

Effect of Viral Double-Stranded RNA on Protein Synthesis in Intact Cells

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Received for publication 17 August 1972

The addition of purified, noninfectious, double-stranded RNA of bovine enterovirus, a picornavirus, to intact cells in culture results in a rapid cessation of cellular polypeptide synthesis. This inhibition is specific for host cell protein synthesis since the translation of picornavirus-specific proteins is not affected by the double-stranded viral RNA.

It has been recently reported that heterologous double-stranded RNAs have an inhibitory effect on *in vitro* protein synthesis (4, 8, 9). We report a similar inhibition of cellular polypeptide synthesis when purified, noninfectious, double-stranded RNA isolated from cells infected with a picornavirus, bovine enterovirus -1 (BEV), is added to intact cells in suspension culture. This inhibition is specific for cell-directed protein synthesis as demonstrated by a lack of inhibition when the viral double-stranded RNA is added to either BEV or Mengo virus-infected cells at the time of maximum viral-specific protein synthesis.

MATERIALS AND METHODS

Cell, virus, and viral infections. Propagation of mouse L cells and Ehrlich ascites tumor (EAT) cells, virus isolation, and infections have previously been described (5, 14, 15).

Isolation and purification of viral double-stranded RNA. After 7 to 7.5 hr of infection, cells were harvested by centrifugation, resuspended in phosphate-buffered saline, and homogenized gently in a glass Dounce homogenizer. Cell debris was removed by centrifugation at $700 \times g$ for 20 min, and RNA was extracted from the supernatant fluid at 37 C by the method of Penman (11). The viral double-stranded RNA was isolated by differential salt precipitation (2), treated with ribonuclease A and T₁ at 15 $\mu\text{g}/\text{ml}$ and 2 $\mu\text{g}/\text{ml}$, respectively, deoxyribonuclease at 20 $\mu\text{g}/\text{ml}$, and Pronase at 1.0 mg/ml. The enzymatic treatment procedure is as described by Stern and Friedman (13). The double-stranded RNA was purified by centrifugation through sucrose gradients as described by Bishop et al. (3) and then dialyzed against 0.15 M NaCl with 0.001 M ethylenediaminetetraacetic acid (EDTA) for 12 hr at 4 C. Radioactively labeled double-stranded RNA was obtained by adding 3.0 μCi of [5-³H]uridine per ml to the viral incubation medium 1 hr postinfection in the presence of 5 μg of actinomycin D per ml.

Infectivity assay of viral double-stranded RNA. Titers of infectious RNA were determined by

a plaque assay in which confluent L-cell monolayers on Falcon plastic dishes (60 mm) were used. Double-stranded RNA, appropriately diluted in 1 M NaCl containing 100 μg of diethylaminoethyl (DEAE)-dextran per ml, was added in a volume of 0.05 ml to the monolayer which had previously been washed with saline and then 0.6 M NaCl. After a 30-min incubation period at 37 C, the monolayers were overlaid with a 1:1 mixture of 1.2% Noble agar and 2 \times Earle minimal essential media. The dishes were incubated for 48 hr, after which the agar overlay was removed and the cell sheet was stained with 0.8% crystal violet in 20% ethanol for the enumeration of plaques.

Determination of amino acid incorporation into polypeptides. Cells in suspension were incubated in a 37 C shaker bath at various concentrations in Hanks minimal essential medium and with various concentrations of radioactive protein precursors as described for each experiment in the legends (Fig. 1, 2, 4, 5, 6). Samples were withdrawn, and incorporation was halted by freezing in a dry CO₂-ethanol bath (-70 C). Samples were thawed, bovine serum albumin was added as carrier, and samples were made to 10% trichloroacetic acid with 20% trichloroacetic acid. Next, the samples were boiled at 100 C for 5 min and the resulting precipitate was washed three times with 5% trichloroacetic acid, dried at 37 C for 15 hr, and solubilized in 0.5 ml of Nuclear-Chicago solvent (New England Nuclear, Boston, Mass.) for 2 hr at 37 C. Samples were counted in a Beckman liquid scintillation counter in a toluene-2,5-diphenyl-oxazole (New England Nuclear, Boston, Mass.) scintillation cocktail. Using the wide-window, fixed ISO-SET, ¹⁴C was counted at 90% efficiency, while ³H was counted at 35% efficiency.

Radioactive materials. Radioactive materials included: [5-³H]uridine, 20 to 30 Ci/mmole; [¹⁴C]-leucine, 58.2 Ci/mmole; [¹⁴C]phenylalanine, 383 Ci/mmole (all three from New England Nuclear); and [¹⁴C]protein hydrolysate, 54 Ci/mA (Amersham/Searle, Arlington Heights, Ill.).

RESULTS

Infectivity of viral double-stranded RNA. The viral double-stranded RNA used in

the experiments to be described was tested for infectivity. From the same isolation, one half of the total RNA initially isolated was prepared as described in Materials and Methods, the other half was treated similarly excepting the ribonuclease treatment. Both 20s sedimenting RNAs were then tested for infectivity by the plaque assay method outlined in Materials and Methods. It can be seen in Table 1 that treatment of the double-stranded viral RNA with ribonuclease drastically reduces (5 log units) the plaque-forming ability of the RNA. Therefore, the following results are not a consequence of the replicative capacity of this viral RNA, since ribonuclease-treated RNA was used in subsequent experiments.

Effect of viral double-stranded RNA on protein synthesis. The effect of viral double-stranded RNA on cellular protein synthesis in intact EAT cells was examined. A suspension of cells in culture, actively incorporating amino acids, was supplemented with purified BEV double-stranded RNA. As demonstrated in Fig 1, the addition of the viral RNA resulted in a cessation in the incorporation of amino acids. This probably reflects an overall inhibition in cellular translation rather than a reduction in the rate of synthesis. The inhibition can be seen to occur within 10 min after the addition of the double-stranded RNA. By 40 min after treatment, the rate of amino acid incorporation has decreased by 70% as compared to the control culture. Also, the level of inhibition induced by the double-stranded RNA is identical to that eventually obtained when cells are infected with complete BEV virus (Fig. 2). The following experiment was carried out to eliminate the possibility that the inhibition by the viral double-stranded RNA was a consequence of an alteration in cell membrane permeability, which would have resulted in suppressed incorporation of protein precursors.

TABLE 1. Infectivity of double-stranded RNA^a

Isolation	RNA (PFU/ μ g) (ribonuclease treatment)	RNA (PFU/ μ g) (untreated)
1	<1	3.2×10^5
2	<1	5.3×10^5

^a Viral double-stranded RNA was isolated and prepared as described in Materials and Methods except, where indicated, ribonuclease treatment was eliminated. The 20s sedimenting RNAs were plated on confluent L-cell monolayers also described in Materials and Methods. Each value presented represents the average of 10 plates at three different dilutions of RNA. PFU, plaque-forming units.

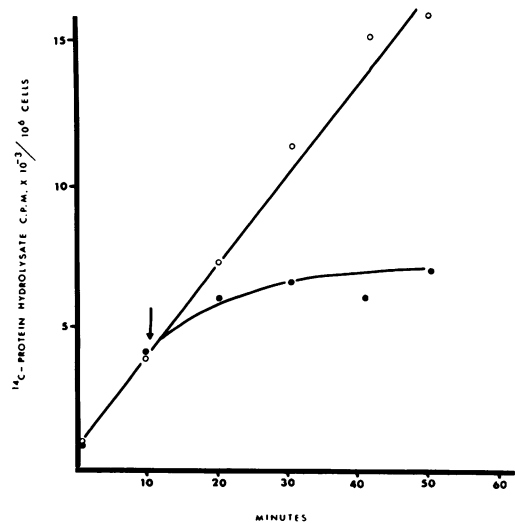


FIG. 1. Effect of viral double-stranded RNA on cellular protein synthesis. ¹⁴C-protein hydrolysate was added to a culture of EAT cells (2.5×10^6 cells per ml) to a final concentration of 2.0μ Ci/ml. The culture was immediately divided. After 10 min of incubation at 37 C with slight agitation, BEV double-stranded RNA, in a small volume of $2 \times$ SSC (SSC = $0.15 M$ NaCl, $0.015 M$ sodium citrate, pH 7.0), was added to one culture to a concentration of 20μ g/ml, and an equal volume of $2 \times$ SSC was added to the control culture. Samples were prepared for analysis of amino acid incorporation as described in Materials and Methods. Symbols: O, untreated control cells; ●, double-stranded RNA-treated cells. Arrow indicates time of RNA addition.

sors. Viral double-stranded RNA-treated cells and untreated cells were monitored for their respective ability to incorporate amino acids. The rate and extent of uptake of radioactive amino acids in Fig. 3 indicates no difference in membrane permeability between cells cultured with or without the viral RNA.

To test the specificity of this inhibition, the effect of BEV double-stranded RNA on viral-specific protein synthesis was investigated. At 145 min postinfection with BEV virus, a time of linear viral-specific protein synthesis (Fig. 4), BEV double-stranded RNA was added to the culture. No inhibition in the accumulation of viral-specific proteins by the viral RNA can be detected, even 60 min after treatment (Fig. 5). To show that this was viral-specific protein synthesis, the culture was assayed at 6 hr after virus infection for virus production. A normal burst size for BEV-infected EAT cells (25 plaque-forming units per cell) was obtained by plaque assay determination.

To further test the specificity of the inhibi-

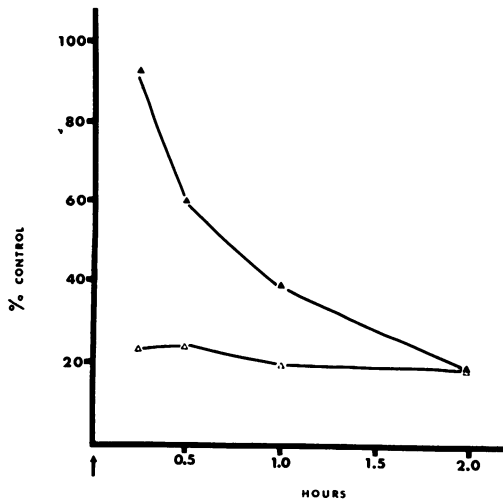


FIG. 2. Inhibition of cellular protein synthesis by viral double-stranded RNA. BEV double-stranded RNA at a concentration of 25 $\mu\text{g}/\text{ml}$ in $2 \times \text{SSC}$ (see Fig. 1 legend) was added to a suspension culture of EAT cells (10^7 cells per ml). After the addition of the RNA, samples of 10^8 total cells were removed and pulse labeled for 10 minutes with 5 μCi of ^{14}C -phenylalanine. The pulse was halted by freezing the sample at -70°C in a dry ice-ethanol bath. Samples were later assayed for hot trichloroacetic acid-precipitable material. The data are expressed as percentages of an identically labeled control to which an equal volume of $2 \times \text{SSC}$ was added. Also shown is the inhibitory effect by BEV virus in a culture handled similarly. (Infection by the virus is described in reference 5.) Each point was determined from the average of duplicate samples. The arrow indicates the initial time of treatment with the double-stranded RNA and virus. Symbols: Δ , double-stranded RNA treated; \blacktriangle , BEV infected.

tor, the effect of BEV double-stranded RNA on the protein synthesis of another picornavirus, Mengo virus, was examined. Adding BEV RNA at a time of increasing accumulation of viral proteins in the replication cycle of Mengo virus (15), no alteration in translation was noted (Fig. 6).

Extent of incorporation of viral double-stranded RNA into whole cells. Using radioactively labeled BEV double-stranded RNA, the rate and amount of RNA associated with whole cells was determined. Cells were incubated in the presence of the double-stranded RNA at 20 $\mu\text{g}/\text{ml}$. Thoroughly washed cell samples were then assayed for uptake of the RNA. A linear increase in cell-associated label could be observed over a 2-hr incubation period (Fig. 7). At this time, this represented approximately 1.8 pg per cell. Similar incorporation

was obtained for virally infected cells (data not shown).

Nature of the cell-associated RNA. Cells were incubated with labeled double-stranded RNA for 30 min at which time they were placed in RNA-free medium and incubated further for various intervals. The cell-associated RNA was then analyzed as to its double- or single-stranded nature on the basis of ribonuclease resistance. After a total of 30 min of incubation, 98.8% of the RNA was found to have retained its double-strandedness. However, at 60 min incubation, only 65% was resistant to ribonuclease, and by 120 min only 25.5% was double-stranded RNA (Table 2). Although the double-stranded RNA appears to become single-stranded (ribonuclease sensitive) with time, it would seem from Fig. 2 that the RNA is not capable of translation, precluding the possibility that a newly synthesized viral protein is the mediator of the inhibition observed.

DISCUSSION

The data presented here further suggest that the inhibition of cellular protein synthesis

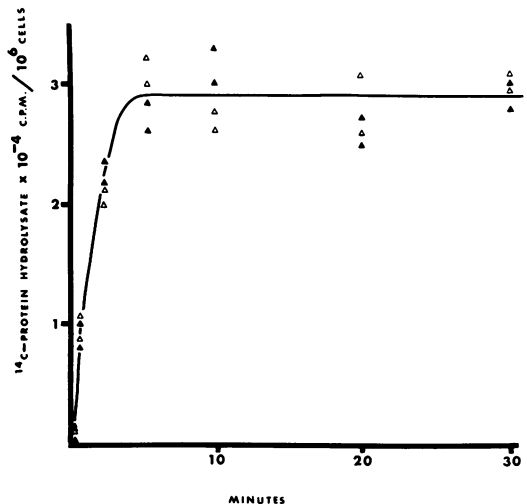


FIG. 3. Effect of viral double-stranded RNA on cell permeability. EAT cells were incubated at 37°C in phosphate-buffered saline at a concentration of 4×10^6 cells per ml for 1 hr. The cells were then pelleted and resuspended to a concentration of 2×10^6 cells per ml in phosphate-buffered saline containing 2 μCi of ^{14}C -protein hydrolysate per ml plus 25 μg of viral double-stranded RNA per ml in $2 \times \text{SSC}$ (see Fig. 1 legend) or an equivalent volume of $2 \times \text{SSC}$. Samples were extracted and diluted 1:100 in saline, washed three times in saline, and the final cell pellet was solubilized in NCS at 37°C overnight. Symbols: Δ , untreated control; \blacktriangle , double-stranded RNA treated.

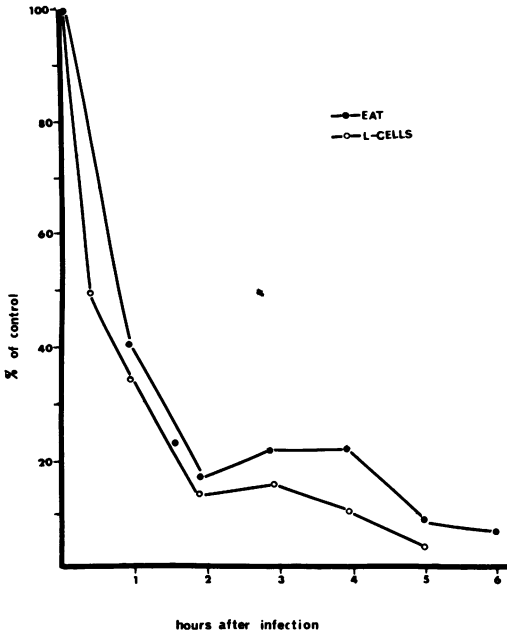


FIG. 4. BEV-induced inhibition of host protein synthesis. Cells were infected as previously described (5). At various time intervals after infection, samples of 2×10^6 cells were pulse labeled for 10 min with $10 \mu\text{Ci}$ of ^{14}C -phenylalanine. Samples were analyzed for hot trichloroacetic acid-precipitable material. Values are reported as percentages of an uninfected control culture pulse labeled in the same fashion.

by picornavirus double-stranded RNA seen in vitro reflects the inhibition occurring in infected cells. This is demonstrated by an inhibition of translation in intact cells when noninfectious viral double-stranded RNA is added to the culture medium. The inhibition is rapid, observable as early as 10 min after treatment with the viral RNA. Allowing 5 min for the incorporation of the double-stranded RNA (2), this inhibition may actually be commencing within 5 min of addition of the inhibitor. The inhibition in the rate of amino acid incorporation was more than 70%, 40 min after the whole cells come in contact with the double-stranded RNA. This inhibition in translation by the viral double-stranded RNA is not a consequence of decreased membrane permeability since the cells show no alteration in the uptake of amino acids after treatment.

Reisolation of the viral double-stranded RNA from treated cells at a time of maximum inhibition indicated that the RNA had retained nearly 99% of its double-stranded nature determined by its resistance to ribonuclease. This suggests that single-stranded viral RNA by itself is not the inhibitor. That viral double-

stranded RNA is responsible for inhibition of host cell translation in virally infected cells is also evidenced, though only indirectly, by the necessity of a functional, single-stranded genome; ultraviolet-inactivated virus (12) or hydroxylamine-treated virus (10) has no effect on cell-specific protein synthesis. Also, Hunt and Ehrenfeld (9) demonstrated that single-stranded viral RNA did not inhibit in vitro synthesis of hemoglobin at concentrations at which the double-stranded RNA is inhibitory.

This inhibition of translation by picornavirus double-stranded RNA appears specific. There is no detectable inhibition of protein synthesis in picornavirus-infected cells by the viral intermediate. This is seen for both homologous (BEV) and heterologous (Mengo virus) polypeptide synthesis employing BEV double-stranded RNA. This is consistent since BEV and Mengo virus can replicate productively in simultaneously infected cells. The rate of incorporation of the double-stranded RNA is identical for both picornavirus-infected and uninfected cells. Approximately 0.8 pg per cell can be found after a 1-hr incubation period reflect-

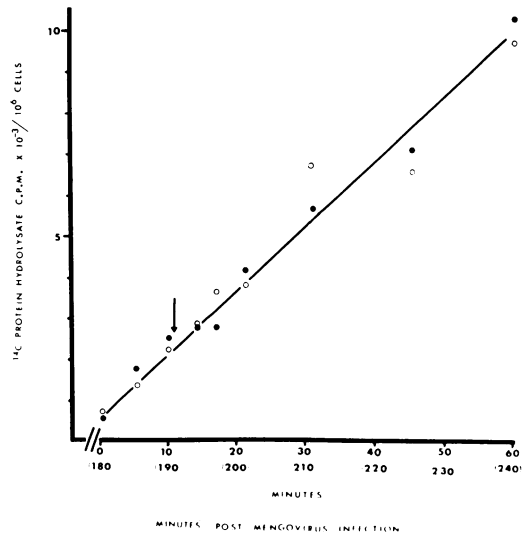


FIG. 5. Effect of BEV double-stranded RNA on protein synthesis in BEV-infected cells. EAT cells were infected with BEV at a multiplicity of infection of 50 as previously described (5). At 135 min postinfection, $2.0 \mu\text{Ci}$ of ^{14}C -leucine per ml was added to the culture. The culture was then divided. After 10 min of incubation at 37°C , BEV double-stranded RNA was added to one culture to a concentration of $20 \mu\text{g/ml}$. The control was treated with an equal volume of RNA suspension buffer. Symbols: \circ , untreated BEV-infected cells; \bullet , double-stranded RNA-treated BEV-infected cells. Arrow indicates time of RNA addition.

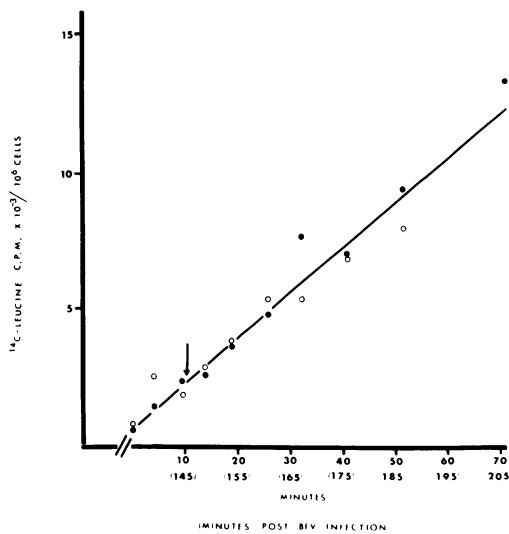


FIG. 6. Effect of BEV double-stranded RNA on protein synthesis in Mengo virus-infected cells. EAT cells were infected with Mengo virus at a multiplicity of infection of 80 as previously described (13). At 180 min postinfection, 1.0 μ Ci of 14 C-protein hydrolysate per ml was added to the culture and then the culture was equally divided. The procedure follows that as described in the legend of Fig. 4. Symbols: \circ , untreated Mengo virus-infected cells; \bullet , double-stranded RNA-treated Mengo virus-treated cells. Time of RNA addition marked by arrow.

ing 20% adsorption. Weigers and Koch report that, in the absence of polycations, approximately 20% of poliovirus double-stranded RNA is adsorbed to HeLa cells after a 15-min incubation period (16). These authors also state that the degree of double-stranded RNA adsorbed is not dependent on RNA concentration and has only a slight dependence on cell concentration. In our system, a 15-min incubation results in about 7 to 9% RNA adsorbed, and this amount increases as the incubation period is lengthened. It should be noted that the poliovirus double-stranded RNA used by Weigers and Koch was not treated with ribonuclease which may increase its adsorption to cells. Whether the BEV double-stranded RNA taken up by cells is intracellular or adheres to the cell surface is currently being investigated. Preliminary evidence suggests it is intracellular.

The mechanism of inhibition of host cell translation by the viral intermediate is unknown. The inhibition observed may represent an interaction between the double-stranded RNA and ribosomes, transfer RNA (tRNA), essential factors, or messenger RNA (mRNA).

Ribosomes and tRNA can be eliminated since such interactions should produce nonspecific inhibition. Thus the possible site of specificity is the mRNA, the essential factor(s), or both. From *in vitro* studies (1, 9), there is indirect evidence that the initiation process of the host is inhibited. It is also suggested by Ehrenfeld and Hunt (9) that an initiation factor required for polypeptide synthesis is depleted during incubation with the viral double-stranded RNA and that its formation or regeneration is pre-

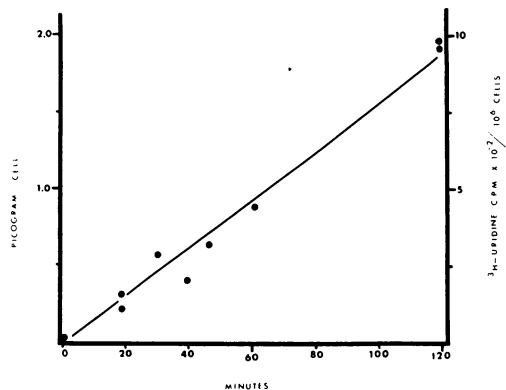


FIG. 7. Rate and extent of incorporation of BEV double-stranded RNA into whole cells. EAT cells, at a concentration of 5×10^6 cells per ml, were incubated in the presence of 20 μ g of 3 H-double-stranded RNA per ml (510 counts per min per μ g). Cell samples were removed and washed three times with saline. Samples were then analyzed for cold trichloroacetic acid-insoluble material by precipitation onto membrane filters (Millipore Corp.).

TABLE 2. Secondary structure of cell-associated RNA^a

Total time of incubation (min)	Untreated (counts/min)	Ribonuclease-treated (counts/min)	% Ribonuclease-resistant RNA
30	987	975	98.9
60	1026	667	65.0
120	1002	256	25.5

^a Ehrlich ascites, at a concentration of 4×10^6 cells per ml in Hanks minimal essential medium were treated with 3 H-double-stranded viral RNA at a concentration of 20 μ g/ml. The suspension culture was kept at 37 C under slight agitation for 30 min, after which the cells were washed with 37 C minimal essential medium, resuspended to the same cell concentration in RNA-free medium, and incubated further. Samples of 8×10^4 total cells were removed, pelleted, resuspended in $2 \times$ SSC, and then phenol extracted at 37 C. The resulting aqueous phase was divided equally; one-half was treated with 10 μ g of pancreatic ribonuclease per ml and 2 μ g of ribonuclease T₁ per ml at 37 C for 20 min, the other half was incubated similarly without ribonuclease. The samples were then analyzed for cold trichloroacetic acid-insoluble material.

vented by this RNA. Further evidence from *in vitro* studies with a hemoglobin protein synthesizing system suggest that, in the absence of mRNA, complexes are formed between the initiator formyl methionyl tRNA (met-tRNA_F) and 40s ribosomal subunits, a reversal of the normal function of codon recognition. In the presence of poliovirus double-stranded RNA this complex formation is prevented (6). This met-tRNA_F subunit complex seems to require an initiation factor(s) (7). Ascione and Vande Woude (1) report the existence of a virus-specific initiation factor isolated only from cells infected with foot and mouth disease virus, another picornavirus. This factor stimulates the binding of *N*-acetyl-aminoacyl-tRNA to viral mRNA in a foot-and-mouth disease virus RNA-dependent system *in vitro*. Host initiation factors isolated from uninfected cells do not permit translation of the viral message. This factor may be insensitive to the translational inhibitory action of the viral double-stranded RNA, whereas a corresponding cellular factor may not.

However, the work of Speyer et al. (4) would suggest that the inhibition seen by double-stranded RNA is specific for the message. In an *E. coli* *in vitro* protein synthesizing system directed by f2 and T₄ mRNAs, the synthetic double-stranded RNA, polyinosinic-polycytidylic acid inhibits only the former system. Also, it was shown that the conformation of the f2 RNA plays a role in the inhibition.

A well defined *in vitro* study employing cellular, viral, and synthetic messages and cell and viral initiation factors may help to elucidate the mechanism of inhibition by picornavirus double-stranded RNAs.

ACKNOWLEDGMENTS

The authors wish to acknowledge the fine technical assistance of Elodee A. Hopper and Mengo virus donated by Suzanne Prather. This work was supported by grants from the Public Health Service, no. CA 10417 from the National Cancer Institute, and from the Damon Runyon Memorial fund (DRG 1018). B. C. S. is a predoctoral trainee supported by Public Health Service genetics training grant GM 12 from the National Institute for General Medical Sciences.

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