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Defective Interfering Passages of Sindbis Virus: Chemical Composition, Biological Activity, and Mode of Interference

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Defective interfering (DI) particles of Sindbis virus, appearing between the eighth and fourteenth passages, cosediment with and have the same buoyant density as standard virus. Virion RNA from such late passages is heterogeneous by polyacrylamide gel electrophoresis, whereas early passage RNA is homogeneous. No differences were found in the virion proteins from such passages. Cells co-infected with early and late passage virus synthesize as much intracellular viral-specific RNA and protein as is made after infection with early passage virus alone, although virus production is inhibited by 90% or more. Such cells synthesize two new intracellular species of RNA with molecular weights of 2.2×10^6 and 0.86×10^6. Nucleocapsid assembly is blocked in these cells, and the amount of intracellular capsid protein made is reduced by 50%. The presence of a new intracellular protein in late passage infection was detected by polyacrylamide gel electrophoresis.

In many viral systems, continuous passaging of plaque-purified (standard) virus leads to the accumulation of defective interfering (DI) particles (5; A. S. Huang, Annu. Rev. Microbiol., in press). Although the events leading to their first appearance and subsequent accumulation are poorly understood, the mechanism by which they inhibit virus production is gradually being elucidated, at least for a few select viruses (2, 3, 6, 9).

Schlesinger et al. (17) recently extended the list of viruses in which this phenomenon occurs to include the togaviruses, specifically Sindbis virus. Serial passaging of this virus in BHK-21 cells resulted in the appearance of DI particles by the eighth or ninth passage. Subsequently, several papers have appeared analyzing various aspects of interference in this system. Shenk and Stollar (19) isolated DI particles from Sindbis virus-infected BHK cells which do not independently initiate viral-specific RNA synthesis and which, upon co-infection with standard virus, inhibit RNA synthesis. The RNA isolated from these defective virions was identical in molecular weight to infectious RNA. Intracellularly, however, a new 12*S* double-stranded RNA was synthesized as well as a number of small, single-stranded RNA species not seen in normal infections.

Eaton and Faulkner (4) also obtained DI particle-containing passages of Sindbis virus grown in BHK cells and found that they induce the synthesis of a 20*S* single-stranded RNA species as well as the double-stranded 12*S* RNA previously observed by Shenk and Stollar (18). They also demonstrated that the production of 20*S* single-stranded RNA did not take place when cells were co-infected with nondefective and UV-irradiated defective passages of Sindbis virus.

We have continued to study the class of DI particles of Sindbis virus first described by Schlesinger et al. (17) and have attempted to answer the following questions related to these particles and their biological activity. (i) What is the nature of the RNA and proteins of the DI particle as compared with infectious Sindbis virus? (ii) What viral synthetic events occur after infection with DI particle-containing preparations of virus? (iii) At what level of virus production does the DI particle exert its inhibitory effect?

Although we were unable to separate DI particles from normal virions, we found that the RNA from viral preparations containing DI particles was heterogeneous, consisting of small-molecular-weight RNA species as well as infectious viral RNA. In contrast, the protein

composition of such mixed virus populations was indistinguishable from that of standard virus alone. While severely inhibiting virus production, DI particle-containing passages do not alter the rate of synthesis of intracellular viral-specific RNA or protein. Nucleocapsid formation, however, is blocked in these cells and there appears to be a defect in capsid protein production.

Cells co-infected with standard and defective virus contain two new single-stranded RNA species not seen in cells infected with standard virus alone. The major new species has a molecular weight of 0.86×10^6 and is probably identical to the 20S single-stranded RNA observed by Eaton and Faulkner (4). The second species, a minor component, has a molecular weight of approximately 2.2×10^6 and may be a derivative of the 3×10^6-molecular-weight RNA found in standard infections but much reduced or absent in mixed infections.

MATERIALS AND METHODS

Cell cultivation and virus growth. Monolayers of BHK-21 cells were maintained in Eagle minimal essential medium supplemented with 6% fetal calf serum. Wild-type Sindbis virus was plaque-purified and passaged according to published procedures (17).

Virus assays. Plaque assays were carried out by using primary or secondary chicken embryo fibroblasts according to procedures described by Pfefferkorn and Hunter (11).

Interference assays involved the co-infection of cells with early- and late-passage virus as described earlier by Schlesinger et al. (17). Confluent monolayers of BHK cells were inoculated with late-passage virus at varying multiplicities of infection (MOI). Immediately thereafter, early-passage virus (MOI = 10) was added to the above infected cells, as well as to control cells not previously infected with late-passage virus. After a 1-h adsorption period, 7 ml of medium was added to each plate and virus was harvested between 10 and 16 h later. The extent of inhibition of virus production was determined relative to control cultures infected with early-passage virus alone.

Virus purification. Labeled virus was purified by centrifugation overnight through a composite velocity and equilibrium gradient as described by Scheele and Pfefferkorn (13). After collection of the gradient, samples from each tube were precipitated with 10% trichloroacetic acid, collected on membrane filters (Millipore Corp.), dried, and counted in a Packard scintillation counter. Fractions containing radioactive virus were pooled.

Preparation of cytoplasmic extracts. (i) Confluent monolayers of BHK cells were chilled and washed twice with TNE (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 7.5). Nonidet P-40 (0.5% in TNE containing 10 μg of polyvinyl sulfate per ml) was added, and the cells were detached by repeated flushing of the plate by using a Pasteur pipette. The unbroken nuclei and cell debris were removed by centrifugation in a Sorvall centrifuge for 10 min at 2,000 rpm.

(ii) A modification of the Dounce homogenization technique of Burge and Strauss (1) was used. Cell monolayers were rinsed twice with cold TNE, scraped from the surface of Falcon plastic dishes with a rubber policeman, and pelleted by low-speed centrifugation. Cells were resuspended in a one-tenth solution of TNE, allowed to swell for 20 min at 4 C, and homogenized with 20 strokes of a Dounce homogenizer. Cell debris was removed by centrifugation at 2,000 rpm for 10 min.

Analysis of cellular nucleocapsids. Cell extracts were prepared by method ii given above. EDTA was added to a final concentration of 0.002 M. A 0.5-ml sample of the cell extract was layered over a 15 to 30%, 12-ml sucrose gradient and centrifuged in an SB206 International rotor at 34,000 rpm for 2 h at 4 C. Viral nucleocapsid was isolated by Nonidet P-40 lysis of purified virions and centrifugation through a 15 to 30% sucrose gradient.

Purification of RNA. Cell and viral RNA were purified in the same manner, except that 0.1 OD_{260} (optical density at 260 nm) unit of unlabeled purified Sindbis virus was added to all virus preparations before extraction. With both types of RNA, polyvinyl sulfate (10 μg/ml), sodium dodecyl sulfate (SDS; 1%), and *Escherichia coli* tRNA (50–100 μg/ml) were added before extraction. Two volumes of a 1:1 mixture of TNE-saturated phenol and chloroform-isoamyl alcohol (24:1) were then added. The mixture was vortexed for 3 to 10 min, and the phases were separated by centrifugation for 10 min at about 1,500 rpm. The phenol phase was re-extracted with 1 ml of TNE. The aqueous phase was re-extracted as before. The two resulting aqueous phases were combined, re-extracted with an equal volume of chloroform-isoamyl alcohol, and precipitated overnight at −20 C with 2.5 vol of 95% ethanol.

Labeling procedures. Cells were infected and exposed to 0.25 to 1.0 μg of actinomycin D per ml for 5 h before labeling. [5-^3H] uridine (24.3 Ci/mmol, 10–50 μCi/ml) was then added to 2 ml of medium per 10^7 cells. Incorporation was terminated after 1, 5, or 7 h by removal of the virus-containing medium and preparation of cell extracts.

In kinetic experiments, 2×10^5 cells attached to the surface of 35-mm Falcon plastic dishes were infected and exposed to actinomycin D for 2 h before labeling. [5-^3H]-Uridine (1 μCi/ml) and unlabeled uridine (2×10^{-5} M) were then added. Incorporation was stopped at hourly intervals and extracts were prepared by method i given above. The entire cell lysate from individual plates was acid-precipitated, filtered, dried, and counted.

To obtain labeled BHK ribosomal RNA, confluent monolayers were exposed to 1.2 μCi/ml of ^{14}C-uridine (422 mCi/mmol) in the presence of 2×10^{-5} M unlabeled uridine for 24 h in a total volume of 8 ml.

For labeling of proteins, cells were infected and grown in the absence of amino acids for 5 h before labeling with ^{35}S-methionine (180 Ci/mmol, 10 μCi/ml) in the presence of 10^{-5} M unlabeled methionine to maintain linear incorporation. When radioactive virus was prepared, the methionine concentration was reduced to 10^{-6} M, and the medium was collected 7 h after the addition of isotope.

Polyacrylamide gel electrophoresis. For the analysis of both viral and cellular RNA, 0.5% agarose-1.8% polyacrylamide gels were prepared according to procedures provided by E. Wimmer (manuscript in preparation). Gels were prepared in a Tris (7.2 mM), phosphate (sodium dihydrogen phosphate, 6 mM) buffer (pH 7.6–7.8) containing glycerol (10%), SDS (0.2%), and disodium EDTA (.02 mM). Separate solutions of monomer (containing 0.04 ml/25 ml of *N,N',N'*-tetramethylenediamine) and agarose (0.25 g/25 ml, "Seakim" agarose, Bausch & Lomb) were mixed at 55 C, 0.25 ml of 10% ammonium persulfate was added, and the resulting solution was poured rapidly into parafilm-sealed perspex tubes (9 cm by 0.6 cm). Gels were polymerized for at least 1 h at room temperature before use. They could be stored at 4 C for later use after application of a layer of Tris-phosphate buffer to the top of the gel and sealing the tubes tightly with parafilm. Gels were pre-electrophoresed for 0.5 to 1 h before use. Electrophoresis was performed at constant voltage and a current setting of 5 mA/gel for 2.5 to 3 h. Gels were frozen and sliced into disks about 1 mm thick. Each slice was incubated with 0.5 ml of concentrated ammonia and dried completely, and the hydrolyzed RNA was dissolved in 0.2 ml of water. Five milliliters of scintillation fluid was added for counting.

Proteins were reduced, alkylated, and analyzed on vertical slab or cylindrical 10% polyacrylamide gels according to published procedures (15).

Isotopes. [5-^3H] Uridine (24.3 Ci/mmol) and ^{35}S-L-methionine (180 Ci/mmol) were purchased from Schwarz BioResearch, Orangeburg, N.Y. Uniformly-labeled ^{14}C-uridine (422 Ci/mmol) was obtained from New England Nuclear Corp.

RESULTS

Sedimentation behavior of early- and late-passage virus. Sindbis virus grown in BHK-21 cells (radioactively labeled with ^{3}H-uridine or with ^{35}S-methionine) was routinely purified in one step by sedimentation overnight through a composite velocity and equilibrium sucrose gradient (see Materials and Methods). A single peak of radioactivity and infectivity was obtained in this gradient. Interfering activity cosedimented with the peak of infectivity using late-passage virus (eighth to fourteenth passages). Early-passage virus preparations (first to sixth passages) showed no interference ability when co-infected with another early-passage virus or, as shown previously, when the multiplicity of infection ranged up to 100 PFU/cell (17). Virus purified in the above manner was subjected to a second equilibrium centrifugation in D_2O-sucrose gradients according to Shenk and Stollar (19). Again, no separation of infectivity from interfering ability was achieved. Figure 1 displays the distribution of radioactivity (in this case, ^{35}S-methionine) on such a gradient for passages 3, 8, and 9. The

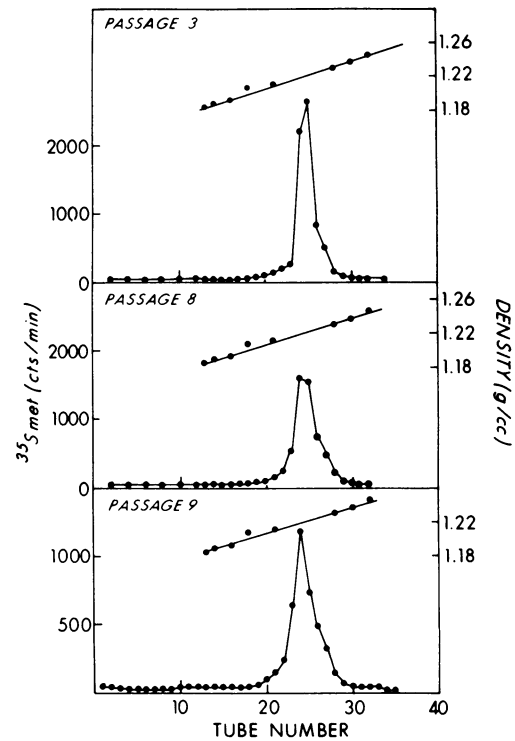

FIG. 1. *Comparison of the buoyant densities of early- and late-passage virus in a 14 to 40% D_2O sucrose gradient. Gradients (12 ml) containing 0.1% fetal calf serum were centrifuged for 14 h at 35,000 rpm in an SB283 International rotor. Density was determined by weighing 0.1-ml samples from selected tubes.*

same distribution was observed when the virus from the fourteenth passage was labeled with ^{3}H-uridine. In general, there was little change in the ratio of infectivity per unit of radioactivity or in interfering potential during the isolation of the viral particles (Table 1).

Because of our inability to physically separate defective from standard virions, an estimate of the relative contribution of each particle to the total population was calculated from the ratio of PFU recovered after purification to acid-insoluble radioactivity (by using either ^{3}H-uridine or ^{35}S-methionine-labeled virus). This quantity was always smaller for late-passage virus, indicating the presence of noninfectious labeled material. The ratio of this quantity for early- and late-passage virus gives a crude estimate of the amount of noninfectious virus produced in a given infection.

Another parameter that was carefully monitored was the degree of inhibition of normal virus production by defective virus-containing passages. These two values, PFU to counts per

TABLE 1. *Recovery of radioactivity, infectivity, and interference potential during purification of early (3)- and late (8 and 9)-passage virus*

Purification step	Passage	PFU/counts per min[a] (×10³)	Interference[b]	
			MOI	Control (%)
None	8		20	15
	9		5	3.5
First gradient	3	8.7		
	8	2.6	26	1.7
	9	0.66	2.3	1.8
Sucrose-D_2O gradient	3	7.1		
	8	1.3	10	2.5
	9	0.65	2.0	3.3

[a] The virus was labeled with ^{35}S-methionine.

[b] Interference was carried out against early-passage virus at a multiplicity of 13. The multiplicity of late-passage virus being assayed for interference ability is given in the table.

minute and interference potential, were reliable indicators of the proportion of DI particles present in any virus sample. As the PFU-to-counts per minute ratio of late-passage virus approached that of early-passage virus, the degree of interference elicited in a standard assay (described in Materials and Methods) decreased. Although interference could be enhanced by increasing the multiplicity of late-passage virus, it was usually tested at low multiplicity (MOI = 1) in order to establish that the preparation had a high proportion of DI particles.

Isolation and comparison of RNA from purified early- and late-passage virus. ^{3}H-Uridine-labeled RNA extracted from gradient-purified early- and late-passage virus was analyzed by electrophoresis on agarose-polyacrylamide gels. The RNA had to be purified before analysis because as much as 90% of the radioactivity was excluded from the gel when SDS disruption alone was used to dissociate RNA from protein. The RNA pattern of standard virus always contained a single species of RNA with a molecular weight of 3.6×10^6 to 4.0×10^6 (calculated according to Peacock and Dingham [8] and using BHK-21 ribosomal RNA as markers). Preparations containing DI particles yielded RNA patterns that were more heterogeneous and had a variable number of smaller RNA fragments. Figure 2 displays the RNA pattern obtained from phenol-extracted passage 2 and passage 8 virus. The sevenfold decrease in the ratio of PFU to counts per minute in passage 8 relative to passage 2 (Table 2) suggests that infectious particles comprise only about 12% of the total particles produced in late-passage infections. The percentage of RNA that is found outside the main viral RNA region for a number of different experiments, including the one shown in Fig. 2, is presented in Table 3 together with virus yield and interference data for each experiment.

It is clear that there is more low-molecular-weight RNA present in purified late-passage virus populations than is ever found in early passages of the virus. It should be stressed that in all of the RNA purifications, 0.1 OD_{260} unit of purified, unlabeled, standard Sindbis virus was added to eliminate possible effects of differences in viral concentration on recovery and intactness of the RNA. Because the defective particles could not be separated from standard

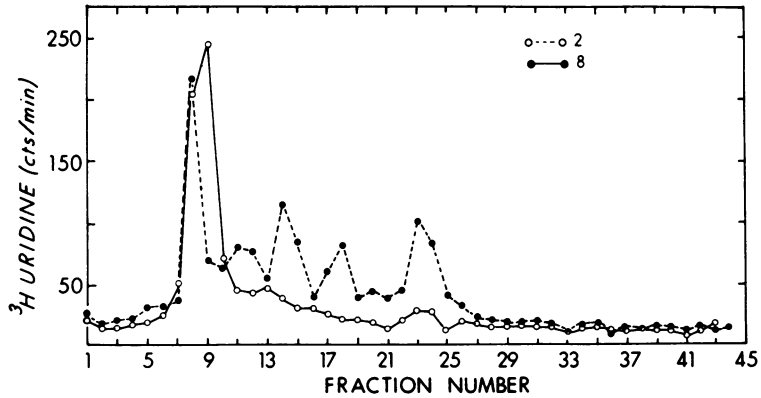

FIG. 2. *Comparison of the RNA from purified early- and late-passage virus by polyacrylamide gel electrophoresis. Phenol-extracted viral RNA from passages 2 and 8 was ethanol-precipitated and resuspended in 50 μliters of electrophoresis buffer (see Materials and Methods) containing 10% glycerol and 0.2% SDS; sucrose and bromophenol blue were added just before electrophoresis. Samples were layered on 0.5% agarose-1.8% polyacrylamide gels and electrophoresis was carried out for 2.5 to 3 h. The origin is at the left.*

TABLE 2. *Virus yields, radioactivity, and PFU-to-counts per minute ratios for early- and late-passage infections*[a]

Passage	PFU/ml (× 10^9)	Counts per min per ml	PFU/Counts per min (× 10^5)
2	30	180,000	1.7
8	0.6	24,300	0.25

[a] Cells were labeled with ^{3}H-uridine (24.3 Ci/mmol, 50 μCi/ml) between 5 and 10 h postinfection. Labeled virus produced during this interval was purified through a sucrose gradient as described in the text.

TABLE 3. *Comparison of early- and late-passage viral RNAs in terms of their degree of heterogeneity*

Passage added	Virus yield (PFU/ml × 10^9)	Interference[a]		Total RNA[b] outside main viral RNA region (%)
		MOI	Control (%)	
2	30			30
8	0.6	1.2	25	70
2	30			20
8	4.0	2.0	57	42
6	1.8			18
14	0.23	1.0	40	46
5	2.2			16
5 + 9	0.23	1.1	49	45

[a] Interference was assayed against passage 5 virus at a multiplicity of 10. The multiplicity of late-passage virus is indicated in the table.

[b] These estimates were made from electrophoretic patterns similar to those illustrated in Fig. 2. In the experiment presented in Fig. 2 (passages 2 and 8 in the first set of data listed above), the infectious viral RNA is assumed to be in fractions 7 through 10.

virus, we have not investigated further the relationship between the new RNA species detected and the interference demonstrated by the virus population from which they were derived.

A comparison of the protein compositions of early- and late-passage virus. Figure 3 shows two polyacrylamide gel patterns of the proteins present in purified early- and late-passage virus. The three viral proteins (14) found in early-passage virus are present in late-passage virus in the same proportions.

Cellular events after infection with early- or late-passage virus. Infection of BHK cells with late-passage virus alone or co-infection with late and early passages led to a marked drop in virus production (Table 2). We observed, however, that cells from such infections incorporated amounts of ^{3}H-uridine into RNA that were completely unrelated to the degree of inhibition of virus production (Table 4). This observation is further documented in Fig. 4, which shows the rate of incorporation of ^{3}H-uridine over a 7-h period after infection with passage 5 (MOI = 10) or passages 5 and 9 (the latter at an MOI of 1, which reduced the virus yield by about 90%).

Despite this large accumulation of RNA in the presence of defective virus, very little of the RNA was encapsidated. Figure 5 presents a

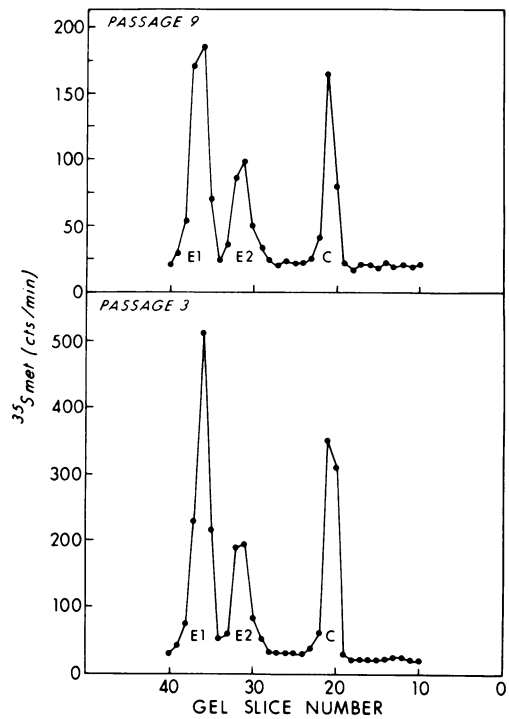

FIG. 3. *Comparison of the proteins present in purified early- and late-passage virus by polyacrylamide gel electrophoresis. E1 and E2 are viral envelope proteins; C is the capsid protein. The numbering of the slices started with the slice containing dye.*

TABLE 4. *Incorporation of ^{3}H-uridine into BHK-21 cells infected with early- and/or late-passage virus*

Passage added	Virus yield (PFU/ml × 10^9)	MOI	^{3}H-uridine incorporation[a] (counts/min)
2	30	20	80,520
8	0.6	42	206,880
5	1.5	10	57,400
5 + 9	0.21	10 + 1	59,600

[a] Incorporation was carried out between 5 and 6 h after infection. Cell extracts were made and acid-precipitable radioactivity measured.

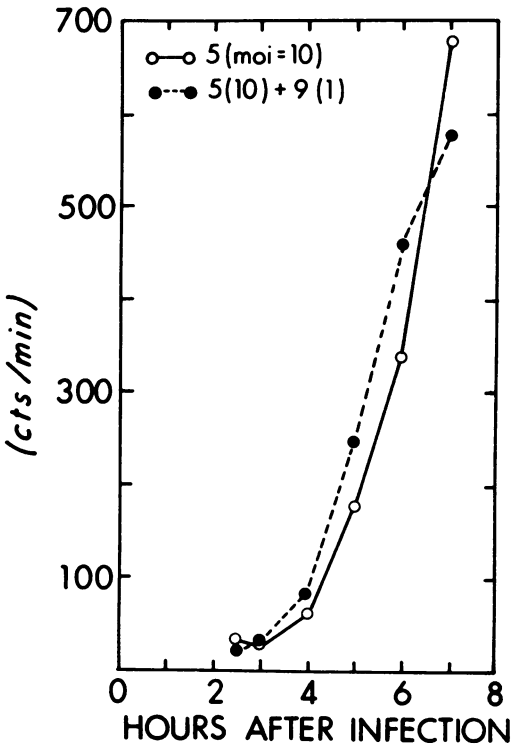

FIG. 4. *Comparison of the kinetics of ^{3}H-uridine incorporation after infection with passage 5 (MOI = 10) or co-infection with passages 5 (MOI = 10) and 9 (MOI = 1). ^{3}H-Uridine (1 μCi/ml, 2 × 10^{-5} M) was added 2 h after infection. Actinomycin D (1 μg/ml) was present from the time of infection.*

sucrose gradient profile of the distribution of viral-specific RNA in cell extracts treated with 0.002 M EDTA to dissociate polyribosomes and permit more clear visualization of the nucleocapsid. ^{14}C-Amino acid-labeled viral nucleocapsid obtained by Nonidet P-40 disruption of purified standard virus was run concurrently to obtain more certain identification of the nucleocapsid. During infection with standard virus (in this case, passage 5), about 40% of the RNA was present in structures sedimenting with viral nucleocapsid. Under interference conditions, in this instance co-infection of cells with passages 5 and 9 as discussed above, a maximum of 8% of the RNA was in the nucleocapsid region. The nonencapsidated RNA synthesized during co-infection with late-passage virus sedimented more slowly than did RNA made in early-passage virus infection.

Acrylamide gel electrophoresis revealed clear differences in the RNA pattern (Fig. 6). Infection with early-passage virus gave rise to at least five or six RNA species in the cell, for convenience called VR1 through VR6. We have not as yet looked at double-stranded RNA directly, but have determined by lithium chloride pre-

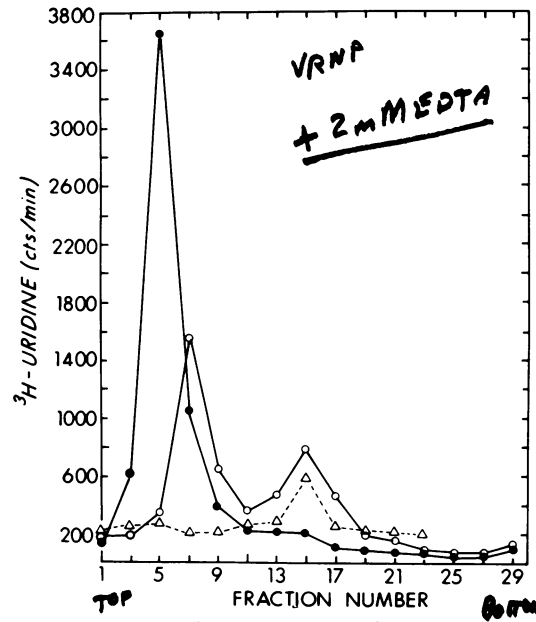

FIG. 5. *Comparison of the distribution of viral-specific RNA in extracts prepared from cells infected with passage 5 or co-infected with passages 5 and 9 under interference conditions. Cells were labeled with ^{3}H-uridine between 1 and 12 h after infection in the presence of 0.25 μg of actinomycin D per ml. Extracts were prepared by method ii (see Materials and Methods), and a portion of each extract was layered onto a 15 to 30% sucrose gradient and centrifuged in an SB206 rotor for 2 h at 34,000 rpm. Passage 5 (○); passage 5 + 9 (●); ^{14}C-amino acid-labeled viral nucleocapsid (△).*

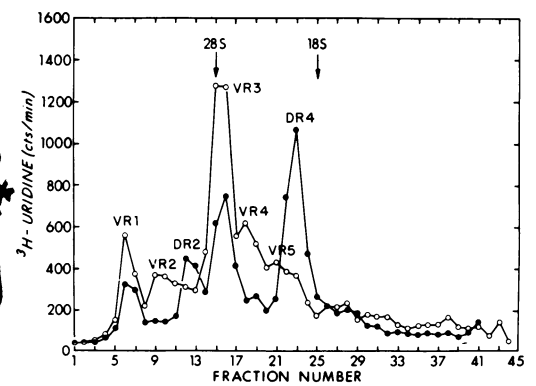

FIG. 6. *Comparison of the viral specific, intracellular RNA species synthesized in cells infected with early- or late-passage virus by polyacrylamide gel electrophoresis. Cells were labeled with ^{3}H-uridine between 5 and 10 h after infection. Actinomycin D (1 μg/ml) was present from the time of infection. Passage 2 (○); passage 8 (●).*

cipitation that 80% of the RNA was precipitated at high salt concentration and that this fraction contained essentially all of the RNA species observed before precipitation. The molecular weights of the various species of RNA are listed in Table 5. VR1 and VR3 correspond to 42 and 26S RNA, the two major single-stranded RNA species found in standard virus infections (20). VR2 may correspond to the 33S RNA observed by others (7, 12). VR4 and VR5 are clearly quite heterogeneous and are undoubtedly a mixed population of RNAs. VR6, which is not well demonstrated in this gel pattern, has been seen more clearly in other experiments. It may be double-stranded since it was not precipitated by 2 M LiCl. It is evident from Fig. 6 that two new RNA species (DR2 and DR4) were synthesized in the presence of DI particles. The major new component has a molecular weight of 0.86×10^6 and is probably identical to the 20S single-stranded RNA identified by Eaton and Faulkner (4). The second new RNA moiety is smaller than VR2, which is absent or much reduced in amount in these defective passage infections, and may be related to it. About 10% of the total radioactivity in both early- and late-passage infections was in the position of genomic viral RNA. For passage 2, about 50% of the radioactivity was in the region of 26S RNA. This value was reduced to 22% in passage 8 infection, and the new 20S RNA species represented about 46% of the total radioactivity. Thus there is a marked drop in the amount of 26S RNA made at the time that the 20S species appears. The relative proportion of 20 to 26S RNA is variable and appears to be dependent on the percentage of DI particles in the infecting inoculum.

In experiments designed to study viral-specific protein synthesis, we found that the rate of incorporation of ^{35}S-methionine into cells infected with early- and late-passage virus was essentially the same (Table 6). An examination of viral-specific proteins by acrylamide gel electrophoresis, however, revealed a difference in the relative amount of radioactive capsid protein formed during early- and late-passage infection. The percentage of radioactivity in the capsid protein was twice as great in cells infected with standard virus as in cells infected with preparations containing defective particles, whereas the percentage of radioactivity in the envelope proteins (E1 and PE2, the precursor of E2 [15]) was identical (Fig. 7 and Table 7).

TABLE 5. *Molecular weights of the RNA species found in cells infected with early (2)- or late (8)-passage virus*

Designation	Mol wt ($\times 10^6$)	Passage	
		2	8
VR1	4	+	+
VR2	3	+	−
VR3	1.6	+	+
VR4	1.4–1.2	+	−
VR5	1.1–0.86	+	−
VR6	0.2	+	+
DR2	2.2	−	+
DR4	0.86	−	+

TABLE 6. *Incorporation of ^{35}S-methionine into BHK-21 cells infected with early- or late-passage virus*

Passage added	Virus yield[a] (PFU/ml $\times 10^9$)	Pulse length (min)	Incorporation of ^{35}S-Methionine (counts per min per ml; $\times 10^5$)
2	2.6	20	1.7
		60	6.0
9	0.03	20	1.4
		60	5.8
5	3.3	40	1.6
11	0.15	40	1.3

[a] Virus yields were obtained 12 h after infection; cells were labeled with ^{35}S-methionine between 5 and 6 h after infection.

At least some of the loss in radioactivity from capsid protein may be accounted for by a new protein clearly visible in autoradiograms of acrylamide slab gels (Fig. 8). This new protein, migrating between B1 (the precursor of the envelope proteins) and PE2 was stable in a pulse-chase experiment (Fig. 8). As expected, radioactivity in B1 was lost during the chase (15).

DISCUSSION

The results reported in this paper suggest that the greatly reduced yield of Sindbis virus that follows co-infection of cells with normal and defective virus reflects the limited amount of nucleocapsid assembled in the cell. Since the rate of viral-specific RNA and protein synthesis proceeds normally under interference conditions, DI-related RNA as well as normal viral RNA are made and both are equally competent to perform all of the functions required for translation.

The finding that less capsid protein is made in cells co-infected with standard and defective virus provides an explanation for the reduced levels of assembled nucleocapsids in these cells. Thus it would appear that defective particle RNA contains a lesion which leads to either a

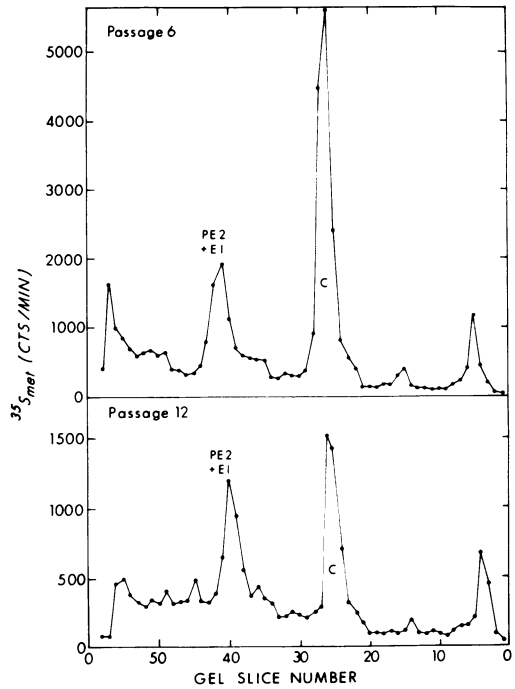

FIG. 7. *Comparison of the viral-specific proteins synthesized in cells infected with passage 5 and passage 11 virus by polyacrylamide gel electrophoresis. (For this experiment, passage 5 virus was first passaged at low multiplicity to dilute out any DI particles that might be present even this early in passaging). Cells were pulse-labeled with* ^{35}*S-methionine (*10^{-5} *M) for 40 min at 5 h after infection, and extracts were prepared with Nonidet P-40. PE2 is the precursor to E2; other viral proteins are identified in Fig. 3.*

total lack of capsid protein synthesis or the synthesis of an unstable capsid protein. Both of these situations have been encountered with the DI particles of poliovirus (2, 3). In that system, infection of HeLa cells with purified DI particles is followed by the synthesis of normal amounts of viral-specific RNA and protein. However, no viral capsid proteins are made. An unstable defective capsid precursor protein was identified and shown to be coded for at the 5′ end of the RNA molecule. We also detected a new protein in defective passage infections. This protein was stable in a pulse-chase experiment under conditions in which the previously identified precursor, B1, was chased. We do not know, as yet, to what extent this protein is related to the normal virion proteins.

In Sindbis virus, the capsid protein is located at the amino terminus of the large polypeptide precursor (16) and, therefore, the gene for this protein may be at the 5′ end of the RNA molecule. Thus, the lesion in Sindbis virus defective RNA appears to be located in the same region as that found for the defective RNA of poliovirus.

The DI particles described by us and those studied by Shenk and Stollar (18, 19) differ in several properties, and the two isolates probably contain different genetic lesions. This would not be surprising, since different defective particles have been obtained by independent passaging in other viral systems (10).

The nature of the viral RNA of the DI particles is not clear. Although the small fragments of RNA are present in large amounts only in preparations of virus showing interference potential, until purification of these particles is achieved their relationship to interference cannot be evaluated. Several explanations for the heterogeneity can be considered: (i) the defective particle population is heterogeneous and each RNA fragment is associated with a unique particle; (ii) the defective population is homogeneous and the smaller RNA fragments are distinct species all packaged into one virion or are weakly complexed to form a single RNA moiety which is cleaved during purification; (iii) the small fragments are unrelated to the defective particles and are nonspecifically associated with virion proteins as a side product of the interference state.

Although most of the defective particles examined thus far contain a single RNA strand, usually smaller than normal, Perrault and Holland (10) recently reported the isolation of heterogeneous RNA from a defective particle population of vesicular stomatitis virus.

The number of single-stranded RNA species produced in togavirus-infected cells is large (7), and more than one of these species is altered after infection with DI particles. It is probable that the 20*S* single-stranded RNA moiety which has a molecular weight about one half that of 26*S* RNA and is synthesized in the presence of DI particles, serves as messenger RNA and contains the critical deletion preventing virion assembly. However, how this RNA relates to the heterogeneous RNA found in late-passage viruses is not known.

TABLE 7. *Incorporation of* ^{35}*S-methionine into BHK-21 cells infected with early- or late-passage virus*

Protein from passage	Counts per min added to gel ($\times 10^5$)	Counts per min recovered ($\times 10^4$) in		Counts per min (%) in	
		Capsid	PE2 + E1	Capsid	PE2 + E1
6	2.46	6.7	1.9	27	7.7
12	1.39	1.8	1.1	13	7.9

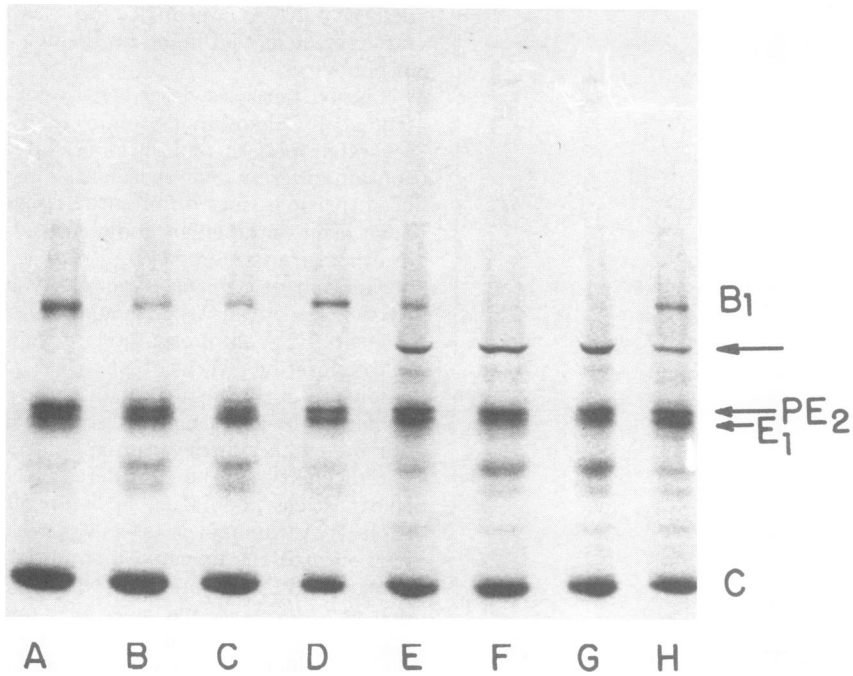

FIG. 8. *Autoradiograms of ^{35}S-methionine-labeled intracellular viral proteins of early- and late-passage virus. Two experiments are included in this gel. The samples labeled D and H refer to a 40-min pulse-labeling of cells infected with passages 5 and 11, and are identical to those analyzed in Fig. 7. Samples labeled A–C and E–G refer to a 30-min pulse, followed by a 30- and 60-min chase with 10^{-4} M methionine, in cells infected with passages 5 and 11, respectively. The arrow indicates the position of the new protein which appears in late-passage infections.*

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