SEPARATION OF ENCEPHALOMYOCARDITIS VIRUS FROM TISSUE COMPONENTS BY MEANS OF PROTAMINE PRECIPITATION AND ENZYMIC DIGESTION

MARVIN L. WEIL, JOEL WARREN, SYDNEY S. BRESEE, JR., SUDIE B. RUSS, AND HELEN JEFFRIES

Department of Virus and Rickettsial Diseases, Army Medical Service Graduate School, Washington, D. C.

Received for publication July 23, 1951

The capacity of protamine sulfate to combine with and precipitate considerable tissue debris from certain virus suspensions without significantly decreasing their infectivity or antigenicity has been reported (Warren, Weil, Russ, and Jeffries, 1949). In the case of certain viruses it has been found that concentrating the protamine-clarified suspensions in the ultracentrifuge yields sediments which can be further purified by enzymic digestion. Further ultracentrifugation of the digest yields preparations of greater purity, as measured by the ratio of infectivity to nitrogen content. Observation in the electron microscope and the analytical ultracentrifuge indicates that the final product has considerable homogeneity.

Encephalomyocarditis virus was chosen for this investigation because of its marked stability, high titer, small particle size, and its resistance to certain proteolytic enzymes. The following is a description of the method employed to obtain highly purified preparations of this particular virus, and also a presentation of certain of the physical and biochemical properties of the particles isolated.

MATERIALS AND METHODS

The brains were removed from 200 to 500 moribund mice, and a 20 per cent suspension was blended in distilled water. After clarification at 3,000 rpm for 15 minutes in the International no. 2 angle head centrifuge, the supernate so obtained was frozen and stored at −40 C. When used, within 1 to 5 days, the material was thawed and thoroughly mixed with dry protamine sulfate (salmine) to give a final concentration of 5 mg per ml of suspension. After allowing the protamine to react with the tissue debris for 30 minutes at 5 C, the preparation was again centrifuged at 3,000 rpm for 15 minutes in the International no. 2 angle head centrifuge, the hemoglobin-tinted supernate removed, and the sediment discarded. This virus-bearing supernatant fluid was immediately spun at 44,770 rpm (140,000 g) for 45 minutes in a Spinco refrigerated ultracentrifuge. The resultant supernatant fluid was decanted, and additional freshly prepared protamine-clarified fluid was added to the same tubes, which were again spun at high speed. By this method the sediment from 50 ml of protamine supernate could be collected in one 12.5 ml centrifuge tube. The final pellets, which were

1 Portions of this paper were presented at the Annual Meeting of the Society of American Bacteriologists, May 1950, Baltimore, Maryland.
small, clear, amber, and gel-like, were resuspended in physiological saline (0.15 M), adjusted to pH 8.0 with 0.02 M phosphate buffer, and frozen overnight at -40 C. The freezing and thawing are not essential but serve as a convenient stopping point in the procedure. This suspension was then thawed and the volume adjusted so that the suspension was 20 to 50X concentrated with respect to an original 10 per cent concentration, after which sufficient dry, twice-crystallized trypsin, containing 50 per cent magnesium sulfate (Worthington Biochemical Company), was added to give a final concentration of pure enzyme of 0.037 per cent or, in later experiments, 0.05 per cent. The suspension was allowed to digest in a water bath at 37 C for 1 hour with frequent shaking, following which it was passed through Whatman no. 40 filter paper into an ice-chilled flask. The water-clear filtrate was centrifuged at 44,770 rpm (140,000 g) for 45 minutes and the resulting pellet resuspended in buffered physiological saline of pH 7.6 to give a concentration of 250 to 450X with respect to an original 10 per cent concentration. Finally, the faintly opalescent suspension was freed of the remaining undispersed material by centrifugation at 900 rpm for 2 to 5 minutes in the International no. 1 horizontal machine.

RESULTS

Influence of suspending media. Since protamine precipitates more of the unwanted brain tissue from suspensions in distilled water than in the presence of sodium chloride (as has been previously noted by Kleczkowski (1946) in the case of other protamine-protein complexes), distilled water was used for the initial blending of the infected brains. For the suspension of the final pellets it was found that physiological saline yielded higher titers (about 1 to 2 log) than suspensions in distilled water, 0.05 M boric acid, 0.05 M glycine, 0.025 M veronal, or 0.025 M sucrose. It is presumed, on the basis of electron micrographs, that the lower titers were due to aggregation in these diluents.

Enzymic digestion as an aid to purification. Electron micrographs of various nondigested encephalomyocarditis infected brain suspensions revealed that although the virus was considerably concentrated, it still remained associated with amorphous debris and smaller particles similar to those occurring in normal mouse brain suspensions ("normal component") prepared with protamine and ultracentrifugation (Warren, 1950). Therefore, an enzyme was sought which might reduce these extraneous tissue particles but would not digest the virus. Furthermore, since small amounts of granular sediment (probably excess protamine) gradually formed in the protamine-clarified tissue suspensions, a proteolytic enzyme was chosen which would remove this sediment. Preliminary studies demonstrated that encephalomyocarditis virus sustained no detectable loss of infectivity at 37 C for 1 hour in the presence of 1 per cent trypsin, 0.1 per cent chymotrypsin, 0.1 per cent ribonuclease, 0.1 per cent desoxyribonuclease, or 1 per cent papain, the highest concentrations studied, when treated at the pH for optimal enzyme activity. The enzyme digestion procedure was performed on the first ultracentrifugation sediment which had been freed of hemoglobin so that degradation products of the latter would not be formed and carried over into the final
encephalomyocarditis virus from tissue concentrate. All five of the proteolytic enzymes reduced the particulate masses which developed upon storage of the protamine-clarified material. Trypsin, ribonuclease, and desoxyribonuclease were selected for further study since they were available in crystalline form.

Comparison of infectivity and nitrogen content. The effectiveness of this method of preparation using tryptic digestion may be gauged by measurement of the infective titer and total residual nitrogen in the crude and final materials. Infectivity was measured by intracerebral titration of 10-fold virus dilutions using 5 to 8 mice per dilution and calculation of the 50 per cent lethal dose. (The mice were 12 to 14 g white Swiss.) Representative results are summarized in table 1. It will be seen that little or no virus is lost in the purification procedure. The 4 purified lots shown have $10^{15.2}$ to $10^{15.9} \text{LD}_{50}$ per gram of nitrogen while a representative crude preparation with $10^{19.9} \text{LD}_{50}$ per ml and 600 μg nitrogen per ml had $10^{12.1} \text{LD}_{50}$ per gram of nitrogen. The residual nitrogen in the final suspensions is 5,000 to 10,000 times less than in the crude while the LD_{50} is increased 1,200 to 1,600 times.

Sedimentation studies in the analytical ultracentrifuge. The sedimentation patterns of the resuspended second ultracentrifugation sediment from encephalomyocarditis infected mouse brains prepared as described, using trypsin digestion, were studied in a Spinco analytical ultracentrifuge. In order to obtain absorption in the near ultraviolet region (365 μm), a Corning no. 5860 glass filter was used in the optical system. Concentrates of infected brain suspended in physiologic saline gave 2 sedimenting boundaries, 1 by refractive index averaging $S_{20} = 151 \times 10^{-13}$ and another by 365 μm absorption averaging $S_{20} = 56 \times 10^{-13}$.

The 151S component occurred in virus preparations only and had sufficient homogeneity and concentration to give a refractive peak. In 5 separate lots examined by the refractive index method, the sedimentation constant ranged from 148S to 159S. This component has been considered due to the virus since it was not found in the control preparations of normal mouse brain.

The 56S component is present in much lower concentration and was occasionally seen in both infected and normal preparations giving only an indistinct absorption boundary. In only 3 of 10 normal mouse brain preparations was it possible to detect this material in the ultracentrifuge. The sedimentation con-

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>LD_{50}/ML (log)</th>
<th>FINAL CONCENTRATE NITROGEN/ML (μg)</th>
<th>LD_{50}/g NITROGEN (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified lot A</td>
<td>8.0</td>
<td>0.06</td>
<td>15.2</td>
</tr>
<tr>
<td>Purified lot B</td>
<td>8.0</td>
<td>0.06</td>
<td>15.2</td>
</tr>
<tr>
<td>Purified lot C</td>
<td>8.7</td>
<td>0.15</td>
<td>15.5</td>
</tr>
<tr>
<td>Purified lot D</td>
<td>8.9</td>
<td>0.09</td>
<td>15.9</td>
</tr>
</tbody>
</table>
Figure 1. Encephalomyocarditis virus from mouse brain. Prepared with protamine and trypsin. Electron micrograph, chromium shadowed, 21,800×.

Figure 2. Encephalomyocarditis virus from mouse brain. Grouped particles. Electron micrograph, chromium shadowed, 29,300×.

Figure 3. Encephalomyocarditis virus from mouse brain. Prepared with protamine and ribonuclease. Electron micrograph, chromium shadowed, 33,000×.
stents of these lots ranged from 51S to 61S. This material is apparently a component of normal mouse brain whose nature and function are unknown.

Appearance in the electron microscope. Examination in the electron microscope of the resuspended second ultracentrifugation sediments corroborates the findings of 2 components with the analytical ultracentrifuge. An electron micrograph of a final sediment from encephalomyocarditis infected mouse brain which had been treated with trypsin is shown in figure 1. The sediment was suspended in distilled water and shadowed with chromium for examination. The major portion of the particles are round, almost spherical, and highly uniform in size. The larger bodies have been found only in encephalomyocarditis infected preparations and are considered the virus particles. The smaller particles have been seen in both infected and normal mouse brain preparations and appear to be a "normal component" of mouse brain tissue.

Figure 2, from another lot of encephalomyocarditis infected tissue prepared in similar fashion, demonstrates regions where the larger particles were grouped in orderly array and exhibit uniformity of size and shape. Some of the smaller particles are also seen.

Figure 3 shows a preparation from encephalomyocarditis infected mouse brain in which 0.1 per cent ribonuclease was used instead of trypsin for the digestion. Although much of the normal component is apparently removed by ribonuclease, some remains, and occasional smaller particles were seen in several electron micrographs of this material from different lots of brain suspension.

Electron micrographs of suspensions of normal mouse brain prepared by the same methods showed small bodies similar to those seen in the infected preparations. It is noteworthy that the normal component shows little tendency to aggregate in the preparations for the electron microscope in contrast to the virus preparations. Furthermore, as was the case with infected brains, 2 preparations of normal mouse brain in which ribonuclease was used for digestion showed considerable decrease in the numbers of smaller particles.

Size. The particle sizes were measured on the photographic plate with a comparator and averaged from measurements of approximately 150 particles. The diameter of individual 151S particles, which are considered to be the virus, was 38.1 ± 2.4 m\(\mu\). According to Kahler and Lloyd (1950), measurement across grouped particles gives an average particle spacing which is independent of the metal coating. Using this method, a diameter of 27.0 ± 2.0 m\(\mu\) was obtained. This is in agreement with the particle size of encephalomyocarditis obtained by means of ultrafiltration (Warren, 1948). The diameter of the smaller 56S component particles measured individually was about 18.5 m\(\mu\).

**DISCUSSION**

A problem frequently encountered in the purification of viruses smaller than 40 m\(\mu\) in diameter is the differentiation of the infectious particle from other sedimentable particles of similar size. Although differential centrifugation alone will occasionally yield concentrates of homogeneous identifiable particles, the repeated centrifugations usually result in small volume yields and low infectivity.
The use of protamine precipitation decreases the need for long cycles of ultracentrifugation as a considerable amount of tissue debris can be removed by this relatively simple method.

Electron micrographs of encephalomyocarditis virus purified by protamine and centrifugation usually revealed a considerable amount of debris even though the infectivity-nitrogen ratio indicated that considerable purification had been achieved. However, when such concentrates were treated with crystalline enzymes, it became a relatively simple matter to secure micrographs of discrete and easily recognizable particles. Absolute purity of the virus has not been obtained since these suspensions frequently revealed 2 particles of differing properties. The larger particle was found only in encephalomyocarditis infected mouse brain suspensions and has been considered the particle responsible for virus activity. On the other hand, the smaller particle was found in both the infected and normal mouse brain suspensions and must be classed in the heterogeneous group of normal components. Furthermore, the purification procedure removed the majority of the extraneous tissue components without reducing the infectivity of the virus.

Previous work (Warren, Smadel, and Russ, 1949; and Dick, 1949) has shown that encephalomyocarditis is indistinguishable from Columbia-SK virus. Working with the latter virus, Jungeblut and Bourdillon (1943) obtained electron micrographs of unshadowed preparations which showed particles of 25 to 30 mμ. This agrees closely with our results. Bourdillon (1944) prepared samples of Columbia-SK virus suspensions by pH precipitation, solvent extraction, and ultracentrifugation which had infectivity-nitrogen ratios similar to those we have described.

**SUMMARY**

Suspensions of encephalomyocarditis virus infected mouse brain with $10^{15.2}$ to $10^{14.9}$ LD$_{50}$ per gram of nitrogen may be prepared by protamine clarification, followed by enzymic digestion and ultracentrifugation. Particles characterized by a sedimentation constant of 151S and an electron microscope image diameter of about 38 mμ individually and 27 mμ in groups are present in such material and are considered to be the virus. Some smaller particles of about 18 mμ in diameter occur in the cleanest preparations of virus; these are regarded as a component of normal mouse brain.

**REFERENCES**


