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A slowly sedimenting infectious component of Rift Valley fever virus

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INTRODUCTION

It is now known that many viruses contain infective particles differing in sedimentation constant. Type II poliomyelitis, for example, consists of a mixture of particles having sedimentation constants of $S_{20} = 170$ and $S_{20} = 100$ (Selzer & Polson, 1954). Of special interest are viruses containing infectious components with exceptionally low sedimentation constants. Hampton (1958) has mentioned the existence of such material in West Nile virus. Kipps, Turner & Polson (1961) estimated the sedimentation constant of the slowly sedimenting fraction of CBO virus as 20*S* and Gessler, Bender & Parkinson (1956) working with Rous sarcoma virus purified with fluorocarbon found high tumour producing activity in the supernatant fluid after centrifugation at 144,000 ***g*** for 20 hr.

In this laboratory it has been observed that on centrifugation of Rift Valley fever virus (RVF), under conditions which sedimented yellow fever (Polson, 1954), African horsesickness (Polson & Madsen, 1954), MEF_1 polio (Polson & Selzer, 1952) and Semliki Forest viruses (Cheng, 1961) from the supernatant fluid, the virus activity could be reduced rapidly by about 99 %. The remaining 1 % of virus could be reduced only slightly by further repeated centrifugations. This apparent inhomogeneity of RVF virus has now been further investigated.

MATERIALS AND METHODS

Viruses

Rift Valley fever

Pantropic Rift Valley fever virus obtained from the Director, Department of Veterinary Research, Onderstepoort, Pretoria, was maintained by intraperitoneal mouse passage. Defibrinated infected blood from adult white mice or a 20 % suspension of liver and spleen from mice 15 to 19 days old were used as sources of virus.

Polio

The MEF_1 strain of type II poliovirus (Selzer, Sacks & van den Ende, 1952) in its 225th passage in mice as a 10 % suspension of 4- to 5-day-old infected unweaned-mouse brains was used.

RVF antiserum

This was prepared in adult mice by eight intraperitoneal injections of a 10 % suspension of infected mouse liver or spleen. The first two injections contained 0·016 % formalin.

Deoxyribonucleic acid and nucleases

Calf thymus DNA, crystalline deoxyribonuclease and ribonuclease were obtained from Seravac Laboratories, Cape Town.

Virus titration

The diluent used for virus suspensions and titrations was a mixture of 5 % (v/v) rabbit serum with 0·85 % (w/v) saline containing 200 units of penicillin and 0·2 mg. of streptomycin per ml.

RVF

Tenfold serial dilutions were made and each was tested in six mice, 3–5 weeks old. Each animal received 0·2 ml. intraperitoneally. Titres are expressed as the negative $\log_{10}$ LD 50 per 0·2 ml., calculated by the method of Reed & Muench (1938). Infectivity units are the antilog values of the negative $\log_{10}$ LD 50.

MEF_1

Each dilution was titrated in a litter of eight unweaned mice, 4–5 days old, 0·02 ml. being injected intracerebrally. In the caesium chloride density gradient centrifugation experiments, 0·03 ml. of each fraction was injected intracerebrally into six 3- to 4-week-old mice. Titres are the negative $\log_{10}$ LD 50 per 0·02 or 0·03 ml.

Complement fixation

Complement-fixation tests were performed as described by Casals & Olitsky (1950). Tenfold or doubling dilutions of antigen were tested against the antiserum diluted 1/10 and 1/100. The diluent (pH 7·2) contained Mg and Ca (Mayer, Osler, Bier & Heidelberger, 1946). Complement was titrated by the method of Casals, Olitsky & Anslow (1951) and 2 units were used in the test. The antiserum was inactivated at 56° C. for 30 min.

The complement-fixing titre or one CF unit is equal to the reciprocal of the highest dilution of antigen giving 50 % fixation.

Sedimentation constants

These were measured in the model LH Spinco centrifuge by either of the following methods.

Method 1, using the no. 40 rotor was described by Polson & Linder (1953).

The average rotor velocity was determined by the method of Polson & Madsen (1954). *Jasus lalandii* haemocyanin (S_{20} = 16) added as a sedimentation reference formed a density gradient which stabilizes the virus boundary. MEF_1 poliovirus was ultracentrifuged simultaneously in another tube as an additional control.

Method II, using the SW 39 rotor was described as method B by Polson & van Regenmortel (1961).

In view of the prolonged centrifugation, the virus suspension was layered over 20 % (w/v) bovine plasma albumin (fraction V) in effective column lengths which ranged between 3 and 8 mm. instead of over 40 % (w/v) sucrose.

Centrifugation in density gradients

Densities were determined in a preformed gradient of caesium chloride (Analar, The British Drug Houses Ltd.), the virus suspension being introduced into the gradient close to its isodensity level (Polson & Levitt, 1963).

Estimation of haemocyanin

Samples containing haemocyanin were assayed by immuno-diffusion as described by Polson & van Regenmortel (1961).

Ultrafiltration

The gradacol membranes used were prepared and calibrated in our laboratory.

Gel diffusion-filtration

The column 45 × 2 cm. containing granulated 7 % Difco agar gel (60 mesh) was described by Polson (1961*a*). The displacement medium (pH 7·0) contained 0·067 M phosphate and 0·073 M-NaCl. Fractions were collected in an automatic collector (Polson, 1961*b*).

The following were used as reference proteins:

(*a*) Haemocyanin of *Burnupena cincta*, S_{20} 89 and 92, molecular weight 6,600,000 and diffusion coefficient $1{\cdot}24 \times 10^{-7}$ cm.^{2}/sec. (Polson & Deeks, 1960).

(*b*) Haemocyanin of *Jasus lalandii*, S_{20} 16, molecular weight 490,000 and diffusion coefficient $3{\cdot}4 \times 10^{-7}$ cm.^{2}/sec. (Joubert, 1954; Polson, 1956).

(*c*) Erythrocruorin of *Arenicola loveni*, S_{20} 60, molecular weight 3,200,000 (Svedberg & Pedersen, 1940).

(*d*) Rabbit or mouse haemoglobin, S_{20} 4·36, molecular weight 68,000 and diffusion coefficient $6{\cdot}5 \times 10^{-7}$ cm.^{2}/sec. (Svedberg & Pedersen, 1940; Polson, 1961*a*).

The opacity near 253 mμ of the column effluent was recorded by an LKB Produkter Uvicord coupled to an Esterline–Angus recording milliammeter.

EXPERIMENTAL AND RESULTS

Preparation of S_{low} *particle material*

To remove all rapidly sedimenting virus, a 20 % suspension of RVF-infected liver and spleen in serum-saline, or infected serum, was centrifuged in the no. 40 rotor four times successively at 33,000 r.p.m. for 60 min. under liquid paraffin. This was added to bind at the interface any lipoids or lipoproteins which might have carried virus towards the surface. After each centrifugation, the supernatant fluid was carefully removed by a pipette held initially half-way between the

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liquid paraffin layer and the bottom of the tube and lowered only enough to remove fluid to a level 1 cm. above the bottom of the tube. The remaining fluid was discarded. The pooled supernatants were centrifuged in clean tubes. The final supernatant fluid was regarded as the S_{low} particle material.

After each centrifugation a sample of the supernatant fluid was titrated in mice for infectivity. Supernatants from liver and spleen which contained more soluble antigen than serum were also titrated for complement fixation. The results are given in Fig. 1. The upper curves show the initial infectivity titres and the changes

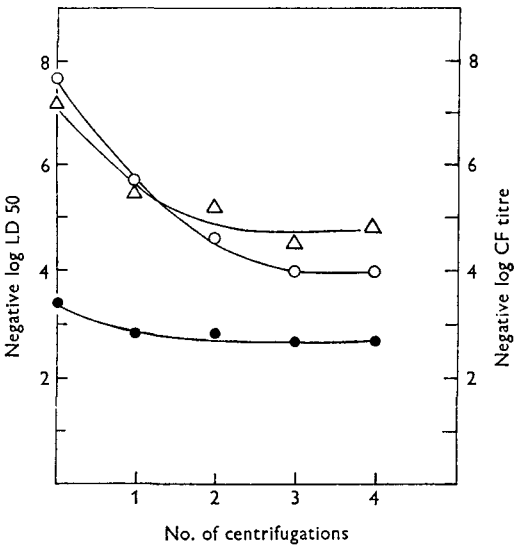

Fig. 1. The changes in infectivity and CF titre of the supernatant fluid during four 1 hr. periods of centrifuging RVF at 33,000 r.p.m. in the no. 40 rotor. The two upper curves are from different experiments. Sedimentation of RVF CF antigen (full circles) and infective virus in two separate experiments (triangles and open circles) on four successive periods of centrifugation at 33,000 r.p.m. for one hour.

in titre of the supernatant fluid after one-hour periods of centrifugation in two experiments. The lower curve shows the initial CF titre and the change in CF titre during successive centrifugations in one experiment.

During the first centrifugation of 1 hr. at 33,000 r.p.m., there was a 100-fold reduction of infective virus in the supernatant fluid. After another slight drop in titre during the second centrifugation, the curve reached a plateau in the region of a negative log LD 50 4·5. The CF antigen showed less reduction in titre during successive centrifugations.

Method I *Determination of sedimentation constants*

Mouse serum infected with RVF was centrifuged twice under liquid paraffin at 30,000 r.p.m. for 60 min. and 9·0 ml. of the supernatant was mixed with 2·0 ml. *Jasus lalandii* haemocyanin. The mixture was placed in a Spinco rotor no. 40 centrifuge tube marked off into ten 0·5 cm. divisions, starting 1·5 cm. from the

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top, the eleventh mark being 1 cm. from the bottom of the tube. The tube was placed in the rotor, which was surrounded by ice until the temperature of the water-balance tube was 2·5° C. The rotor was then spun at 33,000 r.p.m. for 90 min. The rotor velocity was noted at intervals, during acceleration, during the main centrifugation period and during deceleration. Eleven samples were taken from the tube. Each sample was titrated for infectivity in mice and assayed for haemocyanin by the gel precipitin technique.

In this experiment (Fig. 2) there was only slight sedimentation of the haemocyanin and the presence of a faster sedimenting virus component was indicated

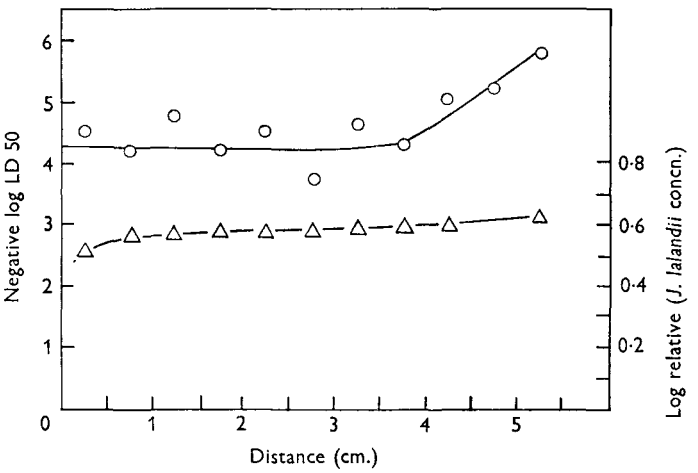

Fig. 2. Sedimentation at 33,000 r.p.m. for 90 min. in the presence of *J. lalandii* haemocyanin, of a suspension of RVF virus which had been previously centrifuged twice at 30,000 r.p.m. for 60 min. The circles represent infectivity titres of successive 1 cm. samples from the tube. The triangles represent the relative concentrations of *J. lalandii* haemocyanin.

by the steep slope of the curve beyond the 4 cm. level in the tube. In succeeding experiments the faster sedimenting virus was eliminated.

In a second experiment a 20 % suspension of liver and spleen infected with RVF was centrifuged for 60 min. at 30,000 r.p.m. The supernatant fluid was removed and submitted to the same treatment. The second supernatant fluid was mixed with *J. lalandii* and centrifuged 90 min. at 33,000 r.p.m. The upper 4 cm. layer of fluid was then carefully removed for the particle size determination. A 10 % suspension of MEF_1 infected mouse brains was treated in the same way to act as a control.

Three centrifuge tubes were marked as described above. The first and second tubes contained RVF and MEF_1—*J. lalandii* haemocyanin mixtures, respectively, the third tube contained 9·0 ml. serum-saline and 2·0 ml. haemocyanin and the fourth, a water balance, was used for temperature measurement. The tubes were centrifuged at 33,000 r.p.m. for 5 hr. and their contents divided into fractions and assayed with the results shown in Fig. 3B. No titres of MEF_1 could be included because it was subsequently found that an MEF_1 suspension of negative log

LD 50 = 5 contained no detectable infective virus after the three preliminary centrifugations to which the RVF had been subjected.

The third experiment resembled the second except that the MEF_1 virus suspension was merely clarified prior to the 5 hr. centrifugation and not subjected to the preliminary three cycles of centrifugation. The results are shown in Fig. 3A.

A fourth experiment was performed on RVF virus alone (Fig. 3C).

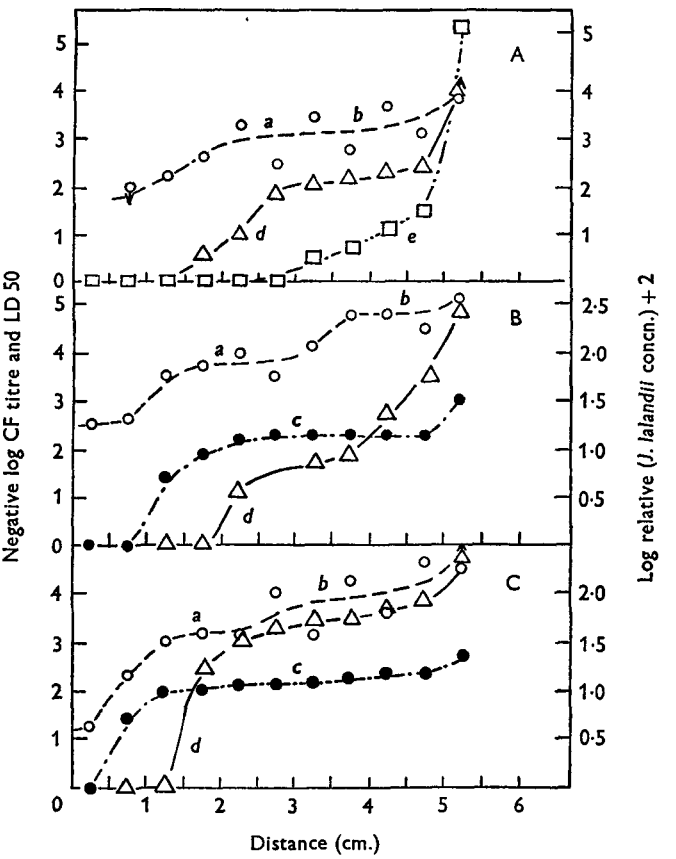

Fig. 3. Sedimentation of S_{low} RVF and MEF_1 poliovirus during 5 hr. at 33,000 r.p.m. in the presence of *J. lalandii* haemocyanin. Diagrams A, B and C refer to different experiments. For the preparation of the S_{low} RVF see text. The points refer to successive 0·5 cm. layers. Curves *a*, *b*—infectivity; curve *c*—complement fixation; curve *d*—relative haemocyanin concentration; curve *e*—infectivity titre of poliovirus.

In Fig. 3, curves (*ab*) show the S_{low} virus content of the samples taken at different levels in the centrifuge tube. Curves (*c*) represent the complement-fixing titres expressed as negative log values. Curves (*d*) indicate the relative haemocyanin concentration expressed logarithmically. Curve (*e*) in Fig. 3A represents the negative log LD 50 values of MEF_1 virus at various levels in the tube.

The sedimentation constants given in Table 1 were calculated from these curves by the formula

$$S_{20} = \frac{2H \sin \alpha}{(2x_1 + H \sin \alpha)\omega^2(t_2 - t_1)} \frac{\eta_T}{\eta_{20}},$$

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where x_1 = distance of the initial boundary from the axis of rotation, α is the angle of inclination of the tube (26°), ω the angular velocity of the rotor, $(t_2 - t_1)$ the effective time of centrifugation (300 min. in these experiments) and η_T/η_{20} the ratio of the viscosity of the solution at the temperature of centrifugation to that of water at 20° C. The position of the sedimenting virus boundary H in the 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 $T = \log \frac{1}{2}(\text{antilog } T_2 - \text{antilog } T_1)$ and T_2 is the virus titre below, and T_1 that of the titre above the boundary zone respectively (Polson & Madsen, 1954).

The infectivity titres obtained with the S_{low} virus samples after ultracentrifugation suggested the presence of more than one component. In Fig. 3B and C the sedimentation diagrams of the S_{low} virus were drawn as two-step curves, but owing to the distribution of points in Fig. 3A, the diagram could only be drawn as a single curve. Sedimentation constants were calculated from the two plateaux *a* and *b* in Fig. 3B and C. The component giving rise to the portion (*a*) of the curves has a sedimentation constant of about 7, while that giving rise to (*b*) appears to have a value between 15 and 19. The CF antigen (curve *c*) has an estimated sedimentation constant between 7 and 8·6.

Variation in the position of the curves in Fig. 3B and C is due to the difference in temperature at which the two ultracentrifugation experiments were conducted (see Table 1).

The *J. lalandii* haemocyanin used for these experiments contained dissociation products which form during storage and give rise to sedimentation curves (*d*) unsuitable for accurate estimate of sedimentation constants.

Table 1. *Calculation of sedimentation constants of RVF* S_{low} *material and complement-fixing antigen*

$X_1 = 5{\cdot}13$ cm.; $t_2 - t_1 = 300$ min.; $\sin_\alpha = 0{\cdot}4384$

Material	Curve	Fig.	Effective rotor velocity (r.p.m.)	Rotor equilibrium temperature (° C.)	H (cm.)	η_T/η_{20}	S_{20}
RVF S_{low}	*a*	3 B	33,230	10·5	1·5	1·312	7·2
RVF S_{low}	*b*				3·5	1·312	15·6
RVF CF antigen	*c*				1·8	1·312	8·6
RVF S_{low}	*a*	3 C	33,020	2·25	1·1	1·691	7·1
RVF S_{low}	*b*				3·2	1·691	18·9
RVF CF antigen	*c*				1·1	1·691	7·1

The haemocyanin, by forming a density gradient in the tube, was valuable as a hindrance to convection and the position of its boundary proved further evidence of the smallness of the sedimentation constants of the slower sedimenting S_{low} (*a*) particle and CF antigen.

The MEF_1 virus (Fig. 3A, curve *e*) had an initial infectivity titre similar to that of the S_{low} material, but after centrifugation for 300 min. at 33,000 r.p.m. had

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sedimented almost completely into a pellet and only traces were detected near the bottom of the tube. In contrast to this, the RVF S_{low} fraction maintained a negative log LD 50 of about 3 below a level 2 cm. from the top of the tube.

Method II

A suspension of RVF S_{low} virus prepared from infected mouse serum was layered over 20% (w/v) albumin in effective column lengths of 3–8 mm. The albumin diffused to form a steep gradient immediately below the effective virus column and prevented the virus from moving back into the effective column by convection. The tubes were centrifuged at 33,000 r.p.m. for 5 hr. at or near 0° C. After centrifugation, the whole of the fluid above the albumin gradient was removed and titrated in mice. The sedimentation constant was calculated from the equation

$$S_{20} = \frac{3{\cdot}50 \log X}{N^2 t} \frac{\eta_T}{\eta_{20}},$$

where η_T and η_{20} are the viscosities of the dispersion medium at the temperature of centrifugation and of water at 20° C., respectively, N the rotor velocity in rev./min., and t the time of centrifugation in minutes,

$$X = \frac{x+l}{x+l(C_t/C_0)},$$

in which x is the distance from the upper meniscus to the centre of rotation, l is the effective column length (i.e. the distance between the upper meniscus and the upper limit of the albumin gradient) and C_t/C_0 the ratio of the final to the initial concentration of virus in the effective column.

Results by this method (Table 2) indicated a value of about 4S for the sedimentation constant of the S_{low} virus fraction.

Table 2. *The sedimentation constant of RVF* S_{low} *virus calculated by centrifugation in columns of different length*

Negative $\log_{10}$ LD 50 of original S_{low} fraction (C_0) = 3·50, N = 33,000 r.p.m., η_{20} = 0·010, η_T = 0·018, t = 300 min., x = 5·85 cm.

Effective column length (cm.)	Negative $\log_{10}$ LD 50 (C_t)	T (° C.)	C_t/C_0	S_{20}
0·3	2·60	0	0·13	3·6
0·4	2·75	0	0·18	4·5
0·5	3·15	0	0·45	3·8
0·6	3·25	0	0·56	—
0·7	3·50	1	1·00	—
0·8	3·25	1	0·56	—
			Average	4·0

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Centrifugation in a density gradient

Using whole infected mouse serum containing both the S_{low} fraction and the sedimentable portion, Polson & Levitt (1963) found a density of 1·23 g./cm.^{3} for the virus. The densities of the S_{low} and sedimentable virus have now been separately determined.

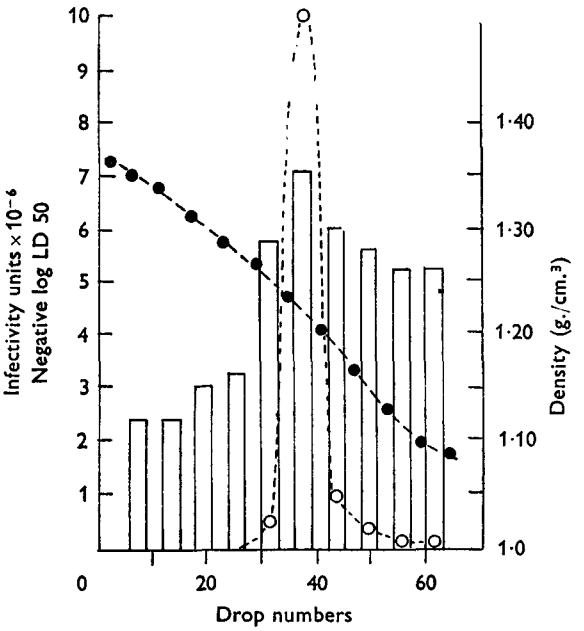

Fig. 4. Density gradient centrifugation of RVF virus (sedimentation constant 452 *S*). The virus was introduced near the centre of a preformed CsCl gradient. Histogram, negative log LD 50 values. Open circles, infectivity units (antilog values). Full circles, distribution of density after centrifugation.

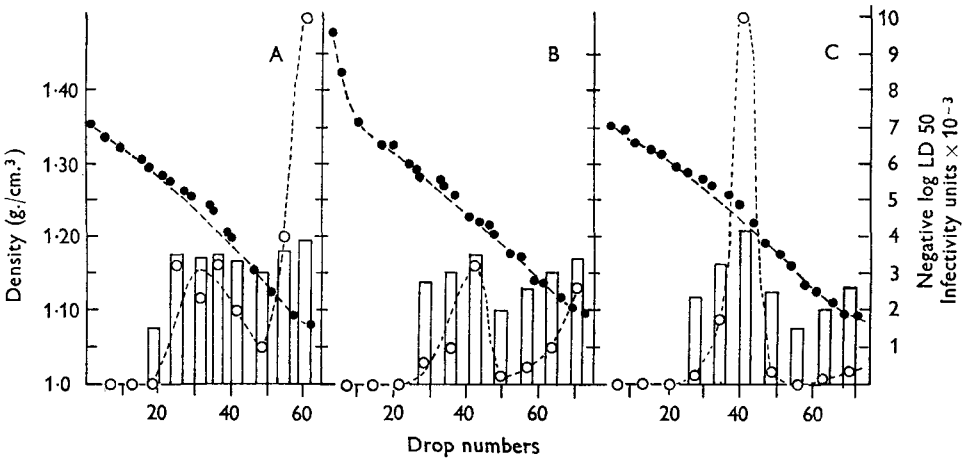

Fig. 5. Density gradient, centrifugation of the S_{low} fraction of RVF. A, B and C refer to separate experiments in which the virus was introduced at the centre of a preformed gradient. Histograms, negative log LD 50 values. Open circles, infectivity units (antilog values). Full circles, distribution of density after centrifugation.

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Mouse serum (10 ml.) infected with RVF virus was centrifuged at 30,000 r.p.m. for 90 min. to sediment the larger virus particles into a pellet. The pellet was resuspended in serum-saline (1 ml.) and the suspension was mixed with enough

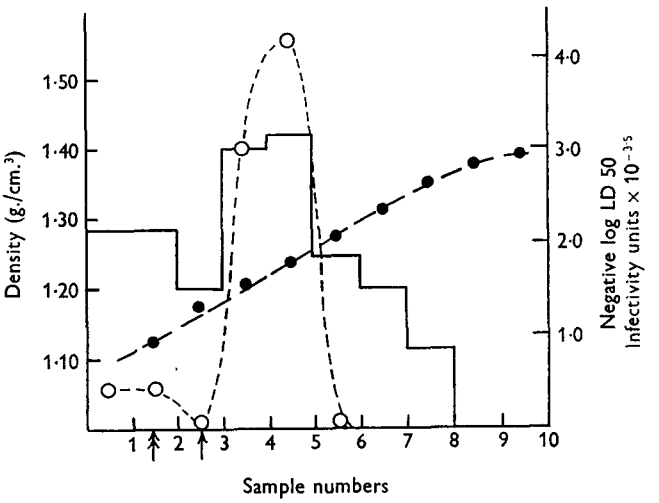

Fig. 6. Density gradient centrifugation of the S_{low} fraction of RVF in the presence of haemoglobin. In this experiment the virus was introduced at a density level of 1·09 g./cm.³ and the tube was sampled from the top. Histogram, negative log LD 50 values. Open circles, antilog values (infectivity units). Full circles, density gradient after centrifugation. Double arrows, point of introduction of virus and haemoglobin. Single arrow, position of haemoglobin after centrifugation.

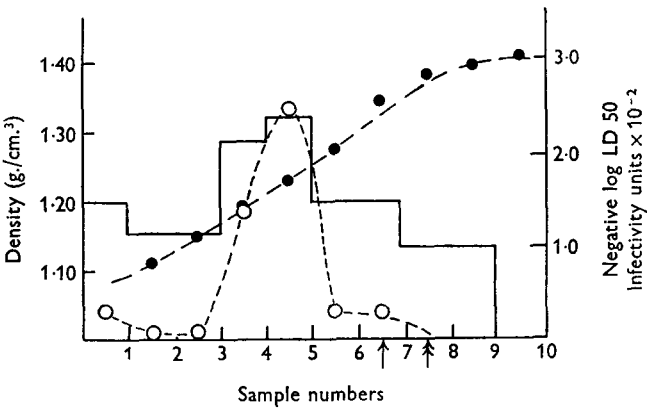

Fig. 7. Density gradient centrifugation of the S_{low} fraction of RVF virus in the presence of haemoglobin. In this experiment the virus sample was introduced at a density level of 1·36 g./cm.³. Samples taken from the top. Histogram, negative log LD 50 values. Open circles, antilog values (infectivity units). Full circles, density gradient after centrifugation. Double arrows, point of introduction of virus and haemoglobin. Single arrow, position of haemoglobin after centrifugation.

caesium chloride solution to bring the density of the mixture to 1·22 g./cm.³ and introduced in the centre of a CsCl density gradient. The tube was centrifuged for 300 min. at 33,000 r.p.m. Samples were taken and examined as described by

Polson & Levitt (1963). The results (Fig. 4) indicated a density of 1·23 g./cm.^{3} for the rapidly sedimenting particles of which the virus is mainly composed.

Fig. 5A, B and C shows the results of three experiments made in the same way but with the S_{low} fraction. There appears to be a main component of density 1·23 g./cm.^{3} and a variable amount of components of lower density.

In an attempt to obtain more definite results, the S_{low} virus sample was introduced into gradients on either side of the expected density level. On this occasion the centrifuge tubes were sampled from the top to ensure that the fractions taken

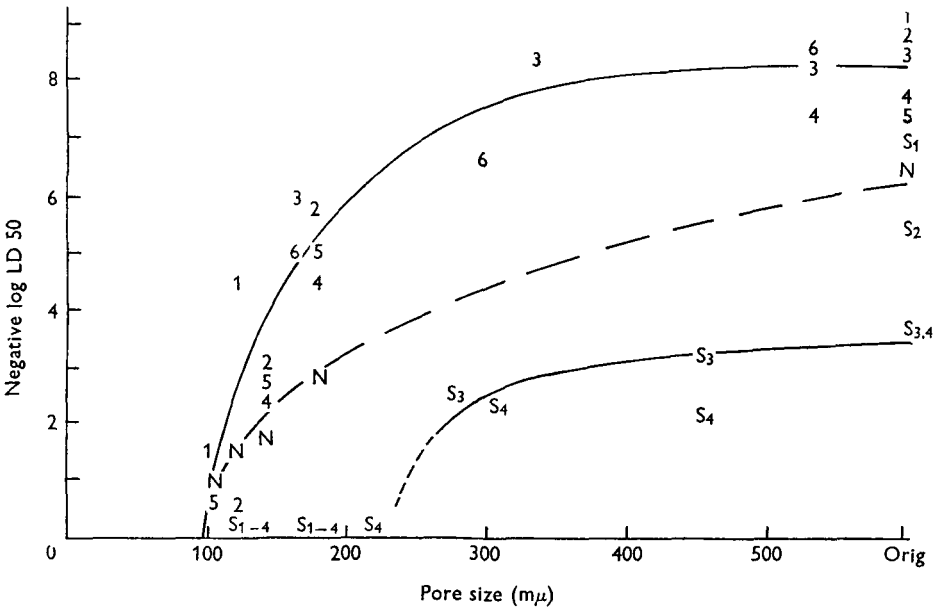

Fig. 8. Results of ultrafiltration experiments on various RVF virus materials. Ordinates are titres in filtrates expressed as neg. log LD 50 values and abscissae membrane pore sizes in mμ. The *S*'s indicate the titre of the S_{low} material and the *N*'s that of the neurotropic strain of RVF. The *S*'s on the base line at log LD 50 = 0 mean that no virus could be detected in the filtrates. The numbers indicate the titres of the filtrates of the 'whole' pantropic virus in different experiments. The titres of the stock virus used are given on the ordinate on the right side of the diagram.

at and near the meniscus were not contaminated with virus from other parts of the gradient. A RVF S_{low} preparation was mixed with CsCl to form two virus suspensions having densities 1·09 and 1·36 g./cm.^{3}. These were introduced into two separate density gradient tubes in place of samples 2 and 8, respectively, and centrifuged in the usual manner. Ten samples were taken from the top of the tube using separate pipettes for each. The results of this experiment are shown in Figs. 6 and 7.

In Fig. 6 it may be seen that most of the infective virus moved down from a density region of 1·09 where it was introduced to a region of density 1·23 g./cm.^{3}. Similarly Fig. 7 shows that most of the virus introduced at a density of 1·36 moved up to a density level of 1·23 g./cm.^{3}. However, it should be noted that in each case a small fraction of virus rose to the surface of the CsCl gradient.

Ultrafiltration

Ultrafiltration experiments were performed on defibrinated RVF infected serum containing the *S* 450 particles as well as on the infected serum freed of the *S* 450 particles. In addition, ultrafiltration experiments were done on the neurotropic strain of the virus, which had received 105 intracerebral passages in mouse brain. In Fig. 8 is given a composite diagram of the results. It will be noticed that the 'whole' virus in the infected serum and the neurotropic strain showed the conventional ultrafiltration curves for viruses. The filtration end-point for both strains was approximately 100 mμ. In contrast to this, the S_{low} material showed a filtration end-point between 219 and 278 mμ, a range of pore sizes through which the 'whole' virus filtered quite readily.

Gel diffusion-filtration

Further information about the particles of the S_{low} fraction of RVF virus was obtained from diffusion-filtration experiments with agar gel.

Mouse serum infected with RVF was centrifuged twice under liquid paraffin at 30,000 r.p.m. for 60 min. The centrifuged serum (1 ml.) was mixed with *Burnupena cincta* haemocyanin (1 ml.) and rabbit haemoglobin (0·1 ml.) and placed on a column (45 × 2 cm.) of granulated agar gel which had passed a 60 mesh sieve. The fraction collector was started when 35 ml. of buffer had flowed from the column and forty-eight fractions of about 1·8 ml. were collected. Every fourth fraction was titrated in mice and the numbers of the fractions containing the reference proteins were noted (Fig. 9A).

In a similar experiment with 2·0 ml. of infected serum which had been pretreated in the same manner, rabbit haemoglobin (0·1 ml.) and *Arenicola loveni* erythrocruorin (0·3 ml.) were added as reference proteins (Fig. 9B).

For comparison an exactly similar experiment was made with MEF_1 poliovirus purified by two cycles of low- and high-speed centrifugation. The reference proteins were rabbit haemoglobin and *A. loveni* erythrocruorin (Fig. 9C). The results showed that whereas poliovirus ($S_{20} = 156$) emerged from the column in a relatively narrow zone very slightly in advance of the *A. loveni* erythrocruorin ($S_{20} = 60$), the RVF virus began to come out before the erythrocruorin and even before the *Burnupena cincta* pigment ($S = 89-92$). RVF virus did not leave the column in as sharp a zone as the MEF_1, but the possibility that the RVF used for these experiments contained some *S* 450 particles cannot be excluded.

RVF S_{low} material which had been centrifuged four times was also tested by gel diffusion-filtration. First, 2·5 ml. of the S_{low} virus suspension derived from liver and spleen, containing haemoglobin and other liver pigments but no added proteins, was applied to the column and fractions collected as before. Every fifth fraction was titrated in mice and tested by complement fixation. When all the protein components had emerged, 1·5 ml. of the S_{low} virus suspension containing 0·5 ml. of the *B. cincta* and 0·5 ml. of the *Jasus lalandii* haemocyanins were run through at the same flow rate and an ultra-violet opacity record of the emerging protein zones were made. The speed of the recording milliammeter chart and that of the fraction

collector were known. By correlating the haemoglobin peak on the chart with the numbers of the fractions in which haemoglobin was visible, it was possible to locate the position of peak virus infectivity and peak CF activity in relation to the reference proteins. In Fig. 10, diagram B shows the ultra-violet opacity of each

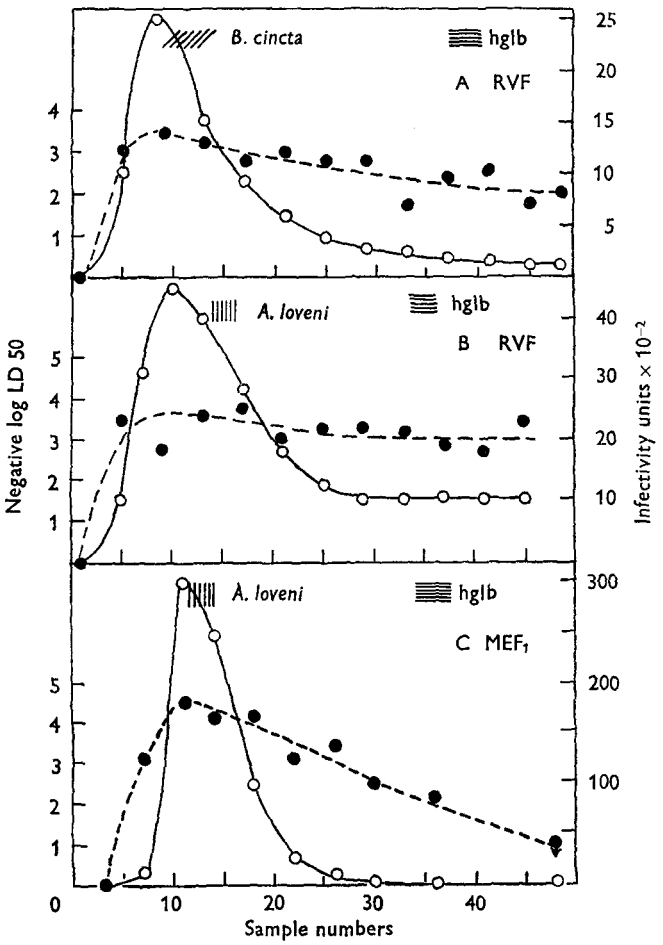

Fig. 9. Diffusion-filtration of, A and B, RVF virus (previously centrifuged twice at 30,000 r.p.m. for 60 min.) and, C, MEF_1 poliovirus in a column of granulated 7% agar gel in the presence of haemoglobin (hglb) and the erythrocruorin of *A. loveni* and haemocyanin of *B. cincta*. Full circles, negative log LD 50 values. Open circles, antilog values (infectivity units). The position of the pigments in the filtrate are shown by shaded areas.

fraction, diagram C the relative virus concentration in every fifth fraction, and diagram D the CF activity of the fraction assayed for virus. The marker pigments responsible for the ultra-violet opacity peaks are indicated in diagram B. Diagram A is the 'elution' curve of *J. lalandii* haemocyanin alone. In this experiment also the virus began to emerge before the *B. cincta* pigment. Most of the CF antigen came out much later than the major part of the virus.

The gel diffusion-filtration behaviour of the denser component of the S_{low} fraction was next investigated.

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The S_{low} fraction from mouse serum was centrifuged in a CsCl gradient as previously described and the fraction containing the virus component of density 1·23 g./cm.^{3} was dialysed to remove CsCl and placed on the agar column. In this experiment rabbit serum (5%, v/v) was added to the displacement medium to stabilize virus infectivity and the column was equilibrated with this mixture before the virus was applied. Every third fraction was titrated in mice.

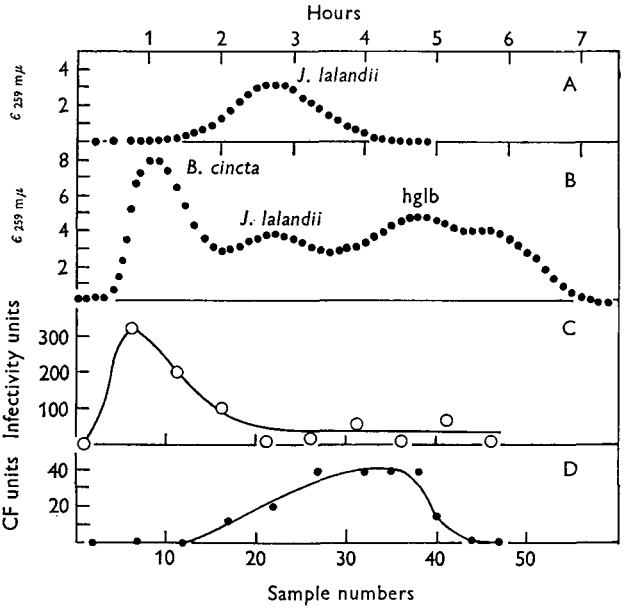

Fig. 10. Gel diffusion-filtration of: A, *J. lalandii* haemocyanin. B, *B. cincta*, *J. lalandii* haemocyanins and haemoglobin (hglb) in a suspension of mouse liver and spleen. C, RVF S_{low} virus fraction. D, RVF complement-fixing antigen present in the S_{low} virus preparation.

The infectivity curve resembled closely that of the whole S_{low} fraction (Fig. 10C), indicating that the denser S_{low} particle diffuses less readily into agar than *B. cincta* haemocyanin and therefore presumably has a lower diffusion constant (Polson, 1961*a*).

In view of the possibility that S_{low} RVF may be filamentous, the gel-diffusion filtration behaviour of deoxyribonucleic acid known to consist of filamentous molecules was examined on a similar agar column (60 × 2 cm.) consisting of 100 mesh 7% Ionagar no. 2 (Oxoid).

Calf thymus DNA of high molecular weight (4 mg.) in 2 ml. of the buffered saline medium was applied to the column and washed through in the usual manner. The ultra-violet opacity diagram (Fig. 11) obtained on gel diffusion-filtration of this substance showed pronounced 'tailing' in a manner reminiscent of the behaviour of RVF S_{low} virus (Fig. 10C). This is in striking contrast to the symmetrical curve obtained with spherical molecules such as *Jasus lalandii* haemocyanin on gel diffusion-filtration (Fig. 10A). The DNA used in these experiments showed a single sedimenting component in the analytical ultracentrifuge. The sedimentation constant found was approximately 26 Svedberg units at infinite dilution.

Effect of immune serum and nucleases

As it seemed possible that the S_{low} fraction might contain infective nucleic acid, the effects of antiserum and nucleases on its infectivity were examined. A 20% suspension of infected mouse liver and spleen was clarified by low-speed centrifugation and the S_{low} fractions prepared from it as previously described. Neutralization tests were done on the clarified suspension and on the S_{low} fractions. Twofold dilutions of antiserum were mixed with equal volumes of test virus diluted to contain 100 to 200 LD 50 per 0·2 ml. The mixtures were left at room temperature for 6 hr. and 0·2 ml. of each was injected intraperitoneally into six mice.

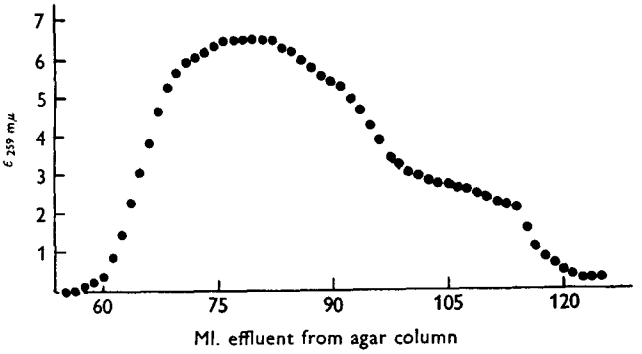

Fig. 11. Diffusion-filtration of high molecular deoxyribonucleic acid in a column of granulated 7% agar gel.

Table 3. *Effect of nucleases on the RVF* S_{low} *virus fraction*

Material tested	Negative $\log_{10}$ LD 50
RVF S_{low} control	4·15
RVF S_{low} and RNAse	3·84*
RVF S_{low} and DNAse	4·17

* The slight difference in titre between the material treated with RNAse and the untreated control is within the limits of the assay.

From the results the antiserum dilutions which protected 50% of the mice were calculated. It was found that 100 LD 50 of the S_{low} fraction required a dilution of 1/64 and 50 LD 50 of the whole virus a dilution of 1/90. There was no evidence for the presence in either virus preparation of an unneutralizable constituent.

To test the effect of nucleases, RVF S_{low} material prepared from a liver and spleen suspension was treated with crystalline RNAse and DNAse (Seravac). Two ml. portions of the virus suspension were treated with 2 ml. of enzyme and incubated at 37° C. for 1 hr. The $MgSO_4$ (2 mg.) was added to the DNAse suspension. A control virus sample was also incubated at 37° C. The three samples were titrated in mice. The results (Table 3) show that the S_{low} material is unaffected by RNAse and DNAse.

In another experiment the S_{low} material was split into two fractions by centrifugation in a CsCl gradient. One virus fraction had a density of 1·23 g./cm.^{3} and the other consisted of the components of lower density. Each fraction was treated with both enzymes. Neither was affected by RNAse or DNAse.

DISCUSSION

Most of the infectivity of RVF virus is associated with a particle having a sedimentation constant of 452 Svedberg units (Naudé, Madsen & Polson, 1954).

It has now been shown that after repeated ultracentrifugation at a velocity sufficiently high to remove all infective virus particles from an MEF_1 poliovirus suspension ($S_{20} = 156$) RVF virus retains a relatively high degree of infectivity.

Cheng (1961), who determined that Semliki Forest virus has a monodisperse infective particle, also noticed traces of virus in the supernatant fluid after high-speed centrifugation, but attributed its presence to convective disturbances. If this were the case the number of infective particles in the supernatant fluid should decrease progressively during successive periods of high-speed centrifugation. This was shown to occur in experiments with monodisperse MEF_1 poliovirus when infective virus was no longer detectable in the supernatant fluid after three cycles of ultracentrifugation. However, under similar conditions, the supernatant fluids of RVF virus maintained a fairly constant infectivity. The particles responsible for this have conveniently been called the S_{low} fraction.

The experimental evidence suggests that the S_{low} virus fraction is composed of a range of particles varying in density and in sedimentation constant. The results of three experiments performed to determine the sedimentation constant of the S_{low} virus by prolonged ultracentrifugation in a *J. lalandii* haemocyanin gradient were presented. In one experiment, the infectivity of the S_{low} virus samples after centrifugation varied to such an extent that only a one-step curve was drawn (Fig. 3A), whereas in the other two experiments (Fig. 3B and C) the distribution of infectivity in the tube suggested the presence of two components. Sedimentation constants obtained from these curves (*a* and *b*, Fig. 3B and C) were about 7 and 15–19 Svedberg units.

A second method of centrifugation using the SW 39 rotor yielded a sedimentation constant of 4 Svedberg units which may be regarded as a minimum value. Although the disparity in results is within the range of experimental error of these techniques, the different sedimentation constant values obtained, together with the results of density gradient centrifugation experiments, suggest inhomogeneity in the S_{low} virus fraction.

The results of centrifugation in CsCl gradients indicated that the greater portion of the S_{low} virus has a density of 1·23 g./cm.^{3}, which is the same as that found for the readily sedimentable portion of RVF virus with a sedimentation constant of 452 Svedberg units. However, in all these experiments, the results suggested the presence of lighter components varying in density which rose to the surface in the tube during centrifugation. Owing to the method of preparing the S_{low} virus fraction (i.e. consecutive centrifugation under a layer of liquid paraffin) it is to be

expected that the virus particles of lower density would vary in amount from one S_{low} preparation to another, causing the variability in the results obtained.

It may be assumed that a smooth spherical particle of sedimentation constant 15–19 and density 1·23 g./cm.^{3} would be about 12 mμ in diameter and, when passed through a column of granulated 7 % agar, would be eluted in a similar position to *J. lalandii* haemocyanin (S_{20} = 16). In gel diffusion-filtration experiments with the denser S_{low} fraction of RVF virus, the infective zone emerged from the column with the solvent front and ahead of the largest standard protein, i.e. *Burnupena cincta* haemocyanin (S_{20} = about 90), indicating that its diffusion constant is smaller than that of either haemocyanin. The relatively low sedimentation and diffusion constants of the S_{low} virus taken together suggest a very high frictional ratio. It is therefore postulated that at least the S_{low} particles having the same density as the sedimentable RVF virus (S_{20} = 452) are filamentous. Ultrafiltration experiments showed that the S_{low} material has a filtration end-point more than double that of the S 452 form of the virus. This may be taken as further evidence for the filamentous nature of the S_{low} particles as spherical particles would filter through a membrane more readily than long filaments.

Further supporting evidence is obtained from the behaviour of high molecular DNA on the granulated 7 % agar column. This substance migrated with the solvent front and showed excessive 'tailing' in its elution curve similar to that of the S_{low} virus fraction. A possible explanation for the 'tailing' effect is that the filamentous particles were trapped in crevices in and/or between surfaces of contact of the agar granules. This is in contrast to the behaviour of spherical particles, which are delayed for shorter periods in these localities due to their higher diffusion rates. The behaviour of RVF in gel columns was shown to contrast strikingly with that of 'spherical' viruses such as MEF_1. It was also shown that the various S_{low} particles were neutralized by antibody and resistant to nucleases. They are therefore unlikely to be free infectious nucleic acid, or nucleic acid encased in a layer of non-antigenic lipoid or other material as postulated by Herriott (1961). Since most of the S_{low} virus has a density similar to that of the bulk of the virus in infective mouse serum, it may be provisionally assumed that its infectious nucleic acid is protected from nucleases in a manner similar to that of the RVF of sedimentation constant, 452 Svedberg units.

The evidence now presented suggests that certain viruses, such as Rift Valley fever, produce infective particles which are not sedimentable under conditions capable of removing most of the virus infectivity from the supernatant fluid and that these particles may be filamentous.

Gessler *et al.* (1956) reported that Rous sarcoma virus purified by their fluorohydrocarbon method was not appreciably sedimented during 20 hr. centrifugation at 144,000 ***g***. in fluid of density 1·004. The supernatant fluid, which appeared to be more infective than the starting material, gave electron micrographs showing particles 10–20 mμ in diameter. These authors inferred that their treatment had broken up larger, loosely packed virus particles into infective subunits. As the density of the 'non-sedimentable' virus was not determined the cause of its resistance to centrifugation is difficult to assess. Low densities of from 1·15 to

1·19 have been assigned to Rous sarcoma virus by other authors (Kahler, Bryan, Lloyd & Maloney, 1954; Crawford, 1960) and attributed to the presence of lipoid or water in the virus particles.

The density of RVF virus (1·23 g./cm.^{3}) and that of the S_{low} virus fraction, which varies from 1·23 to < 1·1 g./cm.^{3}, is lower than the density of about 1·3 attributed to protein particles and probably indicates the presence of lipoid material in conformity with the ether sensitivity of this virus (Andrewes & Horstmann, 1949).

The S_{low} fraction, however, does not behave as a small particle so that the assumption of a filamentous shape seems the simplest explanation of its centrifuged behaviour. Those particles which rise to the surface during density gradient centrifugation may also be filamentous and presumably contain very much more lipoid than the 'normal' virus.

SUMMARY

If Rift Valley fever virus (RVF), of which the main component has a sedimentation constant (S_{20}) of about 450, is centrifuged under conditions adequate to sediment MEF$_1$ poliomyelitis virus (S_{20} = 156) completely, about 1 % of the original infective particles remain in the supernatant fluid. This slowly sedimenting (S_{low}) fraction was shown by centrifugation to contain infective components ranging in sedimentation constant from 4 to 19 Svedberg units. Density gradient centrifugation showed that the densities of these particles varied from 1·23 to < 1·1 g./cm.^{3}. The components were neutralized by RVF immune serum but were not affected by ribonuclease or deoxyribonuclease. In gel diffusion-filtration experiments the S_{low} virus behaved as substances having very low diffusion constants. Its filtration end-point using graded collodion membranes, is approximately two to three times higher than the 'whole' virus. It is suggested that particles comprising the S_{low} virus fraction contain lipoid material and may be filamentous.

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