Effect of Interferon on Synthesis of Eastern Equine Encephalitis Virus RNA[1] (35502)

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The action of interferon is thought to involve inhibition of the translation of viral messenger RNA (1–3), although its precise mechanism is not known. The one system, that of Marcus and Salb (2), which purports to demonstrate the site and mode of such inhibition, by showing *in vitro* that Sindbis virus RNA-chick ribosome complexes from interferon-treated cells did not translate, unfortunately has not been confirmed (4).

As a contribution to the solution of this problem, we decided first to characterize the effect of rabbit interferon on Eastern equine encephalitis (EEE) viral RNA synthesis, an interferon and virus with which we have considerable experience (5, 6). De Somer *et al.* (7) originally showed that chick interferon inhibited the synthesis of phenol extractable infectious RNA and suggested that that may be the primary action of interferon. Ho (5) suggested on quantitative grounds that this was unlikely because interferon inhibited virion replication to a much greater extent than biologically active RNA. Mecs *et al.* (8) presented similar results with Semliki Forest virus, and showed, in addition, that the 20S ribonuclease-resistant RNA and the 26S RNA were even less inhibited by interferon than total viral RNA. The results reported herein that interferon exerts relatively little effect on rate of synthesis of viral RNA synthesis further substantiates the impression that interferon does not primarily act on viral RNA synthesis.

Methods. Cell cultures. A serially-passaged fibroblastic line of cells of rabbit embryo origin designated RE-ICH, initiated in this laboratory, was grown and maintained in Hanks' balanced salt solution supplemented with 0.5% lactalbumin hydrolysate, 2 m*M* L-glutamine, Eagle's vitamin mixture (9), and 8% newborn calf serum. Cultures were prepared in sealed bottles or in plastic petri dishes incubated in a humidified, 5% CO_2 incubator. The preparation of chick embryo cultures (10) and rabbit kidney cultures (6) has been previously described.

Eastern equine encephalitis (EEE) virus. The virus (5) was titrated by plaque formation on chick embryo cell cultures in plastic dishes incubated under 5% carbon dioxide.

Interferon. Interferon used in these experiments was obtained from serum of rabbits induced with NDV (6). The pool titrated 3300 units/ml by a method using 50% VSV plaque reduction in rabbit kidney plates (6). The effect of this interferon on reduction of EEE virus plaque formation is similar to VSV. Its effect on EEE virus replication is presented in Results.

Reagents. Uridine-5-^{3}H sp act 20 Ci/mmole, was obtained from Schwarz Bioresearch. Actinomycin D was supplied through the courtesy of Merck, Sharpe and Dohme. Reticulocyte standard buffer (RSB) (11), was prepared according to Warner *et al.* (12) and contained 10 m*M* NaCl, 1.5 m*M* Mg $(CH_3CO_2)_2$ and 10 m*M* Tris HCl at pH 7.4. NETS buffer (13) used in RNA centrifugation contained 0.1 *M* NaCl, 10 m*M* EDTA, 10 m*M* tris HCl and 0.2% sodium dodecyl sulfate (SDS) at pH 5.3. All solutions were prepared with sterile, distilled, deionized water.

Experimental protocol. Monolayer RE-ICH cultures in petri dishes or prescription bottles were treated overnight at 37° with a suitable dose of interferon diluted in maintenance medium. Doses are expressed in units

[1] This investigation was supported by Public Health Service Research Grant No. ROI AI-02953 from the National Institutes of Health.

per milliliter per 10^6 cells. Eighty min prior to infection the cultures received actinomycin D (2 μg/ml). They were then exposed to EEE at an input multiplicity of 15–30 PFU/cell, still in the presence of actinomycin D. After 40 min of adsorption at 37°, the cultures were washed twice with maintenance medium to remove residual virus. In continuous labeling experiments, fresh medium containing ^{3}H-uridine was added. The time of addition of ^{3}H-uridine, or in the case of pulse-labeled experiments, the time of addition of fresh medium was considered to be zero time and all other times are calculated from this moment. Actinomycin D was continuously present in all reagents used until the cultures were harvested. Pulse-labeling experiments were performed in a walk-in incubator room at 37°. Cells were detached and harvested by scraping into RSB in pulse-labeling experiments, or by the action of 0.25% trypsin in EDTA, 0.2%.

Cytoplasmic extracts. Cells were swollen for 15 min in RSB and broken with a tight-fitting stainless steel Dounce homogenizer. Nuclei and debris were removed by centrifugation for 10 min at 1000*g*. All operations were performed at 0–4°. Extracts for rate-zonal analysis of RNA were made 0.5% with respect to SDS and, if necessary, stored at —70°. Storage for up to 14 days at —70° did not affect subsequent density gradient analysis.

Rate-zonal centrifugation of sucrose density gradients. Fourteen to 27% (w/w) sucrose gradients were prepared in RSB (for cytoplasmic elements) or NETS (for RNA analysis) with a Beckman syringe-type gradient former. One-ml samples were layered on 30-ml gradients in 1 × 3-in. tubes and centrifuged in a Beckman SW 25.1 rotor at 4°. For RNA analysis, gradients were centrifuged at 22°. The gradients were fractionated with an Isco Model D fractionator with an ultraviolet analyser. Absorbance at 254 nm was continuously recorded. Sedimentation coefficients of viral components were estimated from the absorbance peaks of ribosomes or ribosomal RNAs.

Preparation of radioactive samples. The method used was a modification of that described by Staehelin *et al.* (14). Whole cultures solubilized in 0.05% SDS or gradient fractions were precipitated with 6–10% trichloroacetic acid (TCA, final conc) containing 80 mg of Celite and filtered onto a bed prepared by depositing 20 mg of Celite on a 25-mm filter paper disc. The sample was washed twice with 5 ml of 5% TCA, once with 5 ml of 0.5% TCA and once with 5 ml of methanol. The sample was then air-dried and transferred to a scintillation vial with 0.5 ml of an organic base solubilizing agent (NCS, Nuclear-Chicago and Co., or Soluene, Packard Instrument Co.) at 37° for 1 to 5 hr. Ten ml of scintillation fluid (toluene containing 2,5-diphenyloxazole, 5 g/liter) were added and the samples were kept in the dark for 72 hr prior to counting in a Beckman L-100 liquid scintillation counter at ambient temperature.

Ribonuclease treatment. Samples were diluted to contain <0.02% SDS and NaCl concentration was adjusted to 0.15 *M*. Pancreatic ribonuclease (Worthington Biochemical Corp.) was added at 5 μg/ml (18.2 units/ml) and the sample was incubated for 30 min at room temperature.

Results. The effect of interferon on virus growth. Under conditions described in Materials and Methods, EEE virus replicated rapidly in RE-ICH cells in the presence of actinomycin D, reaching a peak titer in 6 to 9 hr. The effect of various doses of interferon is shown in Table I. One hundred units reduced virus yield about 99% and further tenfold reduction was seen with 1000 units. Since virus titers did not significantly increase between 8 and 21 hr after infection, we believe that these growth curves represent

TABLE I. Effect of Dose of Interferon on Growth of EEE.

Dose of interferon (units/ml/ 10^6 cells)	EEE titer at time (hr postinfection)		
	0	8	21
0	4.2×10^{3a}	2.6×10^7	4.7×10^7
10	—	1.0×10^7	1.3×10^7
100	—	3.8×10^5	3.6×10^5
1000	—	2.2×10^4	1.1×10^5
10,000	2.8×10^3	3.6×10^4	5.2×10^4

[a] PFU/0.1 ml, assayed on chick embryo cell monolayers.

single cycles.

Inhibition of viral core formation by interferon. Group A arboviruses are reported to possess cores or nucleo-capsids which sediment at 130–140S in sucrose gradients (15). An experiment was performed to see if formation of these cores was inhibited by interferon. Cytoplasmic extracts were prepared 6 hr after infection of cell cultures treated with 100 units of interferon and centrifuged on sucrose gradients. A prominent peak at about 130S was evident in extracts from infected cells. This peak, which was not present in uninfected cells was about 95% reduced in cultures treated with 100 units of interferon, which is comparable to the reduction in virus yield by this dose of interferon.

The effect of interferon on viral RNA synthesis. Since both infectious virus and viral core synthesis were inhibited by interferon, we next examined the synthesis of virus-specific RNA. Synthesis of RNA in uninfected cultures treated with actinomycin D was negligible. In infected cultures (Fig 1), RNA synthesis commenced soon after infection and was still proceeding at 6 hr, when the experiments were terminated. At this time, cytopathic effects due to both actinomycin D and EEE were present. Pretreatment with interferon inhibited RNA synthesis by about 70% in this experiment, which is less than the amount of inhibition of infectious virus. Treatment with ribonuclease (Fig. 1) showed that inhibition of ribonuclease-resistant RNA was about 54%. In some experiments involving cytoplasmic extracts, no inhibition of ribonuclease-resistant material was seen.

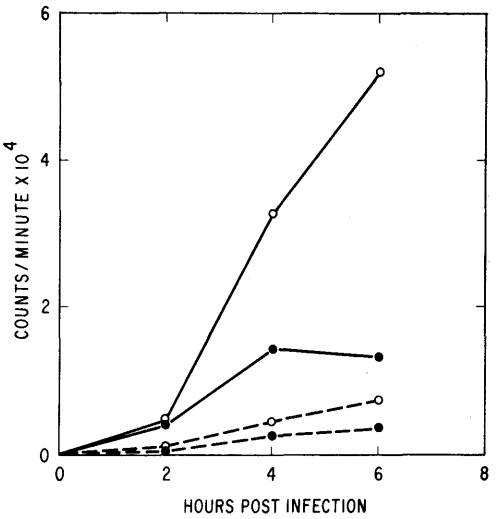


FIG. 1. The effect of interferon on EEE viral RNA synthesis. Each point represents the mean incorporation of ^{3}H-uridine by two 5-cm plate cultures (~3 × 10^6 cells/plate), treated with interferon (300 units/culture) (●); or normal rabbit serum (○). Tritiated uridine (2.5 μCi/ml) was added at 0 time and cultures were harvested at times shown. Total incorporation (—); and ribonuclease-resistant fraction (- - -) were measured.

To see if the decrease in viral RNA accumulation was due to a decrease in the rate

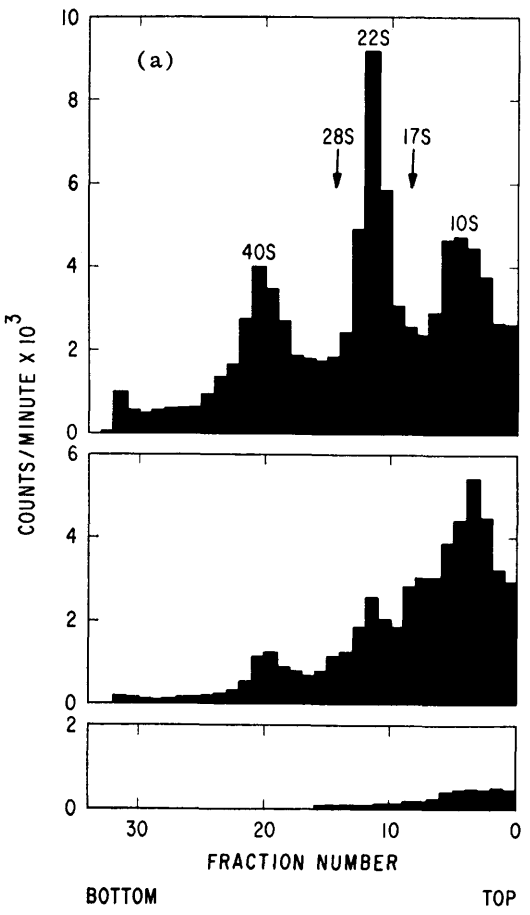
TABLE II. Effect of Interferon Treatment on the Rate of RNA Synthesis in Cells Infected with EEE.

Interferon doses[a] (units)	Pulse[b] (min)	Total incorporation (cpm)	Total incorporation % of control	RNase resistant[c] (cpm)	RNase resistant[c] % of control
0 (control)	3	3978	100	1200	100
	10	23,156	100	2580	100
100	3	5168	129	1195	96
	10	16,116	70	1872	73
3000	3	4072	102	1060	88
	10	12,952	56	1957	76

[a] 16 ml of interferon at indicated units/milliliter was placed on dishes containing 2.4 × 10^7 cells.

[b] Cultures were pulsed with ^{3}H-uridine 3.5 hr after infection. Acid precipitable ^{3}H-uridine was counted.

[c] Per 0.1 ml of cytoplasmic extract, equivalent to 3 × 10^5 cells.



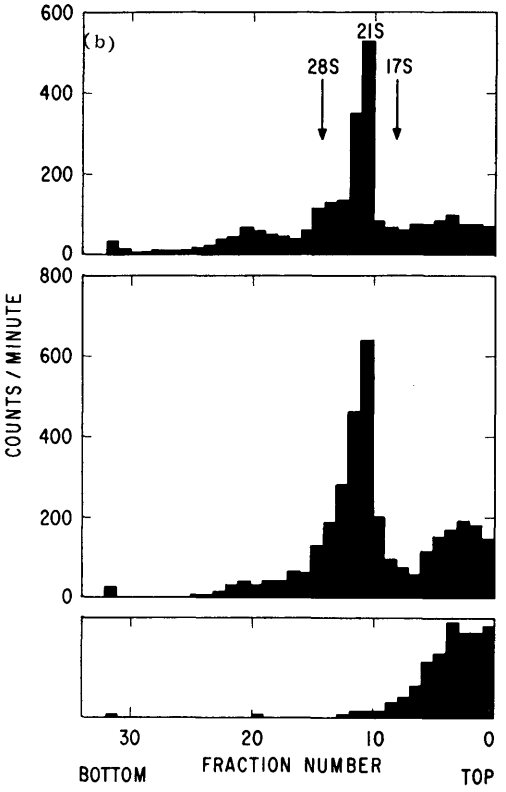


FIG. 2. The effect of interferon on the sedimentation profile of viral RNA. Cytoplasmic extacts from untreated cultures infected with EEE (top histograms); infected cultures pretreated with interferon (100 units/10^6 cells/ml) (middle histograms); and untreated, uninfected cultures (bottom histograms) were prepared and made 0.5% with respect to SDS. The extracts were centrifuged 16 hr at 19,000 rpm on 14–27% sucrose gradients. ^{3}H-Uridine (2.5 μCi/ml) was added at 0 time and the cytoplasmic extracts were prepared at 6 hr. Total (a); and ribonuclease resistant (b) incorporation into each fraction was measured. The positions of the 17S and 28S ribosomal RNAs are shown by vertical arrows.

of synthesis, the following experiment was done (Table II): Four 15-cm dish cultures containing each 2.4 $\times$ 10^7 cells were treated with different doses of interferon and 4 were left untreated. 3.5 hr after infection with EEE virus, when rate of accumulation of viral RNA was maximal (Fig. 1), half of the cultures were pulsed with 600 μCi of ^{3}H-uridine in 6 ml for 3 min, and the other half were pulsed for 10 min. Half of each sample was treated with RNase. Incorporation of ^{3}H-uridine into the TCA-precipitable fraction is shown in Table II. It is apparent that after a 3-min pulse, interferon had essentially no effect on the incorporation of uridine in total or RNase resistant RNA. In contrast, after a 10-min pulse, some inhibition of incorporation was observed, although this was less marked in the RNase-resistant fraction.

We interpret these results to mean that accumulation of viral RNA depends on two competing processes; synthesis and degradation. Rates of synthesis can only be compared in short pulse experiments. Under these conditions interferon does not decrease accumulation and hence has no effect on the rate of synthesis of viral RNA in this system. Separate experiments showed that 3-min pulses yielded identical results in cultures 2 and 6 hr after infection. On the other hand, inhibition of incorporation in 10 min or longer pulses may be caused by an increased

rate of degradation in interferon-treated cultures.

Measurement of the acid-soluble nucleotide fraction shows that although the ^{3}H-uridine does not equilibrate with the intracellular pool for several hours, interferon treatment has no effect on the rate of ^{3}H-uridine uptake. Therefore, the results described above are not due to interferon-induced alterations in the availability of the ^{3}H-uridine.

Density gradient analysis of RNA from infected cells. The distribution of accumulated cytoplasmic RNA of continuously labeled cultures on sucrose density gradients is shown in Fig. 2a. In uninfected cells, a small amount of low molecular weight material can be seen. In infected cells, most of the ^{3}H-uridine is incorporated in three peaks. There is a broad peak centered at about 10S of undetermined significance; a peak of 23S which probably includes the replicative intermediate since, as shown below, it is partially ribonuclease-resistant and a peak at 40S which, by analogy with findings using other group A arboviruses, is likely to be the viral RNA (16–19). Interferon inhibits both 23S and 40S peaks but has no effect on the 10S RNA. Portions of each fraction were treated with ribonuclease and the distribution of ribonuclease-resistant RNA is shown in Fig. 2b. Interferon has little or no effect on the ribonuclease-resistant RNA in the 20–23S region.

Next an analysis of RNA profiles after various pulse periods was undertaken (Fig. 3). After a 1 and 3-min pulse, essentially all the uridine is incorporated in an RNA peak at about 20S. This material is largely resistant to ribonuclease and interferon had no effect on its appearance. After a 10-min pulse both the broad 10S peak and a 40S viral RNA peak have appeared. There may also be a small peak at about 30S (fraction 19–20). In interferon treated cells, only the 40S peak (fraction 26–27) and possibly the 30S peak are inhibited. The 10S material seems to be made in normal amounts. Thus, it appears that in the presence of interferon, RNA initially synthesized in a 20–23S complex is prevented from maturing to the viral 40S form but is released to a 10S form.

Discussion. As shown previously (4), interferon inhibits EEE virus synthesis to a much greater extent than accumulated viral RNA. However, even with massive doses of interferon which inhibited more than 99.9% of virus replication, essentially no inhibition of the rate of RNA synthesis was observed.

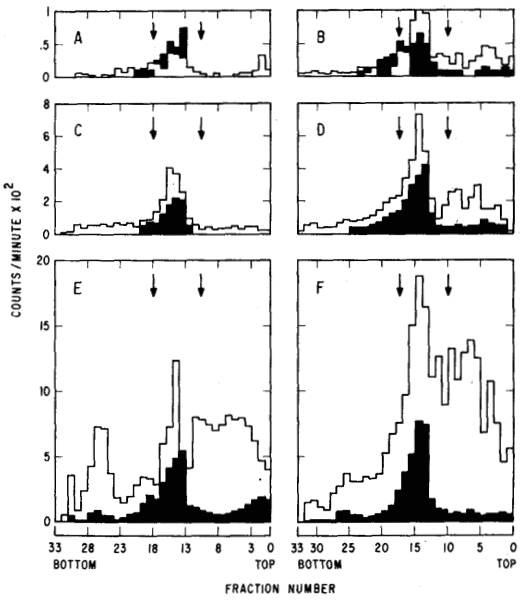


FIG. 3. The effect of interferon on rapidly synthesized EEE viral RNA. Density gradient analysis of cytoplasmic extracts of infected control (A, C, and E) or interferon treated, infected cultures (B, D, and F) pulsed at 3.3 hr with ^{3}H-uridine (200 μCi/ml) for 1 min (A and B); 3 min (C); 4 min (D); or 10 min (E and F). Each fraction was divided into two portions. Total acid precipitable tritium (white histograms); and ribonuclease-resistant tritium (black histograms) were measured. Procedures were as described under Fig. 2. (vertical arrows) denote the 17S and 28S ribosomal RNAs.

Pulse-labeling experiments show that in normal infected cells, the RNA first made is a 20S RNase resistant fraction, a process which is not inhibited by interferon. After longer pulses, the label appears in three RNase-sensitive peaks: one in the 40S region, one about 20–23S and a third heterogeneous class of RNA sedimenting broadly in the 10S region. In interferon treated cells, there is inhibition of accumulation of RNase sensitive material in the 20–23S and 40S region.

These results are best explained by the supposition that the 20–23S complex serves as a

precursor of 40S viral RNA, and this transition is inhibited by interferon. Perhaps in interferon treated cells, a breakdown product represented in the 10S region is made instead. Sonnabend (20) also shows but do not comment on a low molecular weight RNA in interferon-treated, infected cells.

The apparent absence of a 26S "interjacent" RNA found with other group A viruses including EEE (21) is puzzling. Several possible explanations come to mind. Perhaps it is sedimenting too close to the complexes containing 20S RNase-resistant material to be separable on our gradients, or the techniques we used may have converted the 26S RNA to a different form, perhaps 40S material. Finally this type of RNA may not be made in demonstrable amounts in this system.

Our work confirms the interferon resistance of the 20S RNA-resistant complex (8) and further suggests that the actual rate of incorporation of nucleotides as measured in short-pulse experiments, is not reduced by interferon. This result is rather surprising in view of the inhibition of RNA polymerase activity reported by Sonnabend (20). Perhaps the level of enzyme present is not the critical factor controlling RNA synthesis in the intact cell. This suggestion is strengthened by the recent finding of Friedman and Grimley (22) that cycloheximide, which stops synthesis of all proteins, including, presumably, RNA polymerase, had relatively little effect on Semliki Forest virus RNA synthesis.

Summary. Rabbit cell cultures were treated with interferon and then infected with EEE in the presence of actinomycin D. Concentrations of interferon that inhibited virus replication by 99%, inhibited accumulation of viral RNA, as measured by continuous labeling, by about 70%. On the other hand, the rate of synthesis of viral RNA, as measured by short-pulse labeling, was not significantly inhibited by large doses of interferon. Sucrose density gradient analysis of viral RNA from interferon-treated and untreated cells showed that ^{3}H-uridine was rapidly incorporated into a ribonuclease-resistant structure sedimenting at 20S. This incorporation was unaffected by interferon; but interferon reduced accumulation of 40S virion RNA and ribonuclease sensitive RNA sedimenting at 23S. We conclude that the observed inhibition of accumulation might be due to an increased rate of turnover which may be associated with the well-known ability of interferon to inhibit viral protein synthesis.

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Received Nov. 9, 1970. P.S.E.B.M., 1971, Vol. 137.