# THE FILTRATION AND CENTRIFUGATION OF THE VIRUSES OF RABBIT FIBROMA AND RABBIT PAPILLOMA

By M. SCHLESINGER and C. H. ANDREWES

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

It seemed important to us to estimate the sizes of the viruses of rabbit fibroma (Shope, 1932) and rabbit papilloma (Shope, 1933) because of their interesting relationship to the virus-tumour problem. The fibroma virus produces proliferative lesions in some ways intermediate between tumours and inflammatory reactions; the growths caused by the papilloma virus have the characters of tumours (Rous & Beard, 1934) and frequently become actual carcinomata (Rous & Beard, 1935).

## Material and methods

The fibroma virus used in these studies was the OA strain described by Andrewes (1936). Rabbits were inoculated with virus intratesticularly, and killed 3–7 days later; their testes were then minced and ground up with powdered Pyrex glass to give a 5 per cent suspension in a 1 : 1 broth-Ringer mixture. This was centrifuged, filtered through paper pulp and sand and then through a Gradocol (Elford, 1931) collodion membrane of average pore size (A.P.D.) 1$\mu$ to give a stock filtrate for further study. Fluids to be tested were inoculated intradermally into the shaved flanks of rabbits in falling tenfold dilutions. The resulting skin lesions were examined daily between 3 and 8 days later and the virus titres of the fluids estimated accordingly.

As a source of papilloma virus we used warts from cottontail rabbits kindly sent in 50 per cent glycerol by Dr R. E. Shope. This material readily infected domestic rabbits but, like some other observers, we have had difficulty in transmitting the virus serially in tame rabbits. The warts were washed in saline to remove some of the glycerol; then 5 per cent suspensions in broth-Ringer were clarified by filtering through asbestos pulp, and stock filtrates obtained by filtration through a Gradocol membrane of A.P.D. 0·70–1$\mu$. The asbestos pulp gave a better clarification than paper pulp and sand; it was satisfactory for the papilloma virus but not for that of the fibroma, too much of which was lost by adsorption. Papilloma virus was titrated by rubbing dilutions into scarified areas on the flanks of rabbits. For this purpose areas of skin were closely clipped, but to avoid contamination of one site from another ridges of unclipped hair were left between the bare areas.

Filtrations through graded collodion (Gradocol) membranes were carried out as described in papers by W. J. Elford and his collaborators (Elford, 1931; Elford & Andrewes, 1932, 1935). Positive pressure of air or nitrogen, not greater than 1 atmosphere, was used.

Centrifugation was carried out in an Ecco centrifuge by the method described by Bechhold & Schlesinger (1931) and Schlesinger (1932). Virus suspensions were placed in wide flat-bottomed tubes and when these were centrifuged the virus was deposited on to a disk of thick filter-paper at the bottom of the tube. The method gives results in good agreement with those obtained by Elford (1936) by a different technique. In his paper Elford (1936) describes and compares the two methods. When Schlesinger's technique is employed the virus diameter is calculated from the formula

$$\alpha = 6{\cdot}15 \times 10^8 \sqrt{\left(\frac{\eta h \log C_0/C_t}{(\sigma_p - \sigma_m) RTN^2}\right)},$$

where $\alpha$ = particle diameter in m$\mu$, $\eta$ = viscosity of medium in c.g.s. units, $\sigma_p$ = density of particle, $\sigma_m$ = density of medium, $h$ = height of liquid column, $R$ = distance of filter-paper from axis of rotation, $N$ = r.p.m.

In the experiments dealt with in this paper $\eta = 0{\cdot}011$, $\sigma_m = 1{\cdot}009$ and $R = 8$ cm.

## Filtration of fibroma virus

Table I shows the results of experiments on filtration of the fibroma virus.

It will be seen that the stock filtrates never had a high titre; usually 0·1 c.c. failed to infect intradermally when diluted more than 1 : 1000. Thus loss of virus by adsorption can probably be held responsible for the rather irregular results obtained.

Table I. *Filtration of fibroma virus*

| Exp. | Rabbit no. | Titre of stock filtrate | Amount filtered c.c. | A.P.D. of membrane | Virus in filtrate | Percentage passing filter |
|---|---|---|---|---|---|---|
| 1 | 100 | $10^3$ | 10 | 0·72 | +(undil.) | 0·1 |
| | 101 | $10^3$ | 10 | 0·72 | 0 | <0·1 |
| 2 | 102 | $10^4$ | 5 | 0·72 | $10^2$ | 1 |
| | 102 | $10^4$ | 30 | 0·72 | $10^3$ | 10 |
| | 103 | $10^3$ | 5 | 0·72 | $10^3$ | 100 |
| | 103 | $10^3$ | 30 | 0·72 | $10^3$ | 100 |
| 3 | 72 | $10^3$ | 7·5 | 0·51 | +(undil.) | 0·1 |
| | 73 | $10^3$ | 7·5 | 0·51 | +(undil.) | 0·1 |
| 4 | 64 | $10^3$ | 5 | 0·42 | 0 | <0·1 |
| | 65 | $10^3$ | 5 | 0·38 | +(undil.) | 1 |
| | 65 | $10^3$ | 5 | 0·38 | +(undil.) | 1 |
| 5 | 116 | $10^2$ | 10 | 0·27 | 0 | <1 |
| | 117 | 10 | 10 | 0·27 | 0 | <1 |

Elford & Andrewes (1932) showed that vaccinia virus was held back by membranes of very different size according to the virus titre of the fluid filtered. When they used stock filtrates having a titre of only about $10^3$, as in the above experiments with fibroma, vaccinia virus was usually retained

by a membrane of A.P.D. 0·4$\mu$. Yet with a vaccinia filtrate of titre $10^6$ they obtained an active filtrate through a 0·25$\mu$ membrane. From the results in Table I it would be difficult to give a precise estimate of the size of the fibroma virus. If, however, one compares the findings with those of vaccinia filtrates having a similar titre one can probably infer that its diameter is not very different from that of vaccinia, estimated by Elford & Andrewes at 125–175 m$\mu$. This result is in accord with Paschen's (1936) estimate, on optical grounds, of the size of the fibroma elementary body as 150 m$\mu$.

### FIBROMA CENTRIFUGATION EXPERIMENTS

The results of centrifuging fibroma stock filtrates in the Ecco centrifuge are given in Table II.

Table II. *Centrifugation of fibroma virus*

| | | | Particulars of centrifugation | | | | |
|---|---|---|---|---|---|---|---|
| Exp. | Rabbit no. | Titre of stock filtrate | Time min. | Speed r.p.m. | Height of column of fluid (cm.) | Titre of supernatant | Percentage remaining in supernatant |
| 6 | 58 | $10^2$–$10^3$ | 42 | 10,000 | 2·0 | 10 | 1–10 |
| | 58 | $10^2$–$10^3$ | 42 | 10,000 | 0·5 | ? + undil. | ?0·1–1 |
| 7 | 65 | $10^3$ | 30 | 10,000 | 2·0 | $10^3$ | 10–100 |
| | 65 | $10^3$ | 60 | 10,000 | 1·6 | 10 | 1 |
| | 66 | $10^3$ | 30 | 10,000 | 2·0 | $10^3$ | 10–100 |
| | 66 | $10^3$ | 60 | 10,000 | 1·6 | 10 | 1 |
| 8 | 82 | $10^2$ | 30 | 10,000 | 2·0 | 10 | 10 |

In Exp. 7 the fluid was centrifuged for 30 min.; then a sample was taken and the supernatant spun again for 30 min. Another sample of supernatant fluid taken at the end of that time was reckoned as the result of 1 hour's spinning.

The specific gravity of the virus was estimated by centrifuging suspensions made up in saccharose solutions of different concentrations and therefore of different specific gravities. The aim was to find a medium of such density that no deposition of virus would occur. In order to obtain greater centrifugal force than was obtainable with the Ecco centrifuge we used the Sharples closed bowl technique described by Schlesinger (1936). In this method the inner wall of the rotating cylinder is first coated with a thin layer of agar, which is allowed to set, and then a comparatively small amount of virus-containing fluid is introduced. When the cylinder is rotating this forms a thin layer over the agar and the particles have a very small distance to traverse before meeting and becoming stuck to the agar. A force equal to 20,000 × gravity is readily obtained. Preliminary experiments showed that the method was as readily applicable to viruses as large as that of the fibroma as to the bacterio-phages studied earlier. The control fluid was kept, for the duration of the centrifugation, in a thin layer in a Petri dish over agar containing sugar in the same proportions as in the fluid being centrifuged. Centrifugation experiments

with the fibroma virus in various sugar solutions showed that after spinning for 75 min. at 33,000 r.p.m. there was still just detectable deposition in a medium of specific gravity 1·25. The specific gravity of the virus was therefore taken as 1·3. No attempts at direct calculation of the virus size from experiments with the Sharples centrifuge were made.

From the data in Table II the size of the virus could be calculated by means of the formula given earlier. The values for the diameter of the virus obtained were 126 m$\mu$ (Exp. 7) and 141 m$\mu$ (Exp. 8). These figures agree well with those obtained by filtration.

### Filtration of papilloma virus

In Table III are seen the results of experiments on filtration of the papilloma virus. In many experiments the filtrates were not titrated; it is, however, possible in work with this virus to estimate roughly the relative amounts of virus present in two fluids by comparing the incubation periods and extent of the lesions.

Table III

| Exp. | Rabbit no. | Titre of stock filtrate | Amount filtered c.c. | A.P.D. of membrane $\mu$ | Virus in filtrate | Percentage passing filter |
|---|---|---|---|---|---|---|
| 9 | 59 | 100 | 5 | 0·34 | + | 10–100 |
| | 60 | 100 | 5 | 0·34 | + | 10–100 |
| | 61 | 1000 | 5 | 0·34 | + | 10–100 |
| 10 | 68 | 1000 | 7·5 | 0·27 | + | 10–100 |
| | 68 | 1000 | 7·5 | 0·23 | + | 10–100 |
| | 69 | 1000 | 7·5 | 0·27 | + | 10–100 |
| | 69 | 1000 | 7·5 | 0·23 | + | 10–100 |
| 11 | 74 | 10 | 20 | 0·16 | + | 10–100 |
| | 75 | 100 | 20 | 0·16 | + | 10–100 |
| 12 | 98 | 10 | 10 | 0·11 | 0 | <10 |
| | 99 | 10 | 10 | 0·11 | + | 1–10 |
| 13 | 104 | 10 | 10 | 0·11 | + | 1–10 |
| | 105 | 100 | 10 | 0·11 | + | 10 |
| 14 | 133 | 1000 | 10 | 0·08 | + | 0·1 |
| | 134 | 100 | 10 | 0·08 | + | 1 |
| 15 | 147 | 1000 | 9 | 0·088 | +(1 : 10 neg.*) | 0·1 |
| | 147 | 1000 | 9 | 0·072 | +(1 : 10 neg.*) | 0·1 |
| | 148 | 100 | 9 | 0·088 | 1 : 10 | 10 |
| | 148 | 100 | 9 | 0·072 | +(1 : 10 neg.*) | 1 |

* Negative.

It is clear from the table that down to a membrane of A.P.D. 0·16 $\mu$ nearly all the virus passed through, even though its initial titre was low. With tighter membranes progressively more and more was held back until filtrates through membranes of A.P.D. 0·072 $\mu$ produced in each of two rabbits a single wart after an incubation period of 37 days. This steady fall in the amount of virus passing membranes from 0·16 to 0·072 $\mu$ probably indicates that the true end-point is not far below an A.P.D. of 0·072 $\mu$. Actually no tighter membranes were tested as there was no more virus available of the only batch which in our hands would infect in fairly high dilutions. Virus of a much less active

batch was wholly retained by filters of 0·11 $\mu$ A.P.D. These data indicate for the virus diameter a value of 23–35 m$\mu$, assuming, as Elford does (1933), that the ratio of the limiting pore-size to the virus diameter in this range is between 3 : 1 and 2 : 1. We may be criticized for calculating the virus-size directly from the filtration data in the case of the papilloma virus while refraining from doing so with the fibroma data, though the two viruses were active to about the same titre. The reason is that the results of filtration through asbestos and through coarser membranes suggested that adsorption played a large part in the fibroma experiments, while there was little evidence of this with the papilloma virus.

### CENTRIFUGATION OF PAPILLOMA VIRUS

The results of centrifuging filtrates of papilloma virus are seen in Table IV.

Table IV. *Centrifugation of papilloma virus*

| | | | Particulars of centrifugation | | | | |
|---|---|---|---|---|---|---|---|
| Exp. | Rabbit no. | Titre of stock filtrate | Time hr. | Speed r.p.m. | Height of column of fluid (cm.) | Titre of supernatant | Percentage remaining in supernatant |
| 16 | 84 | 1000 | 1 | 10,000 | 1 | >100 | >10 |
| | 85 | 10 | 1 | 10,000 | 1 | 10 | >10 |
| 17 | 98 | 10 | $2\frac{1}{3}$ | 9,500 | 1 | 0 | <10 |
| | 99 | 10 | $2\frac{1}{3}$ | 9,500 | 1 | 10 | >10 |
| 18 | 133 | 100 | 2 | 9,500 | 0·9 | Undil. + | ?10 |
| | 133 | 100 | 4 | 9,500 | 1·0 | Undil. | 1 |
| | 134 | 100 | 2 | 9,500 | 0·9 | 10–100 | >10 |
| | 134 | 100 | 4 | 9,500 | 1·0 | Undil. | 1 |

In Exp. 18 the filtrate was spun for 2 hours, sampled and the supernatant again centrifuged for a further 2 hours. The object was to test whether the particles in the first supernantant fluid would deposit at the same rate as those in the stock filtrate, i.e. whether the virus particles were of fairly uniform size. It appears that they were uniform, so far as the limits of accuracy of the method permit us to judge.

An attempt was made to estimate the specific gravity of the virus by centrifugation in the Sharples centrifuge in sugar solutions of different densities as described earlier. Such experiments showed deposition of about 99 per cent of the virus after centrifugation for 2 hours at 32,000 r.p.m. in a medium of specific gravity 1·25. Its specific gravity is therefore possibly rather greater than that of the fibroma virus and calculations have accordingly been made both for a specific gravity of 1·3 and for one of 1·4. If it is 1·3, calculation from the data in the table gives values for the virus diameter of 49 m$\mu$ (Exp. 17), 50 and 37 m$\mu$ (Exp. 18). If the specific gravity is taken as 1·4 these figures become 42, 43 and 32 respectively. The outside limits are thus 32–50 m$\mu$. These values will be seen to be rather higher than those (23–35 m$\mu$) obtained by filtration. Papilloma virus purified by Beard & Wyckoff (1937) and called by them a high molecular weight protein was

thought by them, on the basis of its sedimentation rate, to have a particle diameter of about 40 mμ. It is of interest that the papilloma virus seems to have a size appreciably smaller than that of the Rous sarcoma (70–100 mμ—Elford & Andrewes, 1935).

SUMMARY

The diameter of the virus of rabbit fibroma has been estimated by filtration through graded membranes to be about the same as that of vaccinia (125–175 mμ) and by centrifugation to be 126–141 mμ.

That of the rabbit papilloma virus appeared by filtration to be 23–35 mμ and by centrifugation 32–50 mμ.

Dr Schlesinger's tragic death took place after the experiments above reported had been completed but before they had been described in writing. I am deeply grateful to my colleague Dr W. J. Elford for making some determinations of specific gravity and viscosity on virus suspensions, for making calculations from the data in the tables and also for providing the Gradocol membranes used. (C. H. A.)

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