Particle Size of the MEF_1 Lansing Strain of Poliomyelitis Virus. (19826)

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There is no uniformity of opinion about the morphology of the viruses of the poliomyelitis group. Particle sizes have been determined frequently, but the results reported are widely divergent. Thus using ultrafiltration technics Theiler and Bauer(1) found the size of poliomyelitis virus to be 10-15 mμ. This diameter approximates that found by Elford, Galloway and Perdrau(2) of 8-12 mμ. Theiler and Gard(3) established a diameter of 9-13 mμ for Theiler's virus, and Sanders and Jungeblut (4) found Columbia SK virus to be similar in size. Using other physico-chemical methods Gard and Pedersen(5) estimated the size of Theiler's virus to be 640 x 14 mμ. Tiselius and Gard(6) and Gard(7) found filamentous particles with a diameter of 15-20 mμ on examining purified Theiler's virus in the electron-microscope. Similar structures were found by Jungeblut and Bourdillon(8) in fluid from tissue cultures in which sk virus was propagated, and in purified preparations from mouse brain virus these authors found spherical particles with diameters ranging from 25-30 mμ. Loring, Schwerdt and Marton(9) reported a particle size of 15-20 mμ for purified MV strain of poliomyelitis virus. Particles of a similar magnitude were observed by Loring(10) in preparations of Lansing and Y-SK strains of poliomyelitis virus. Recently Leyon, Gard and Eklund(11) reported measurements made on electron photomicrographs of purified Theiler's virus. The most reliable measurements indicate that the elementary particles of Theiler's virus are approximately spherical and have a diameter of 28 mμ. Reagan, Shenk and Bruecker(12) published electron photomicrographs of virus particles extracted from monkey cords infected with the Leon strain of poliomyelitis. From their photographs the elementary particle appears to be a short rod. The width is 12-15 mμ and length 30-35 mμ. In view of the controversial results obtained by previous workers it was decided to reinvestigate the particle size of poliomyelitis virus with the ultracentrifugation technic of Polson and Linder(13).

Material and methods. The MEF_1 Lansing strain of poliomyelitis adapted to suckling mice by repeated intracerebral passage was used (Casals, Olitsky and Anslow(14), and Selzer, Sacks, and van den Ende(15)). At the time the virus was used in these experiments it had undergone 34 serial transfers in suckling mice. Its identity was confirmed by complement fixation and neutralization tests with a Lansing immune serum supplied by Dr. Bodian, Johns Hopkins Hospital. For centrifugation a 10% emulsion of brains from virus infected suckling mice was used. The suspensions were made in phosphate buffer at pH 8.2 and clarified by centrifugation for 1 hour at 2800 r.p.m. in a refrigerated horizontal centrifuge. The slightly opalescent supernatant fluid was placed in a graduated spinco angle centrifuge tube and the height of the meniscus noted. It was then spun in a rotor which had previously been brought to equilibrium temperature for the required speed. The total time of centrifugation included the period of acceleration and deceleration. The average speed of centrifugation is obtained by dividing the total number of revolutions by the total time. Immediately after the rotor stopped the temperature of water in the tube used for balancing the virus suspension was determined and the tube containing the virus then transferred to a metal stand at the bottom of a large container of water at the same temperature as the centrifuge rotor, care being taken that the fluid water in the container was at a level above that of the meniscus in the centrifuge tube. With a special sampling device the fluid in the tube was removed in successive layers of 1 cm. Thus the contents of the tube was usually divided into 6 samples. Each sample was titrated by intracerebral inoculation of 10-fold dilutions into 3 to 4 weeks old mice. Fifty percent end-points were determined by the method of Reed and

TABLE I. Ulracentrifugation of Poliomyelitis Virus, MEF$_1$ in 1% *C. sincta* Haemocyanin.

r.p.m.	t_2-t_1 in min.	T°C	η/η_{20}	LD_{50} of samples at 1 cm	2 cm	3 cm	4 cm	5 cm	Bottom	LD_{50} of original material
30000	105	12	1.24	0	T*	0	T*	0	—	4
22840	100	9.5	1.32	2.1	1.6	2	2.5	2.5	4.5	4.6
20000	100	9	1.345	1.6	.8	1.7	1.6	3	3.7	4

* T = Trace.
$a = 26°$; $x_1 = 5.31$ cm

Muench(16). The titres of the different samples were plotted against the relative positions in the centrifuge tube. The position of the sedimenting virus boundary in the centrifuge tube, which corresponds to the 50% concentration point in the Svedberg light absorption technic is determined from the position of a line drawn through a point representing a titre T, where

$$T = \log\left(\frac{\text{antilog } T_2 - \text{antilog } T_1}{2}\right) \quad (1)$$

and T_2 is the virus titre below, and T_1 that of the virus above the boundary zone respectively. From the position of the sedimenting boundary thus determined the sedimentation constant is calculated using the equation of Svedberg and Pedersen:

$$S = \frac{2\,H \sin a}{(2X_1 + H \sin a)\,\omega^2\,(t_2 - t_1)}\,\eta/\eta_{20} \quad (2)$$

where X_1 = distance of initial boundary from the axis of rotation, a the angle of inclination of the tube with the axis of rotation, ω the angular velocity of the centrifuge rotor and $(t_2 - t_1)$ the time of centrifugation. X_1 the distance of the meniscus from the centre of rotation must be corrected for errors due to distortion which the tube undergoes during centrifugation. η/η_{20} is the ratio of viscosities of the solution at the temperature of centrifugation to that of water at 20°C.

To minimize heat convection currents during and after ultracentrifugation the virus was centrifuged in the presence of approximately 1% of whelk (*Caminella sincta*) haemocyanin. On centrifuging haemocyanin it was noticed that below the sedimenting boundary a protein gradient formed which had a function of overcoming heat convection disturbances similar to that of the sugar gradient used in the centrifugation experiments of Pickles(17).

Results. The results of experiments conducted at different rotor velocities are recorded in Table I.

The haemocyanin used in this experiment had a sedimentation constant of 100 Svedberg units and at 20000 r.p.m. for 100 min., its boundary moved 2.7 cm down the tube. From the above table it is evident that the virus boundary had reached the bottom of the tube at rotor velocities greater than 22840 r.p.m. At 20000 r.p.m. the boundary did not extend down the full length of the centrifuge tube but reached a level between 3.5 and 5.5 cm below the initial meniscus. The existence of this boundary is indicated by the sudden increase in the titres of the samples taken below the 3.5 cm level. From equation (2) it can be shown that the sedimentation constant of the virus lies between 129 and 189 Svedberg units. The most likely position which the boundary will occupy in the tube is that which can be calculated from equation (1).

This boundary is indicated by the vertical interrupted line in Fig. 1. This line cuts the

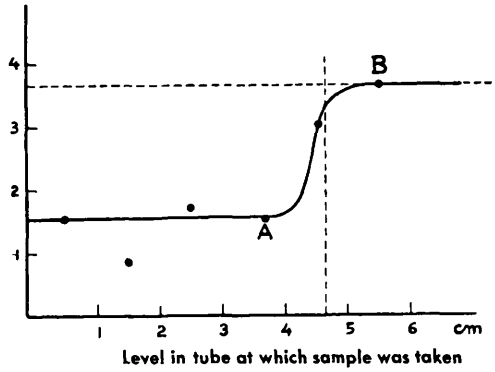

FIG. 1. Titres expressed in neg log LD_{50} of samples taken at different levels in centrifuge tube of virus spun at 20000 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD_{50}.

TABLE II.
Ultracentrifugation Experiments of Virus Suspension Clarified by Different Methods.

Suspension	LD_{50} of samples taken at the following levels 1 cm	2 cm	3 cm	4 cm	5 cm	6 cm	LD_{50} of material used at the start	S_{20}
After centrifugation only	1.2	1.3	2.1	3	3.3	4	4.3	183
After centrifugation and ether extraction	T*	T*	T*	2.8	2.9	4.2	3.7	170
After centrifugation, ether extraction and tryptic digestion	0	0	1.2	1.2	<2	4	3.4	>208

* T = Trace.
$x_1 = 5.31$ $\alpha = 26°$ r.p.m. 19740 time = 100 min. T°C = 9 $\eta/\eta_{20} = 1.345$

abscissa at 4.65 cm from the initial meniscus. The sedimentation constant calculated from this value is 163.1 Svedberg units which is in agreement with the value of 160-170 S reported by Gard and Pedersen(5) for Theiler's virus. From this value of the sedimentation constant the particle size was calculated using the modified Stokes equation

$$r^2 = \frac{9}{2} \frac{S\eta}{(d-\rho)}$$

where r is the radius of the particle, S the sedimentation constant, d the density of the particle assumed to be 1.33 g/cc η the viscosity and ρ the density of the dispersion median. The calculated size of 29.8 mμ or approximately 30 mμ is in agreement with that found on electron micrographs of SK virus, Jungeblut and Bourdillon(8) and Theiler's virus Leyon, Gard and Eklund(11). Owing to the dependency of the above sedimentation constant on the virus titrations in mice, no great precision is claimed for this value of the particle size of the MEF_1 strain of poliomyelitis. However, from the result of experiments, such as those marked in Fig. 1 it can be concluded with confidence that the sedimenting boundary must lie within the range of changing virus concentration. (Portion A B of graph) From the position of A and B it can be calculated that the size of the virus particle is within the range of 26 to 32 mμ.

To establish that the poliomyelitis virus does not exist as a smaller particle attached to lipoid or other brain components, experiments were performed on infected mouse brain extracts previously clarified by ether extraction or by ether extraction and tryptic digestion.

MEF_1 infected mouse brains were emulsified in 1/15 M phosphate buffer at pH 8.1 and clarified by centrifugation at 2800 r.p.m. for 1 hour in a refrigerated centrifuge. The supernatant was then shaken up in an equal amount of anaesthetic ether in the cold and again centrifuged at 2800 r.p.m. for an hour in the refrigerated centrifuge. The aqueous layer was removed, placed in a vacuum and the major portion of the dissolved ether thereby removed. The residual ether was removed by dialysis overnight against a large volume of buffer at pH 8.1. The solution was divided into two equal volumes. To one portion crystalline trypsin, 0.01 g per 20 ml brain extract was added and allowed to digest at 37°C for 30 min., after which 4 ml normal rabbit serum was added immediately to inhibit further tryptic action. The solution was slightly opalescent after ether extraction but cleared completely after 15 minutes digestion with trypsin.

Separate experiments were carried out to determine the proteolytic activity of the trypsin used. After ether treatment, but before tryptic digestion, the protein content of the virus solution was 0.296 g/100 ml. After 15 and 30 minutes tryptic digestion it was 0.205 g and 0.195 g/100 ml respectively.

The following samples were then centrifuged simultaneously in the Spinco angle centrifuge, each in the presence of 1% whelk haemocyanin. 1) Brain emulsion clarified by centrifugation at 2800 r.p.m. for 1 hour. 2) Brain emulsion clarified by centrifugation and

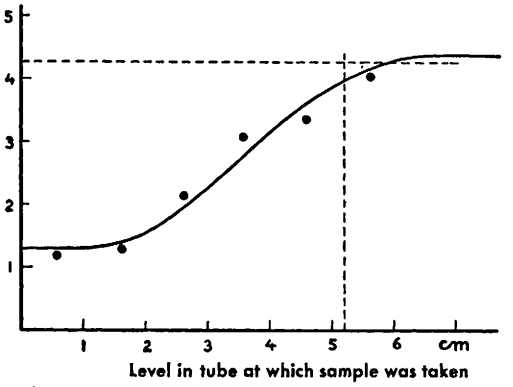

FIG. 2. Titres expressed in neg log LD_{50} of samples taken at different levels in centrifuge tube of virus spun at 19740 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD_{50}.

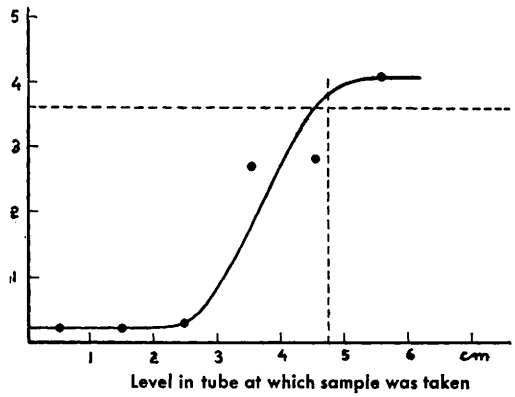

FIG. 3. Titres expressed in neg log LD_{50} of samples taken at different levels in centrifuge tube of virus after ether extraction and spinning at 19740 r.p.m. for 100 min. The horizontal interrupted line represents titre of original unspun material and the vertical interrupted line, position of virus boundary as estimated from equation (1). Ordinate = titre in neg log LD_{50}.

ether extraction. 3) Brain emulsion clarified by centrifugation, ether extraction and tryptic digestion. Successive layers were then removed from each of the tubes, and separately titrated for virus in order to determine the level of the sedimenting virus boundary. The results are recorded in Table II.

In all cases the haemocyanin boundary had moved down 2.7 cm. In Fig. 2 and 3 are recorded the virus titres (LD_{50}) of the different samples plotted against the levels in the centrifuge tubes from which the samples were taken. The vertical interrupted lines indicate the calculated position of the sedimentation boundaries.

In the case of ether treated suspensions less residual virus remains in the samples taken above the sedimenting boundary Fig. 3 than in the case of untreated suspensions. This observation had been confirmed in subsequent experiments. This may indicate that lipoids in untreated suspensions by adhering to the wall of the centrifuge tubes impedes the downward movement of the virus along the sides of the tubes during centrifugation.

It appears that the virus treated with ether and trypsin has been almost completely sedimented whereas in the other samples the virus has not reached the bottom of the tubes. There is certainly no evidence that ether treatment and tryptic digestion reduces the size of the infective virus particle as the sedimentation constant and consequently the particle size is again of the same order of magnitude as found in the previous experiment.

Summary and conclusions. 1. Using a Spinco preparative ultracentrifuge (Model L) a sedimentation constant of 163 S has been determined for the MEF$_1$ strain of poliomyelitis virus adapted to suckling mice. This sedimentation constant corresponds to a particle size of approximately 30 mμ when a particle density of 1.33 g/cc is assumed. This size is larger than that previously reported especially by investigators using ultrafiltration technics. 2. The high sedimentation constant is apparently not due to adsorption of the virus to tissue fragments. Thus preliminary clarification by ether and tryptic digestion did not reduce the speed of sedimentation of the virus during centrifugation. On the contrary such preliminary clarification, if anything, appeared to have increased the sedimentation constant of the virus. 3. The size calculated from results of the centrifugation experiments are in agreement with those obtained from electron photomicrographs and are probably closer to the correct value than those obtained by ultrafiltration.

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1. Theiler, M., and Bauer, H., *J. Exp. Med.*, 1934, v60, 767.
2. Elford, W. J., Galloway, I. A., and Perdrau, J. R., *J. Path. and Bact.*, 1935, v40, 135.
3. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, v72, 49.
4. Sanders, M., and Jungeblut, C. W., *J. Exp. Med.*, 1942, v75, 631.
5. Gard, S., and Pedersen, K. O., *Science*, 1941, v94, 493.
6. Tiselius, A., and Gard, S., *Naturwissenschaften*, 1942, v30, 728.
7. Gard, S., *Acta Med. Scand.* Suppl. 1943, 143.
8. Jungeblut, C. W., and Bourdillon, J., *J.A.M.A.*, 1943, v123, 399.
9. Loring, H. S., Schwerdt, C. E., and Marton, L., *Phys. Rev.*, 1944, v65, 354.
10. Loring, H. S., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 366.
11. Leyon, H., Gard, S., and Eklund, G., *Bioch. Acta*, 1950, v4, 385.
12. Reagan, L. R., Shenk, D. M., and Brueckner, A. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 42.
13. Polson, A., and Linder, A., In press.
14. Casals, J., Olitsky, P. K., and Anslow, R. O., *J. Exp. Med.*, 1951, v94, 111.
15. Selzer, G., Sacks, M., and van den Ende, M., *S.A.M.J.*, 1952, v26, 201.
16. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

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Bioautographic Analysis of Blood for Growth Factors* Active for *L. leichmannii* 4797. (19827)

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Previous observations by Couch *et al.*(1) on vit. B_{12} content of blood from various species have shown that the vit. B_{12} activity of turtle, chicken and turkey blood is higher than that of other species with the exception of the rabbit. These workers reported further that autoclaving blood samples from the chick, turkey and turtle at pH 12 destroyed only a part of the *L. leichmannii* activity. Such activity was explained on the basis of the fact that the latter species have nucleated erythrocytes and that the *L. leichmannii* response of the alkali treated and autoclaved blood samples was due to thymidine. A later study (2) reported values on vit. B_{12} content of blood from various species which were within the range of those reported by Couch *et al.*(1). The B_{12} content of blood from different breeds of cows has been determined in this laboratory(3). Blood samples from the Jersey breed contained the least B_{12} while those from Guernsey, Holstein and Brahman breeds were significantly higher.

The present study was designed to determine whether blood from species with nucleated erythrocytes contained growth factors for *L. leichmannii* 4797 other than vit. B_{12}.

Experimental. Five hundred ml of whole blood was collected from the cow, sheep and pig in oxalated containers. Blood samples from the chick, turkey and turtle were each pooled in order to obtain 500 ml of whole blood. In all cases blood samples were diluted with an equal volume of water, layered with toluene and incubated at 37°C for 24 hours. The autolysates were adjusted to pH 7 and autoclaved for 5 minutes at 120°C. After cooling, the samples were homogenized, centrifuged and the centrifugates were concentrated *in vacuo* to 20 ml. The vit. B_{12} content of each autolyzed blood sample (prior to concentration *in vacuo*) was determined according to the titrimetric method of Skeggs *et al.*(4) with *Lactobacillus leichmannii* 4797 as the test organism and crystalline vit. B_{12} as the standard. A 10 ml portion of each of the

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