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Modified Method of Mammalian Cell Synchronization Improves Yield and Degree of Synchronization

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A modified method to synchronize CHO and HeLa cells is developed based upon a combined shaking-off and chemical blockage. This method has effectively blocked quiescient cells, which is the main obstacle of high degree synchronization. Flowcytometry data show the improvement on the degree of synchronization and yield compared to two previously used methods. © 1991 Academic Press, Inc.

INTRODUCTION

Cell synchronization, when used to obtain a large quantity of cells at some specific phases of the cell cycle, is essential for research on cell cycle-dependent events in cell biology. Mitotic shaking and chemical agent block are methods commonly used for cell synchronization. Thymidine (TdR) and hydroxyurea (HU) are the frequently used chemical agents [1–3]. Obtaining mitotic cells by mechanically shaking culture flasks is based upon the fact that during mitosis the cells are less adherent [4–6]. TdR block results from an inhibition of the enzymatic mechanism for the synthesis of deoxycytidine triphosphate from cytidine-5′-phosphate due to excess thymidine [7]. HU has both inhibitory and cytotoxic action on cells grown *in vitro* [8, 9]. During a short exposure, HU is selectively lethal to S-phase cells, cells which are synthesizing DNA. It prevents G_1 cells from entering S phase but does not affect the viability of these cells. Cells resume DNA synthesis after HU removal. HU at concentrations used for cell synchronization is not toxic to G_2 cells, which proceed through mitosis and division.

It was also reported that a combination of methods, i.e., obtaining mitotic cells by shaking and then blocking them with chemical agents, improves synchronization [3, 10] by either increasing the yield or improving the degree of synchronization.

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To study effects of nonionizing electromagnetic radiation on the mammalian cell cycle, a high degree (i.e. >90%) of synchronization was required. Previously reported approaches were inadequate. Consequently a modified method was developed based upon a combined shaking-off and chemical blockage. Attention was focused on blocking quiescient cells, which either remained in a pre-DNA synthesis stage or very slowly moved through the cell cycle [11]; such cells decrease the degree of synchronization. The combined method improves synchronization of both Chinese hamster ovary (CHO) and HeLa cells. The greater adherence of HeLa cells, however, led to a slight reduction in synchronization efficiency relative to CHO.

MATERIALS AND METHODS

Cell Culture

CHO cells (ATCC No. CCL61) were obtained from American Type Culture Collection (Rockville, MD) and monolayer cultured with DME/F12 medium (Sigma Chemical Co., St. Louis, MO) plus 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. HeLa S-3 cells kindly supplied by Dr. James M. Collins, Biochemistry Department, Medical College of Virginia were monolayer cultured with DMEM medium (Sigma) plus fetal calf serum, penicillin, and streptomycin as for CHO culture. Both cell lines were incubated in 5% CO_2 at 37°C. Under these culture conditions the generation time of CHO cells was 13 h (5.5 h for G_1 phase, 5 h for S phase, and 2.5 h for G_2/M phase). The generation time of HeLa cells was 23 h (11 h for G_1, 9 h for S, and 3 h for G_2/M). HeLa cells were also cultured in spinner flasks at a spin speed of 60 rpm at concentrations between 3×10^5/ml and 10^6/ml, under the same conditions used for monolayer culture. In this culture the generation time was approximately 24 h.

CHO and HeLa cell generation times were determined from growth curves obtained by cell counting. Cell cycle phase durations were determined from flow cytometry data and growth curves of partially synchronized cell cultures by double TdR block [5, 17]. Data obtained in our laboratory were consistent with that of Tobey *et al.* [5] and Thilly *et al.* [17].

Cell Synchronization

Method 1. Double or multiple TdR block: CHO and HeLa cells were synchronized by double thymidine block following the reports by Tobey *et al.* [5]. Thymidine was added to random monolayer cell cultures to a final concentration of 5 m*M* and incubated for a period somewhat longer than the sum of G_2 plus M plus G_1 portions of the cycle (9 h for CHO, 14 h for HeLa). The cultures were released from thymidine block by reculture in normal medium for a period that

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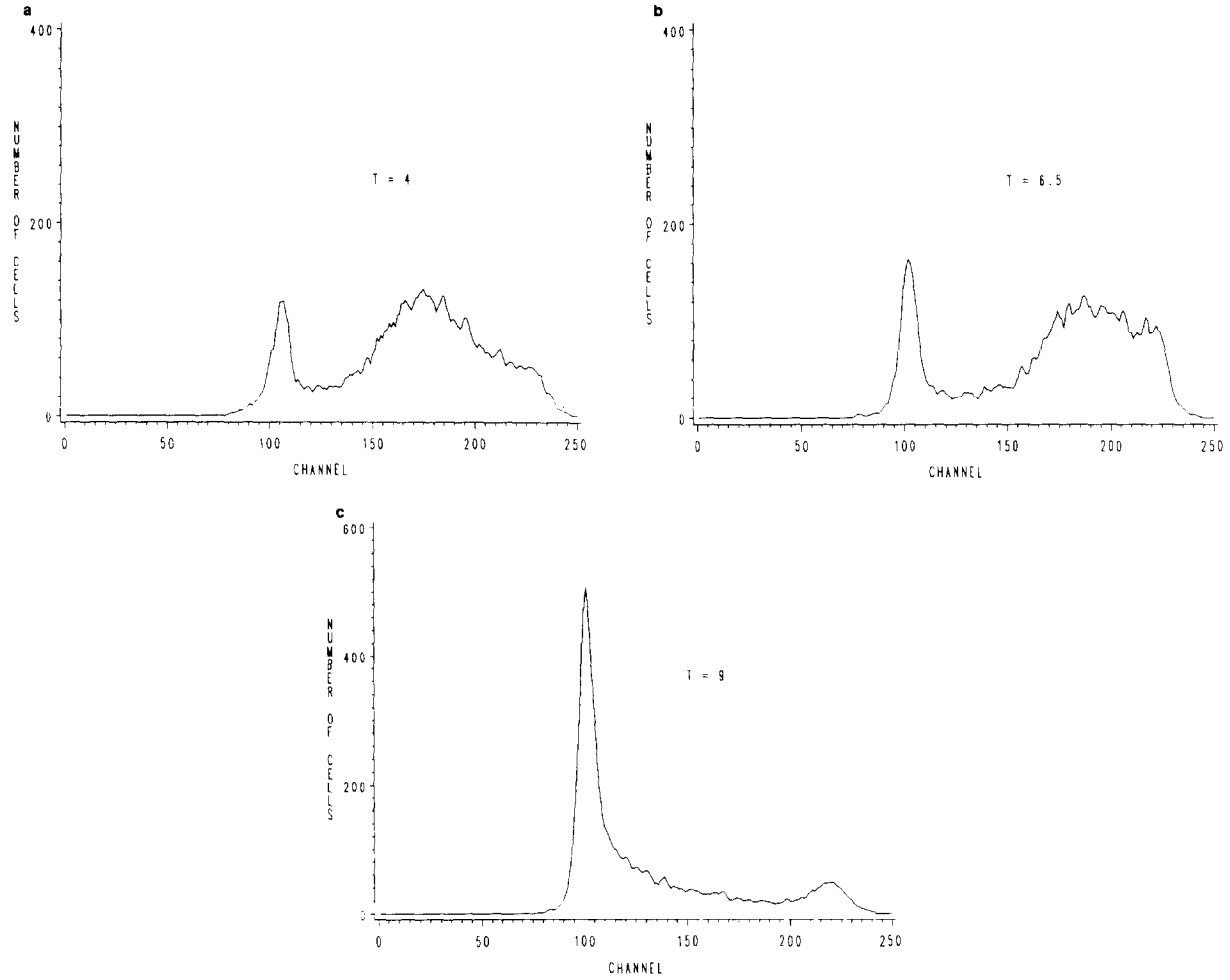

FIG. 1. CHO cells synchronized by double thymidine block, (a) S-phase cells; (b) G_2/M-phase cells; (c) G_1-phase cells.

slightly exceeded S phase (5.5 h for CHO and 9.5 h for HeLa). This procedure was repeated for a second cycle block. Synchronization of suspension-cultured HeLa cells was attempted by multiple thymidine block following the method of Thilly *et al.* [12] and Collins [2] with some timing modifications. The cells underwent repeated cycles of a 14-h exposure to 2 m*M* TdR followed by a 10-h exposure to normal medium for periods of up to 4 weeks. After the final release cells were assayed cytofluorometrically [2] to check synchronization.

Method 2. Thymidine block, mitotic shake-off and HU block: This method for CHO synchronization was described in detail by Meyn *et al.* [10] and Grdina *et al.* [3]. CHO cells were seeded into culture dishes and allowed to grow for 18 h. Thymidine (5 m*M*) was added and cells were incubated for 9 more h and then released from blockage by change of medium. Six hours after the release, mitotic cells were selectively detached from the monolayer by gentle agitation and collected from the decanted medium. This shake-off procedure was repeated two to four times with 15-min intervals to increase cell yield. More shake-off gave higher yield but increased the percentage of nonmitotic cells. Mitotic cells were held at 4°C during collection. Cells were incubated in the presence of 2 m*M* HU for 9 h after collection and then sampled for degree of synchronization after release.

Method 3. Modified method: This method consisted of double thymidine block followed by mitotic shake-off and hydroxyurea block. Double thymidine block, as described above, was used to induce a large mitotic index and hence increase the yield from the shake-off procedure. Gentle shaking of treated CHO cells yielded up to 15% of total cells. HeLa cells were more adherent. Gentle shaking yielded a small number of mitotic cells, whereas violent shaking detached cells in phases other than M phase. To avoid this, a low trypsin concentration (0.05%; 1/5 of the concentration for normal trypsinization) was used to treat cell cultures for a maximum of 3 min at room temperature. Regular medium with 10% FCS was added and shaking was performed. Mitotic cells collected from shaking were then cultured with 2 m*M* HU, 9 h for CHO cells and 12 h for HeLa cells. HU prevented cells from entering S phase but did not otherwise alter their physiological state. After 1 h the medium was changed to remove unattached cells and culture was continued in the presence of 2 m*M* HU.

Two millimolar HU was selected for synchronization after different concentrations were tested. We found that 0.1 m*M* HU did not effectively block cells at the G_1/S boundary. The 2 m*M* concentration we used was significantly lower than the 7.5 m*M* concentration reported by Grdina *et al.* [3] to induce cell synchronization.

Flowcytometry

Flowcytometry was used to determine the relative DNA contents of cells to check the degree of cell synchronization. Monolayer-cultured CHO or HeLa cells were trypsinized, washed with calcium/magnesium-free phosphate-buffered saline, pH 7.0, and centrifuged for 10

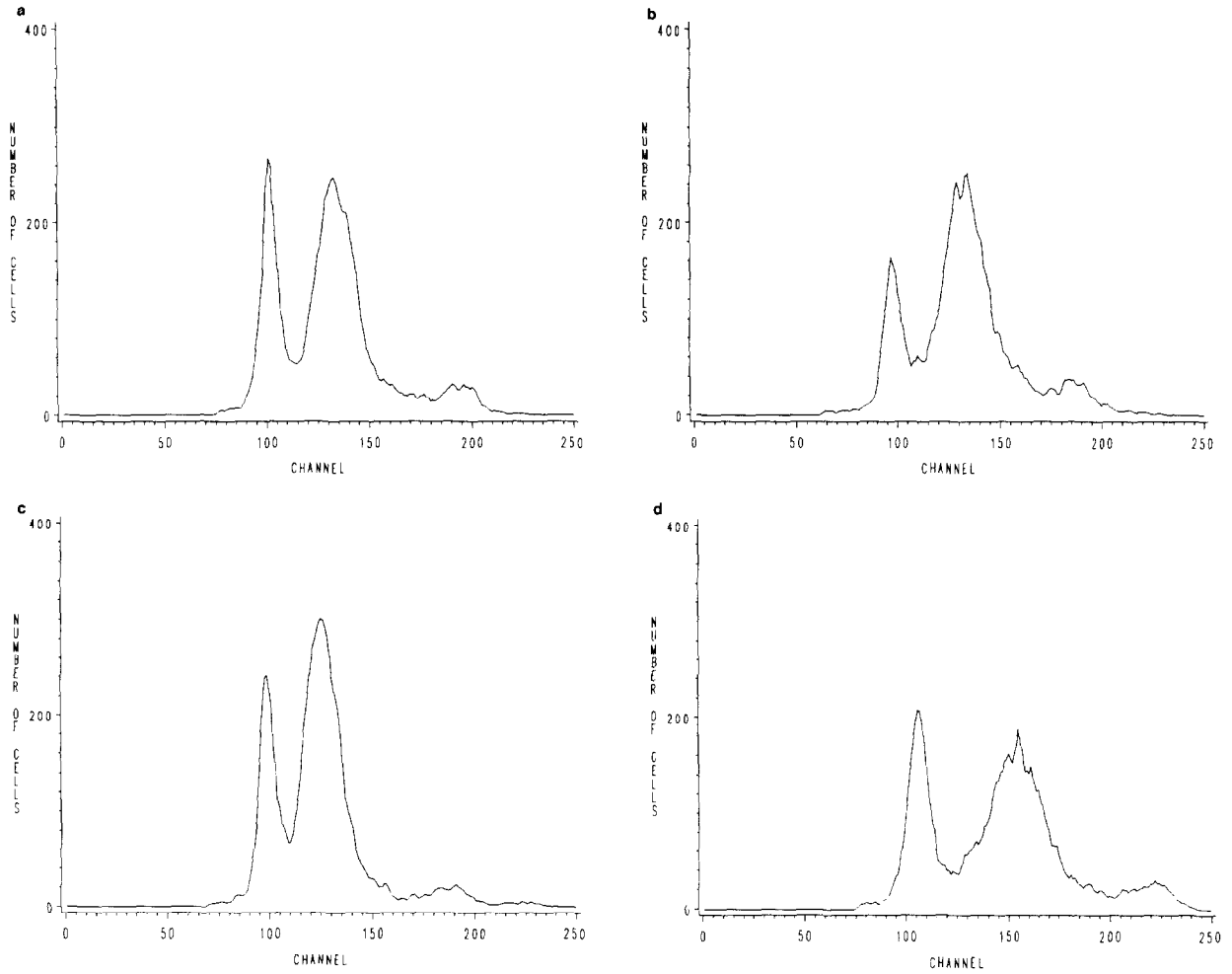

FIG. 2. HeLa cells synchronized by multiple thymidine block, sampled after (a) 1 week of treatment; (b) 2 weeks of treatment; (c) 3 weeks of treatment and (d) 4 weeks of treatment. Cells underwent repeated cycles of a 14-h exposure to 2 m*M* TdR followed by a 10-h exposure to normal medium for the entire treatment period.

min at 500 *g*. Cell pellets were vortexed, dyed with PIF stain [2, 13], and filtered through a 37-μm screen filter. The final cell concentration was adjusted to 10^6/ml. Cell samples were analyzed by an EPICS753 flow cytometer (Coulter Electronics, Hialeah, FL) with an excitation wavelength of 488 nm. The flourescence was measured at wavelengths above 630 nm. Data were collected and analyzed by an MDADS microcomputer and DNA distributions were plotted.

RESULTS

Double TdR block (Method 1) and shake-off plus HU block (Method 2) are relatively simple methods, but did not yield a high degree of synchronization in our laboratory. Compared to Method 1, Method 2 produced better synchronization but a low cell yield, especially for HeLa cells.

DNA distributions obtained using these methods are shown in Fig. 1–3. Figure 1 summarizes the results of CHO cells treated by double TdR block (Method 1). Cells were sampled and dyed for DNA analysis 4, 6.5, and 9 h after the second release for S-phase (Fig. 1a), G_2/M-phase (Fig. 1b), and second generation G_1-phase (Fig. 1c) samples. The degree of synchronization was not adequate. The peaks at channel 100 in (a) and (b) represent the portion of cells which remained in G_0/G_1 phase and did not progress through the cycle (i.e., quiescent cells).

Figure 2 indicates the results of suspension-cultured HeLa cells treated with multiple TdR blocks (Method 1). In all cases (a)–(d), cells were sampled 3 h after release with: (a) cells treated for 1 week, (b) cells treated for 2 weeks, (c) 3 weeks of treatment and, (d) 4 weeks of treatment. Quiescent cells were not eliminated by this synchronization method. As in Fig. 1, the portion of quiescent cells, shown as the peaks at channel 100, did not decrease even after 4 weeks of treatment. Elimination or reduction of the quiescent G_0 cell fraction appeared to be a major limitation of these methods of cell synchronization.

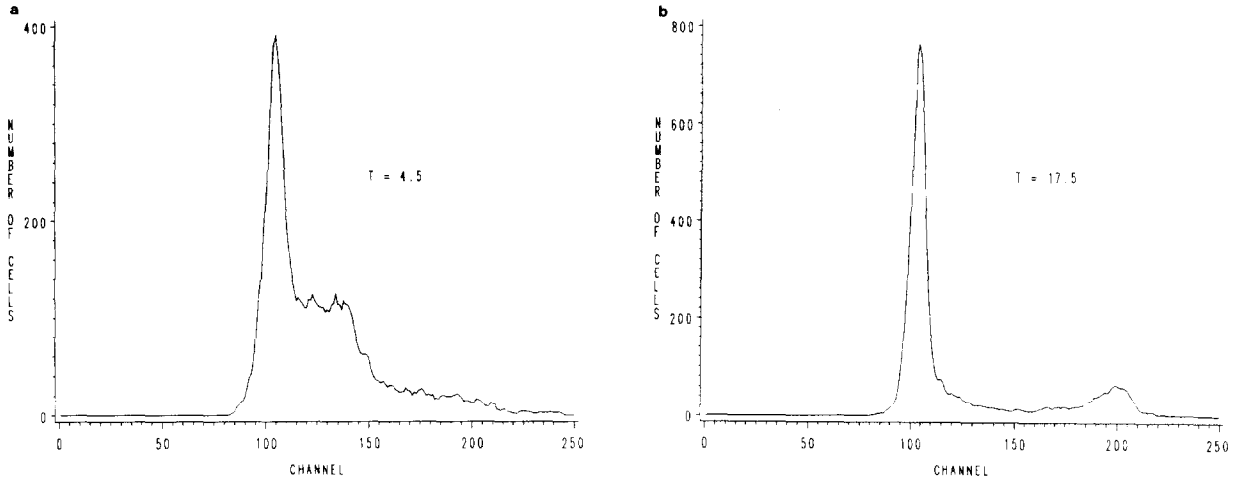

FIG. 3. CHO cells synchronized by shake-off plus hydroxyurea block, sampled (a) 4.5 h and (b) 17.5 h after release from HU block.

Results of Method 2 are shown in Fig. 3. CHO cells were sampled, 4.5 h (a) and 17.5 h (b) after release from HU block. The synchronization was not satisfactory as shown by the relatively large fraction of G_0/G_1 cells in the S-phase sample (Fig. 3a). This may have been due to inadequate separation of mitotic cells obtained from

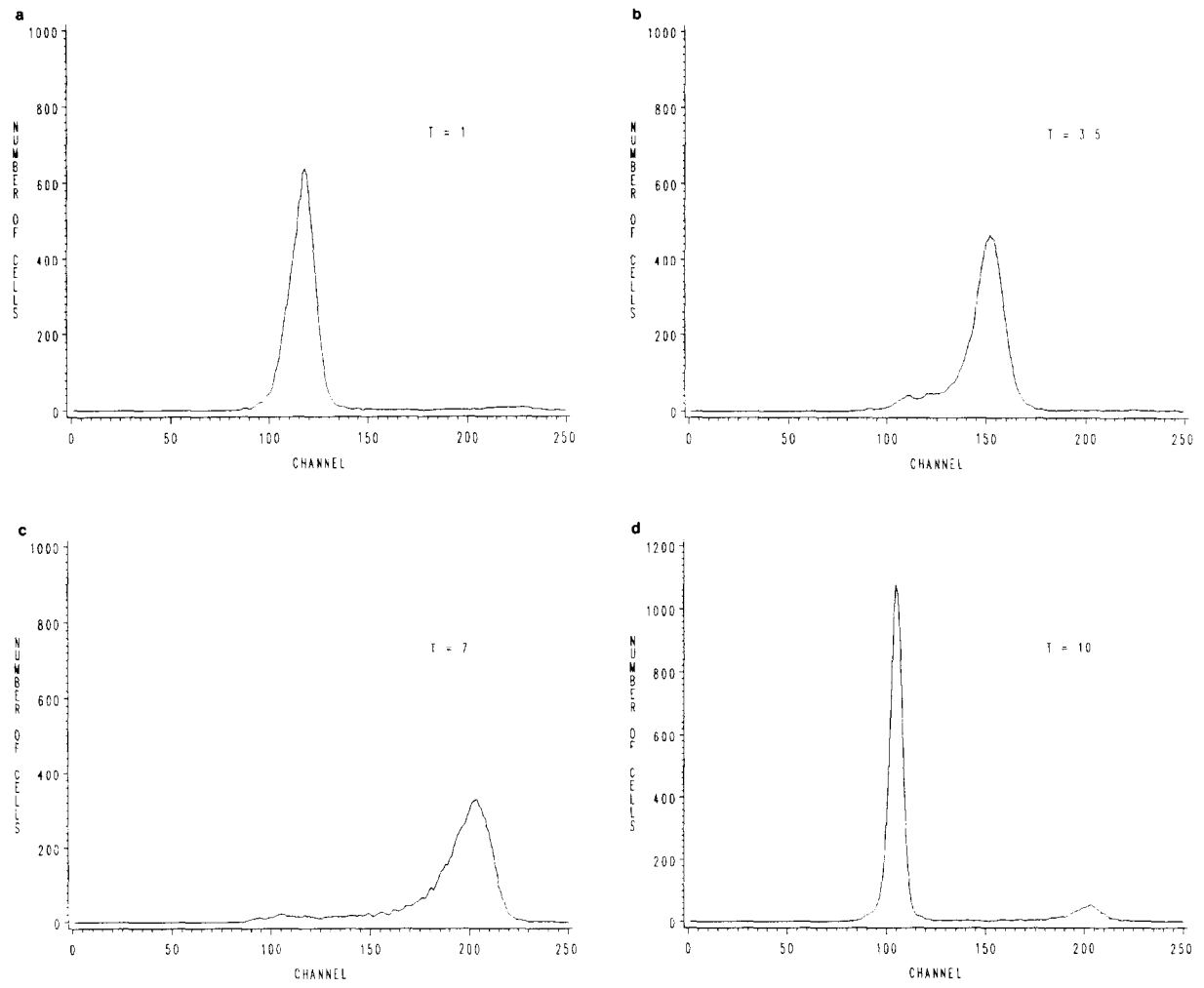

FIG. 4. CHO cells synchronized by the modified method.

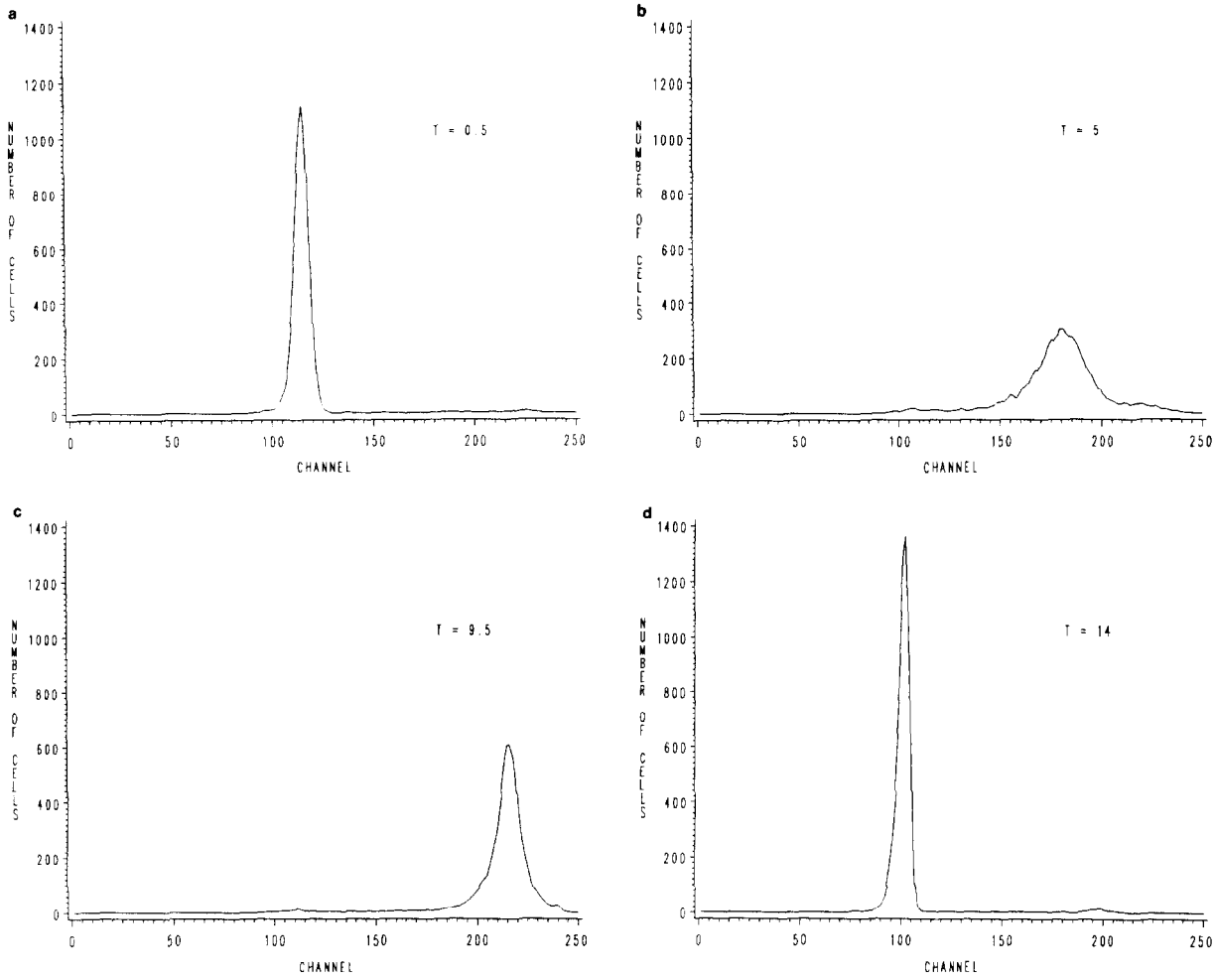

FIG. 5. HeLa cells synchronized by the modified method.

random phase cell cultures by shaking and the absence of subsequent cell mitotic blockage.

The modified method, as stated under Materials and Methods, utilized a double TdR block and shake-off plus HU blockage. This improved the degree of synchronization since: (a) double TdR block induced a high mitotic index, which increased the yield; (b) discarding the medium with unattached cells after cells have settled and using low concentration trypsin to treat the HeLa cell culture before shaking increased the yield and purity of M-phase cells. For both CHO and HeLa cells the yield was high (>15% of total cells) and the synchronization was significantly improved, as shown in Fig. 4 for CHO and in Fig. 5 for HeLa. In both cases cells were sampled and stained for DNA analysis by flowcytometry at the times shown after the final release from

TABLE 1

Percentage of CHO Cells in Specific Phases in a Sample Synchronized by Method 3

Sampling time after release (h)	G_1	S	G_2/M
3.5		93.4	
6.5			86.9
10.0	83.4		

Note. The data are shown in Fig. 4.

TABLE 2

Percentage of HeLa Cells in Specific Phases in a Sample Synchronized by Method 3

Sampling time after release (h)	G_1	S	G_2/M
5		89.8	
9.5			92.4
14	96.0		

Note. The data are shown in Fig. 5.

HU block. From these figures it can be seen that the cells entered S phase shortly after release and progressed through the normal cycle. By sampling at various times after release, synchronized cell samples can be obtained in any phase. For example, at 2–4 h after release S-phase CHO cells were obtained as shown in Fig. 4b.

To evaluate the degree of synchronization the percentage of cells in a given phase can be obtained by integrating the curve within a predetermined range of channels. This procedure will result in a small error relative to the method, using a Gaussian distribution function to model the distribution of G_1 or G_2/M cells [14] for curves of well-synchronized samples. Tables 1 and 2 summarize the percentage synchronization for CHO and HeLa cells, respectively. Mathematical methods were suggested to quantitate the degree of cell synchronization. Calculations of the degree of synchronization were based upon data from growth curves of synchronized cells [15] and the variation of DNA distribution with time [16] for a culture period at least one cycle long. In our case, however, the high degree of synchronization and symmetrical DNA distributions obviated the need for mathematical analysis and permitted simple and direct calculation of the degree of synchronization by relative DNA distribution area analysis.

As for the case of other cell synchronization methods, Method 3 maintains synchronization for only the first and second generations. The DNA distributions of third generation CHO or HeLa cells synchronized by Method 3 approach the distribution of random phase cultures. However, Method 3 synchronized cells exhibit a greater degree of synchronization than obtained using Methods 1 or 2 even after two generations.

It should be noted that cell synchronization methods should not cause DNA mutation or other cellular functional disorders. Highly synchronized cells with disturbed cellular functions are of limited general use. We have tested the viability and growth characteristics of postsynchronized CHO and HeLa cells after release from synchronization by the method described herein. Trypan blue assay indicated no effect on cell viability. Cells were subcultured after they returned to random phase (three to five generations after synchronization). There were no significant differences between postsynchronized cultures and normal cultures as indicated by comparison of growth curves (unpublished data).

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