

## Effect of pH on the Protective Action of Interferon in L Cells

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The pH of the solution in which interferon was applied to L cells determined the level of resistance developed against challenge with vesicular stomatitis virus (VSV). No inhibition of challenge virus was observed when interferon was applied to cells at pH 6.0. At pH 6.5, partial inhibition of VSV replication was observed and inhibition was maximum at pH 7.0. Evidence was obtained that interferon interacted with L cells at pH 6.0, but that resistance did not develop until the cells were placed in a medium at pH 7.0. These effects were explained by data showing that exposure of cells to a medium at pH 6.0 reversibly inhibited both ribonucleic acid and protein synthesis.

Levine (8) demonstrated that, although the initial interaction between interferon and chick embryo cells occurs at 4 C, the development of resistance to virus challenge is a second step that requires incubation at 37 C. This work was extended by Friedman (2), who showed that treatment of cells with trypsin, after the initial interaction with interferon in the cold, inhibited the subsequent development of antiviral activity. He concluded that interferon is bound initially to some superficial cell site, and that this binding is a necessary prerequisite for the development of resistance to virus. Several groups of workers have shown that ribonucleic acid (RNA), synthesis as well as protein synthesis, is necessary for the development of resistance (3, 4, 8, 10, 13).

As part of a study of the initial interaction of mouse interferons and L cells, the influence of the pH of the medium in which interferon is applied was investigated. This report shows that although pH had no effect on the initial reaction of interferon and L cells it had a marked influence on the subsequent development of resistance to challenge with virus.

### MATERIALS AND METHODS

**Viruses.** The Indiana strain of vesicular stomatitis virus (VSV) was propagated in L-cell cultures as previously described (14). Virus pools were kept at -60 C for use as challenge virus in interferon assays.

**Cell cultures.** A continuous line of L cells (clone 929) was grown in 32-oz (907-g) prescription bottles for 7 days with one change of Eagle's minimal essential medium supplemented with 4% calf serum. Cell suspensions, obtained by scraping the cells from the glass with a rubber policeman, were prepared in growth medium. Cell counts were adjusted so that cultures in

60-mm petri dishes or in 2-oz (56.7-g) prescription bottles were initiated with  $3 \times 10^6$  cells in 4 ml of medium or with  $10^6$  cells in 7 ml of medium, respectively. L-cell cultures were used for interferon assays after 24 hr of incubation at 37 C, and after 48 hr for virus assays.

**Interferons.** L-cell interferon was prepared by inoculating the cultures with Newcastle disease virus (NDV) as described by Youngner et al. (14).

Interferon that appeared in the circulation of mice after intravenous injection of *Escherichia coli* endotoxin (endotoxin interferon) was prepared as described by Stinebring and Youngner (12).

Assays of interferon were carried out by the plaque reduction method with VSV as the challenge virus. The details of this assay have been recorded (14).

**Preparation of solutions at different pH values.** Phosphate-buffered saline (PBS; 0.01 M with respect to phosphate at pH 7.2) was prepared as described by Merchant et al. (11). The pH of the buffer was adjusted to values less than 7.2 by the addition of small amounts of 1.0 or 0.1 M HCl. The pH was measured at 25 C by use of a Beckman Zeromatic pH meter calibrated with standard buffers. Solutions of PBS at pH values greater than 7.0 were prepared by adding 1 M NaOH.

**Treatment of cells with interferon solutions at various pH values.** Monolayer cultures of L cells to be exposed to interferon in PBS at a certain pH were washed twice with 3 ml of PBS at the same pH before adding the interferon solution. For the sake of brevity, the following convention has been adopted: PBS5 refers to PBS in which the pH has been adjusted to 5.0; PBS6 means PBS adjusted to pH 6.0; etc. After incubation at 37 C in air containing 5% CO<sub>2</sub>, the cultures were washed with Eagle's medium containing 4% calf serum (pH 7.2) and then challenged with VSV (0.5 ml) at an input multiplicity of infection of 10. After incubation for 1 hr at 37 C, the unadsorbed virus was drawn off and the cultures were washed twice with 3.0 ml of growth medium. An additional

3.0 ml of medium was added, and the infected cultures were incubated for 6 hr at 37 C. The culture fluids were collected and frozen at -60 C until the infectivity titers were determined by a plaque assay. The effectiveness of the interferon applied at different pH values was determined by the amount of inhibition of yield of challenge virus during a single cycle of replication (7). The yield of challenge virus was compared to the yield in two types of controls: (i) cultures treated with identical interferon dilutions in either PBS7 or Eagle's medium, and (ii) cultures treated with PBS at the same pH or with Eagle's medium, but containing no interferon.

The buffered interferon solutions from pH 7.0 to pH 5.5 were stable for the 5-hr incubation period. In PBS4.5 and PBS5.0, however, the pH values rose to 4.9 and 5.2, respectively. For shorter incubation periods, no increase in pH was observed.

**Incorporation studies.** To measure the incorporation of  $^{14}\text{C}$ -leucine or  $^3\text{H}$ -uridine into L cells, a modification of the method of Fujioaka et al. (5) was used.  $^{14}\text{C}$ -leucine (specific activity, 180 mc/mmmole) and  $^3\text{H}$ -uridine (specific activity 2.0 c/mmmole) were purchased from Schwarz Bio Research, Inc., Orangeburg, N.Y. The  $^{14}\text{C}$ -leucine was diluted in Hanks' balanced salt solution (BSS) to a final concentration of 0.25 mc/ml, and the  $^3\text{H}$ -uridine was diluted to 0.5 mc/ml in the same medium. Cultures pretreated at each pH were drained and pulsed at 37 C with 1 ml of isotope solution (10-min pulse for  $^3\text{H}$ -uridine, and 20-min pulse for  $^{14}\text{C}$ -leucine). Incorporation was stopped by placing the petri dishes on ice. The isotope solution was removed; the cells were washed with cold Hanks' BSS, and then scraped with a rubber policeman into 2 ml of cold BSS. The suspended cells were washed twice more with 10 ml of Hanks' BSS and then centrifuged in 15-ml centrifuge tubes, at 1,500 rev/min, in the angle head of an International (PR-2) refrigerated centrifuge. The cell pellet was resuspended in 2 ml of Hanks' BSS, and the cells were disrupted by treatment for 8 min at maximum amperage in a Raytheon 10-kc sonic oscillator, model DF-101. After precipitation with trichloroacetic acid, the insoluble material was collected by filtration and the radioassay was carried out in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

## RESULTS

**Influence of pH on the plaque inhibition titer of interferon.** Interferon made in L cells infected with NDV was serially diluted in PBS6, PBS7, or Eagle's medium, and 3 ml of each dilution was added to duplicate L-cell cultures. Control cultures were treated with the same diluents without interferon. After different times of incubation at 37 C, the interferon dilutions and the control diluents were removed, and the cultures were challenged with 40 to 60 plaque-forming units (PFU) of VSV and overlaid with agar as described previously (14). The results in Fig. 1 show that the 50% plaque inhibition titers increased with time when the interferon was applied in PBS7 or in

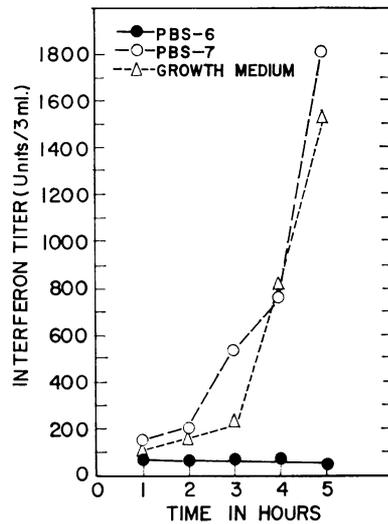


FIG. 1. Influence of pH on interferon titer determined by the plaque inhibition method. Duplicate L cell cultures were washed and inoculated with interferon diluted in PBS6, PBS7, or Eagle's medium. After different times of incubation at 37 C, the cultures were washed with growth medium and challenged with 40 to 60 PFU of VSV.

Eagle's medium. In contrast, there was no detectable increase in resistance to virus in the cultures exposed to interferon diluted in PBS6. It should be noted that the average plaque counts in cultures exposed to PBS6, PBS7, or to Eagle's medium without interferon were not significantly different.

**Influence of pH range on interferon action.** To determine the range of pH values over which inhibition of interferon action could be observed, L-cell cultures were exposed to interferon diluted in PBS4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 8.0. For convenience, interferon activity was measured by the yield inhibition method in these experiments. Duplicate cultures were washed twice with PBS at each pH and then exposed to 3 ml of interferon diluted in PBS at the corresponding pH; each culture received 400 units of interferon from NDV-infected L cells. Control cultures at each pH were treated similarly, except that the PBS contained no interferon. After 5 hr of incubation at 37 C, the cultures were washed and challenged with VSV at an input multiplicity of 10. After 1 hr of adsorption, the inoculum was removed, the cultures were washed twice, and 3 ml of Eagle's medium was added. The fluids from infected cultures were harvested after an additional 6 hr of incubation at 37 C. The results of this experiment are presented in Fig. 2. When interferon was applied in PBS7 or in PBS8 (not shown), the yield of progeny virus after challenge was effec-

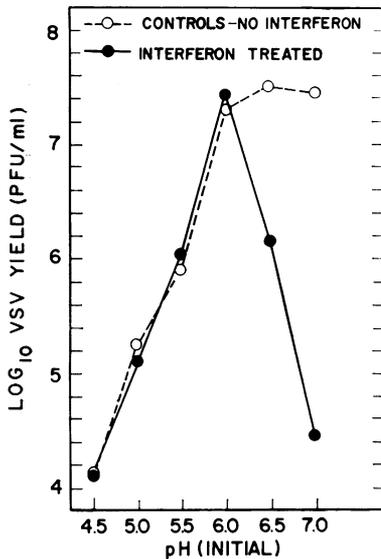


FIG. 2. Effect of pH range on interferon action in L cells. Cultures were incubated for 5 hr with 400 units of interferon in PBS at each pH. After removal of the interferon, the cultures were washed and challenged with VSV. Virus yields were determined in fluids harvested after 6 hr of incubation of the cultures in growth medium.

tively inhibited; at pH 6.5 a partial inhibition of the development of resistance was observed. At pH 6.0 no resistance developed. The virus yields in control cultures in PBS6 that lacked interferon were not significantly different from the yields from controls in PBS7 and PBS8. By contrast, the decrease in virus yield in cultures exposed to PBS below pH 6.0, with or without interferon, resulted from the increasing toxicity for L cells of the acidified PBS solutions.

To determine whether the effects described could be reproduced with an interferon other than that induced in L cells by NDV, similar studies were conducted using the inhibitor released into the circulation of mice after injection of *E. coli* endotoxin (endotoxin interferon). Duplicate cultures were treated with 100 units of endotoxin interferon diluted in PBS6 or in PBS7 as previously described. After incubation at 37 C for 1, 3, and 5 hr, the cultures were washed, challenged with VSV, and the yield of progeny virus was determined. Control values were obtained from cultures treated with PBS at the same pH but without interferon. The results of these studies (Table 1) showed that endotoxin interferon behaved similarly to interferon from NDV-infected L cells. Resistance to challenge with VSV did not develop in cultures treated with interferon in PBS6.

TABLE 1. Comparison of effect of PBS6 and PBS7 on the protective action of endotoxin-stimulated mouse interferon in L cells<sup>a</sup>

Diluent added to L cells	Interferon dose	Log <sub>10</sub> VSV yield (PFU/ml) 6 hr after challenge at		
		1 hr	3 hr	5 hr
PBS-6	None	7.45	7.70	7.65
PBS-6	100 units	7.65	7.25	7.18
PBS-7	None	7.42	7.70	7.75
PBS-7	100 units	7.20	6.65	5.70

<sup>a</sup> Duplicate cultures were exposed to 100 units of endotoxin interferon in PBS6 or in PBS7; control cultures were exposed to diluent only. After 1, 3, or 5 hr at 37 C, the solutions were removed, the cultures were washed with growth medium, and they were then challenged with VSV. After incubation for 6 hr in growth medium at 37 C, the released virus was harvested and assayed.

The possibility that the data obtained above were the result of inactivation of interferon in PBS6 at 37 C was eliminated by diluting the L-cell interferon in PBS6, PBS7, or Eagle's medium, and incubating it at 37 C or at 4 C for 5 hr. No significant change in the titer of the inhibitor was observed under any of these conditions.

*Influence of pH 6.0 on the initial interaction of interferon with L cells.* A second explanation for the lack of development of resistance at pH 6.0 is, possibly, that the initial reaction between interferon and L cells was blocked at this pH. To test this possibility, cultures exposed to 400 units of interferon from NDV-infected L cells in cold PBS6, PBS7, or Eagle's medium were incubated for 15 hr at 4 C. After this time, the interferon was removed and the cultures were washed three times with cold Eagle's medium and transferred to 37 C. The resistance to challenge virus was measured by the yield inhibition method at 0, 2, 4, 6, and 8 hr after the transfer of the cultures to 37 C. The results summarized in Fig. 3 show that resistance to the virus challenge developed at the same rate regardless of the pH at which the interferon solution was applied. These data indicate that the initial interaction of interferon with L cells takes place at pH 6.0, even though resistance does not develop under these conditions.

*Maintenance of cell resistance at pH 6.0.* Another possible explanation for the failure of cell resistance to develop at pH 6.0 is that the metabolic products required to express the anti-viral action of interferon (13) were unstable at this pH. If interferon protection were dependent upon the so-called "second protein" and if this material were unstable in cells exposed to pH 6.0,

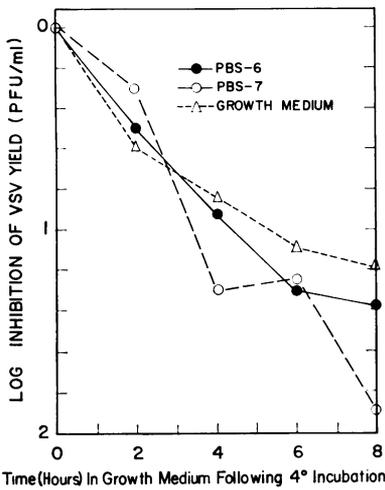


FIG. 3. Lack of effect of pH 6.0 on initial interaction of interferon and L cells. L cells were incubated for 15 hr at 4 C with interferon in PBS6, PBS7, or in Eagle's medium. After this period, the cultures were washed three times with cold medium, 3 ml of Eagle's medium were added, and the cultures were transferred to 37 C. At the times shown, the cultures were challenged with VSV by the yield inhibition method. Virus yield in control cultures without interferon was  $2.1 \times 10^7$  PFU/ml.

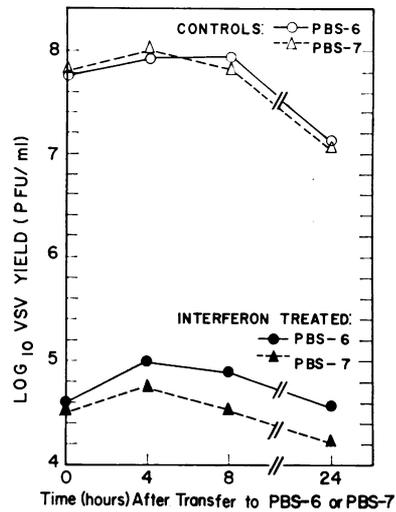


FIG. 4. Maintenance of cell resistance at pH 6.0. Cultures treated for 15 hr at 37 C with 270 units of interferon in Eagle's medium were washed and transferred to PBS6, PBS7, or to fresh Eagle's medium, and incubation was continued. After various times in these media, the cultures were challenged with VSV according to the yield inhibition method. The results with Eagle's medium were similar to those in the other diluents and are not shown.

cells in which interferon protection had been established would become more susceptible to infection when held at this pH. To test this possibility, L-cell cultures were inoculated with 270 units of L-cell NDV interferon in Eagle's medium and incubated for 15 hr at 37 C. The cultures were then washed with PBS6, PBS7, or Eagle's medium, covered with 3 ml of the same solution, and incubated again at 37 C. After 0, 4, 8, and 24 hr of additional incubation at 37 C, the cultures were challenged with VSV and the yield of progeny virus was determined as described previously. The results of this experiment, plotted in Fig. 4, show that interferon protection, once established, is equally stable in cells exposed to PBS6 or PBS7. The results from cultures exposed to Eagle's medium were identical to the data obtained with PBS6 and PBS7.

**Influence of PBS6 on macromolecular synthesis in L cells.** Another possible explanation for the failure of cells treated with interferon at pH 6.0 to develop resistance to virus is that macromolecular synthesis might be inhibited at this pH, thus preventing the formation of significant amounts of "second protein." This possibility was tested by measuring the difference in the rate of incorporation of  $^{14}\text{C}$ -leucine and  $^3\text{H}$ -uridine into protein and RNA, respectively, as a function of the pH of the PBS to which the cells were exposed. Replicate cultures of L cells were treated

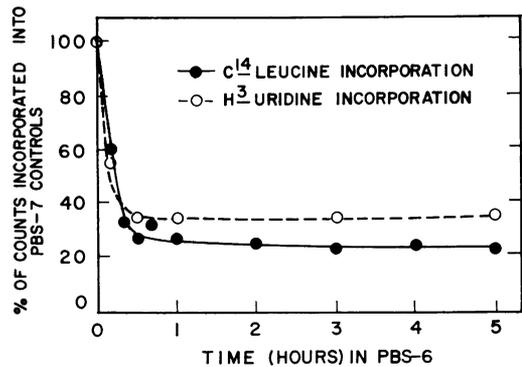


FIG. 5. Influence of exposure of L cells to PBS6 on protein and RNA synthesis. Cultures were treated with PBS6 or PBS7 for the intervals shown, then pulsed with  $^{14}\text{C}$ -leucine or  $^3\text{H}$ -uridine. At each time interval, the trichloroacetic acid-precipitable counts in the cells exposed to PBS6 were compared to the counts in cells in PBS7. (Average of counts incorporated into PBS7 control cultures:  $^{14}\text{C}$ -leucine, 1,717 counts per min per culture;  $^3\text{H}$ -uridine, 524 counts per min per culture.)

with either PBS6 or PBS7 and after intervals of 10 min to 5 hr they were pulsed with radioactive precursors as described above. The results of these experiments, summarized in Fig. 5, clearly demonstrated that the rates of both  $^{14}\text{C}$ -leucine and  $^3\text{H}$ -

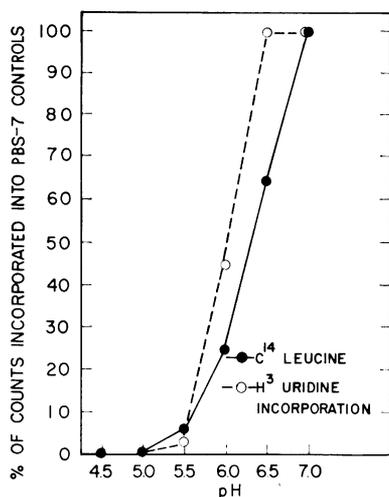


FIG. 6. Influence of PBS at different pH on macromolecular synthesis in L cells. Cultures were incubated at 37 C at the pH shown for 5 hr, then pulsed with either  $^{14}\text{C}$ -leucine or  $^3\text{H}$ -uridine. The trichloroacetic acid-precipitable counts were compared to cultures incubated for 5 hr in PBS7. (Counts incorporated into PBS7 control cultures:  $^{14}\text{C}$ -leucine, 1,247 counts per min per culture;  $^3\text{H}$ -uridine, 1,801 counts per min per culture.)

uridine incorporation were decreased by about 70% within 20 min of exposure to PBS6.

Additional incorporation studies were carried out to determine the effect on macromolecular synthesis of exposure to a range of pH. These experiments at pH values from 4.5 to 7.0 were conducted as described for PBS6, except that a single 5-hr incubation period at 37 C was applied at each pH. The results of these studies (Fig. 6) show that the incorporation of both  $^{14}\text{C}$ -leucine and  $^3\text{H}$ -uridine was progressively reduced at decreasing pH. In PBS5 and PBS5.5, no significant synthesis of either RNA or protein was observed.

#### DISCUSSION

The data presented show that exposure of L cells to PBS at pH 6.0 results in a rapid and reversible inhibition of both RNA and protein synthesis. Control of macromolecular synthesis by exposure to PBS at pH 6.0 offers certain advantages over the use of the usual chemical or antibiotic inhibitors. In addition to the speed and reversibility of the inhibition of synthesis, the treatment does not involve entry into the cell of molecules or ions that are not ordinarily present. The mechanism by which exposure of L cells to PBS6 depresses RNA and protein synthesis is under investigation; preliminary studies indicate

that exposure of L cells for 20 min to PBS6 causes a disaggregation of polysomes. Parallel studies of chick embryo cell cultures showed that these cells are more resistant to the effects of lowered pH than are L cells. The basis for the species and cell differences is being explored.

The results confirmed that interferon can interact fully with L cells even though resistance does not develop (3, 9). We used a lowered range of pH to demonstrate a model system for separating the initial interferon-cell interaction from the synthetic processes essential for the development of resistance.

Previous studies of the effect of change of pH on the action of interferon have been reported by Gifford (6) and by De Maeyer and De Somer (1). The pH range covered in these reports was from 6.8 to 7.6. Gifford (6) concluded that in chick embryo cells the plating efficiency of the vaccinia virus challenge was altered by changes in pH, but that the action of interferon was not significantly affected. De Maeyer and De Somer (1), using a continuous line of rat tumor cells, reported an increase in interferon titer at pH 6.8 compared to pH 7.2. However, since both papers covered only a narrow physiological range of pH, the inhibition of interferon activity at the low pH used in this study was not observed.

#### ACKNOWLEDGMENT

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