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Infective Virus Substructure from Vesicular Stomatitis Virus

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Treatment of suspensions of vesicular stomatitis virus with Tween-ether results in a rapid and considerable loss of infectivity (ca. 4 logs in 2 min), but the residual infectivity is comparatively stable to further treatment with ether. The infectivity remaining after the short exposure to Tween-ether is not due to virus for the following reasons. (i) It is much less infective for tissue cultures than for mice, whereas the intact virion is equally infective for both hosts. (ii) The residual infectivity is much less stable than virus infectivity in both sucrose and tartrate gradients. (iii) Virus immune serum does not neutralize its activity. (iv) The infectivity is associated with material which sediments further in sucrose gradients and has a greater buoyant density in tartrate gradients than the virion. Experiments with ^{32}P-labeled virion showed that the infective substructure contains ribonucleic acid with the same sedimentation characteristics as that extracted from the virion. Electron microscopy shows that the infective component has the same overall bullet-like structure as the virion but lacks the outer envelope and fringe structure.

The infectivity of suspensions of vesicular stomatitis virus (VSV) is rapidly reduced by a brief treatment with ether. As the inactivation is far from complete, it seemed possible that the residual infectivity could be due to a virus substructure rather than to virus. The experiments described in this paper were designed to explore this possibility.

MATERIALS AND METHODS

Virus growth and titration. Virus of the Indiana type (strain Ind. C) was used. Monolayers of BHK-21 cells (4) were inoculated at a virus-cell ratio of ca. 0.01 to minimize interference effects (Crick, 1967), and then were incubated for 18 hr at 37 C in Eagle's medium. After this period of incubation, the monolayers had been destroyed and the titer of infective virus was maximal (ca. $10^{9.5}$ plaque-forming units per ml). For the preparation of ^{32}P-labeled virus, the infected monolayers were washed three times with Earle's saline, in which the phosphate had been replaced with 0.01 M tris(hydroxymethyl)aminomethane, *p*H 7.6. The phosphate concentration of this medium was less than 4×10^{-5} M. Incubation of the infected monolayers was then continued in the phosphate-low medium containing 1 μg of actinomycin D per ml and $^{32}PO_4$ until the monolayer left the glass. The medium was separated from the cell debris by centrifugation at $500 \times g$ for 5 min and was used for the isolation of the virion (see below).

Infectivity determinations were made either by intracerebral inoculation of 7-day-old mice with serial 10-fold dilutions of the virus or by plaque titration on BHK-21 cell monolayers. The virus titers obtained by the two methods are in good agreement.

Isolation of ^{32}P-labeled virion. Saturated ammonium sulfate solution was added to the virus suspension to give a final 60% saturated solution. The precipitate was separated by centrifugation at $2,500 \times g$ for 15 min, resuspended in one-tenth of the initial volume of the virus suspension, and filtered through Sephadex G-25 charged with 0.04 M phosphate buffer (*p*H 7.6) to remove the small molecules. The virus was then sedimented into 23.5 ml of a 15 to 45% linear gradient of sucrose in 0.04 M phosphate (*p*H 7.6) for 2 hr at 20,000 rev/min in the SW25 rotor of a Spinco ultracentrifuge. Fractions (1 ml) were collected from the bottom of the tube and tested for infectivity and radioactivity. The most infective fractions were combined.

Tween-ether disruption of virus. Virus suspensions were mixed with a stock Tween 80 solution to give a final concentration of 3 mg of Tween per ml. This suspension was then shaken gently at room temperature with an equal volume of ether, the organic layer removed, and the dissolved ether was blown off in a stream of nitrogen. In the experiments to determine the rate of inactivation of virus infectivity, portions of the aqueous layer were immediately diluted for titration without removal of the ether so that the time of contact with the solvent could be more accurately controlled.

Sucrose gradient centrifugation. Two types of gradient were used. For fractionation of the virion and Tween-ether disrupted preparations, the material was centrifuged into 23.5 ml of 15 to 45% gradients in 0.04 M phosphate (*p*H 7.6) for 2 hr at 20,000 rev/min,

by use of an SW25 rotor. RNA was fractionated by centrifugation at 20,000 rev/min for 15 hr through 23.5 ml of 5 to 25% gradients prepared in 0.1 M sodium acetate (*p*H 5.0). In each instance, 1-ml fractions were collected from the bottom of the tubes.

Tartrate gradient centrifugation. The ^{32}P-virion and the Tween-ether disrupted virion were layered onto 4.5 ml of preformed potassium tartrate gradients (d = 1.25 to 1.02) and centrifuged for 2 hr at 35,000 rev/min in the SW39 rotor of a Spinco ultracentrifuge. The contents were then collected as single drops from the bottom of the tubes. Every third drop was used for the measurement of density. The remainder were diluted in buffer solution, and a portion of each individual fraction was counted. Samples of successive pairs of fractions were combined for the determination of infectivity.

Electron microscopy. A drop of the purified virion or Tween-ether treated preparation was mixed with an equal volume of 3% phosphotungstic acid adjusted to *p*H 6.5; a drop of the mixture was placed on a carbon-Formvar grid. Excess fluid was removed by means of a filter paper, and the grid was examined immediately in a Siemens Elmiskop I electron microscope. Fractions from the sucrose gradients were first dialyzed against 0.1 M ammonium acetate to remove sucrose before being mixed with the phosphotungstic acid.

RESULTS

Treatment of virus with Tween-ether. Unfractionated virus grown in BHK cells was mixed with Tween 80 in a glass-stoppered tube to give a final Tween concentration of 3 mg/ml. The mixture was then shaken gently with an equal volume of ether by slow and repeated inversion of the tube. The infectivity in mice of portions of the aqueous layer removed at different times after addition of the ether is shown in Fig. 1. After the very rapid fall in infectivity in the first 1 to 2 min, there was no further decrease during the next 13 to 14 min.

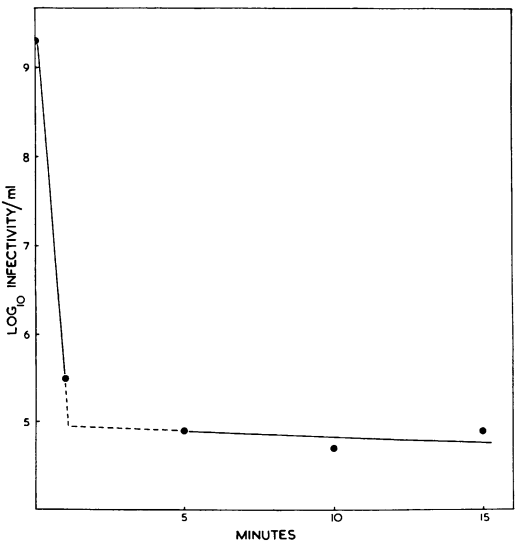

FIG. 1. *Effect of Tween-ether on the infectivity of vesicular stomatitis virus in mice.*

TABLE 1. *Relative infectivity for mice and BHK cell monolayers of three different samples of VSV before and after Tween-ether treatment*

Sample	Titer in mice (log ID_{50}/ml)	Titer in cells (log plaque-forming units/ml)
Virus	9.4	9.6
Tween-ether virus	5.1	3.3
Virus	9.6	9.2
Tween-ether virus	4.8	2.7
Virus	8.6	8.4
Tween-ether virus	4.8	3.3

The infectivity of the aqueous layer 5 min after addition of the ether was measured in mice and in BHK cells. The results in Table 1 show the relatively low infectivity of the Tween-ether virus for BHK cells compared with its infectivity for mice. This low infectivity for cells was observed whether the measurement was made by plaque assay or by observing the cytopathic effect of the preparation.

In these tests, the preparations were allowed to adsorb onto the cell monolayers for 30 min at 25 C before addition of the medium or agar overlay. It seemed possible that, if the ether-resistant infectivity was not due to virus, a slow rate of adsorption of the material to the cells might be the reason for its low infectivity for cells compared with mice. To test this possibility, 0.1-ml samples of dilutions of virus or Tween-ether virus were added to replicate monolayers of BHK cells and allowed to adsorb for different times at 37 C before addition of the agar overlay. The results in Fig. 2 show that the number of plaques obtained with the virus inoculum did not increase after 5-min adsorption, whereas the number obtained with the Tween-ether virus was still increasing at 80 min. Even with this period of adsorption, however, the titer of the Tween-ether virus was still much lower than it was in mice.

Resistance to antiserum of the Tween-ether treated virus. The foregoing results suggested that the residual infectivity of virus suspensions treated with Tween-ether may not be due to intact virus particles. This was confirmed by the observation that hyperimmune guinea pig serum

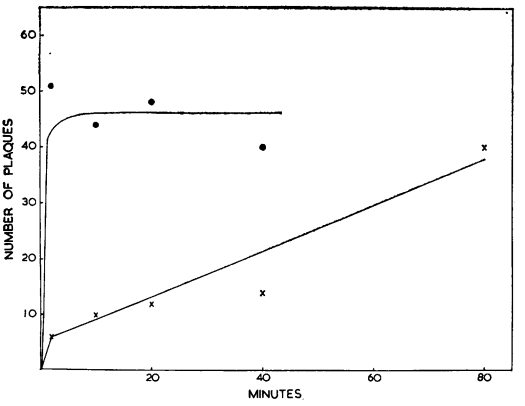

FIG. 2. *Rate of adsorption of VSV and Tween-ether treated VSV to monolayers of BHK cells. Symbols:* ● = *virus;* × = *Tween-ether treated virus.*

TABLE 2. *Effect of virus antiserum on the infectivity of virus and Tween-ether disrupted virus*[a]

Sample	Infectivity titer in BHK cells (plaque-forming units/ml)
Virus	2.6×10^7
Virus + 1:1,000 antiserum	2.1×10^3
Virus + Tween + 1:1,000 antiserum	1.4×10^3
Tween-ether virus	2.1×10^2
Tween-ether virus + 1:1,000 antiserum	3.1×10^2

[a] Dilutions of each preparation were mixed with an equal volume of 1:1,000 antiserum before being added to monolayers of BHK cells.

at a dilution of 1:1,000 did not decrease the plaque count of the Tween-ether virus, whereas the same dilution of antiserum neutralized 4 logs of virus infectivity (Table 2). The possibility that the ether-resistant fraction was due to the presence in the virus stocks of a contaminating virus was eliminated by the demonstration that the product obtained by infecting cells with the ether-resistant material was indistinguishable from normal harvests of VSV.

Stability of Tween-ether disrupted virus. Volumes of 2 ml of (i) virus, (ii) Tween-ether disrupted virus, and (iii) a mixture of virus diluted 1,000-fold and Tween-ether disrupted virus were centrifuged for 2 hr at 20,000 rev/min through 15 to 45% gradients in the SW25 rotor, and the entire contents of the tubes were mixed and titrated in mice. Whereas about 50% of the virus infectivity in the first and third preparations was recovered, only 1% of the infectivity of the Tween-ether preparation was recovered. The recovery of infectivity of the Tween-ether preparation from tartrate gradients was also extremely low (ca. 1%), whereas more than 50% of the virus infectivity could be recovered. These results provide further evidence that the ether-resistant fraction of VSV is not residual virus.

Sedimentation properties of the virion and Tween-ether virion. Virus which had been grown in the presence of ^{32}P and actinomycin D was used for these experiments. The purified virion isolated from a sucrose gradient was used as starting material. Figure 3 shows the distribution of ^{32}P and infectivity when (A) ^{32}P virion alone, (B) Tween-ether treated ^{32}P-virion, and (C) a mixture of Tween-ether treated ^{32}P-virion and unlabeled virion were centrifuged in three separate gradients. The untreated virion gave fairly

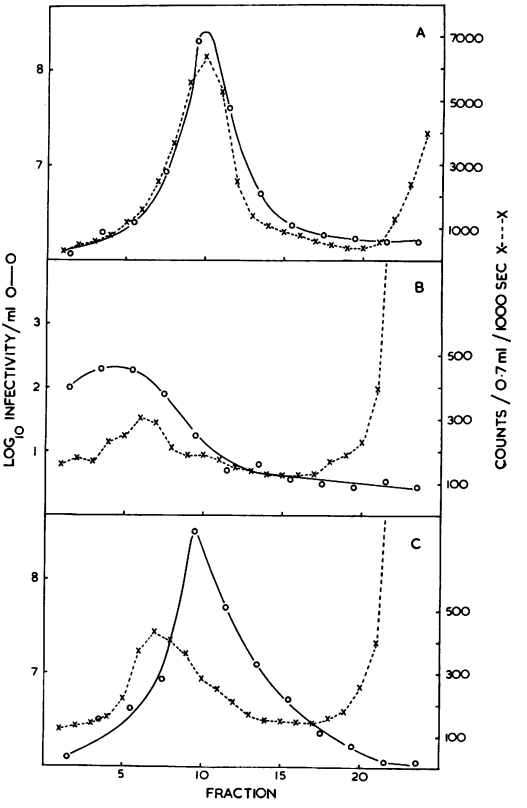

FIG. 3. *Sucrose density gradient centrifugation of ^{32}P-virion and Tween-ether treated virion. (A) ^{32}P-virion alone; (B) Tween-ether treated ^{32}P-virion; (C) mixture of Tween-ether treated ^{32}P-virion and unlabeled virion. Infectivity was determined by titration in mice.*

sharp coinciding peaks of radioactivity and infectivity at fractions 9 and 10 (Fig. 3A). The radioactivity at the top of the tube is probably due to disruption of a portion of the virion during its isolation.

The Tween-ether treated virion gave a completely different distribution of ^{32}P and infectivity (Fig. 3B). Most of the ^{32}P was found at the top of the tube, but about 10 to 15% was located as a fairly broad band with a peak at fraction 6. The infectivity profile had a similar spread, suggesting that the infectivity was due to a heterogeneous mixture of particles.

The profile of infectivity and ^{32}P obtained by centrifuging a mixture of Tween-ether treated ^{32}P virion and untreated, unlabeled virion (Fig. 3C) confirms that the virion sediments to its usual position in the gradient even in the presence of the Tween-ether disrupted virion. The low infectivity of the Tween-ether treated virion does not make a significant contribution to the overall infectivity of the mixture.

The lower position in the gradient of the infective material produced by the Tween-ether treatment compared with that of the untreated virion is probably due to an increase in density caused by the removal of lipid. This was confirmed by sedimenting the untreated virion and Tween-ether treated virion in preformed potassium tartrate gradients. The virion had a buoyant density of 1.14 g/ml (Fig. 4A). However, the distribution in the gradient of the radioactivity and infectivity of the Tween-ether disrupted virion was completely different (Fig. 4B). About 10 to 15% of the radioactivity was present at a density of 1.21 g/ml, with the remainder at the top of the tube. The peak of infectivity of the Tween-ether treated virion coincided with the radioactive peak at 1.21 g/ml.

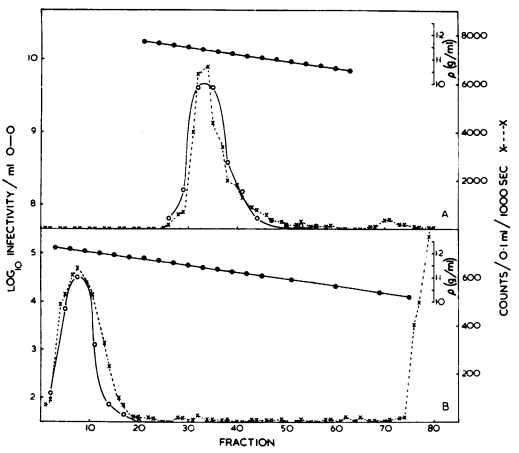

FIG. 4. *Tartrate gradient centrifugation of (A) ^{32}P-virion and (B) Tween-ether disrupted ^{32}P-virion. Infectivity was determined by titration in mice.*

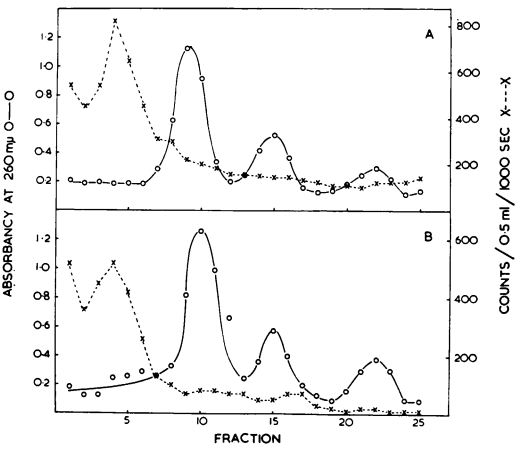

FIG. 5. *Sucrose density gradient centrifugation of RNA from (A) ^{32}P-virion; (B) infective component of Tween-ether treated ^{32}P-virion. BHK cell RNA was added as marker.*

Nature of the ether-resistant infective component. Tween-ether treated ^{32}P virion was sedimented in a sucrose gradient, and the fractions containing the infective component were isolated. These were diluted with 0.01 M phosphate (*p*H 7.6) and extracted with 1% sodium dodecyl sulfate and phenol. About 50% of the radioactivity in the preparation was found in the aqueous layer. This relatively low recovery of ^{32}P activity in the aqueous layer, compared, for example, with one of 80% which is obtained when ^{32}P-labeled foot-and-mouth disease virus is extracted by the same procedure (1), suggests that some of the ^{32}P in the viral substructure is associated with molecules other than ribonucleic acid (RNA). Using the same conditions of extraction, with untreated virus, 10 to 15% of the radioactivity of ^{32}P-labeled VSV virion is left in the aqueous layer.

The preparations obtained by phenol-sodium dodecyl sulfate extraction of virion and the viral substructure were mixed with BHK cell RNA (to act as marker) and centrifuged through separate 5 to 25% sucrose gradients. The distribution of ^{32}P and absorbancy at 260 mμ (Fig. 5) show that the two preparations have similar sedimentation coefficients. Each preparation was completely hydrolyzed to slowly sedimenting material when treated with 0.01 μg of ribonuclease per ml before centrifugation, showing that all of the radioactivity was present as RNA.

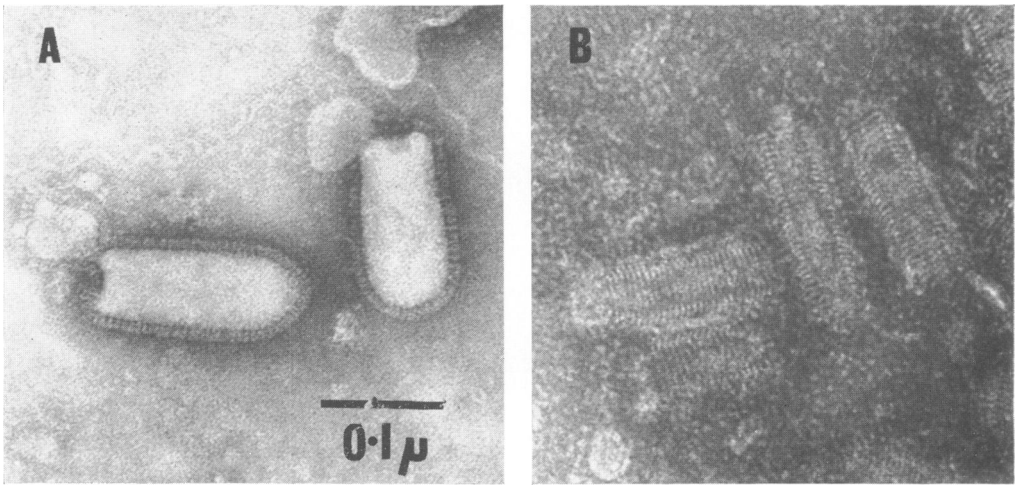

FIG. 6. *Electron micrographs of (A) virion and (B) virion disrupted with Tween-ether.*

Electron microscopy. Treatment of purified virion preparations with Tween-ether resulted in the production of large numbers of skeleton-like structures with the general overall shape of the virion (Fig. 6). A number of rosette-like structures similar to those found in untreated harvests of VSV were also seen (2). The penetration of the phosphotungstic acid into the skeletons was very pronounced, presumably owing to removal of the lipid from the virions. Particle counts were made on each of 24 fields of the untreated and treated preparations after mixing with a suitable dilution of 0.188-μ latex particles. More than 2,500 particles from each preparation were counted, and it was found that the number of skeletons in any sample was not significantly different from the number of virions in the sample from which it was prepared. The skeletons clumped if they were stored in phosphate buffer for more than a few hours at 2 C. In consequence, the Tween-ether disrupted preparations were generally examined as quickly as possible after preparation. After Tween-ether treatment, the skeleton-like structure was found in large numbers in the most infective fractions obtained from sucrose gradients, lending support to the concept that the ether-resistant infectivity is associated with this structure.

DISCUSSION

The data presented in this paper provide evidence that controlled disruption of the virion of VSV can lead to an infective viral substructure. It seems unlikely that the infectivity is due to residual traces of virion because (i) the rate of adsorption of the infectivity component to BHK cell monolayers is much slower than that of the virion, (ii) the infectivity for BHK cells is about 1% as great as it is for mice, whereas the virion is equally infective for both cells and mice, and (iii) it is not neutralized by viral antiserum. This third observation is similar to the finding that infective viral RNA is not neutralized by viral antibody (*see, for example*, 3), although this substructure is not free RNA because its infectivity is unaffected by ribonuclease.

The infective substructure sediments to a lower position in sucrose gradients than the virion, and its density in tartrate gradients is 1.21 g/ml, compared with 1.14 g/ml for the virion. This increase in density would be expected if the Tween-ether treatment had removed the lipid coat of the virion. Removal of the lipid coat also removes 85 to 90% of the radioactivity of ^{32}P-virion. At least 50% of the ^{32}P in the substructure is present as RNA, which can be extracted by phenol and sodium dodecyl sulfate. This RNA has the same sedimentation characteristics as the RNA of the virion, suggesting that the entire infective potential of the virion is still present in the structure. As the structure also contains most of the RNA of the virion, its low infectivity is likely to be due to the removal of specific attachment sites by the Tween-ether treatment.

ACKNOWLEDGMENTS

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