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

I. Reversible Inhibition of Cell Proliferation and Effects on the Uptake of Labeled Precursors of Nucleic Acid and Protein

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SUMMARY

β-Phenethyl alcohol (PEA) was found to be an effective inhibitor of the proliferation of mouse L cells in suspension culture. Both its action as an inhibitor and its toxicity were found to be critically dependent on concentration and duration of exposure. The inhibition of proliferation caused by exposure of cells to 0.10% PEA (8.2×10^{-3} M) was almost fully reversible even after periods of exposure to PEA as long as 12 days. This concentration of PEA caused a rapid decrease in the uptake of labeled precursors of DNA, RNA, and protein into mouse L cells. For each precursor, the initial reduction in uptake was complete within 20 minutes after the addition of PEA. Following this drop, there was a residual low level of DNA synthesis, as measured by thymidine-^{3}H incorporation, which depended on the concentration of PEA used. The rate of DNA synthesis remained depressed over the next 5 days in the presence of PEA. The sudden initial depression of uridine-^{14}C and leucine-^{14}C uptake in PEA-treated cells was accompanied by a rapid reduction in the number of cytoplasmic polyribosomes with a concomitant increase in the number of single ribosomes. In the continuing presence of PEA, the depression of uridine-^{14}C and leucine-^{14}C uptake was succeeded by a transient recovery to higher levels of incorporation, reaching a maximum about 10 hours after the addition of PEA.

These results indicate that PEA is not a specific inhibitor of DNA synthesis in mammalian cells.

INTRODUCTION

β-Phenethyl alcohol (PEA) has been found to inhibit the proliferation of bacteria (1, 2), bacterial and animal viruses (3–6), molds (7), plants (8), and mammalian cells (9). Concentrations of PEA that arrest the proliferation of microbial cells have also been observed to affect the synthesis of cellular macromolecules. In their initial studies on this agent, Berrah and Konetzka (2) found that a concentration of 0.25% PEA, which stopped growth and inhibited DNA synthesis in *E. coli*, caused no apparent change in RNA or protein synthesis. Similarly, on the basis of their studies of the effects of 0.5% PEA on mouse L cells, Leach *et al.* (9) concluded that the major site of action of PEA is concerned with DNA. In contrast, recent evidence from the work of Rosenkranz, Carr and Rose (10), Lester (7), and Prevost and Moses (11) has drawn attention to the fact that PEA-induced inhibition of microbial growth is almost certainly accompanied by effects on RNA and protein synthesis be-

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sides those on DNA synthesis. It was therefore of interest to determine whether or not PEA could cause a specific depression in the rate of DNA synthesis in mammalian cells, in the absence of any concomitant effects on RNA or protein synthesis. The results obtained indicate that although the major effect of 0.10% PEA on L cells is to inhibit DNA synthesis, prompt, transient effects on protein and RNA synthesis are also observed. These results provide further support for the view that the action of PEA is not simply to cause a specific inhibition of DNA synthesis.

METHODS

A subline, L 60, of Earle's L cells, conditioned to growth in medium CMRL 1066 (12) lacking thymidine and coenzymes, was used in all experiments. The medium was supplemented with 10% horse serum (Connaught Medical Research Laboratories, Toronto) and antibiotics. Cells were maintained in the exponential growth phase, in spinner flasks (O. H. Johns Glass Co., Toronto), or in roller tubes (13) at 37°.

Cell concentrations in the spinner flasks were determined with a Coulter particle counter (Coulter Electronics, Hialeah, Florida). Under optimal conditions, the cell population doubled in number every 15–20 hr. For determinations of colony-forming ability, appropriate numbers of cells were deposited in 60-mm plastic petri dishes (Falcon Plastics, Los Angeles, California) containing CMRL 1066 and 20% horse serum (12). Prior to the latter step, samples of cells taken from PEA-treated cultures were diluted approximately 10^3- to 10^5-fold so that the resultant drug concentration had negligible effects on cell growth. After an incubation period of 10–14 days at 37°, the colonies were stained with a 0.2% methylene blue solution and counted.

PEA was obtained from Matheson, Coleman and Bell, Cincinnati, Ohio. The lots of PEA used were tested for the presence of impurities (14), an F and M Model 810-29 Analytical Gas Chromatograph being used, and were found to contain 0.1–0.2% phenylacetaldehyde plus smaller quantities of an unidentified second contaminant. Because PEA was diluted approximately 1000-fold for use in experiments reported here, the concentration of phenylacetaldehyde to which the cells were exposed was in the vicinity of 0.0001%. It was found that at least 10-fold greater concentrations of phenylacetaldehyde were required to produce any detectable effects on the proliferation of L cells.

The rates of incorporation of thymidine-methyl-^{3}H (^{3}H-TdR), uridine-2-^{14}C (^{14}C-UR) or uridine-6-^{3}H (^{3}H-UR) and L-leucine-^{14}C (^{14}C-leucine) were used to determine rates of DNA, RNA, and protein synthesis, respectively. For use in measuring cellular uptake of radioactive leucine, medium was prepared in which the leucine content was reduced to one-fifth of the amount usually present. Cells growing in the thymidine-free medium routinely used in all experiments were transferred to this modified medium before the beginning of each incubation period.

The following radioisotopes were purchased from the New England Nuclear Co., Boston: thymidine-methyl-^{3}H (specific activity 6.7 C/mmole); uridine-2-^{14}C (24.2 mC/mmole); and L-leucine-^{14}C (24.7 mC/mmole). Uridine-6-^{3}H (7.53 C/mmole) was purchased from the Radiochemical Centre, Amersham, England. Two-milliliter samples of cells were exposed to these radioactive precursors for a short period lasting from 5 min to 1 hr, and the incubation was terminated by rapidly cooling the cell samples to 4°. The cells were subsequently collected on Millipore filters (Millipore, Inc., Montreal, 0.45 μ pore size) and washed with 5 ml of cold phosphate-buffered saline (PBS) (12) and with 10 ml cold (4°) 5% trichloroacetic acid. The filters were then transferred to counting vials, dried, immersed in 10–15 ml of scintillation fluid which contained 6 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per liter of toluene, and counted on a Nuclear Chicago model 722 scintillation counter.

In one experiment, grain counts were used to measure ^{3}H-UR incorporation into PEA-treated cells. At the beginning of the experiment PEA was added to the cells at a

final concentration of 0.10% v/v (8.2×10^{-3} M). At intervals thereafter, samples were removed from the culture and incubated at 37° for 30 min with ^{3}H-UR (final concentration, 2 μC/ml). The cells were then prepared for autoradiography according to methods previously described (15). Grains were counted over 100 cells on each slide preparation.

The methods used to investigate changes in cytoplasmic polyribosome formation induced by PEA were as follows (16): Cells in the exponential growth phase were sedimented by centrifugation (1500 rpm for 10 min) and resuspended in a reduced volume of medium CMRL 1066 lacking thymidine, coenzymes, and leucine. The cell concentration was approximately 2.5×10^{5} cells per milliliter. After a 15-min conditioning period in this medium, sufficient PEA was added to provide a final concentration of 0.15%. The cells were then exposed to the PEA for periods lasting from 1 to 5 min, and ^{14}C-leucine (0.5 μC/ml final concentration) was introduced into the culture for the final minute of each exposure interval. Control cultures received ^{14}C-leucine only. Rapid arrest of the incubation was accomplished by pouring the spinner cultures into ice-cold PBS. The cells were then sedimented (1500 rpm for 10 min at 0°), and the cell pellet was resuspended in 30 ml of ice-cold PBS. This procedure was repeated 3 times. The final pellet was resuspended in 1.5 ml of hypotonic reticulocyte standard buffer (RSB) (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.1 M Tris-HCl, pH 7.4) and allowed to stand at 4° for 0.5 hr. After this interval, 0.15 ml of a 5% solution of Brij-58 (Atlas Chemical Industries, Inc., Wilmington, Delaware) was added to the cell preparation, and cells were ruptured in a Dounce homogenizer (10 strokes). Cell nuclei were sedimented out of suspension (1000 rpm, 15 min, at 4°) in a clinical centrifuge. Sodium deoxycholate (Matheson, Coleman and Bell, Cincinnati, Ohio) was added to the cytoplasmic extract (final concentration 0.5%). A 1.5-ml aliquot was layered on 13 ml of a preformed 30–15% (w/v) linear sucrose gradient. The gradient was centrifuged for 120 min at 24,000 rpm at a setting of 25°F using a black S.W. 25.1 rotor in a Spinco Model L ultracentrifuge. A peristalsis pump was used to withdraw the gradient from the bottom of the centrifuge tube. The material was passed through an absorbance recorder (Model 2000, Gilford Instruments, Oberlin, Ohio), which provided a continuous tracing of the absorbance at 260 mμ. Successive fractions of approximately 1 ml each were collected as the gradient emerged from the absorbance recorder. One drop of 0.05% bovine serum albumin was added to each fraction shown by the absorbance measurements to contain polyribosomes. The RNA was precipitated with cold (4°) 10% trichloroacetic acid. Further processing for the counting of radioactivity was the same as for the whole-cell preparations described above.

RESULTS

Toxicity of PEA for Mouse L Cells in Suspension Cultures

A preliminary test of the toxicity of PEA for L cells grown in suspension cultures was carried out as follows: Four separate spinner flask cultures were each treated with a PEA concentration of 0.08%, 0.12%, 0.16%, or 0.20% v/v. One additional culture served as an untreated control. At various times after the addition of PEA, 1-ml samples of cells were removed from these cultures, diluted 1000-fold, and tested for colony-forming ability. The results of this experiment are shown in Fig. 1. Each point represents the average of the number of colonies on 2 dishes. The average number of colonies formed by cells from the control culture doubled in 15 hr. The number of colonies formed by cells from the treated cultures remained constant for several hours, depending on the concentration of PEA. The number of colonies formed subsequently fell in cultures treated with 0.12, 0.16, and 0.20% PEA while a small increase in colony number was observed in the cultures treated with 0.08% PEA. These results indicated that relatively little loss of colony-forming ability would be expected to occur in cultures exposed to 0.10% PEA.

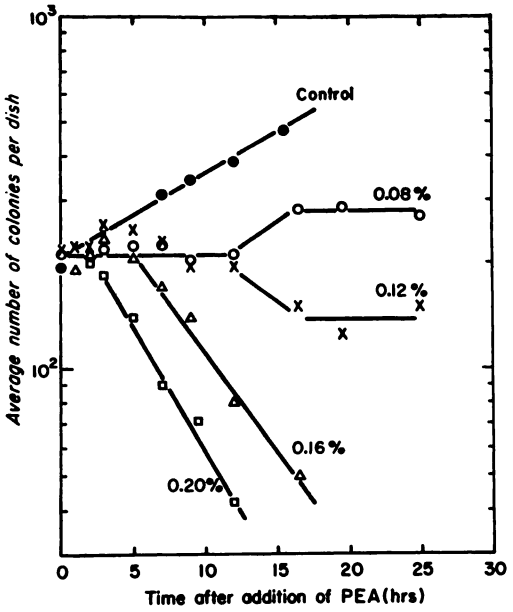

FIG. 1. *Effects of various concentrations of PEA on the colony-forming ability of L cells in suspension culture*

Cells were diluted 1000-fold prior to test. Each point represents the average number of colonies on 2 dishes, each of which received 1 ml of the diluted culture.

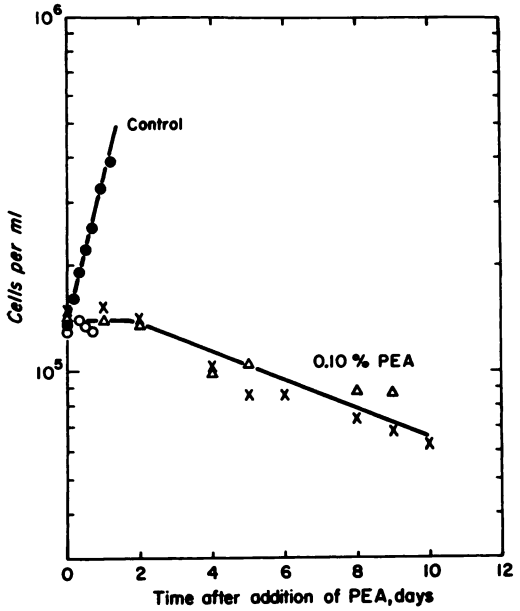

FIG. 2. *Inhibition of cell growth by 0.10% PEA*

Inhibition of Cell Growth by 0.10% PEA

On the basis of the results described above, a test was made of the inhibition of cell proliferation in cultures exposed for

TABLE 1

Colony-forming ability as a function of time for L cells treated with phenethyl alcohol

Time (days)	Experiment 1			Experiment 2		
	Plating efficiency,[a] untreated control[b]	Plating efficiency,[a] 0.10% PEA	Per cent survival	Plating efficiency,[a] untreated control	Plating efficiency,[a] 0.10% PEA	Per cent survival
0	32	32	100	98	90	92
1	100	81	81	72	86	120
2	82	71	87	132	84	64
3	37	16	43	113	91	80
4	68	51	75	75	76	101
5	55	49	89	98	95	97
6	40	25	63	69	79	113
7	97	61	63	93	92	99
8	85	74	87			
9	82	79	97			
10	32	22	69			
11	62	51	82			
12	68	59	87			

[a] Number of colonies formed per 100 cells tested. Mean values for 6–10 dishes, each of which received an average of 100 cells

[b] Maintained in the exponential growth phase and not exposed to PEA.

various periods of time to a concentration of PEA of 0.10%. The results obtained are presented in Fig. 2 and Table 1. It is apparent from Fig. 2 that the cell concentration remained constant for the first 2 days of exposure of the cells to 0.10% PEA, and then decreased slowly over an 8-day period, probably as a result of mechanical disruption of the cells in the spinner cultures. Table 1 gives values for the percentage survival of colony-forming ability of cells exposed to 0.10% PEA, obtained by dividing the plating efficiency of cells treated with PEA by the plating efficiency of cells from an untreated control culture. It is apparent that little loss of colony-forming ability occurred in the treated cells, even after the cells had been exposed to PEA for periods as long as 12 days. Thus, 0.10% PEA effectively arrests the proliferation of L cells in suspension culture, and the arrest of proliferation is almost fully reversible even after extended exposure of the cells to this concentration of the agent.

Inhibition of the Incorporation of Labeled Precursors of DNA, RNA, and Protein into PEA-Treated Cells

The results presented above show that PEA at a final concentration of 0.10% v/v reversibly arrests the growth of suspension cultures of mouse L cells. To test for the effects of this concentration of PEA on the synthesis of DNA, RNA, and protein by L cells, the incorporation of ^{3}H-TdR, ^{3}H-UR, and ^{14}C-leucine into PEA-treated cells was examined. Successive cell samples were removed from PEA-treated and untreated control spinner cultures and labeled by a brief exposure to one of the labeled precursors.

The duration of the labeling was 10 min for ^{3}H-TdR and ^{3}H-UR, and 5 min for ^{14}C-leucine. Labeling was terminated and the radioactivity incorporated into acid-insoluble material was determined, as described under Methods. The results obtained are shown in Fig. 3. Figure 3A shows that the uptake of ^{3}H-TdR decreased to a value about one-sixth of the control value within 20 min after addition of PEA to the culture. Figures 3B and 3 C show that even more rapid decreases in the rates of incorporation of ^{3}H-UR and ^{14}C-leucine occurred, minimum levels of uptake being reached in both cases approximately 10 min after the addition of PEA. It is apparent from these results that PEA inhibits not only the uptake of ^{3}H-TdR, but ^{3}H-UR and ^{14}C-leucine as well, so the inhibitory effects of 0.10% PEA are not specific for DNA synthesis alone.

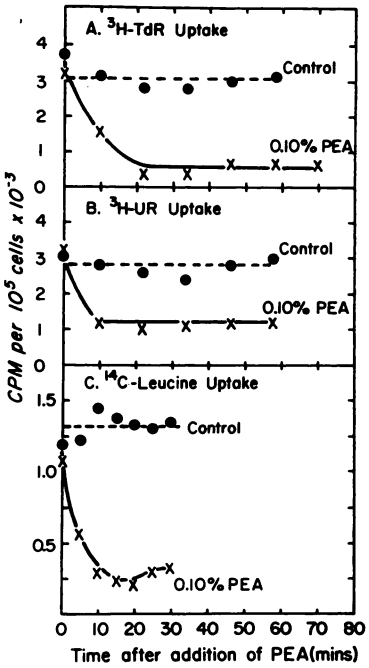

FIG. 3. *Effect of 0.10% PEA on the uptake of labeled precursors of DNA, RNA, and protein*

Samples from the cultures were pulse-labeled as follows: (A) ^{3}H-TdR, 10 min at 4 μC/ml; (B) ^{3}H-UR, 10 min at 8 μC/ml; (C) ^{14}C-leucine, 5 min at 0.4 μC/ml. The points are plotted at the time corresponding to the end of the labeling interval. The broken lines indicate mean control values.

Effects of Longer Exposures to PEA on the Uptake of Labeled Precursors

Since the inhibition of cell proliferation by 0.10% PEA was found to be reversible after periods of exposure to PEA as long as 12 days (Table 1), it was of interest to determine whether or not the depression of incorporation of labeled precursors of DNA, RNA, and protein shown in Fig. 3 persisted

over longer periods of time. Accordingly, cells were tested for their ability to incorporate labeled precursors at various times during a 5-day exposure to 0.10% PEA.

At various times after the addition of PEA, cell samples were removed from treated and untreated cultures and incubated with the appropriate labeled precursor for 30 or 45 min. The untreated control culture was diluted daily with fresh growth medium to maintain it in the exponential growth phase for the duration of the experiment. The results obtained are given in Fig. 4. It may be seen from Fig. 4A

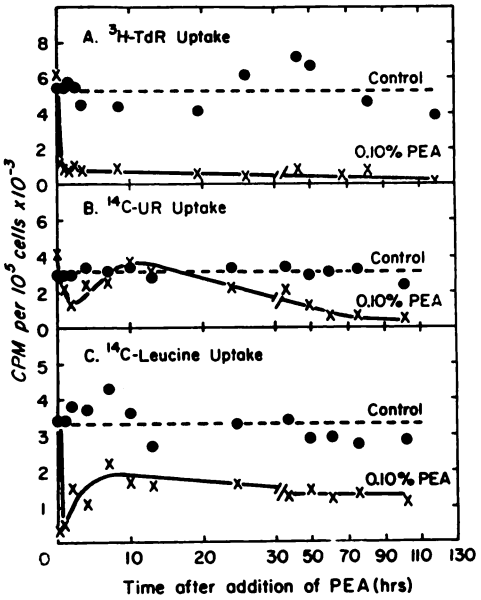

FIG. 4. *Effect of prolonged exposures to 0.10% PEA on the uptake of labeled precursors*

Samples from the cultures were pulse-labeled as follows: (A) ³H-TdR, 30 min at 2 μC/ml; (B) ¹⁴C-UR, 45 min at 0.4 μC/ml; (C) ¹⁴C-leucine, 45 min at 0.4 μC/ml. The broken lines indicate mean control values.

that, as before, the uptake of ³H-TdR decreased abruptly during the first 30 min of exposure of the cells to PEA. This reduced rate of incorporation of ³H-TdR persisted throughout the entire duration of the experiment. Fluctuations in residual uptake have been observed in different experiments and probably are related to small variations in dosage of PEA, since even minor variations in PEA concentration can have a profound effect on the rates of uptake of ³H-TdR (Fig. 6).

Very different results were obtained when the incorporation of the other two labeled precursors was studied (Figs. 4B and 4C). In each case, the initial rapid decrease in rate of incorporation was followed by a return toward normal values, reaching a maximum approximately 10 hr after addition of PEA. In the case of ¹⁴C-UR uptake (Fig. 4B), the maximum rate of incorporation at 10 hr was equal to the control values. After 10 hr, the rate of uptake of ¹⁴C-UR and ¹⁴C-leucine gradually decreased, approaching a second minimum 80–100 hr after the addition of PEA to the cultures. The secondary decrease was more pronounced in the case of ¹⁴C-UR uptake (Fig. 4B) than ¹⁴C-leucine uptake (Fig. 4C). These results show that the initial reduction in ³H-UR and ¹⁴C-leucine uptake does not last for more than 2 hr after the addition of PEA. During the next 8–10 hr the incorporation of both precursors increases; after this time it begins to decline again. No such transient increase in ³H-TdR uptake was seen in the continuing presence of PEA.

Autoradiography of ³H-UR Labeled Cells

It was possible that the transient decrease and increase in ¹⁴C-UR incorporation observed during the first 10 hr after the addition of PEA (Fig. 4B) was limited to only a portion of the cell population, and that, in some of the cells, a normal rate of uridine uptake persisted throughout this interval. To test this possibility, ³H-UR uptake into PEA-treated cells was examined by autoradiographic methods. Counts were made of the numbers of grains per cell in autoradiographs prepared from cells exposed to PEA for different periods of time and then labeled for 30 min with ³H-UR. The distributions of grain counts obtained are given in Fig. 5. The frequency distribution for untreated cells (panel A) is reasonably symmetrical about a mean count of 22 grains/cell. In the cells exposed to PEA for 30 min (panel B) the distribution of grain counts is shifted to a

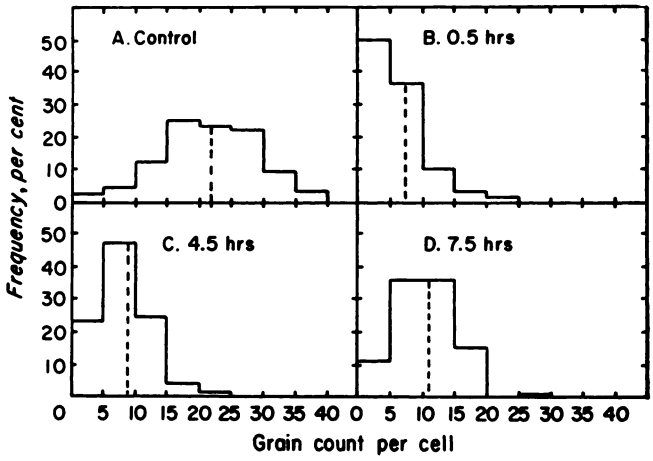

FIG. 5. *Grain count distributions for cells pulse-labeled with ^{3}H-UR after various intervals of exposure to 0.10% PEA*

Cell samples used for autoradiography were taken from PEA-treated cultures which were exposed to 2μC/ml of ^{3}H-UR for 30 min. The broken lines represent the means of the grain count distributions.

range substantially below that of the control cells. For this distribution the mean is 7 grains/cell. A second peak near the mean control count was not found. This indicates that relatively few, if any, cells were present in which ^{3}H-UR uptake was unaffected by exposure to PEA. For the cells exposed to PEA for 4.5–7.5 hours, the mean grain count began to increase toward that found for the untreated control population, as expected from the data of Fig. 4B.

Effect of Various Concentrations of PEA on the Uptake of ^{3}H-TdR

A persistent low level of incorporation of ^{3}H-TdR into PEA-inhibited cells was observed even after prolonged exposure of the cells to 0.10% PEA (Fig. 4A). In order to determine whether or not higher concentrations of PEA would suppress this residual incorporation, the uptake of ^{3}H-TdR was studied in cultures exposed to 0, 0.08, 0.12, 0.16, and 0.20% PEA. Successive cell samples were removed and incubated with ^{3}H-TdR for 30 min. The results are shown in Fig. 6. It may be seen that the extent of the initial decrease in the rate of incorporation of ^{3}H-TdR was greater as the concentration of PEA was increased, and that the differences observed persisted throughout the duration of the experiment.

The results in Fig. 6 indicate that the low level of ^{3}H-TdR incorporation which persists in cells exposed to 0.10% PEA (Fig. 4A) can be almost completely suppressed by increasing the concentration of PEA to 0.20%. Since 0.20% PEA is toxic

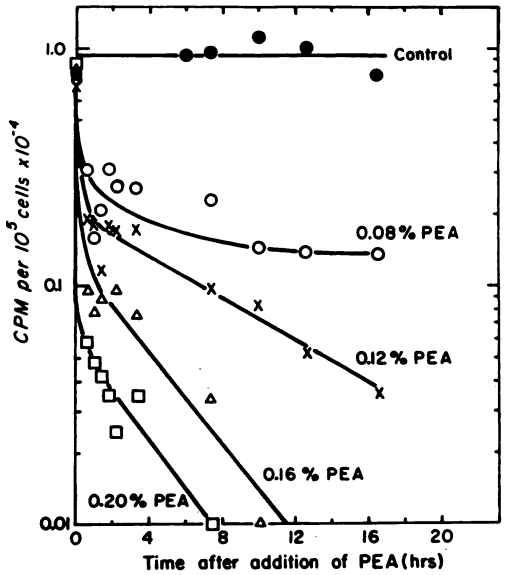

FIG. 6. *Effect of different concentrations of PEA on the incorporation of ^{3}H-TdR*

At successive intervals after addition of PEA to the cultures, cell samples were exposed to a 30-min pulse of ^{3}H-TdR at 2 μC/ml.

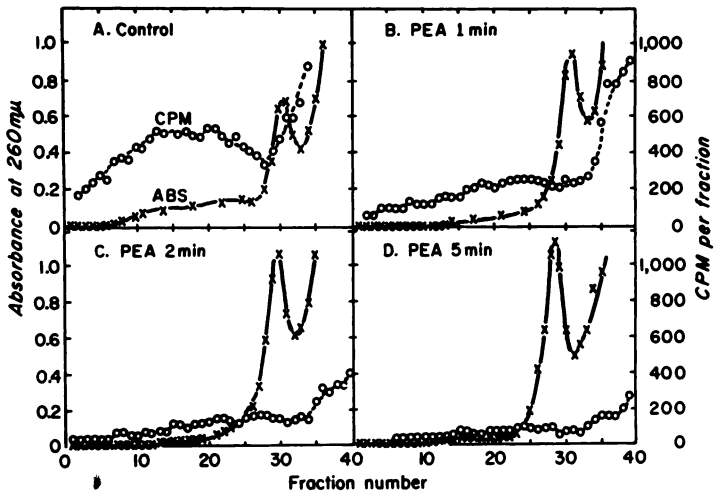

FIG. 7. *Effect of 0.15% PEA on polyribosomes*

Cells were exposed to PEA for various periods of time, ^{14}C-leucine at 0.5 μC/ml being present during the last minute of each period.

for L cells (Fig. 1), it appears that complete suppression of ^{3}H-TdR incorporation is not possible at concentrations of PEA which cause a reversible inhibition of cell proliferation. It is also apparent that substantial differences in the levels of residual DNA synthesis will result from small variations in dose of PEA.

Effects of PEA on Polyribosomes

The occurrence of PEA-induced rate changes in the uptake of ^{14}C-leucine (Figs. 4B and 4C) indicated a possible malfunctioning of the mechanisms involved in protein synthesis. As a first step in the study of the effects of PEA on the protein synthesizing system, cells treated with 0.15% PEA were examined for their content of ribosomes in single and aggregate states. The cells were labeled with ^{14}C-leucine during the final minute of a brief exposure to 0.15% PEA. Cytoplasmic extracts were prepared from treated and untreated cells and subjected to sucrose gradient analysis is described under Methods. Some typical results are presented in Fig. 7. In each panel of Fig. 7, the distributions of ultraviolet light-absorbing material and of ^{14}C radioactivity are shown as a function of the position of the material in the sucrose gradient. Figure 7A shows the results obtained for cells not exposed to PEA. The polyribosomes appeared in fractions 10–27, and the later fractions contained single ribosomes in a symmetrical peak. ^{14}C-Leucine was distributed over the polyribosome region. The radioactive material in the fractions containing single ribosomes was not specifically associated with the monomer absorbance peak and probably represented labeled protein formed during the incubation period.

Figure 7B shows the results obtained for cells exposed to 0.15% PEA for 1 min. The absorbance of the polyribosome region was noticeably decreased and the peak corresponding to single ribosomes was increased. The quantity of radioactive material in the polyribosome region was also substantially reduced. After 2 and 5 min of exposure to PEA (Figs. 7C and 7D), a further reduction in polyribosomes and a further increase in single ribosomes was observed. At these times the amount of radioactivity in the polyribosome region was reduced to a very low level. No label which was specifically associated with the monomer absorbance peak appeared in any of the distributions shown in Fig. 7. These results show that PEA caused a rapid loss of polyribosomes

from the cytoplasm with a corresponding increase in single ribosomes.

DISCUSSION

At an appropriate concentration, PEA is an effective, yet almost fully reversible, inhibitor of the proliferation of L cells in suspension cultures, for periods as long as 12 days. The finding that its effects on cell proliferation are critically dependent on concentration (Fig. 1), is in agreement with the results of Leach *et al.* (9), who found that 0.2% PEA inhibited division in suspension cultures of Ehrlich IIB cells, while 0.1% and 0.05% PEA did not completely arrest cell proliferation. The results presented above do not, however, support the suggestion by these workers (9) that the early major site of action of PEA is concerned with DNA. The concentration of PEA (0.1%) which reversibly inhibited the proliferation of L cells also caused a prompt decrease in the rates of uptake of precursors of RNA and protein as well as a precursor of DNA.

The effects of PEA on the uptake of RNA and protein precursors did differ in at least one important respect from the effect on uptake of the DNA precursor. While the uptake of ^{3}H-TdR was at a low level during the entire duration of the exposure of cells to PEA, the initial depression in the uptake of ^{14}C-UR and ^{14}C-leucine was followed by a transient recovery to higher rates of incorporation (Fig. 4). The occurrence of a similar recovery of RNA and protein synthesis has recently been reported by Prevost and Moses (11) in PEA-treated *E. coli* cells. The basis for this phenomenon is still unclear. It is likely that the inhibitory action of PEA on the uptake of ^{14}C-leucine resulted from an effect on protein synthesis, which in turn was related to the rapid disappearance of polyribosomes from the cytoplasm. The loss of polyribosomes indicates that the reduced uptake of ^{14}C-leucine is not simply the result of an effect remote from protein synthesis such as a change in the kinetics of amino acid utilization from cell pools.

It is not clear whether the inhibition of nucleic acid synthesis by PEA occurs independently, or as a result of, the effect on protein synthesis. Until the relationships between these changes are understood, the action of PEA cannot be attributed with certainty to any single biosynthetic pathway.

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