Effects of β-Phenethyl Alcohol on Mouse L Cells in Suspension Culture

II. Effects on the Cell Division Cycle

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SUMMARY

The cell division cycle of mouse L cells in suspension culture was changed markedly by exposure of the cells to 0.10% β-phenethyl alcohol (PEA). The progress of cells from the G1 phase into the S phase, from the S phase into the G2 phase and from the G2 phase into mitosis was found to be almost completely stopped for at least 40 hr after the addition of PEA.

Cell populations exposed to PEA for 24 hr and then washed free of PEA showed a partial synchronization of cell proliferation. This partial synchronization appeared to be mainly due to a delay in the recovery of G1- and S-phase cells in PEA-treated populations, rather than to an accumulation of cells in any particular phase of the cell cycle.

The results obtained indicate that PEA has at least two separate effects on the cell division cycle of mammalian cells, an inhibition of the initiation of DNA synthesis, and an interference with the onset of cell division. They support the view that PEA is not simply a specific inhibitor of DNA synthesis in mammalian cells.

INTRODUCTION

Arrest of cell proliferation and inhibition of DNA synthesis are two effects which have been observed repeatedly when microorganisms (1–4) or mammalian cells (5, 6) have been exposed to β-phenethyl alcohol (PEA). It seems clear that PEA is not a specific inhibitor of DNA synthesis, since prompt inhibitory effects on RNA and protein synthesis were also seen in PEA-treated cells (6). However, it was still possible that the growth inhibition of mammalian cells seen in the presence of PEA was an indirect consequence of the suppression of DNA synthesis by PEA. Such an indirect inhibition of cell proliferation occurs, for example, with another inhibitor of DNA synthesis, 5-fluoro-2′-deoxyuridine (FUdR) (7). In cultures of mammalian cells exposed to FUdR, the progress of cells through the cell cycle is arrested as they attempt to initiate DNA synthesis, and the cells accumulate at this point in the cycle. Cell proliferation ceases in the FUdR-treated cultures only because the cells fail to pass through DNA synthesis and reach mitosis. To test whether or not a similar indirect inhibition of cell proliferation was occurring in PEA-treated cell populations, the progress of cells through the division cycle of mouse L cells was studied at different times after exposure of the cells to PEA. It was found that the arrest of cell proliferation in PEA-treated L-cell cultures is not due solely to an inhibition of DNA synthesis. The results also indicate that the partial synchronization that is seen

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in PEA-treated cultures after removal of the PEA is due primarily to differential delay in the resumption of DNA synthesis, rather than to an accumulation of cells in one phase of the cell cycle.

METHODS

A subline, L60, of mouse L cells was used in all experiments. The growth medium and the methods used to propagate the cells in suspension cultures were the same as previously described (6). The durations of the phases of the division cycle (8) under the growth conditions used were approximately as follows: Phase of DNA synthesis (S-phase), 7–8 hr; premitotic phase (G2 phase), 3–4 hr; postmitotic phase (G1 phase), 5–8 hr; mitosis (M), less than 1 hr; total cycle time, 15–20 hr.

Thymidine-^{3}H (^{3}H-TdR) labeling and autoradiography were used to detect cells in the S-phase. Two types of labeling experiments were carried out. In the first ("continuous labeling"), the cells were exposed to ^{3}H-TdR (New England Nuclear Company, Boston, Massachusetts, 6.7 C/mmole) at an activity of 0.1 μC/ml for 30 hr, and samples of cells were removed at various times during this period and prepared for autoradiography as described previously (8). In the second type of experiment ("pulse labeling"), the cells were exposed to 1.0 μC/ml of ^{3}H-TdR for 30 or 60 min, whereupon sufficient unlabeled TdR was added to bring the concentration of TdR to 100 μg/ml. The resulting reduction in the specific activity of the ^{3}H-TdR was sufficient to yield negligible further labeling of the cells. At various times after pulse labeling, samples of cells were prepared for autoradiography. In both types of experiments, percentages of labeled cells were determined, based on the results obtained by scoring cells in the autoradiographs.

To estimate the relative number of cells in the G1 and G2 phases, microspectrophotometry was combined with autoradiography as described by Mak (9). Cells were pulse-labeled for 1 hr with ^{3}H-TdR, fixed, and stained by the Feulgen technique. Autoradiographs of the cells were prepared and the relative DNA content of the individual unlabeled cells was determined by microspectrophotometry using the two-wavelength technique (10).

To determine the rates of entry of cells into mitosis, colcemide (courtesy of Dr. C. A. Schaffenburg, Ciba Co., Ltd., Dorval, Quebec) was added to the cultures to a final concentration of 2×10^{-7} g/ml, and the accumulation of mitotic figures in the cultures was followed. Samples of cells were removed at various times after the addition of colcemide, dilated with hypotonic saline, fixed in 3:1 ethanol-acetic acid, and stained with acetic orcein. Counts were made of the number of mitoses per 1000 cells in each sample.

In most experiments, PEA (Matheson, Coleman and Bell, Cincinnati, Ohio) from the same lots used previously (6), was added to the cultures at a concentration of 0.10% v/v (8.2×10^{-3} M). In some experiments, cell growth kinetics were studied following the transfer of PEA-treated cells to medium without PEA. In such experiments, the PEA-treated cells were sedimented (1000 rpm, 5 min at room temperature), resuspended in fresh medium at 37°, sedimented again, and then returned to suspension culture in fresh warm medium.

RESULTS

Inhibition of the Progress of Cells from G1 into S

Previous results (6) had shown that one of the major effects of 0.10% PEA on L cells is an inhibition of DNA synthesis. Such an inhibition would be expected to prevent the entry of cells from the G1 phase into the S phase, as well as to inhibit the progress of cells through the S phase. To test for these effects, two suspension cultures of cells were exposed to 0.10% PEA, while a third culture, not exposed to PEA, was used as a control. One of the treated cultures, and the control, were subjected to continuous labeling with ^{3}H-TdR, and the other treated culture was pulse-labeled at various times with ^{3}H-TdR (see Methods). The percentage of labeled cells in each culture was determined as a func-

tion of time by means of autoradiography. The results are shown in Fig. 1. In the untreated control, which was subjected to continuous labeling with ^{3}H-TdR (curve *A*), approximately 45% of the cell population incorporated radioactive precursor immediately. This percentage represented the proportion of cells initially present in the S phase of the cell cycle. With the subsequent uninterrupted flow of cells from the G1, M and G2 phases into S, the proportion of labeled cells increased until finally, after a time lapse of approximately 14 hr, equal to the combined durations of G1, M, and G2 (see Methods), all cells had taken up ^{3}H-TdR.

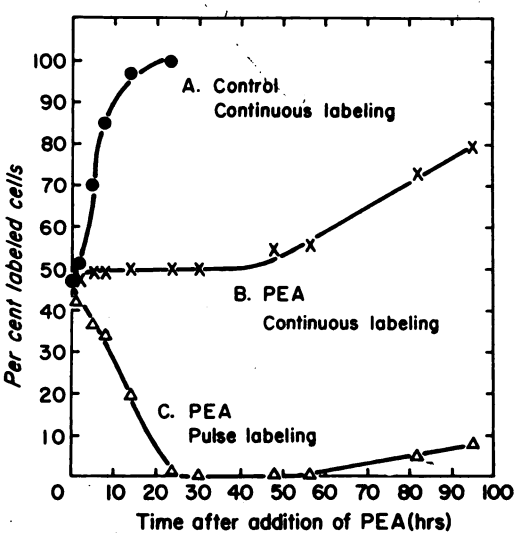

FIG. 1. *Effect of 0.10% PEA on the percentage of cells labeled with ^{3}H-TdR*

L cells in suspension culture were labeled with ^{3}H-TdR at various times after addition of PEA. Curves *A* and *B*, 0.1 μC/ml of ^{3}H-TdR continuously present; curve *C*, pulse-labeled with 1.0 μC/ml of ^{3}H-TdR for 60 min.

Figure 1, curve *B*, shows the results obtained with the PEA-treated culture which was continuously labeled with ^{3}H-TdR, beginning immediately prior to addition of PEA to the culture. With this procedure, about 45% of the cells became labeled initially, but subsequent to the addition of PEA, no further labeling took place for at least 30 hr. This is the result that would be expected if PEA prevented cell progression from G1 into S. When the duration of the exposure of the cells to PEA was extended beyond 40 hr, a slow accumulation of labeled cells began to take place. The basis for this accumulation is not known, but it could have been due to a gradual decrease in the effective concentration of PEA in the medium.

It was possible that the failure to detect the progress of cells from the G1 phase into S in Fig. 1, curve *B*, was due to inhibition of uptake of ^{3}H-TdR by the PEA present in the cultures to levels below the threshold sensitivity of the autoradiographic assay. This might prevent detection of the progress of cells from G1 into S even if such progression occurred. Whether the amount of ^{3}H-TdR incorporation was sufficient to be detected by autoradiography was determined from a study of the ability of S-phase cells to incorporate ^{3}H-TdR in the presence of PEA. Samples of cells from a treated culture were pulse-labeled with ^{3}H-TdR at various times after the addition of PEA. The percentage of labeled cells in each sample was plotted as a function of the exposure time of the cells to PEA (Fig. 1, curve *C*). The first sample withdrawn showed slightly more than 40% labeled cells. This proportion dropped steadily in later samples until it became negligible at 24 hr. A small number of labeled cells was again detected at times greater than 40 hr. The labeled cells seen during the first 24 hr were probably S-phase cells in which a low level of DNA synthesis persisted even in the presence of 0.10% PEA. A residual uptake of ^{3}H-TdR in the presence of this concentration of PEA was observed in earlier experiments (6). These results indicate that the autoradiographic technique used should have been sensitive enough to detect S-phase cells for approximately 24 hr. As a consequence, any progression of cells from G1 into S would have been associated with an increasing fraction of labeled cells with time in the presence of ^{3}H-TdR. However, since the fraction of labeled cells was constant under these conditions for over 24 hr (curve *B*), the entry into S of cells from the G1 phase must have been blocked. The absence of this transition

suggests that PEA acts to prevent the initiation of DNA synthesis. It is also apparent from curve *C* that progress of cells through the S phase may continue in the presence of PEA, though at a greatly reduced rate. It is therefore necessary to consider the possibility that at least some cells are able to complete DNA synthesis and enter the G2 phase in PEA-treated cultures. Evidence that this does not in fact occur will be presented below (Figs. 3 and 4).

Effect of PEA on the Progress of Cells from G2 into Mitosis

Previous work (6) had shown that PEA causes an almost immediate cessation of the proliferation of L cells. This growth arrest could be due to an effect of PEA on the passage of cells from G2 into mitosis or to an effect on the completion of mitosis.

To distinguish between these two possibilities, the rate of progress of cells into mitosis was studied in two PEA-treated cultures and an untreated control culture. Colcemide (2×10^{-7} g/ml) was added to one of the PEA-treated cultures and to the control, in order to arrest in mitosis any cells entering M from G2 (11). At hourly intervals, mitotic indices were determined. The results are shown in Fig. 2. In the control culture which was treated with colcemide (curve *A*), the mitotic index rose to 25% over a 10-hr period. In the culture treated with PEA but not with colcemide (curve *C*), mitotic figures disappeared within 1 hr after addition of PEA to the culture, indicating an immediate arrest by PEA of the progress of cells from G2 into mitosis. In the presence of both PEA and colcemide (curve *B*), the mitotic index showed no increase, but instead, decreased slowly to negligible values over a 5- to 6-hour interval. The gradual decrease of curve B is presumably due either to disintegration of mitotic cells arrested for several hours by colcemide, or to an escape of mitotic cells from the inhibitory effects of this agent.

These results show that no detectable flow of cells from G2 into M occurred after the addition of PEA to the cultures. The

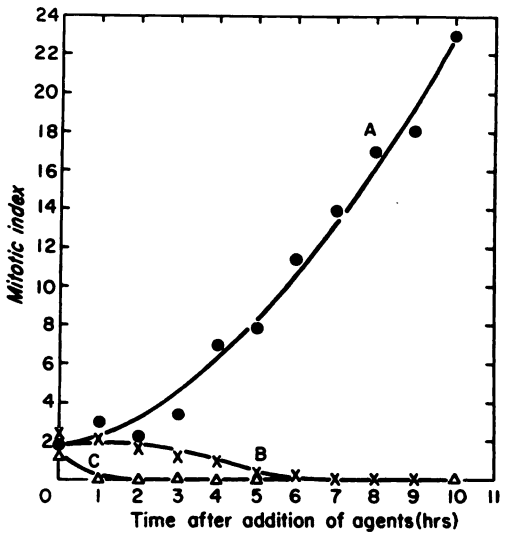

FIG. 2. *Effect of 0.10% PEA on the accumulation of mitotic figures by colcemide*

Curve *A*, 2×10^{-7} g/ml colcemide, no PEA. Curve *B*, 0.10% PEA plus 2×10^{-7} g/ml colcemide. Curve *C*, 0.10% PEA only.

only cells seen in mitosis after this time appeared to be those which were already in mitosis at the time PEA was added. The results shown as curve *C* indicate that cells already in M at the time of addition of PEA are still able to complete mitosis, or the cells disintegrate and are lost for counting as mitotic figures.

Absence of Accumulation of PEA-Treated Cells in the G2 Phase

The results presented in Fig. 2 indicated that the progress of cells from G2 into M was arrested by PEA. Results presented earlier (Fig. 1) raised the possibility that at least some cells may be able to complete DNA synthesis and enter G2 in the presence of PEA. If this were to occur, these cells would be expected to accumulate in G2, since their progress out of G2 into M would be inhibited. Alternatively, the residual DNA synthesis which occurs in PEA-treated cultures over the first day after exposure of the cells to PEA (curve *C*, Fig. 1; and Fig. 6, ref. 6) may not be sufficient to permit cells to synthesize a complete complement of DNA, and they may be arrested

in S in a state of incomplete DNA replication.

It is possible to distinguish between these two alternatives by determining the number of cells in G2 in cultures exposed to PEA for at time equivalent to the time required for the residual DNA synthesis to drop to negligible values. From curve *C* of Fig. 1, this time is approximately 24 hr. The proportion of cells in the culture which were in G2 at this time was estimated as follows.

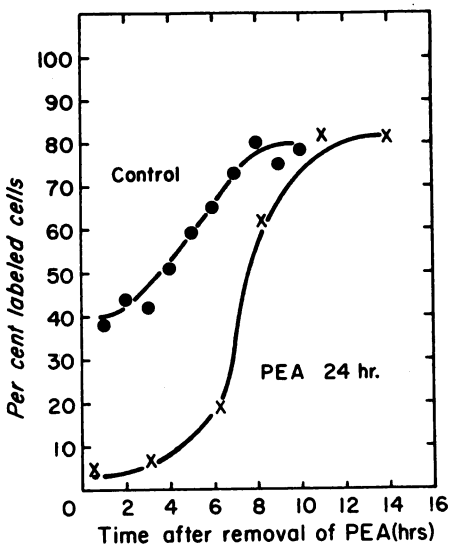

FIG. 3. *Labeling of cells with ³H-TdR after removal of PEA*

Cells were exposed to 0.10% PEA for 24 hr. After removal of the PEA, ³H-TdR at 0.1 μC/ml and colcemide at 2×10^{7} g/ml were added to the cultures.

After a 24-hr exposure to PEA, the cells were washed free of PEA and incubated for 14 hr in the presence of ³H-TdR and colcemide. Under these conditions, the only cells in the culture which would remain unlabeled would be those which were in G2 at the time the PEA was removed from the culture. These cells would be prevented by the colcemide from continuing around the cell cycle and entering S. All other cells would pass through at least a portion of S and would become labeled. The percentage of labeled cells as a function of time in this experiment is shown in Fig. 3. After a delay of 4–5 hr, this percentage increased from the low value characteristic of cells inhibited for 24 hr with PEA (see Fig. 1, curve *C*) to a maximum of 80%. Thus, the 20% of the cells in the culture which remained unlabeled, represented the cells that were in G2 and M at the time of removal of PEA from the culture. This percentage corresponds closely to the percentage of cells in G2 and M in a normal exponentially multiplying culture of L cells with a generation time of 20 hr (8).

It may be concluded that this experiment failed to provide any evidence for an accumulation of cells in the G2 phase in a PEA-treated culture. It seems likely, therefore, that in the presence of PEA, S-phase cells are unable to complete DNA synthesis, and are arrested in a state of partial DNA replication.

Relative Numbers of Cells in G1 and G2, Determined by Microspectrophotometry

The results presented in Figs. 1 and 3 indicate that little progression of cells from G1 through S and into G2 occurs in PEA-treated cultures of L cells. If these findings are correct, it would be expected that no detectable increase would occur in the number of cells in G2 relative to the number in G1 after treatment of a cell population with PEA. This prediction was tested as follows: An exponentially multiplying cell population was pulse-labeled with ³H-TdR and then exposed to 0.10% PEA. Hence, at the time of addition of PEA, all unlabeled cells were either in G1 or in G2. Since G2 cells have a DNA content double that of G1 cells, it is possible to distinguish between cells in these two phases of the cell cycle on the basis of their content of DNA, measured by microspectrophotometry (9). Any progression of cells from G1 through S and into G2 in the presence of PEA would appear as an increase in the number of cells with the higher DNA content relative to those with the lower DNA content. The results are presented in Fig. 4 for various durations of exposure of the cell population to PEA.

The data are presented in the form of histograms which show the relative numbers of unlabeled cells possessing a given

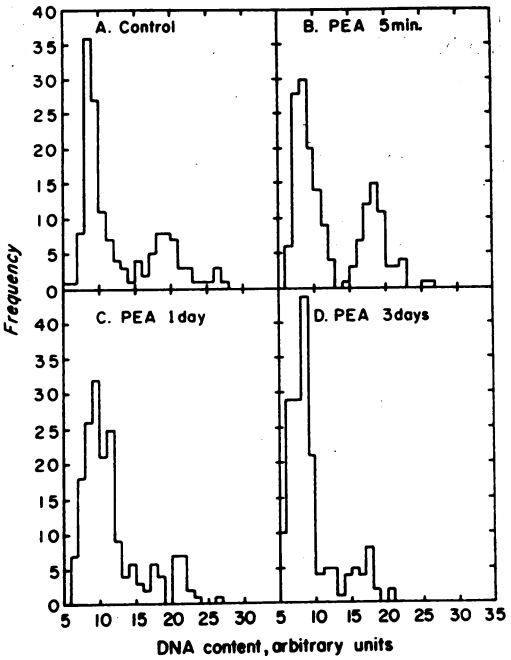

FIG. 4. *Effect of 0.10% PEA on the DNA content of individual cells*

Cultures were pulse-labeled with 1.0 μC/ml of ^{3}H-TdR for 60 min and then exposed to 0.10% PEA for various periods of time. The DNA content of unlabeled cells in autoradiographs was determined by microspectrophotometry (9).

DNA content, measured in arbitrary units for DNA per cell. Two peaks are apparent in the distribution for the untreated control (A) and for the culture exposed to PEA for only 5 min (B). The peak at approximately 9 units of DNA per cell corresponds to the G1-phase cells and represents 60% of the unlabeled cells. The peak at approximately 18 units of DNA per cell corresponds to the G2-phase cells and represents the remainder of the unlabeled cells. Comparison of the results obtained after 1 and 3 days of exposure of the cells to PEA (histograms C and D) with those obtained immediately before or after addition of PEA to the culture (histograms A and B) shows that no increase in the G2-phase subpopulation occurred relative to the G1-phase subpopulation. In fact, the opposite result was obtained, in that the relative number of cells in G2 appeared to decrease in populations exposed to PEA for periods of a day or longer. This loss of G2-phase cells in this experiment could have been due to a very low rate of residual cell division in the cultures, analogous to the residual DNA synthesis seen in cultures treated with 0.10% PEA (6) or to a preferential loss of G2-phase cells from the suspension cultures due, for example, to mechanical damage. In either case, these results indicate that no detectable numbers of cells moved from G1 through S into G2.

Partial Synchronization of G1-Phase Cells after Removal of PEA

Since cells in the presence of PEA are unable to initiate DNA synthesis (Fig. 1) but appear to be capable of appreciable synthesis of RNA and protein (6), it was possible that PEA-treated cells might accumulate at the end of the G1 phase immediately prior to the S phase. This type of accumulation has been shown to occur in FUdR-inhibited cultures (7), in which DNA-synthesis is inhibited, but RNA and protein synthesis continue.

An experiment was carried out to test for an accumulation of cells in G1 immediately prior to S, in PEA-treated cultures. Cells were pulse-labeled with ^{3}H-TdR so that cells that were in the S-phase could be identified subsequently. PEA was then added to a concentration of 0.10%. At various times after this, the PEA was removed from the cultures, and further ^{3}H-TdR, along with colcemide, was added. Under these conditions, an increase in the percentage of labeled cells would be the result of a progression of cells from G1 into S. Cells in G2 at the time of removal of PEA would be prevented from entering S due to the presence of colcemide. If the cells in G1 in the presence of PEA were poised ready to initiate DNA synthesis, then these cells should become labeled very rapidly after removal of PEA and addition of ^{3}H-TdR. The results are shown in Fig. 5. Figure 5A shows the results obtained for the control culture not exposed to PEA. From an initial value of 40% labeled cells, representing the proportion of cells in S at the time of pulse-labeling with ^{3}H-TdR, the percentage of labeled cells increased to a

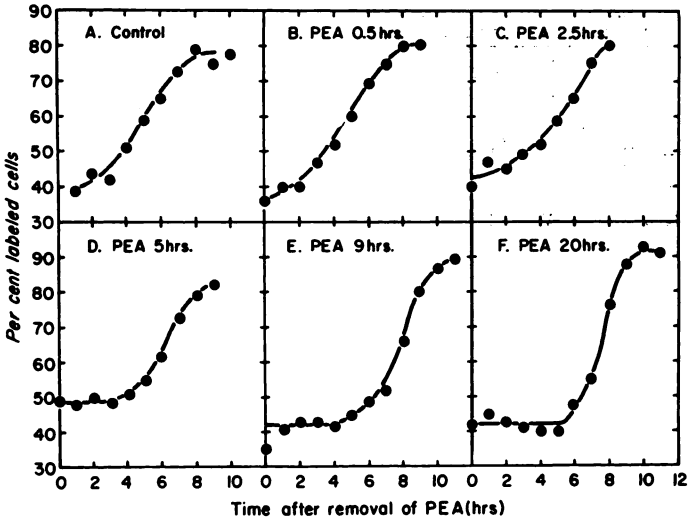

FIG. 5. *Delayed resumption of DNA synthesis in PEA-treated cells*

Cells were pulse-labeled with 1.0 μC/ml of ^{3}H-TdR to label S-phase cells prior to the addition of 0.10% PEA. PEA was removed after the times indicated, and the cells were exposed continuously to ^{3}H-TdR at 0.1 μC/ml to label cells entering S from G1. Colcemide was present at 2×10^7 g/ml to prevent progress of G2-phase cells into G1.

maximum of 80% after 8 hr. This increase was due to the entry of cells into S from G1. The remaining 20% of the population which did not become labeled was trapped in G2 by the action of colcemide (Fig. 3). In cells exposed to PEA for 20 hr (Fig. 5F), a different result was obtained. For a period of 5 hr after removal of the PEA, the percentage of labeled cells did not increase above the initial value of 40% which resulted from the labeling of S-phase cells prior to addition of the PEA. After the 5-hr delay, the percentage of labeled cells increased rapidly, and attained a value of 90% over the succeeding 5-hr period. The remaining 10% of the cells which did not become labeled probably represented a partially depleted G2-phase population (Fig. 4) which was prevented from proceeding around the cycle because of the presence of colcemide.

This result demonstrates that a partial synchronization of cells occurred in the G1 phase in the presence of PEA, but that the entry of this partially synchronizated population into the S phase was delayed for 5 hr after removal of PEA from the culture. Cells exposed to PEA for periods shorter than 20 hr (Fig. 5 B–E) showed shorter delays in entering S, and decreased partial synchronization.

Partial Synchronization of Cell Proliferation after Removal of PEA

The results given in Fig. 5 showed that the entry of cells from G1 into S was delayed for about 5 hr after removal of PEA from a culture which had been exposed to the inhibitor for 20 hr. A similar delay in the recovery of DNA synthesis for both G1 and S cells was seen in experiments described above (Fig. 3), in which the percentage of labeled cells began to increase toward normal values only 4–5 hours after removal of PEA from cultures exposed to 0.10% PEA for 24 hr. Since both the initiation of DNA synthesis in G1 cells and the resumption of DNA synthesis in S cells were delayed following removal of PEA from 24-hr inhibited cultures, it was of interest to examine the progress of cells into mitosis under similar conditions. Cells were treated with 0.10% PEA for 24 hr and then washed free of PEA. Colcemide was added immediately after removal of the PEA, and successive cell samples were

taken for mitotic index determinations. The results are shown in Fig. 6A. No mitotic figures were observed for 2 hr after removal of PEA. The mitotic index then increased sharply to approximately 15% and remained constant until about 13 hr, when it began to increase again, reaching a value of 85% at 19 hr after removal of the PEA.

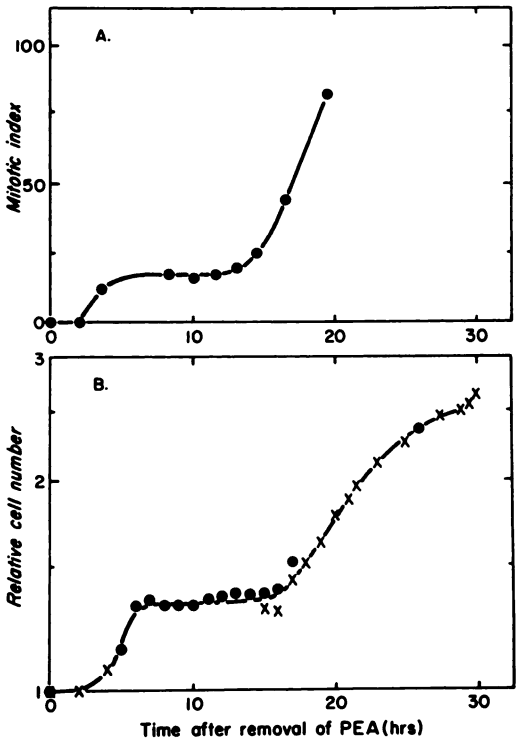

FIG. 6. *Partial synchronization of cell growth in cultures treated with PEA*

Cells were exposed to 0.10% PEA for 24 hr. (A) Mitotic index in the presence of 2×10^{-7} g/ml colcemide, added after removal of PEA. (B) Cell counts, relative to those obtained immediately after removal of PEA. Results of 2 experiments.

Since it was known that G1- and S-phase cells were delayed after removal of PEA, and since the early increase in the mitotic index corresponds approximately to the expected proportion (20%, see Fig. 3) of cells in the G2 phase after a 24-hour exposure to PEA, it was assumed that the cells that were in G2 at the time of removal of PEA entered mitosis with relatively little delay. It is likely that the rise in mitotic index between 13 and 19 hr was due to the arrival at mitosis of cells that were in S or G1 at the time PEA was removed from the culture. The degree of synchronization of G1 cells (Fig. 5F) partly accounts for the rapidity of the increase in the mitotic index.

The increase in cell number as a function of time after removal of PEA from a culture exposed to 0.10% PEA for 24 hr is shown in Fig. 6B. In this experiment, no colcemide was added to the culture. It may be seen that the cell number increased by about 25% between 2 and 6 hr after removal of PEA, then remained constant between 6 and 15 hr, and finally increased by a factor of 2 between 15 and 30 hr after the PEA was removed from the culture.[3] Thus, the results shown in Fig. 6B are in good agreement with those shown in Fig. 6A. It may be concluded that PEA produced a residual effect which delayed the progress of G1- and S-phase cells around the cycle after its removal from the culture, and that little residual effect of PEA on G2-phase cells occurred.

DISCUSSION

The results presented in this paper show that the inhibition of DNA synthesis in mouse L cells by 0.1% PEA is not solely responsible for the suppression of cell proliferation, since PEA also inhibits the entry of cells from the G2-phase into mitosis (Fig. 2). This latter effect is not obviously related to the action of PEA on DNA synthesis, though it is still possible that PEA acts on a biosynthetic pathway common to both DNA synthesis and mitosis. For example, both processes may be inhibited as a consequence of the alteration in the rate of the protein synthesis which occurs in the presence of PEA (6).

Previous studies have shown that inhibitors of cell proliferation may accumulate cells at a particular point in the cell cycle. For example, cells treated with 5-fluoro-2′-deoxyuridine (FUdR) accumulate in G1 immediately prior to S (7), cells in the

[3] No such synchronization of cell growth was apparent in control cultures not treated with PEA (see Fig. 2).

presence of colcemide accumulate in mitosis (11) and cells exposed to ionizing radiation accumulate in the G2 phase (12). Under circumstances where recovery from inhibitory effects takes place, the accumulation of cells in one phase of the cycle may result in a degree of synchronization of subsequent progress through the cell cycle. Although a partial synchronization of the cell population was seen in PEA-treated cultures after removal of the PEA, (Figs. 3 and 6), this synchronization appears to have been mainly due to a delayed recovery of cells in the G1 and S phase, rather than to an accumulation of cells in either of these phases.

Studies of the effects of PEA on bacteria have indicated that the inhibitor acts by preventing the initiation of DNA synthesis. Once synthesis of a molecule of DNA is successfully initiated, it appears that synthesis of that molecule can be completed even in the presence of PEA (13). Inhibition of the initiation of DNA synthesis also occurs in mammalian cells exposed to PEA (Fig. 1). However, since the mammalian chromosome appears to have multiple initiation sites (14), it would be expected that some DNA synthesis could go on in mammalian cells in the presence of PEA, at least until the replication process began to require new initiation sites. At this time, DNA synthesis would cease, and a complete complement of DNA would not be synthesized. This expectation is in accord with the results described above. In order to obtain a more rigorous test of this model for the action of PEA on mammalian cells, it will be necessary to study in detail the mode of replication of DNA on individual chromosomes, in the presence and absence of PEA.

ACKNOWLEDGMENTS

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