Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 16, *Numbers* 1 *and* 2, *January and April*, 1941.

Printed in the Union of South Africa by the
Government Printer, Pretoria.

The Particle Size of African Horsesickness Virus as Determined by Ultrafiltration and Ultracentrifugation.

By ALFRED POLSON, Section of Protozoology and Virus Diseases, Onderstepoort.

THE methods available for the determination of the particle size of virus have been described by Elford (1938). This excellent critical review needs no elaboration so that, in any study at the present time, it is necessary merely to record details of technique together with any modifications essential for the adaptation of a particular method to a given virus.

It is considered advisable to check and confirm results by using at least two different methods. The choice of methods will depend upon the apparatus and facilities available as well as the characteristics of the virus to be studied. In the case of horsesickness virus, several different strains of mouse neurotropic virus were used, and determinations were made by filtration through gradocol membranes and by Elford's inverted capillary modification of the Bechhold and Schlesinger centrifugation method.

A. GRADOCOL FILTRATION.

The series of graded membranes used were prepared and calibrated in this laboratory. For this preparation a small room was fitted with a double door to eliminate draughts, arrangements were made for the thermostatic control of temperature to within 0·5° C., humidity was accurately controlled and ventilation was provided by means of an electric fan fitted to a vent in such a way that no noticeable draught was produced. For the rest the apparatus was exactly that described by Elford and demonstrated repeatedly during the course of visits to his laboratory in London.

At the commencement of the work it was decided to use the nitro cellulose solution supplied by Nobel Chemical Finishes Ltd., under the commercial name Necol No. 356 A/9 supplied in sealed metal 1-gallon cases. Although Elford's technique was followed with meticulous care it was soon apparent that constant and reproducible results could not be obtained. The reason for this is quite obscure but it may be related to prolonged storage of the collodion solution in the cans. In the meantime a supply of Parlodion had been

ordered from the Mallinckrodt Chemical Works, New York, as recommended by Bauer and Hughes (1935). When this was used for the preparation of the parent collodion solution excellent results were obtained immediately. Merck's reagents were used throughout. The absolute ethyl alcohol was dried by shaking with calcium oxide and distilling. Anhydrous ether was dried over sodium wire and distilled. Glacial acetic was dried by fractional freezing four times. Amyl alcohol was used as supplied by Merck without further purification.

The pore sizes of the membranes were determined by the rate of flow of the water method. The apparatus used was manufactured by Messrs. Chemical Glassblowers, Gray's Inn Road, London, to specifications kindly furnished by Elford and was inspected by him prior to shipment to South Africa. The thickness of the membranes was measured by means of a Zeiss micrometer gauge. The porosity of three discs from each large membrane was measured and if a variation greater than 4 per cent. was obtained, all the discs were discarded. In addition the porosity of stored discs were checked from time to time to ascertain whether any change had taken place.

As an additional check as soon as the approximate size of the virus had been determined, a series of membranes was ordered from the vaccine department of St. Mary's Hospital, London. The results of rate of flow of water determinations and of the virus filtration end point with these membranes corresponded well within the limits of experimental error.

Virus strains used and the Preparation of Stock Filtrates.

Six antigenic different strains of neurotropic alternated virus were used in the experiments. These are the strains at present in use for the preparation of routine horsesickness vaccine; quite arbitrarily they have been labelled 449 (mouse generation 151), 1180 (generation 201), Vryheid (generation 125), KA, OD (generation 103) O (generation 150). These strains were used to relieve the monotony of repeated checking of results with one strain, and to determine whether there was any appreciable difference in the size of virus particles of different antigenic structure.

Virus emulsions were prepared by triturating the brains of mice, destroyed by ether anaesthesia when moribund after intracerebral infection, in 80 cc. of Hartley's broth pH 7·6. The suspensions were then clarified by centrifugation for 20 minutes at a speed of 10,000 revolutions per minute in a water cooled Ecco Ultima centrifuge. This procedure produced a remarkably clear emulsion but it was soon apparent that sufficient colloidal material remained in suspension to seriously hamper filtration even through very coarse membranes. Further clarification of the stock emulsion was attained by filtration through either sand and paper pulp, asbestos pulp, Seitz pads or Berkefeld candles. The method used depended upon the results of preliminary trials to determine primarily the reduction in virus titre of the filtrate since it was found that there were marked differences in the behaviour, even of different strains of the same virus. An illustration of this point is shown in the results detailed in

Table I. One emulsion of strain 449 and one of strain Vryheid after preliminary centrifugation, were filtered through Seitz E.K. discs under identical conditions. In each case 20 cc. of Hartley's broth was passed through the disc as a preliminary washing before filtering the virus emulsion.

TABLE I.

Titration of Strains Vryheid and 449 before and after Seitz Filtration.

Dilution.	Strain Vryheid.		Strain 449.	
	Unfiltered Emulsion.	Seitz Filtrate.	Unfiltered Dilution Emulsion.	Seitz Filtrate.
10^1	4; 4; 4;	5; 5; 5;	3; 4; 4;	3; 4; 4;
10^2	4; 4; 4;	6; 0; 0;	4; 4; 4;	4; 4; 4;
10^3	4; 5; 6;	×; 0; 0;	4; 4; 5;	4; 5; 5;
10^4	0; 0; 0;	0; 0; 0;	4; 5; 0;	4; 5; 5;
10^5	—	—	—	—

In this and all subsequent tables the numeral indicates the number of days after intracerebral injection the mouse died.
0 = Survived.
× = Death from some cause other than horsesickness usually traumatic injury.
— = Injection not done.

It will be seen that Seitz filtration resulted in at least 100 fold reduction in titre of strain Vryheid, whereas the titre of strain 449 was not affected. This finding with strain Vryheid was confirmed on several occasions.

Briefly it may be stated:—

(*a*) Clarification through sand and paper pulp filters caused little or no decreases in virus titre but the filtrates were found to contain an appreciable amount of material in suspension.

(*b*) Asbestos pulp filters were more satisfactory. The filtrates were clear and the reduction in virus titre was negligible.

(*c*) The behaviour of Berkefeld candles was variable. Usually the filtrates were clear but frequently absorption of virus was excessive.

(*d*) Seitz filtration produced the clearest filtrates but as illustrated above the reduction in virus titre might be too great.

Usually a bacteria free stock filtrate was prepared by filtration of the clarified emulsion through a coarse gradocol membrane.

Technique of Gradocol filtration.

A battery similar to that described by Bauer and Hughes was erected. All-metal stainless steel membrane holders of 25 c.c. capacity

were used. A disc of Whatman hardened filter paper No. 541 was interposed between the membrane and the perforated metal supporting-plate to increase the effective filtering area. Positive pressure (usually 1-2 atmospheres) was supplied from a cylinder of compressed nitrogen through an accurate valve. At least 5 c.c. of Hartley's broth was passed through each membrane before adding the clear virus emulsions. A minimum of 10 c.c. of fluid was passed through each membrane before removing a sample for determination of the presence or absence of virus. Filtrations of virus control were carried out by intracerebral injection of decimal dilutions into white mice obtained from the locally bred stock of proved susceptibility. The dose injected was 0·05 c.c.

Experimental.

A. *Filtration of Strain* 449.

The stock virus emulsion after centrifugation was clarified by filtration through a Seitz E.K. disc. It was then passed through a membrane of APD 268 mμ. The results of filtration through the series of gradocol membranes are given in Table 2, recording 3 different experiments.

Table 2.

Filtration of Strain 449 *through Gradocol Membranes.*

Dilution.	APD of Membranes.					
	268 mμ.	171 mμ.	126 mμ.	115 mμ.	104 mμ.	80·6 mμ.
1	4, 5, 5	5, 5, 5	5, 5 ×	5, 5, 6	8*, 0, 0	0, 0, 0
10^1	5, 5, 5	4, 5, 7	5, 6, 6	5, 0, 0	×, 0, 0	—
10^2	5, 5, 5	**6, 6, 0**	**5, 7, 0**	**5, 5, 0**	—	—
10^3	6, ×, ×	0, 0, 0	×, 0, 0	0, 0, 0	—	×
10^4	6, ×, ×	×, ×, 0	0, 0, 0	—	×	×
10^6	6, 0, 0	×, 0, 0	—	—	—	—
1	4, 4, 4, 5	5, 5, 5, 5	5, 5, 6, 6	**4, 7, 0, 0**	**6, 6, 7*, 9**	0, 0, 0, 0
10^1	4, 5, 5, ×	5, 5, 5, 5	**6, 6, 0, 0**	4, 0, 0, 0	0, 0, 0, 0	—
10^2	5, 5, 5, 5	**9, 9,** ×, 0	5, ,0 0, 0	—	—	—
10^3	5, 5, 6, 7	0, 0, 0, 0	—	—	—	—
10^4	7, 0, 0, 0	—	—	—	—	—
1	4, 4, 4	4, 4, 4	4, 4, 5, 5	4, 5, 5, 5	5, 5, 5, 5	—
10^1	4, 4, 4	4, 4, 4	—	—	—	—
10^2	4, 4, 5	5, 5, 5	—	—	—	—
10^3	5, 5, 5	5, 5, 0	—	—	—	—
10^4	**5, 5, 0**	**5, 6, 0**	—	—	—	—

* These mice were destroyed *in extremis* on the 8th and 7th day respectively. A brain emulsion subinoculated into other mice caused 100 per cent. mortality on day 3 and 4, thus indicating the presence of horsesickness virus.

Results.

The stock filtrate (that which had been passed through the 268 mμ membrane) contained virus in comparatively high titre, and was well suited for further filtration. There was a progressive decrease in virus titre on filtration through membranes of smaller average pore diameter. Virus was detected in low concentration in the filtrate that passed the 104 mμ membrane but was retained completely by the 80·6 mμ membrane.

In Figures 1 and 2 the titres of the filtrates are plotted against the four sizes of the respective membranes used in Experiments 1 and 2 above. The significance of these graphs is described later.

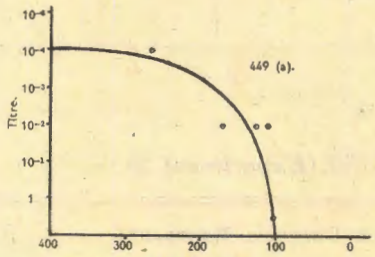
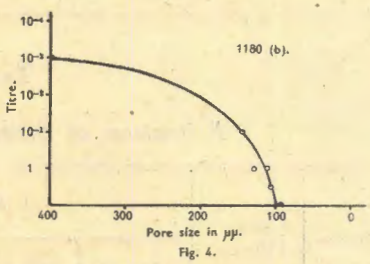


Fig. 1.



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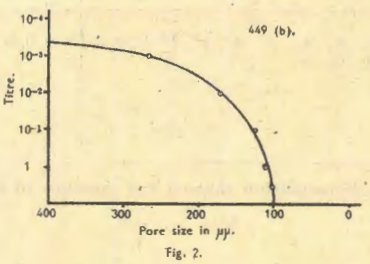
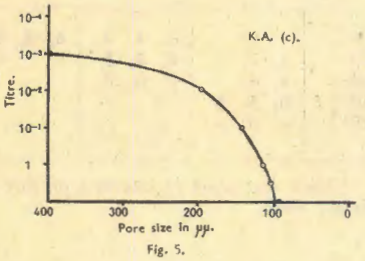


Fig. 2.



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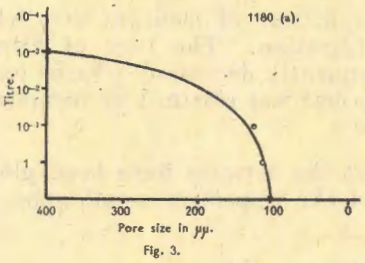

Fig. 3.

B. *Filtration of Strain* 1180.

Preliminary experiments showed that Seitz filtration did not materially reduce the titre of this strain of virus. The clear Seitz filtrate was used as stock emulsion without further clarification. The results of two experiments are shown in Tables 3 and 4.

TABLE 3.

Filtration of Strain 1180 (*Experiment* 1).

Dilution.	Seitz Filtrate.	APD of Gradocol Membranes.			
		126 mμ.	115 mμ.	104 mμ.	86·7 mμ.
1	—	5, 5, 5	**4, 5, 0**	0, 0, 0	0, 0, 0
10^1	4, 4, ×	**5, 6, 0**	0, 0, 0	—	—
10^2	**4, 4, 5**	0, 0, 0	—	—	—
10^3	**5, 6, 0**	6, 0, 0	—	—	—
10^4	0, 0, 0	—	—	—	—

TABLE 4.

Filtration of Strain 1180 (*Experiment* 2).

Dilution.	Seitz Filtrate.	APD of Gradocol Membranes.				
		145 mμ.	131 mμ.	113 mμ.	106 mμ.	92 mμ.
1	—	4, 4, 5	**4, 5, 0**	4, 4, 5, ×	4*, 4*, 4*, 5, 0, 0	0, 0, 0, 0, 0, 00
10^1	3, 5, 5	**5, 5, 5**	6, 0, 0	0, 0, 0	—	—
10^2	4, 4, 5	5, 0, 0	—	—	—	—
10^3	**4, 5, 6**	—	—	—	—	—
10^4	5, 0, 0	—	—	—	—	—

* Mice destroyed *in extremis* on day 4. Subinoculation showed the presence of horsesickness virus.

Results.

Seitz filtration produced a clear filtrate of medium titre which was eminently suited for further filtration. The titre of filtrates which passed the fine membranes frequently decreased. Virus passed through a membrane of APD 106 mμ but was retained by membranes of 104 mμ and smaller pore diameter.

In Figures 3 and 4 the titres of the filtrates have been plotted against the average pore diameter of the respective membranes.

C. *Filtration of Strain KA.*

Clarification by Seitz filtration of emulsions of this strain of virus caused too pronounced a reduction in the titre that the filtrates were quite unsuitable for further investigation. Eventually it was found that suitable emulsions could be prepared by passage through a Berkeveld V candle, followed by a membrane of 402 mμ APD.

TABLE 5.

Filtration of Strain KA.

Dilution.	APD of Gradocol Membranes.					
	402 mμ.	193 mμ.	143 mμ.	115 mμ.	104 mμ.	92 mμ.
1	4, 4, 4	4, 4, 6	4, 4, 4	**5, 5, 0**	5, 6*, 6*, 0, 0, 0	0, 0, 0, 0, 0, 0
10^1	4, 4, 5	5, 6, 6	**5, 5, 6**	0, 0, 0	—	—
10^2	4, 5, 6	**5, 6, 0**	6, 0, 0	—	—	—
10^3	**5, 5, 5**	0, 0, 0	0, 0, 0	—	—	—
10^4	7, 0, 0	—	—	—	—	—
10^4	7, 0, 0	—	—	—	—	—

* Horsesickness confirmed by subinoculation.

In Figure 5 the titre of the filtrates is plotted against the average pore diametre of the respective membranes.

In a second experiment the titre of the various filtrates was not determined; mice were injected with undiluted filtrate to determine the presence or absence of virus. All mice died that received filtrates which had passed membranes of 193, 145 and 113 mμ APD; 3 out of 9 mice that received 108 mμ filtrate died, the period of incubation being lengthened by about 3 days; there were no deaths amongst 9 mice that received the 92 mμ filtrate.

Results.

Again it was shown that a small amount of virus was able to squeeze through a membrane with APD of 104 mμ but all virus was retained by the 92 mμ membranes.

D. *Filtration of Strain Vryheid.*

The marked adsorption of this strain of virus by Seitz discs, made this method of preliminary clarification quite unsatisfactory. One experiment was completed with a Seitz filtrate as stock emulsion. The titre of this stock emulsion was less than $^1/_{100}$, a trace of virus was shown to pass a membrane with an APD of 170 mμ but all virus was retained by the 107 mμ and smaller membranes. This experiment clearly indicates not even the desirability but the necessity of commencing with a stock filtrate of high titre if any weight is to be attached to the results of gradocol filtration experiments.

In a second series of experiments the stock filtrate was clarified by filtration through asbestos pulp followed by Berkefeld V filtration followed by a membrane with an APD of 488 mμ. Both filtrates had a titre of 10^4. The titres of other filtrates were not determined accurately. All the mice that received material which

had passed the 170, 145, 128 and 112 mμ membranes died; 1 out of 4 mice that received filtrate from a 107 mμ membrane and 1 out of 3 from a 104 mμ membrane died and the presence of horesickness virus was demonstrated by passage. No virus passed an 87 mμ membrane.

E. *Filtration of Strain O.*

The stock was clarified by passage through a membrane of 464 mμ APD after preliminary clarification through a Berkefeld V candle. In one experiment 2 out of 4 mice that received 113 mμ filtrate died but all virus was retained by the 107 mμ membrane. In a second experiment all 3 mice that received 107 mμ filtrate died but no virus succeeded in passing the 92 mμ membrane.

F. *Filtration of Strain OD.*

Out of six attempts a single successful experiment was carried out with this strain. The stock emulsion was clarified by filtration through an asbestos pulp filtrate. Of six mice that received filtrate which passed through a membrane of 107 mμ APD , 3 died; no virus passed a 92 mμ membrane.

Discussion.

In considering the results of filtration experiments on strains of neurotropic horsesickness virus characterized by different antigenic structure using a standard technique with gradocol membranes of decreasing porosity, it is evident that if there is any difference in the size of virus particles, it is of an order which cannot be demonstrated by this method. All of the strains were able to pass a membrane with an average pore diameter of 107 to 104 mμ even though in exceeding low concentration; no virus was found capable of passing a membrane with an APD of 92 mμ or less. From this it may be concluded that the limiting membrane is one with a porosity of approximately 100 mμ. This contention is supported by co-ordination of figures 1-5. In these figures the titres of the filtrate of 3 virus strains are plotted against the average pore diameter of the respective membranes. These end-point curves show that the abscissa axis is intersected at approximately the point 100 mμ which may be taken as the end point porosity.

Applying Elford's factor for the relation of the pore diameter to the end point porosity it is found that the diameter of neurotropic horsesickness virus is $100 \times 0{\cdot}4$ to $0{\cdot}6 = 40$ mμ to 60 mμ with a mean of 50 mμ.

B. Determination by the inverted Capillary Tube method of Centrifugation.

Again Elford's review of the application of this method to the determination of the particle size of viruses is so comprehensive that

it is necessary merely to note details of technique and to describe some modifications which were found to be desirable for the application to neurotropic horsesickness virus.

The capillary centrifuge cell was turned out of M.V.C. aluminium silicon alloy, the dimensions being such as to fit the cups of an Ecco ultima water cooled centrifuge. In the end of each cell 7 capillaries were drilled each 2 mm. in diameter to a depth of 1 cm. thus the contents of the capillaries in each cell amounted to approximately 0·2 c.c. Preliminary experiments showed that contact of the virus emulsion with the M.V.C. alloy had some harmful effect upon the virus. This was overcome by coating the cell and capillaries with a fine layer of brushing duco which remained firmly attached and had no tendency to flake off under the centrifugal force during spinning. Immediately prior to use, the cells were dipped into molten paraffin wax, any excess of wax being removed by vigorous shaking. This additional film of wax was renewed before each experiment. By this method it was found that there was no decrease in the titre of an emulsion after more than 6 hours contact. The capillaries were filled by means of finely drawn Pasteur pipettes. At the conclusion of one experiment it was found more convenient and more accurate to determine the amount of fluid withdrawn from the capillaries by weighing. Since the virus is more stable in 10 per cent. horse serum saline than in Hartley's broth, all brain emulsions were prepared in serum saline. Before use, the serum was clarified by filtration through a Berkefeld V filter and was inactivated at 56° C. for 30 minutes. The density of the serum saline was kept constant at 1·006 gm./c.c. even though this entailed slight variations in the percentage of serum; density determinations were made by means of a hygrometer. Prior to centrifugation the virus emulsions were clarified by spinning at 9,000 revolutions per minute for 20 minutes followed by filtration through Seitz E.K. discs (strain 449) or through asbestos pulp filters (all other strains).

Constancy of temperature during centrifugation is a most important factor. It was found that in spite of the cooling jacket of circulating tap water there was a tendency for the temperature of the fluids in the cups to rise several degrees during the course of a run of only half an hour. This was overcome by setting up the centrifuge and allowing it to run at 10,000 revolutions per minute for about half an hour. It was found that the temperature rose a few degrees to a point dependent upon the room temperature and the temperature of the circulating water, at which heat it remained constant. The capillaries were then filled and the critical experiment allowed to proceed.

The speed of centrifugation was determined by means of a stroboscopic arrangement similar to that described by Bauer and Pickles (1936). In order to be certain that the correct stroboscopic pattern was being viewed it was found advantageous to have a series of 50 slots on the periphery of the revolving disc and an inner series of 25 holes. By viewing the different fathoms through both sets of apertures the characteristic double fathom could be picked up with care and certainty.

For calculation of the particle diameter in mμ Elford's formula was used, viz:

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log \times}{\Delta \sigma \, N^2 t}}$$

$$\text{Where } \times = \frac{x_1 + l}{x_1 + l} \frac{C_t}{C_o}$$

In this equation:—

d = diameter of the particle in mμ.

η = viscosity of the dispersion medium in Pois.

x_1 = distance of the upper closed end of capillary from the axis of rotation = 6·7 c.m.

l = length of capillaries = 1 c.m.

C_t/C_o = relation of the titre after centrifugation to the titre before centrifugation.

N = number of revolutions per minute of the centrifuge.

t = time of centrifugation in minutes.

$\Delta\sigma$ = difference in density between the particle and the dispersion medium.

Elford found that the sedimentation within the capillaries followed Stokes law where the relation Ct/Co was small namely in the neighbourhood of 0·1 which is the limit of accurate titration of the majority of viruses. In the case of neurotropic horsesickness viruses it has been shown and confirmed repeatedly (Alexander 1935) that it is possible to titrate in mice to two-fold limits with reasonable accuracy. Consequently this procedure was adopted in the experiments to be described, the end points of titration being taken as the dilution which killed half of the injected mice, taking into consideration the sum of the results with material from each of the two capillary cells used. The speed and time of centrifugation was regulated so that the reduction in titre varied from 4 to 20 times. In fig. 6 the calculated size of the virus particles is plotted against the reduction in titre after centrifugation. It is seen that within this range the graph is a straight line parallel with the abscissa so that the conclusions drawn from the experiments in this range are valid.

The viscosity of the dispersion medium, i.e., the 10 per cent. serum saline specific gravity 1·006 gm. per c.c. was measured over a range of temperatures by means of an Ostwald viscosimeter. In fig. 7 the viscosity in Pois is plotted against temperature in degrees Centigrade. By substitution of the temperature of centrifugation in the curve the viscosity can be read off directly.

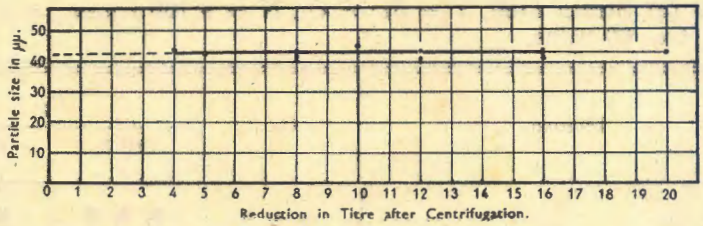

Fig. 6.

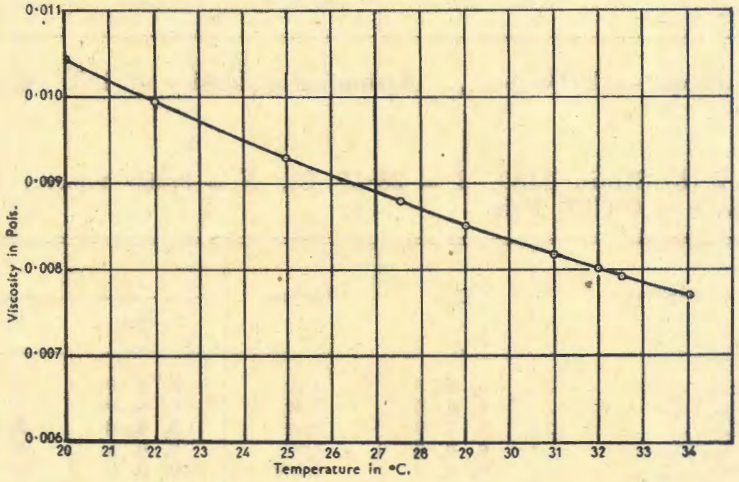

Fig. 7.

Experimental.

1. Strain 449, T = 33·7° C., N = 11,500 r.p.m., η = 0·0076 Pois, t = 32 mins.

Dilution.	C_o.	Dilution.	C_t. Cell 1.	C_t. Cell 2.
1/100	×, **6, 7**	1/10	5, 0, 0	5, 6, 0
1/200	5, 0, 0	1/20	**6, 0, 0**	**6, 0, 0**
1/400	0, 0, 0	1/40	0, 0, 0	6, 0, 0
1/800	0, 0, 0	1/80	0, 0, 0	0, 0, 0

Result.—The reduction in titre is 5 fold thus C /C = 1/5. Assuming a density of the virus particle of 1·3, d = 42·8 mμ.

2 (*a*) Strain 1180, T = 31·5° C., N = 9,700 r.p.m., η = 0·0081 Pois, t = 60 mins.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 4, 0	**6, 6, 0**	**5, 6, 0**
1/20	5, 5, 5	0, 0, 0	6, 0, 0
1/40	5, 5, 0	0, 0, 0	0, 0, 0
1/80	4, 4, 5	0, 0, 0	0, 0, 0
1/160	**6, 6, 0**	—	—
1/320	5, 0, 0	—	—
1/640	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3. d = 41·7 mμ.

2 (*b*) Strain 1180, T = 28·5° C., N = 9,560 r.p.m., t = 60 min., η = 0·0086 Pois.

Dilution.	C_o.	Dilution.	C_t.	
			Cell 1.	Cell 2.
1/100	5, 5, 5	1/10	5, 6, 0	5, 6, 0
1/200	6, 6, 6	1/20	6, 6, 0	6, 5, 0
1/400	6, 7, 0	1/40	**5, 6, 0**	**5,** ×, ×
1/800	**6, 6, 0**	1/80	0, 0, 0	0, 0, 0
1/1,600	0, 0, 0	1/160	0, 0, 0	5, 0, 0

Result.—Ct/Co = $^1/_{20}$. Assuming a density of 1·3. d = 42·5 mμ.

2 (*c*) Strain 1180, T = 34·0° C., N = 11,000 r.p.m., t = 31 min., η = 0·0077 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 5	4, 6, 0	5, 6, 0
1/20	5, 5, 0	**4, 0, 0**	**5, 5, 0**
1/40	5, 5, 6	0, 0, 0	0, 0, 0
1/80	**6, 6, 0**	0, 0, 0	0, 0, 0
1/160	0, 0, 0	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $\frac{1}{4}$. Assuming a density of 1·3, d = 44·2 mμ.

3 (*a*) Strain Vryheid. T = 30° C., N = 10,000, t = 60 min., η = 0·00834 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 7	**5, 7, 0**	**6, 6, 0**
1/20	4, 5, 6	7, 9, 0	0, 0, 0
1/40	5, 5, 6	0, 0, 0	0, 0, 0
1/80	5, 5, 6	0, 0, 0	0, 0, 0
1/160	**6, 8, 0**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3. d = 40·9 mμ.

3 (*b*) Strain Vryheid, T = 28° C., N = 10,000 r.p.m., t = 60 min., η = 0·0087 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	4, 4, 5	**8, 0, 0**	**7, 7, 0**
1/20	4, 6, 6	0, 0, 0	6, 0, 0
1/40	5, 5, 5	0, 0, 0	0, 0, 0
1/80	5, 6, 6	0, 0, 0	0, 0, 0
1/160	**4, 5, 0**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3. d = 42·4 mμ.

4 (*a*) Strain OD, T = 28° C., N = 10,000 r.p.m., t = 60 min., η = ·0087 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 5	**7, 0, 0**	**5, 8, 0**
1/20	4, 5, 5	6, 0, ×	5, 0, 0
1/40	6, 6, 0	0, 0, 0	0, 0, 0
1/80	7, 7, 0	0, 0, 0	0, 0, 0
1/160	**6, 7, 9**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3. d = 41·8 mμ.

4 (*b*) Strain OD, T = 28·9° C., N = 9,950 r.p.m., t = 60 min., η = 00855 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 6	**6, 7, 0**	**6, 0, 0**
1/20	5, 6, 6	0, 0, 0	7, 0, 0
1/40	6, 6, 7	0, 0, 0	0, 0, 0
1/80	6, 7, 0	0, 0, 0	0, 0, 0
1/160	**7, 7, 0**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3, d = 41·8 mμ.

5 (*a*) Strain KA, T = 27° C., N = 9525 r.p.m., t = 60 min., η = 0·0089 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 5	0, 0, 0	6, 7, 0
1/20	5, 5, 6	**5, 6, 0**	**7, 0, 0**
1/40	5, 5, 5	0, 0, 0	5, 0, 0
1/80	4, 6, 0	0, 0, 0	0, 0, 0
1/160	**5, 7, 0**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_8$. Assuming a density of 1·3, d = 42·8 mμ.

5 (*b*) Strain KA, T = 28° C., N = 9675 r.p.m., t = 60 min., η = 0·0087 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 6	**6, 6, 0**	**5, 5, 6**
1/20	5, 6, 6	6, 0, 0	0, 0, 0
1/40	5, 5, 6	0, 0, 0	0, 0, 0
1/80	5, 5, 6	0, 0, 0	0, 0, 0
1/160	**5, 6, 0**	—	—
1/320	0, 0, 0	—	—

Result.—Ct/Co = $^1/_{16}$. Assuming a density of 1·3, d = 43·3 mμ.

6 (*a*) Strain O, T = 28·4° C., N = 10,000 r.p.m., t = 60 min., η = 0·00862 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 7, ×	8, 8, 0	7, 9, 9
1/20	6, 6, 7	**8, 8, 0**	**8, 9, 10**
1/40	6, 7, 10	0, 0, 0	0, 0, 0
1/80	7, 8, 8	0, 0, 0	0, 0, 0
1/160	**8, 8, 0**	—	—
1/320	7, 0, 0	—	—

Result.—Ct/Co = $\frac{1}{8}$. Assuming a density of 1·3, d = 40·2 mμ.

6 (*b*) Strain O, T = 29·3° C., N = 9800 r.p.m., t = 60 min., η = 0·00845 Pois.

Dilution.	C_o.	C_t.	
		Cell 1.	Cell 2.
1/10	8, 8, 9	8, 0, 0	8, 8, 0
1/20	7, 7, 0	**9, 0, 0**	**8, 9, 0**
1/40	7, 7, 9	0, 0, 0	0, 0, 0
1/80	8, 8, 0	0, 0, 0	0, 0, 0
1/160	**8, 8, 0**	—	—
1/320	7, 0, 0	—	—

Result.—Ct/Co = $\frac{1}{8}$. Assuming a density of 1·3, d = 40·3 mμ.

In Table 6 below, the results of 12 experiments on 6 different strains of virus are summarized.

TABLE 6.

The Particle size of Horsesickness Virus determined by inverted Capillary Centrifugation.

Strain.	DIAMETER OF VIRUS PARTICLE IN mμ.			
	Exp. (*a*).	Exp. (*b*).	Exp. (*c*).	Average.
449	42·8	—	—	42·80
1180	41·7	42·5	44·2	42·80
Vryheid	40·9	42·4	—	41·65
OD	41·8	41·8	—	41·80
KA	42·8	43·3	—	43·05
0	40·2	40·3	—	40·25
				42·06

Comment.—These determinations have all been made on the assumption that the density of the virus is 1·3 gm. per c.c., i.e., the density of ordinary proteins, and that the particles are spherical in shape.

There is no significant difference in the calculated diameter of virus of the six strains. The slight variation in size shown in the table above is well within the boundary of experimental error when due consideration is paid to the fact that a biological test is necessary to determine the virus titres of the different emulsions.

C. Determination of the Density of the Virus Particles.

Since it is impossible by present methods to obtain pure virus in weighable amounts the pyknometric method of density determination is excluded. The method of zero sedimentation by centrifugation in cane sugar solutions of sufficient density to reduce the rate of sedimentation to zero was considered impracticable because of the long time of centrifugation and possible harmful effect of concentrated sugar solutions on the virus. Consequently the density was calculated from measured rates of sedimentation in two media of different density using a modification of Elford's formula shown below.

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta_1 \log \times_1}{(\sigma - \sigma_1)\, N_1^2\, t_1}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots a_1$$

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta_2 \log \times_2}{(\sigma - \sigma_2)\, N_2^2\, t_2}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots a_2$$

Where σ is the density of the particles and σ_1 and σ_2 the densities of the two different media.

Dividing a_1 by a_2 gives the equation:—

$$\frac{(\sigma - \sigma_1)\, N_1^2\, t_1}{(\sigma - \sigma_2)\, N_2^2\, t_2} = \frac{\eta_1 \log \times_1}{\eta_2 \log \times_2}$$

from which the value may be calculated.

Data for the rate of sedimentation in a medium of one density was obtained from the centrifugation experiments detailed above, e.g., in normal horse serum saline of density 1·006 gm./cm.$^{-3}$. Two experiments were selected which gave mean value of d which approximated most closely to the mean of all the 12 recorded experiments, viz., Vryheid *b* and *a*. To obtain data applicable to centrifugation in a medium of different density, a clarified virus emulsion was diluted with cane sugar solution in sodium chloride until the final concentration was 18 per cent. cane sugar and 1 per cent sodium chloride. The density of this solution was 1·072 gm. per c.c. and preliminary experiments showed that it had no harmful effect upon the virus. The viscosity was determined by means of an Ostwald viscosimeter. Strain 449 was used in all the determinations.

Experimental.

1. T = 25·5° C., N = 10,000 r.p.m., t = 120 min., σ_1 = 1·072 gm. per c.c, η_1 = 0·0162 pois.

Dilution.	C_0.	C_t.	
		Cell 1.	Cell 2.
1/10	4, 4, 6	4, 4, 5	5, 5, 6
1/20	4, 4, 5	5, 5, 6	5, 5, 6
1/40	4, ×, ×	4, 4, ×	5, 7, 0
1/80	4, 5, 0	**6, 0, 0**	**6, 6, 7**
1/160	4, 5, 5	7, 0, 0	0, 0, 0
1/320	**6, 6, 0**	0, 0. 0	0, 0, 0

Result.—Ct/Co = $\frac{1}{4}$.

2. T = 28° C., N = 10,000 r.p.m., t = 120 min., σ_1 = 1·072 gm. per c.c., η_1 = 0·153 pois.

Dilution.	C_0.	C_t.	
		Cell 1.	Cell 2.
1/10	4, 4, 4	5, 5, 5	4, 5, 7
1/20	5, 5, 5	5, 5, 0	5, 6, ×
1/40	4, 5, 5	5, 6, 6	5, 5, 7
1/80	5, 5, 6	**6, 7, 0**	**5, 6, 0**
1/160	5, 5, 6	6, 0, 0	5, 0, 0
1/320	**5, 6, 0**	0, 0, 0	0, 0, 0

Result.—Ct/Co = $\frac{1}{4}$.

3. T = 27° C., N = 10,000 r.p.m., t = 135 min., σ_1 = 1 072 gm. per c.c. η_1 = 0·0156 Pois.

Dilution.	C_0.	C_t.	
		Cell 1.	Cell 2.
1/10	5, 5, 5	4, 4, 0	4, 5, 5
1/20	4, 4, 4	4, 5, 5	4, 4, 6
1/40	4, 5, 5	**4, 5, 7**	**5, 7, 0**
1/80	5, 5, 5	5, ×, 0	0, 0, 0
1/160	4, 4, 4	0, 0, 0	0, 0, 0
1/320	**4, 6, 0**	0, 0, 0	0, 0, 0

Result.—Ct/Co = $\frac{1}{8}$.

By substitution in the above formula the density of horsesickness virus particles is calculated as follows, using the data of experiment, Vryheid (*b*) and 1180 (*a*):—

	Vryheid (*b*)	1180 (*a*)
Experiment 1	1·26 gm./c.c.	1·27 gm./c.c.
Experiment 2	1·227 gm./c.c.	1·233 gm./c.c.
Experiment 3	1·257 gm./c.c.	1·266 gm./c.c.

Mean 1·252 gm. per c.c.

Using this value of 1·25 gm. per c.c. and recalculating *d* in all the experiments in part 2 it is found that a mean value 45·4 mμ is obtained.

Summary.

(1) Details of the technique employed for the preparation of gradocol membranes are given together with the technique employed in the filtration of neurotropic horsesickness virus.

(2) From data collected from the filtration of 6 strains of virus it is found that the limiting membrane is one with a porosity of 100 mμ. Hence the diameter of the virus particles is from 40-60 mμ with a mean of 50 mμ.

(3) Details of the technique used for determinations by the inverted capillary method of centrifugation are given.

(4) The method used for determining the density of the virus particles is given. By this method a density of 1·25 gm. per c.c. was calculated.

(5) By the cetrifugation method a mean value of 45·4 mμ for the particle diameter was calculated.

(6) The particle diameter determined by both methods agree closely.

(7) There was no significant difference in the size of the virus particles of 6 strains of different antigenic structure.

Acknowledgments.

In conclusion I wish to acknowledge my great indebtedness to Dr. R. A. Alexander for his guidance, helpful advice and criticism. Further I am greatly indebted to Dr. P. J. du Toit for enabling this work to be carried out.

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