JOURNAL OF VIROLOGY, July 1968, p. 728-737

Vol. 2, No. 7
Printed in E.S.A.

Maturation Defects in Temperature-sensitive Mutants of Sindbis Virus[1]

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Received for publication 3 April 1968

Temperature-sensitive mutants of Sindbis virus, which synthesize viral ribonucleic acid (RNA) but not mature virus at the nonpermissible temperature, were selected for the study of viral maturation. Of these, three mutants which complement each other genetically were used. Two major proteins, the nucleocapsid and membrane proteins, located, respectively, in the viral nucleoid and membrane, were found in intact virions. In cells infected with wild-type Sindbis virus, four distinct types of viral RNA with sedimentation coefficients of 40*S*, 26*S*, 20*S*, and 15*S* were detected in constant distribution. The 20*S* RNA was ribonuclease-resistant, whereas the other types were ribonuclease-sensitive. The 40*S* RNA, identical to that obtained from the virion, was found associated with nucleocapsid protein as a subviral particle, which was assumed to be the nucleoid. Viral materials from cells infected with the mutants under nonpermissive conditions were compared with those from cells infected with wild-type virus, in terms of (i) the distribution of the different types of RNA, (ii) the association of infectious viral RNA into subviral particles, and (iii) the ability of infected cells to hemadsorb goose erythrocytes. According to these criteria, each of the three mutants demonstrated different maturation defects. Defective nucleocapsid proteins and membrane proteins may each account for one of the above mutants. The third mutant may have defects in a minor structural protein or possibly a maturation protein which is involved in the assembly of Sindbis virus.

Sindbis virus belongs to the group A arboviruses (23). It is a small, lipid-containing ribonucleic acid (RNA) virus (15) with a central nucleoid (ribonucleoprotein core) enclosed by a lipoprotein membrane (13).

Temperature-sensitive (*ts*) mutants of Sindbis virus (2) were divided into RNA^- and RNA^+ categories. At the nonpermissive temperature, RNA^- mutants make neither RNA nor virions, whereas RNA^+ mutants make viral RNA but not virions. Burge and Pfefferkorn (3) separated the RNA^+ mutants into three nonoverlapping complementation groups. It is assumed that each contains a single defect in a different cistron. The viral protein determined by that cistron is defective and is rendered nonfunctional at the nonpermissive temperature. One RNA^+ mutant from each complementation group was chosen for the present study. Since these mutants make viral RNA but not virions, they offer a unique opportunity to study the processes of viral maturation.

[1] Contribution no. 1426, Central Research Department, Experimental Station, E. I. du Pont de Nemours and Co.

MATERIALS AND METHODS

Cells. Primary cultures of chick embryo (CE) cells were prepared according to the method of Dulbecco and Vogt (6) and were grown in Eagle's medium with 3% calf serum. Each 100-mm petri dish contained approximately 6×10^6 cells.

Virus. The strain of Sindbis virus which we used for most of our earlier work was obtained from B. Sagik, Austin, Tex. The virus used was the large plaque variety. The stock was purified by two successive single-plaque selections. Temperature-sensitive mutants and a heat-resistant, wild-type (HR) virus were kindly given to us by E. R. Pfefferkorn, Hanover, N. H. Stock virus preparations were produced in CE cells. The two wild-type strains of virus possessed similar one-cycle growth characteristics.

Infection of cells and labeling. Monolayer cultures of CE cells were washed twice with phosphate-buffered saline (PBS) and then were infected by the addition of 1 ml of PBS solution containing 5 μg of actinomycin D per ml and sufficient virus to provide an input of 20 plaque-forming units (PFU) per cell. The virus was allowed to adsorb for 45-min at 37 C. Medium

used for virus growth contained 3% rabbit serum instead of calf serum. Radioactive compounds were usually added to the culture at 3 to 4 hr after infection, when the rate of viral production was maximal. For short pulse-labeling of viral RNA, cells were scraped from the plates, resuspended in a small volume of medium, and kept suspended by stirring at 37 C until the radioactive material was added. Radioactive labeling was terminated by the immediate addition of reagents for RNA extraction, as described below.

Purification of labeled virus. The supernatant medium containing the mature labeled virus released from infected cells was collected. The virus was precipitated by the slow addition of $(NH_4)_2SO_4$ to 50% saturation at 4 C and *p*H 7.4 (Sreevalsan, *personal communication*). The precipitate was redissolved in 1% of the original volume of 0.05 M borate buffer (*p*H 9.0) containing 0.12 M NaCl (borate saline). $(NH_4)_2SO_4$ was then removed by dialysis, and the virus was centrifuged on a 5 to 35% sucrose gradient in borate saline. The fraction which contained the greatest titer of infectious virus and the most radioactivity was collected for use as partially purified virus.

Preparation of cytoplasmic extract. CE cells were washed three times with PBS, scraped from the petri dishes, and centrifuged at 800 × *g* in a Sorvall preparative centrifuge for 3 min. Cells were resuspended and allowed to swell for 8 min in the following solution (solution D): 0.01 M tris(hydroxymethyl)aminomethane (Tris) (*p*H 7.4), 0.01 M KCl, and 10^{-4} M $MgCl_2$. Cells were then ruptured with four strokes of a Dounce homogenizer. Sucrose was added to a concentration of 5%, and four additional strokes were applied. Cell debris was separated by centrifugation at 800 × *g* for 3 min. The pellets were washed twice with small volumes of solution D. The supernatant fluid plus added washings were cleared by centrifugation at 4,000 × *g* for 10 min and then were centrifuged on a sucrose gradient. The recovery of radioactivity was maximal (90%) when the Mg ion concentration in the homogenization solution and in the sucrose gradient was 10^{-4} M. An increase in Mg concentration or the presence of ethylenediaminetetraacetic acid (EDTA) decreased the recovery. In earlier experiments, RSB solution (0.01 M KCl, 0.01 M Tris, 1.5 × 10^{-3} M $MgCl_2$, *p*H 7.4) was used to prepare cytoplasmic extracts. Although less radioactivity was recovered, the patterns in both solutions were identical.

RNA extraction. Two methods were used at different times; each gave the same results. One method was the extraction of the whole cells by hot phenol and 0.5% sodium dodecyl sulfate (SDS; 18). The RNA was analyzed at 4 C on a 5 to 20% sucrose gradient in Tris buffer containing 0.1 M NaCl, 0.01 M Tris, 0.04% EDTA (*p*H 6.7). The other method used was the incubation of the cytoplasmic extract with 1% SDS at 37 C (14). The RNA was then analyzed on a 15 to 30% sucrose gradient containing 0.01 M Tris, 0.1 M NaCl, 5 × 10^{-3} M EDTA, 0.5% SDS, *p*H 7.4 (SDS buffer), at 25 C.

Sucrose density gradients. The sucrose density gradients were prepared by a Buchler gradient-forming chamber, and fractions were collected from the bottom of the gradient. Concentration of sucrose solutions was expressed as per cent (w/v). Sedimentation values were estimated by the method of Martin and Ames (11). Trichloroacetic acid-insoluble material was collected on filters (Millipore Corp., Bedford, Mass.), and the radioactivity was counted in a liquid scintillation counter.

Assay of infectious RNA. Infectious RNA was assayed by the plaque technique on CE cells which had been washed with hypertonic saline (16).

Viral plaque assay. The viral plaque assay was performed on CE cells under agar overlay according to the method of Dulbecco and Vogt (6).

Acrylamide gel electrophoresis. The protein components of the virion and subviral particles were analyzed by electrophoresis, according to the procedure of Summers et al. (22).

Hemagglutination of viruses. Hemagglutination of viruses was performed according to the method of Clarke and Casals (5). A 0.2% suspension of goose erythrocytes was mixed with the virus diluents to give a final *p*H of 5.8. After a 1-hr incubation period at 37 C, the hemagglutination titer was determined as the highest dilution of virus in which a positive pattern was present.

Hemadsorption. The capacity of the infected monolayer cultures to adsorb goose erythrocytes was determined as described by Burge and Pfefferkorn (4). A minor modification was made in determining the concentration of erythrocytes. The monolayers, together with the adsorbed red cells, were scraped into phosphate-borate buffer, *p*H 5.8. A 10% solution of Saponin in 0.9% saline was then added in 1% of the volume of cell suspension to lyse the erythrocytes. After removal of cell debris, the hemoglobin content of the supernatant fluid was read at 410 mμ in a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.).

Reagents. Actinomycin D was kindly supplied by Merck Sharp and Dohme, Rahway, N. J. ^{3}H-uridine (16.3 c/mmole) was purchased from the Nuclear-Chicago Corp., Des Plaines, Ill. ^{14}C-amino acid mixture (10 mc/mmole) and ^{14}C-choline methyl chloride (52 mc/mmole) were purchased from the New England Nuclear Corp., Boston, Mass.

RESULTS

Viral specific RNA in cells infected by wild-type Sindbis. A one-step growth curve of wild-type Sindbis virus at 37 C is shown in Fig. 1. Log-phase growth started about 2 hr after infection, and the rate of viral production was maximal at 3 to 4 hr after infection. The RNA obtained after a 15-min pulse with ^{3}H-uridine at the time of maximal viral growth consisted of several types which sedimented at different rates on a sucrose gradient (Fig. 2). By comparison with CE ribosomal RNA as markers, sedimentation coefficients of the several types of viral RNA were calculated to be 40*S*, 26*S*, 20*S*, and 15*S*. The occurrence of several forms of viral RNA in other group A arbovirus-infected cells has been reported (7, 19, 21). The 40*S* RNA sedimented at the same rate as the RNA

extracted from purified virus. Assay of infectious viral RNA indicated that the 40S RNA contained over 95% of the infectivity of the total viral RNA extracted. The 20S RNA was probably a double-stranded structure since, under specific conditions, it was resistant to ribonuclease digestion (10). The nature of the 15S RNA is unknown. The 26S RNA had identical base composition to that of the 40S RNA (T. Sreevalsan et al., *in press*); however, definite knowledge of its role in the infected cell is still lacking.

Protein components in the wild-type virion. HR virus, labeled with ^{14}C-amino acids, was purified on a sucrose gradient as described in Materials and Methods. The radioactive peak fraction was treated with 1% SDS, 0.5 M urea, and a 10% volume of acetic acid to disrupt the virus. The mixture was then dialyzed against buffer containing 0.1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol, and was subjected to acrylamide gel electrophoresis (22). The purified virus contained two proteins with different electrophoretic mobilities (Fig. 3). The radioactivity of the slow-moving component was about 2.4 times that of the fast-moving component. Strauss et al. (20) have recently reported the same findings. However, the possibility that the virion contains another minor protein too low in concentration to be detected by gel electrophoresis has not been ruled out.

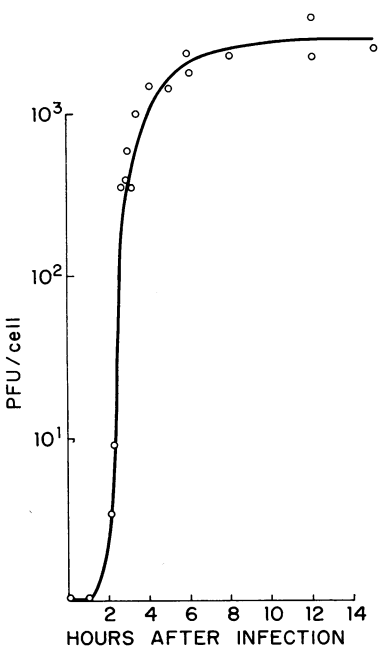

FIG. 1. *Growth curve of wild-type Sindbis at 37 C. The production of virus was calculated as the number of plaque-forming units per CE cell.*

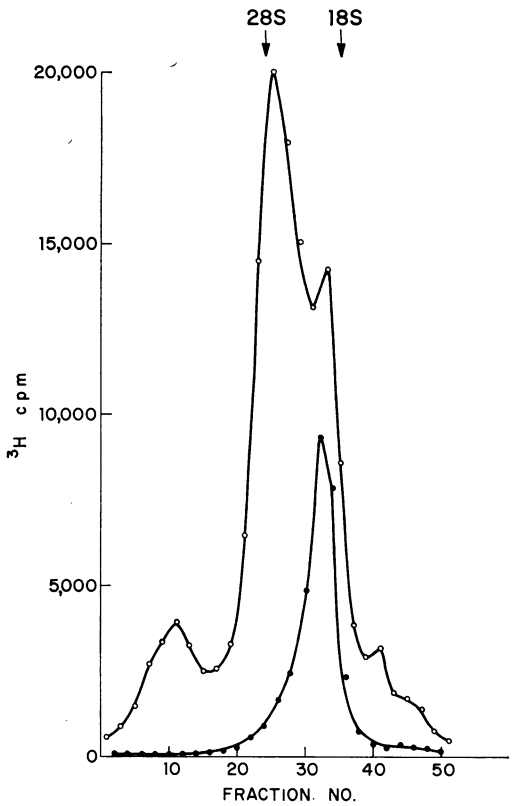

FIG. 2. *Separation of four types of Sindbis-specific RNA on sucrose density gradient. Ten plates of CE cells were pulsed with 1 mc of ^3H-uridine for 15 min, 3.5 hr after infection at 37 C. RNA was extracted by hot phenol and SDS method and was analyzed on a 5 to 20% sucrose gradient containing 0.1 M NaCl, 0.01 M Tris, and 0.04% EDTA (pH 6.7). Gradients were centrifuged for 15 hr at 50,400 × g in a SW25.3 rotor at 4 C. The contents of even-number tubes were first digested by pancreatic ribonuclease at a concentration of 1 μg/ml in the presence of 0.15 M NaCl at 37 C for 10 min. Symbols: ○, trichloroacetic acid-insoluble counts; ●, ribonuclease-resistant trichloroacetic acid-insoluble counts.*

Subviral particle in cytoplasmic extract. Studies of the development of other group A arboviruses indicated the presence of particles, presumably precursors to mature virions and thought to be the viral nucleoid (1, 8, 12). It was reasonable to assume that similar particles would be found in cells infected with Sindbis virus. Cytoplasmic extracts of the infected cells were, therefore, scrutinized for a fast-sedimenting particle which contained infectious RNA. Cytoplasmic extracts were made from cells which had been labeled for

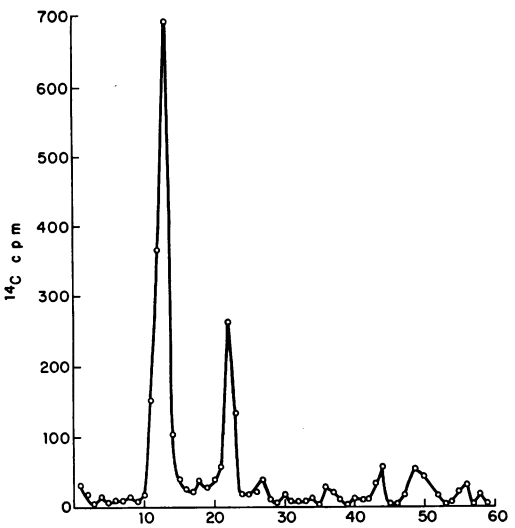

FIG. 3. *Gel electrophoresis patterns of proteins derived from Sindbis virus. Two plates of monolayer cultures were infected with HR and actinomycin D at 37 C. Medium contained no serum and only 25% of the amount of amino acids. Five hr later, 35 μc of ^{14}C-amino acids was added per plate. The medium containing virus was harvested 20 hr after infection and was purified on a sucrose gradient. Virus in the radioactive peak fraction was disrupted and subjected to acrylamide gel electrophoresis (17 hr, 3 ma per 8-cm tube). Migration is from left to right.*

1 to 1.5 hr during the period when viral growth was most rapid. These extracts were then analyzed on sucrose gradients. As indicated in Fig. 4, two broad peaks of radioactivity with sedimentation coefficients of approximately 140*S* and 65*S* were observed in infected cells and were absent from the uninfected control. To discount the possibility that the fast-sedimenting complex consisted of dimers of ribosomes with short pieces of viral RNA attached, the experiment was repeated under conditions in which dimers of ribosomes would not exist. As shown in Fig. 5, after incubation with ribonuclease or in the presence of EDTA, the 140*S* particle persisted and was therefore considered to be a viral-specific structure. The 65*S* peak was labile under these conditions. In one preliminary experiment, viral RNA extracted by phenol was mixed with uninfected CE cell cytoplasmic extract and was analyzed on a sucrose gradient; no 65*S* peak was observed. Therefore, although the nature of the 65*S* particle is not clear at present, it is not likely to be an artifact, as described by Girard and Baltimore (9).

The addition to infected cultures of ^{14}C-amino acids or ^{14}C-choline as short pulses demonstrated (Fig. 6) that both the 140*S* particle and the 65*S* particle contained RNA and protein but no significant amount of lipid.

RNA was extracted from those fractions containing the greatest amount of 140*S* and 65*S* particles. As shown in Fig. 7, the RNA from 140*S* particles sedimented mainly as 40*S* RNA, some as 26*S* RNA. RNA from 65*S* particles was mostly 26*S* RNA.

The patterns of gel electrophoresis of ^{14}C-amino acid-labeled proteins which were derived from the 140*S* and 65*S* particles were compared to those of the proteins derived from the virions. Purified virions contained two detectable protein peaks with different electrophoretic mobilities (Fig. 3). The 140*S* particles contained exclusively the fast-moving protein. The 65*S* particles contained mostly the fast-moving protein, but did contain some other protein which was found in the virions (Fig. 8). This could be contamination by ribosomal protein, since ribosomes sedimented at about the same rate as the 65*S* particles.

The nature of the 65*S* particles is uncertain at present. The 140*S* particles appear to be precursor particles to the virions (viral nucleoid). Synthesized only in the Sindbis virus-infected cells, they contained infectious viral RNA (40*S*) and one of the viral proteins. If we assume that the two proteins in the virions are membrane protein in the

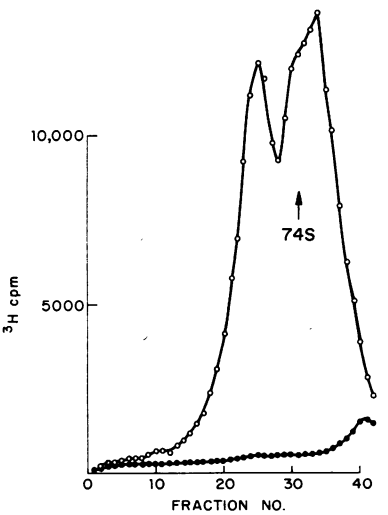

FIG. 4. *Sedimentation pattern of cytoplasmic extracts from Sindbis-infected cells. Two plates of cells were pulsed with 100 μc of ^{3}H-uridine for 1 hr, 4 hr after infection at 37 C. Cytoplasmic extracts were analyzed on 5 to 30% sucrose gradients in RSB buffer in a SW25.1 rotor at 14,700 × g for 11 hr. Symbols: ○, infected cells; ●, control, uninfected cells treated with actinomycin D and pulsed in same manner.*

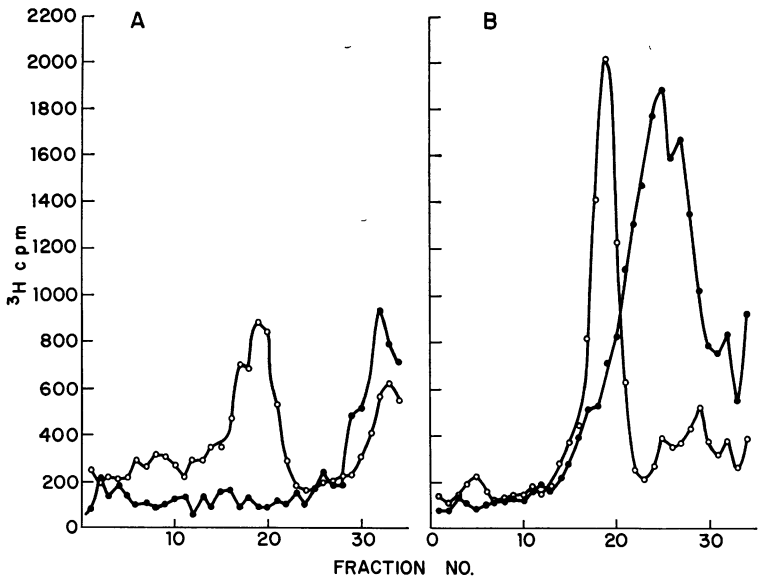

FIG. 5. *Sedimentation patterns of cytoplasmic extract treated with ribonuclease and EDTA. The extract was prepared as in Fig. 4, in solutions containing (A) 0.01 M Tris, 0.01 M EDTA (pH 7.4); (B) 0.01 M Tris, 0.001 M Mg, 1 μg of ribonuclease per ml; and was then analyzed on a 5 to 30% sucrose gradient in corresponding buffers. Centrifugation was done in a SW25.3 rotor at 12,600 × g for 16 hr. Symbols: ○, infected cells; ●, control, uninfected cells not treated with actinomycin D and pulsed in same manner.*

lipid membrane and nucleocapsid protein in the nucleoid, then the fast-moving protein is the nucleocapsid protein since it was the only protein detected in the precursor of the virions. The location of the two viral structural proteins in the virion has also been confirmed by Strauss et al. (20). Freidman and Berezesky (8) and Sreevalsan (*personal communication*) reported similar subviral particles in cytoplasmic fractions of cells infected by two other arboviruses, Semliki Forest virus and Western equine encephalomyelitis virus.

Characterization of ts *RNA*$^+$ *mutants.* The stocks of RNA$^+$ *ts* mutants and HR were grown at the permissive temperature, 27 C. A yield of approximately 2,000 to 3,000 PFU/cell was produced by all mutants and HR after 48 hr at 27 C, and by HR after 12 hr at the nonpermissive temperature, 40 C. The growth curve of HR at 40 C was similar to that of the wild type at 37 C (Fig. 1), except that the eclipse phase was shortened to 1 hr. The mutants were characterized as to their reversion frequency and degree of leakiness. Plaque assays were performed simultaneously at 40 and 27 C, and the reversion frequency was the ratio of PFU determined at 40 C to that determined at 27 C. The degree of leakiness was calculated by determining the PFU at 27 C produced by growth at 40 C (2). These characteristics, as tabulated in Table 1, showed that, at the nonpermissive temperature, virus yields from the mutants were less than 0.1% of the yield obtained at the permissive temperature, or less than 1 PFU/cell; therefore, in our subsequent analyses, the possible error from the growth of back mutants to wild type or leakiness of the mutants at 40 C was negligible. These characteristics were stable upon storage at −20 C for several months.

RNA and 140S particles in cells infected by RNA$^+$ ts *mutants.* At 40 C, the RNA$^+$ mutants produced viral RNA but no infectious virus. To determine the form in which RNA accumulates and whether it is free or associated with the structures previously described, it was first necessary to determine if any newly synthesized viral RNA or 140*S* particles were released from the cells in the absence of virus formation. Medium highly labeled with ^{3}H-uridine was added to cells infected with the mutant viruses at 3.5 hr after infection and was allowed to remain for an additional 3 hr. The medium was then collected and analyzed for 40*S* RNA and 140*S* particles. Neither component was found in the medium. Thus, in cells infected with the mutant viruses and incubated at the nonpermissive temperature, most of the viral RNA made remained inside the cell. Cells infected with the HR strain of virus constantly released viral RNA into the medium at both temperatures. This reflects the

production and release of mature virions. The viral RNA extracted from the cells during maximal rates of viral production and release nevertheless consistently exhibited 40S and 26S RNA in a ratio of approximately 1:3. This ratio was estimated by determining the amount of radioactivity contained in each RNA peak.

The 40S RNA and 140S particles which accumulated in the cells were determined for each of the mutant strains of virus under nonpermissive conditions. Cells were infected with equal input of virus (20 PFU/cell) by use of each of the three mutant strains and the HR strain of virus. Cytoplasmic extracts of the infected cells were prepared according to the method described, and each was divided into two equal volumes. One portion was analyzed for its content of 140S particles, whereas the other portion was analyzed for RNA. The results of these experiments are shown in Fig. 9. The total radioactivity recovered from the RNA gradients was about 90% of that recovered from the cytoplasmic extract gradient.

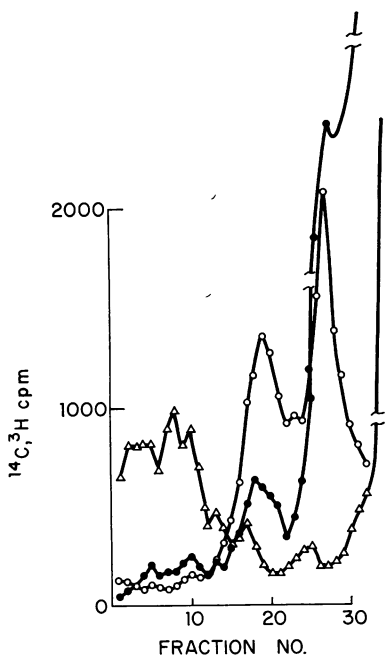

FIG. 6. *Sedimentation patterns of cytoplasmic extract labeled with ^{3}H-uridine, ^{14}C-amino acid, and ^{14}C-choline. Four plates of infected cells were pulsed with 50 μc of ^{3}H-uridine (○) and 50 μc of ^{14}C-amino acids (●) simultaneously for 1 hr, 4 hr after infection at 37 C. Cytoplasmic extract was centrifuged as in Fig. 5. Another four plates of cells were pretreated with 10 μc of ^{14}C-choline methyl chloride (△) for 12 hr before infection. Cytoplasmic extract was made at 4 hr after infection and was analyzed on a separate gradient.*

TABLE 1. *Reversion frequency and degree of leakiness of mutants*

Mutants	Titer of stock at 27 C	Reversion frequency	Degree of leakiness
	PFU/ml		*PFU/ml*
ts-2.....	7.3×10^{8}	$<4 \times 10^{-5}$	$<5 \times 10^{4}$
ts-20.....	8.3×10^{8}	$<1 \times 10^{-5}$	$<4 \times 10^{4}$
ts-23.....	2.3×10^{8}	$<10^{-7}$	$<2 \times 10^{5}$
HR.....	7.0×10^{8}	1	7.0×10^{8}

The amount of 40S and 26S RNA was estimated by the radioactivity under respective peaks on the surcose gradient. It is evident that the distribution of types of viral RNA extracted from the cells infected with the three different mutant strains of virus differed from each other and from the wild type at 40 C. For instance, as shown by the relative amount of 40S to 26S RNA (first column, Table 2), *ts*-2-infected cells produced only a small amount of 40S RNA and a very large amount of 26S RNA. The ratio of 40S to 26S RNA was 1:10; *ts*-20-infected cells produced an equal amount of 26S and 40S RNA; and *ts*-23-infected cells produced 40S and 26S RNA in a ratio of approximately 1:2.

40S RNA was found associated with 140S particles in cytoplasmic extracts from cells infected with the wild-type strain of virus. We endeavored to compare the amounts of 40S RNA and 140S particles in the cytoplasmic extracts of cells infected with each of the three *ts* mutants and HR under nonpermissive conditions. The amounts of 140S particles were roughly estimated by the radioactivity under their peaks relative to the total radioactivity on the gradients. The amounts of 40S RNA were estimated similarly. These percentages are tabulated in Table 2. A positive correlation between these quantities existed.

Hemadsorption of infected cells. An equal number of PFU from each of the viruses was added to sets of five monolayer cultures. These cultures, plus an uninfected control, were then incubated either for 6 hr at 40 C or for 30 to 40 hr at 27 C. Hemadsorption capacity was then determined for each set of virus-infected cultures as previously described, and was expressed as a percentage of that of the HR-infected cells in Table 3.

To rule out the possibility that the difference in hemadsorption resulted from unequal numbers of remaining cells on each petri dish after growth for 40 hr at 27 C, the numbers of remaining cells were determined in all cases. They were approximately equal for mutant and HR-infected cultures.

Hemagglutination of viruses grown at 27 C. Medium of infected cells which were grown at

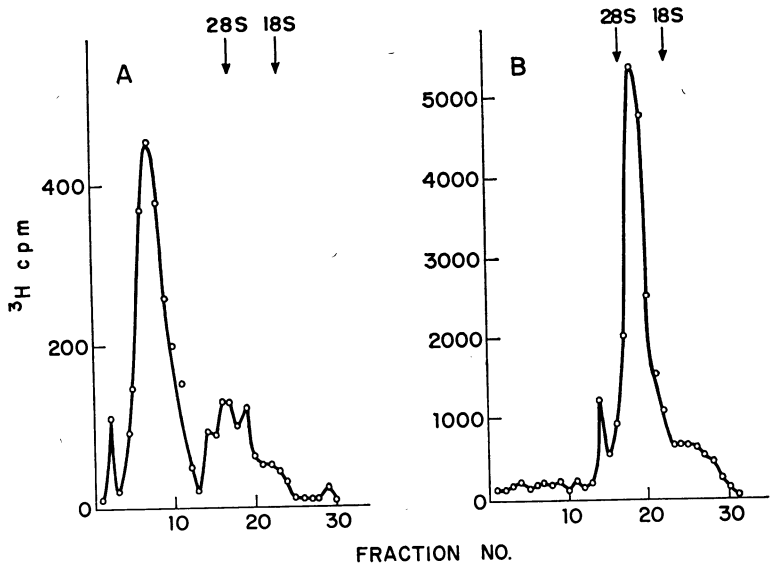

FIG. 7. *RNA extracted from subviral particles. Peak sucrose fractions of (A) 140S particles and (B) 65S particles were made 1% with SDS, incubated for 10 min at 37 C, then diluted to less than 15% sucrose with SDS buffer, and analyzed on 15 to 30% sucrose gradient containing SDS buffer. Centrifugation was performed at 25C in a SW25.3 rotor at 61,000 × g for 16 hr. Arrows are 28 and 18S ribosomal RNA markers.*

TABLE 2. *Summary of production of viral RNA and 140S particles of infected cells at nonpermissive temperature*

Mutants	40S RNA/ 26S RNA[a]	140S particle/total cytoplasmic extract[b]	40S RNA/ total RNA[a]
		%	%
ts-2.....	0.1	5	9
ts-20.....	1.1	36	45
ts-23.....	0.65	31	37
HR.....	0.35	26	24

[a] Averages of three experiments.
[b] Averages of two experiments.

TABLE 3. *Summary of hemadsorption capacity of infected cells*

Mutants	40 C[a]		27 C[b]	
	Optical density (410 mμ)	Hemadsorption	Optical density (410 mμ)	Hemadsorption
		%		%
ts-2.....	0.10	12	0.73	61
ts-20.....	0.09	11	0.53	44
ts-23.....	0.08	10	0.10	10
HR.....	0.80	100	1.20	100
Uninfected control...	0.08	10	0.11	10

[a] Averages of two experiments.
[b] Averages of three experiments.

TABLE 4. *Hemagglutination (HA) of viruses grown at 27 C*

Mutants	HA units	PFU	PFU/HA units
ts-2.....	32	1.0×10^8	3×10^6
ts-20.....	32	1.2×10^8	3×10^6
ts-23.....	<2	1.0×10^8	—
HR.....	32	1.1×10^8	3×10^6

27 C for 40 hr was collected and filtered through a 0.22-μ pore size filter to eliminate cell debris. Infectivity and hemagglutination of each mutant and HR virus were then determined as described in Materials and Methods. The results are shown in Table 4.

DISCUSSION

Burge and Pfefferkorn (3) demonstrated that the RNA$^+$ mutants can be classified into three nonoverlapping complementation groups. If each group corresponds to one cistron, then three virus-specific proteins are involved in the maturation process. A logical assumption is that two of these are the major structural proteins of the virus, one located in the lipoprotein membrane, the other located in the nucleoid. The third defective protein may be a minor structural protein, as yet undetected by acrylamide gel electrophoresis, or a maturation factor responsible for the

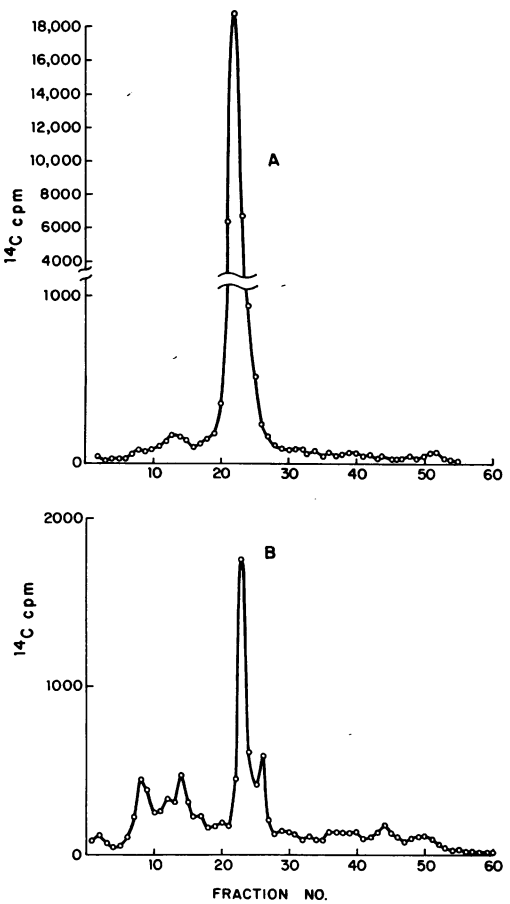

FIG. 8. *Gel electrophoresis patterns of proteins derived from subviral particles. Eight plates of cells were infected with HR virus and actinomycin D. Growth and labeling were identical to Fig. 3, except that cells were harvested 15 hr after infection at 37 C. Cytoplasmic extract was prepared and sedimented on a sucrose gradient as in Fig. 6. Peak fractions of (A) 140S and (B) 65S were further analyzed by gel electrophoresis (17 hr, 3 ma per 8 cm of gel), as in Fig. 3. Difference in total counts between (A) and (B) was due to difference in counts contained in the original samples.*

assembly process (17). The differences found among the RNA$^+$ mutants from different complementation groups support the above assumption.

The three RNA$^+$ mutants and the wild-type strain of Sindbis virus differed in (i) the amounts of the different types of RNA produced at 40 C, (ii) their ability to accumulate 140*S* particles at 40 C, and (iii) their hemadsorption capacity at 27 C. The maturation defect of each of these mutants is discussed in terms of viral proteins.

Few 140*S* particles, if any, were formed in *ts*-2-infected cells. Some 40*S* RNA was formed. Viral maturation appeared to be blocked at the stage of nucleoid formation and probably was a result of defective nucleocapsid protein. Thus, the protein failed to complex with the 40*S* RNA to form 140*S* particles.

Infection of cells with the mutants *ts*-20 and *ts*-23 resulted in similar accumulations of 140*S* particles. Viral maturation appeared to be blocked at the step after nucleoid formation and before the assembly of the nucleoid with the lipoprotein membrane. Since these two mutants belong to different nonoverlapping complementation groups, it is likely that two proteins are involved in this step. We reasoned that one of the two mutants might demonstrate nonfunctional membrane proteins. Preliminary data indicated that viral membrane proteins from cells infected with each of the three mutants and HR migrated identically when they were subjected to acrylamide gel electrophoresis.

Since hemagglutinin is believed to be located in the membrane of the Sindbis virion, it presumably is the membrane protein (or part of it). Therefore, viral membrane protein might be altered in one of the mutants so that it cannot hemagglutinate goose erythrocytes. The hemadsorption capacities of cells infected with each of the three mutants were compared with that of cells infected with the HR strain. At the nonpermissive temperature, cells infected with any of the mutants acquired very little hemadsorption capacity as compared to HR-infected cells.

However, cells infected with the mutant viruses and grown at the permissive temperature showed a substantial difference in their hemadsorption capacities. Cells infected with mutant strains *ts*-2 and *ts*-20 had hemadsorption capacities of 61 and 44%, respectively, of that of the HR-infected cells. Cells infected with mutant strain *ts*-23 failed to hemadsorb. At the permissive temperature, significant yields of infectious *ts*-23 viruses were produced and these also failed to hemagglutinate goose erythrocytes. The cells and the virions must, therefore, contain a defective hemagglutinin. If the hemagglutinating activity is a function of the membrane protein, this function cannot play a significant role in the maturation of the virion at the permissive temperature. However, at the nonpermissive temperature, a defective membrane protein like that of *ts*-23 virus may fail to function in the maturation of the virion.

From the data concerning nucleoid accumulation and hemadsorption capacity, we hypothesize that (i) mutant *ts*-2 produced nonfunctional nucleocapsid protein, (ii) mutant *ts*-23 produced nonfunctional membrane protein, and (iii) mu-

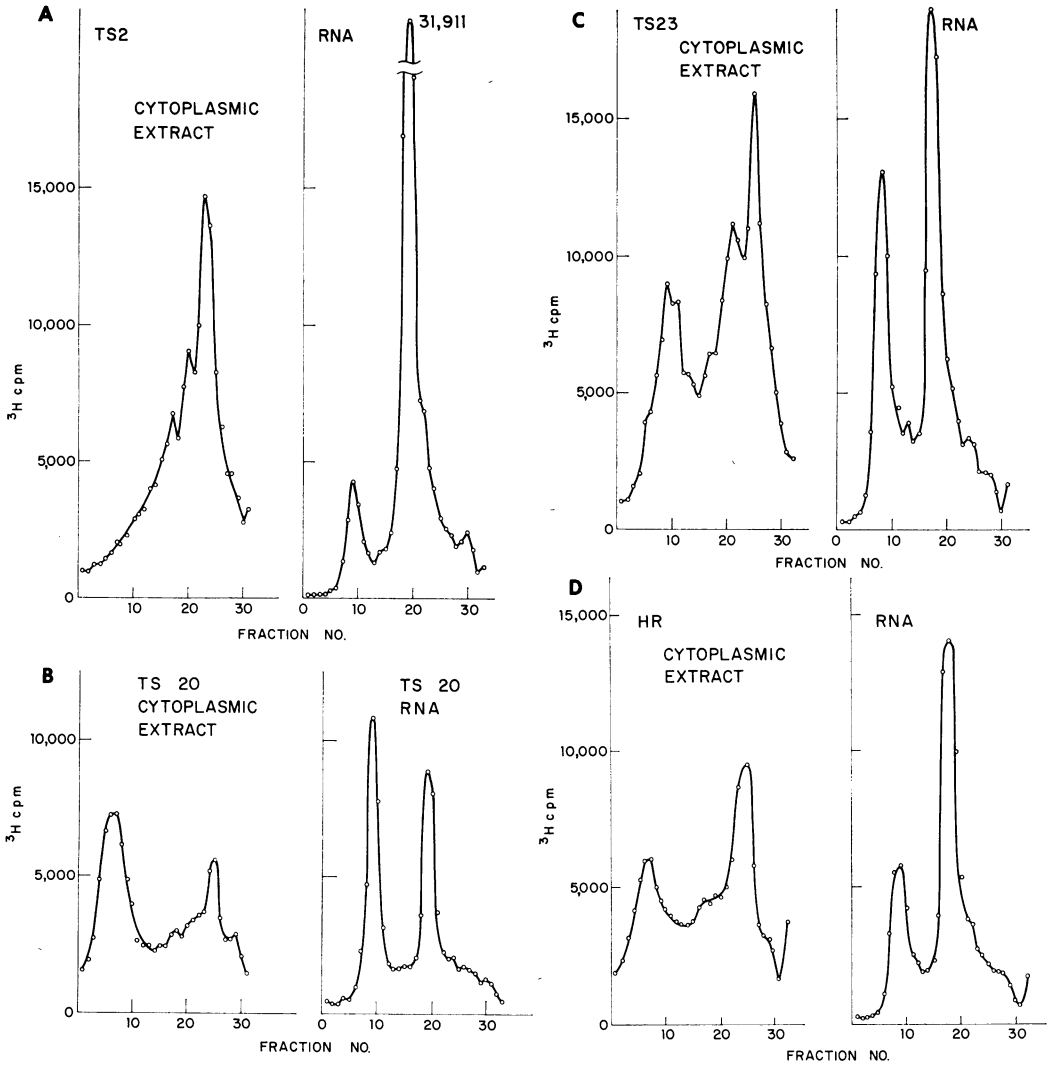

FIG. 9. *Sedimentation patterns of cytoplasmic extract and RNA of cells infected by (A) ts-2, (B) ts-20, (C) ts-23, and (D) HR at 40 C. Two plates of cells were infected with a multiplicity of 20 PFU/cell of each strain of virus. A 200-μc amount of ^{3}H-uridine per plate was added 3 hr after infection and was allowed to remain for an additional 2 hr. Cytoplasmic extract and RNA were prepared as described in the text. Centrifugation of cytoplasmic extract: 15 to 30% sucrose gradient in solution D; SW25.3 rotor, 50,400 × g/f for 15 hr. Centrifugation of RNA, same as in Fig. 7.*

tant *ts*-20 produced functional nucleocapsid as well as membrane protein. It may produce a nonfunctional minor structural protein or a maturation protein.

This explanation is consistent with data obtained in an experiment in which heat lability and conditional lethality of the mutant virions were studied. Burge and Pfefferkorn (3) showed that mutant *ts*-2 and *ts*-23 virions were very heat-labile as measured by the loss of infectivity after heating at 60 C for 5 min. A defective viral structural protein, either nucleocapsid protein or membrane protein, would be expected to result in heat lability of the virion. They further showed that mutant *ts*-20 is particularly resistant to inactivation by heat, a condition expected if both major structural proteins of the virus are unaffected. This finding is in agreement with our hypothesis.

Since completing this study, we have become aware of the similar study by B. W. Burge and

E. R. Pfefferkorn. They were most generous in forwarding their manuscript to us prior to its publication in the *Journal of Molecular Biology*.

ACKNOWLEDGMENTS

We thank T. Sreevalsan for help with tissue culture during the initial phase of this work, and Joan Wilson and Barbara Stone for expert technical assistance.

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