THE SIZE OF THE VIRUS OF RABIES ("FIXED" STRAIN) BY ULTRAFILTRATION ANALYSIS

By I. A. GALLOWAY and W. J. ELFORD

From the National Institute for Medical Research, London, N.W. 3

The early literature on experimental rabies contains many records of unsuccessful attempts to filter the virus through the ordinary grades of bacteriological candle, Berkefeld and Chamberland. However, Remlinger (1903) found that the virus ("fixed" strain) would pass through Berkefeld V candles, but was completely retained by the Berkefeld N and W and Chamberland B (L 10) and F (L 5) grades. The material filtered was an untreated suspension in tap water of infective rabbit brain which had been ground well in a mortar. Cultures of *Pasteurella avicida* in broth medium were added to the suspension in certain experiments to control the impermeability of the candle to bacteria. Remlinger (1904) considered the failure of the virus particles to pass the less porous filters might be due to the blocking of the pores by tissue substances present in the crude suspension rather than to the large size of the virus particles themselves. This view he was able to substantiate, for after filtering the suspension through a Berkefeld V candle, the walls of which had been thinned by scraping, he found that the virus contained in the active filtrate could then be filtered successfully through the N and W grades of Berkefeld candle. The filterability of the "street" rabies virus was similar to that of the "fixed" strain.

More recently Marie & Urbain (1930), using a clarified (centrifugation) 10 per cent suspension of infective brain tissue in Martin's broth at *p*H 7·2, found that "fixed" rabies virus would pass a Chamberland L 3 candle, whereas the results of many of their earlier attempts to filter the virus using water as the suspension medium had been negative.

While other investigators too have reported the successful filtration of rabies virus through bacteria-proof candles, the observations of Remlinger and of Marie & Urbain have been referred to in some detail as affording a further illustrative role of the important factors to be recognized in filtering viruses, firstly the role of the medium, and secondly the necessity for freeing the suspension of relatively large tissue particles by preliminary centrifugation or filtration.

In the present studies the method of ultrafiltration analysis using the graded collodion membranes (gradocol), described by Elford (1931), has been employed to provide evidence of the particle size of rabies virus. The general lines of the technique have followed closely those already outlined in previous

studies from this laboratory (see Barnard & Elford, 1931; Galloway & Elford, 1931; Elford, 1933; and Elford & Galloway, 1933), and need not be repeated in detail here.

EXPERIMENTAL METHODS

Strain of virus. The strain of "fixed" rabies virus used was that of the Pasteur Institute, Paris. Rabbits inoculated with this strain of virus are generally *in extremis* in 8–10 days. With extremely small doses of virus the period of incubation is a little longer.

Stock filtrates. A weighed amount of infective rabbit brain was ground up with sterile quartz powder in Hartley's broth at *p*H 7·6 to give a 2 per cent suspension. (More concentrated suspensions than this were tried—up to 10 per cent—but the most consistent results were obtained with 2 per cent systems.) This was centrifuged for 15 min. at 2500 r.p.m. and the supernatant fluid then passed through a sand and pulp filter. Such preliminary treatment furnished a liquid which was slightly opalescent in appearance, and by subsequent filtration through a suitable membrane a bacteria-free stock filtrate of the virus was obtained. Eight such stock filtrates were prepared with membranes of grades 0·5–0·9 μ.

Method of testing filtrates. Two healthy young Himalayan rabbits, 1200–1500 g., were used for each test, the animals receiving 0·25 c.c. of the liquid by intracerebral inoculation. Such tests were made on serial tenfold dilutions of the filtrate. Surviving animals were tested for immunity. In two experiments where the rabbits died late (after 14 days), subinoculations were made into two fresh rabbits, and the presence of the virus established.

RESULTS OF FILTRATION EXPERIMENTS

The first stock filtrates prepared were very low in titre, the limiting infective dilution being only 1:10. An experiment was made in which the virus suspension was tested at each stage in the treatment. Thus after the preliminary centrifugation, the fluid was infective in a dilution 10^{-4} but not at 10^{-5}; after passing the sand and pulp filter it was infective at 10^{-3} but not at 10^{-4}; and finally the stock membrane filtrate (0·78 μ) was infective in a dilution 10^{-1} but not 10^{-2}. This suggested that the virus might be associated with cellular débris of varying degrees of dispersion, and from which the free virus particles become dissociated or disentangled only with difficulty. Experiments in the hope of finding conditions that would ensure a stock filtrate of greater potency were made with (*a*) glycerinated infective rabbit brains which had been stored at 0° C. for 1 month—the stock filtrate in this case was infective in a dilution of 10^{-2} but not at 10^{-3}; (*b*) portions of infected rabbit brains which were repeatedly frozen and thawed to facilitate the liberation of the virus from the tissue cells by autolysis—in this case too the limiting infective dilution of the stock filtrate was only 10^{-2}. These efforts failed to reveal a method that would be an improvement on the normal procedure of using fresh brain material which, as the data in the table show, furnished

stock filtrates varying in potency from a titre of 10^{-1} to 10^{-3}. The procedure adopted in the later experiments was to pool portions from several brains of infected rabbits. The titre of the sand and pulp filtrate from a broth suspension of this material was found to be 10^{-4}; that of a primary stock membrane filtrate ($0{\cdot}78\,\mu$) was 10^{-3}; and that of a secondary filtrate through a $0{\cdot}5\,\mu$

Table I. *Filtration of rabies virus ("fixed" strain) through membranes of graded porosities*

Date of exp.	A.P.D. membranes for stock filtrates μ	Titre of stock filtrates	A.P.D. of membranes for secondary filtrates μ	Vol. filtered c.c.	Test of secondary filtrates	Day of death of inoculated rabbits days	Subsequent control tests
29. vi. 33	0·78	10^{-3}	0·50	10	10^{-3}	9	—
21. iv. 33	0·80	10^{-1}	0·50	10	10^{-1}	14	Passage +
9. v. 33	0·90	10^{-2}	0·40	10	(1) +	(1) 10	—
					(2) +	(2) 10	
13. vi. 33	0·78	10^{-2}	0·35	10	(1) +	(1) 9	—
					(2) +	(2) 9	
2. vi. 33	0·78	10^{-1}	0·25	10	(1) +	(1) 10	—
					(2) +	(2) 14	Passage +
9. v. 33	0·90	10^{-2}	0·25	10	(1) +	(1) 9	—
					(2) +	(2) 9	—
21. iv. 33	0·80	10^{-1}	0·25	10	(1) 0	—	Not immune
					(2) 0	—	,,
13. vi. 33	0·78	10^{-2}	0·20	8	(1) 0	—	Not immune
					(2) 0	—	,,
2. vi. 33	0·78	10^{-1}	0·20	7·5	(1) 0	—	Not immune
					(2) 0	—	,,
29. vi. 33	*0·50	10^{-3}	0·20	12	(1) 0	—	Not immune
					(2) 0	—	,,

* $0{\cdot}50\,\mu$. This stock filtrate was obtained by preliminary filtration of the sand and pulp filtrate through a membrane of A.P.D. $0{\cdot}78\,\mu$.

\+ = rabbit died of rabies.

Filtrates through membranes A.P.D. $0{\cdot}20$–$0{\cdot}40\,\mu$ were tested only undiluted.

10^{-x} = limiting infective dilution or titre.

membrane also 10^{-3}. A similar observation was made in another experiment, in which the primary stock filtrate possessed a titre of only 10^{-1}, that the virus particles contained in a filtrate through a $0{\cdot}78\,\mu$ membrane may pass in undiminished amount through a second membrane of A.P.D. $0{\cdot}5\,\mu$. This suggests that the dispersion of the virus particles in the stock membrane filtrate is relatively uniform, and hence the big loss in infectivity during the preliminary clarification treatment is most probably due to the removal of coarse tissue particles with which virus remains associated. In other words, there is a very intimate connection between virus and cell, and the amount of virus that can be freely dissociated is small. Examination of the results given in the table shows that active filtrates were obtained through membranes of porosities ranging from $0{\cdot}5\,\mu$ down to $0{\cdot}25\,\mu$. Membranes of A.P.D. $0{\cdot}25\,\mu$ yielded active filtrates in two experiments out of three. Even under the most favourable conditions, no virus was detected in filtrates from membranes of A.P.D. $0{\cdot}2\,\mu$, and this porosity is therefore taken as the filtration end-point.

DISCUSSION AND SUMMARY

The filtration end-point of $0{\cdot}2\mu$ leads us to assign according to Elford (1933) a value 100–150 mμ for the particle diameter of the virus of rabies. This figure we have previously reported in *Ann. Report Med. Res. Council* (1934) and also in a discussion held by the Royal Society of Medicine (Galloway, 1936). Recently the Japanese workers, Yaoi *et al.* (1936), using similar graded collodion membranes (gradocol) and the same general technique as employed in the present experiments, have studied independently another strain of "fixed" rabies virus "Fukuoka". Their stock filtrates appear to have been more potent than ours, their figures for the limiting infective dilution being 10^{-3} to 10^{-4}. The incubation period in rabbits inoculated with the "Fukuoka" strain is also shorter than in the case of rabbits inoculated with Pasteur Institute (Paris) strain. Even so, they find exactly the same value for the filtration end-point $0{\cdot}2\mu$ as found by us for the Pasteur strain, thus indicating the particle size of these two "fixed" strains of rabies virus to be the same.

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