PARTICLE SIZE DISTRIBUTION OF AFRICAN HORSESICKNESS VIRUS

by

ALFRED POLSON AND THELMA MADSEN

South African Council for Scientific and Industrial Research and the University of Cape Town
Virus Research Unit, Department of Pathology, University of Cape Town (South Africa)

During the course of our work on the measurements of physico-chemical properties of animal viruses, particularly those occurring in Africa, it was noticed that in suspensions of some of the viruses there were particles of at least two different sizes, both particles being infective. The work reported here is that done on neurotropic African horsesickness virus.

EXPERIMENTAL

The strains of horsesickness virus on which the work was performed were 1180, 1178, Vryheid and A 501 (ALEXANDER, personal communication). Virus emulsions were prepared as 10% suspensions of virus-infected mouse brains in 10% rabbit serum saline. The crude suspensions were spun at 2600 r.p.m. for 1 hour and the supernatant fluids used in the ultracentrifugation studies. Although it is possible to obtain sedimentation diagrams without the use of density gradients in the centrifuge tube as was shown by BRADISH *et al.*[1] and POLSON AND LINDER[2], they were nevertheless used as a safeguard against possible heat convection currents. The use of cane sugar solutions in establishing density gradients was avoided because of the difficulty of correcting for viscosity in the determination of sedimentation rates in this medium. Instead reliance was placed on haemocyanine, which itself has a high sedimentation constant and which is known to provide an effective gradient. The haemocyanine of Caminella sincta of sedimentation constant 100 *S* was found useful for this purpose (POLSON AND LINDER[2]).

Infected brain suspensions in 10% rabbit serum saline containing 1.5% haemocyanine were placed in 10 ml graduated Spinco centrifuge tubes and spun in the rotor which had previously been brought to the centrifugation equilibrium temperature for the required rotor velocity (POLSON AND LINDER[2]). The rotor velocity was carefully noted at intervals during acceleration, during the main centrifugation run and during the deceleration period. The average effective rotor velocity during the period of centrifugation, which was 100 minutes in these experiments, was determined by plotting the square of the actual rotor velocities at different intervals against time and calculating the average effective velocity according to the formula r.p.m. $= \left(\frac{\text{Area included by curve}}{100}\right)^{1/2}$. The method is similar to that described by BRADISH *et al.*[1].

Immediately after the rotor stopped the temperature of the water in the balancing tube was determined and the tube containing the virus transferred to a metal holder at the bottom of a large container made of 8 mm thick "perspex" and filled with water at the same temperature as the centrifuge rotor, care being taken that the water in the container was at a level above that of the meniscus in the centrifuge tube. With

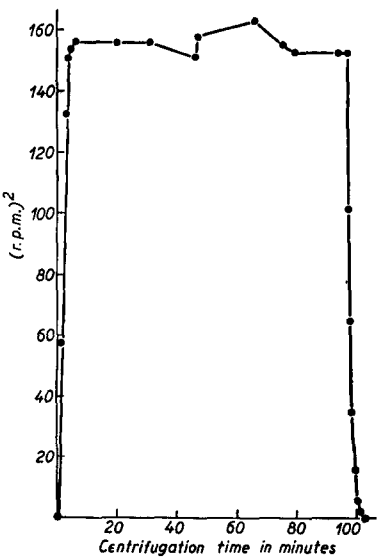

Fig. 1. Showing the square of rotor velocity against time in minutes. Average speed calculated from this curve is 12,220 r.p.m.

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a sampling device the fluid in the tube was removed in successive layers of 0.5 or 1 cm. Thus the contents of the tubes were divided into 8 or 10 samples. Each sample was titrated by intracerebral inoculation of tenfold dilutions into 3 to 4-weeks old mice. The mice were kept under observation for 8 days. Fifty percent end-points were determined by the method of REED AND MUENCH[3]. The titres of the different samples were plotted against the relative positions in the centrifuge tube. The position of the sedimenting virus boundary in the centrifuge tube which corresponds to the 50% concentration point in the Svedberg light absorption technique is determined from the position of a line drawn through a point representing a titre T where $T = \text{Log}\left(\frac{\text{Antilog } T_2 - \text{Antilog } T_1}{2}\right)$ and T_2 is the virus titre below, and T_1 that of the titre above the boundary zone respectively. From the position of the sedimenting boundary H thus determined, the sedimentation constant was calculated using the equation of Svedberg and Pedersen as modified for sedimentation in the angle centrifuge

$$S = \frac{2\,H.\,\text{Sin}\,\alpha}{(2x_1 + H\,\text{Sin}\,\alpha)\,\omega^2\,(t_2 - t_1)}\,\eta/\eta_{20} \qquad (1)$$

where X_1 = distance of initial boundary from the axis of rotation, α is the angle of inclination of the tube, ω the angular velocity of the rotor, $(t_2 - t_1)$ the effective time of centrifugation (100 minutes in the present experiments), and η/η_{20} the ratio of viscosities of the solution at the temperature of centrifugation to that of water at 20° C.

The sedimentation constant can also be calculated from a knowledge of the distance sedimented by a substance of known sedimentation constant under identical conditions of centrifugation. For this purpose the following equation is used

$$S_2 = S_1\,\frac{H_2}{H_1}\,\frac{(2x_1 + H_1\,\text{Sin}\,\alpha)}{(2x_1 + H_2\,\text{Sin}\,\alpha)} \qquad (2)$$

where S_2 = the sedimentation constant of the substance under investigation, S_1 the sedimentation constant of the reference substance, H_2 the distance which the substance under investigation sedimented and H_1 the distance which the reference substance sedimented.

The advantages of using this equation are several. No accurate knowledge of the effective rotor velocity is necessary nor the temperature of the rotor during centrifugation. Tube distortions during centrifugation do not play a role either in computing sedimentation constants. If the reference substance has no effect on the material under investigation it can be mixed with the material and centrifuged in the same tube. In the experiments with horsesickness virus Caminella sincta haemocyanine with a sedimentation constant 100 Svedberg units was mixed with the virus prior to centrifugation. The haemocyanine served a dual purpose, firstly, in establishing a density gradient and secondly, in serving as a reference substance.

RESULTS

In Fig. 2 are given diagrams showing the virus content of the samples taken at different levels in the centrifuge tube in three centrifugation experiments on strain 1180 of horsesickness virus. The virus was subjected to centrifugation at an effective speed of 11,000 r.p.m. for 100 minutes. It will be seen that the sedimentation diagrams are similar in all three cases and that there are at least three components of different sedimentation constants present in horsesickness virus. The boundary of the component with the lowest sedimentation constant moved down to 1.25 cm while the intermediate component sedimented to approximately the 4 cm level. The presence of an additional very rapidly sedimenting component is suggested by the fact that the original emulsion has a higher virus titre than any portion of the supernatant fluid recovered after this degree of centrifugation.

Several experiments were conducted to show that the inhomogeneity of the virus is real and that it is not due to unrecognised artifacts.

a. The component with the lowest sedimentation rate is not due to virus coated with a thick layer of lipoid which would produce a particle of low specific gravity. If this was the case the particle would show negative sedimentation on centrifugation in a medium of high density. Such an experiment was performed. A suspension of virus was

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centrifuged at 20,000 r.p.m. for 100 minutes in a cane sugar medium of density 1.17 g/ml. The titrations of the samples taken at different levels in the centrifuge tubes after centrifugation showed that some sedimentation had still occurred and that no virus moved to the top levels in the tube thus indicating that the "lighter" component is not virus coated with a heavy layer of lipoid.

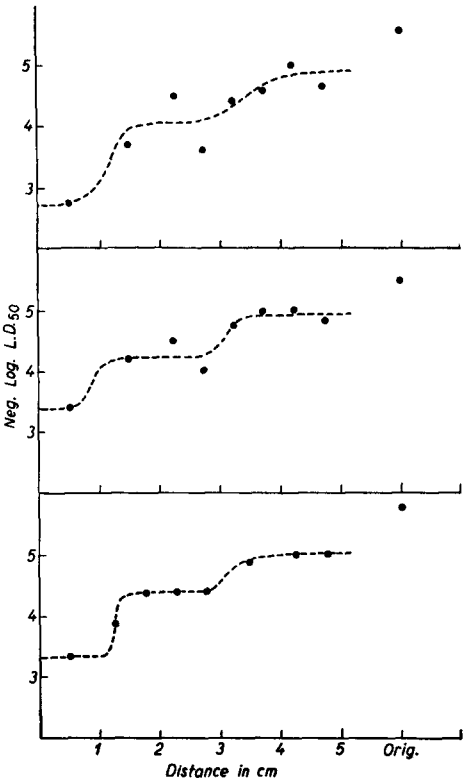

Fig. 2. Titre of samples taken at various levels in the centrifuge tubes in three different experiments conducted with approximately constant rotor velocity and time of centrifugation. Strain 1180 of horsesickness virus.

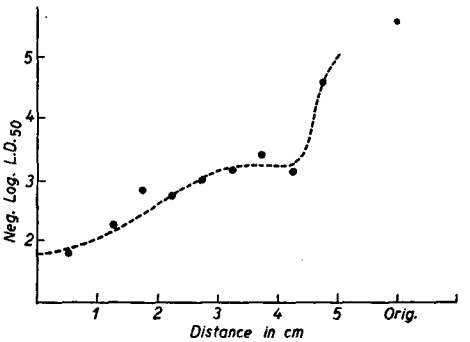

Fig. 3. Titres of samples taken at various levels in the centrifuge tube in an experiment in which material was centrifuged at 14,900 r.p.m. for 100 minutes.

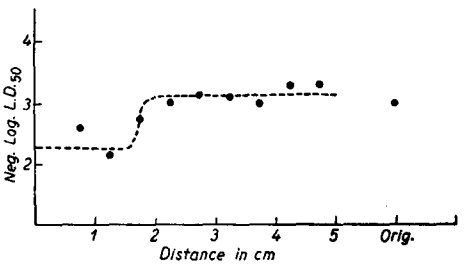

Fig. 4. Titres of samples taken at various levels in the centrifuge tube after centrifugation of the "lighter" component at 11,000 r.p.m.

By centrifugation in media of different densities by the method of LAUFFER, TAYLOR AND WUNDER[4] the hydrated density of the "light" particle has been found to be 1.28 g/ml. This aspect of the work however needs confirmation by experiments carried out with a range of media of different densities.

b. The "lighter" component is not due to an artifact arising out of spinning at the relatively low speed of 11,000 r.p.m. It is also demonstrable in fluids subjected to centrifugation at 15,000 r.p.m. but has of course at the higher speed sedimented through a greater distance (Fig. 3).

c. The different components present in the emulsion can exist independently. The "lighter" component was separated from the "heavier" components by centrifugation at 20,000 r.p.m. for 30 minutes and upon centrifugation of the supernatant at 11,000 .p.m. only a single sedimentation "boundary" was obtained (Fig. 4). The "heavy"

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component, recovered from the deposit after centrifugation at 20,000 r.p.m., was washed six times in 10% rabbit serum saline, each time being recovered by centrifugation. Finally it was tested by centrifugation at 11,000 r.p.m. for 100 minutes and a sedimentation diagram was obtained which showed only the presence of the "heavy" components (Fig. 5). There is therefore no equilibrium between the "light" and "heavy" components.

d. The "light" component does not result from dissociation brought about by the haemocyanine. The absence of such an effect was evident when it was found that a cane sugar gradient could be substituted for haemocyanine (Fig. 6).

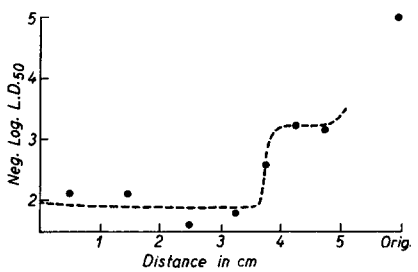

Fig. 5. Titres of samples taken at various levels in the centrifuge tube after centrifugation of the "heavier" components at 11,000 r.p.m.

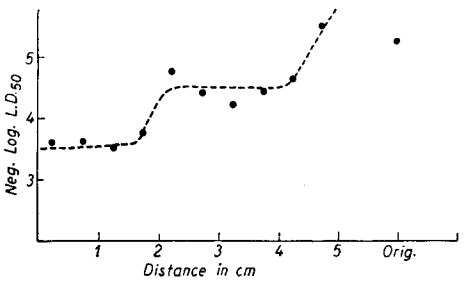

Fig. 6. Titres of samples taken at various levels in the centrifuge tube after centrifugation at 14,770 r.p.m. in the presence of a sugar gradient ranging from 0–16%.

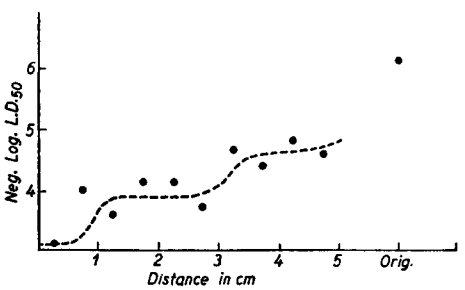

Fig. 7. Titres of samples taken at various levels in the centrifuge tube after centrifugation at 11,000 r.p.m. of the "light" component separated by repeated centrifugation.

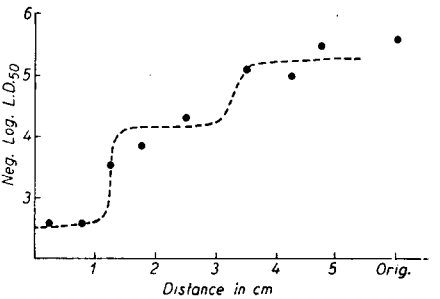

Fig. 8. Titres of samples taken at various levels in the centrifuge tube after centrifugation at 11,000 r.p.m. of the "light" component separated by repeated passage at limit dilutions.

e. Attempts were made to obtain a "light" virus free from the "heavy" components by alternative separation of the "light" component by centrifugation and mouse passage. After 9 such alternate centrifugations and passage a suspension of the virus was ultracentrifuged at 11,000 r.p.m. for 100 minutes and a result obtained (Fig. 7) which was not different from that with the original virus suspension.

An alternative method was also employed to produce virus consisting only or predominantly of the "light" component. After the "heavy" virus was removed by centrifugation serial tenfold dilutions of the supernatant fluid containing the "light" component were injected into mice. The brains of mice which were moribund after receiving the highest infective dilution of the virus suspension were collected. Suspensions

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were prepared and once more freed of "heavy" virus by centrifugation, after which they were titrated in mice. The alternative centrifugation and "limit dilution" passage was repeated 3 times. After the third passage the sedimentation rate of virus in infected brain emulsions was determined in the usual way. The results (Fig. 8) revealed no difference from the original virus.

f. The "light" component is not peculiar to strain 1180 alone. In Fig. 9 are given sedimentation diagrams obtained in experiments with the strains 1178, A501, Vryheid and 1180. All were centrifuged at approximately the same speed. Unfortunately the centrifugation temperatures were different in the different experiments as a result of which different sedimentation distances were obtained. The haemocyanine, with which the virus was mixed, however showed corresponding differences in boundary movement. It could therefore be concluded that the sedimentation constant of the corresponding components in the different strains were the same, and that the only difference amongst the strains was in the relative amounts of the different components present.

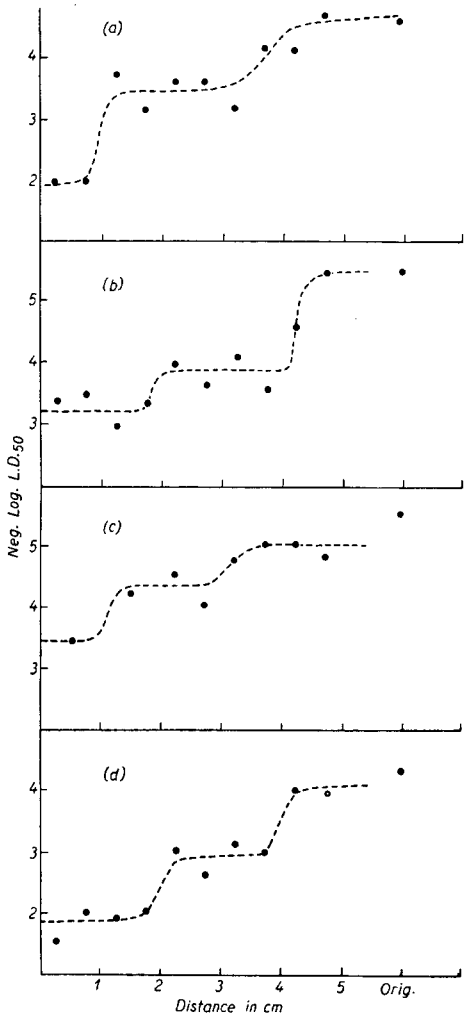

Fig. 9. Titres of samples taken at various levels in the centrifuge tubes after centrifugation at approximately the same speed of four different strains of horsesickness virus: a. 1178; b. A501; c. 1180; d. Vryheid.

Sedimentation constants and particle sizes of the components of horsesickness virus

As the amount of sedimentation of the "light" components was very small no reliable sedimentation constant could be calculated for it from the diagrams obtained at 11,000 r.p.m. However an appreciable migration of the "light" component to warrant the calculation of a reasonably accurate sedimentation constant was obtained when the virus was centrifuged at 14,900 r.p.m. for 100 minutes. The sedimentation diagram is shown in Fig. 3.

In Table I are given the experimental data from which the sedimentation constant was calculated.

The sedimentation constant of the "light" component was also calculated with the aid of equation 2 and the data in Table II. The haemocyanine boundary (sedimentation constant 100 *S*) moved down 1.5 cm during centrifugation. From these data a sedimentation constant of 173 *S* was calculated. Using the modified Stokes equation and assuming spherical particles of density 1.33 g/ml particles diameters of 50.8 mμ and 31.2 mμ were calculated for the two components of horsesickness.

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TABLE I

ULTRACENTRIFUGATION OF DIFFERENT STRAINS OF AFRICAN HORSESICKNESS VIRUS

(Sedimentation constant of the "heavy" component)

$X_1 = 5.35$, t_1–t_2 = 100 minutes, Sin $\alpha = 0.4384$

Strain	*T °C*	η/η_{20}	*H in cm*	*r.p.m.*	*S in Svedbergs*
1180	9.0	1.39	3.7	10,800	477
1180	6.0	1.52	3.2	11,060	441
1180	7.0	1.46	3.2	10,800	438
1180	6.75	1.48	3.3	10,830	460
1180*	6.8	1.47	3.75	10,920	499
A501	8.0	1.42	4.2	10,830	530
Vryheid	9.5	1.37	4.0	11,136	471
1178	8.0	1.42	3.8	10,800	496
					Average 476

* After removal of "light" component by centrifugation.

TABLE II

SEDIMENTATION CONSTANT OF THE "LIGHT" COMPONENT OF STRAIN 1180 OF AFRICAN HORSESICKNESS VIRUS

$X_1 = 5.35$, t_1–t_2 = 100 minutes, Sin $\alpha = 0.4384$

T °C	η/η_{20}	*H in cm*	*r.p.m.*	*S in Svedbergs*
7.5*	1.46	1.20	10,800	177
9.0	1.39	2.60	14,900	183
				Average 180

* Average values of 5 experiments.

The soluble antigen of horsesickness virus

It was found that, after complete removal of the infective virus particles from solution by ultracentrifugation at 30,000 r.p.m. for 2 hours, the supernatant fluid still contained about one half of the original complement fixing power. From analytical diffusion measurements by the method of POLSON[5] it was found that the particle size of antigen remaining in the supernatant fluid is approximately 12 mμ in diameter.

DISCUSSION

From the work reported in this paper it appears that tissues infected with neurotropic horsesickness contain at least two particles differing significantly in size but both being infective for mice. Assuming spherical particles of density 1.33 g/ml sizes of 31.2 mμ and 50.8 mμ were calculated for the "lighter" and "heavier" particles. Brains of mice infected with strain 1180 appear to contain particles bigger than 51 mμ in diameter as there is always appreciably more virus in the crude emulsions than in any samples of supernatant fluid recovered after centrifugation. The presence of this com-

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ponent could not be detected in the other strains examined. The relative amounts of "light" and "heavy" components differ for the different strains. Whether this is a genuine difference or whether it is dependent on the number of intracerebral passages in mice must still be determined.

A point of great interest is that the sizes of the two components fall within the size classiffcation of the animal viruses (POLSON[6]) the "lighter" being approximately ¼ in weight of the "heavier" component. As far as the writers are aware this phenomenon of the inhomogeneity of the infective particles has not been observed before; a possible explanation for this is that most of the centrifugation work of other workers in this field has been done on purified virus preparations in which case the analytical ultracentrifuge fitted with the optical system was used. The slower sedimenting component is usually in too low concentration to be observed on the sedimentation diagram when this technique is used.

From the experiments reported above it would appear that the "lighter" virus is not the "heavy" virus coated in a thick layer of lipoid. At the moment no proof can be given that the "heavy" particle is not the "light" particle attached to fragments of tissue protein, but in view of the relatively sharp sedimentation boundaries obtained and the constant size relationship between the two the concept that the "heavy" particle is an aggregate of four "light" particles must be favoured. In previous work where the capillary centrifugation technique of ELFORD[7] was used a particle size of 45 mμ was obtained for African horsesickness virus by POLSON[8]. Our present findings indicate that the size must be assumed to be midway between those of the two components, or if adsorption to tissue fragments is assumed that of the smaller component must give a closer measure of the true dimensions of the horsesickness virus.

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SUMMARY

The existance of infective particles showing at least two sedimentation constants have been observed in suspensions of neurotropic African horsesickness virus. Assuming a density of 1.33 g/ml, particle sizes of 31.2 mμ and 50.8 mμ were calculated for the two particles respectively. In addition to these particles a complement fixing, but non-infective particle of diameter 12 mμ has been found in suspensions of the virus.

RÉSUMÉ

Dans des suspensions de virus neurotropique Africain du cheval s'observent des particules infectieuses présentant au moins deux constantes de sédimentation. En leur attribuant une densité de 1.33 g/ml, les deux types de particules mesureraient respectivement 31.2 mμ et 50.8 mμ. En outre, il existe dans les suspensions du virus des particules non infectieuses, fixant le complément et d'un diamètre de 12 mμ.

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ZUSAMMENFASSUNG

In Suspensionen des Virus der neurotropen afrikanischen Pferdesterbe konnten infektiöse Partikel mit mindestens zwei Sedimentationskonstanten nachgewiesen werden. Unter der Annahme einer Dichte von 1.33 g/ml werden für die Teilchen Grössen von 31.2 mμ und 50.8 mμ errechnet. Darüberhinaus wurde in den Suspensionen des Virus ein komplementbindendes aber nicht infektiöses Teilchen von 12 mμ Durchmesser gefunden.

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