J. Hyg., Camb. (1964), **62**, 239
With 3 plates
Printed in Great Britain

Physical and serological investigation of Rift Valley fever antigens

By JULIA LEVITT and A. POLSON
C.S.I.R. and U.C.T. Virus Research Unit, Medical School, Cape Town, South Africa

(*Received* 13 *December* 1963)

INTRODUCTION

When the infective particles have been centrifuged from preparations of MEF_1 poliovirus, rabies, Rift Valley fever, bluetongue and horse sickness viruses, complement-fixing 'soluble' antigens remain in the supernatant fluids (Kipps *et al.* 1957). By centrifugation in the preparative ultracentrifuge, it was found that the soluble antigens of MEF_1 poliovirus and neurotropic Rift Valley fever virus are approximately 12 mμ in diameter and that the complement-fixing antigen of bluetongue is associated with several components of which the smallest is 8 mμ in diameter.

Several new techniques which lend themselves to the study of soluble antigens are now available. Filtration through columns of granulated agar gel (Polson, 1961*a*; Andrews, 1962; Steere & Ackers, 1962) provides an additional method of particle size determination, and zone electrophoresis in a density gradient has proved effective in the purification (and classification) of certain viruses (van Regenmortel, 1961; Polson & Deeks, 1962). The application of these and other established techniques to the non-infective antigens of pantropic and neurotropic Rift Valley fever virus is now described.

MATERIALS AND METHODS

Virus strains

One pantropic and three neurotropic strains of Rift Valley fever virus were kindly supplied by the Division of Veterinary Services, Onderstepoort. The neurotropic strains consisted of neurotropic Rift Valley fever (NRVF) derived from Smithburn's original neurotropic virus (Smithburn, 1949) which had had 103 intracerebral passages in adult mice; a mouse-egg-mouse strain (MEM) which had had 102 mouse, 56 egg and an additional 17 mouse passages; and a tissue-culture strain (R 120) derived from the serum of a lamb infected with a fully virulent field strain of virus passaged serially in lamb kidney tissue culture. After 50 passages in tissue culture, this strain was no longer hepatotropic but remained neurotropic (Weiss, personal communication).

In our laboratories, these viruses were injected intracerebrally into 3- to 5-day-old mice and the brains of all sick mice were harvested after 48 hr. Stock virus suspensions were prepared to eliminate the RVF virus inhibitor present in mouse

brain (Polson & Madsen, 1955). The diluent used was a mixture of 5% (v/v) rabbit serum with 0·85% (w/v) saline containing 200 units penicillin and 0·2 mg. of streptomycin per ml. A 10% (w/v) suspension of infected brain tissue was clarified by centrifugation and then dialysed in a cellophane bag in saline at 4° C. for 2–3 days to remove the virus inactivator. After dialysis, the virus suspension was stored at 4° C. as stock virus. In an alternative method, the virus was removed from infected brain suspensions by alternate cycles of low- and high-speed centrifugation. Virus pellets were finally suspended in a convenient volume of serum-saline and stored at 4° C. as stock virus. Fresh stock virus suspensions were prepared about every 3 months.

The fully virulent pantropic virus (PRVF) was maintained by intraperitoneal mouse passage of infected liver or blood.

Phosphate buffered saline (PBS), 0·85% (w/v) NaCl, 0·01 M-phosphate (pH 7).

Agarose was prepared from Ionagar no. 2 (Oxoid) by precipitation with polyethylene glycol (Russell, Mead & Polson, 1964).

Preparation of antigens

Antigens of the neurotropic strains were derived from the brain tissue of infant mice infected intracerebrally when 3–5 days old with 0·02 ml. stock virus diluted 1/10. The incubation period was 2–3 days. The pantropic antigen was derived from liver and spleen of mice approximately 16 days old infected by intraperitoneal injection of 0·1 ml. stock virus diluted 10^{-3}, which had an incubation period of 30 hr. Infected tissue was extracted by either of the following methods.

Method 1. About 10 g. of infected tissue was ground in a mortar with cold PBS (50 ml.) and the suspension in portions of 25 ml. was subjected to ultrasonic treatment at 20 kcyc./sec. in a M.S.E. Mullard ultrasonic disintegrator for 15–20 min. The suspension was centrifuged at 33,000 rev./min. for 60 min. in the no. 40 rotor of a Spinco model L ultracentrifuge to remove tissue debris and most of the virus. All centrifugations were done under a layer of liquid paraffin to trap lipoid floating to the surface. The supernatant fluid was pervaporated to half its volume, dialysed against PBS which had been diluted 1/2 with distilled water and pervaporated further to about one-eighth of the original volume. A precipitate which formed during pervaporation was removed by centrifuging at 12,000 rev./min. for 15 min. The solution was finally dialysed against undiluted PBS, stored at 4° C. in the presence of penicillin and streptomycin, and used with the least possible delay.

Method 2. Infected tissues were homogenized in a chilled Waring blender and extracted with acetone and ether (at a temperature between 0° and 4° C.) as described by Clarke & Casals (1958). To obtain concentrated antigen extracts, the dried tissue was rehydrated in 2 ml. cold PBS for every 1 g. of original tissue, left overnight at 4° C., and centrifuged at 30,000 rev./min. for 30 min. The supernatant fluid was removed and the sediment, redispersed in the same volume of PBS as before, was subjected to ultrasonic treatment at 20 kcyc./sec. for 20 min. The suspension was centrifuged at 30,000 rev./min. for 40 min. The supernatant fluid was removed and the residue extracted once more in the same way but with ultra-

sonic treatment for 10 min. only. The three extracts were combined and dialysed overnight against distilled water at 4° C. to remove impurities such as salt. The solution was freeze-dried in 6 ml. amounts and stored at −20° C.

Preparation of antisera

Neurotropic strains. Adult mice received six or seven intraperitoneal 0·2 ml. injections of either a 10% emulsion of infected mouse brains or virus which had been centrifuged from a 10% emulsion at 30,000 rev./min. for 60 min. and resuspended in saline. The injections were given at 5-day intervals and the mice were bled 14 days after the last dose.

Pantropic virus. Unimmunized mice do not survive intraperitoneal injection of the pantropic virus. The adult mice used for production of antisera were therefore protected by two intraperitoneal injections of either the neurotropic virus (Smithburn, 1949) or pantropic virus inactivated with formalin, before receiving six injections of the live pantropic virus. PRVF antiserum I was obtained from mice preimmunized with a neurotropic strain and PRVF antiserum II from mice preimmunized with formalin inactivated pantropic virus.

Gel diffusion-filtration

The columns (60 × 2 cm.) were prepared (Polson, 1961*a*) from granulated 7% (w/v) agarose which had passed a 100-mesh sieve. The displacement medium, 0·067 M-phosphate buffer and 0·073 M-NaCl, pH 7·2, was saturated with chloroform to inhibit microbial growth. The following were used as reference proteins (Polson, 1961*a*):

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

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

(*c*) Mouse haemoglobin, molecular weight 68,000 and diffusion coefficient $6{\cdot}5 \times 10^{-7}$ cm.²/sec.

The effluent from the column passed through an LKB 'Uvicord' apparatus which recorded its opacity to ultraviolet light of wavelengths near 2537 Å. and fractions were collected automatically in the apparatus of Polson (1961*b*).

Zone electrophoresis in sucrose density gradients

The apparatus of Polson & Cramer (1958) was used. Experiments were done under standardized conditions of pH, potential and concentration gradients, and ionic strength as described by Polson & Deeks (1962). To facilitate exact comparison between the results of different runs, the mobility of the antigens was in each case compared with the mobilities of phenol red (added to the sample) and haemoglobin which the antigen preparations always contained. Electrophoresis was continued for about 24 hr. with a current of about 25 mA. and a potential gradient of 4·5 V./cm. During this period, the haemoglobin migrated about 4 cm. and the phenol red 20 cm. Afterwards the contents of the column were fractionated as described by Polson & Cramer (1958).

The 'protein' content of each fraction was measured by the light scattering method of Mead (1962). Three ml. dilute perchloric acid (66 ml. A.R. 60%, w/w, acid diluted to 500 ml. in distilled water) were added to 1 ml. of a 1/100 dilution in saline of each fraction. The mixtures were examined in the light-scattering apparatus and the galvanometer readings which are proportional to 'protein' content were recorded as turbidimetric units.

Fractions to be tested by complement fixation and gel precipitation were dialysed against three changes of PBS containing thiomersal 0·01% (w/v) over a period of 3–5 days to remove the sugar. Fractions were concentrated when required, by dialysis overnight against PBS diluted 1/4 in distilled water, followed by freeze drying and solution of the residue in 0·5 ml. distilled water.

Complement-fixation (CF) titrations

Dilutions of antigen were made in the solution recommended by Mayer, Osler, Bier & Heidelberger (1946). Antisera were inactivated at 56° C. for 30 min. Titration was by the method of Casals & Olitsky (1950). Complement was titrated by the method of Casals, Olitsky & Anslow (1951) and two units used in the test. The complement-fixing titre is the reciprocal of the highest dilution of antigen giving 50% fixation.

Ultracentrifugation

Approximate sedimentation constants were determined by the methods of Polson & Linder (1953) and Polson & Madsen (1954). In this technique, a protein of appropriate sedimentation constant is added to form a density gradient which hinders convection.

Determination of particle density

Densities were determined by centrifugation in a preformed gradient of caesium chloride by the method of Polson & Levitt (1963*a*).

Gel precipitation

Qualitative gel-precipitin tests (Ouchterlony, 1953) were done on flat glass plates in a 3 mm. layer of 1·5% (w/v) washed Difco agar dissolved in phosphate buffer containing sodium ethylene-diamine tetra-acetate and sodium azide (Allison & Humphrey, 1960). A cutter was used forming wells 4 mm. in diameter and 5 mm. apart, parallel to a trough 3 mm. wide and 4 mm. distant from the wells. Antigen samples were placed in the wells and antisera in the troughs. A hexagonal arrangement of wells 7 mm. in diameter and 4 mm. apart (Mansi, 1957) was also used.

Quantitative gel-precipitin tests were performed in a perspex apparatus as described by Polson (1958).

By means of a microcomparator, the distance of the precipitation bands from the antibody meniscus and the band widths after various time intervals were measured. The microcomparator consisted of a telescope mounted on the platform of a Hilger–Watts travelling microscope with a vernier scale. The perspex apparatus was held in place in a special adjustable clamp on an optical bench, at the focal point of the telescope. The cross-hairs of the telescope were focused on the perspex apparatus and the measurements made.

EXPERIMENTAL AND RESULTS

Gel diffusion-filtration through granulated 7 % agarose

To determine the elution position of PRVF antigen in relation to those of the three reference proteins, 2 ml. of antigen prepared by Method 1 which already contained haemoglobin was mixed with 0·5 ml. of a 4 % solution of *B. cincta* haemocyanin and 0·5 ml. *J. lalandii* haemocyanin, placed on the agarose column and washed through with 0·067 M-phosphate, 0·073 M-NaCl, pH 7·2. When about 60 ml. had passed through, 50 fractions of 2 ml. were collected.

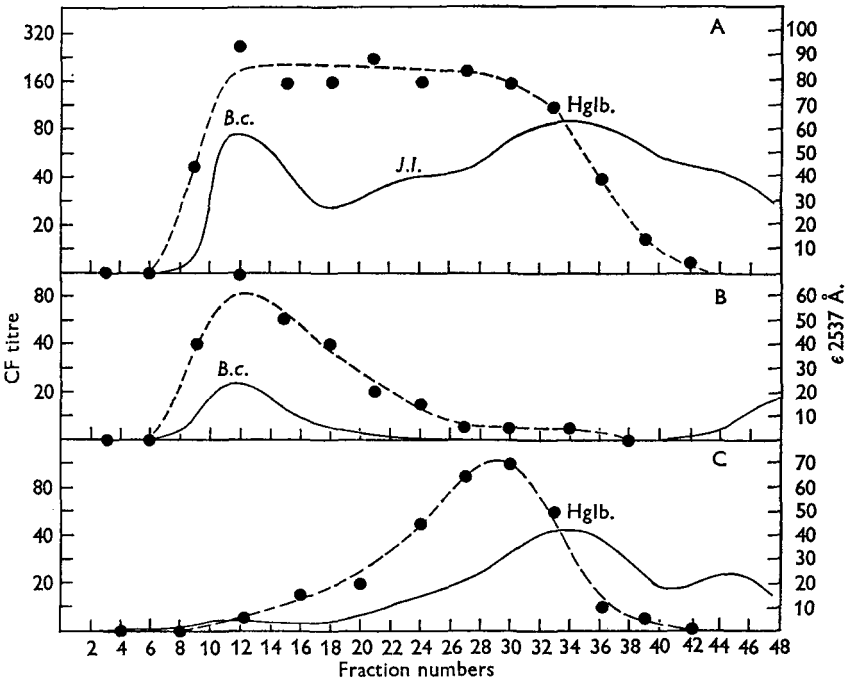


Fig. 1. A. Gel diffusion-filtration of PRVF antigen through granulated 7% agarose showing the complement-fixing titre of the filtrate (broken line) in relation to the peaks in the Uvicord trace (solid line) corresponding to *B. cincta* (*B.c.*) haemocyanin, *J. lalandii* (*J.l.*) haemocyanin and haemoglobin (Hglb.) respectively (reading from the left). B. Fractions 7–14 from A, pooled concentrated and refiltered through the column. C. Fractions 27–35 from A, pooled concentrated and refiltered through the column.

Every third fraction was titrated by complement fixation against the homologous antiserum diluted 1/50. Fig. 1 A shows the CF titre and the Uvicord trace. As the latter indicates opacity and not extinction it considerably underemphasizes the height and therefore the resolution of the protein peaks. The results show that while the haemocyanins and the haemoglobin came out in distinct though only partially separated peaks, the antigen emerged with an almost constant titre in a large volume comprising the two haemocyanins and about half the haemoglobin.

To confirm the inhomogeneity in particle size of the antigen suggested by this

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press

result, fractions 7–14 containing antigen and *B. cincta* haemocyanin were mixed to form pool A and fractions 27–35 containing antigen and haemoglobin were mixed to form pool B. Pools A and B were separately dialysed against 0·017 M-phosphate, concentrated by pervaporation to 3 ml., clarified by centrifugation at 8000 rev./min. for 10 min. and run through agarose columns as described above. The elution curves of pool A (Fig. 1B) and pool B (Fig. 1C) confirm the particle size inhomogeneity and suggest that neither aggregation of the smaller particles nor dissociation of the larger ones was proceeding rapidly under the experimental conditions.

It appeared therefore that the soluble antigen of PRVF virus consists of particles ranging in diameter (or at least in diffusibility) from that of *B. cincta* haemocyanin (or greater) to a size slightly larger than that of haemoglobin. Partial classification of such a mixture on a particle size basis should be effected by centrifugation. To select the smaller particles, a solution of crude PRVF antigen was centrifuged four times successively under liquid paraffin in the no. 40 rotor at 33,000 rev./min. for 60 min. (cf. Polson & Levitt, 1963*b*). After each centrifugation, the supernatant fluid was removed by a pipette held initially half-way between the 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 pervaporated from 13 to 2 ml., dialysed against 0·034 M-phosphate and applied to the agarose column. The elution diagram (Fig. 2) confirmed that centrifugation had removed most of the larger particles of antigen.

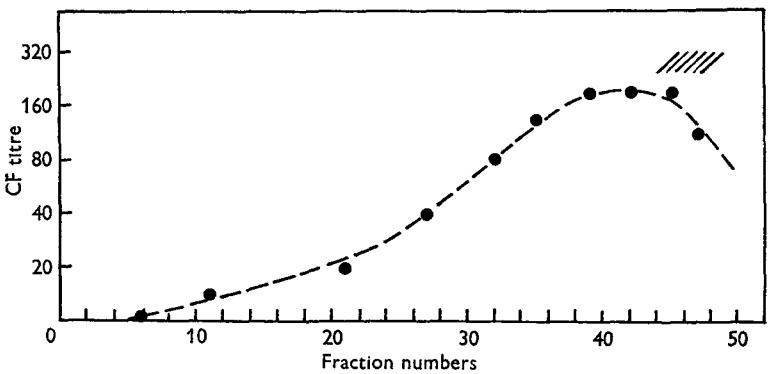


Fig. 2. Gel diffusion-filtration of the small particle fraction of PRVF antigen separated by centrifugation. The broken line shows the complement-fixing (CF) titre of the filtrate and the hatched area, the fraction in which haemoglobin was visible.

Antigen from a neurotropic strain (NRVF) mixed with the two haemocyanins and subjected to gel diffusion-filtration in the same way gave almost identical results (Fig. 3).

The CF titre curves in Figs. 1A and 3 appear to have three modest peaks suggesting the presence of three overlapping components. In an attempt to confirm this, a gel diffusion-filtration experiment on the pantropic antigen was performed

in which the dilutions in the CF test followed the series 1/50, 1/100, 1/150, 1/200. Although subsequent work with other methods supported the existence of three components, no confirmation was obtained on this occasion. The results are shown in Fig. 4. This experiment in which haemocyanins were not added indicates the elution characteristics of the ultraviolet absorbing constituents of the tissue extract and shows that some purification of the antigen could be achieved by this technique.

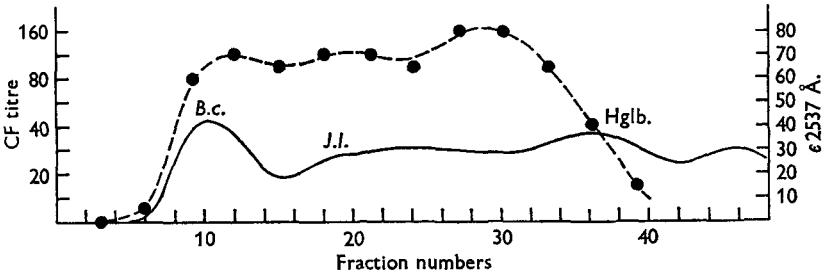


Fig. 3. Gel diffusion-filtration of NRVF antigen in relation to *B. cincta* (*B.c.*) and *J. lalandii* (*J.l.*) haemocyanins and haemoglobin (Hglb.). The solid line indicates the ultraviolet absorption and the broken line, the complement-fixing (CF) titre of the filtrate.

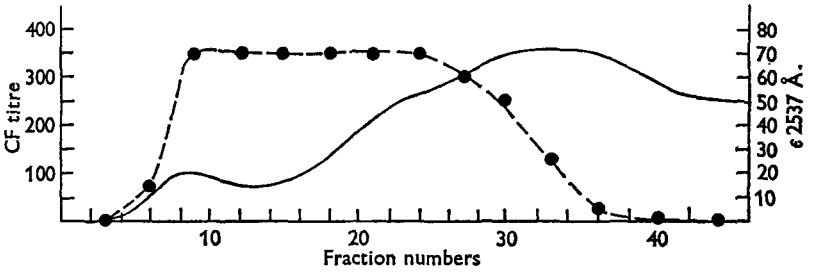


Fig. 4. Gel diffusion-filtration of PRVF antigen showing the ultraviolet absorption of the tissue extract in the absence of haemocyanins (solid line) and the CF titre (broken line).

Centrifugation in a protein density gradient

Ultracentrifugation experiments were performed in a further attempt to group the antigens according to size. Antigen suspensions were prepared by Method 1 but were pervaporated only to half their original volume before dialysis against PBS. Nine ml. of antigen suspension, mixed with 2 ml. *J. lalandii* haemocyanin, were placed in a graduated centrifuge tube and spun at 33,000 rev./min. for 180 min. in the no. 40 rotor. After centrifugation, the contents of the tube were removed in 10 fractions as described by Polson & Linder (1953). The CF titres of the fractions were plotted against their positions in the tube to give the sedimentation diagram for the soluble antigen of the pantropic virus shown in Fig. 5. There appear to be three complement-fixing components, of which one (*c*) sedimented to the bottom of the tube. The two slower sedimenting components (*a* and *b*) are indicated by the two steps in the curve. The sedimentation constants of (*a*) and

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press

(*b*) calculated by the method of Polson & Madsen (1954) were 7·8 and 28·8 Svedberg units respectively.

An attempt was made to estimate the sedimentation constant of the largest component (*c*) by centrifuging an antigen suspension at 30,000 rev./min. in the presence of *B. cincta* haemocyanin for 40 min. The results were inadequate for exact calculation but indicated that the sedimentation constant of the largest antigen particle may exceed 100 Svedberg units.

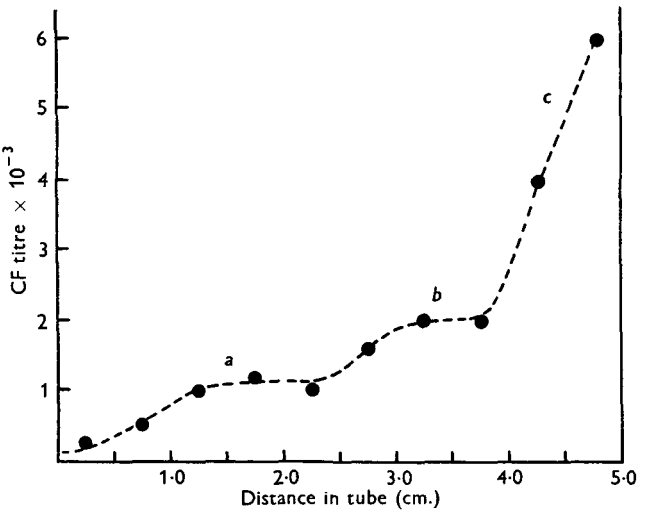


Fig. 5. The vertical distribution of CF titre in a tube containing PRVF antigen mixed with *J. lalandii* haemocyanin which had been centrifuged at 33,000 r.p.m. for 180 min.

A similar experiment with an antigen suspension prepared from a neurotropic strain (NRVF) yielded sedimentation constants of 8·4 and 28·9 Svedberg units for components (*a*) and (*b*) respectively. Here again there was evidence of a component of sedimentation constant greater than 100 Svedberg units.

Gel precipitation and immunological identity

The fractions obtained from gel diffusion-filtration experiments on the pantropic and neurotropic virus antigens (cf. Figs. 1A and 3) were pooled in groups of three (6 ml.), freed of excess salt by dialysis against PBS which had been diluted 1/5 in distilled water, and freeze-dried to concentrate them for gel precipitation tests. The dry residues were redissolved in 0·2 ml. distilled water and tested for immunological identity against both a pantropic and neurotropic antiserum diluted 1/2 with the results shown in Plate 1*a*. The precipitation lines are continuous except at the terminal wells where line splitting and crossing occurred. These effects were found to be artifacts caused by the complex diffusion system at the ends of the rows and disappeared when the samples showing the effect were placed in wells in the central region. The continuity of the lines indicates that the antigens of the different size groups are immunologically identical.

It was further shown that antigen preparations of all four strains of the virus were indistinguishable when tested in neighbouring wells. Plate 1*b* demonstrates this and also reveals the presence of a second smaller antigen. Normal liver and brain preparations were also included in the experiment and showed no precipitation lines.

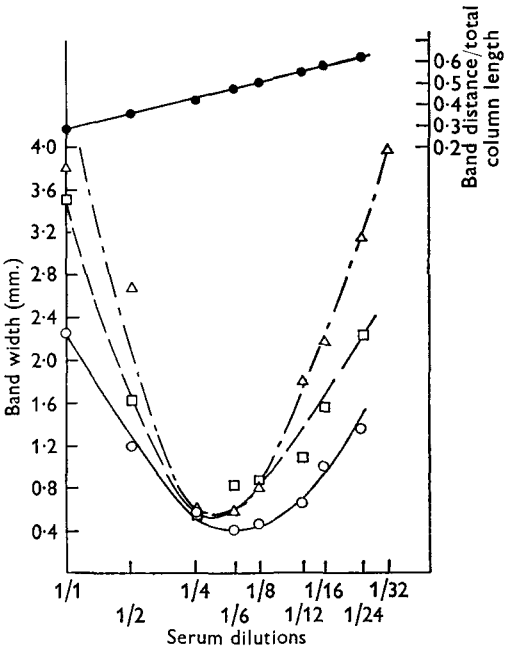


Fig. 6. Results of a quantitative gel precipitin test of polydisperse PRVF antigen against PRVF antiserum (I) diluted 1/2. The parabolic curves relate antigen dilution to band width and the diagonal curve to band distance from the antibody meniscus after 8 days (○ and ●), 14 days (□) and 21 days (△).

Quantitative gel-precipitin tests

To determine the diffusion constant of the pantropic antigen, several quantitative gel-diffusion experiments (Polson, 1958) were performed. Fig. 6 shows a typical diagram obtained with antigens prepared by Method 2. Similar results were obtained from antigens prepared by Method 1. These antigen preparations are now known to be inhomogeneous with regard to particle size and it should be noted that the band width curves are parabolic in contrast to the V-shaped curves obtained by van Regenmortel (1959) and Polson & Hampton (1960) using monodisperse systems. The former curves do not allow the diffusion coefficient to be accurately determined.

To obtain a suspension of more nearly monodisperse PRVF antigen, a preparation made by method 1 was centrifuged for 5 hr. at 33,000 rev./min. under liquid paraffin. The upper portion of the supernatant fluid was removed and tested against PRVF antiserum (II) diluted 1/10. The results are shown in Fig. 7. In this instance the band width measurements produced V-shaped curves indicating at their intersection the position that the precipitation line would occupy if derived

from antigen and antibody in exactly optimal proportions. The distance (x_b) of such a line from the antibody meniscus could be obtained by interpolation. By subtracting x_b from the total column length, the distance from the band to the antigen meniscus (x_g) could be determined. The diffusion coefficient of the antigen (D_g) was calculated from the equation

$$\frac{D_g}{D_b} = \frac{x_g^2}{x_b^2}.$$

There is unfortunately some doubt about D_b, the diffusion coefficient of mouse γ-globulin, which is stated by Allison & Humphrey (1959, 1960) to be the same as

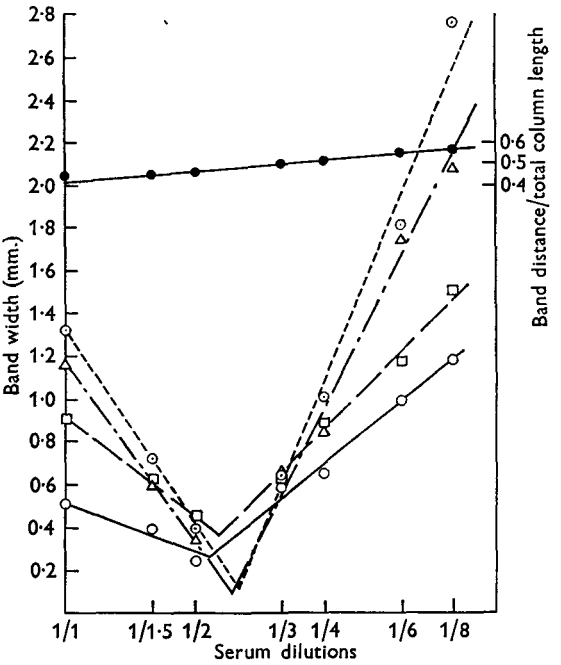


Fig. 7. Results of a quantitative gel precipitin test of the small fraction of PRVF antigen against PRVF antiserum (II), diluted 1/10. The V-shaped curves relate antigen dilution to band width and the diagonal curve to band distance from the antibody meniscus after 6 days (○ and ●), 8 days (□), 11 days (△) and 12 days (⊙).

that of human and rabbit γ-globulin for which they give a value of $3{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. A higher value, $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. was obtained by Largier (1959) for horse tetanus antitoxin which was shown to be free of traces of macroglobulins. This value is also similar to that calculated for the diffusion coefficient of the precipitating antibody against a haemocyanin prepared in the rat, the rabbit and the guinea-pig (Polson & Deeks, 1960). In addition, a value of $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. for macroglobulin-free human γ-globulin has been obtained by Polson & Potgieter (1964), who attributed the lower results of earlier investigators to the presence of macroglobulin in their preparations.

If the diffusion coefficient of mouse γ-globulin is provisionally assumed to have the higher value of $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec., the smallest particles of PRVF antigen

have a diffusion coefficient of $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. for which a particle diameter of 7 mμ is obtained by the Stokes–Einstein equation. If the diffusion coefficient of mouse γ-globulin has the lower value $3{\cdot}8 \times 10^{-7}$ cm.^{2}/sec. given by Allison & Humphrey, the diffusion coefficient of the smallest antigen particles is $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. and the corresponding diameter, 9 mμ.

Plate 2*a* shows the appearance of precipitation bands from which measurements were made, 7 days after the experiment had been set up. After a further 25 days, the precipitation bands on either side of the optimal proportions region had split as shown in Plate 2*b*. This splitting occurred both with unfractionated antigen and with the more homodisperse fraction obtained by centrifugation. Plate 2*c* shows an Ouchterlony test set up with a series of twofold dilutions of PRVF antigen prepared by Method 2. It may be seen that a single band of precipitate split into two on dilution.

The density of PRVF antigen

To determine the density of the most rapidly sedimenting fraction, PRVF antigen prepared by Method 1 (but without pervaporation or dialysis) was centrifuged at 33,000 rev./min. for 90 min. in the no. 40 rotor. The pellets were resuspended in 3 ml. buffer and clarified at 8000 rev./min. for 8 min. Small portions were adjusted to densities of 1·08, 1·22 and 1·33 for insertion at appropriate levels in preformed density gradients of caesium chloride in separate tubes as described by Polson & Levitt (1963*a*). The tubes were centrifuged for 4 hr. at 33,000 rev./min. The contents of each tube were divided into 10 fractions by pipetting from the top and the fractions tested by gel precipitation. The average density of all fractions containing antigen was 1·27. The density of the antigen is therefore very close to 1·27 g./ml. During centrifugation, a visible band of a non-antigenic fraction formed at a density level of 1·19 g./ml. in each tube.

Another experiment was performed to obtain the densities of the slower sedimenting antigens. A preparation of PRVF antigen was separated on 7% agarose as described above (cf. Fig. 1) and the fractions containing *B. cincta* haemocyanin and haemoglobin were pooled separately. These will be referred to as *A* and *C* respectively since pool *B* was formed by the intermediate fractions in this instance. The three pools were concentrated to 0·5 ml. by pervaporation and dialysis as described and each was placed in the middle of a preformed caesium chloride gradient at a density level of 1·22 g./ml. Centrifugation was for 16 hr. at 30,000 rev./min. Ten fractions were taken from each tube and tested by gel precipitation with the results shown in Plate 2*d*. The average density of the antigen from each pool was again close to 1·27 g./ml.

Zone electrophoresis

Antigen extracts prepared by Method 2 were submitted to zone electrophoresis in a sucrose gradient to find out whether antigens from different strains differed in mobility and to investigate electrophoresis as a means of purification.

The results obtained with the three neurotropic strains and the pantropic strain are presented in Fig. 8*a* and *b* respectively. In all cases the complement-fixing

antigen had approximately the same migration rate as the haemoglobin which was contained in the 'protein' peak 3–5 cm. from the origin in the light scattering histograms. A second 'protein' peak occurs between 9–13 cm. from the origin. The complement-fixing antigens of the four strains are therefore indistinguishable by zone electrophoresis.

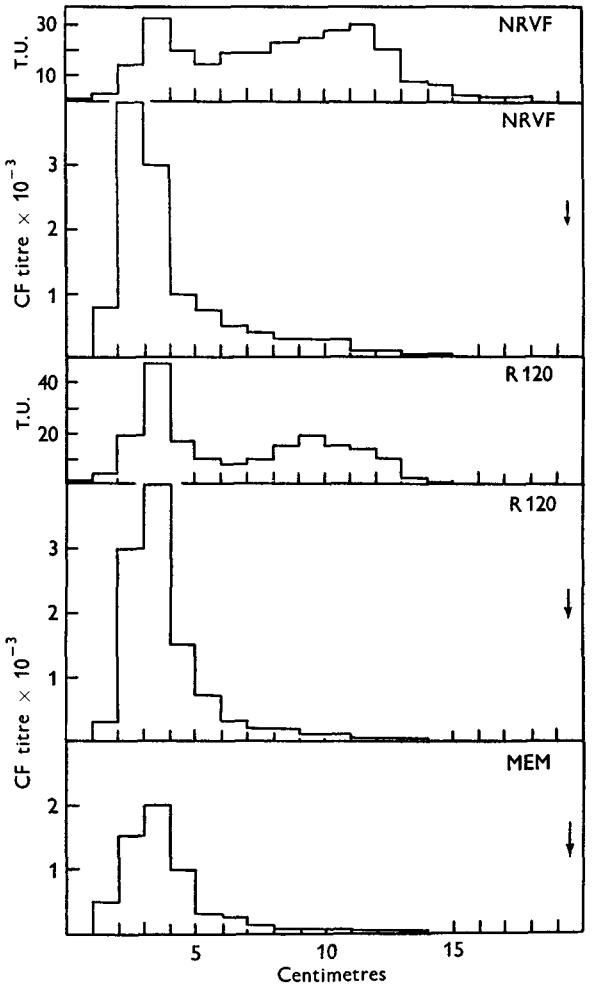


Fig. 8*a*. Zone electrophoresis diagrams of the antigens of the neurotropic strains. The CF titres and 'protein' content (turbidimetric units, T.U.) of successive fractions are plotted as histograms. The haemoglobin is contained in the 'protein' peak 3–5 cm. from the origin. The arrow indicates the position of the phenol red band.

Consecutive 1 cm. samples from the electrophoresis column were also tested by gel precipitation against antisera to all the strains. With all four strains of RVF, precipitation bands occurred in the same region as the peak complement-fixing activity. A second faint line of precipitation was detected when fractions 11 and 12 of PRVF were tested against the homologous antiserum (Plate 3*a*). This faint line of precipitation was detectable when fractions 11–15 of NRVF antigen were tested

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press

against antisera to R 120 and PRVF and when fractions 11–14 of MEM antigen and fractions 11–13 of R 120 antigen were tested against PRVF and the homologous antiserum. Fractions 9–13 of R 120 antigen showed this precipitation band when tested against MEM antiserum. This precipitation line appears to be due to a second faster migrating antigen which may account for the 'tailing' of the CF titre into this region of the electrophoresis diagrams. Further evidence for the existence of this second antigen will be given.

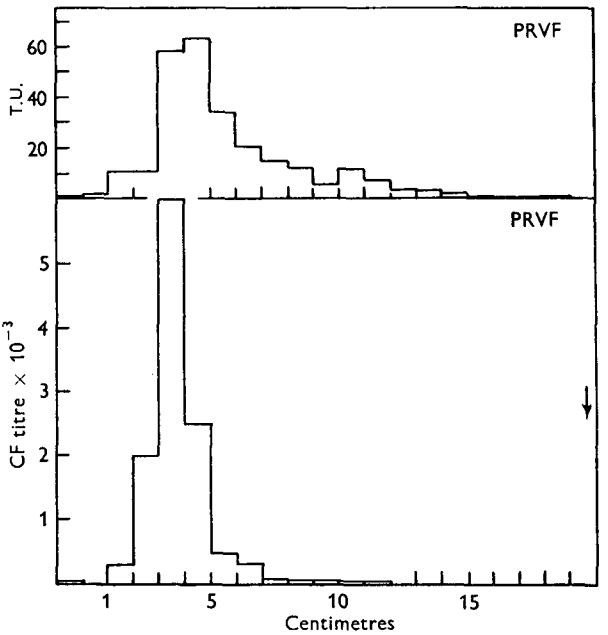


Fig. 8*b*. Zone electrophoresis diagram of the pantropic antigen. The curves have the same significance as in Fig. 8*a*.

The neurotropic antigen fractions having the highest CF titre showed splitting of the precipitation bands when tested with various antisera. A greater degree of splitting was shown by the pantropic antigen (Plate 3*a*), but there is however no other evidence for the presence of more than one antigen in this region.

The faster migrating antigen was not demonstrable when fractions from the electrophoresis column were tested in wells 7 mm. in diameter except with the R 120 strain. A precipitation band occurred with fractions 8–15 which coincided with the inner band formed by antigen which had not been submitted to electrophoresis. However, the faster migrating component of PRVF antigen was demonstrated by testing in wells 2 mm. in diameter against the homologous antiserum (Plate 3*b*). This test also showed that the bands produced by fractions 6 and 11 are not continuous indicating that two distinct antigens are involved.

A preparation of PRVF antigen which had not been exposed to acetone and ether was also shown to migrate with the haemoglobin.

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press

DISCUSSION

Extracts from organs of mice infected with pantropic Rift Valley fever or with any of three strains of neurotropic Rift Valley fever contain, in addition to virus which may be removed by centrifuging, at least two 'soluble antigens'. These antigens are detectable and distinguishable from each other by the Ouchterlony test with mouse antisera and at least one of them is titratable by complement fixation. One of these antigens which appears to be quantitatively less important since it is not easily detectable has a higher electrophoretic mobility at pH 8·6 than the principal antigen but too little is known about its properties to merit further discussion.

Filtration of virus-free antigen preparations from infected mice through granulated 7 % agarose suggested the presence of antigenic particles ranging in size or diffusibility from that of haemoglobin to at least that of *B. cincta* haemocyanin. Early fractions (presumed to contain large or slowly diffusing antigen particles) and later fractions (presumed to contain smaller antigen particles) retained their properties when reconcentrated and refiltered separately through the column. This not only confirms the polydisperse nature of the antigen, but shows that neither aggregation of the smaller particles nor breakdown of the large ones took place perceptibly under the conditions of the experiment.

Centrifugal analysis indicated that the antigen though polydisperse is not a mixture of particles varying randomly in size but of particles in three size groups defined by sedimentation constants of about 8, 29 and more than 100 Svedberg units. Particles of the smallest size group of PRVF antigen prepared by repeated centrifugation showed, on diffusion-filtration through granulated 7 % agarose, peak CF activity in fractions which came out just before the haemoglobin, corresponding to an antigen fraction to which a sedimentation constant of 8 had been assigned by Polson & Levitt (1963*b*).

Densities of the antigens in the three particle size groups were determined by prolonged centrifugation in a preformed gradient of caesium chloride. The value of 1·27 g./ml. found for all the antigens may indicate the presence of lipid as most protein molecules have densities of about 1·33 g./ml. Spherical particles of sedimentation constant 100 and density 1·27 would be about 26 mμ in diameter according to the modified Stokes equation. The smaller particles having the same density would have diameters, if spherical, of 7–8 mμ and 14 mμ. There is no evidence to suggest that aggregation or dissociation of antigen molecules took place under the particular experimental conditions, but the possibility that the larger antigenic components are composed of 7–8 mμ diameter units is not ruled out.

A value of $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. was obtained for the diffusion coefficient of the smallest particles of antigen (from PRVF virus) separated by differential centrifugation when the diffusion coefficient of mouse γ-globulin was assumed to be $4{\cdot}81 \times 10^{-7}$ cm.^{2}/sec. Substitution of this result in the Stokes–Einstein equation gives a particle diameter of the order of 7 mμ in good agreement with the value estimated by centrifugal analysis. If, however, the diffusion coefficient of mouse

γ-globulin given by Allison & Humphrey (1960) is assumed to be correct, the diffusion coefficient of PRVF antigen becomes $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. and the corresponding particle size is 9 mμ.

Band splitting occurred after about 2 weeks in quantitative gel precipitin tests (including one on a preparation rendered at least approximately homodisperse by differential centrifuging) at concentrations of antigen higher and lower than that at the optimal proportions region. This was not observed in experiments with haemocyanin (Polson & Deeks, 1960) or with turnip yellow mosaic virus (van Regenmortel, 1959) but with poliovirus, band splitting occurred after a prolonged period (Polson & Hampton, 1960). This was also demonstrated with RVF antigens in Ouchterlony tests when antigen concentrations were not at optimal proportions. The formation of multiple precipitation lines with a single antigen–antibody system in the presence of an excess of one of the reactants has also been observed amongst others by Wilson & Pringle (1954) and Grabar (1957).

Zone electrophoresis of the antigens from three neurotropic and a pantropic strain of RVF virus showed that in each instance the complement-fixing activity migrated together with or slightly behind the haemoglobin at pH 8·6. This technique therefore did not serve as a means of separating the antigens from haemoglobin which is invariably found in preparations from infected mouse brain or liver. However, separation from impurities at or near the origin of electrophoresis and midway between the origin and the phenol red band had occurred. The mobility of infective pantropic RVF virus in zone electrophoresis is approximately half that of phenol red under the same conditions (Levitt, Naudé & Polson, 1963). The infective virus of the neurotropic strains also migrates faster than haemoglobin (Polson, unpublished results). Thus the antigens of RVF virus migrate at a rate different from that of the infectious virus, whereas with MEF_1 poliovirus, both virus and its soluble antigen migrate at the same rate (Polson, Selzer & van den Ende, 1957).

A close correlation between complement fixation and gel precipitation was established, both tests showing peak antigen activity in the haemoglobin region of the electrophoresis column. Similarity between the gel diffusion reaction and the pattern produced in complement fixation was also demonstrated by Pereira, Pereira & Allison (1959) with adenovirus.

The antigens from pantropic and neurotropic strains of RVF could be distinguished neither by gel precipitin tests nor by electrophoresis. Antigen from the pantropic strain however had a higher complement-fixing titre and showed a greater degree of band splitting in gel precipitin tests. It appears therefore that greater amounts of pantropic antigen are produced in the liver than neurotropic antigen in the brain and that any difference between the antigens from the two sources is quantitative rather than immunological.

SUMMARY

1. Extracts containing non-infective soluble antigens have been prepared from the tissues of mice infected with a pantropic or any of three neurotropic strains of Rift Valley fever virus. The antigens were detectable by complement-fixation and gel-precipitin tests using antisera prepared in mice.

2. The extracts appeared to contain at least two antigens separable by electrophoresis and distinguishable by Ouchterlony tests. The faster migrating minor antigen occurs sparsely and was not further examined.

3. No distinction, physical or immunological, was observed between the major antigen derived from the four strains.

4. In gel diffusion-filtration experiments with granulated 7% agarose, the major antigen appeared to be polydisperse containing particles with diffusion rates ranging from at least as low as that of *Burnupena cincta* haemocyanin to almost as high as that of haemoglobin.

5. Centrifugal analysis indicated the presence of particles having sedimentation constants of about 8, 29 and greater than 100 Svedberg units. These particles have a density of about 1·27 g./ml. If all the particles of the major antigen are spherical, with a density of 1·27 g./ml., the three size groups have diameters of about 7–8, 14 and > 26 mμ.

6. The diffusion coefficient of the smallest particle was estimated to be $6{\cdot}06 \times 10^{-7}$ cm.^{2}/sec. corresponding to a sphere 7 mμ in diameter, or $4{\cdot}78 \times 10^{-7}$ cm.^{2}/sec. corresponding to a sphere 9 mμ in diameter depending on which of two values for the diffusion coefficient of mouse γ-globulin is used in the calculation.

7. In density gradient zone electrophoresis at pH 8·6, the principal antigen migrated at about the same rate as haemoglobin and appreciably slower than the virus.

The authors are indebted to Prof. A. Kipps for his continued interest in this work, and to Dr T. H. Mead for valuable criticism of this paper. This investigation was supported in part by a Public Health Service research grant AI 04044–02 from the National Institute of Health, Bethesda, U.S.A.

REFERENCES

ALLISON, A. C. & HUMPHREY, J. H. (1959). Estimation of the size of antigens by gel diffusion methods. *Nature, Lond.*, **183**, 1590.

ALLISON, A. C. & HUMPHREY, J. H. (1960). A theoretical and experimental analysis of double diffusion precipitation reactions in agar and its application to characterization of antigens. *Immunology*, **3**, 95.

ANDREWS, P. (1962). Estimation of molecular weights of proteins by gel filtration. *Nature, Lond.*, **196**, 36.

CASALS, J. & OLITSKY, P. K. (1950). A complement-fixation test for poliomyelitis virus. *Proc. Soc. exp. Biol., N.Y.*, **75**, 315.

CASALS, J., OLITSKY, P. K. & ANSLOW, R. O. (1951). A specific complement-fixation test for infection with poliomyelitis virus. *J. exp. Med.* **94**, 123.

CLARKE, D. H. & CASALS, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Amer. J. trop. Med. Hyg.* **7**, 561.

GRABAR, P. (1957). Agar-gel diffusion and immunoelectrophoretic analysis. *Ann. N.Y. Acad. Sci.* **69**, 591.

KIPPS, A., NAUDÉ, W. DU T., POLSON, A., SELZER, G. & VAN DEN ENDE, M. (1957). The size distribution of specific antigens in virus-infected tissues and their significance. *Ciba Foundation symposium on the nature of viruses*, p. 224. Ed. G. E. W. Wolstenholme and E. C. P. Millar. London: J. and A. Churchill Ltd.

LARGIER, J. F. (1959). A purification and investigation of tetanus antitoxin. *Arch. Biochem. Biophys.* **77**, 350.

LEVITT, J., NAUDÉ, W. DU T. & POLSON, A. (1963). Purification and electron microscopy of pantropic Rift Valley fever virus. *Virology*, **20**, 530.

MANSI, W. (1957). The study of some viruses by the plate gel diffusion precipitin test. *J. comp. Path.* **67**, 297.

MAYER, M. M., OSLER, A. G., BIER, O. G. & HEIDELBERGER, M. (1946). The activating effect of magnesium and other cations on the hemolytic function of complement. *J. exp. Med.* **84**, 535.

MEAD, T. H. (1962). Purification of rabies soluble antigen. *J. gen. Microbiol.* **27**, 397.

OUCHTERLONY, C. F. (1953). Antigen-antibody reactions in gels. IV. Types of reactions in coordinated systems of diffusion. *Acta path. microbiol. scand.* **32**, 231.

PEREIRA, M. S., PEREIRA, H. G. & ALLISON, A. C. (1959). Use of gel diffusion precipitation test in the diagnosis of adenovirus infections. *Lancet*, i, 551.

POLSON, A. (1958). A method for determination of diffusion coefficients by the gel precipitin technique. *Biochim. biophys. Acta*, **29**, 426.

POLSON, A. (1961*a*). Fractionation of protein mixtures on columns of granulated agar. *Biochim. biophys. Acta*, **50**, 565.

POLSON, A. (1961*b*). A simplified fraction collector for gradient elution chromatography. *J. Chromatog.* **5**, 116.

POLSON, A. & CRAMER, R. (1958). Zone electrophoresis of type 1 poliomyelitis virus. *Biochim. biophys. Acta*, **29**, 188.

POLSON, A. & DEEKS, D. (1960). The diffusion constants of rabbit, guinea-pig and rat antibodies to a single common antigen. *Biochim. biophys. Acta*, **39**, 208.

POLSON, A. & DEEKS, D. (1962). Zone electrophoresis of entero-viruses. *J. Hyg., Camb.*, **60**, 217.

POLSON, A. & HAMPTON, J. W. F. (1960). Determination of the diffusion constant of poliovirus by the gel precipitin technique. *Biochim. biophys. Acta*, **44**, 18.

POLSON, A. & LEVITT, J. (1963*a*). Density determination in a preformed gradient of caesium chloride. *Biochim. biophys. Acta*, **75**, 88.

POLSON, A. & LEVITT, J. (1963*b*). A slowly sedimenting infectious component of Rift Valley fever virus. *J. Hyg., Camb.*, **61**, 451.

POLSON, A. & LINDER, A. M. (1953). The determination of sedimentation constants of proteins and viruses with the help of the Spinco preparative ultracentrifuge. *Biochim. biophys. Acta*, **11**, 199.

POLSON, A. & MADSEN, T. (1954). Particle size distribution of African horsesickness virus. *Biochim. biophys. Acta*, **14**, 366.

POLSON, A. & MADSEN, T. (1955). A brain factor influencing the viability of neurotropic Rift Valley fever. *Nature, Lond.*, **176**, 645.

POLSON, A. & POTGIETER, G. M. (1964). Determination of diffusion constants by a multiunit analytical method. *Nature, Lond.* (in the Press).

POLSON, A., SELZER, G. & VAN DEN ENDE, M. (1957). The electrophoretic mobilities of adapted MEF_1 poliomyelitis virus and its soluble antigen. *Biochim. biophys. Acta*, **24**, 600.

RUSSELL, B., MEAD, T. H. & POLSON, A. (1964). A method of preparing agarose. *Biochim. biophys. Acta* (in the Press).

SMITHBURN, K. C. (1949). Rift Valley fever. Neurotropic adaptation of the virus and the experimental use of the modified virus as a vaccine. *Brit. J. exp. Path.* **30**, 1.

STEERE, R. L. & ACKERS, G. K. (1962). Restricted-diffusion chromatography through calibrated columns of granulated agar gel; a simple method for particle size determination. *Nature, Lond.*, **196**, 475.

VAN REGENMORTEL, M. H. V. (1959). Determination of diffusion coefficient of turnip yellow mosaic virus by the gel precipitin technique. *Biochim. biophys. Acta*, **34**, 553.

VAN REGENMORTEL, M. H. V. (1961). Zone electrophoresis and particle size of cucumber mosaic virus. *Virology*, **15**, 221.

WILSON, M. W. & PRINGLE, B. H. (1954). Experimental studies of the agar-plate precipitin test of Ouchterlony. *J. Immunol.* **73**, 232.

EXPLANATION OF PLATES

PLATE 1

a. Gel precipitation test on pantropic and neurotropic antigens after gel diffusion-filtration. The fractions pooled in groups of three are in the wells. The antisera in the troughs are diluted 1/2.

b. Gel precipitation test on (1) PRVF, (2) NRVF, (3) MEM and (4) R120 antigens showing immunological identity of the main antigens and the presence of a second probably smaller antigen. Wells 5 and 6 contained normal brain and liver extracts respectively. PRVF antiserum (II) was used.

PLATE 2

a, *b*. Quantitative gel precipitin test on dilutions of PRVF antigen against NRVF antiserum diluted 1/4 showing the appearance of the bands after (*a*) 7 and (*b*) 32 days. C = control tube containing normal liver antigen.

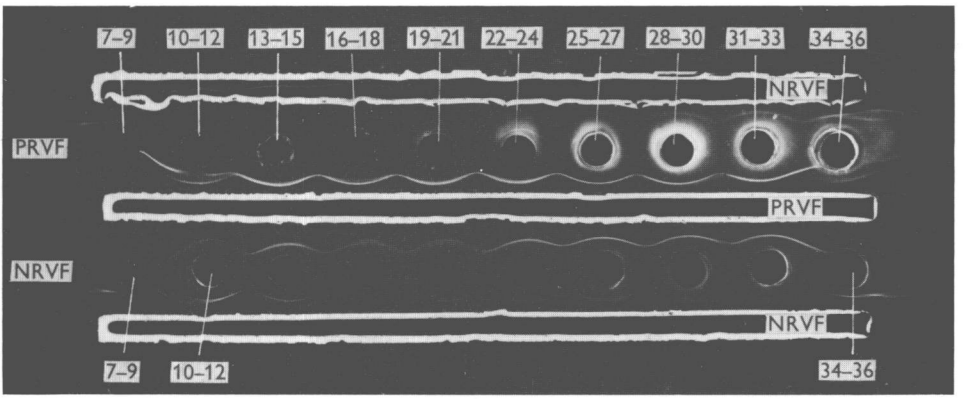
c. Gel precipitin test of doubling dilutions of PRVF antigen against PRVF antiserum (I) showing line splitting.

d. Estimation of the particle density of PRVF antigen. Pools *A*, *B* and *C* (see text) of antigen were introduced into preformed density gradients as fraction 5 (density 1·22) and centrifuged for 16 hr. at 30,000 rev./min. Successive fractions from each tube were submitted to the gel precipitin test with the results shown. After centrifugation the relevant fractions had the following densities: Pool *A*, fraction 7, 1·278; pool *B*, fraction 6, 1·256, fraction 7, 1·286; and pool *C*, fraction 5, 1·238, fraction 6, 1·278 and fraction 7, 1·286. The average density is close to 1·27.

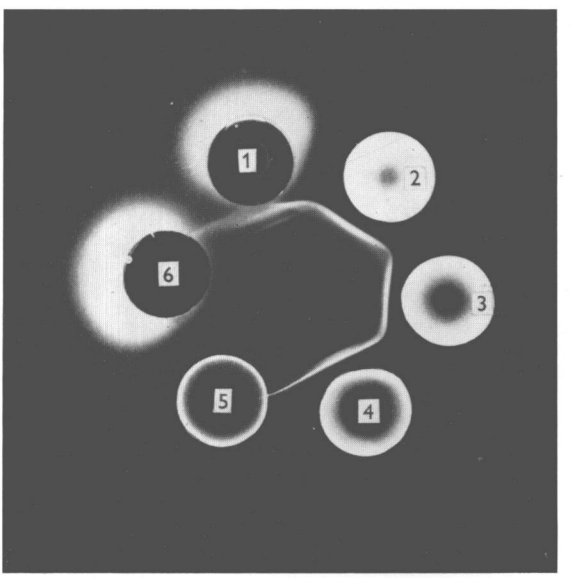
PLATE 3

a. Consecutive 1 cm. fractions of PRVF antigen after zone electrophoresis, tested by gel precipitation. The various antisera in the troughs are diluted 1/2 except R 120[1] and MEM[1] which are undiluted. Sample 0 containing opalescent material was taken 1 cm. below the origin of electrophoresis.

b. Gel precipitation test of fractions from zone electrophoresis, showing non-identity of the antigens in fractions 6 and 11.

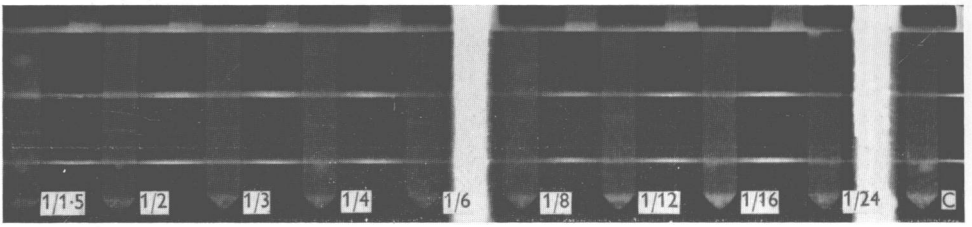


a

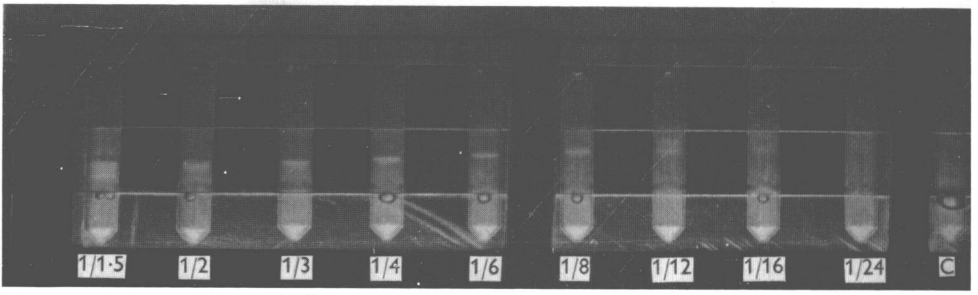


b

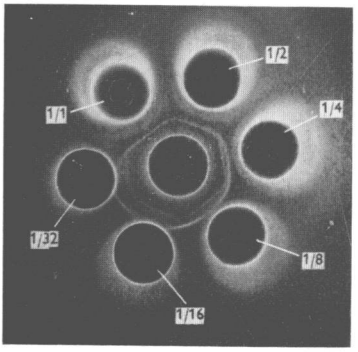
https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press



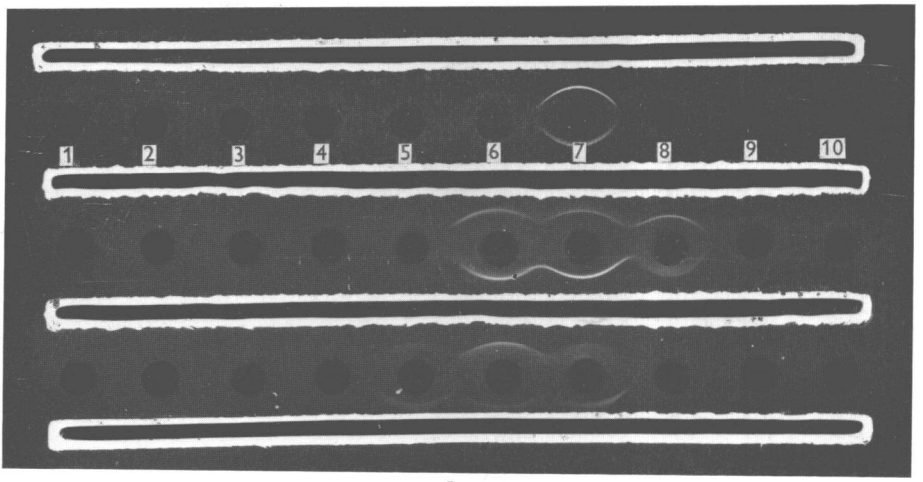
a



b



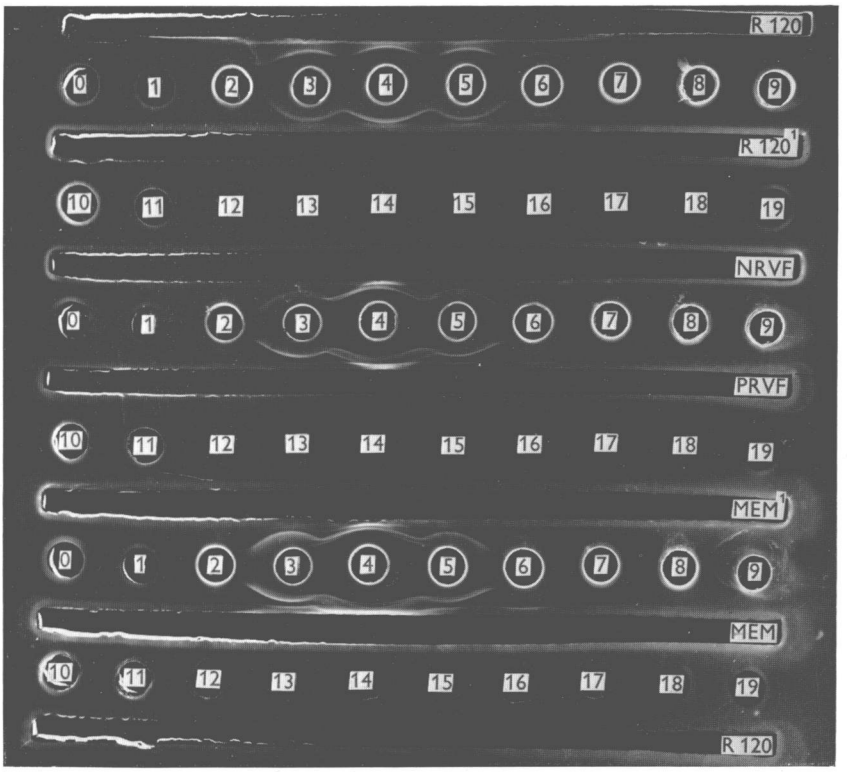
c



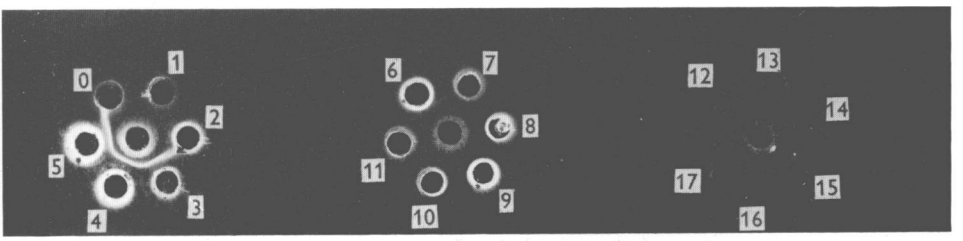
d

JULIA LEVITT AND A. POLSON

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press



a



b

https://doi.org/10.1017/S0022172400039966 Published online by Cambridge University Press