 20 mM) for 8-hr periods, followed by resuspension for 8 hr in normal F10 Medium before application of the subsequent 20-mM thymidine blockade. Samples were removed for determination of FMF patterns at the sixth hr after reversal of thymidine blockade.

cycle traverse in synchrony, first synthesizing DNA and subsequently dividing (Tobey and Ley, 1971) A culture of CHO cells was maintained for 30 hr in isoleucine-deficient medium (Fig 3 A) and then was given isoleucine to allow reinitiation of cycle traverse. Synchronized cultures undergo appreciable synchrony decay during traverse of the entire cell cycle, owing to different rates of traverse by individual cells in the population, thereby complicating analysis of noncycling fractions that might be present In order to maximize the probability of detection of a nontraversing fraction at 9 hr after administration of isoleucine, Colcemid was added (to 0.2 μg/ml) to prevent rapidly traversing cells from reentering G_1 In Colcemid the traversing population was arrested in metaphase and, therefore, exhibited the DNA content of mitotic cells. Results obtained from a sample prepared for FMF analysis at 18 hr indicated that once again a fraction of the population had stopped in the early S phase and existed as a population discrete from the bulk of normal traversing cells [1] During further

[1] A shoulder in the traversing fraction curve indicating cells with DNA content greater than in mitotic cells represents cells which have spontaneously escaped Colcemid-induced metaphase arrest and have reinitiated cycle traverse *in the absence of an intervening division.* These cells have commenced their second round of DNA replication without dividing; therefore, they contain more DNA than those cells still arrested in metaphase Given sufficient time, most of the traversing cells will accumulate with a tetraploid or greater DNA content at an approximate scale

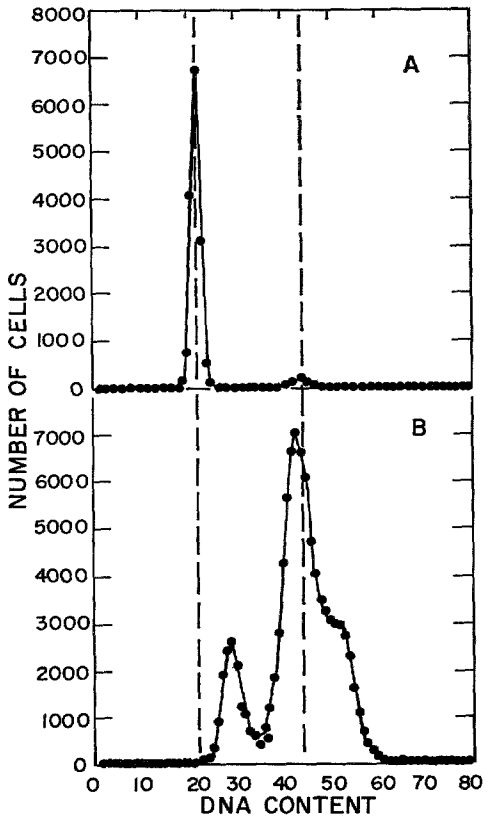

FIGURE 3 DNA distribution patterns showing nontraversing fraction of cells after reversal of isoleucine-mediated G_1 arrest. Broken lines represent values for G_1 and G_2 + M DNA calculated from controls. Cells were maintained for 30 hr in isoleucine-deficient medium (DNA pattern shown in A); then reversal of arrest was accomplished by resuspension of cells in fresh isoleucine-containing medium at t = 0. Colcemid was added at t = 9 hr (final concentration 0.2 μg/ml), and a sample was removed for analysis of the DNA distribution pattern at t = 18 hr (shown in B). The numbers of cells examined in (A) and (B) were 18,000 and 95,000, respectively.

incubation the DNA content of these S-phase cells did not increase, indicating that after reversal of isoleucine-mediated G_1 arrest a significant fraction of the population (12.4% in Fig. 3 B) is able to initiate but unable to complete genome replication.

Perhaps the least disruptive method of synchronizing cells currently available is mitotic selection. No drugs are employed in our standard protocol, nor is trypsinization or preliminary cold shock employed (Tobey et al, 1967; Petersen et al., 1968). Thus, biochemical perturbation is minimal with this technique Cells were prepared by mitotic selection (Fig. 4 A) and allowed to traverse the cell cycle in suspension culture As in cultures released from isoleucine-dependent G_1 arrest, Colcemid was added at 9 hr to prevent traversing cells from subsequently reentering G_1. The FMF pattern obtained from a sample collected at 18 hr reveals that a small fraction of the population (5.5% in Fig. 4 B) is stopped in G_1, unable to initiate DNA synthesis. In a series of similar experiments, the fraction of nontraversing cells ranged from 4.7 to 5.9%. These values should be com-

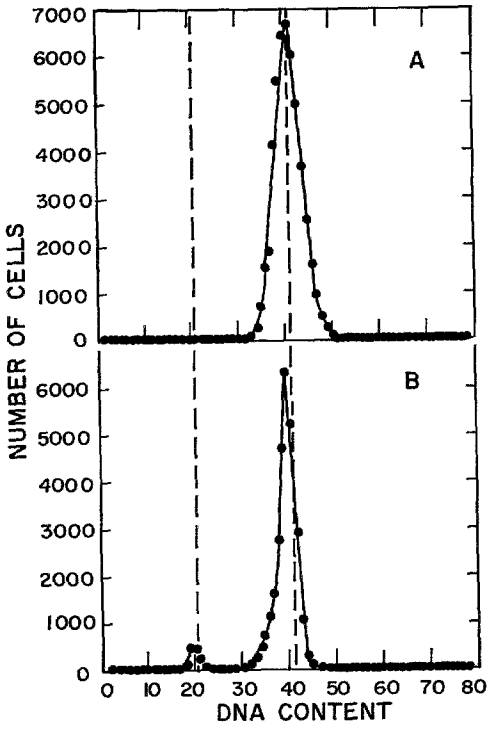

FIGURE 4 DNA distribution patterns showing nontraversing fraction of cells in culture prepared by mitotic selection. Broken lines represent values for G_1 and G_2 + M DNA calculated from controls. Cells were prepared by mitotic selection with no drugs or chemicals (DNA pattern shown in A) and were allowed to continue cell cycle traverse in suspension culture. Colcemid was added at 9 hr (0.2 μg/ml), and a sample was analyzed for the DNA distribution pattern at t = 18 hr (shown in B). The numbers of cells in (A) and (B) were 52,000 and 32,000, respectively.

reading of 88. Spontaneous dissolution of the mitotic apparatus in Colcemid-treated strain DON Chinese hamster cells was reported previously by Stubblefield (1964).

pared with nontraversing fractions of 17 and 12 4% induced by double-thymidine blockade and isoleucine deficiency techniques, respectively The nontraversing fraction failed to initiate genome replication even after culture for 48 hr.

Because the yield of mitotic cells from a single detachment is so low due to the small number of cells in mitosis in exponential populations at any given time, we frequently accumulate larger quantities of mitotic cells by storing cells from successive detachments in an ice bath. The chilled cells are unable to complete mitosis. After accumulation of sufficient quantities of cells for biochemical studies, the chilled samples are pooled, centrifuged, and resuspended in fresh, warm medium to allow the cells to commence cycle traverse (Tobey et al., 1967). This treatment could potentially compromise the "normality" of the cells, although to a lesser degree than alternatives. To determine the effects of cold storage on induction of nontraversing populations, cells prepared by mitotic selection were chilled immediately after collection and stored in an ice bath for varying periods before resuspension in warm medium After Colcemid addition at 9 hr, samples were collected for FMF analysis at 18 hr after transfer to warm medium The results shown in Table II indicate that storage of cells for at least 4 hr at 0°C does not increase

TABLE II

Effect of Cold Storage on the Nontraversing Fraction of Cells Prepared by Mitotic Selection

Hr stored at 0°C	Nontraversing fraction after resuspension in fresh, warm medium	Increase in nontraversing fraction relative to nonchilled control value
		%
0	0 055	—
2	0 057	3.6
4	0.050	—
8	0.081	47
10	0 100	82

Cells were prepared by mitotic selection (Tobey et al., 1967; Petersen et al., 1968) and either spun down and resuspended in fresh medium at a concentration of 200,000 cells/ml or were chilled in an ice bath immediately after detachment. After periods of cold storage as indicated above, cells were spun down and resuspended in fresh, warm medium at a concentration of 200,000 cells/ml Colcemid was added at 9 hr, and samples were prepared for analysis with the FMF at 16 hr after resuspension.

the fraction of nontraversing cells upon subsequent reinitiation of cycle processes. Prolonged cold storage does appreciably increase the fraction of nontraversing cells (see also Nagasawa and Dewey, 1972, Ehmann and Lett, 1972).

Finally, questions arise concerning the effects on cell cycle traverse of synchronizing protocols used in combination For example, for studies of late interphase, cells may be accumulated in the S phase by use of double-thymidine blockade, but the conditions will produce a nontraversing fraction of nearly 17% after removal of thymidine. However, reversal of G_1 arrested cells results in a nontraversing fraction of 12.4% Some improvement in thymidine blockade might be possible by first synchronizing cells in G_1 and then resynchronizing them in S by imposition of a single, brief thymidine blockade Indeed, this is the case Cultures were treated with thymidine between 4 and 14 hr after reversal of isoleucine-dependent G_1 arrest (Fig. 5 A). At 7 hr after removal of thymidine (immediately before the first signs of increase in cell number), the nontraversing fraction amounted to13 4%, a value much improved over the 17% fraction observed after release from double-thymidine blockade. The other factors being equal, we would select the isoleucine deficiency/single-thymidine block protocol over the double-thymidine blockade technique, since the latter procedure induces a larger nontraversing subpopulation In similar fashion, numerous other combinations of synchrony-induction protocols can be examined for effects in cell cycle traverse.

DISCUSSION

The data presented herein suggest that various protocols for synchrony induction known to induce specific biochemical-biophysical alterations in cellular processes affect in differential fashion the fraction of cells which subsequently carries out cycle-related operations. Simply stated, the results suggest that the greater the amount of perturbation introduced into a population by a specific treatment protocol, the greater the subsequent fraction incapable of carrying out cycle processes Therefore, we propose that the term "traverse perbation index" be defined as the fraction of cells which is converted to an abnormal or a noncell cycle traversing state as the result of experimental manipulation Determination of the traverse perturbation index allows direct comparison of effects on cell cycle traverse either induced by different

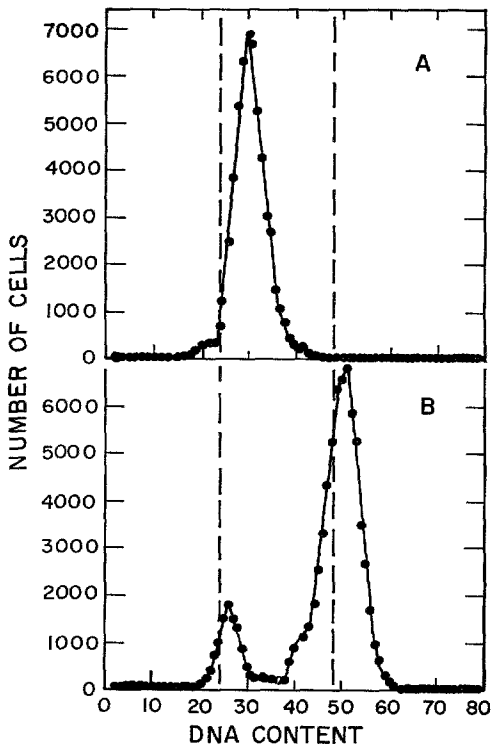

FIGURE 5 DNA distribution patterns showing nontraversing fraction of cells in culture prepared by combined use of the isoleucine deficiency technique and a single-thymidine blockade Broken lines represent values for G_1 and G_2 + M DNA calculated from controls. A culture maintained for 30 hr in isoleucine-deficient medium was resuspended in fresh complete medium containing isoleucine. Thymidine was added 4 hr later (final concentration of 20 mM), and after 10 hr the cells were resuspended in fresh medium (DNA pattern shown in A). 7 hr later a sample was removed for determination of the DNA distribution pattern (shown in B). The numbers of cells in (A) and (B) were 56,000 and 76,000, respectively.

protocols in the same cell line or induced by a common protocol in different cell lines In this report we have examined perturbation indices of the former type: perturbation indices of synchrony-induction protocols in the CHO cell In the following discussion, the term perturbation index refers to effects of techniques for synchronizing CHO cells, however, note that the term by definition may be utilized in comparison of effects of any treatment protocol upon posttreatment cell cycle traverse.

In the CHO cell, the order of decreasing perturbation indices is double-thymidine blockade > isoleucine-mediated G_1-arrest > mitotic selection, with increases in perturbation index induced by application of multiple-thymidine blockades or storage at 0°C for prolonged periods. Techniques with a high perturbation index may still be profitably employed in studies of cell cycle parameters, provided that appropriate allowance is made for nontraversing populations in data interpretation. Of particular importance in this regard is the biochemical activity of nontraversing cells; this fraction is not comprised of dead cells, since trypsin was employed for preparation of monodisperse populations before fixing and staining for FMF analysis. Cells incapable of continuing maintenance operations and membrane integrity are destroyed by this treatment (DeLuca, 1965). Thus, nontraversing cells may well remain biochemically active for several days but, for reasons unknown, have lost reproductive capacity. Under normal growth conditions, the fate of these cells is unclear, since traversing cells continue dividing, grossly complicating observation of nontraversors. To facilitate detection of the nontraversing fraction, it was necessary in this study to employ Colcemid, an agent known to produce multiple effects upon cell cycle processes when administered at sufficiently high concentrations (see review by Petersen et al, 1969) Although we cannot rigorously exclude Colcemid-induced effects in our systems, it seems unlikely that Colcemid is responsible for induction of the nontraversing fraction, since biphasic DNA population distributions are routinely observed before addition of Colcemid, indicating a prior segregation into traversing and nontraversing subpopulations.

Perhaps the most interesting aspect of the perturbation index data presented in this report is the finding that, in cultures prepared by mitotic selection (prepared essentially free of biochemical-biophysical perturbation), there was still a small but reproducible fraction of cells which were irreversibly arrested with a G_1 DNA content. Evidence that the nontraversing fraction with G_1 DNA content was not the result of stress introduced by the mitotic selection protocol was provided by studies with asynchronous cells from an exponentially growing culture. Asynchronous cells were treated with Colcemid, and samples were removed for FMF analysis at daily intervals; a subpopulation containing 7% of the total cells present maintained a G_1 DNA content for 5 days (i e , was stopped in G_1 and nontraversing), while traversing cells continued to increase their DNA content throughout the experimental period (Kraemer et al., 1972).

Results obtained with both synchronized and unsynchronized cells suggest that the generation of a nontraversing subpopulation with G_1 DNA content may be an intrinsic growth property of the populations studied.

The 5.5% possibly represents a fraction which is damaged during transition from mitosis to G_1, although the cells are not killed since, in that event, they should have been destroyed by the dispersal technique employing trypsin Alternatively, the nontraversing fraction could represent a very unique and interesting subpopulation The mitotic selection technique selects cells capable of traversing into mitosis between successive detachments, yet from this essentially pure traversing population there arises a consistent, stable, nontraversing fraction with a G_1 DNA content This sudden conversion from a traversing to a nontraversing state is at least superficially similar for model systems within the whole animal for generation of G_0 cells, cells which presumably enter a unique physiological state in which they remain for indefinite periods without undergoing division or differentiation but retain the potential for carrying out either of these processes (see review by Epifanova and Terskikh, 1969) That is, we raise the speculative possibility that nontraversing cells with G_1 DNA content may represent a fraction of cells in the G_0 phase, derived from a continuous line culture A definitive decision regarding the nature of these cells must await further biochemical characterization of this potentially unique fraction We are currently studying other diploid and aneuploid cell lines to define further the speculative generalities discussed above.

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REFERENCES

BOSTOCK, C J., D. M. PRESCOTT, and J. B. KIRKPATRICK. 1971. An evaluation of the double thymidine block for synchronizing mammalian cells at the G_1-S border. *Exp. Cell Res.* **68**:163.

CULLING, C, and P. VASSAR. 1961. Desoxyribose nucleic acid. *Arch. Pathol.* **71**:76.

DELUCA, C. 1965. The use of trypsin for the determination of cellular viability. *Exp. Cell Res.* **40**:186.

EHMANN, U. K., and J. T LETT. 1972. Effect of cold on metaphase cells: Extended generation times and aberrant cytokinesis *Exp. Cell Res.* In press.

ENGER, M. D., and R. A. TOBEY. 1972 Effects of isoleucine deficiency on nucleic acid and protein metabolism in cultured Chinese hamster cells: Continued RNA and protein synthesis in the absence of DNA synthesis *Biochemistry.* **11**:269.

EPIFANOVA, O I., and V V. TERSKIKH. 1969. On the resting periods in the cell life cycle. *Cell Tissue Kinet* **2**:75.

GALAVAZI, G., and D. BOOTSMA. 1966 Synchronization of mammalian cells *in vitro* by inhibition of the DNA synthesis. *Exp. Cell Res* **41**:438.

KRAEMER, P. M , L L. DEAVEN, H A. CRISSMAN, and M. A VAN DILLA. 1972. DNA constancy despite variability in chromosome number. *In* Advances in Cell and Molecular Biology. E. J DuPraw, editor Academic Press Inc., New York. **2**:47.

MERCHANT, D J., R H. KAHN, and W H. MURPHY, JR. 1960. *In* Handbook of Cell and Organ Culture. Burgess Publishing Co., Minneapolis, Mn.

NAGASAWA, H , and W. C. DEWEY. 1972. Effects of cold treatment on synchronized Chinese hamster cells treated in mitosis *J. Cell Physiol.* In press

PETERSEN, D F , E C. ANDERSON, and R. A. TOBEY. 1968. Mitotic cells Source of synchronized cultures. *In* Methods in Cell Physiology. D M. Prescott, editor. Academic Press Inc., New York. **3**:347.

PETERSEN, D. F., R A. TOBEY, and E. C. ANDERSON 1969. Synchronously dividing mammalian cells. *Fed. Proc* **28**:1771.

STUBBLEFIELD, E. 1964. DNA synthesis and chromosomal morphology of Chinese hamster cells cultured in medium containing *N*-deacetyl-*N*-methylcolchicine (Colcemid). *In* Cytogenetics of Cells in Culture. R. J C. Harris, editor. Academic Press Inc , New York. 223

STUDZINSKI, G. P., and W. C. LAMEERT. 1969. Thymidine as a synchronizing agent. I. Nucleic acid and protein formation in synchronous HeLa cultures treated with excess thymidine. *J. Cell Physiol.* **73**:109.

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.

TOBEY, R. A , and K. D. LEY. 1971. Isoleucine-mediated regulation of genome replication in various mammalian cell lines. *Cancer Res.* **31**:46.

VAN DILLA, M. A , T. T. TRUJILLO, P. F. MULLANEY, and J. R. COULTER. 1969. Cell microfluorometry: A method for rapid fluorescence measurement. *Science (Wash. D. C.).* **163**:1213.