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Double-Stranded Poliovirus RNA Inhibits Initiation of Protein Synthesis by Reticulocyte Lysates

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ABSTRACT **Infection of HeLa cells with poliovirus results in an inhibition of host-cell protein synthesis. Cytoplasmic extracts from infected cells contain an activity that inhibits initiation of protein synthesis *in vitro* by rabbit reticulocyte lysates. This inhibitory activity has been purified and characterized, and is shown to reside in poliovirus double-stranded RNA. The intact double-stranded molecule is not essential for inhibitory activity.**

Many lytic viruses inhibit protein synthesis in their host cells prior to the onset of virus-directed protein synthesis (for review, see ref. 1). In cells infected with poliovirus, or other picornaviruses, there is a concomitant rapid disaggregation of polysomes (2). A functional viral genome seems to be required for inhibition to occur, since infection with UV-inactivated virus does not affect the rate of cellular protein synthesis (3).

We have reported that a heat-treated cytoplasmic extract from poliovirus-infected HeLa cells inhibits the initiation of protein synthesis in a rabbit reticulocyte lysate (4). The inhibition results in the disaggregation of reticulocyte polysomes to single ribosomes after the completion of several rounds of polypeptide chain synthesis. The inhibitory activity appeared in infected cell extracts at a rate identical to that at which host-cell protein synthesis was inhibited. We have now identified the inhibitory component as double-stranded poliovirus RNA.

MATERIALS AND METHODS

Preparation of inhibitor

HeLa S3 cells, grown in suspension culture in Eagle's minimum essential medium supplemented with 7% calf serum, were infected with purified poliovirus type 1 at 100 PFU (plaque-forming units)/cell (5). The infected cells were incubated for 5 hr at 37°C, collected by centrifugation, swollen in hypotonic buffer, and disrupted by Dounce homogenization. Nuclei and cell debris were removed by low-speed centrifugation, and the cytoplasm was clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was heated to 100°C for 5 min, and the centrifugation was repeated to remove the resulting precipitate. The supernatant extract from 7.5×10^6 cells was layered onto a linear 5–20% sucrose gradient prepared in 10 mM Tris·HCl (pH 7.2)–10 mM KCl–1.5 mM $MgCl_2$ and was centrifuged for 5 hr at 45,000 rpm at 4°C in the SW-65 rotor. Fractions of 0.3 ml were collected by needle puncture of the tubes, and portions of each fraction were assayed for their ability to inhibit protein synthesis by a rabbit reticulocyte lysate.

Assay of cell-free protein synthesis by rabbit reticulocyte lysates

Reticulocytes were obtained from rabbits made anemic by four successive daily bleedings from the ear of about 30 ml. On the fourth day, 0.5 ml of Imferon (gift of Lakeside Laboratories, Inc.) was injected intramuscularly. 2 days later, the hematocrit had risen to about 30%, and reticulocytes constituted 25–35% of the cells present in the blood. Cells were harvested and washed and then lysed in an equal volume of water (6). The stroma were removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was frozen in small aliquots in liquid nitrogen.

The assay mixture contained in 3 ml: 1.75 ml of lysate, 33 μM hemin, 10 mM Tris·HCl (pH 7.3), 70 mM KCl, 1.5 mM $MgCl_2$, 1 mg/ml creatine kinase (EC 2.7.3.2) (Boehringer), 6.5 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, amino acids at 1/10 the concentration specified by Borsook *et al.* (7), and 10 μCi of [^{14}C]alanine, 123 Ci/mol. To 0.1 ml of this mixture was added 0.01 ml of the sucrose-gradient fractions to be assayed. The complete assay mixture was incubated at 33°C for 20 min and portions of 0.02 ml were removed and pipetted into 1 ml of 1.0 N NaOH containing 1 mg/ml of unlabeled alanine. These samples were incubated for 15 min at 37°C, then protein was precipitated with 5% TCA before filtration on Whatman GF/C glass-fiber filters. After drying, filters were counted with 20% efficiency in a Nuclear Chicago low-background proportional counter.

Preparation of poliovirus double-stranded RNA

Poliovirus double-stranded RNA was prepared by either of two methods: (*a*) HeLa cells were infected with poliovirus at a multiplicity of 100 PFU/cell in the presence of 10 μg/ml of actinomycin D (gift of Merck), and 0.5 μCi/ml of [^{14}C]uridine (40 Ci/mol). 5 hr after infection, the cells were harvested and washed with Earle's salts solution, suspended in 0.05 M sodium acetate (pH 5.5)–0.02 M EDTA, and extracted two times with phenol at 65°C. The aqueous phases were pooled, made 0.3 M in NaCl, and nucleic acids were precipitated with 2 volumes of EtOH at − 70°C. The precipitate was collected by centrifugation, suspended in 2 M LiCl, and stored overnight at 4°C (8). The LiCl supernatant, containing double-stranded RNA, was diluted and layered on a 15–30% sucrose gradient prepared in 10 mM Tris (pH 7.2)–10 mM KCl and was centrifuged for 17 hr in the SW-25.1 rotor at 22,000 rpm at 4°C. Gradients were pumped through a Gilford recording spectrophotometer.

Abbreviation: TCA, trichloroacetic acid.

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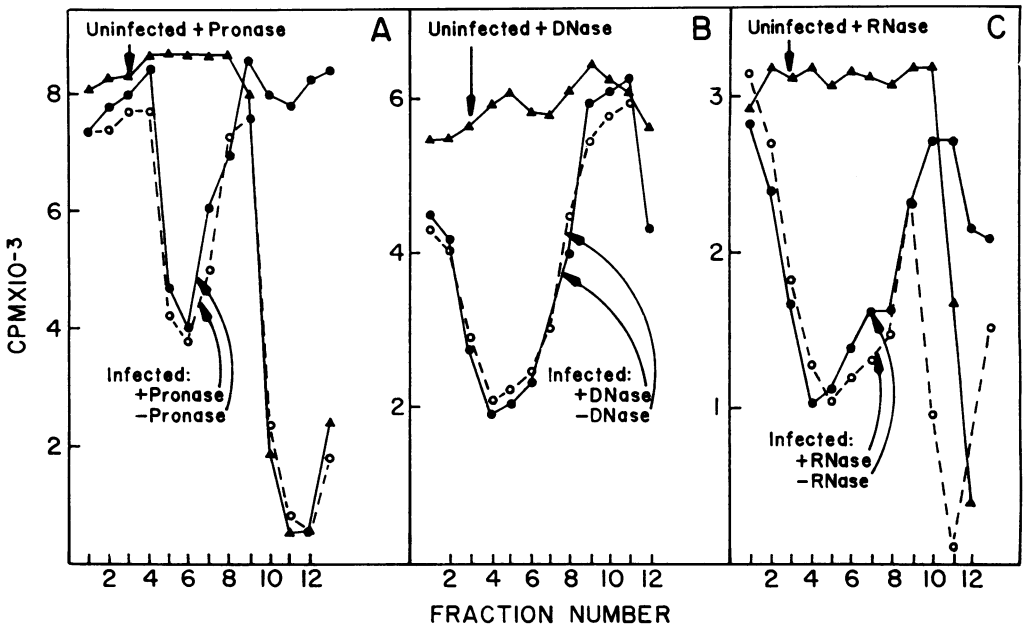

FIG. 1. Failure of pronase, deoxyribonuclease, and pancreatic ribonuclease to affect the inhibitory activity of sucrose gradient fractions of poliovirus-infected and uninfected HeLa extracts. Heated cytoplasmic extracts were prepared from uninfected and poliovirus-infected HeLa cells. Portions of the infected and uninfected extracts were treated for 30 min at 37°C with (*a*) 100 μg/ml of pronase, (*b*) 10 μg/ml of DNAse, or (*c*) 10 μg/ml of pancreatic RNase in the presence of 0.3 M NaCl–0.03 Na citrate. Portions of the infected cell extract were incubated untreated. The samples were chilled and layered onto sucrose gradients, centrifuged, and assayed. The ordinate indicates the incorporation of [^{14}C]alanine into TCA-insoluble material by a reticulocyte lysate in the presence of fractions from the sucrose gradient indicated on the abscissa. Each frame represents the results of separate experiments.

1-ml fractions were collected and aliquots were precipitated onto filters with TCA in the presence of bovine serum albumin as a carrier. The filters were washed, dried, and counted at 90% efficiency in Triton X-100–toluene scintillation fluid in a Beckman scintillation spectrometer.

(*b*) Poliovirus-induced RNA polymerase was prepared from infected HeLa cells as was described (9), and the enzyme was then used to catalyze the synthesis of poliovirus-specific RNA *in vitro*. The reaction mixture was incubated at 37°C for 30 min. Pancreatic ribonuclease (10 μg/ml) was then added, and the incubation was continued for 15 min. The reaction mixture was made 1% in sodium dodecyl sulfate and layered onto a sucrose gradient as described above.

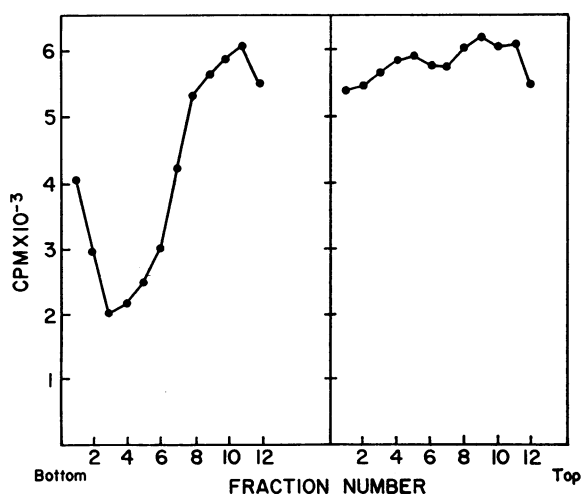

FIG. 2. Effect of digestion with micrococcal nuclease on inhibitory activity of sucrose gradient fractions of poliovirus-infected HeLa extracts. Inhibitory extract was prepared, and half of the sample was incubated for 30 min at 37°C with 100 μg/ml of micrococcal nuclease (*right*) in the presence of 1 mM $CaCl_2$–0.01 M Tris·HCl, pH 8.5. The remainder was incubated in the same buffer without nuclease (*left*). The samples were chilled and layered onto sucrose gradients, centrifuged, and assayed. Data plotted as in Fig. 1.

Materials

Trypsin, pancreatic ribonuclease, deoxyribonuclease, and micrococcal nuclease were purchased from Worthington Biochemicals, Inc. Pronase (Protease) was the product of Sigma. All radioisotopes were obtained from Schwarz BioResearch, Inc.

RESULTS

Characterization of the inhibitory component

Experiments to determine the nature of the inhibitory component in poliovirus-infected HeLa cytoplasm were facilitated by its relatively high sedimentation coefficient, which enabled us to treat the extract with potent hydrolases and subsequently to separate the inhibitor from the digestive enzymes by centrifugation. Initial attempts to identify the inhibitor were nevertheless discouraging. Treatment of the cytoplasmic

TABLE 1. *Properties of poliovirus-induced inhibitor of protein synthesis*

1.	Excluded from Sephadex G-25
2.	Not dialyzable
3.	Binds to DEAE-cellulose
4.	Precipitable with $(NH_4)_2SO_4$
5.	Resistant to iodoacetamide
6.	Resistant to periodate
7.	Sensitive to acid hydrolysis
8.	Sensitive to alkali hydrolysis

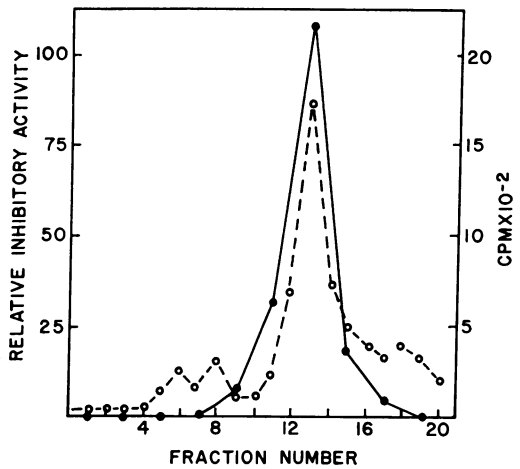

FIG. 3. Sedimentation and inhibitory properties of poliovirus double-stranded RNA. Labeled poliovirus double-stranded RNA was prepared by LiCl extraction. Portions of the sucrose gradient fractions were assayed for inhibitory activity in the reticulocyte lysate protein-synthesizing system. ●—● relative inhibitory activity; O- -O radioactivity in LiCl supernatant. Serial dilutions of fractions 9–17 were assayed to obtain the relative inhibitory activity of each fraction.

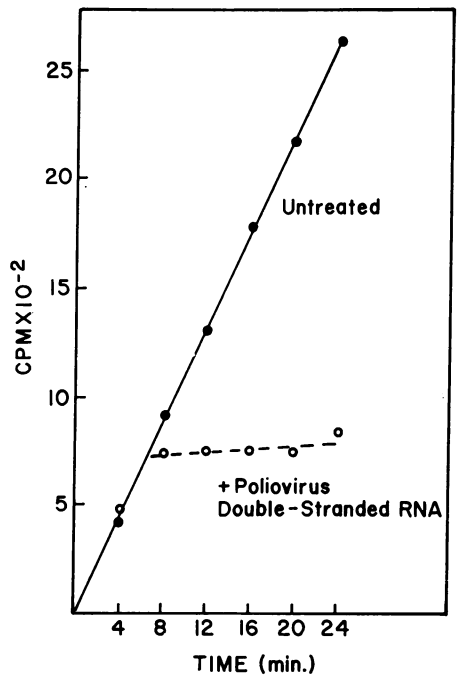

FIG. 4. Effect of poliovirus double-stranded RNA on the synthesis of protein by rabbit reticulocyte lysate. 0.02 ml of Fraction 13 from the sucrose gradient shown in Fig. 3 was added to 0.2 ml of the reticulocyte lysate assay mixture. The untreated assay mixture contained no additions, and gave identical results as samples incubated in the presence of Fraction 5. At the indicated times, 0.02-ml portions were removed, and acid-precipitable radioactivity was determined.

extract with pronase, deoxyribonuclease, or pancreatic ribonuclease did not destroy its inhibitory activity (Fig. 1). Incubation with amylase (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20), or neuraminidase (EC 3.2.1.18) also had no effect on the sedimentation rate or activity of the inhibitory component. Lowry-positive or ultraviolet-absorbing material could not be detected in that fraction of the sucrose gradient demonstrating maximum activity. Additional properties of the inhibitor are listed in Table 1.

Although pancreatic ribonuclease did not destroy the inhibitory activity, treatment of the heated cytoplasmic extract with this enzyme at low salt concentrations frequently resulted in an apparent decrease in its sedimentation coefficient. These conditions favor the attack by pancreatic ribonuclease on double-stranded RNA (10). In addition, the accumulation of a relatively heat stable form of poliovirus double-stranded RNA during the infectious cycle has been established (11). Treatment of the inhibitory extract with high concentrations of micrococcal nuclease, which attacks double-stranded nucleic acids (12), completely abolished its capacity to inhibit reticulocyte protein synthesis (Fig. 2).

Effect of purified poliovirus double-stranded RNA

Labeled poliovirus double-stranded RNA was prepared, and sedimented through a sucrose gradient; portions of the gradient fractions were assayed for their ability to inhibit protein synthesis by the reticulocyte lysate. A peak of inhibitory activity coincided with the poliovirus double-stranded RNA (Fig. 3). Fractions from sucrose gradients that contained poliovirus single-stranded RNA demonstrated no inhibitory activity. The kinetics of inhibition by purified double-stranded RNA, shown in Fig. 4, were identical with those previously reported for the cytoplasmic inhibitor of protein synthesis (4).

Further proof of the identity of the active component in infected cell extracts was obtained by cosedimentation with purified double-stranded RNA. The cytoplasmic inhibitor was prepared from cells infected with poliovirus in the presence of [^{14}C]uridine and actinomycin D in order to label only poliovirus-specific RNA. When this inhibitory extract was mixed with double-stranded RNA synthesized *in vitro* with [^{3}H]GTP, the two peaks of radioactivity sedimented identically (Fig. 5). Thus, intact poliovirus double-stranded RNA is the factor produced in infected cells that inhibits initiation of protein synthesis in a cell-free system.

Specificity of the inhibition reaction

In an effort to determine whether the entire length of the poliovirus double-stranded RNA is required for inhibitory activity, labeled molecules were treated with low concentrations of micrococcal nuclease for short times to partially digest the RNA. The extracts were then sedimented through sucrose gradients and the radioactivity, as well as inhibitory activity, was measured. Fig. 6 shows that although most of the radioactivity became acid-soluble or sedimented as small pieces of nucleic acid, peaks of inhibitory activity were evident with sedimentation values less than that of the intact molecule (as the concentration of nuclease was increased, the inhibitory fractions shifted to lower S values, see *bars*). These data indicated that the entire poliovirus double-stranded RNA molecule was not essential for inhibition. Preliminary experiments suggest that double-stranded RNA isolated from purified reovirus, as well as low concentrations (about 0.01 μg/ml) of the synthetic polymers poly(A·U) and poly(I·C), inhibit protein synthesis by the reticulocyte lysate. Higher concentrations (about 10 μg/ml) of the synthetic copolymers did not inhibit the lysate.

DISCUSSION

The mechanism by which poliovirus double-stranded RNA inhibits protein synthesis by reticulocytes is obscure. The absence of detectable ultraviolet-absorbing material in the

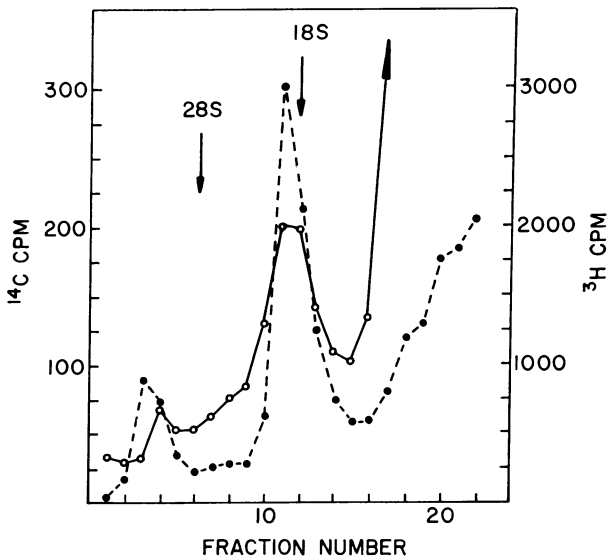

FIG. 5. Cosedimentation of poliovirus double-stranded RNA with inhibitory extract. Poliovirus-induced RNA polymerase was prepared and incubated with [^{3}H]GTP (9). The reaction products were extracted with 1% sodium dodecyl sulfate (SDS) and mixed with a heated cytoplasmic extract from HeLa cells infected with poliovirus in the presence of 10 μg/ml of actinomycin D and 1 μCi/ml of [^{14}C]uridine (40 Ci/mol). The mixed sample was layered onto a 15–30% sucrose gradient prepared in NETS buffer (0.1 M NaCl–1 mM EDTA–5 mM Tris·HCl (pH 7.4)–0.2% SDS) and centrifuged for 17 hr at 23,500 rpm at 20°C in the SW-25.1 rotor. ●– –● ^{3}H; product of poliovirus RNA polymerase reaction; O—O ^{14}C; inhibitory extract.

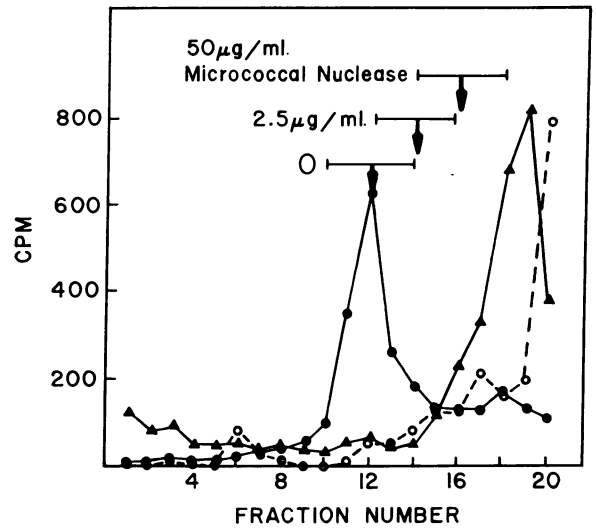

FIG. 6. Inhibitory activity and sedimentation properties of poliovirus double-stranded RNA incompletely digested with micrococcal nuclease. Labeled poliovirus double-stranded RNA was prepared by LiCl extraction. Before sucrose gradient sedimentation, portions were incubated for 10 min at 37°C with 0, 2.5, or 50 μg/ml of micrococcal nuclease in the presence of 1 mM $CaCl_2$ at pH 8.5. The samples were then chilled, layered on sucrose gradients, and centrifuged. 0.01-ml portions of each gradient fraction were assayed for inhibitory activity in the reticulocyte lysate assay system; the remainder of each fraction was precipitated with 5% TCA in the presence of bovine serum albumin, and the radioactivity was determined. The bars (——) indicate fractions with inhibitory activity for each concentration of nuclease. In this experiment, the radioactivity incorporated (per 20 min) by the reticulocyte lysate treated with the peak inhibitory fractions was as follows: Fract. 2 (control), 4010 cpm; Fract. 12 (about 20S), ●—●, no nuclease, 645 cpm; Fract. 14 (about 16S) ▲—▲, 2.5 μg/ml; nuclease, 1262 cpm; Fract. 16 (about 12S), O– – –O, 50 μg/ml nuclease, 1300 cpm.

sucrose gradient fractions permits only the rough estimate that when maximal inhibition is achieved, there is only one molecule of double-stranded RNA per polyribosome in the lysate. Previous results (4) demonstrated that even at maximal concentrations of the inhibitor, several rounds (less than 10) of polypeptide chain synthesis were completed at the uninhibited rate before amino acid incorporation ceased. At submaximal concentrations, the normal rate of protein synthesis continued for a longer period of time, at which point incorporation stopped abruptly. These data suggest that a factor required for initiation of polypeptide chain synthesis is depleted during the incubation, and that its formation or regeneration is prevented by the double-stranded RNA.

The inhibition of protein synthesis by poliovirus double-stranded RNA apparently does not require the entire double-stranded genome. In fact, it is likely that various double-stranded RNAs with different specificities can shut-off cellular protein synthesis. It is curious that the same type of molecule induces interferon in some host cells (13), whereas in others protein synthesis may be inhibited before sufficient interferon can be produced to stop viral infection. However, there is as yet no direct evidence that these events compete in the manner suggested here.

The question arises as to whether or not the inhibition by poliovirus double-stranded RNA of reticulocyte protein synthesis in a cell-free extract is, in fact, the same mechanism by which host cell protein synthesis is inhibited after infection. This is difficult to prove conclusively; however, the rate of appearance of the reticulocyte inhibitor in infected cells coincides precisely with the rate of reduction of protein synthesis in the infected cells (4). In addition, several agents that prevent or slow down the inhibition of host-cell protein synthesis during infection also cause a proportional decrease in the inhibitory activity of the infected cell extracts on the reticulocyte system (unpublished observations).

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