Certain Physical Properties of a Herpangina Strain and a Pleurodynia Strain of Coxsackie Virus. (20283)

SYDNEY S. BREESE, JR., AND ANGELA BRIEFS. (Introduced by J. Smadel.)

From the Department of Health, Education, and Welfare, Public Health Service, National Microbiological Institute, National Institutes of Health, Bethesda, Md.

The present study was initiated to determine whether 2 members of the Coxsackie group of viruses which had been isolated originally from clinically different types of illness and which were known to be serologically distinct from one another possessed discernible differences in physical properties which might further serve to distinguish them. Strain No. 1816(1) (Huebner type H_3), which is immunologically identical with Dalldorf Group A, Type 10(2), was used as the representative Herpangina virus in the current work and strain No. 4520(3), which is identical with Dalldorf Group B, Type 3, was used as the Pleurodynia virus. Strain No. 1816 had been employed previously in electron microscopic and ultracentrifugal studies(4) and had been shown to be indistinguishable in size and sedimentation constant from another Herpangina (Group A) Coxsackie virus.

Several technics which have been reported elsewhere were adapted to the present study. These include the use of Attaclay in purification(5), estimation of sedimentation constant using concepts of pseudo-velocity and effective velocity(6), and pseudo-replication of agar blocks for electron microscope studies(7).

Materials and methods. Coxsackie virus, strain No. 1816, was propagated in 4-day-old mice and was prepared for examination in the electron microscope by the methods previously reported(4). The No. 4520 strain was propagated in 2-day-old mice and was purified in the same manner. The experiments with the separation cell required larger quantities of virus suspension than had previously been prepared, and a modification in the purification procedure was made. Following the general method of Hiatt *et al.*(5) the crude 10% suspension of infected mouse muscle was added to Attaclay SF* to give a 30% concentration of Attaclay. The mixture was shaken by hand and placed in a 2°-4°C refrigerator for a short time until it could be centrifuged at 3000 RPM for 10 minutes. The supernatant fluid was carried through the regular purification procedure starting with the ether extraction as described previously(4). *Infectivity* was determined by inoculating 0.04 ml amounts of several 10-fold dilutions of the material to be tested into each of eight 4-day-old mice. All mice were observed for 10 days after which the 50% end-point (LD_{50}) was calculated by the method of Reed and Muench (8). *Sedimentation* in the ultracentrifuge was recorded by both Schlieren and ultraviolet (2500 Å) absorption methods in a Model "E" Spinco machine. The separation cell(9) used in these experiments was made by replacing the regular analytical cell center section with one made of Lucite which had a dural barrier plate mounted in it. The Lucite center section was used without gaskets which facilitated assembly of the cell. All separation cell experiments were run at 17,250 RPM.

The concept of pseudo-velocity proposed by Epstein and Lauffer(6) has been used in the estimation of the sedimentation rate of the 4520 strain of virus since visible boundaries have not been observed with this agent. The pseudo-velocity is the ratio of the meniscus-to-barrier distance to the effective time of sedimentation. Since the sedimentation constant is defined as the velocity of the boundary per unit field of force at a given time and a given distance from the center of rotation of the rotor, we may make a calculation of the sedimentation constant from the pseudo-velocity rather than the measured velocity of a visible boundary. The formula for calculation is as follows: $S = \frac{\text{pseudo-velocity (cm/sec.)}}{\text{centrifugal force (dynes/g)}}$ where the centrifugal force is calculated at the distance of the barrier from the center of rotation. This value may then be corrected for the viscosity of the solution and the tem-

* Attapulgus Clay Co., Philadelphia, Pa.

perature of the experiment to give the value S_w^{20} usually reported. This method was used to calculate the sedimentation constant for the shortest times of sedimentation for which there was 10-fold or greater difference between the infectivity titer of the starting material and the material sampled from the top of the cell. The times of sedimentation used for the 4520 strain were chosen so that the boundary would not pass the barrier in certain runs but would be through the barrier in others. In the case of the strain 1816 separation cell experiments the visible boundary sedimentation constant was calculated as well as the sedimentation constant from the pseudo-velocity. Specimens were prepared for electron microscopy by *pseudo-replication* from agar blocks. Two per cent agar in distilled water was poured in petri dishes to a depth of 0.5 cm 12-24 hours before use. Small blocks 1 sq cm were cut from the agar and mounted on clean glass slides. A drop or two of virus suspension was allowed to evaporate to dryness in air on each block. The block was then flooded with a 0.5% solution of parlodian in amyl acetate. The glass slide was held vertically to drain off the excess parlodian. When the parlodian film was dry it was floated off on distilled water and 3 to 4 stainless steel screens were placed on each film which was then lifted from the water, film side up, on aluminum strips. The specimens were shadowed with chromium at arc tang 1/5. An RCA model EMU microscope with objective aperture and extended range lens was used.

Results. Use of Attaclay. The use of Attaclay reduced the time required for processing a virus suspension for use in these experiments by about 30% since it eliminated overnight storage in the refrigerator and the freezing, thawing, and centrifugation used prior to the ether extraction in the purification procedure outlined previously(4). Two Group A and 2 Group B viruses were treated with Attaclay and the preparations used as antigens in complement-fixation tests and also in many of the separation cell ultracentrifuge experiments. The method was most effective when applied to suspensions having a nitrogen content of 0.7-1.0 mg/ml. The use of a 30% concentration of Attaclay was found to be optimal both for reduction of nitrogen content and for maintenance of complement-fixation and infectivity titers. For example with a preparation of 1816 virus the nitrogen was reduced from 2.67 mg/ml to 1.07 mg/ml with the infective titer remaining at 7.2. The infective titers were not significantly reduced, but the nitrogen was reduced about 60%. Furthermore, the use of Attaclay removed the pink color of the crude suspensions (due to hemoglobin) and also reduced to about one-third the amount of ether needed in the subsequent extraction of the supernate since adsorption on Attaclay apparently removes large quantities of lipoid material.

Electron microscopy. The electron micrographs of both strains of Coxsackie virus show spherical particles which are notably uniform. In both cases the particles have formed clusters which are hexagonal or diamond arrays. The diameter of the particles measured singly is approximately 37 mμ. Fig. 1 and Fig. 2 show the 4520 strain in small diamond-shaped regular arrays. The 1816 strain formed even larger clusters and, as is seen in Fig. 3 and Fig. 4, the groupings are hexagonal pseudo-crystals which have two or three layers or sheets of particles. The smaller particles of about 18 mμ diameter seen in the background are assumed to be a normal component of mouse muscle suspensions(4).

Ultracentrifugation. The sedimentation constant of the 1816 strain of Coxsackie has been determined previously(4) as being approximately 150S. Using the separation cell, a series of sedimentation runs was made with the 1816 strain in which the refractive index boundary was seen to approach and then pass through the cell barrier. The results of these experiments are shown in Table I. For these same runs the sedimentation constant has been calculated using the pseudo-velocity and the centrifugal force at the distance of the barrier from the center of rotation, *i.e.,* 6.723 cm. For estimation by this method the 2 shortest periods of centrifugation for which there was an infective titer reduction of 1 log or more in the fluid of the top compartment were used for calculating the sedimentation constant. In the experiments with the 1816

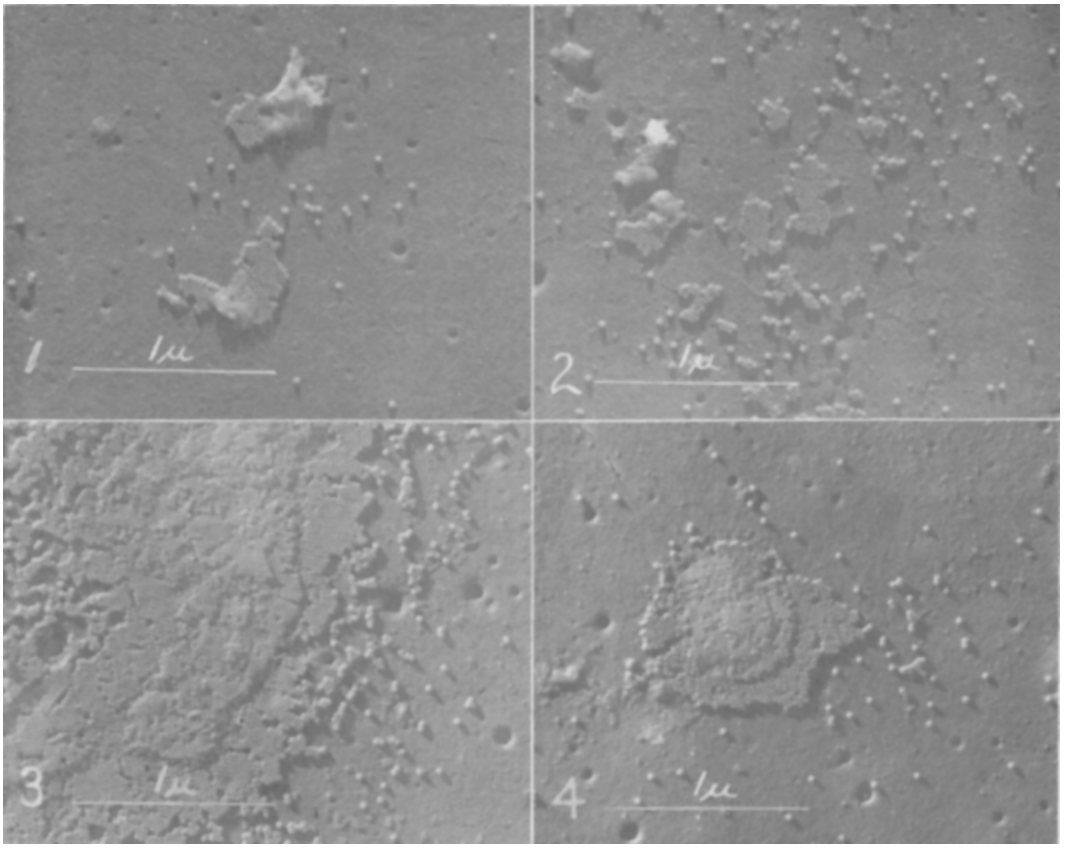

FIG. 1. Coxsackie virus—Strain 4520—pseudo replica from agar, chromium shadowed.
FIG. 2. Coxsackie virus—Strain 4520—pseudo replica from agar, chromium shadowed.
FIG. 3. Coxsackie virus—Strain 1816—pseudo replica from agar, chromium shadowed.
FIG. 4. Coxsackie virus—Strain 1816—pseudo replica from agar, chromium shadowed.

strain the average of the two sedimentation constants calculated from the pseudo-velocity and the infectivity difference is 167S compared with an average of 156S from the visible boundary calculation. Thus the sedimentation constant calculated indirectly is 7% greater than the observed value for the 1816 strain. Employing the pseudo-velocity technic on the 4520 strain provided a sedimentation constant of 175S. Assuming that this method of interpolation could be at least 10% in error, the range of sedimentation constant for the 4520 strain would be from 157S to 193S.

Discussion. The size and sedimentation constants of a Herpangina strain and a Pleurodynia strain of Coxsackie virus do not differ appreciably when estimated by procedures employing the electron microscope and the separation cell in the analytical ultracentrifuge. The electron micrographs of particles from both sources reveal uniformly spherical bodies. Using the concept of pseudo-velocity and the change in infectivity in the top compartment of the separation cell for estimating the sedimentation constant, there is a difference of less than 5% between the values for the two strains of virus. In the case of 1816 strain this method of estimation is some 7% higher than the sedimentation constant calculated from the visible boundary.

The application of pseudo-velocity used here differs from that of Epstein and Lauffer (6). In their experiments the pseudo-velocity was used to identify the visibly sedimenting particle as the infectious agent. The assumption in this study is that a significant change in infectivity in the separation cell

TABLE I. Data on Separation Cell Experiments with Coxsackie Viruses.

Coxsackie strain	Effective time of sedimentation, sec.	℄ to Meniscus, cm	Pseudo velocity, cm/sec.	Difference in titer starting mat'l and top of cell log	S_w^{20*} calc.	S_w^{20}† obs.
1816 (Group A)	600	5.93	1.32×10^{-3}	.1	524	144
	960	5.98	.777	.8	307	155
	1740	5.95	.444	1.5	171	153
	1680	6.05	.403	2.5	163	159
	1080	6.32	.374	2.1	152	
4520 (Group B)	540	6.00	1.35	.6	584	
	960	5.98	.777	.8	335	
	1920	5.90	.428	1.6	202	
	2400	5.96	.317	1.7	148	
	3000	5.95	.257	2.0	117	

* Using pseudo velocity and centrifugal force at barrier distance 6.723 cm.
† Calculated from visible boundary on photographic plates.

indicates that a boundary exists and has crossed the barrier. When this boundary is not visible the pseudo-velocity may be used to estimate the sedimentation constant of the infectious agent. The difficulty in preparing sufficiently concentrated preparations of relatively pure animal viruses to produce refractive index boundaries in the ultracentrifuge makes an indirect method based on biological measurements, such as infectivity, particularly desirable.

The electron micrographs presented in this study show the virus particles in uniform clusters. These formations have occurred only in those specimens made by pseudo-replication of agar blocks. Previous electron micrographs(4) of the 1816 strain, for example, were made from specimens prepared by placing a drop of suspension on parlodian coated screens and drying in air, and in no case did the particles form clusters. The present electron micrographs show clusters very similar to those previously seen in purified plant viruses(9). Further investigation of the technic including the effect of pH change in the agar and varied concentration of virus may reveal the optimum conditions for cluster formation.

Summary. 1. There is no appreciable difference between the particle diameters of the 2 Coxsackie viruses—Strain 1816 (Herpangina) and Strain 4520 (Pleurodynia). The diameter is approximately 37 mμ for single particles and about 28 mμ for particles measured in rows. 2. The sedimentation constants, estimated indirectly, are 167S for the 1816 strain and 175S for the 4520 strain. For the 1816 strain the visible boundary measurements give 153S in this series of experiments. 3. Attaclay is useful in the procedure for preparing partially purified virus from suspensions of infected mouse muscle. 4. The technic of pseudo-replication from agar blocks has led to electron micrographs showing the virus particles in orderly hexagonal arrays.

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