Increased immunogenicity can be related to several properties of cells after treatment with VCN. (i) The VCN removes sialic acid from the cell surface (*14*). This sialic acid may offer a steric hindrance to the perception of the antigen itself or may sterically interfere with contact between antigen-bearing and immunocompetent cells. (ii) The VCN reduces the negative charge on the cell surface (*14*). A negatively charged antigen-processing or antigen-responsive cell might be more attracted to a less highly charged cell. (iii) Reducing the negative charge on the cell surface also reduces the rigidity of the cell surface, because the mutually repellent negative charges on the cell surface are removed (*15*). Increased deformability of the antigenic cell itself might increase the area of contact between it and the antigen responsive cell. (iv) Treatment of cells with VCN renders them more easily phagocytized (*16*) and the phagocytosis of antigens may facilitate antigen processing and the development of immunity. Whether the increased susceptibility of VCN-treated cells to complement lysis (*17*) plays a role here or not is unknown.

This present immunotherapy model can probably serve as a model for the study of other variables in cancer immunotherapy. It also provides a reasonable basis for experimental cancer immunotherapy in man where BCG has already been utilized (*1*). The fact that mitomycin treatment of the VCN-treated cells prevents the growth of the tumor challenge and does not interfere with the regression of tumors induced by the VCN-treated cell may provide the basis for a VCN-treated tumor vaccine that can be used in patients in whom conventional therapy has been ineffective.

RICHARD L. SIMMONS
ANGELYN RIOS
*Department of Surgery, University of Minnesota, Minneapolis 55455*

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5. Neuraminidase from *Vibrio cholerae* (VCN) was obtained either from General Biochemicals, Chagrin Falls, Ohio, or from Behringwerke Ag-Marburg-Lahn, West Germany. The latter preparation is described by the manufacturer as having no detectable proteinase, aldolase, or lecithinase activity. Both contain 500 units of enzyme per milliliter. One unit of neuraminidase activity is defined by both manufacturers as being equivalent to the release of 1 $\mu$g of *N*-acetyl neuraminic acid from a glycoprotein substrate at 37°C at 15 minutes at *p*H 5.5. Since no differences in activity in the two products could be detected, the former material was used in most experiments. In the concentrations utilized in these experiments VCN released approximately 250 nanomoles per $10^9$ viable MC-42 tumor cells under the conditions of these experiments; VCN in these concentrations has no effect on cell viability. The enzymatic activity of these VCN preparations can be destroyed by heating to 56°C for 30 minutes or 100°C for 10 minutes.
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28 June 1971; revised 23 August 1971

## Interferon Action: Inhibition of Vesicular Stomatitis Virus RNA Synthesis Induced by Virion-Bound Polymerase

Abstract. *The particle-bound RNA polymerase activity of vesicular stomatitis virus (VSV) can be demonstrated in vivo. Linear synthesis of viral RNA persists for 5 to 6 hours at 34°C in infected monolayers of chick embryo cells treated with cycloheximide and actinomycin D to block synthesis of protein and cell-specific RNA. At least 55 percent of the RNA made under these conditions is complementary to virion RNA. RNA synthesis mediated by VSV polymerase activity is inhibited in cells first treated with chick-derived interferon or polyriboinosinate·polyribocytidylate, but not by mouse interferon. The RNA product of VSV polymerase activity is present throughout the cytoplasm, and its synthesis is inhibited by the interferon system, as judged by autoradiographs that show the physical distribution, in cells, of RNA produced by virion polymerase in the absence of translation—a demonstration of the transcription product of the viral genome.*

The presence of RNA polymerase in the virion of a virus sensitive to interferon permits a direct test of the interferon system on the first virus-specific synthetic process thought to occur within the infected cell. We now describe conditions that demonstrate the synthesis of viral RNA by the virion-bound polymerase of vesicular stomatitis virus (VSV) (*1*) in chick embryo cells and its inhibition by the interferon system.

Synthesis of VSV-RNA by virion polymerase was demonstrated in chick cells as follows. Chick embryo cell monolayers were prepared from 9-day-old embryos and used as primary or secondary cultures containing 1 to $2\times10^6$ cells for each 35-mm petri dish (Falcon) after incubation at 37°C for 1 to 3 days (*2*). Cells for autoradiographs were seeded as single cells at 1 to $2\times10^5$ cells for each 35-mm dish. The NCl (Nutrient Colorado + Inositol) medium plus 6 percent calf serum (*3*) was used throughout—as cell growth medium, as a vehicle for virus attachment, and as a general diluent. Dialyzed calf serum was used in all reaction mixtures that contained tritiated uridine. Monolayers of chick embryo cells were infected with VSV stocks prepared in cultures of chick embryo cells under conditions that produced high yields of B-type particles (*4*). Diethylaminoethyl (DEAE)-dextran (10 $\mu$g/ml) was added to all stocks of VSV to maximize plaquing efficiency on the monolayers. Virus attachment occurred with 0.5 ml of virus stock for 30 to 40 minutes at 4°C to achieve an effective multiplicity of about 10 to 20 plaque-forming particles (PFP) per cell and to provide a means of synchronizing the time of virion entry into the cell (*5*) and the initiation of polymerase activity. After

attachment and removal of unadsorbed virus, the cell monolayers were treated with 1.0 ml of warm (37° to 39°C) reaction mixture, kept at 37°C for 20 minutes, and incubated at 34°C. The standard reaction mixture consisted of NCI medium plus dialyzed calf serum (6 percent) containing the following reagents: actinomycin D (1 $\mu$g/ml), cycloheximide (50 $\mu$g/ml), [5-$^3$H]uridine (5 to 10 $\mu$c/ml; specific activity, $\simeq$ 25 c/mmole). For autoradiography the concentration of [$^3$H]uridine was increased to 25 to 50 $\mu$c/ml. Synthesis of virus RNA was measured at intervals during incubation in duplicate or triplicate. The reaction mixture was removed, and the virus-infected cell monolayer was solubilized by adding 1 ml of a solution of 1 percent sodium dodecyl sulfate (SDS) and 1 m*M* ethylenediaminetetraacetate (EDTA) in phosphate-buffered saline (without $Mg^{2+}$ and $Ca^{2+}$) and incubating for 10 minutes at 37°C. The solution containing the solubilized cell monolayer was placed in a Falcon plastic tube (17 by 100 mm), and the process was repeated with another 1 ml of the SDS buffer. The washings were combined, 0.5 ml of cold ether was added to the contents of the tube, and the tubes were agitated with a Vortex mixer. The ether treatment insured reproducible results and minimal background. After the phases separated, 2 ml of 10 percent trichloroacetic acid (TCA) was added to each tube. Vortex mixing was repeated, and the contents of the tubes were plated on Whatman glass fiber filters (GF/A) and washed with 5 percent TCA. The filters were dried and placed in plastic vials containing 5 ml of scintillation solution (toluene-BBOT); the radioactivity was then measured (Packard spectrophotometer, 3320). Controls to establish background [$^3$H]-uridine incorporation consisted of mock-infected cells treated in an identical manner. Independent controls established that cycloheximide in the reaction mixture (50 or 100 $\mu$g/ml) reduced the rate of protein synthesis to 5 percent or less of control values within less than 10 minutes.

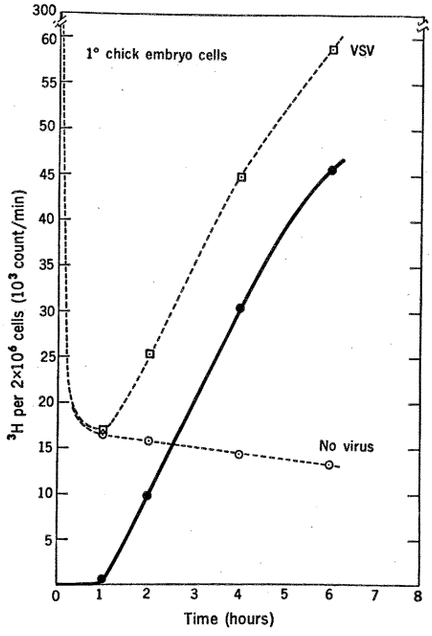


Fig. 1. The time course of [$^3$H]uridine incorporation into acid-insoluble material induced by VSV polymerase in monolayers of primary chick embryo cells that were treated with cycloheximide (50 $\mu$g/ml) (*6*), actinomycin D (1 $\mu$g/ml), and [$^3$H]-uridine (10 $\mu$c/ml) (solid line). The dotted line from time zero to 1 hour represents data from a mock-infected culture. The zero point was obtained by incubating the cells with [$^3$H]uridine in reaction mixture minus actinomycin D. Thus, the initial part of the curve represents the rapid loss of [$^3$H]uridine incorporation into acid-precipitable material characteristic of cells treated with actinomycin D. All other points represent the accumulation of acid-precipitable [$^3$H]uridine-labeled material produced by cells in the presence of actinomycin D and cycloheximide in the standard reaction mixture. The lower dotted line, from 1 hour on, shows the incorporation of [$^3$H]-uridine into acid-precipitable material in mock-infected cells, whereas the upper dotted line, from 1 hour on, demonstrates the cumulative increase of $^3$H (count/min) into acid-insoluble material after infection of the monolayer with a multiplicity of 30 plaque-forming particles per cell. The solid line is derived from the difference between VSV-infected and mock-infected cultures and is taken as representing VSV-RNA synthesis induced by virion polymerase.

A typical experiment (Fig. 1) illustrates the effect of cycloheximide on virion-induced RNA synthesis. The lag period, before significant incorporation of [$^3$H]uridine into acid-insoluble material, varied from ¼ to 2 hours, but averaged about 1 hour from the time that the reaction mixture was added (*6*). The lag period terminated with a linear increase in the cumulative incorporation of [$^3$H]uridine. The different rates of accumulation observed may reflect variability in the physiology of the cell monolayers (*7*). Linear accumulation of [$^3$H]uridine into acid-insoluble material persists for 5 to 6 hours. The background levels of incorporation are high in most tests, but the increase in acid-insoluble counts incorporated in cells infected with VSV is significant, especially since the background counts in mock-infected cells tend to stabilize or decrease slightly in cumulative label experiments (Fig. 1), and VSV may actually produce "background" levels below those of controls because of its capacity to turn off cell RNA synthesis (*8*). Hence, if RNA synthesis in VSV-infected cells is calculated as the difference between $^3$H (count/min) in the acid-insoluble fraction of the background and that of infected cells, the value so obtained is probably minimal.

The acid-insoluble, $^3$H-labeled product synthesized in cells infected with VSV and incubated for 5 hours under standard conditions was extracted to determine whether it represented the complementary product of functional polymerase from the vesicular stomatitis virion. The $^3$H-labeled product was annealed to unlabeled virion RNA as follows. Several 60-mm plates of chick embryo cells were infected with VSV

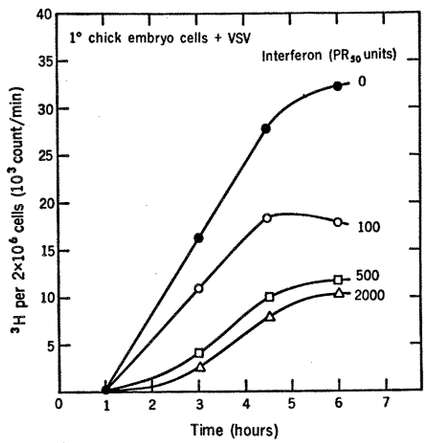


Fig. 2. Effect of interferon-mediated interference on VSV-RNA synthesis induced by the virion polymerase. Primary chick embryo cell monolayers were "aged" in vitro in attachment solution for 3 days at 37°C (*14*), and then fresh medium containing various amounts of chick-derived interferon was added in a volume of 1.5 ml per 35-mm plate. Incubation with interferon or mock interferon was carried out for 20 to 24 hours at 40°C. The medium was aspirated and 0.5 ml of VSV stock [the usual titer was 1 to 2 × 10$^8$ plaque-forming particles per milliliter, of the B-type (*4*)] was adsorbed for 30 to 35 minutes at 4°C. Unadsorbed virus was removed by aspiration, 1.0 ml of the standard reaction mixture was added, and the plates were held at 37°C for 20 minutes before they were placed at 34°C for the remainder of the experiment. Duplicate plates were processed to measure $^3$H radioactivity in material precipitable in cold acid (*12*). The number of PR$_{50}$ (VSV) units of chick cell interferon is shown. The curves represent the difference in radioactivity between virus-infected and mock-infected cells treated with real and mock interferon. Background activity was the same, within ± 15 percent, for cells treated with real or mock interferon.

(Fig. 1, legend) and incubated in the standard reaction mixture for 5 hours. Cells from these monolayers were solubilized in SDS-EDTA solution, and the [$^3$H]RNA was extracted with phenol and purified through a Whatman CF-11 cellulose column. Large RNA molecules were absorbed to the column at 25°C in 35 percent ethanol in TSE buffer (*9*) and were eluted in TSE alone, a procedure that yields only large RNA molecules, both single- and double-stranded. When this fraction was treated with ribonuclease A in high salt [1 × SSC (0.15*M* sodium chloride, 0.015*M* sodium citrate), *p*H 7.4], 99 percent of it was digested, an indication that essentially all of the RNA synthesized and accumulated in 5 hours in VSV-infected cells was single-stranded (*10*). The annealing reaction was performed in 1 × SSC at 121°C (autoclave) for 5 minutes by mixing unlabeled virion RNA [extracted with phenol-SDS from partially purified VSV (*11*)] with the $^3$H-labeled acid-insoluble product (prepared as described in Table 1 from either VSV-infected or mock-infected chick embryo cells). The mixture was cooled to 85°C, kept at that temperature for 2 hours, and finally cooled to 40°C for 4 hours. The resultant products were assayed for resistance to ribonuclease A in high- and low-salt solutions. In the uninfected chick embryo cell there was no RNA that could anneal with anything in the cell itself (Table 1, lines 1 and 2). About 33 percent of the RNA extracted from VSV-infected cells anneals to vesicular stomatitis virion RNA—that is, is resistant to ribonuclease A in 1 × SSC (Table 1, lines 3 and 4). This ribonuclease-resistant material is sensitive to the nuclease in low-salt and resistant in high-salt solutions, indicating that it is in a double-stranded form (Table 1). Thus, in contrast to RNA from uninfected chick embryo cells, a significant amount of the RNA from VSV-infected cells anneals to virion RNA. Further tests were made to determine whether optimum annealing conditions had been attained. The annealing mixture was subjected to cellulose CF-11 column chromatography to separate single- and double-stranded molecules (*9*). Thirty-one percent of the label from VSV-infected cells was located in material that produced a discrete peak that was completely sensitive to ribonuclease in low-salt and was 87 percent resistant in high-salt solution, an indication of predominantly double-helical material. The remaining label, 67 percent of the total, was in material that produced a second discrete peak and was totally sensitive to digestion by ribonuclease A, an indication of single-stranded material. Thus, the annealing reaction produces two species of RNA, one which is mostly double helical and one which is entirely single stranded. Material from the peak of single-stranded RNA was tested by repeating the annealing procedure with freshly added unlabeled virion RNA.

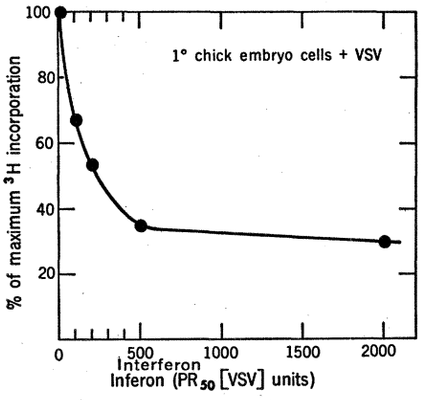


Fig. 3. Dose dependence of interferon and the inhibition of vesicular stomatitis virion–induced RNA synthesis. Experimental conditions as described in Fig. 2, but the dose of chick cell-derived interferon was varied and all plates were sampled at 4½ hours after infection with VSV. Each point is derived from radioactivity measurements (count/min) of duplicate plates and represents the difference between cells treated with mock interferon or interferon and infected with VSV (the multiplicity was 20 PFP).

The second annealing rendered 36 percent of the single-stranded material resistant to ribonuclease A. Thus, at least 55 percent (31 plus 36 percent of 67 percent) of the product was complementary to VSV-virion RNA. The results imply that the failure to anneal the remaining 45 percent of single-stranded RNA does not necessarily mean that the RNA is other than VSV-specific. The observed results could be explained by an interference of the annealing process due to a vast excess of unlabeled chick cell RNA in the reaction mixture. Hence, at least 55 percent of the product extracted from VSV-infected chick cells is complementary to the virion RNA of VSV. This product, therefore, is the result of VSV-RNA polymerase activity of the infecting virions functioning in the virtual absence of viral and cellular protein synthesis and during inhibition of cellular RNA transcription.

The effect of interferon-mediated interference on this VSV-specific RNA synthesis was determined. When chick embryo cell monolayers are first treated with interferon derived from chick cells and infected with VSV, the incorporation of [$^3$H]uridine into acid-precipitable material is reduced (Fig. 2) (*12*). There is a lag in the appearance of virion polymerase–induced RNA synthesis and continued decrease in the apparent rate of its accumulation until no net synthesis is apparent (5 to 6 hours).

Table 1. Test for annealing of vesicular stomatitis virion RNA with the $^3$H-labeled product from chick embryo cells (CEC) infected with VSV (the multiplicity was = 10 PFP per cell) in the presence of actinomycin D (1 μg/ml) and cycloheximide (50 μg/ml). One hour after infection and addition of the standard reaction mixture, [$^3$H]uridine (10 μc/ml) was added to each 60-mm plate of cells, and incubation was continued for 4 hours at 34°C. Mock-infected and VSV-infected plates averaged 8,467 and 24,673 count/min, respectively, of material precipitable by cold trichloroacetic acid per plate in this experiment. This material was extracted five times at room temperature with redistilled phenol saturated with phosphate-buffered saline. The resultant aqueous phase was precipitated at −20°C after the addition of 0.1 vloume of 20 percent (volume per weight) sodium acetate and 2 volumes of absolute alcohol. The precipitate was resuspended in water, lyophilized, and resuspended in 5 ml of 1 × SSC (*p*H 7.4). Mock-infected cells yielded 87,500 count/min from 14 60-mm plates (5 to 7 × $10^6$ cells per plate) and gave a specific activity of about 3,800 count/min per absorbancy (260 nm) unit. The 14 plates of VSV-infected cells yielded 303,000 count/min and gave a specific activity of about 20,000 count/min per absorbancy unit. This material was purified furtherthrough cellulose chromatography and tested as described in a 1.0 ml volume at different salt concentrations and in the presence or absence of ribonuclease A. Reaction mixtures were incubated for 30 minutes at 35°C.

| Test condition | Solution | | Source of [$^3$H]RNA | | Ribonuclease A (μg) | $^3$H acid precipitable (count/min) |
|---|---|---|---|---|---|---|
| | Type | Amount (ml) | Cell | Amount (ml) | | |
| 1 | 1 × SSC | 0.6 | Mock-infected CEC | 0.4 | | 561 |
| 2 | 1 × SSC | .6 | Mock-infected CEC | .4 | 10 | 5 |
| 3 | 1 × SSC | .6 | VSV-CEC | .4 | | 4254 |
| 4 | 1 × SSC | .6 | VSV-CEC | .4 | 10 | 1301 |
| 5 | $H_2O$ | .9 | VSV-CEC | .1 | | 1014 |
| 6 | $H_2O$ | .9 | VSV-CEC | .1 | 10 | 3 |
| 7 | 1 × SSC | .9 | VSV-CEC | .1 | 10 | 306 |

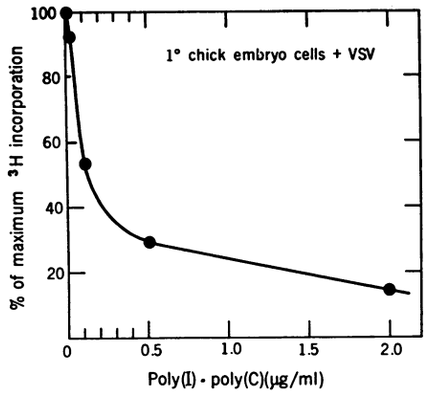


Fig. 4. Dose dependence of poly(I) · poly(C) and the inhibition of RNA synthesis induced by vesicular stomatitis virion. Experimental conditions were like those described in Fig. 2, but the dose of poly(I) · poly(C) was varied and all plates were sampled at 4½ hours after infection with VSV. The DEAE-dextran (10 μg/ml) was added to all plates treated with poly(I) · poly(C). Incubation was for 24 hours at 40°C. Each point represents a duplicate sampling as in Figs. 1 to 3, with replicates agreeing within ± 15 to 20 percent of the mean value.

It is important to ascertain whether the reduction in uridine incorporation into macromolecules observed in interferon-treated cells reflects a real decrease in RNA synthesis or an apparent change resulting from an effect on the uridine transport system as reported recently for chick and other cells (*7*). The acid-soluble uridine in cells was determined by adding uridine (100 m*M*) and [³H]uridine (10 μc/ml; standard reaction mixture) to monolayers for 20-minute periods every hour after infection, and then extracting the cells for 5 minutes with cold 5 percent TCA (*7*). The uridine thus incorporated into the acid-soluble material of the infected cell is not affected by prior treatment with interferon—over a 5-hour period averaging 11,480 and 11,300 count/min for each 20-minute labeling period per $1 \times 10^6$ infected cells treated with mock or real interferon, respectively. Individual determinations agreed within ± 15 percent of the average. Similar results were obtained in uninfected cells treated with real or mock interferon. Hence, interferon-mediated viral interference does not significantly change the permeability of the cell membrane for uridine—an indicator molecule for such changes (*7*).

The dose dependence of interferon on inhibition of virion polymerase–induced RNA synthesis is shown in Fig. 3. In this experiment, 250 $PR_{50}$ VSV units (*13*) of interferon reduced virion-RNA synthesis, and the capacity to produce infectious virus by 50 and 98 percent, respectively. High concentrations of mouse interferon [5000 $PR_{50}$ VSV units (*13*)] had no significant effect on VSV polymerase activity in chick embryo cells, attesting to the specificity of the interferon effect.

Prior treatment of chick embryo cells, aged for 3 to 7 days in vitro (*14*), with double-stranded polyinosinate·polycytidylate [poly(I)·poly(C)] induces a similar type of dose-response curve (Fig. 4). In the experiment shown, 0.1 μg of poly(I)·poly(C) per milliliter induced a 50 percent inhibition of virion-RNA synthesis and a 99 percent reduction in the capacity of the cells to produce infectious virus. The dose-dependency curves for interferon action and that of poly(I)·poly(C) in interfering with virion-induced RNA synthesis are similar, as might be expected if the basic process were common to the two different agents inducing the interference. Both curves show an initial period of responsiveness followed by reduction in the efficacy of increasing doses of interferon or poly(I) · poly(C) as inhibitors of viral RNA synthesis. These curves may indicate that there are two different states

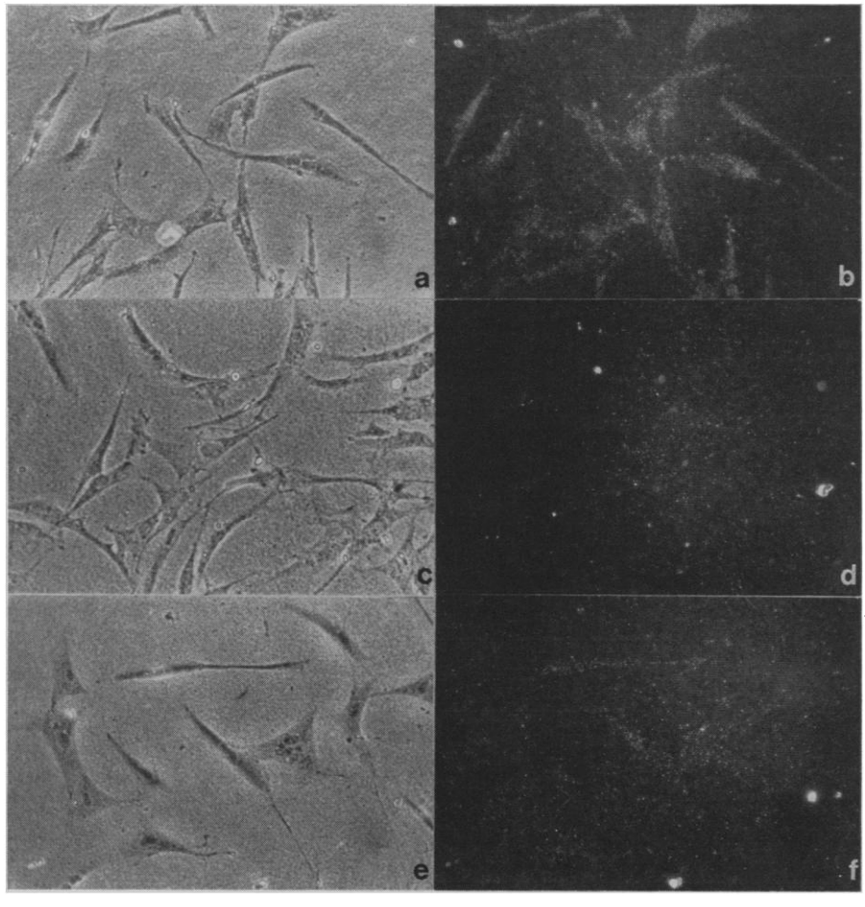


Fig. 5. Phase-contrast photomicrographs of chick embryo cells and their corresponding autoradiographs, which demonstrate RNA synthesis induced (a and b) by VSV virion–bound polymerase, (c and d) in the absence of VSV infection, and (e and f) by virion polymerase after prior treatment of cells with chick-derived interferon. Cells (a and b) received mock interferon + VSV ($m = 20$); (c and d) received mock interferon only; and (e and f) received interferon (≃ 1000 $PR_{50}$-VSV units) + VSV ($m = 20$). Chick embryo cells (1 to $2 \times 10^5$/ml for each 35-mm plate) were treated with chick-derived interferon or mock interferon [the supernatant fluid of chick embryo culture after 24 hours at 40°C purified through $Zn^{2+}$ precipitation procedure (*14*)], infected with VSV ($m =$ 20 PFP), and incubated in the standard reaction mixture lacking isotope. At 4½ hours after infection, 25 μc of [³H]uridine were added to the 1.0 ml of medium in each dish, and incubation at 34°C was continued for 20 minutes. The cells in the dishes were fixed with a mixture of acetic acid and ethanol, rinsed with phosphate-buffered saline, extracted twice (30 minutes each time) with 2 percent perchloric acid at 2°C, and rinsed with water. Each plate was dipped in Kodak NTB3 liquid emulsion and exposed for 6 days prior to development for silver grains. Dark-field optics were used to photograph the autoradiographs.

of virion RNA–polymerase complexes within the cell and that the refractory material may represent RNA-enzyme complexes from nonproductive particles physiologically or topologically isolated from the interferon system (*15*).

Autoradiographs confirm the virion polymerase activity in vivo and its inhibition by the interferon system. They illustrate for the first time the physical distribution of RNA produced by virion polymerase [mRNA = (+) strands (*16*)] in cells blocked in translation. The ubiquitous distribution of this viral RNA in the cell cytoplasm is revealed in autoradiographs produced from cells labeled for 20 minutes with [$^3$H]uridine 4½ hours after treatment with cycloheximide and actinomycin D, and infection with VSV (Fig. 5, a and b). In contrast, uninfected cells showed virtually all radioactivity localized in the nucleus—presumably reflecting residual cellular DNA–dependent RNA synthesis (Fig. 5, c and d). Compared to uninfected cells, many of the infected cells show fewer silver grains over the nuclear region—implying a synergistic effect of VSV and actinomycin D on the turning off of cell RNA synthesis (*8*). This finding indicates that viral RNA synthesis, as judged by gross differences in acid-precipitable radioactivity (count/min) from [$^3$H]uridine incorporation in uninfected and infected cells probably provides a minimum measure of the synthetic activity. The reduction in virion polymerase activity, indicated by a reduction in grain counts, that results from prior treatment with interferon or poly(I)·poly(C), is illustrated in Fig. 5, e and f. The distribution of grains indicates that many cells in the population have an interferon-refractory source of virion-induced RNA synthesis (Fig. 3). Examination of the autoradiographs at several planes reveals that there are silver grains over the nuclei of VSV-infected cells, but this number is significantly less than in the controls. This difference is not readily apparent from photomicrographs taken at a single plane of focus (Fig. 5, b and f).

The discovery of virion-associated polymerases (*1, 17*) provides a new dimension in the molecular biology of animal viruses, and its demonstration as a functioning system in vivo in reovirus-infected cell populations (*18*) and herein with VSV, extends the usefulness of this discovery to complex systems in the intact cell—including analysis of individual cells. After attachment and entry of the VSV genome and its attendant polymerase into a host cell, the genome's transcription—synthesis of a complementary RNA strand—is, most likely, the first virus-specific synthetic event (*1*). Our studies show that, even in the absence of an amplification of this event by translation, the molecules of virion polymerase and RNA that enter the cell at the time of infection are stable enough to provide substantial synthesis of RNA for several hours, spanning the equivalent of much of the growth cycle of the virus. Whether the rate of viral RNA synthesis from parental strands and its quality are tempered by subsequent translational events in the normal infective process remains to be studied. However, results from the autoradiographs indicate that interferon acts to reduce the rate of virion-induced RNA synthesis.

The inhibitory effect of the interferon system on VSV virion polymerase activity provides the first evidence of interference with the function of a specific viral enzyme in the absence of translation. This finding raises several new questions concerning the molecular basis of interferon action. Our results appear to conflict with earlier evidence from interferon-treated cells for an inhibition of viral RNA translation (*19–21*) and a lack of inhibition of vaccinia mRNA transcription (*20*). The report (*21*) of a reduction in the synthesis of early RNA in vaccinia-infected chick embryo cells through interferon action suggests inhibition of the viral transcriptase. The conflicting report obtained with mouse L cells may have resulted from the exaggerated cytotoxic effect of vaccinia virus on L cells first treated with interferon (*20*). However, Bialy and Colby (*22*), using our experimental conditions, have shown that the interferon system inhibits transcription by the DNA-dependent RNA polymerase of the vaccinia virion.

There is no reason for supposing that the interferon system consists of a single molecular species with a single action. The interferon system might encompass reactions that inhibit viral translation (*19–21*), viral RNA transcription, or a transcription-translation–coupled system. If the last were a truer description of the site of action, then experiments designed to measure an effect on each reaction separately might provide equally affirmative results. In that sense, our experiments do not preclude translation of VSV mRNA as another possible site of action by the interferon system. Until more is known about the efficiency of the transcriptase reaction in vitro and in vivo, a necessary coupling of transcriptional and translational events cannot be ruled out.

Speculation on interaction of the postulated antiviral protein of interferon action, whether a translation inhibitory protein (*19*) or a transcription inhibitory protein, or both, requires substantiation by experiments on transcription-translation reactions—coupled and uncoupled. A regulator molecule of viral RNA synthesis may be a prime candidate for the putative antiviral protein, but selective nuclease activity as an alternative mode of action is also possible. It has been suggested that interferon action may involve inhibition of transcription of early SV40 mRNA (*23*), and that translation of the input genome strand of Sindbis virus may not be subject to control by the interferon system (*24*).

PHILIP I. MARCUS
DEAN L. ENGELHARDT
JOHN M. HUNT
MARGARET J. SEKELLICK
*University of Connecticut, Storrs 06268*

### References and Notes

1. D. Baltimore, A. S. Huang, M. Stampfer, *Proc. Nat. Acad. Sci. U.S.* **66**, 572 (1970).
2. We first used 60-mm dishes containing 5 to $7 \times 10^6$ cells at confluency. However, the acid-precipitable material from this large number of cells was difficult to filter. The yield from the 35-mm plates was easier to filter and more economical of cells, medium, and radioisotopes. Reproducibility of isotope counting was high in the 35-mm dishes (standard deviation of ± 15 to 20 percent of the mean value).
3. P. I. Marcus and D. H. Carver, *Science* **149**, 983 (1965), ref. 3.
4. A. S. Huang, J. W. Greenawalt, R. R. Wagner, *Virology* **30**, 161 (1966); A. S. Huang and D. Baltimore, *Nature* **226**, 325 (1970).
5. S. S. Silverstein and P. I. Marcus, *Virology* **23**, 370 (1964).
6. Prior treatment of monolayers with cycloheximide for 5 minutes at 37°C and the drug's continued presence during virus adsorption at 4°C produced a slightly longer lag period but results were otherwise similar to Fig. 1. Puromycin (50 μg/ml) was used successfully in the reaction mixture in place of cycloheximide, making it unlikely that some unique type of protein synthesis was responsible for the observed results. *p*-Fluorophenylalanine (750 μg/ml) may be used instead of cycloheximide although the apparent RNA synthesis was less. All three reagents reduced the yield of infectious VSV to essentially background (~ $10^5$-fold reduction). Sindbis virus, which requires the synthesis of polymerase for RNA replication, was tested in the standard reaction mixture as an additional control on the effectiveness of the block to protein synthesis. No viral RNA synthesis was detected 6 hours after infection ($m = 20$), but when cycloheximide was omitted, 20,400 count/min had accumulated. Background averaged 2400 count/min.
7. Young cell monolayers infected with VSV show greater uridine incorporation into the acid-insoluble fraction than do cells aged in vitro—perhaps because of decreased efficiency in the uridine transport system of chick embryo cells at high population densities

[M. J. Weber and H. Rubin, *J. Cell. Physiol.* **77**, 157 (1971)]. The transport of uridine into cells may be the rate-limiting step for its accumulation [P. G. W. Plagemann and M. F. Roth, *Biochemistry* **8**, 4782 (1969); T. L. Steck, Y. Makata, J. P. Bader, *Biochim. Biophys. Acta* **190**, 237 (1969); M. J. Weber and H. Rubin. *J. Cell. Physiol.* **77**, 157 (1971).

8. A. S. Huang and R. R. Wagner, *Proc. Nat. Acad. Sci. U.S.* **54**, 1579 (1965). More pertinent are autoradiographic experiments with actinomycin D and cycloheximide. They indicate that cellular RNA synthesis insensitive to this mixture—revealed as intranuclear silver grains after labeling for 20 minutes with [$^3$H]uridine—decreases in VSV-infected cells whether first treated with interferon or not. Initial studies with VSV indicate that the RNA-synthesizing capacity of the virion in vivo is about four times more resistant to ultraviolet radiation than is infectivity (plaque formation) and exposure to 20 to 30 lethal hits (1 hit = 37 percent survival of infectivity), while sufficient to eliminate detectable RNA synthesis, produces a background of acid-insoluble radioactivity about one-half that of mock-infected control cells. Thus, the synthesis of VSV-RNA under our conditions provides a minimal value and the true value may be twice that reported.
9. R. M. Franklin, *Proc. Nat. Acad. Sci. U.S.* **55**, 1504 (1966). (TSE: tris, saline, EDTA.)
10. Similar results obtained from mock-infected cells demonstrate that the high background of acid-insoluble. [$^3$H]uridine-labeled material in the chick embryo cells treated with actinomycin D and cycloheximide is not due to significant amounts of double-stranded RNA or DNA-RNA hybrid molecules.
11. J. A. Mudd and D. F. Summers, *Virology* **42**, 328 (1970).
12. The curves in Fig. 2 do not show the original data for the mock-infected and mock-interferon controls, and for the VSV-infected cells with and without prior interferon treatment. All uninfected cell controls show results similar to the lower dotted curve in Fig. 1.
13. $PR_{50}$ means plaque reduction assay where 1 $PR_{50}$ unit (VSV) represents the concentration of interferon that will reduce the plaque titer of VSV by 50 percent in a standard assay system [R. R. Wagner, *Virology* **13**, 323 (1961)]. Mouse interferon purified by zinc acetate (Dr. C. Colby) showed 50,000 $PR_{50}$-VSV unit/ml assayed on mouse L cells. Interferon or mock interferon prepared from chick embryo cells aged in vitro (*14*) and preparations purified with zinc acetate usually contained 10,000 $PR_{50}$-VSV unit/ml assayed on 3-day monolayers of chick embryo cells. Preparations obtained after perchloric acid precipitation and neutralization with KOH produced comparable results.
14. D. H. Carver and P. I. Marcus, *Virology* **32**, 247 (1967); R. Z. Lockart, Jr., *Med. Appl. Virol.* **2**, 45 (1968).
15. This explanation follows from the large ratio of physical to infectious particles in a stock preparation of B-type VSV [A. J. Hackett, *Virology* **24**, 51 (1964), which implies that many virions of VSV do not initiate a successful infection. However, they may function as enzymatically active, biologically nonproductive, transcribing complexes. D. H. L. Bishop has shown that at least 25 percent of the VSV B-type particles possess functional transcriptase [*J. Virol.* **7**, 486 (1971)]. Some nonproductive transcribing complexes may not be accessible to inhibition mediated by the interferon system and hence register as refractory to its action—even under conditions where inhibition of the biologically important enzymatic activity may be virtually complete so as to produce a precipitous drop in yield of infectious virus.
16. By the usual convention the virion (parental or genome) strand of RNA is termed plus (+). The translating or messenger strand of most RNA viruses is a (+) strand or messenger RNA (mRNA). However, in VSV there is evidence that the translating or message strand has a base composition complementary to virion RNA [F. L. Schaffer, A. J. Hackett, M. E. Soergel, *Biochem. Biophys. Res. Commun.* **31**, 685 (1968)]. If the message strand, rather than the virion strand, on polysomes of infected cells is a common reference point, then almost all viruses contain a (+) strand (mRNA) in the virion. However, by this convention, VSV contains a (−) strand in the virion and the virion polymerase produces a (+) strand or mRNA. As noted by Baltimore *et al.* (*1*) the virion polymerase of VSV is probably a transcriptase rather than a replicase.
17. Vaccinia virus: J. R. Kates and B. R. McAuslan, *Proc. Nat. Acad. Sci. U.S.* **58**, 134 (1967); W. E. Munyon, E. Paoletti, J. T. Grace, Jr., *ibid.* **58**, 2280 (1967). Reovirus: A. J. Shatkin and J. D. Sipe, *ibid.* **61**, 1462 (1968); Y. Borsa and A. F. Graham, *Biochem. Biophys. Res. Commun.* **33**, 895 (1968). Vesicular stomatitis virus; D. Baltimore, A. S. Huang, M. S. Stampfer (*1*); RNA tumor viruses: H. Temin and S. Mizutani, *Nature* **226**, 1211 (1970); D. Baltimore, *ibid.*, p. 1209. Newcastle disease virus: A. H. Huang, D. Baltimore, M. A. Bratt, *J. Virol.* **7**, 389 (1971).
18. Y. Watanabe, S. Millward, A. F. Graham, *J. Mol. Biol.* **36**, 107 (1968).
19. W. A. Carter and H. B. Levy, *Science* **155**, 1254 (1966); *Biochim. Biophys. Acta* **155**, 437 (1968); H. B. Levy and W. A. Carter, *J. Mol. Biol.* **31**, 561 (1968); P. I. Marcus and J. Salb, *Virology* **30**, 502 (1966); I. M. Kerr, *J. Virol.* **7**, 448 (1971).
20. W. K. Joklik and T. C. Merigan, *Proc. Nat. Acad. Sci. U.S.* **56**, 558 (1966).
21. S. Ohno and T. Nozima, *Acta Virol.* **10**, 310 (1966).
22. H. Bialy and C. Colby, in preparation.
23. M. N. Oxman and M. J. Levin, *Proc. Nat. Acad. Sci. U.S.* **68**, 299 (1971).
24. P. I. Marcus and H. L. Zuckerbraun, in *The Biology of Large RNA Viruses*, R. D. Barry and B. W. J. Mahy, Eds. (Academic Press, New York, 1970), p. 455 and figures 16 and 17.
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# Light Adaptation in the Rat Retina: Evidence for Two Receptor Mechanisms

Abstract. *Light adapting the rat retina with transient white flashes too dim to bleach a substantial amount of visual pigment produces a change in electroretinogram spectral sensitivity and an increase in flicker fusion frequency. Increment threshold curves obtained with a long wavelength adapting stimulus and a short wavelength test stimulus show rod saturation.*

Although it is generally agreed that some of the visual receptors in the rat differ from typical rods, experiments on the rat eye are frequently interpreted as if responses reflected the activity of rods alone (*1*, *2*). This report provides new evidence that at higher levels of light intensity a second type of receptor contributes to the rat electroretinogram (ERG). The starting point for these experiments was two aspects of rat vision which differ from rod vision in the human eye.

First, light adaptation produces a spectral sensitivity that differs from the rod rhodopsin spectral sensitivity of the dark-adapted animal in that it is more sensitive in the long wavelength region (*3*, *4*). In man such shifts signify a shift from rods to cones. Second, increment thresholds increase proportionately with increases in background (*5*) over a range of intensities which far exceeds those found for the rods in man, cat, and monkey. The rods in these latter animals saturate under moderate conditions of illumination (*6*–*8*). The shift in spectral sensitivity and the failure to find rod saturation might be interpreted as evidence for a second receptor; however, there are other possible explanations. For example, as Dowling (*2*) has pointed out, at low levels the visual pigment in the rods is likely to act as screening pigment in front of the reflective postretinal tissues. Adapting the retina to levels that bleach a significant amount of visual pigment could remove this screen and produce a shift in spectral sensitivity due to the increased reflectance from postretinal tissues. Another possibility is that the bleaching of visual pigment produces photoproducts (*9*) which filter the incident light.

Not only might bleaching explain spectral sensitivity changes, it could also account for the failure to find rod saturation. As has been shown for human cones by Alpern *et al.* (*10*), pigment bleaching by a steady background

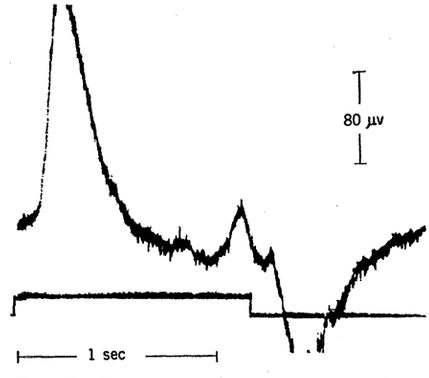


Fig. 1. Electroretinogram recorded from albino rat in response to a test flash which follows 1 second after the onset of a brief adapting stimulus. The lower tracing marks the duration of the adapting stimulus. The onset of the adapting stimulus produced a large *b*-wave response that carried the tracing off the oscilloscope screen. The smaller positive response appearing just before the adapting light turns off is the incremental response to the test stimulus.