Effect of Interferon on Polymerization of Single-Stranded and Double-Stranded Mengovirus Ribonucleic Acid

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ABSTRACT

GORDON, IRVING (University of Southern California, Los Angeles), SARA S. CHENAULT, DOUGLAS STEVENSON, AND JEAN D. ACTON. Effect of interferon on polymerization of single-stranded and double-stranded mengovirus ribonucleic acid. *J. Bacteriol.* 91:1230-1238. 1966.—The effect of interferon on actinomycin-resistant mengovirus ribonucleic acid (RNA) replication in L cells was investigated to determine whether defective or partially polymerized RNA products were made and whether synthesis of any specific class of virus RNA was prevented. RNA labeled with uridine-C¹⁴ was extracted in hot and cold phenol and analyzed by zonal sucrose density centrifugation. Both single- and double-stranded infectious RNA peaks were identified. Interferon treatment caused almost complete depression of uridine-C¹⁴ incorporation throughout linear sucrose gradients except in the 4S region, and no infectivity was detectable in any fraction. These inhibitory effects are attributable to the action of interferon, because they were reversed when cultures were treated with actinomycin D simultaneously with interferon. The results, with those of other investigators, indicate that the step at which interferon interrupts virus multiplication is between the events immediately after uncoating and the formation of template "minus" strands; under the conditions of our experiments, no partially polymerized virus RNA products were made.

The mechanism by which interferon inhibits virus synthesis is still unknown, but information concerning the step at which it interrupts the virus replicative sequence has been obtained in the past few years. Since interferon protects against infection induced by virus ribonucleic acid (RNA; 16), it must exert its effect after virus uncoating, but Levy (23) has demonstrated that repression of cellular protein synthesis, one of the earliest events detectable after mengovirus infection, is not affected by interferon treatment. It is also known that infectious virus RNA is not made in interferon-treated cells (9, 14, 27) and that virus RNA synthesis is depressed (5, 24, 25). Friedman and Sonnabend (13) recently reported that interferon suppresses synthesis of a virus RNA which has characteristics of double-stranded RNA. The inhibition by interferon of virus RNA synthesis is prevented, however, when actinomycin D is administered simultaneously with interferon (32).

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The aim of our studies was to determine whether treatment with interferon blocks formation of any specific class of virus RNA, whether double-stranded RNA appears in extracts, and whether defective or partially polymerized virus RNA products are made during interferon inhibition. Experiments were done in L cells infected with mengovirus. The specificity of the effect of interferon was investigated by comparing polymerization of viral-specific RNA in cultures treated with interferon and actinomycin D simultaneously with that in cultures first treated with interferon, then with actinomycin D.

MATERIALS AND METHODS

Cells. Experiments were done with the 929 strain of L cells, obtained from Richard M. Franklin. They were maintained in 1-liter Blake bottles containing 80 ml of Earle's solution supplemented with 10% calf serum. No antibiotics were used. The cells were shaken or scraped off the glass and dispersed in growth medium for passages. Within 2 to 3 days at 37 C, bottles contained about 10⁸ cells.

Chick embryo fibroblasts were used for assay of

Newcastle disease virus (NDV). Ten-day-old chick embryos were decapitated, forced through a syringe, and treated with 0.25% trypsin at 37 C; successive supernatant fluids were combined and plated in 4-ml amounts in 60-mm petri dishes (Falcon Plastic Co., Los Angeles, Calif.). Monolayers were ready for use after incubation for 2 days at 37 C in Eagle's medium plus 2% calf serum.

Viruses. Mengovirus resistant to inactivation at 37 C was also obtained from Richard M. Franklin. Stocks of the virus were made by infecting L cells growing in Blake bottles; yields were in 24 hr at 37 C. The virus was stored frozen at -65 C.

NDV was obtained from Frederick Wheelock. The virus was grown in the allantoic sac of 10-day-old chick embryos. Allantoic fluid was harvested after 2 days of incubation at 37 C and centrifuged lightly, and the supernatant fluid was used as inoculum.

Virus assays. Monolayers were grown for 2 or 3 days at 37 C in 60-mm petri dishes (Falcon Plastic Co.). After growth medium was removed, the virus inoculum was allowed to adsorb for 1 hr at 37 C in 0.1 ml adjusted with 7.5% filtered NaHCO_3 to pH 8.0. This pH is optimal for survival and adsorption of mengovirus to L cells (6). The cells were then overlaid with 4 ml of double-strength Earle's medium containing 2% calf serum in 1% agar (Difco). Overlay medium for mengovirus assays contained in addition protamine sulfate (British Drug Houses) at a final concentration of 0.02%, which increased plaque size (7). After 3 days of incubation at 37 C, 4 ml of overlay medium containing neutral red (final concentration, 1:40,000) was added, and plaques were counted on the same or the following day. Mengovirus assays were carried out in L cells; NDV assays were carried out in chick embryo fibroblasts.

To determine whether infectious mengovirus RNA was present, 0.5 ml of the sample to be assayed was diluted 1:2 with 1.5 M phosphate-buffered saline (pH 7.4), and 0.5 ml of the diluted sample was allowed to adsorb for 30 min on L cell monolayers at 37 C. The cells were then washed twice with Hanks' balanced salt solution (BSS; pH 8.0) and incubated at 37 C for 2 days in growth medium. To distinguish between cell damage resulting from treatment with hypertonic salt solution and that due to virus infection, and to increase the sensitivity of the assay, cell sheets were frozen and thawed for three cycles and passaged to fresh monolayers to develop plaques, the technique being that used for mengovirus assay.

Preparation of interferon. Allantoic fluid containing $>10^9$ plaque-forming units (PFU) of NDV per ml was irradiated under a GE sterilamp at a distance of 12 inches (30.5 cm) for 5 min, which reduced its infectivity to approximately 10^5 PFU/ml. L-cell bottles were inoculated with ultraviolet (UV)-treated NDV diluted fivefold in BSS. After adsorption for 4 hr at 37 C, sheets were washed with BSS, the medium was replaced with Earle's solution plus 2% calf serum, and the bottles were incubated for 20 hr at 37 C. Only the supernatant fluid was harvested. It was concentrated fivefold in a dialysis bag immersed in Carbowax (Union Carbide Chemical Co., New York, N.Y.), dialyzed 18 hr at 4 C against approximately 100 vol-

umes of isotonic saline, and the pH was brought to 2.0 with 2 N HCl. The material was stored at 4 C at this pH for 18 hr, and then brought to neutrality with 2 N NaOH. This crude preparation contained no infectious virus or hemagglutinin. Control fluid consisted of medium from L-cell bottles subjected to the same procedure.

Assay of interferon. Growth fluid was removed from monolayers of L cells in petri dishes. To each of three monolayers, 0.5 ml of twofold dilutions of interferon was added. After 2 hr, each plate received 3.5 ml of Earle's solution plus 2% calf serum and was incubated overnight at 37 C. At 24 hr after the beginning of interferon treatment, the fluid was removed, and each plate was inoculated with 0.1 ml of mengovirus containing approximately 50 PFU. The dilution of interferon that induced 50% inhibition of plaque formation was considered to contain 1 unit. All preparations used contained 128 or more units of interferon per ml.

Virus growth experiments. In confirmation of the findings of Colter et al. (6), we found that for a given multiplicity of infection, rate of adsorption at pH 8.0 was more rapid and complete and was less affected by variations in cell and virus concentration in monolayers than in suspended cell culture. All experiments reported were therefore performed with monolayer cultures. For experiments to determine kinetics of production of PFU or virus RNA, bottles containing approximately 5×10^7 cells per bottle were employed. When RNA was to be extracted by the sodium dodecyl sulfate (SDS)-phenol technique, Blake bottles containing approximately 10^8 cells per bottle were used. All procedures, including treatment with interferon or control fluid, treatment with actinomycin D, and virus adsorption, were carried out at 37 C until virus replication was terminated by quick freezing at -65 C. Medium was Hanks' salt solution plus 2% calf serum, amino acids, vitamins, and glutamine (10 ml for small bottles, 40 ml for Blake bottles). Washing was done with BSS adjusted to pH 8.0. The adsorption period was 1 hr. Monolayers were drained before they were inoculated with virus, and adsorption was followed by three washings with BSS.

A dose of 128 units of interferon, or an equivalent concentration of control fluid, was used in all experiments. Monolayers were drained of medium, and then interferon or control fluid was added (1 ml for small bottles, or 4 ml for Blake bottles) and allowed to interact with cells for 2 hr before the original volume of the fluid phase was restored with additional medium. Medium containing one or more chemicals replaced the medium in the bottles when chemicals were administered. Actinomycin D was furnished through the kindness of the Drug Development Branch, Cancer Chemotherapy National Service Center. The dose was 3 $\mu\text{g}/\text{ml}$ for all experiments, and this concentration was maintained throughout all procedures from the time of administration of the drug until the cells were frozen. Uniformly labeled uridine- C^{14} (specific activity 230 mc/mole) was obtained from Nuclear-Chicago Corp., Des Plaines, Ill., and uridine-2- C^{14} (specific activity 30 mc/mole) was purchased from Schwarz Bio Research, Orangeburg,

N.Y. SDS-phenol extractions (see below) were carried out on monolayers washed with BSS and frozen at -65°C . Assays for PFU were carried out after three cycles of freezing and thawing.

Radioactivity assay. Assays for radioactivity were carried out in a Nuclear-Chicago liquid scintillation spectrometer (720 series). To 0.5 ml (or less) of the sample to be counted was added 6 ml of absolute ethyl alcohol and 9 ml of scintillation fluid which consisted of a mixture of 6 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 2-*p*-phenylene-bis(5-phenyloxazole) (POPOP) dissolved in 1 liter of toluene. PPO and POPOP were obtained from Nuclear-Chicago Corp. Background counts varied from 20 to 25 counts/min. Quench was determined by the channel ratio method (4).

Extraction of RNA from *L* cells infected with mengovirus. The method of Homma and Graham (18, 19) was employed with minor modifications (SDS-phenol extraction). The infected cells were pelleted by centrifugation in 0.14 M NaCl and stored at -65°C . After thawing at 37°C , 3 ml of 0.14 M NaCl in 0.01 M acetate buffer (pH 5.0) was added, and then 0.3 ml of 5% aqueous solution of SDS was added. The mixture was shaken vigorously for 15 sec, and to it was added 3 ml of 80% phenol plus 0.25% ethylenediaminetetraacetate in phosphate-buffered saline (PBS; pH 7.4) that had been prewarmed to 60°C . The mixture was shaken for 3 min in a water bath (60°C), chilled in an ice bath, centrifuged (2,000 rev/min for 10 min), and the aqueous phase was removed. Two further extractions with chilled phenol were similarly done at 4°C . The RNA was precipitated from the aqueous phase with 6 volumes of cold ethyl alcohol while the mixture was held in an ice bath for 60 min. The supernatant fluid was removed, and the precipitate was dissolved in 2 ml of ice cold LTM buffer, pH 7.6 [0.14 M LiCl, 10^{-3} M tris(hydroxymethyl)aminomethane (Tris) at pH 7.6, and 10^{-3} M Mg^{++}]. The solution was added gradually to a Sephadex G-25 column and eluted with LTM buffer. Fractions containing approximately 12 ml were collected at 4°C . Determinations of optical density at 260 $\text{m}\mu$ (Beckman DU) and assays for radioactivity were performed on each fraction. The elution profile closely resembled those reported by Homma and Graham (18). The first peaks of optical density and radioactivity, respectively, were consistently concurrent in the third to fourth fractions. In a cold room (4°C), the RNA in the peak fraction(s) was reprecipitated with approximately 6 volumes of ice-cold ethyl alcohol during storage overnight, the supernatant fluid was removed after centrifugation, and the precipitate was redissolved slowly in 0.5 ml of LTM buffer.

Zonal sucrose density centrifugation. Linear sucrose gradients from 5 to 20% were prepared in LTM buffer by the method of Martin and Ames (28) and stabilized overnight at 4°C . RNA extracts were layered over them and centrifuged at 4°C in the SW 39 head of a Spinco model L centrifuge at 37,000 rev/min for 2.5 hr. Each tube was placed in an Isco model D density gradient fractionator (Instrumentation Specialties Co.), and 70% sucrose was forced by a

motor-driven syringe through a hole pierced in the tube near the bottom. As the density gradient column floated through an analyzer, optical density at 254 $\text{m}\mu$ was continuously recorded (Isco model UA), and 0.25-ml fractions were collected. When larger total volumes than 0.25 ml were required for subsequent analysis, fractions were diluted with LTM buffer. *S* values for RNA peaks were calculated from the position in gradients of marker RNA whose sedimentation coefficient was known.

Trichloroacetic acid precipitations. Total acid-precipitable radioactivity incorporated during a uridine- C^{14} pulse was determined on cells scraped into BSS. After a portion was removed for assay for infectious virus, the remaining cells were pelleted by centrifugation and suspended in 5% trichloroacetic acid. Trichloroacetic acid precipitation of RNA extracted by the SDS-phenol method was also carried out on portions of sucrose density gradient fractions after treatment with ribonuclease (crystalline pancreatic ribonuclease purchased from C. F. Boehringer, Mannheim, Germany) or LTM buffer. To 0.5 ml of each fraction was added 10 μg of ribonuclease in 0.5 ml of LTM buffer, or 0.5 ml of buffer alone. After 20 min of incubation at 37°C , tubes were chilled in cracked ice, 1 mg of cold carrier RNA (purchased from Nutritional Biochemicals Corp., Cleveland, Ohio) was added in 1 ml of buffer, and then the RNA was precipitated with trichloroacetic acid (final concentration, 5% by volume). Precipitates were collected by centrifugation, washed three times with 5% trichloroacetic acid, and redissolved in 1 N NaOH, and 0.2 ml was assayed for radioactivity.

RESULTS

Effect of actinomycin D and crude interferon on the time course of mengovirus replication. The kinetics of cell-associated PFU production in our monolayer cultures of *L* cells resembled those described by Homma and Graham (18, 19) for suspension cultures. As reported by them and by others (11, 31), mengovirus RNA synthesis and infectious virus output were unchanged when cell RNA synthesis was stopped by addition of actinomycin D. To determine the effect of crude interferon on the kinetics of virus replication, monolayers were treated with interferon or control fluid 18 hr before infection, actinomycin D (3 $\mu\text{g}/\text{ml}$) was added 1 hr before infection, and this concentration was maintained thereafter. Virus RNA production was measured as trichloroacetic acid - precipitable radioactivity incorporated during successive 1-hr pulses of uridine- C^{14} administered during the multiplication cycle to different cultures. Assays for PFU and radioactivity were done concomitantly. The eclipse period was between 5 and 6 hr (Fig. 1). Virus multiplication was complete at 9 hr. Synthesis of virus RNA preceded formation of infectious virus by 0.5 to 1 hr. Production of infectious virus in the pres-

FIG. 1. Effect of interferon on the kinetics of mengo-virus PFU production (A) and incorporation of uridine- C^{14} (B) in L cells treated with actinomycin D. Monolayers were treated with interferon or control fluid for 2 hr and medium was added. It was replaced 15 hr later with medium containing 3 μ g/ml of actinomycin D. Cells were infected at different multiplicities ($m = 0.5$ and $m = 25$) 1 hr thereafter and the cells were again washed at the end of the adsorption period. Uridine-2- C^{14} (1 μ c per culture, specific activity 30 mc/mmole) was administered for successive 1-hr pulses during the multiplication cycle to different cultures. At the end of each pulse, cells were assayed for PFU and trichloroacetic acid-precipitable radioactivity as shown. Cumulative counts per minute (cpm) and PFU for $m = 25$ are compared as percentages of maxima attained in control cultures (B).

ence of control fluid equaled that of untreated cultures, approximately 300 PFU per cell being made during a single burst. Crude interferon induced 95 to 99% inhibition of cell-associated PFU output. Up to 20% of the total radiouridine in control monolayers was incorporated during the first 5 hr. During this period, incorporation in interferon-treated cultures almost equaled that in cultures treated with control fluid. Most of the RNA in control cultures, however, was made after the 5th hr, whereas there was scarcely any increase in the RNA produced in interferon-treated cultures after this time.

Reversal of crude interferon action by simultaneous administration of actinomycin D. Although treatment with control fluid had no effect on the virus multiplication cycle, showing that the spent L-cell medium concentrated by the procedures used for interferon preparation was noninhibitory, as a further control it was necessary to demonstrate that the suppressive effect of the crude interferon on virus multiplication could be reversed by simultaneous addition of actinomycin D, since synthesis of new cellular messenger RNA (mRNA) appears to be required for expression of the inhibitory activity of interferon (22, 26, 32, 33).

The output of cell-associated infectious virus at 7 hr was determined for cultures that had and had not received actinomycin D at the time of administration of control fluid or interferon (Table 1). Every culture was treated with actinomycin D at the end of the adsorption period, however, because the results reported later were obtained with virus grown in the presence of the drug. The inhibitory effect of the crude interferon preparation was absent when the actinomycin D was simultaneously administered to cultures. The impurities present in the interferon failed to block viral synthesis in the presence of actinomycin D, and the inhibitory effect of crude interferon was therefore due solely to the interferon content.

Relative size of RNA synthesized during interferon inhibition. To determine whether interferon inhibited the synthesis of only a single class of virus RNA, or conversely whether polymerization of only a given class of RNA took place in the presence of interferon, SDS-phenol extracts from infected and uninfected L cells treated, respectively, with interferon or control fluid were analyzed by zonal sucrose density centrifugation (Fig. 2). Monolayers were exposed to interferon or control fluid 18 hr before infection ($m = 25$), and 3 μ g/ml of actinomycin D was added 1 hr before infection. Uniformly labeled uridine- C^{14} (5 μ c per culture) was administered at the end of the ad-

TABLE 1. Prevention of interferon-induced inhibition of mengovirus growth in L cells by simultaneous addition of actinomycin D with interferon

Expt	Treatment of cells 18 hr preinfection	PFU 7 hr postinfection*
1	Control fluid†	4.4×10^7
	Interferon†	4.6×10^5
	Control fluid + actinomycin D*	3.2×10^7
	Interferon + actinomycin D	2.7×10^7
2	Control fluid	1.6×10^8
	Interferon	2.2×10^6
	Control fluid + actinomycin D*	2.2×10^8
	Interferon + actinomycin D	2.5×10^8

* Actinomycin D (3 μ g/ml) was added to each monolayer at the end of adsorption (1 hr after infection, $m = 25$) and also 18 hr preinfection where indicated.

† Crude concentrate; see Materials and Methods.

sorption period and remained in the cultures between 1 and 7 hr postinfection, or for a corresponding 6-hr pulse in uninfected cultures. This period was that during which almost all trichloroacetic acid-precipitable uridine- C^{14} incorporation occurred in interferon-treated cultures, and included the period of maximal rate of RNA synthesis in uninhibited cultures (Fig. 1). At the end of the radiouridine pulse, RNA extracted by the SDS-phenol technique was layered on a 5 to 20% sucrose linear gradient, centrifuged in the Spinco SW 39 rotor (37,000 rev/min for 2.5 hr), and optical density and radioactivity along the gradient were determined.

As expected (17, 30), soluble RNA (sRNA) synthesis was not completely shut down by treatment with actinomycin D, but there was otherwise little radiouridine incorporated into the RNA of uninfected cultures (Fig. 2a). There was also the expected accumulation of newly synthesized virus RNA at the bottom of the gradient in infected cultures treated with control fluid, together with a second peak in the 16S region (Fig. 2b). Interferon treatment did not inhibit 4S RNA synthesis, but did inhibit synthesis of all other classes of RNA equally; neither accumulation nor absence of a given density class was discernible. These data are representative of the results obtained in other experiments, in two of which the gradients were layered with concentrations of RNA high enough to merge the 16S and 28S ribosomal RNA optical peaks (3). The over-

FIG. 2. Analysis by zonal sucrose density centrifugation of RNA extracted from uninfected (A) and infected (B) L cells treated with actinomycin D and interferon and pulse-labeled with uridine- C^{14} for 6 hr. Monolayers were treated with interferon or control fluid for 2 hr and medium was added. It was replaced 15 hr later with medium containing 3 μ g/ml of actinomycin D. Cells were infected ($m = 25$) 1 hr thereafter and washed at the end of the 1-hr adsorption period. Uniformly labeled uridine- C^{14} (5 μ c per culture; specific activity, 230 mc/mmole) was administered between 1 and 7 hr postinfection, or for a corresponding period in uninfected cultures. At the end of the radiouridine pulse, RNA was extracted by the SDS-phenol technique, layered on a 5 to 20% sucrose linear gradient, and centrifuged in the SW 39 rotor at 37,000 rev/min for 2.5 hr. Optical density at 254 $m\mu$ was continuously recorded, and fractions were collected and assayed for radioactivity.

loaded gradients therefore contained correspondingly more radioactivity than those that were more lightly loaded; however, the counts in preparations made with RNA from interferon-treated cultures remained not only markedly depressed but also were apportioned equally between fractions below the 4S region to the bottom of the gradient.

Inhibition by interferon of formation of double-stranded mengovirus RNA. The location of a virus-specific RNA peak in the 16S region of uninhibited cultures suggested that this RNA might be double-stranded; that is the region in which it has been demonstrated in extracts of cells infected with other viruses, including encephalo-

myocarditis (EMC; 8, 29) and ME (15) viruses, which are considered to be closely related to mengovirus. Evidence to support the assumption that the 16 to 22S virus RNA was double-stranded was obtained by comparing its susceptibility to ribonuclease with that of the rapidly sedimenting RNA in cultures treated with neither interferon nor control fluid. Actinomycin D was added 1 hr after infection ($m = 25$). The uridine- C^{14} pulse ($5 \mu\text{C}$ per culture) was administered between the 5th and 7th hr after infection, when the rate of radiouridine incorporation into mengovirus RNA was maximal in our system (Fig. 1b). The sucrose gradients were loaded with SDS-phenol extracts to the maximal amount that did not cause merging of the 16S and 28S ribosomal peaks. Portions of each 0.25-ml sucrose gradient fraction were incubated with ribonuclease and with buffer for 20 min at 37 C; then, after chilling and addition of carrier RNA to each sample, trichloroacetic acid-precipitable radioactivity was determined. Under these conditions, both the 16 to 22S and 40 to 60S virus RNA peaks were demonstrable as radiouridine incorporated into acid-insoluble form in the samples treated with buffer (Fig. 3). Ribonuclease treatment ablated the RNA peak at the bottom of the gradient. In contrast, approximately 35% of the acid-insoluble RNA in the 16 to 22S peak was ribonuclease-resistant, upholding the supposition that the 16 to 22S RNA was double-stranded.

FIG. 3. Zonal sucrose density centrifugation analysis of ribonuclease-sensitive and -resistant RNA in SDS-phenol extracts from infected cultures not treated with interferon. Actinomycin D ($3 \mu\text{g/ml}$) was added 1 hr after infection ($m = 25$), and a 2-hr pulse of uridine- C^{14} ($5 \mu\text{C}$ per culture) was administered between the 5th and 7th hr after infection. At the end of the pulse, the RNA was extracted, layered on a 5 to 20% sucrose linear gradient, and centrifuged in the SW 39 rotor at 37,000 rev/min for 2.5 hr. Optical density at 254 $m\mu$ was continuously recorded, and 0.25-ml fractions were collected. Samples of each fraction were incubated with either buffer or ribonuclease for 20 min at 37 C, chilled, cold carrier RNA was added, and trichloroacetic acid-precipitable radioactivity was determined.

It was also of interest to determine the effect of interferon on virus-specific RNA synthesis when it was occurring at its maximal rate in control cultures, particularly since the rate of RNA synthesis in interferon-treated cultures was minimal during this period. The uridine- C^{14} pulse was therefore administered between the 6th and 7th hr after infection. The effect on virus-specific RNA synthesis of administration of actinomycin D simultaneously with interferon 18 hr before infection was also ascertained. In this series of experiments, each gradient fraction was assayed for infectious RNA as well as for radioactivity.

When actinomycin D was added to cells at the time of infection, a radiouridine peak was again found in both the 16 to 22S and 40 to 60S regions of the gradient from cultures treated with control fluid (Fig. 4a). The RNA in both peaks was infectious, whereas RNA from other portions of the gradient was not infectious. In contrast, there was little new RNA synthesized in the interferon-treated cells, even in the 4S region; counts of radioactivity approached background values for most fractions, and none was infectious.

Other cells were exposed to actinomycin D from the time interferon or control fluid was administered 18 hr before infection until the RNA was extracted at the end of the radiouridine pulse. Although these cell sheets showed the microscopic evidence of damage and some destruction that regularly occurred when they were exposed to $3 \mu\text{g/ml}$ of actinomycin D for this period of time, there were large radiouridine peaks in the 40 to 60S region of gradients from cells treated with either interferon or control fluid, and the RNA from these peaks was infectious (Fig. 4b). This indicates that the actinomycin D blocked interferon inhibition. There was much less radiouridine incorporation in the 16 to 22S region in cells treated with control fluid and actinomycin D 18 hr before infection than was observed in cells treated with control fluid but not with actinomycin D until infection, and infectious RNA was demonstrable in only one 16 to 22S fraction. There were still fewer counts in the 16 to 22S region when cells received interferon and actinomycin D simultaneously, but some 16 to 22S RNA was made.

DISCUSSION

The experiments in L cells reported here were all carried out in the presence of actinomycin D, which shuts down synthesis of L-cell ribosomal RNA completely (31) but inhibits neither terminal additions to 4S RNA (10, 17, 30) nor mengovirus replication (11, 18, 31). Protocols for our experiments were the same in principle as those em-

FIG. 4. Zonal sucrose density centrifugation analysis of RNA extracted from L cells treated with actinomycin D and interferon and pulse-labeled with uridine- C^{14} between the 6th and 7th hr postinfection. Interferon or control fluid was added to monolayers 18 hr before infection. Medium was added 2 hr later. To one set of cultures (B) actinomycin D (3 μ g/ml) was added with the interferon or control fluid; the other set received no actinomycin D (A). Medium from both sets was replaced with medium containing 3 μ g/ml of actinomycin D when cells were infected ($m = 25$). Uniformly labeled uridine- C^{14} (5 μ c per culture; specific activity, 230 mc/mmole) was administered between the 6th and 7th hr after infection. At the end of the pulse, the RNA was extracted by the SDS-phenol technique, layered on a 5 to 20% sucrose linear gradient, and centrifuged in the SW 39 rotor at 37,000 rev/min for 2.5 hr. Optical density at 254 $m\mu$ was continuously recorded, and fractions were collected and assayed not only for radioactivity but also for infectious RNA. Infectivity is indicated by +, lack of demonstrable infectivity by -.

ployed by Taylor (32, 33), who utilized actinomycin D to study virus-specific RNA synthesis in chick embryo fibroblasts infected with Semliki Forest virus. She found that incorporation of adenosine- H^3 into Schmidt-Tannhauser extracts was markedly reduced when cells were exposed first to interferon purified by a modification of the procedure of Lampson et al. (21), then to

actinomycin D. When the cells were treated with actinomycin D before they were treated with interferon, however, ensuing actinomycin-resistant virus RNA synthesis was not impeded. Others have reported similar reversal of interferon inhibition of virus replication when cells were preincubated with actinomycin D or puromycin (22, 26) or *p*-fluorophenylalanine (12). The virus-inhibitory activity of other substances that can be present in impure interferon preparations, however, is not affected by exposure of cells to actinomycin D. The crude interferon used in our experiments did not contain such substances, since it blocked neither plaque formation (Table 1) nor infectious RNA synthesis (Fig. 4) when cells were treated simultaneously with interferon and actinomycin D before they were infected.

Our results show that interferon inhibited the polymerization of all classes of RNA distinguishable by sucrose gradient centrifugation analysis that could be presumed to be coded by the mengovirus genome. There appeared to be neither a specific ablation of an RNA class nor an accumulation of noninfectious, defective, or partially polymerized RNA as a result of interferon inhibition. Radiouridine incorporation into the ribonuclease-sensitive infectious RNA that sedimented in the 40 to 60S region, and into the ribonuclease-resistant infectious RNA that sedimented in the 16 to 22S region, was equally and markedly inhibited. Whether the observed double-stranding, presumably the result of pairing between parental "plus" and template "minus" RNA strands, is or is not an artifact of extraction, the results indicate that neither "plus" nor "minus" strands were synthesized.

Judgment must be reserved as to whether interferon inhibited sRNA formation; spreading of the 16S infectious RNA peak due to non-ideal sedimentation (3) might have accounted for the larger amount of radiouridine in the 4S region of gradients from cultures treated with control fluid than in cultures treated with interferon (Fig. 2b, 4a). Although the use of serial transfer increased the sensitivity of our assay for infectious RNA over methods that rely on direct plaqueing or direct determination of infectious center formation, infectivity was demonstrable in no sucrose gradient fraction containing RNA from interferon-inhibited cultures.

Evidence to support the conclusion that the more slowly sedimenting infectious mengovirus RNA was double-stranded consists of its location in the 16 to 22S region, and the fact that approximately one-third of it was ribonuclease-resistant. During the experiments reported here, however, it was not feasible to determine its thermal transi-

tion characteristics or its base composition as additional criteria of double-strandedness. The careful studies of Tobey (34) failed to reveal double-stranded mengovirus RNA, but Homma and Graham (19) found ribonuclease-resistant 19S RNA in the mengovirus system. Double-stranded virus RNA was discovered in EMC virus (29) which is closely related to mengovirus and thereafter was studied in other viruses (1, 2, 20, 35). Its characteristics have subsequently been further delineated in EMC (8) and ME viruses (15). Mengo, EMC, and ME viruses possess common antigens and are members of the Columbia-SK group.

Friedman and Sonnabend (13) have reported evidence that interferon inhibits the appearance of Semliki Forest virus RNA having characteristics of a double strand. Sucrose density centrifugation analysis revealed that a more slowly sedimenting actinomycin-resistant virus RNA synthesized 2 to 3 hr after infection of chick embryo fibroblasts predominated over a more rapidly sedimenting component. Interferon treatment caused incorporation of adenosine- H^3 into acid-insoluble RNA to be markedly depressed in all sucrose gradient fractions when the pulse was given 2 to 3 hr postinfection. A pulse 6 to 8 hr postinfection also revealed considerable depression of incorporation into the slow component, but less so than into the fast one. The discrepancy between the latter finding and the marked inhibition of all virus RNA polymerization that we found when interferon-treated L cells infected with mengovirus were pulse-labeled late in the infectious cycle might be attributable to differences in technique, including duration of the pulse, differences in the kinetics of replication of Semliki Forest virus and mengovirus, or differences in interferon activity.

Virus-specific radiouridine incorporation in cells treated simultaneously with control fluid and actinomycin D before infection was not as great as that in cells treated only with control fluid and exposed to actinomycin D only after they were infected, particularly in the 16 to 22S region (Fig. 4). Close analysis of these data seems unfruitful at present, however, because actinomycin D was somewhat deleterious to the cells. In spite of this fact, when it was administered 18 hr before infection the output of infectious virus was undiminished (Table 1). A decision as to whether the apparent discrepancy between virus-specific RNA synthesis and infectious virus output is worth further study might be guided by the outcome of additional experiments.

Levy (23) studied the action of interferon on early events in mengovirus infection of L cells.

Shortly after infection, L-cell protein and RNA synthesis both cease (11). Levy (23) showed that interferon treatment capable of preventing mengovirus replication did not avert cutoff of cell protein synthesis but did delay inhibition of cell RNA synthesis by about 1 hr. He and his colleagues L. Snellbaker and S. Baron have recently found that interferon similarly interdicts the inhibition of cellular RNA synthesis in chick embryo cells by Sindbis virus (*personal communication*). The precise relationship between these findings and the more general phenomenon of inhibition of viral RNA synthesis by interferon is still under study, but, if they are connected, these data support the concept that interferon acts on an early phase of virus replication.

Our results, together with those of other investigators, therefore put the point of action of interferon between the events immediately after virus uncoating and the formation of template "minus" RNA strands, and show that partially polymerized virus RNA products were not made in interferon-inhibited cells.

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LITERATURE CITED

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