EFFECT OF ACID pH ON MACROMOLECULAR SYNTHESIS IN L CELLS

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ABSTRACT

Eagle's medium adjusted to pH 6 was found to inhibit the rate of RNA and protein syntheses in monolayer cultures of L cells. Incubation of the cells at pH 6 decreased the rate of incorporation of amino acids into nascent peptide chains and caused a disaggregation of polyribosomes. Messenger RNA seemed to persist during the exposure of the cells to medium adjusted to pH 6, since protein synthesis resumed when the cells were transferred to recovery medium containing actinomycin D. The inhibitory effects of pH 6 on macromolecular synthesis were reversible and the viability of the cells exposed to pH 6 did not decrease. The permeability of the cells was not altered by the exposure to pH 6.

The pH of the solution in which interferon was applied to L cells was shown by Hallum et al., (2) to determine the level of the antiviral activity which developed. Incubation of L cells with phosphate-buffered saline adjusted to pH 6 (PBS-6) resulted in complete inhibition of the protective action of interferon in L cells. Exposure to pH 6 did not inhibit the initial interaction of cells with interferon nor did it influence the maintenance of the antiviral state, once established. However, the rate of protein and RNA syntheses was inhibited by 70% of that observed in cells incubated in PBS-7. The present work investigates the mechanisms involved in the inhibition of macromolecular synthesis in cells exposed to pH 6. The mechanisms investigated are the following: the effect of pH 6 on translation, on changes in the permeability of the cells, and on the stability of cellular mRNA.

MATERIALS AND METHODS

Media

(*a*) Phosphate-buffered saline adjusted to pH 6 or pH 7 was prepared as described by Hallum et al. (2).

(*b*) BME, Basal Medium Eagle (Grand Island Biological Company, New York) (0.85 mM with respect to phosphate), was supplemented with 4% calf serum. The buffering capacity was increased by raising the phosphate salt concentration 30-fold (med. 30 ×). The pH obtained was 6.8 and was adjusted to 7.0 by adding 0.2 ml of 10 N NaOH to 1 liter of med. 30 ×. To obtain med. 30 ×-6, 1.4 ml of concentrated HCl was added to 1 liter of med. 30 × to adjust the pH to 5.8. Within 30 min of incubation of this medium with cells, the pH shifted to 6.0 and was maintained at this level for the time of the experiment. Therefore this buffer is referred to as med. 30 ×-6. The pH was measured at 25°C by the use of a Beckman Zeromatic pH meter calibrated with standard buffers.

(*c*) MEM, Minimal Essential Medium (Grand Island Biological Company, New York), was supplemented with 4% calf serum.

Cells

Monolayer cultures of L cells (clone 929) were grown in 32-oz prescription bottles and maintained as described by Hallum et al. (2). Cells to be incubated at pH 6 or 7 were prepared in 4-oz prescription bottles, each containing 5×10^6 cells in 8 ml of MEM, 24 hr before the experiment. Cells to be used

for polyribosome extraction were subcultured from 7-day-old cells 24 hr before the experiment so that each 32 oz bottle contained 1.2×10^7 cells in logarithmic phase.

Isotopes

L-Amino acid-^{14}C mixture in 0.1 N HCl solution was purchased from New England Nuclear Corp., Boston, Mass. The mixture contained the following amino acids; SA in mCi/mmole is shown in parentheses: L-alanine (120), L-arginine (240), L-aspartic acid (160), L-glutamic acid (200), glycine (80), L-histidine (240), L-isoleucine (240), L-leucine (240), L-lysine (240), L-phenylalanine (360), L-proline (200), L-serine (120), L-threonine (160), L-tyrosine (360), and L-valine (200). 5-Uridine-^{3}H with an SA of either 28 Ci/mmole, or 20 Ci/mmole was purchased from Schwarz Bio Research Inc., Orangeburg, N. Y.

Radioactivity Assays

INCORPORATION OF LABELED PRECURSORS INTO INTACT CELLS: Isotopes were diluted in PBS-6 or PBS-7 to the desired concentrations. Amino acid-^{14}C mixture was diluted to 1 μCi/ml and uridine-^{3}H was diluted to 2 μCi/ml. Cultures in 4-oz bottles were drained and incubated at 37°C with 2 ml of uridine-^{3}H solution for 20 min. During the last 3 min of this incubation 2 ml of amino acid-^{14}C solution were added. Incorporation was stopped by adding cold PBS-6 or PBS-7 and placing the bottles in an ice bath. The cells were scraped from the glass with a rubber policeman and centrifuged for 10 min at 1500 rpm in the angle head of an International (PR-2) refrigerated centrifuge. The cell pellet was suspended in 5 ml of cold PBS-6 or PBS-7, recentrifuged, drained, and resuspended in 2 ml of PBS-6 or PBS-7. The cells were disrupted by treatment for 8 min at max amperage in a Raytheon 10-kc sonic oscillator (Raytheon Co., Waltham, Mass.), model DF-101. After precipitation with 10% trichloroacetic acid (TCA) the insoluble material was collected by filtration, and assayed for radioactive counts.

DETERMINATION OF TOTAL COUNTS AND TCA-SOLUBLE COUNTS IN INTACT CELLS: Total counts were determined by placing a sample of the disrupted cell preparation into a scintillation vial and adding 10 ml of Bray's solution. Soluble counts were determined by assaying the supernatant obtained after centrifugation of a solution containing 1 ml of disrupted cell preparation and 1 ml of 10% TCA in the angle head of an International (PR-2) refrigerated centrifuge at 2000 rpm for 15 min. A sample of the supernatant was placed into a scintillation vial and 10 ml of Bray's solution was added.

DETERMINATION OF AMINO ACIDS-^{14}C IN CYTOPLASMIC EXTRACTS: Amino acids-^{14}C were diluted in PBS-6 or PBS-7 to a concentration of 2 μCi/ml. A volume of 5 ml was used for each 32 oz bottle of L cells, and three bottles were used per sample. The cells were labeled for 3 min at 37°C and the incorporation was stopped as described above. Cells were collected, washed, and fractionated on sucrose gradients as described below. The TCA-precipitable material in each fraction was collected and assayed for radioactive counts.

LABELING OF RIBOSOMAL RNA: Uridine-^{3}H was diluted in MEM to a concentration of 5 μCi/ml. Monolayers of 1-day-old L cell cultures containing 1.4×10^7 cells per 32 oz bottle were divided into two 32-oz bottles containing 10 ml of labeled MEM and 40 ml of unlabeled MEM and incubated at 37°C for 48 hr (2 generations). The cells were washed twice with med. 30 ×-6 or med. 30 ×-7 to free them of residual radioactivity in the medium, and were further incubated for 0.5, 2, or 5 hr in either med. 30 ×-6 or med. 30 ×-7. Cells were scraped and fractionated as will be described later. TCA-precipitable material was collected and assayed for radioactivity in each fraction.

All the radioassays were carried out in a Tri-Carb liquid scintillation spectrometer model 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.).

Fractionation of the Cells and Isolation of Polyribosomes

Polyribosomes were isolated by a modification of the method of Scharff (4). Monolayers of L cells ($1.0–1.2 \times 10^7$ cells per 32 oz bottle) were incubated for different periods of time with med. 30 ×-6 or med. 30 ×-7, and labeled for 3 min with amino acid-^{14}C mixture as previously described. Cells were scraped from the glass with a rubber policeman and centrifuged at 1500 rpm for 2 min in the angle head of an International (PR-2) refrigerated centrifuge. The cell pellet was suspended in 10 ml of cold Earle's salt solution and recentrifuged. Cells were drained and resuspended in isotonic solution containing 1.5 ml RSB (0.01 M Tris, 1.5×10^{-3} M $MgCl_2$, 0.145 M NaCl), 0.1 ml PVS (potassium salt of polyvinyl-sulfuric acid; 30 μg/ml) and 0.16 ml 5% Nonidet P-40 (Shell Chemical Co., New York). The suspension was mixed thoroughly at 4°C for 15 min. A pellet containing cell debris and nuclei was obtained by centrifuging at 1500 rpm for 10 min. The entire supernatant fluid was layered over 25 ml of linear sucrose gradient (15%–30% w/w in RSB). The gradients were prepared in 3 × 1-inch cellulose nitrate tubes, by using an instrument similar to that described by Britten and Roberts (1). With constant stirring, 13 ml of 30% sucrose

and 12.4 ml of 15% sucrose were mixed. The gradients were allowed to stabilize overnight at 4°C. The gradients, carrying the sample on top, were centrifuged in a Spinco model L-2 ultracentrifuge with an SW 25.1 rotor for 90 min at 24,000 rpm at 4°C. 1-ml fractions were collected from the bottom of the tube and the OD at 260 mμ was measured with a Gilford flow cell recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio).

RESULTS

Effect of pH 6 on the Rate of Proteins and RNA syntheses in L cells

L cell monolayer cultures were washed twice with med. 30 ×-6 or med. 30 ×-7 and were incubated at 37°C with the same media. After 0, 0.5, 2, or 5 hr of incubation, the media were removed and the cells were labeled with uridine-^{3}H and with amino acids-^{14}C as previously described. The label was added in PBS of the same pH used for incubation of the cells. The cells were then collected, washed, and disrupted. The TCA-precipitable material was collected and counted in a scintillation counter as described in Materials and Methods.

The results of this experiment are plotted in Fig. 1 and show the per cent incorporation of uridine-^{3}H and amino acids-^{14}C in cells incubated in med. 30 ×-6 compared to that observed in cells incubated in med. 30 ×-7. Rate of incorporation of amino acids and uridine was inhibited to 40% and 70%, respectively, of the controls (med. 30 ×-7).

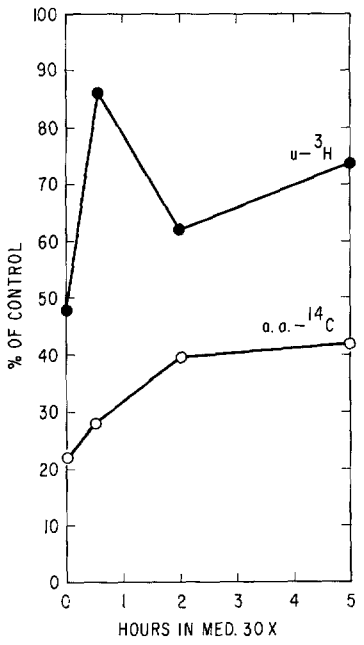

FIGURE 1 Effect of incubation in med. 30 ×-6 on RNA and protein syntheses. L cell monolayer cultures were incubated with either med. 30 ×-6 or med. 30 ×-7 for the times shown, then labeled with uridine-^{3}H for 20 min (●——●) and with amino acid-^{14}C mixture for 3 min (○——○). At each interval the TCA-precipitable counts in the cells incubated in med. 30 ×-6 were expressed as the per cent of counts in cells incubated with med. 30 ×-7.

The Permeability of the Cells at pH 6

Since ion permeabilities were shown to be affected by changes of pH in the crayfish axon (5), one possible explanation for the inhibition of protein synthesis could be that exposure of cells to pH 6 caused a change in permeability. This change in permeability could either decrease the uptake of amino acids or increase their leakage from the cells at pH 6.

The uptake by the cells was studied by measuring the accumulation of radioactive amino acids inside the cells. L cells were washed twice with 2.5 ml of med. 30 ×-6 or med. 30 ×-7, drained dry, and incubated with 2 ml of amino acid-^{14}C mixture (0.5 μCi/ml) in PBS-6 or PBS-7 at 37°C. At 0, 5, 10, and 15 min the incorporation was stopped and the cells were collected, rinsed, and disrupted as previously described. The TCA-precipitable and the TCA-soluble materials were collected and assayed for radioactivity as described in the Materials and Methods section. Another group of L cells were incubated for 5 hr with med. 30 ×-6 or med. 30 ×-7, and were then labeled with amino acids-^{14}C and treated in the same way as described for the previous group of cells. The results of the experiment are described in Fig. 2. It is shown that while the TCA-soluble counts, which represent free amino acids, accumulate at the same rate at either pH 6 or pH 7 (Fig. 2 *a*), there is an inhibition of incorporation of amino acids into proteins at pH 6 (Fig. 2 *b*). The same results were found after the 5 hr period of incubation at either pH 6 or pH 7. These findings indicate that the inhibition observed is not due to changes in uptake of amino acids by the cells at pH 6.

Leakage of amino acids and proteins from the cells was studied by assaying radioactive counts

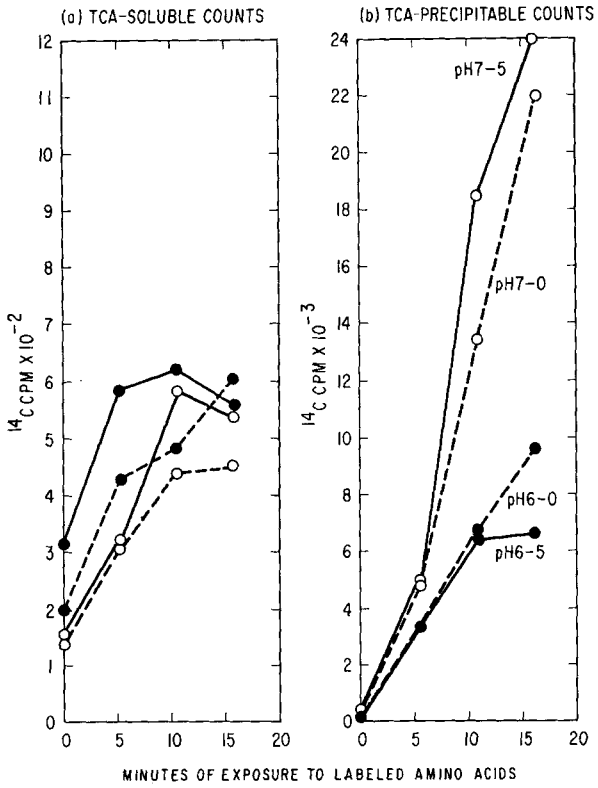

FIGURE 2 The permeability of L cells at pH 6 and pH 7. L cells were rinsed with med. 30 ×-6 or med. 30 ×-7 and incubated with amino acid ^{14}C mixture in PBS-6 or PBS-7. At the times shown, cells were collected, disrupted, and assayed for TCA-soluble (*a*) and TCA-precipitable (*b*) counts. Another group of L cells were incubated for 5 hr with med. 30 ×-6 or med. 30 ×-7, and were then labeled with amino acids-^{14}C and treated in the same way as described for the previous group of cells. ●——●, cpm, med. 30 ×-6, 0 time; ●——●, cpm, med. 30 ×-6, 5 hr; ○——○, cpm, med. 30 ×-7, 0 time; ○——○, cpm, med. 30 ×-7, 5 hr.

which leaked into the medium at pH 6. L cells were labeled with amino acids-^{14}C in PBS-7 for 20 min, washed to remove the label, and further incubated with med. 30 ×-6 or med. 30 ×-7. After 0, 0.5, 2.5, and 5 hr of incubation the culture medium was sampled and assayed for total radioactivity as described in the Materials and Methods section. At the same time intervals, cells from which the media had been removed and assayed were then washed, collected, and disrupted to determine the radioactivity left in the cells. The results in Fig. 3 demonstrate that the same amount of radioactivity leaks into the medium at either pH 6 or pH 7 (Fig. 3 *a*). Corroboratively, the number of counts left in the cells is the same at either pH (Fig. 3 *b*). These results indicate that there is no difference in leakage from cells exposed to medium adjusted to either pH 6 or pH 7.

Effect of pH 6 on Polyribosome Profiles

The effect of pH 6 on translation was investigated by analyzing polyribosome profiles and measuring the incorporation of radioactive amino acids into nascent polypeptide chains following treatment of the cells at pH 6. Cells were washed and incubated in either med. 30 ×-6 or med. 30 ×-7 for 0.5, 2, or 5 hr. The cells were then labeled for 3 min with an amino acid-^{14}C mixture prepared in PBS-6 or PBS-7, and were fractionated as described. Fractions were collected and the OD at 260 mμ, as well as the TCA-precipitable counts, were determined.

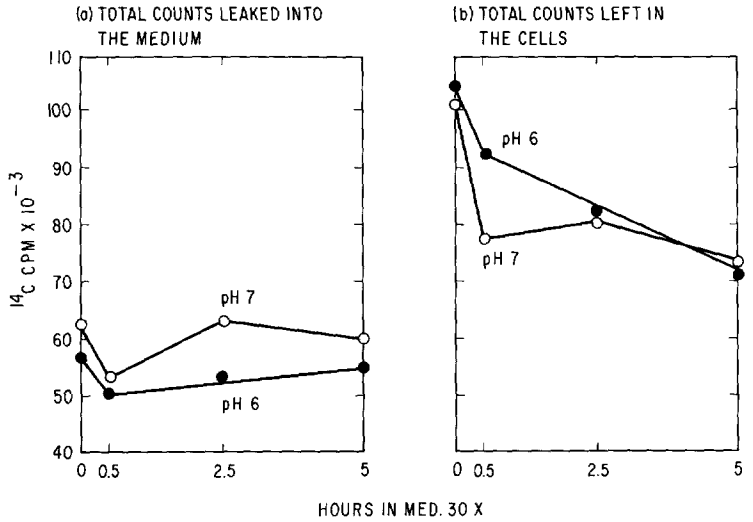

FIGURE 3 Leakage of radioactive counts from L cells at pH 6 and pH 7. L cells were labeled with amino acid-^{14}C mixture in PBS-7 for 20 min, then washed and further incubated with med. 30 ×-6 or med. 30 ×-7. At the time intervals shown, the medium was assayed for total radioactive counts (*a*). At the same time the cells from the same cultures were collected and disrupted. The disrupted cell preparations were assayed for total radioactive counts (*b*). ●——●, cpm, med. 30 ×-6; ○——○, cpm, med. 30 ×-7.

The polyribosomal profiles obtained are shown in Fig. 4. In this figure, disaggregation of the polyribosomes can be seen as early as 0.5 hr after incubation at pH 6. This disaggregation is accompanied by a decrease in the translational activity of the polyribosomes, as shown by the reduced level of incorporation of amino acids into nascent chains (Fig. 4) and by the lower rate of translation (Table I). The rate of translation is estimated, as described by McCormick and Penman (3), from the ratios of the radioactivity of TCA-precipitable material at the top of the gradient (which represents soluble proteins) to the radioactivity in the nascent chains in the polyribosomal region.

The profiles obtained by OD (260 mμ) measurements were not sufficiently detailed in the polyribosomal region. Therefore, another method was used in which radioactivity of prelabeled ribosomal RNA (rRNA) was determined. L cells were labeled for 48 hr (2 generations) with uridine-^{3}H. The radioactive medium was removed and the cells were washed twice and further incubated at 37°C with med. 30 ×-6 or med. 30 ×-7. After 0, 0.5, 2, and 5 hr of incubation, the cells were collected and fractionated as previously described. Each fraction was assayed for TCA-precipitable radioactive counts, and the results are described in Fig. 5. After 0.5 hr of incubation in med. 30 ×-6, a peak of light polyribosomes is demonstrated in fraction 19 (Fig. 4 *a*). The polyribosomes later dissociate to enlarge the single ribosome fraction (Figs. 5 *b* and 5 *c*).

Persistence of mRNA

One of the possible reasons for the disaggregation of polyribosomes after pH 6 treatment was a breakdown of mRNA. To determine if the cellular mRNA persisted during pH 6 exposure, the following experiment was carried out. Cells were washed and incubated for 5 hr in either med. 30 ×-6 or med. 30 ×-7. Cells were then washed twice with BME containing 4% calf serum (1 ×) and were allowed to recover from the treatment by further incubation in the same medium, either with or without actinomycin D. At 0.5 and 2 hr after transfer to BME (1 ×), samples were labeled with uridine-^{3}H and with amino acids-^{14}C in PBS-7 as previously described. The TCA-precipitable radioactivity was then counted as before. The results of this experiment are summarized in Fig. 6. Fig. 6 *a* shows that there is a rapid

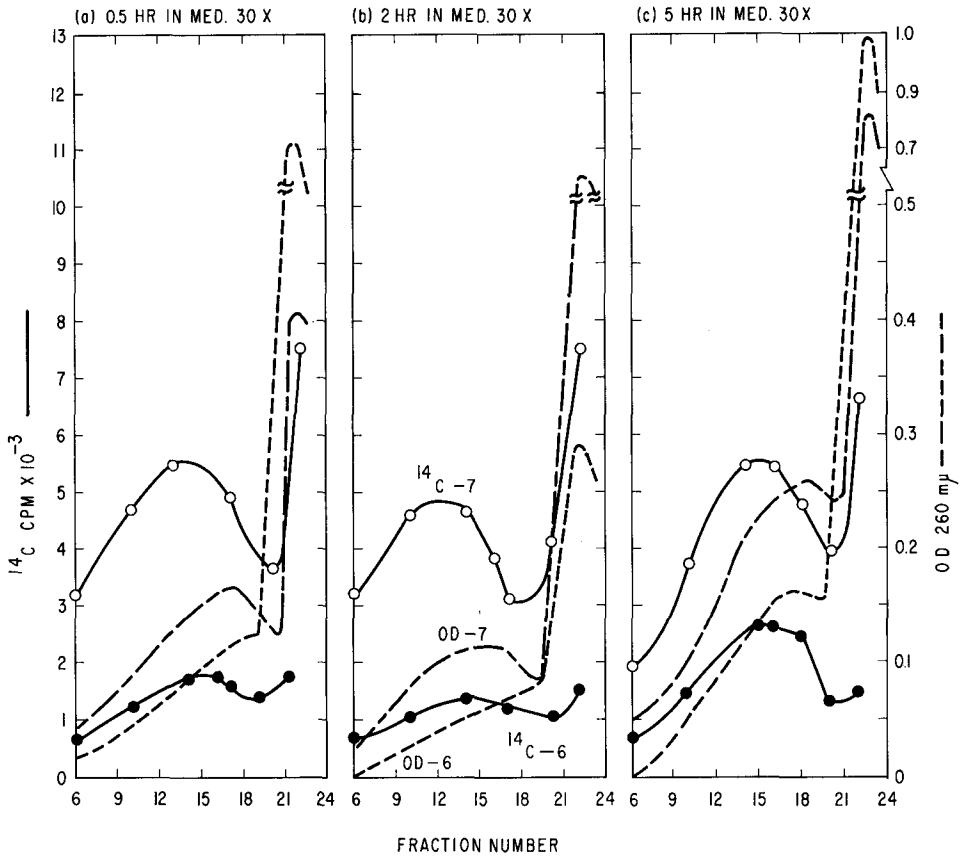

FIGURE 4 Changes in polyribosome profiles resulting from incubation of L cells in med. 30 ×-6 or med. 30 ×-7. L cell monolayer cultures were incubated in either med. 30 ×-6 or med. 30 ×-7. At the time intervals shown in (*a*)–(*c*), cells were labeled with amino acid-^{14}C mixture for 3 min. Cytoplasmic extracts were then obtained and prepared for centrifugation on 25 ml of 15–30% sucrose–RSB gradients as described in the Materials and Methods section. Fractions were collected and the OD at 260 mμ and the counts in the TCA-precipitable material were determined for the fractions. ●——●, cpm, med. 30 ×-6; ○——○, cpm, med. 30 ×-7; - - -, OD, med. 30 ×-6; — — —, OD med. 30 ×-7.

recovery of RNA synthesis from the pH 6 treatment. However, when actinomycin D is present in the recovery medium, no incorporation of uridine is observed, as expected. Protein synthesis, as can be seen from Fig. 6 *b*, also recovers rapidly when cells are transferred from pH 6 to recovery medium, either in the absence or in the presence of actinomycin D. The rapid and full recovery of protein synthesis that takes place in the presence of actinomycin D shows that the mRNA survived the pH 6 exposure. It was shown by a separate experiment that exposure to pH 6 did not affect the inhibitory activity of actinomycin D. Actinomycin D (1 μg/ml) inhibited 90% of RNA synthesis in cells incubated with either med. 30 ×-6 or med. 30 ×-7 compared to control cells which were incubated with med. 30 ×-7 in the absence of actinomycin D.

DISCUSSION

The studies presented in this paper were designed to investigate further the inhibitory mechanism of PBS-6 described by Hallum et al. (2). To eliminate side effects which resulted from the lack of amino acids and serum in the PBS solutions, BME supplemented with 4% calf serum and adjusted to the desired pH was used. Since this medium could not be maintained at pH 6 over the time of the experiment, the buffering capacity was increased by raising the phosphate salt concentra-

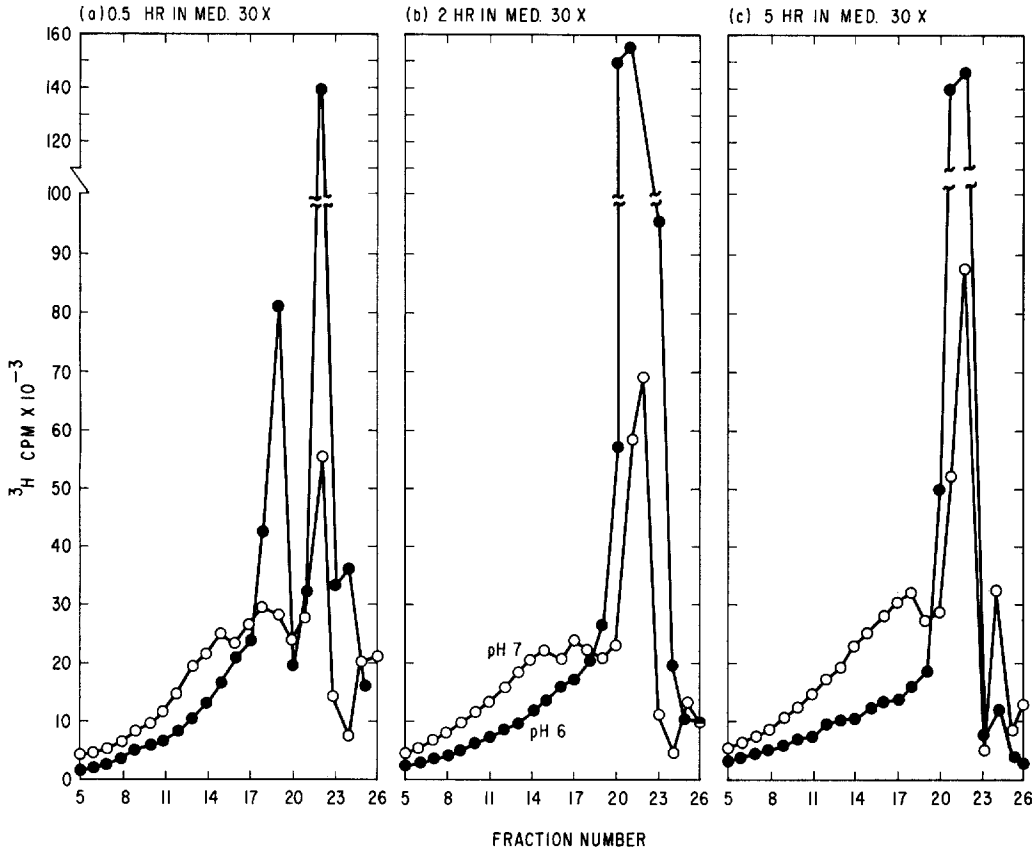

FIGURE 5 Changes in polyribosome profiles resulting from incubation of L cells in med. 30 ×-6 or med. 30 ×-7. L cells were labeled for 48 hr with uridine-^{3}H and then incubated with med. 30 ×-6 or med. 30×-7. After 0.5, 2, and 5 hr of incubation, cells were collected and fractionated as described in the legend for Fig. 4. The counts in the TCA-precipitable material were determined in each fraction. ●——●, cpm, med. 30 ×-6; ○——○, cpm, med. 30 ×-7.

tion 30-fold (med. 30 ×). Incorporation of uridine-^{3}H and amino acids-^{14}C or polyribosome profiles in cells incubated with med. 30 × were not significantly different from those in cells incubated in BME containing 4% calf serum (1 ×).

Protein synthesis was found to be inhibited by the action of pH 6 on the translation. Disaggregation of polyribosomes was demonstrated by optical density (260 mμ) as early as 0.5 hr after incubation at pH 6 (Fig. 4). This disaggregation was associated with a considerable inhibition of incorporation of amino acids-^{14}C into nascent polypeptide chains as compared to pH 7 controls. The rate of translation, as calculated from the ratio of counts in the released proteins to counts in the nascent polypeptide chains (Table I), was also inhibited by pH 6. Disaggregation of polyribosomes was also demonstrated by a more sensitive measurement in which the profiles were obtained by assaying ^{3}H-labeled ribosomal RNA following a pH 6 treatment. After 0.5 hr at pH 6 the profiles demonstrated polysomes (Fig. 5 *a*) which dissociated to enlarge the single ribosome fraction after 2 and 5 hr of further incubation at pH 6 (Figs. 5 *b* and 5 *c*).

The disaggregation observed after exposure to pH 6 could result from a degradation of mRNA. The results eliminated this possibility. It was concluded that the mRNA persisted during the incubation at pH 6 since a recovery of protein synthesis (Fig. 6) was observed in the presence of actinomycin D in the recovery medium. Under

TABLE I

Rate of Translation in L cells at pH 6 and pH 7

Time of incubation	pH of med. 30 X	Ratio (cpm-top of gradient/ cpm-nascent chains)	Rate of translation (pH 7) / (pH 6)
(*hr*)			
0.5	6	1.4	2.64
	7	3.7	
2	6	2.2	2.13
	7	4.7	
5	6	1.2	2.00
	7	2.4	

L cells were incubated in med. 30 ×-6 or med. 30 ×-7. At the times shown the cells were fractionated and the radioactivity in each fraction was determined as described in Materials and Methods. Rate of translation is calculated from the ratio of counts at the top of the gradient to counts in the nascent chains.

these circumstances no new mRNA could be synthesized, and therefore the observed recovery of protein synthesis is due to preexisting mRNA which survived the pH 6 treatment.

Another possible explanation for the inhibition was that the exposure to pH 6 caused changes in the permeability of the cells to precursors. Uptake was measured by assaying the amino acids-^{14}C counts accumulated in the cells. Leakage was measured by assaying the ^{14}C counts which leaked into the medium. The results described in Figs. 2 and 3 demonstrated no change either in uptake or in leakage due to the pH 6 treatment. Therefore it was concluded that pH 6 did not affect the permeability of L cells.

The inhibitory effects of exposure of L cells to pH 6 are reversible. L cells which had been exposed to pH 6 for 5 hr fully recovered their ability to incorporate amino acids or uridine within 0.5 hr after transfer to BME growth medium. The cells are also viable after exposure to pH 6. A growth curve of the cells incubated with MEM following 5 hr of incubation at pH 6 demonstrated a lag of 36 hr followed by an exponential growth at the same rate as the control. The lag in multiplication, in contrast to the rapid recovery of RNA and protein synthesis, is very long, indicating that there is another mechanism which is even more sensitive to pH 6 and is important for the multiplication of the cells. It is suggested a priori that this factor could be the sensitivity of DNA synthesis to pH 6. It will be of further interest to determine if DNA synthesis is also affected by pH 6.

The findings described in these studies provide an additional tool to control macromolecular syntheses. Control of macromolecular synthesis by exposure to pH 6 offers certain advantages over the use of chemicals or drugs. In addition to the speed and the reversibility of the inhibition of synthesis, the treatment does not involve entry into the cells of foreign molecules or ions. Since the inhibition of synthesis was not complete, it

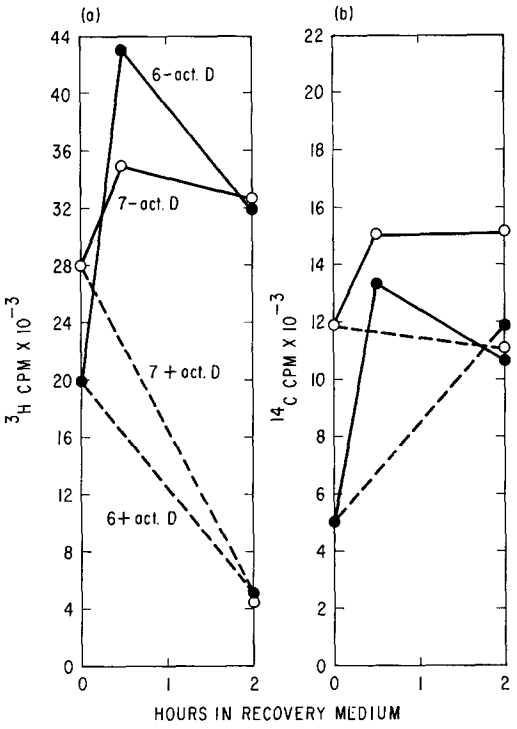

FIGURE 6 Recovery of RNA and protein syntheses in L cells following a 5 hr exposure to pH 6. L cells were incubated for 5 hr with med. 30 ×-6 or med. 30 ×-7. The cells were then transferred to growth medium with or without actinomycin D (1 μg/ml). At the times shown, the cells were labeled with uridine-^{3}H for 20 min and with amino acid-^{14}C mixture for 3 min, collected, disrupted, and assayed for TCA-precipitable radioactivity. ●——●, cpm, med. 30 ×-6, without actinomycin D; ○——○, cpm, med. 30 ×-7, without actinomycin D; ●— —●, cpm, med. 30 ×-6, with actinomycin D; ○— —○, cpm, med. 30 ×-7, actinomycin D.

has yet to be determined if the remaining incorporation was a specific residual synthesis. Therefore, it will be of further interest to investigate the site of the effect of pH 6 on macromolecular synthesis and to determine if this effect is partitioned in the cells. For example, it has yet to be determined if mitochondrial or nuclear synthetic events escape inhibition by pH 6. The results could be utilized for further research on synthesis of macromolecules in the different cellular compartments.

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