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Ultrafiltration and Electron Microscopy of Three Types of Poliomyelitis Virus Propagated in Tissue Culture.* (20881)

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Previous work on filtration of poliomyelitis virus through gradocol membranes(1-3) was performed with Type 2 strains (MV, Lansing) propagated in the nervous system of monkeys or mice, and the extracts or stock filtrates actually used in the critical filtration experiments contained as little as one and not more than 100 infective doses. Using Elford's formula and different methods of estimating the average pore diameter (APD) which held back the virus, the size was variously reported as 8-12 mμ(2), 10-15 mμ (1,4), and 15-23 mμ(3). Since filtration endpoints may be affected both by the concentration of virus and the presence of extraneous tissue particles, it appeared possible that the size of the poliomyelitis viruses might actually prove to be smaller in tests with water-clear tissue culture fluids containing 10^6 to 10^8 infective doses per ml. Poliomyelitis virus of all 3 immunologic types, propagated in cynomolgus kidney tissue cultures, was studied by filtration through gradocol membranes and by electron microscopy.

Materials and methods. With one exception, the virus used in these tests was grown in 250 ml centrifuge bottles in a roller drum. The washed, minced cynomolgus kidney tissue, suspended in chick embryo extract, was embedded over the entire inner surface of the bottles previously coated with chicken plasma. The medium consisted of 0.5% lactalbumin hydrolysate (enzymatic—Nutritional Biochemicals Corp., Cleveland), Simms ox serum ultrafiltrate, Earle's balanced salt solution, 0.05 mg of crystalline soybean trypsin inhibitor, 100 units of penicillin and 0.1 mg of streptomycin per ml; 40 ml of this medium were used per bottle. After 7 days in the roller drum at 35°C, the bottles were inoculated with 0.1 ml of undiluted, virus-containing tissue culture fluid. Virus was harvested twice at 2- or 3-day intervals. In some tests only the first harvest was used, in others a pool of the two. The virus was stored in the frozen state in a chest containing solid CO_2. Virus titrations were performed in roller tubes of cynomolgus kidney tissue cultures. The gradocol membranes were purchased from St. Mary's Hospital, London, England. Prior to filtration of the tissue culture fluid, brain and heart infusion, proteose-peptone broth (Difco) was passed through the membranes—10 ml for membranes with an APD of more than 100 mμ and 5 ml for those less than 100 mμ. Filtration was performed under positive pressure of nitrogen—10 lb per square inch for membranes over 100 mμ and 15 lb for

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those under 100 mμ. The tissue culture fluid was centrifuged at about 2,000 rpm for 10 minutes on an ordinary horizontal centrifuge. The Type 1 and Type 2 poliomyelitis virus culture fluids passed through the 220 mμ membranes within a few minutes without evidence of clogging and the resulting filtrate was the stock used for the other membranes. The Type 3 culture fluids, however, quickly clogged even 1140 mμ membranes after filtration of the first 15 to 20 ml. Accordingly in the last test with this virus, a separate 1140 mμ membrane was used for each 20 ml of tissue culture fluid. The 1140 mμ filtrate did not clog the 680 mμ membrane, but the 680 mμ filtrate still clogged the 290 mμ membranes and 4 of these membranes had to be used to collect 94 ml of stock filtrate. The virus titer of the 290 mμ stock filtrate was about the same as that of the unfiltered tissue culture fluid. The amounts filtered through the other membranes varied from 10 to 18 ml. The diameter of the effective membrane surface was 2.7 cm. The identity of the virus preparations used in these tests was confirmed by immunologic tests.

Results of ultrafiltration. The data presented in Table I indicate that, despite the high virus content of the tissue culture fluids, the estimated size of the 3 types of poliomyelitis virus turned out to be larger rather than smaller than the 8-12 mμ(2) and 10-15 mμ (1,4) reported in the tests with extracts of monkey spinal cords of very low potency. In 2 of the present tests, in which the virus failed to pass the 50 mμ membranes, the stock filtrates had titers of $10^{5.2}$ and $10^{5.4}$ TCD_{50} (50% tissue culture cytopathogenic doses). Passage through the 44 mμ, and in one instance the 39 mμ, membrane was obtained only with stock filtrates of much higher potency. In all the tests, except the last with Type 3 virus, the inoculum in each tube was 0.2 ml and the number of tubes used for each filtrate was only 4; in the last test with Type 3 virus, in which passage through the 39 mμ membrane was achieved, the inoculum was one ml per tube and 5 tubes were used for each filtrate. With the variations just noted, the filtration end-point for all 3 types of poliomyelitis virus appears to be about the same and, according

TABLE I. Gradocol Membrane Filtration of 3 Types of Poliomyelitis Virus Propagated in Kidney Tissue Culture.

Membrane used		Type 1 (Mahoney) KP54-$10^{7.4}TCD_{50}$/ml			Type 2 (Y-SK) KP10-$10^{6.2}TCD_{50}$/ml		KP34-$10^{7.6}TCD_{50}$/ml		Type 3 (Leon) KP3-$10^{5.9}TCD_{50}$/ml		KP58-$10^{6.7}TCD_{50}$/ml	
APD, mμ	Thickness, mm	Amt filtered, ml	Result		Amt filtered, ml	Result	Amt filtered, ml	Result	Amt filtered, ml	Result	Amt filtered, ml	Result
290	.145										94	$10^{6.5}TCD_{50}$/ml
220	.174	62	1, 1, 1, 1*		55	$10^{5.2}TCD_{50}$/ml	80	1, 1, 1, 1	61	$10^{5.4}TCD_{50}$/ml		
79	.172						15	2, 2, 2, 3				
60	.151	15	1, 1, 1, 1				15	2, 2, 2, 2	10	2, 3, 6, 0	18	1, 1, 1, 1, 1
50	.158	15	2, 2, 2, 2		5	0, 0, 0, 0	15	2, 2, 2, 3	12	0, 0, 0, 0	18	1, 1, 1, 1, 2
44	.137	15	2, 2, 2, 2						10	0, 0, 0, 0	18	1, 1, 1, 1, 1
39	.123	15	0, 0, 0, 0				15	0, 0, 0, 0	13	0, 0, 0, 0	18	2, 2, 2, 2, 3
36	.116				12	0, 0, 0, 0					18	0, 0, 0, 0, 0
28	.092				12	0, 0, 0, 0	15	0, 0, 0, 0	13	0, 0, 0, 0		
Size based on Elford's formula		13-20 mμ			13-20 mμ				12-18 mμ			
Size of particles seen with electron microscope		30 mμ			27 mμ				41 mμ			

* Each figure refers to number of days after inoculation that a complete or almost complete cytopathogenic change was observed in one culture tube.

to Elford's formula, the size would be in the range of 12 to 20 mμ. It should be noted here that Elford, Galloway, and Perdrau(2) demonstrated unequivocal passage of virus only through membranes having an APD of 40 mμ or more, and based their estimate of size on the assumption that, if their virus preparations had been more potent, the membrane just failing to permit passage would have been 24 mμ. In the experiments of Theiler and Bauer(1) the virus passed regularly through membranes with an APD of 90 mμ or more, irregularly in the range of 67 to 40 mμ, and only once in 4 tests through a 35 mμ membrane, the filtrates infecting only one of 7 inoculated monkeys.

It appeared desirable, therefore, to determine by filtration through the same batch of membranes whether or not the same strain of virus propagated in the nervous system of monkeys might yield a certain proportion of particles that would be smaller than those produced in kidney tissue culture. A 20% uncentrifuged, frozen suspension of cynomolgus spinal cords, containing the Y-SK virus that initiated the kidney tissue cultures used in the present tests, was diluted to 1% with the tissue culture medium previously described. After centrifugation at 10,000 rpm in the No. 30 Spinco head (8,700 g) for 30 minutes in the cold and *in vacuo,* 96 ml of the supernatant liquid passed through a single 680 mμ membrane without any evidence of clogging in about 2 minutes. This filtrate then passed through a single 290 mμ membrane in about 8 minutes and the resulting stock filtrate was used for all the other membranes. The results shown in Table II are based on tests in kidney tissue culture tubes inoculated with one ml amounts of filtrate. Comparative titrations of this virus in monkeys and kidney tissue culture tubes indicated that the *in vitro* method may even be slightly more sensitive. The irregular results, which are similar to those reported by Theiler and Bauer(1). do not permit the conclusion that poliomyelitis virus propagated in the nervous system of monkeys can be smaller than that developing in tissue culture.

Observations with the electron microscope. Preparations from the following lots of tissue culture fluids were studied: a) *Control uninoculated fluid* consisting of a pool of 3 consecutive harvests made 11, 14, and 18 days after preparation of a large kidney tissue culture bottle in which medium No. 199 was used instead of the lactalbumin hydrolysate medium previously described; b) *Type 1 (Mahoney), kidney passage 30,* consisting of a pool of 2 successive harvests, which had $10^{7.9}$ TCD_{50} of virus per ml, the lactalbumin hydrolysate medium being used here; c) *Type 2 (Y-SK), kidney passage 10,* consisting of a pool of 2 successive harvests, which had $10^{6.2}$ TCD_{50} of virus per ml, the medium being No. 199 and an aliquot of this lot being used in the gradocol membrane filtration test; and d) *Type 3 (Leon), kidney passage 3,* consisting of a pool of 2 successive harvests, which had $10^{6.2}$ TCD_{50} of virus per ml, the medium being lactalbumin hydrolysate and an aliquot of this lot, twice diluted with Hanks' balanced salt solution, being used in the gradocol membrane filtration test. Each of these pools was centrifuged at low speed and stored in the frozen state in a chest containing solid CO_2 for several weeks before the specimens were prepared for electron microscopy.

TABLE II. Gradocol Membrane Filtration of Type 2 Poliomyelitis Virus (Y-SK) Propagated in Nervous System of Cynomolgus Monkeys. Titer of unfiltered virus = $10^{6.2}TCD_{50}$/g or $10^{4.2}TCD_{50}$/ml of 1% suspension.

APD of membrane, mμ	Amt filtered, ml	Time of filtration, min.	Result
290	96	8	$10^{8.5}TCD_{50}$/ml 2, 2, 2
60	12	20	6, 11, 0, 0, 0
50	20	50	0, 0, 0, 0, 0
44	20	50	3, 4, 6, 7, 10
39	20	60	6, 0, 0, 0, 0
36	20	74	0, 0, 0, 0, 0
Size based on Elford's formula			12-18 mμ

A total of 111 ml of each fluid was distributed in 3 chilled, stainless steel centrifuge tubes and spun at 8,700 g for 30 minutes in a chilled Spinco No. 30 rotor. A barely perceptible sand-gray sediment was present after the supernatant liquid was decanted. This supernatant liquid was again centrifuged in a cold Spinco No. 30 rotor at 70,000 g for one

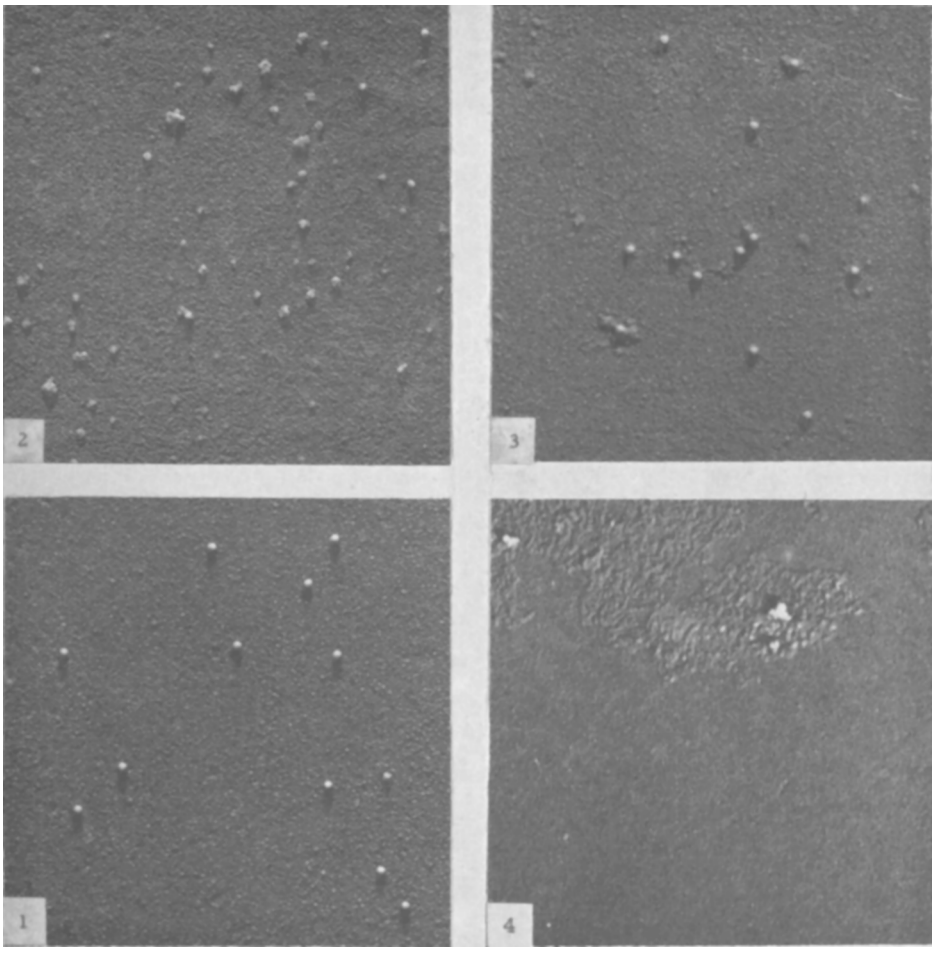

FIG. 1-4—Gold-manganin shadowed × 31667.

FIG. 1. Particles in tissue culture fluid of Type 1 poliomyelitis virus (Mahoney).
FIG. 2. Particles in tissue culture fluid of Type 2 poliomyelitis virus (Y-SK).
FIG. 3. Particles in tissue culture fluid of Type 3 poliomyelitis virus (Leon).
FIG. 4. Amorphous material found in some screens prepared from control uninoculated, but incubated, tissue cultures.

hour. The supernatant liquid was now discarded and, after allowing the inverted tubes to drain dry on gauze, they were carefully examined but no pellet of any kind was discernible. The tubes were rinsed with 5 ml of Seitz-filtered distilled H_2O, using a round-end capillary pipette forcibly to spray the sides and base of the tube. At the same time the tip of the pipette was gently rubbed along the surface of the tube to dislodge the invisible sediment. This "wash-fluid" was then diluted to 12 ml with water and centrifuged at 6,600 g for 30 minutes in steel tubes in a Spinco No. 40 rotor to remove any aggregates. The resulting supernatant liquid was then transferred to other tubes which were spun at 92,000 g for one hour. After discarding this supernatant liquid, 0.5 ml of water was used to wash each tube as before. This was water-clear, devoid of gross particles, and represented a 222-fold concentration of the original fluid. The final concentrates were not titered but, if one assumes that little or no virus was lost during this manipulation, the Type 1 material would have had $10^{10.2}$ TCD_{50} per ml, the Type 2 and Type 3—$10^{8.5}$ TCD_{50} per ml. The concentrates were placed on collodion-coated screens, shadowed with gold-

TABLE III. Size of Particles in Electron Microscope Photographs of Concentrates from Tissue Culture Fluids Containing 3 Types of Poliomyelitis Virus.

Type of poliomyelitis virus	No. of particles measured	Range of size, mμ	Mean size, mμ
1 (Mahoney)	117	28.9-33.3	30.3
2 (Y-SK)	103	23.2-29.6	26.9
3 (Leon)	115	37.1-43.5	41.2

manganin at arc tan 1/5 and observed with the RCA, EMU electron microscope. The electron micrographs were prepared by Mr. Lucien Caro.

Fig. 1-3 illustrate typical fields of the preparations from the virus-containing tissue culture fluids. Screens of the control, uninfected tissue culture fluid concentrate were generally free of significant debris; the appearance of occasional areas of amorphous material is shown in Fig. 4. The diameters of these particles as they appear on the negatives (not the prints) were measured with a precision measuring engine and the results are shown in Table III. The difference between the mean size of 27 mμ of the Type 2 particles and 30 mμ of the Type 1 particles is probably not significant. It is noteworthy that, after this work was completed, Stanley, Bachrach, and Schwerdt(5) reported finding particles having a diameter of 28 mμ in preparations from 2 other Type 2 strains of poliomyelitis virus, Lansing and MEF_1, and that the property of infectivity resides solely in the 28 mμ particle. Since no other significant particles were found in our tissue culture preparations, it is highly probable that those presented and measured in the accompanying photographs represent the virus. The somewhat smaller size of the poliomyelitis viruses obtained by gradocol membrane filtration using Elford's formula may have several explanations. It may be that the particles spread out and appear larger when they dry on the collodion film or it may mean that Elford's formula needs revision. In this respect it should be noted that both Leyon and Gard(6) and Melnick, Rhian, Warren, and Breese(3), on the basis of sedimentation data, assigned a size of 28 mμ to the Lansing strain of Type 2 poliomyelitis virus.

One still needs to consider, however, the marked discrepancy in the size of the Type 3 particles, mean of 41 mμ, particularly when the gradocol membrane filtration data were similar for all 3 types. While there is no obvious explanation for this discrepancy, it is perhaps noteworthy that the Type 3 cultures were found to have a "mucinous" or other "sticky" substance which quickly glazed and clogged the gradocol membranes. If this substance, either a part of the virus particles or adsorbed on them, were responsible for more extensive gold-manganin shadowing, it might explain the seemingly larger size of the Type 3 particles in the photographs obtained with the electron microscope. If one compares only the white spheroids, the Type 3 and Type 1 particles are more nearly alike in size.

Summary. When the concentration of virus in stock filtrates of the tissue culture fluids was greater than 10^6 TCD_{50} per ml, membranes with an APD of 39 mμ to 44 mμ allowed a certain proportion of poliomyelitis virus particles to pass. Membranes with an APD of 36 to 39 mμ held back the virus and, according to Elford's formula, the size of all 3 types of poliomyelitis virus is estimated at 12-20 mμ. After removal of gross amorphous material by preliminary centrifugation, the tissue culture fluids yielded practically pure suspensions of virus particles which measured, on the average, 30 mμ for Type 1 (Mahoney), 27 mμ for Type 2 (Y-SK), and 41 mμ for Type 3 (Leon). The seemingly larger size of the Type 3 particles may be an artifact.

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