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The Particle Size of Bluetongue Virus as determined by Ultrafiltration and Ultracentrifugation.

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A FULL description of the technique used for a similar investigation of physical characteristics of the virus of horsesickness has been given previously (Polson 1941). Since that time the bisectable capillary method of centrifugation as a modification of or improvement upon Elford's inverted capillary method has been described (Polson 1941). In the experimental work to be recorded in this article the same methods have been used so that it is necessary to detail only those modifications of technique which have been adopted with a view to ease of procedure and greater accuracy. These are concerned chiefly with improvements in the methods of preparing infective emulsions for filtration and centrifugation.

MATERIALS AND TECHNIQUE.

Virus.

A single antigenic strain of bluetongue virus was used, that known as the "Bekker" strain [Bekker, De Kock and Quinlan, (1934), Neitz (1948)]. When the investigation was commenced the virus was available only in the sheep virulent form in the 15th passage through sheep after its original isolation from cattle. Later the chick embryo adapted virus [Alexander (1947)] became available and this was used for a considerable extension of the work.

Preliminary Clarification of Virus Infected Tissue Emulsions.

(*a*) *Sheep Virulent Virus.*—Sheep were destroyed approximately 48 hours after the first rise in temperature after infection with stored virulent blood, since it has been found experimentally that at that stage the virus titre is at its maximum. The spleens were removed with aseptic precautions and passed through a latapie mincer. To the resulting pulp sufficient saline was added to make an approximate 10 per cent. emulsion which was thoroughly agitated and rapidly frozen and thawed several times to disintegrate the cellular material prior to clarification for preparation of the stock emulsion. Preliminary investigation showed that clarification by the standard method of filtration through sand and paper or asbestos pulp filters either left so much tissue debris in suspension that the gradocol membranes were clogged and gave false filtration end points, or alternately reduced the

virus titre so much that the filtrate was of little value for further filtration or centrifugation. Consequently two further methods of clarification were adopted.

1. *Clarification by aluminium Hydroxide.*—The aluminium hydroxide used was prepared as follows: A 5 per cent. $Al_2(SO_4)_3$ solution was heated to 60° C. and excess 10 per cent. ammonia added. The precipitated $Al(OH)_3$ was filtered off and washed on the filter paper until the test for sulphate ions was negative. The precipitate was scraped off the filter paper and stored as a gel in the refrigerator at 4° C. The crude 10 per cent. saline spleen emulsion was centrifuged for 3 hours at 3,000 r.p.m. in a Clay Adams angle centrifuge. The supernatant fluid was decanted and diluted with an equal volume of 0·133 M phosphate buffer in saline pH 7·9. To 40 c.c. of this buffered emulsion 1·5 gm. of aluminium hydroxide gel was added, thoroughly mixed and placed in the refrigerator for half an hour. The mixture was then spun for 10 minutes in a horizontal centrifuge at 3,000 r.p.m. The clear, slightly opalescent supernatant fluid was passed through a coarse gradocol membrane with an average pore diameter of 700-800 mμ to provide the final bacteria free clarified virus suspension. By this procedure apparently the coarser tissue debris is adsorbed to the gel leaving the finely dispersed virus particles in a state of considerable purity in suspension.

2. *Clarification by Trypsin Digestion.*—The supernatant fluid obtained after angle centrifugation of the crude 10 per cent. saline spleen emulsion for 3 hours at 3,000 r.p.m. was adjusted to pH 8. This slightly turbid fluid was warmed to 37° C., Merck's crude trypsin in powder form added to make a final concentration of 0·01 per cent. and digestion allowed to continue in the incubator at 37° C. for 20 minutes. The fluid which still contained fatty materials in suspension was shaken up with an equal volume of ethyl ether. After it has stood for a few minutes in a separating funnel the bottom layer was separated and centrifuged for 10 minutes at 3,000 r.p.m. The fatty materials separated off as a thick white layer on top of the clear watery solution in the lower parts of the centrifuge cell. The ether dissolved in the watery solution was removed by suction. This fluid which could then be passed through a 700 mμ gradocol membrane with great ease was used as the stock filtrate.

With the sheep virulent virus no opinion can be expressed as to whether either of these procedures resulted in a significant reduction in virus titre since it was not possible to carry out comparative infectivity titrations. At least stock filtrates were produced which contained an adequate virus concentration for the further experimental work, and the minimum quantities of particulate matter other than virus particles present did not interfere with further filtration. This was indicated by the ease of filtration through all membranes with A.P.D.'s down to that in the vicinity of the virus limiting membrane.

(*b*) *Chick Embryo Adapted Virus.*—Embryos after 8 days preliminary incubation were infected with egg adapted virus by injection into the volk sac. After 24 hours incubation at 35° C. followed by transfer to 32° C. for 48 hours the dead embryos were harvested and emulsified without the addition of any diluent to provide a tissue pulp of high virus titre (Alexander 1947). The supernatant fluid obtained by angle centrifugation of this pulp

was clarified either by aluminium hydroxide adsorption or trypsin digestion as above to furnish the requisite stock filtrates. As it was possible to carry out this work in eggs strictly on a quantitative basis it was early apparent that clarification by aluminium hydroxide adsorption did not reduce the virus titre more than the best results obtained with sand and pulp filtration.

Gradocol membranes.—Elford's gradocol membranes were prepared and calibrated in the laboratory by the usual technique (Polson 1941).

Centrifugation.—High speed centrifugation was carried out in an Ecco Ultima water-cooled centrifuge the speed being measured stroboscopically.

Experimental.

A. *Estimation of Particle Size by Gradocol Membrane Filtration.*

1. *Sheep Virulent Virus.*

In table 1 are given the results of a series of experiments on sheep. To test for the presence or absence of virus sheep were given a subcutaneous injection of 5 c.c. of the filtrates obtained from membranes of progressively decreasing A.P.D. The subsequent febrile and clinical reactions were recorded daily and the results were confirmed by applying an immunity test approximately 28 days later. For the immunity test virulent sheep blood was used in a dose of 2 c.c. subcutaneously. The reactions produced in those sheep which did not react to the injection of a filtrate served to control the avirulence of that filtrate as well as the virulence of the blood used for the immunity test.

Table 1.

Estimation of Particle Size by Gradocol Membrane Filtration Sheep Virulent Virus.

Method of Clarification.	A.P.D. of Gradocol Membranes in mμ.										
	700	554	376	350	260	226	212	200	195	189	166
Aluminium hydroxide.....	R.I.	—	—	R.I.	R.I.	—	—	—	-	N.R.	N.R.
Aluminium hydroxide.....	R.I.	—	R.I.	—	—	—	—	—	—	N.R.	N.R.
Aluminium hydroxide.....	R.I.	—	—	—	—	—	R.I.	—	N.R.	—	- -
Trypsin digest.....	R.I.	R.I.	R.I.	—	R.I.	R.I.	--	—	N.R.	—	—
Trypsin digest.....	R.I.	R.I.	—	—	—	R.I.	—	N.R.	—	N.R.	--
Seitz filtration.....	R.I.	—	—	—	R.I.	N.R.	—	—	N.R.	--	—

Note.—R.I. = reacted to injection of filtrate and later found immune.
N.R. = did not react to injection of filtrate and later proved susceptible.

Result.

Using a stock filtrate clarified by Seitz filtration the virus was found to pass the 260 mμ membrane but was retained by the 226 mμ membrane. As this was the only occasion on which the virus was

retained by a 226 mμ membrane it is assumed that the discrepant result was due to an initial low virus titre of the stock filtrate. On the one occasion when a 200 mμ membrane was used the filtrate was not infective. Filtrates from all other membranes of higher or lower porosity were active or inactive as would have been anticipated.

Conclusion.

From this series of experiments it is concluded that the average pore diameter of the limiting membrane for sheep virulent bluetongue virus is approximately 200 mμ. Applying Elford's formula for the relation between pore size and particle diameter (Elford 1938) the diameter of the virus is calculated as 100-150 mμ.

2. *Chick embryo adapted virus.*

All this work was carried out quantitatively. The virus titres of the various filtrates were determined by serial 10-fold dilution in eggs after 8 days preliminary incubation and then maintained at 33·6° C. for 7 days (Alexander 1945); 6 eggs were used for each dilution and dead embryos in the vicinity of the 50 per cent. lethal end point were harvested for subinoculation to determine whether death was due to the specific action of the virus or not. This procedure greatly increased the accuracy of the titrations. Virus titres were calculated by the method of Reed and Muench and are expressed logarithmically. The results of a series of experiments are given in Table 2.

TABLE 2.

Estimation of Particle Size by Gradocol Membrane Filtration. Chick Embryo Adapted Virus.

Method of Clarification.	L.D. 50 of Gradocol Membrane filtrates (A.P.D. in mμ).							
	880	350	250	230	200	195	191	178
Asbestos filter.....	4·364	2·6364	0·5000	—	—	0	—	0
Asbestos filter.....	3·6525	2·7500	—	0	0	—	—	—
Asbestos filter.....	4·6110	4·0000	Trace.	0	0	—	—	—
Aluminium hydroxide.....	4·0000	—	1·9036	0·5262	0	—	—	—
Aluminium hydroxide.....	3·8752	—	>2·0000	1·0000	0·5000	—	—	—
Aluminium hydroxide.....	4·8447	—	—	>2·0000	>1·0000	—	0	—

NOTE.—Trace: 1 out of 6 eggs which received undiluted filtrate died and on subinoculation was found to contain virus in high titre.

Result.

When filtration through asbestos pulp was used for preliminary clarification the average pore diameter of the limiting membrane was found to be between 230 and 250 mμ. Since the virus titre of the stock emulsion had not been materially decreased the results of the 3 experiments, which

are obviously incorrect in the light of all the other determinations, have been disregarded, and are believed to be due to reduction of the pore diameter of the membranes by clogging with particulate cellular debris. When adsorption on to aluminium hydroxide was used for clarification the initial virus titre was approximately the same, yet in all those experiments, virus passed through the 230 mμ membrane; in two out of three experiments virus in relatively low concentration was present in the 200 mμ filtrate and on the only occasion tested was absent from the 191 mμ filtrate.

Conclusion.

It is concluded that the limiting membrane is slightly less than 200 mμ, the virus particle having a calculated diameter of slightly less than 100-150 mμ.

Comment.

The results of this series of experiments once again emphasises the necessity for taking the greatest care with the preliminary clarification of stock virus emulsions if entirely erroneous conclusions are to be avoided. Although the work on the sheep virulent virus can hardly be regarded as comprehensive due entirely to the excessive number of sheep which would be required for such an investigation, yet the results obtained agree closely with those carried out in greater detail with the egg adapted variant.

B. Estimation of the Particle Size by Centrifugation.

The method of bisectable capillary centrifugation (Polson 1941) was used in preference to Elford's inverted capillary method (Elford 1938), the chief reason being that with the inverted capillary technique calculations based on reductions of titres greater than ten-fold give values for the particle diameter which are too low. [Elford (1938), Markham, Smith and Lea (1942)].

The formula developed by Elford for calculating the size of a virus particle from the rate of sedimentation in inverted capillaries was applied namely:—

$$d = 7{\cdot}94 \times 10^7 \sqrt{\frac{\eta \log X}{\triangle \sigma N^2 t}} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

$$\text{where } X = \frac{x_1 + l}{x_1 + l} \frac{C_t}{C_a}$$

x_1 = distance of axis of ritation from the perifery of the cell.

η = viscocity of the medium.

N = revolutions per minute.

t = time of centrifugation in minutes.

$\triangle \sigma$ = difference in density between particle and medium.

$\frac{C_t}{C_a}$ = relation between virus concentration after and before centrifugation.

d = diameter of the particle sedimented.

l = length of the capillary.

(1) *Sheep Virulent Virus.*

Sheep would have been required in far greater numbers than were available if this investigation was carried out strictly quantitatively i.e. if the virus concentrations before and after centrifugation were to be determined accurately. Consequently a somewhat crude modification was adopted.

Previous titrations of the infectivity of sheep have shown that a 10 per cent. emulsion of spleen from sheep destroyed 48 hours after the first rise in temperature of a bluetongue reaction contains constantly about 10,000 M.I.D.'s of virus per c.c. (Alexander personal communication). With the bisectable capillary the reduction in titre when the boundary of the sedimenting virus just passes into the lower segment is more than 100-fold. The suspensions after centrifugation for varying lengths of time were diluted 1 in 100 before injection in 1 c.c. amounts subcutaneously into sheep. The shortest period of centrifugation that was required to produce an inactive diluted virus suspension was assumed to be the time required to sediment the virus boundary just into the lower capillary segment. Therefore in the equation Ct/Co was taken as 0 to give a value for Σ of 1·27 ($x_1 = 3·7$). The data obtained from two experiments are given in Table 3.

TABLE 3.

Centrifugation of Sheep Virulent Virus.

Exp.	N = r.p.m.	t.	T°C.	7	Reaction in Sheep.
1.....	10,500	15	24·0	0·0095	R.I.
	10,500	30	24·0	0·0095	R.I.
	10,700	45	24·6	0·0094	N.R.
	10,900	90	24·6	0·0094	N.R.
	—	—	—	—	R.I.*
2.....	10,500	50	20·5	0·0104	N.R.
	10,500	60	21·0	0·0102	N.R.
	10,500	70	21·0	0·0102	N.R.
	—	—	—	—	R.I.*

* Controls for infectivity of original suspension.

$x = 3·7$ c.m. $l = 1$ c.m. $\triangle\sigma = 0·106$. g.m./c.c. (see later).

Result.

In experiment 1 it is assumed that the virus boundary was sedimented into the lower segment of the bisectable capillary in from 30 to 45 minutes. This gives a calculated particle diameter of 108-133 mμ. The data from experiment 2 is inconclusive but it is calculated that the particle size is greater than 107 mμ since the virus had been sedimented in less than 50 minutes. At this stage the work with sheep was discontinued in favour of a more comprehensive and more accurate examination of the egg adapted virus.

(2) *Egg Adapted Virus.*

An essential prerequisite to the calculation of the diameter of the virus particle is the accurate determination of its specific gravity. The method of centrifugation in media of different densities was used [Elford (1938), Polson (1941)] and from the data collected both values were calculated.

Technique.

Egg adapted virus emulsions were clarified by angle centrifugation for 1 hour followed by filtration through asbestos pulp. This method was adopted because in centrifugation studies removal of all particulate debris has no bearing on the final results comparable to that in filtration studies.

The method of centrifugation in media of high densities may introduce a serious source of error if the densities of the media are raised by low molecular substances like salts or sugars as these substances might diffuse into the structure of the virus and so increase its density.

This effect is demonstrated below with bluetongue virus and cane sugar. A medium of high density was used in the form of 25 per cent. saccharose solution in 0·85 per cent. saline containing 5 per cent. horse serum; "Merthiolate" in a concentration of 1:10,000 was added as a preservative. The low density was 0·85 per cent. saline. Clarified chick embryo emulsion was added in the proportion of 1 part of emulsion to 3 parts of medium. This produced final densities of 1·075 and 1·00 gm./c.c. respectively, determined pyknometrically. Preliminary centrifugation runs showed that in the sugar solution a ten-fold reduction in titre could be expected after 80 minutes. Data from 6 experiments are given in Table 4.

TABLE 4.

Exp.	Medium.	Density. σ	T°C	N.	Viscosity. η	Virus titre and time of centrifugation min.				
						C_0.	t_1	C_1	t_2	C_2
1.....	Sugar	1·075	22·5	10,200	·0165	3·3602	80	2·3111	90	2·6521
2.....	Sugar	1·075	21·0	10,200	·0170	3·3121	90	2·8521	100	2·1610
3.....	Sugar.	1·075	24·5	10,200	·0157	3·5000	90	1·3732	100	1·8000
4.....	Saline	1·0	24·0	10,200	·0095	4·2500	30	2·6600	40	2·0000
5.....	Saline	1·0	20·0	10,200	·0101	3·4010	30	2·5111	35	1·8301
6.....	Saline	1·0	20·0	10,200	·0101	4·0210	30	2·7630	35	2·6710

NOTE.—The captions in the table are explained by reference to the formulae in the text.

Calculations.

(*a*) *Density.*—Calculations of the density of the virus particle were made from the formula.

$$\sigma = \frac{\sigma_2 N^2_2 t_2 \eta_1 \log x_1 - \sigma_1 N^2_1 t_1 \eta_2 \log x_2}{N^2_2 t_2 \eta_1 \log x_1 - N^2_1 t_1 \eta_2 \log x_2}$$

where σ_1 = density of the medium of low specific gravity.

σ_2 = density of the medium of high specific gravity.

* "Merthiolate" = Sodium ethyl mercuri thiosalicylate, Eli Lilly & Co.

The other symbols have the meanings noted previously.

(*b*) *Particle Diameter.*—Calculations of the particle diameter were made by substitution of the average density obtained namely 1·147 gm./c.c. in the previously mentioned formula:—

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

The results are given in tabular form in Table 5.

TABLE 5.

Egg Adapted Virus. Calculated Density and Particle Diameter in Concentrated Sugar and in Saline Solutions.

Exp.	Time of Centrifugation.	x	Density g.m./c.c.	Particle diameter in mμ.
1.....	80	1·23	1·170	157
	90	1·21	1·140	144
2.....	90	1·16	1·120	122
	100	1·24	1·146	145
3.....	90	1·27	1·169	155
	100	1·26	1·139	145
4.....	30	1·26	—	118
	40	1·27	—	—
5.....	30	1·23	—	115
	35	1·26	—	113
6.....	30	1·26	—	122
	35	1·27	—	113
			Average 1·147	Average 132

NOTE.—The density of the particle was determined from the data obtained from the 30 and 35 minutes centrifugation experiments 5 and 6 and those of experiments 1, 2 and 3. The solutions were centrifuged immediately after they were mixed to minimize the time of contact of the virus with the sugar.

From Table 5 it can be seen that there is a marked difference in the particle sizes when calculated from the data obtained from the experiments using saccharose medium and those using saline. This can be explained on the assumption that sugar diffused into the structure of the virus thereby increasing its density, thus causing an increase in sedimentation rate. This increase in sedimentation rate is reflected in the larger particle size in sugar medium as calculated from formula (1).

This difficulty was overcome by centrifuging the virus in a concentrated horse serum albumin solution. Serum albumin was chosen as its molecular weight is high and the viscosity of concentrated solution is reasonably low.

To increase the rate of sedimentation of the virus in this medium a special centrifuge cell was constructed on similar lines to the cell used above, but which could be placed on the bottom of the centrifuge cup to get the greatest possible radius of centrifugation. The following apparatus sketched in Fig. 1 was constructed.

Three circular metal sections 3·8 cm. in diameter were cut and holes 2 mm. in diameter drilled through all except the bottom section into which the holes extend about 5 mm. The flat faces of these sections were well ground. The sections turn on a central bolt which screws into the bottom section. In a certain position the holes coincide to form 12 capillaries. The top section acts as a lid, the central section which is 1 cm. thick as test section and the bottom as receiver for the virus centrifuged out of the central section. When in use this cell is supported on the bottom of the centrifuge cup by a thin piece of rubber sheet. In this position the radius of centrifugation is increased from the previous value x = 3·7 cm. to x = 5·76 cm.

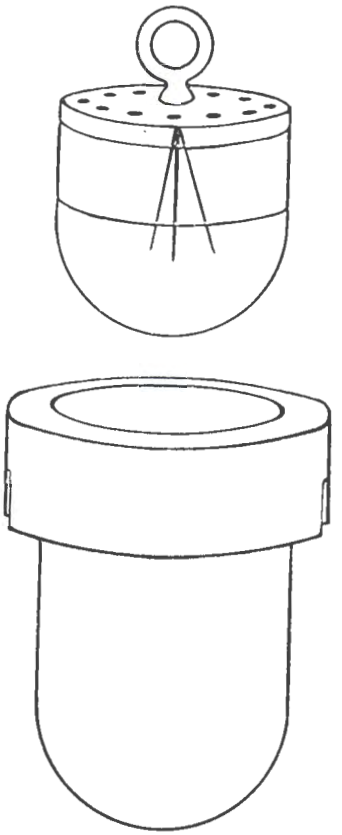

Fig. 1.

The centrifuge run was made as follows:—The virus solution was placed in each of the 12 capillaries. The lid is turned slightly to close the cavities and the cell is placed in the centrifuge cup. Sufficient virus solution was then let in to cover the lid. This solution counteracts the hydrostatic pressure inside the cell thus preventing the solution from leaking out of the capillaries when the cell in centrifuged. The top of the cup is then closed and the cell centrifuged for different periods of time and the virus content of the central section determined.

Data from two different experiments are given in Table 6. The virus was suspended in 24·6 per cent. serum albumin solution in saline at pH 7·3.

TABLE 6.

Exp.	Medium.	Density. σ	T°C.	N.	Viscosity. η	Virus titre and time of centrifugation min.				
						Co.	t_1	C_1	t_2	C_2
7.....	Albumin	1·062	24·5	10,200	0·0329	3·243	150	2·600	180	2·183
8.....	Albumin	1·062	25·2	10,200	0·0324	3·500	150	2·835	180	1·604
5.....	Saline	1·00	20·0	10,200	0·0101	3·401	30	2·5111	35	1·8301
6.....	Saline	1·00	20·0	10,200	0·0101	4·021	30	2·7630	35	2·6710

The results are given in tabular form in Table 7.

TABLE 7.

Calculated Density and Particle Diameter in Concentrated Serum Albumin Solutions.

Exp.	Time.	X	σ	Particle diameter in mμ.
7.....	150	1·129	1·126	126·0
	180	·1156	1·101	126·0
8.....	150	1·132	1·094	127·0
	180	1·150	1·105	123·0
5.....	30	1·231	—	131·0
	35	1·260	—	128·0
6.....	30	1·262	—	139·0
	35	1·271	—	130·0
			Average 1·106	Average 128

The particle sizes calculated from the data of the high and low density media used in this experiment agree very closely which indicates that no increase in density of the particle occurred in the concentrated albumin solution. Sharp, Taylor, McLean, Beard and Beard (1945) reported a similar result with influenza virus. They found that in concentrated sugar solution the density of the particle increased. True densities were found when concentrated bovine albumin solutions were used.

SUMMARY.

1. The particle diameter of bluetongue virus (sheep virulent and egg adapted virus) was determined by gradocol membrane filtration.

2. Details of the technique for clarifying infective emulsions by aluminium hydroxide adsorption and trypsin digestion are given.

3. The density of the egg adapted virus was calculated from data obtained from centrifugation in media of low and high specific gravity and was found to be 1·147 gm./cm.^{3} in strong cane sugar and 1·106 gm/cm.^{3} in serum albumin.

4. The particle diameter of the sheep virulent virus was determined approximately, and of the egg adapted virus accurately by centrifugation. The results may be summarized as follows:—

Virus.	Method of Determination.	
	Ultrafiltration.	Ultracentrifugation.
Sheep virulent.....	100–150 mμ.	108–133 mμ.
Egg adapted.....	100–150 mμ.	128 mμ.

Acknowledgments.

In conclusion I wish to acknowledge my great indebtedness to Dr. R. A. Alexander for helpful advice and criticism, to Mr. D. A. Haig for determining the virus titres of the solutions and to Dr. P. J. du Toit for enabling this work to be carried out.

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