The Largest Rabies-specific Antigen in Extracts of Infected Suckling Mouse Brains

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SUMMARY

Extracts of rabies-infected suckling mouse brains purified by precipitation at pH 4.5, freed from smaller antigens by sedimentation at 161,180 g and digested with RNase, DNase and trypsin show in the ultracentrifuge a component of $S_{20} \approx 16$ to 18 which is lacking in extracts of normal suckling mouse brains similarly treated. The largest rabies soluble antigen ('outer antigen': Mead, 1962b) has a sedimentation constant $S_{20} \approx 16$ estimated by the 'biological' method of Polson & van Regenmortel (1961). The purified antigen appears to consist of rings or possibly single-turn helices about 100 Å in diameter containing about 0.57 μg pentose (as ribose) per μg total nitrogen. The antigen also appears to contain deoxypentose. It is resistant to pancreatic RNase, DNase, trypsin and chymotrypsin, has a density of about 1.34 g./cm³ in CsCl and an electrophoretic mobility about 7/8 that of rabbit serum albumin at pH 8.5.

Preparative density-gradient centrifugation in the analytical rotor of the Model E Spinco centrifuge is described. This allows the method to be applied to smaller particles than can be treated in the S.W. 39 rotor.

INTRODUCTION

Virus-free extracts of rabies-infected suckling mouse brains produce at least four lines of precipitate in the Ouchterlony test with concentrated mouse anti-rabies serum (Mead, 1962b). Two lines had previously been seen by Villemot & Provost (1959) using rabies-infected tissue. Mead (1962a, b) reported evidence for the separate identities of the antigens producing three of the lines and some progress towards the separation of the antigens from brain material and from each other. The precipitation line formed farthest from the antiserum well was shown to be due to an antigen ('outer antigen') which is more readily sedimented than the others and, unlike the others, is resistant to trypsin. When an analytical centrifuge became available this antigen was identified with the substance forming the fastest sedimenting boundary in purified extracts of rabies-infected mouse brains. The antigen appears to be a nucleoprotein containing ribose and deoxyribose and forms small rings or single turns of a helix. Its purification and some of its properties are described in this paper.
Apart from the following, the methods of this study were described by Mead (1962a).

**Buffered saline A**, NaCl 8.5 g, 0.2 M-Na₂HPO₄ 327 ml, 0.2 M-KH₂PO₄ to pH 7.5, thiomersalate 1 g, Triton X-100 (Rohm & Haas Co.) 1 g in 10 l.

**pH 4.5 saline**, NaCl 8.5 g, acetic acid 0.6 ml in 900 ml, adjusted to pH 4.5 with 2 N-NaOH, diluted to 1 l.

**Tris-saline pH 8.4**. In 400 ml, 2-amino-2-hydroxymethylpropane-1,3-diol 2.42 g, NaCl 2.34 g, Triton X-100 0.04 g, thiomersalate 0.04 g, N-HCl to pH 8.4 (about 7 ml).

Agarose was prepared by the method of Russell, Mead & Polson (1964).

**Haemocyanin**. Blood from male rock lobsters (*Jasus lalandii*) was allowed to clot. The serum, clarified by light centrifugation and preserved with thiomersalate (0.01 %) at 4°C, remained unaltered for many months.

**Turnip yellow mosaic virus** free from top component was kindly provided by Dr M. H. V. van Regenmortel of the Department of Microbiology, University of Stellenbosch.

**Ribonuclease** (RNase) 5 x crystallized, salt- and protease-free; **deoxyribonuclease** (DNase), salt-free with 36 % of the activity of the crystalline enzyme; **trypsin**, 2 x crystallized, salt-free; **α-chymotrypsin**, 3 x crystallized, salt-free and **deoxyribonucleic acid** (DNA, from calf thymus) were from Seravac Laboratories, Cape Town. **Yeast ribonucleic acid** (RNA), highly polymerized, was a British Drug Houses product. **Trypsin inhibitor** soy bean, 5 x crystallized, was from General Biochemicals, U.S.A.

**Spectrophotometry** was done in a Model SP 500 Unicam instrument. The two cells for sample and distilled water blank were carefully matched and used in fixed carrier positions. Transmission differences were based on the mean of at least three sets of observations.

**Preparative centrifugation** at forces (measured at the centre of the tube) between 4000 g and 95,000 g was done in Model L or LH Spinco centrifuges. For lower forces a refrigerated MSE machine was used.

Centrifugation at 161,180 g was done in the J rotor of the Spinco model E ultracentrifuge at 50,740 rev./min. As this rotor can be maintained rather exactly at a predetermined temperature during runs, it seemed worth while to find out the temperature (sometimes assumed to be 4°C) at which the buffered salt solutions used as suspension media were most dense and at which thermal convection should be least. This was done with a glass dilatometer holding about 750 ml in a well-stirred and insulated thermostat cooled by the circulation of alcohol from a refrigerating machine. The results were: 0.14 M-NaCl 2·12°C; 0.07 M-NaCl 3·0°C; buffered saline A 1·88°C; 0.14 M-sucrose 1·67°C. The results for NaCl are in good agreement with those of Wright (1919).

In spite of precautions against convection it was essential to trap the centrifuged pellet of antigen in a small button of Hyflo-super-cel (Hyflo). On one occasion when no Hyflo was added, 60 % of the antigen was removed with the supernatant fluid although the conditions of centrifugation were adequate to sediment it completely into a small Hyflo button. The Hyflo-super-cel was extracted with HCl and washed with water by decantation repeatedly to remove the finest particles and then with redistilled acetone. As the centrifuged button from 0·1 g. of Hyflo occludes about 0·3 ml of fluid, as little as possible was used and the additions were roughly weighed.
into the centrifuge tubes in amounts appropriate for the size of pellet expected. Pellets, unless quite colourless, could be seen easily in the pure white Hyflo and supernatant fluids could be removed rapidly and with confidence down to the limits of the Hyflo button.

Analytical centrifugation was done in the An-D rotor of a Model E Spinco centrifuge equipped with a temperature controller and schlieren and ultraviolet (u.v.) optics.

Gradient isodensity centrifugation. To rid the purified antigen (S\textsubscript{20} \approx 18) of possible traces of nucleic acid or other impurity of high molecular weight and different density, equilibrium isodensity centrifugation in an analytical cell was used instead of the usual method employing a swinging bucket rotor. A higher gravitational field could be used commensurate with the small size of the antigen, the progress of separation could be seen during the run and division of the sample into a multitude of fractions was avoided. Withdrawal of the antigen band from the cell contents at the end of the run remained a problem. Best results were obtained with the separation cell of Tiselius, Pedersen & Svedberg (1937) which employs a Kel-F centrepiece and a fixed partition of filter paper supported on a perforated plastic plate. To remove denser and less dense impurities with this apparatus two runs are needed at different initial densities. In the first run a density is chosen which bands the substance to be purified above or below the partition and as close to it as possible. The density of the fluid from that half of the cell which contains the substance after the first run is readjusted so that in the second run the substance bands on the opposite side of the partition. Under our conditions the initial densities of the solutions used for the two runs differed by about 0.04 g./cm.\textsuperscript{3}, but by using other salts to produce the density gradient, a thinner partition, and longer centrifugation periods it is possible that finer separations could be achieved. The conditions needed to improve separation have been discussed by Ifft, Voet & Vinograd (1961).

Preliminary experiments were done with \textit{Jasus lalandii} haemocyanin having S\textsubscript{20} = 17.1 (Polson & Linder, 1953) and an apparent density in CsCl = 1.32 g./cm.\textsuperscript{3} (Polson & Levitt, 1963) close to the values for the antigen. The haemocyanin was usually diluted 1/20 with CsCl buffered with 0.02 M-phosphate to give a final pH of 7.5 and densities between 1.26 g./cm.\textsuperscript{3} and 1.36 g./cm.\textsuperscript{3} which were determined pycnometrically at the temperature of centrifugation (20° or 22°). The speed was 50,740 rev./min. and the phase plate angle 70°. For measurement, the band of haemocyanin was taken to lie between the upper schlieren peak and the lower (reversed) schlieren peak, although of course appreciable amounts would lie outside these limits. The band width was taken as the distance between the peaks and the band position as the distance from the axis of rotation of the point midway between the peaks. Normally the band narrowed to 1 to 2 mm. during the first 5 hr of centrifugation. Further narrowing was extremely slow so that a period of 5 to 6 hr was used in most experiments although the density gradient may not have reached equilibrium in this time. The relations between the band position (after 5 to 6 hr) and the initial density for haemocyanin and the antigen were about linear (Fig. 1). Departures from linearity may have been due partly to experimental error and partly to the fact that the cell was not always filled to exactly the same level. There was reasonable agreement between values determined in the plain cell and those found in the partition cell, indicating little interference by the partition. The final position of the band was the same when haemocyanin was centrifuged in a solution of initial density 1.327 g./cm.\textsuperscript{3}, containing 4.8% (w/v) NaCl (density 1.302 g./cm.\textsuperscript{3}
without NaCl) as when it was centrifuged in a solution of density 1.327 g./cm.\(^3\) containing only CsCl and the usual 0.02 M-phosphate.

To check the efficacy of the partition, \textit{Jasus lalandii} haemocyanin (dialysed free of thiomersalate), turnip yellow mosaic virus, DNA and buffered CsCl solution were mixed and centrifuged at 50,740 rev./min. for about 6½ hr in the fixed partition cell. The initial density of the mixture was 1.307 g./cm.\(^3\). After the run the contents A of the upper compartment nearest the axis of rotation were removed with a plastic-tipped syringe and this compartment and the syringe were rinsed with 4 small portions of water which were added to A. The cell was resealed, rebalanced, returned to the rotor upside down and centrifuged at about 3000 rev./min. until the contents of the lower compartment had been transferred completely to the upper one. The upper compartment was emptied with a clean syringe and rinsed as before to give fraction B. Ultraviolet absorption curves were constructed of the buffered CsCl (d = 1.317 g./cm.\(^3\)), the original mixture, and of fractions A and B. There was a fair separation of the mixture into nucleic acid and non-nucleic acid containing parts (Fig. 2). When in later experiments the antigen was expected to be in the lower compartment, the upper part
The largest soluble rabies antigen was emptied and rinsed 4 times and the lower compartment emptied as described. Water (about 0.15 ml.) was then put in the upper compartment, centrifuged into the lower part and then back into the upper part and removed to ensure thorough rinsing of the partition. This was followed by one or two further rinsings of the upper part.

After this work had been finished a photograph showing the banding of a virus in a density gradient in the fixed partition cell was published by Markham (1966).

The micro-estimation of pentose. As very little pure antigen was available, ribose was estimated in a region below the normal limits of the test, 0.025 μg. ribose in 0.25 ml. being the lowest amount regularly used in establishing the calibration curve for the reagent. Duplicate estimations at this level agreed within 10%. The method was based on Ceriotti’s (1955) modification of the Barrenscheen & Peham (1942) procedure. The Cu reagent has the advantage of giving practically colourless blanks whereas the FeCl₃ in Bial’s solution would probably have obscured the brown and pink colours sometimes formed by substances interfering with the test. Orcinol was purified as follows. Redistilled benzene (about 10 ml./g.) was added to the commercial hydrate (B.D.H.) and distilled off on the water bath, the last part of the distillate being clear. The last traces of benzene were removed in a current of air and the brown crystalline anhydrous residue was twice sublimed at 100° in an apparatus having a water-cooled condenser about 1 cm. above the crude orcinol. The apparatus was evacuated through a solid CO₂ acetone trap by a mechanical pump. The product (m.p. 106°) could be used for several weeks but eventually became discoloured. The sensitivity of the reagent was more than doubled without serious effect on the blank by raising the orcinol concentration from 2 mg./ml. to 8 mg./ml. The reagent was prepared immediately before each test by dissolving orcinol (200 mg.) in concentrated HCl (about 10 ml.) in a 25 ml. volumetric flask, adding 2.5 ml. of 0.004 M-CuCl₂ in concentrated HCl, and diluting to 25 ml. with the same solvent. The reagent, though apparently clear, was filtered with gentle suction through a grade 4 sintered glass filter previously rinsed with concentrated HCl and two portions of the reagent. For the test, 0.25 ml. of standard solution, of unknown or of water, was mixed with 0.25 ml. reagent in each of a series of Pyrex tubes (12 × 150 mm.) which were sealed at about 4 cm. from the open end (to prevent evaporation) and heated in a boiling water bath for 40 min. After cooling, the absorption was measured in microcells against water (see spectrophotometry) at 670 mp. Solutions of RNA were used as standards in some experiments but normally, and for the antigen analysis, standard solutions containing 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μg. ribose/ml. were employed. The standards were individually prepared, not serial dilutions, and, with the blank, were set up in duplicate for each test. Great care was necessary in sealing the tubes. A mixture of coal gas, air and oxygen giving a ‘roaring’ flame was necessary. A silent flame containing less air caused discoloration and erratic results. As the solution of purified antigen to be analysed was kept in an atmosphere saturated with the vapour from a mixture of water and redistilled chloroform the effect of chloroform on the reaction was tested and found undetectable.

The micro-estimation of DNA. Burton’s (1956) method was modified to use 0.2 ml. amounts of solutions containing between 0.2 and 2 μg. DNA. The standard contained DNA (5 mg.) in 0.005 N-NaOH (50 ml.). At fortnightly intervals this solution (10 ml.) was diluted with N-HClO₄ (50 ml.) and 0.005 N-NaOH (40 ml.) and heated at 70° for 15 min. Further dilutions in 0.5 N-HClO₄ were made to give the working standards containing between 1 μg./ml. and 10 μg./ml. For the test, 0.2 ml. volumes of each
standard or of 0.5 N-HClO₄ (for the blank) were mixed with 0.4 ml. of Burton's reagent and incubated 17 hr at 31.5 ° in tubes closed by purified rubber stoppers. The standards and blank were set up in duplicate. The antigen solution (0.1 ml.) was diluted with N-HClO₄ (0.1 ml.) and heated at 70 ° for 15 min. and cooled before addition of the reagent. The absorptions at 600 mμ of blanks, standards and unknown were measured in a single microcell against water. As the blank increased during storage of the reagent this was preferably prepared fresh daily.

The micro-estimation of total nitrogen. The method of Jacobs (1962, 1964) was modified in the following respects:

(i) SnCl₂.2H₂O was recrystallized from warm 2 N-HCl and half of the proportion recommended by Jacobs was added to the reagent.

(ii) The reagent contained 75 % methyl cellosolve.

(iii) The reagent was kept under N in the dark for about 2 hr and was filtered through a grade 4 sintered-glass filter before use.

(iv) The sensitivity was doubled by using 2 cm. spectrophotometer glass cells.

Glycine (twice recrystallized from water) was normally used as reference standard and gave results in good agreement with those obtained with the ammonium sulphate standard employed in experiments with the ninhydrin reagent. Histidine, RNA and DNA were used to confirm the adequacy of the digestion. Analyses were set up in duplicate. The standards contained 8, 16 and 32 μg N/ml. A difficulty with this method is the relatively large reading of the blank. Reagents containing added hydrindantin instead of SnCl₂ were more sensitive but seemed less reliable and gave equally high, or higher, blank readings. Some improvement was gained by recrystallising the citric acid from water. The extinction coefficient of the blank in a 2 cm cell measured against water varied between 0.12 and 0.14 whereas the gross extinction of the standard (8 μg N/ml.) was about 0.2.

Exclusion chromatography. Sephadex G-75 and G-200 were used in accordance with the maker's directions. Agar and agarose columns were at first made by Polson's (1961) method. More effective columns were later made from pearl-form agarose (Hjertén, 1964) prepared with Emulphor EL. A 300-mesh copper sieve was used in this preparation. The pearls were washed with ether on the sieve until the filtrate was clear and then freed from ether, washed and wet sieved as described by Hjertén but without centrifugation. It was found essential to decopper any column prepared with sieved agar or agarose by percolating it with either EDTA solution or glycine at pH 9. Before use columns were run on a mixture of rabbit haemoglobin and phenol red which showed up any irregularities in packing. The effluent from exclusion chromatography columns passed through a Uvicord apparatus to a fraction collector fitted with an event marker. The solvent used was buffered saline A containing thiomersalate which absorbs strongly at 254 mμ, the wavelength of filtered mercury light which passes through the effluent in the Uvicord apparatus. The apparatus was adjusted to allow for this additional absorbance, and enough saline A passed through to saturate the column with thiomersalate before beginning an experiment. The Uvicord traces (representing % absorption) were often converted to rough plots of extinction against fraction number, by measuring the % transmission (T) at the mid point between marks indicating emptying of the fraction collector syphon, and using the expression E = log (1/T).

Electron microscopy. An EM 3 Metropolitan-Vickers instrument was used.
RESULTS

The origin and nature of the antigenic extracts

The preparation of acid-precipitation purified (APP) extracts of rabies-infected suckling mouse brains described in detail by Mead (1962a) was improved by washing the pH 4.5 precipitate with pH 4.5 saline before extraction of the antigens from it with tris-saline pH 8.4 instead of the phosphate buffered saline previously used. The yield from 200 brains was about 200 mg. of white extract redissolving to a pale yellow solution which, if sufficiently concentrated, gave three lines of precipitation in the Ouchterlony test using a concentrated anti-rabies mouse serum. Such extracts, which were usually clear when fresh but became cloudy on storage at 4°C in the presence of thiomersalate, had a complement fixing titre (CFT) of about 300 to 600 at a volume of 60 ml. and in contrast to the crude extracts before acid precipitation which had an absorption peak in the ultraviolet at 275 mμ (minimum at 255 mμ) absorbed maximally at 260 mμ with a minimum at 240 to 245 mμ.

Treatment with ribonuclease and deoxyribonuclease

In an attempt to remove the nucleotide-like material provisionally assumed to be impurity, the freeze-dried residue from 5 ml. of an APP extract was dissolved in 5 ml. 0.2 M-tris buffer of pH 7.6. The solution was clarified (20 min. at 6590 g in the Spinco no. 40 rotor) and 0.8 ml. portions placed in each of 5 dialysis sacs. Two sacs received 0.4 ml. of aqueous RNase solutions giving final enzyme concentrations of 100 μg./ml. and 10 μg./ml. and 0.4 ml. of water. Two sacs received 0.4 ml. of aqueous DNase solutions giving final enzyme concentrations of 100 μg./ml. and 10 μg./ml. and 0.4 ml. of aqueous MgSO₄ to give a final Mg²⁺ concentration of 0.005 M. The control sac received 0.4 ml. water and 0.4 ml. MgSO₄. Each sac was surrounded by 1.6 ml. of 0.1 M-tris and incubated 1 hr at 38°C. The sacs were placed separately in 200 ml. volumes of 0.1 M-tris for 4 hr and were then left in water overnight. The solutions were diluted to 4 ml. with water for spectrophotometry (water blank) and afterwards each was freeze dried after the addition of 0.1 ml. normal rabbit serum to prevent carry-over in the vapour stream. The residues, dissolved in 0.05 ml. amounts of CFT saline, (Mead, 1962a) were applied to an Ouchterlony plate with mouse anti-rabies antibody. The ultraviolet absorption curves of the four treated solutions (as well as that of the control) had peaks at 260 mμ and minima at 245 mμ in spite of the nuclease treatment followed by dialysis. The ratios E₂₇₅/E₂₆₀ for the control and DNase-treated solutions were about the same while those of the RNase-treated solutions were almost identical with each other and appreciably higher than that of the control. The treated materials gave reactions of identity with control material in immuno-diffusion tests. In another similar experiment trypsin-digested crude extract was incubated with DNase. No change in CFT was observed. It was provisionally concluded that only the RNase had any appreciable effect on the nucleotide impurities and that neither enzyme affected the principal antigens. In another experiment with DNase, DNA was added to the brain extract before the enzyme. The increase in E₂₆₀ during subsequent treatment with DNase appeared to show that the brain extract contained little or no DNase inhibitor. Although RNase evidently had some effect on the impurities it was disappointing that so little of the digestion products could be eliminated by dialysis.
The actions of trypsin and chymotrypsin

The destruction of the inner and probably at least one of the intermediate antigens by trypsin and the survival of the outer antigen have already been reported (Mead, 1962b), but a further experiment was done with an APP extract to confirm as far as possible the complete immunity of the larger antigen to this treatment (Fig. 3). About 23% of the complement fixing titre of this particular extract was probably due to the large antigen. The decrease in titre of 6 other batches of APP extract digested with trypsin varied between 30% and 70%.

Fig. 3. Change in CF titre of an APP extract during digestion with trypsin. •—•, control solution in 0.1 M-tris buffer pH 7.6; ■—■, experimental solution as the control but containing trypsin 1 mg./ml. Both solutions were incubated at 37°; samples taken at intervals were treated with trypsin inhibitor 1 mg./ml.

In a similar experiment with chymotrypsin (1 mg./ml.) and another APP extract the enzyme was responsible for loss of part but not all of the original CF activity. The effect of chymotrypsin was confirmed by a similar experiment in which samples from incubated and unincubated controls and the chymotrypsin digest were analysed by immunodiffusion 5, 30 and 90 min. after addition of the enzyme. The agarose gel contained 0.1 M-β-phenylpropionic acid, a known chymotrypsin inhibitor (Bettelheim & Neurath, 1955). Only the inner and outer antigen lines were exhibited by the controls in this experiment. The inner line had disappeared after 30 min. but not after 5 min. of
The largest soluble rabies antigen

digestion. Chymotrypsin, therefore, in spite of a different specificity, closely resembled trypsin in its effects on the two major antigens.

Extracts subjected to enzyme treatment as a step in the purification of the large antigen normally received RNase first, followed by DNase + Mg2+, trypsin and trypsin inhibitor. This sequence had no effect on the antigen as far as could be determined by CF titration or immunodiffusion. No change in CF titre occurred during RNase digestion preceded by trypsin treatment. Valentine & Isaacs (1957a) showed that influenza A virus became susceptible to trypsin digestion after treatment with 0.1 N-HCl. An experiment was therefore done with a solution containing the large antigen freed from all or most of the smaller antigens by centrifugation. Exposure to 0.1 N-HCl for 30 sec. at room temperature abolished its complement-fixing activity.

**Treatment with ethanolamine**

Encephalomyocarditis virus or an antigen (mol. wt. 350,000) obtained from cells infected with the virus were converted by ethanolamine at pH 10.5 into an antigen of mol. wt. 60,000 (Hamilton, Kerr & Work, 1962). Solutions containing the largest and the inner antigen were therefore separately dialysed at 4°C against 0.03 M-ethanolamine containing 0.87% NaCl at pH 10.6 for 24 hr. Controls were dialysed simultaneously against glycine buffer pH 10.5 and buffered saline A. After further prolonged dialysis against phosphate buffer pH 7 followed by water, the solutions and controls were freeze dried and the residues in small volumes of buffered saline were analysed by immunodiffusion with mouse anti-rabies serum as antibody source. Neither ethanolamine nor exposure at pH 10.5 had any effect on the pattern of precipitation lines of either antigen.

**Sedimentation behaviour**

A concentrated APP extract freed from small molecular material by fractionation on Sephadex G-75 was examined in the analytical ultracentrifuge using the schlieren optical system. Two peaks were seen, the first very small (S20 = 17.1), the second heterogeneous and very much larger (S20 ≈ 3). Confirmation that the S20 ≈ 17.1 boundary was probably due to the large antigen was obtained by estimating the sedimentation constant by method A of Polson & van Regenmortel (1961). In the method as ordinarily used for viruses, the boundary is stabilized by a sucrose density gradient. The large antigen, having a relatively small sedimentation constant, needs longer centrifugation during which the stabilizing sucrose gradient might be lost by diffusion. At the suggestion of Dr Polson, a 25% (w/v) solution of bovine serum albumin was placed in the bottom of the tube to trap sedimented antigen and form a density gradient by upward diffusion. In all other respects the method of Polson & van Regenmortel was followed. Estimation of the temperature during the run by subsequent insertion of a pre-cooled thermometer into the balance tube was subject to considerable error. The antigen preparation used for these experiments was an APP extract which had been treated with RNase and trypsin and then clarified for 30 min. at 17,600 g on the SW 39 rotor before being diluted 1/50 with buffered salt solution A. Prior removal of other antigens by trypsin treatment was of course essential as the method depended on CF titrations (Table 1). The same solution (undiluted) was examined in the analytical centrifuge and found to contain much impurity barely sedimenting at 56,100 rev./min. and a small boundary of S20 = 16.1.
The agreement between the results of the two methods appeared satisfactory. Another reason for the provisional assumption that the $S_{20} 16$ to $18$ boundary seen in the analytical centrifuge was caused by the larger antigen was that although a similar boundary was sometimes seen in undigested extracts from normal suckling-mouse brains, it never appeared when these had been treated with trypsin. The impurities in preparations of the large antigen gave peaks of variable size and rate of sedimentation generally below 6 dependent on the history and purity of the sample examined.

Table 1. Determination of the sedimentation constant of the largest antigen by the method of Polson & van Regenmortel (1961)

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Average</th>
<th>Time</th>
<th>Temp.</th>
<th>$C_0$</th>
<th>$C_t/C_0$</th>
<th>$S_{20}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31,030</td>
<td>240</td>
<td>4°</td>
<td>160</td>
<td>0.031</td>
<td>15.7</td>
</tr>
<tr>
<td>2</td>
<td>31,050</td>
<td>120</td>
<td>6°</td>
<td>153</td>
<td>0.459</td>
<td>16.0</td>
</tr>
<tr>
<td>3</td>
<td>31,140</td>
<td>154</td>
<td>1°</td>
<td>96</td>
<td>0.448</td>
<td>14.9</td>
</tr>
<tr>
<td>4</td>
<td>31,170</td>
<td>200</td>
<td>1°</td>
<td>102</td>
<td>0.162</td>
<td>17.8</td>
</tr>
</tbody>
</table>

Average: 16.1

Effective column $l = 1.0$ cm. Distance of meniscus from centre of rotation $x = 5.81$ cm. $C_0 = \text{complement fixing activity of original solution (mean of duplicates). } C_t = \text{mean residual complement fixing activity in effective columns from duplicate tubes.}$

Preparative sedimentation of the largest antigen

Preparative centrifugation in the J rotor of APP extracts before enzyme treatment allowed the smaller antigens to be recovered in the supernatant fluid for later investigation and effected a preliminary separation of the largest antigen from impurities of low molecular weight. While considerable and often heavy losses of antigen occurred during most stages in the purification (Mead, 1962a) this first preparative centrifugation frequently gave an apparent recovery exceeding 100% based on reciprocal CF titre x volume. It appeared that one or more of the antigens could fix more complement when the largest was separated from the others.

The purification of the largest antigen

In the first attempt the smaller antigens were removed in a preliminary centrifugation. The pellet fraction from this was treated with RNase, DNase and trypsin and the digest was then fractionated by repeated centrifugation until nothing further was eliminated in the supernatant fluids.

Three batches of APP extract from a total of 616 infected suckling mouse brains were combined for this experiment. Three batches of APP extract from a total of 612 normal suckling mouse brains were also combined. The extracts from the infected and normal brains were treated in the same manner and as far as possible simultaneously throughout the experiment but the treatment of the infected extract only will be outlined. Details are given in the flow diagram.

As no CF activity had been detected in the original extract from normal brains no further CF titrations were done on the fractions from its later treatment. Immuno-diffusion showed the presence of the large and small antigens in the original extract from infected brains and in $P_1$. Only the small antigen was detectable in SNF$_1$, SNF$_2$ and $P_2$. Analytical centrifugation revealed an antigen peak only in the extract from infected brains. The sedimentation constant in this highly impure solution was 14.0.
The largest soluble rabies antigen

APP extract (24 ml. CFT 2400)

Centrifuge (4 hr, 161,180 g, 2°) with 0·2 g. Hyflo in each 'J' rotor tube

Pellets and Hyflo extracted 5x with buffered saline A

P1 (vol.=12 ml., CFT 3200)

Dilute and pool total vol.=25 ml. digest at 37° for 45 min. with each enzyme in turn

RNase (0·5 mg./ml.) DNase (1 mg./ml. + 0·005 M-Mg.) and trypsin (1 mg./ml.)

Trypsin inhibitor (1 mg./ml.)

Centrifuge (40 min. at 6600 g).

Three-quarters SNF removed. Remaining quarter recentrifuged as above.

Pool SNF's. (CFT 475—40% recovery from P1 and P2)

Four ml. concentrated to 0·8 ml. and examined in the analytical centrifuge (S20 = 15·5).

(Much impurity of low S)

Dialyse against buffered saline A and dilute to 24 ml.

Centrifuge (4 hr, 161,180 g, 2°) with 0·15 g. Hyflo in each 'J' rotor tube

SNFα

Pellets and Hyflo resuspended in 12 ml. buffered saline A and recentrifuged as above with 0·15 g. Hyflo in each 'J' rotor tube

Extract Pβ was clarified at 17,570 g for 15 min. (SW 39)

Centrifuge (4 hr, 161,180 g, 2°) with 0·15 g. Hyflo in one 'J' rotor tube

Pellets and Hyflo suspended in 1 ml. 0·02 M-P04 and centrifuge for 10 min. at 17,570 g

SNFPγ examined in analytical centrifuge (S20 = 19·0)

Hyflo extracted as above until Pγ = 5·5 ml.

Two further cycles of preparative centrifugation at 161,180 g

SNFε (discard)

Pε = 5 ml. (CFT = 890. Yield 66% from digested pellets P1 and P2).
The run was repeated with a 1/5 dilution of the contents of each cell (Pl. 1, fig. 1); $S_{20}$ was then 179. No antigen peak could be detected in P2. No CF activity was detected in any of the supernatant fluids at a dilution of 1/10 except SNFe where a trace was recorded. Progress of the purification can be seen from Fig. 4, showing the decline in the total nitrogen removed in the successive supernatant fluids. The supernatant fluids all had an ultraviolet absorption peak at 275 m$\mu$ and the decrease in the extinction at this wavelength of successive fluids followed the same pattern as Fig. 4. A similar picture was presented by the series of analytical centrifugation traces of which the last, that of Pe is shown in Pl. 1, fig. 2. It is evident that no further substantial purification could be achieved by this method. The inadequacy of the procedure was, however, clearly shown by the persistent appearance of a small pellet at each centrifugation of the normal brain extract. The ultraviolet absorption curves of the final pellets from the
The largest soluble rabies antigen

extracts of infected and normal brains are shown in Fig. 5. The slight maximum at 260 μν in the curve for the extract of infected brains (found later in preparations of higher purity) was lacking in the curve for the normal brain extract. Although the antigen prepared by repeated sedimentation was unlikely to have been quite pure, it appeared adequate for an electrophoresis experiment described later.

As centrifugation alone was inadequate, experiments were done on digested and undigested brain extracts in which centrifugation was combined with either chromatography on DEAE cellulose or exclusion chromatography on agar, agarose or Sephadex G-200. Exclusion chromatography on agarose gave the best results. The first experiments were done with columns containing agar gels of different concentrations granulated by sieving. Later, agarose, also granulated by sieving, was employed. With columns made from gels of concentrations between about 4 % and 10 %, enzyme-treated APP extracts of normal or rabies-infected suckling mouse brains gave chromatography diagrams with two well-defined peaks of which the first probably represented material too large to enter the gel pores. The second peak was often prolonged and occasionally appeared in two distinct sections. The antigen emerged between these peaks and was detected by CF titration of the fractions. A specimen of the largest antigen from which the electron micrograph shown in Pl. 1, fig. 3, was taken was obtained by a combination of exclusion chromatography and preparative centrifugation. The freeze-dried APP extract from 193 brains was dissolved in 10 ml. 0.2 M-tris pH 7.6. The solution was dialysed against the buffer and then digested as shown in the previous flow diagram. The cloudy solution was dialysed against buffered saline A and centrifuged 30 min. at 17,570 g in the S.W. 39 rotor. The opalescent supernatant fluid was pervaporated to 3 ml. and dialysed against buffered saline A. During 2 days storage at 4 ° the solution deposited more precipitate which was discarded. The supernatant solution in portions of 0.7 ml. was run through a column 1.2 x 45 cm. filled with 4.5 % agarose which had passed a 100-mesh sieve. The column was fed with buffered saline A and 2.75 ml. fractions were collected. The Uvicord trace showed peaks with maxima at fractions 7, 13 to 14 and 17 to 18, the last being by far the largest and the middle one very small. From previous experience with this column, fractions 11 to 17 were presumed to contain the antigen and these fractions from three of the runs were pooled, concentrated to 2 ml. and again run through the column in two runs of 1 ml. each. The Uvicord trace showed that the first peak material emerging at fraction 7 had been eliminated and that the 13 to 14 peak was larger in comparison with the 17 to 18 fraction peak. Fractions 9 to 14 from the two runs were pooled, concentrated to 1 ml. and examined in the analytical centrifuge. In addition to the antigen (S_{20} = 16·1), impurities of S_{20} = 5·5 and 3·4 were also present. The preparation was diluted to 6 ml. with buffered saline A and centrifuged 5 hr at 161,180 g in the J rotor but without Hyflo. The supernatant fluid SNF 1 which was removed rather completely was found by titration to contain 60 % of the large antigen present. The pellet was resuspended in 0.02 M-phosphate pH 7·8 to 6 ml. and recentrifuged as before. The supernatant fluid which was not entirely removed on this occasion contained less than 5 % of the initial antigen. The pellet was resuspended in 0.02 M-phosphate to a volume, including rinsings, of 1 ml.

The solution after clarification for 30 min. at 17,570 g in the S.W. 39 rotor contained 13 % of the antigen present in the agarose column effluent. In the analytical centrifuge only one peak (S_{20} = 20) was detectable, and by immunodiffusion analysis one line was formed identical with the one line given by SNF 1. Portions of the solution mixed
with 2 % sodium tungstate were applied to carbon-coated electron microscope grids. Typical ringlike structures varying in apparent diameter from about 87 to about 124 Å were seen (Pl. 1, fig. 3). The ultraviolet absorption curve with a minimum $E = 0.366$ at 250 mÅ and a very flat maximum $E = 0.385$ at 265 mÅ resembled that of other purified preparations of the antigen (Fig. 5).

APP extract dissolved in 24 ml. 0.2M-tris pH 7.6

Centrifuge (4 hr, 161,180 g, 2.5°) with 0.2 g. Hyflo in each 'J' rotor tube

Pellets and Hyflo extracted 7x with tris buffer
(total vol. = 12 ml. 78 % CF activity)

Digest at 37° for 45 min. with each enzyme in turn

RNase (0.5 mg./ml.), DNase (1 mg./ml. + 0.005 M-Mg) and trypsin (1 mg./ml.)

Add trypsin inhibitor (1 mg./ml.) and centrifuge (40 min. 6590 g)

Two-thirds of SNF removed. Remaining third was recentrifuged as above

SNF's pooled (total vol. = 12 ml. — 61 % recovery from digestion and clarification)

Dialyse against buffered saline A and dilute to 18 ml.

Centrifuge (4 hr, 161,180 g, 2.5°) with 0.15 g. Hyflo in each 'J' rotor tube

Hyflo and pellets extracted 6x with 0.2 M-tris (total vol. = 9 ml. — 60 % recovery)

Concentrate to 3 ml. by pervaporation at 4°, dialyse against water and freeze dry

The introduction of agarose pearls (Hjertén, 1964) led to greatly improved separation on a single column. The extracts from 613 infected and about the same number of normal suckling mouse brains were treated alike as shown in the above flow diagram.

The dried product was dissolved in 1 ml. of buffered saline A and applied to a column 1.5 × 100 cm. filled with 7 % agarose pearls fed with the same solvent. The effluent passed through a Uvicord and was collected in 2.75 ml. fractions, fraction 1
being taken during the application of the sample. The extracts gave two main peaks (Fig. 6) which were well separated by the taller columns of pearl agarose. The extract of infected but not that of normal brains showed a third, smaller, intermediate peak occupying about the same part of the elution diagram as the haemocyanin of *Jasus lalandii* which has approximately the same sedimentation coefficient as the antigen. Experiments were also done with 3%, 5%, and 10% agarose pearls, but the 7% concentration was chosen for its better separation of the first and antigen peaks. The first peak impurity common to extracts from normal and infected brains appeared to contain a substance or substances prone to denature or aggregate because, although the solutions applied to the column were perfectly clear, the contents of the fraction collector tubes which held this first peak material were always distinctly turbid.

![Exclusion chromatography](image)

**Fig. 6.** Exclusion chromatography on a 1.5 x 100 cm. column of agarose pearls of *Jasus lalandii* haemocyanin (○—○), digested and centrifuged extracts of normal brains (●—●), and a similar extract of infected brains (■—■).

Attempts to examine the corresponding fraction from a 4·5% agarose column in the analytical ultracentrifuge only showed traces of rather slowly sedimenting material ($S_{20} = 4·3$) suggesting that complete precipitation of any substance of high molecular weight had occurred while the fraction was being concentrated for examination. It seems likely that ‘first peak’ impurity may be responsible for the tendency of brain extracts before this stage of purification to become cloudy on standing and to give turbid solutions when pellets are resuspended after centrifugation. From previous results with this column it was anticipated that most of the antigen would be in fractions 32 to 40 which were pooled and treated as shown in the flow diagram below.

The extract of normal brains left no visible residue but the ampoule was nevertheless rinsed with phosphate-buffered caesium chloride of density 1·34 g./cm.$^3$ (0·8 ml.) and
Freeze-dried product (see above)
redissolved in 1 ml. buffered saline A

Exclusion chromatography 7% agarose pearl
column (1.5 × 100 cm.)

Fractions 32 to 40 pooled (35% recovery) and
centrifuged (4 hr, 161,180 g, 2.5°) with
0.04 g. Hyflo in each 'J' rotor tube

Pellets and Hyflo extracted
SNF (discard)
total vol. = 2 ml. (52% recovery)

Second cycle of exclusion chromatography
on 7% agarose pearls

Fractions 33 to 41 pooled (77% recovery)
centrifuge (4 hr, 161,180 g, 2.5°) with
0.03 g. Hyflo in each 'J' rotor tube

Hyflo and pellets extracted total
SNF (discard)
total vol. = 2 ml. (48% recovery)

Dialyse against 0.02 M-phosphate pH 7.5
and examine in spectrophotometer
(Fig. 7)

Dialyse against water and
freeze dry

the solution centrifuged at 187,000 g and 20° in the fixed partition cell. No band was
detectable by the schlieren optical system at the end of the run. The fractions of the
solution taken from above and below the partition (see Methods) were separately
dialysed and examined in the spectrophotometer. Both gave identical 'curves' with a
maximum extinction of 0.042 at 240 mµ. This absorption may have arisen partly from
a non-diffusible impurity in some samples of CsCl and partly from u.v. absorbing
material liberated by imperfectly washed dialysis sacs. The freeze-dried extract from
infected brains (after the two cycles of exclusion chromatography described above)
was dissolved in 0.185 ml. of 0.02 M-phosphate pH 7.5 and 60% (w/v) CsCl solution
(0.615 ml.) was added. The solution (density 1.343 g./cm.³ at 20°) was centrifuged for
5.5 hr at 187,000 g and 20° in the fixed partition cell. Only one schlieren band was
detected; at the end of the run it was about 1.2 mm wide and situated above the
partition about 6.47 cm. from the axis of rotation. The solutions and rinsings taken
from above and below the partition were dialysed for 2 days against phosphate buffer
and for 1 day against water. Both had ultraviolet absorption curves with poorly
defined maxima at 260 mµ and E275/E260 ratios of about 0.9 but most of the absorbing
material was in the upper part of the cell. The solutions were freeze dried and the
residue from that taken from above the cell partition was redissolved in buffered CsCl
to a density of 1.314 g./cm.³ and recentrifuged as before. The band now appeared to coincide with the partition. The cell contents were withdrawn, diluted to a density of 1.304 g./cm.³ and again centrifuged. On this occasion there was no very convincing evidence from the schlieren system of a band in any part of the cell. The fluids from above and below the partition were treated as before. The ultraviolet absorption of both fractions was now very small but that of the solution from below the partition showed a flat peak at 260 mμ whereas that of the solution from above the partition was greatest at about 270 mμ. The solution taken from below the partition was freeze dried. The residue was dissolved in a drop of 0.02 M-phosphate pH 7.8. A drop of 2% sodium tungstate was added and the mixture applied to carbon-coated electron microscope grids and electron micrographs made (Pl. 2, fig. 4).

Preparation of the large antigen for analysis

Extracts from 1600 infected suckling-mouse brains were purified in the same way as the sample used for preparing the electron micrograph (Pl. 2, fig. 4). The first density gradient centrifugation at a density of 1.308 g./cm.³ occupied 6 hr and a readily detectable band appeared in the upper part of the cell (distance from axis 6.39 cm., width 0.8 mm.) in addition to the antigen band of which most, but not all, was below the partition (Pl. 2, fig. 5). About three-quarters of the recovered complement-fixing antigen was in the fraction taken from below the partition. The two fractions contained about equal amounts of ultraviolet absorbing material, maximal absorption being at 260 mμ for the fraction below the partition and at 265 to 270 mμ for the upper fraction. The solution from the lower part of the cell was dialysed, freeze dried and recentrifuged in CsCl of density 1.340 g./cm.³ for 6 hr. There was one band only, in the upper part of the cell 6.48 cm. from the axis. The two fractions were dialysed, titrated and examined in the spectrophotometer. No CF activity was detected at 1/50 dilution in the fraction from below the partition. The titre of the upper fraction was 300, indicating a yield of only 15% based on the CF activity in the fluid taken from below the partition in the run at density 1.308 g./cm.³. Although good recoveries of Jasus lalandii haemocyanin (estimated by absorption at 275 mμ) have been obtained with this technique, yields of antigen were disappointing possibly owing to adsorption by the filter paper forming the partition. The u.v. absorption curves of the two density gradient fractions showed maxima at 265 mμ but only the fraction from above the partition absorbed appreciably at 230 mμ. The nucleoprotein-like antigen may have been separated from a trace of free nucleic acid by this operation. After a final dialysis and freeze drying the antigen was dissolved in about 1 ml. 0.02 M-phosphate pH 7.5. The solution was kept at 4° in an open ampoule in a closed bottle containing water and chloroform and portions were removed for analysis as required.

The ribose content of the solution of purified antigen

Two estimations were done on different days. For the first experiment 0.1 ml. of the antigen solution and 0.15 ml. water were mixed with 0.25 ml. reagent and for the second 0.15 ml. of antigen and 0.1 ml. water were used. On each occasion duplicate blanks and a series (in duplicate) of ribose standards were run simultaneously (Fig. 8). The results were 1.6 and 1.8 μg. ribose/ml. As dialysed effluents from agarose columns even after long periods of use give a positive anthrone test for carbohydrate, dialysed column rinsings were mixed with a standard ribose solution and submitted to the
orcinol test. No additional ribose was detected. In another experiment human serum albumin was added to a solution of RNA in the proportion previously found to give a mixture with a u.v. absorption curve matching that of the antigen. The protein had no effect on the ribose estimation. Assuming that only purine-bound ribose is estimated and that the molecular ratio of purine to pyrimidine in the antigen RNA is 1:1 the results suggest that the antigen solution contained about 7.3 \( \mu \)g./ml. of RNA which, assuming 16.3 \% N in RNA, would account for 1.18 \( \mu \)g./ml. of nitrogen.

The total nitrogen content of the solution of purified antigen

Duplicate 0.1 ml. amounts of antigen solution were analysed simultaneously. Blanks and standards consisting of 0.1 ml. amounts of solutions of glycine containing 8, 16 and 32 \( \mu \)g. N/ml. were done in duplicate at the same time—also 0.1 ml. amounts of the mixture of RNA and human serum albumin used to match the u.v. absorption curve of the antigen. The latter mixture had been analysed with very similar results on several previous occasions. The antigen solution appeared to contain about 3.0 \( \mu \)g. N/ml. (Fig. 9). From this, 1.18 \( \mu \)g. must be deducted for the RNA, leaving 1.82 \( \mu \)g./ml. If the remainder had been derived from a protein containing 16 \% N its concentration would have been 11.4 \( \mu \)g./ml. As this is lower than would be expected from the ultraviolet absorption curve, samples of the RNA and DNA were analysed to make sure that the conditions of digestion were adequate for this class of substance. The result for the RNA was within 8 \% of the maker's estimate while the DNA appeared to contain 14.8 \% N in fair agreement with the expected value.
Evidence for the presence of DNA in the antigen

In the first test, standards containing 0.5, 1, 2, 4, 6 and 8 μg. DNA/ml. and blanks were put up in duplicate simultaneously with the antigen. The latter gave the same extinction as the standards containing 2 μg. DNA/ml. As 0.1 ml. of antigen solution had been diluted with 0.1 ml. HClO₄ for hydrolysis before the test, the result indicated a DNA content of 4 μg./ml. in the antigen solution. The points given by the standards in this experiment, however, were so scattered that the result was regarded as an indica-

Fig. 8. Estimation of ribose in the solution of purified soluble antigen. •—•, extinction at 670 μm (corrected for blank) of duplicate mixtures prepared with standard ribose solutions. ■, corrected extinction at 670 μm of a single reaction mixture prepared with 0.15 ml. of antigen solution and 0.1 ml. of water. The extinction of each of the duplicate blanks was 0.014.

Fig. 9. Estimation of total nitrogen in the solution of purified soluble antigen (Jacobs's method). The digestions were set up in duplicate and the ninhydrin colorimetry on each digest was repeated with fresh reagent on the following day to give four values for the extinction of the blank, the antigen (■), and each standard (●—●).

tion of the presence rather than the concentration of DNA. In an attempt to confirm this indication, 6 blanks were set up and 6 tests prepared with a solution containing 2.0 μg. DNA/ml. The extinctions of the blanks varied between 0.064 and 0.066 while those of the tests varied between 0.076 and 0.078, the difference being the same as the net reading of the antigen in the first experiment. In the second test using a series of 6 standards there was good agreement between duplicates and the extinctions given by the standards lay on or very near to a straight line when plotted against DNA concentration, but unfortunately this line did not pass through the origin. On this
occasion also the antigen solution appeared to contain 4 µg. DNA/ml., again pointing to the presence of DNA rather than indicating its concentration.

The lines in Fig. 1 of Burton (1956) are either curved over our range of DNA concentrations or fail to pass through the origin. In our experience calibration lines down to a concentration of 1 µg. DNA/ml. were usually straight and frequently but not invariably passed through the origin. The diphenylamine test for DNA does not seem so readily adaptable to very low concentrations as the orcinol test for RNA.

![Ultraviolet absorption curves](image)

**Fig. 10.** The ultraviolet absorption curves of (a) an APP extract of infected brains purified by centrifugation, enzyme treatment, two cycles of exclusion chromatography and iso-density centrifugation in CsCl of density 1.343 g./cm.³ (■—■); (b) a mixture containing 180 µg./ml. of human serum albumin and 9.35 µg./ml. of RNA (○—○); (c) human serum albumin 500 µg./ml. (●—●); (d) and RNA 40 µg./ml. (△—△). Solvent 0.02 M-phosphate pH 7.8.

Some properties of the largest rabies soluble antigen

The ultraviolet absorption curve (Fig. 10) of a specimen of the antigen which had been purified by digestion, centrifugation, two cycles of exclusion chromatography through 7% agarose pearls and centrifugation in a CsCl solution of density 1.343 g./cm.³ was duplicated fairly closely in the 230 to 280 mµ region by a mixture containing 180 µg./ml. of purified human serum albumin (Lister Institute) and 9.35 µg./ml. of
yeast nucleic acid ('highly polymerized', B.D.H.). The ultraviolet absorption curves of these substances by themselves are also shown. The increasing divergence between the curves of the antigen and the mixture in the region beyond 270 m\(\mu\) into the 290 to 300 m\(\mu\) region where the usual protein constituents might be expected to have little absorption suggests that the antigen may contain, as an integral part or impurity, a substance absorbing at longer wavelengths. Unfortunately this possibility was not explored although it was noticed that pellets of the purest preparation when sedimented into buttons of pure white ‘Hyflo’ retained some reddish pigment. This might have been haemoglobin though it seems unlikely that this could have survived the purification sequence.

The electrophoretic mobility in agarose of the specimen purified by repeated centrifugation was compared with those of the constituents of normal rabbit serum (Pl. 2, fig. 6). A layer of 1 % agarose in about 0-01 M-tris buffer of pH 8-5 was poured on a thin glass sheet previously coated with a film of dried agarose. Five holes about 1 to 2 mm. in diameter were punched in a row across the sheet at about one-third of its length from the end to be connected to the negative electrode. A narrow trough was also cut in the position shown in the photograph. The holes were filled as indicated—LP\(e\) being a concentrated solution of the pellet from the fifth successive preparative centrifugation of enzyme-treated APP extract of infected suckling-mouse brains and KP\(e\) being the corresponding solution from the extract of uninfected brains. After electrophoresis for 45 min. at 7.5 v/cm. the agar was cut in a direction parallel to its length so as to separate the part intended for immunoelectrophoresis from the part to be stained for protein. The former was left on the glass slide, and with its trough filled with concentrated mouse antirabies serum, was kept in a damp chamber until the precipitin line had developed. The remainder of the agar was fixed for 10 min. with a solution containing 300 ml. alcohol, 10 ml. acetic acid, 190 ml. water and 2 g. HgCl\(_2\) and was then stained with 0.002 % nigrosine. The part used for immunoelectrophoresis was washed in saline for 2 days and also stained with nigrosine. Although KP\(e\) still contained sedimentable material it failed to give any protein spot whereas LP\(e\) gave a single spot at the same distance from the origin as the single line of precipitation shown by the immunoelectrophoretogram. The mobility of the antigen under the conditions of the experiment was about seven-eighths of that of rabbit serum albumin. A similar result was obtained at pH 8.2 when the mobility of another preparation of the large antigen giving a single electrophoretic spot was compared with that of bovine serum albumin. At pH 6.2, however, the mobility of the antigen, though much less, equalled or slightly exceeded that of the albumin. Solutions of APP extract of infected brains from which the largest antigen had been removed by two 4 hr centrifugations at 161,180 g were also submitted to simultaneous agar electrophoresis and immunoelectrophoresis. The former revealed several protein spots and the latter a single line of precipitation extending over a larger fraction of the migration path than that given by the large antigen, suggesting by its position a lower electrophoretic mobility for the smaller antigen than the large one though a direct comparison was not made. Comparison of these results with those obtained with crude extract (Mead, 1962b) suggest that in the earlier experiments the pores in the agar may have been partially blocked by some of the impurities.

The sedimentation coefficient of the antigen boundary was measured at 56,100 rev./min. on 28 occasions with results varying between \(S_{20}^\text{w} = 13.6\) and \(S_{20}^\text{w} = 20.2\). Part of
this spread may be attributed to difficulty in measuring the precise position of very small peaks which subsided rather rapidly, but the main cause was undoubtedly the marked retarding effect of more slowly sedimenting impurities. The concentration of the antigen itself was probably never high enough to demonstrate the usual concentration-dependence of the sedimentation coefficient but the effect of the concentration of impurities was evident first from the finding that the purest preparations invariably gave the highest values and secondly, from the results of centrifuging three different impure preparations at different concentrations. In one such experiment $S_{20}$ for the antigen increased from 14 to 17.9 when a crude preparation was diluted 1/5 and in another the $S_{20}$ for the antigen in a different preparation diminished from 17.2 to 15.5 when the solution was concentrated $\times$ 5. Five measurements of $S_{20}$ for what were considered to be fairly pure specimens of the antigen giving schlieren diagrams with only one detectable boundary gave values between 18.0 and 20.2, the average being 18.9.

The sedimentation constant of the small antigen was roughly measured for comparison by a modification of method A of Polson & van Regenmortel (1961) in which a compacted mass of Hyflo super cell trapped the antigen sedimented out of the effective column which on account of the small size of the antigen was only about 3.7 mm. long. The Hyflo was first suspended in buffered saline containing 1% bovine serum albumin and was compacted by repeated centrifugation at 85,050 g in the S.W. 39 rotor before use in the experimental run at the same force. The solution had been freed from the largest antigen by two 4 hr centrifugations at 161,180 g. Although it gave only one precipitin line on immunoelectrophoresis it may have contained traces of ‘intermediate’ antigen which could have affected the CF titrations. The result, which must be regarded as approximate, was $S_{20} = 3.6$.

The apparent buoyant density of the antigen was first roughly determined by centrifugation in preformed gradients of CsCl in the S.W. 39 rotor using the method of Polson & Levitt (1963). Although centrifugation was continued for 5 hr at 85,050 g the antigen may not have been perfectly equilibrated in the gradient. The results suggested that the density of the antigen was about 1.32 g/cm.$^3$; that is, about the same as that of Jasus lalandii haemocyanin measured under the same conditions by Polson & Levitt. Density-gradient centrifugation in the partition cell of the analytical centrifuge at 187,000 g (see Methods) soon revealed, however, that the antigen was denser than the haemocyanin by about 0.02 g/cm.$^3$ as can be seen from its final position/initial density plot (Fig. 1). The lines for the antigen and haemocyanin appear to differ in slope, suggesting perhaps that the degrees of hydration of the two substances are differently affected by the concentration of CsCl.

Electron microscopical appearance. Batches of antigen purified by different methods have been examined at intervals during several years, usually without the discovery of any characteristic structure, but on the occasions when collections of particles of the same shape and size have been observed they were invariably ringlike. An example of one of the earlier photomicrographs of a negatively stained preparation is shown in Pl. 1, fig. 3, which is a photographic negative. The instrumental magnification was not known very precisely but the ‘rings’ appear to vary in diameter from about 87 to 124 Å. When similar ‘rings’ and little else were seen in electron micrographs (e.g. Pl. 2, fig. 4) of a preparation purified by a process capable of eliminating all constituents from an extract of uninfected brains, it was assumed that these ‘rings’ may have represented the true structure of the antigen. In one or two of the earlier micrographs the apparent
The largest soluble rabies antigen stacking of 'rings' to form short helices was seen on rare occasions. A spherical particle of density 1.34 g./cm.³ and $S_{20, w} = 18.9$ would have a diameter of about 10 μm equal to the observed diameter of the 'rings', but without knowing the frictional ratio of ring-shaped particles it is impossible to state whether this agreement is fortuitous.

**DISCUSSION**

The particles isolated from infected suckling-mouse brains appear to be rabies-specific because they are detectable by complement fixation or the precipitin test with antisera made by immunizing adult mice with rabies-infected suckling mouse brains but not with antiserum to normal suckling mouse brains prepared in the same way (Mead, unpublished). No similar particles were found in extracts of the brains of normal suckling mice. Evidence that the particles of the largest antigen that survived the final purification sequence were fairly 'pure' depends on electrophoretic, immunoelectrophoretic and sedimentation behaviour, uniform appearance in the electron microscope and the fact that everything seemed to be eliminated from a control extract of uninfected brains. The presence of pentose and probably deoxypentose and the shape of their ultraviolet absorption curve suggest that they contain nucleotides, while the presence of polypeptide is indicated by the formation of a nigrosine-stained spot on electrophoresis, the shape of the ultraviolet absorption curve and possibly by their serological behaviour; though the latter might be due to specific carbohydrate, the presence of which has not been excluded. The particles react serologically as haptens but true antigenicity has not been proved. There is uncertainty about the relative amounts of nucleotide and polypeptide in the particles. The ultraviolet absorption curve is matched fairly well by a mixture containing only about 5 % of RNA with human serum albumin. If a protein containing a higher proportion of aromatic amino acids than human albumin had been used, a lower protein/RNA ratio would have given a better match both at 230 mμ and in the region above 265 mμ. The ultraviolet absorption curve of poliovirus containing about 25 % to 30 % RNA has $E_{235}/E_{260}$ and $E_{275}/E_{260}$ ratios of about 0.97 and 0.75 (Schwerdt & Shaffer, 1955). The corresponding ratios for the rabies antigen are 1.7 and 0.9, suggesting a lower RNA content.

The analytical results for pentose and total nitrogen, on the other hand, point to a much higher content of RNA—between 20 % and 40 % or more depending on the proportion of purine-bound or orcinol-susceptible ribose in the nucleotide. Admittedly both analyses had to be done very near the lower limits of detection by the methods used, but as at least 8 weeks were spent entirely on rehearsals with each method before it was applied to the precious antigen, the results are not thought likely to be subject to errors large enough to explain the discrepancy between the compositions suggested by the u.v. and analytical data. It is, of course, conceivable that the particles may contain pentose unattached to purine or pyrimidine bases. The rabies antigen seems to contain some DNA (or other substance reacting with the diphenylamine reagent) but there is not enough evidence to estimate the proportionate amount. A nucleoprotein containing 40 % of RNA and perhaps DNA as well might be expected to have a density greater than 1.34 g./cm.³, but the values given by several viruses (see for example Mattern, 1962) show that such expectations are not always realized.

Virus-specific particles found in extracts of infected tissue may be subunits not yet incorporated into complete virus particles, fragments of such subunits or of particles
broken up during the extraction process or subsequent treatment or they may represent misfits incapable for some reason of being included in infective particles, cf. short rods of tobacco mosaic virus (Commoner, Shearer & Yamada, 1962). Another possibility is that they may represent non-viral structures on which various virus constituents are synthesized or assembled. One or more of the smaller rabies antigens may be enzymes formed specially for virus reproduction. As our rings have very nearly the same diameter as the tightly coiled rabies helix (Pinteric, Fenje & Almeida, 1963) it is tempting to assume that they represent single turns, although it seems doubtful whether single turns would retain the diameter of the helix as this appears in the micrographs to have a tendency to unwind. Nucleoprotein helices are of course prominent and possibly the only internal features of many myxoviruses (see, for example, Cruickshank, 1964; Rott & Schäfer, 1964). The internal antigen of type A influenza virus contains 5.3% RNA and no other carbohydrate (Frisch-Niggemeyer & Hoyle, 1956) while those of Newcastle disease virus and mumps contain 5.7% and 9.6% RNA respectively (Schäfer & Rott, 1959).

If the rings that we have isolated do in fact represent single turns of nucleoprotein helix, explanations are needed (a) for the formation of single turns rather than short lengths of several turns or segments of a single turn, (b) the exceptionally high apparent ratio of nucleotides to protein and (c) the resistance of the antigen to trypsin, chymotrypsin and pancreatic nucleases.

Single turns might arise as a definite stage in the formation of complete helices or represent a relatively stable stage in their breakdown. A few single turns of the nucleoprotein helix can be seen in some photographs of the influenza internal antigen liberated by ether treatment (Hoyle, Horne & Waterson, 1961 and also Fig. 3 in Cruickshank, 1964), SV5 virus (Choppin & Stoeckenius, 1964) and rabies virus (Pinteric et al. 1963) but they are not a prominent feature. If our rings originate from partially or completely formed helical strands, breakdown may have been caused either mechanically during the disintegration of the brains or during the brief ultrasonic treatment. Alternatively it may have been brought about by tissue enzymes in spite of attempts to keep the extract cold in the early stages of preparation or possibly during precipitation at pH 4.5. The fastest sedimenting boundaries detected in two extracts of infected brains which had not been treated with trypsin or nucleases had sedimentation constants of 14 (17.9 after dilution 1/5) and 15.5—not significantly different from that of the antigen in crude digested preparations. Although a very small boundary of the same sedimentation constant was noticed in the diagram given by the parallel extract of normal brains in one of these experiments (this had disappeared after digestion), it seems unlikely that breakdown of the helix to single turns occurred during digestion with trypsin and nucleases—a conclusion supported by the complete identity of the antigen before and after digestion in immunodiffusion tests. Apart from density gradient centrifugation which was not used in the preparation of specimens for many early electron micrographs or analytical centrifugations, the only individual stage in the purification involving a major loss was the acid precipitation and it is conceivable that this might have represented the loss of helices of more than one turn. The later stages were designed to remove mainly small particles. Large particles might have been expected to separate in the early fractions from exclusion chromatography but these had no complement fixing activity. If our rings are formed by the rupture of a helix it must be assumed that there is a weak point in each turn.
The resistance of the antigen to trypsin, chymotrypsin and pancreatic nucleases must now be considered. Polynucleotides are attacked by pancreatic ribonuclease at the ester bond linking pyrimidine nucleoside 3 phosphates to other nucleotides and in the unlikely event of the complete absence of such groups would presumably be resistant to the enzyme. Double stranded RNA, which is a constituent of some virus particles and probably occurs in small amounts in tissues infected with many RNA viruses (Ralph et al. 1965) is also at least relatively resistant to pancreatic RNase (Gomatos & Tamm, 1963) but the enzyme concentrations used for the test have usually been in the μg./ml range rather than the mg./ml used to purify the rabies antigen.

From electron microscope studies Valentine & Isaacs (1957a, b) concluded that the inner structure of several myxoviruses consisted of trypsin-resistant ribonucleo-protein accessible to ribonuclease only after acid and trypsin treatment and owing its trypsin resistance to RNA which appeared to form 3 to 6 threads attached to the protein structure and parallel to its long axis.

In the case of the largest rabies antigen neither trypsin nor ribonuclease appeared to have any effect in whatever order they were applied. If the RNA in the myxovirus core was adequate to protect the protein from trypsin the same mechanism might apply in the case of the rabies antigen. Resistance to ribonuclease would also be understandable on the Valentine & Isaacs model if the complete helix of the rabies internal component carried its RNA in extended threads with only single nucleotides attached at one or more points on each individual turn. The possibility that the genome of influenza virus (in contrast to that of Newcastle disease) consists of more than one strand of RNA has been suggested by Hirst (1962) on genetic grounds and considered as a structural possibility by Cruickshank (1964). Rings so modelled with only individual nucleotides attached to one turn of a helical protein backbone would presumably be RNase resistant.

Taken together with the presence of DNA, these considerations seem to render it unlikely that the antigen rings are single turns of nucleoprotein helix and as an alternative hypothesis it is suggested that they may represent structures specially formed for the synthesis of rabies virus RNA or of the protein with which the RNA is combined in the internal component. Evidence that host cell DNA may be implicated in the synthesis of some RNA viruses including influenza and fowl plague but not Newcastle disease (Rott & Scholtissek, 1964; Barry, 1964) depends mainly on the effect of actinomycin D on virus production. Although this evidence has lost some of its force from the discovery (Gomatos, Krug & Tamm, 1964) that reactions dependent on double-stranded RNA are also inhibited by actinomycin, the involvement of cell DNA has not been excluded. Some cell nuclei contain complexes of protein with RNA and DNA which can incorporate amino acids but differ from ribosomes. Their activity is unaffected by RNase but only partially resistant to DNase (Wang, 1965). It is conceivable that the function and structure of the largest antigen may be related to particles of this type.

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As the senior author retires on completion of this paper, this Unit has for free disposal to scientists intending to investigate the smaller rabies antigen, sealed ampoules each containing the freeze-dried supernatant fluid from the sedimentation of the largest antigen from about 200 suckling-mouse brains.

REFERENCES


The largest soluble rabies antigen


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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Ultracentrifuge patterns of APP extracts of infected and normal brains after preliminary centrifugation to remove the smaller antigens. The photograph was taken 9 min. after a speed of 56,100 rev./min. had been reached. Sedimentation from left to right. Upper trace extract from infected lower trace extract from normal brains. Both extracts diluted 1/5.

Fig. 2. Ultracentrifuge patterns of digested APP extracts of infected and normal brains after five cycles of preparative centrifugation. The photograph was taken 10 min. after a speed of 56,100 rev./min. had been reached. Sedimentation from left to right. Upper trace extract from infected, lower trace extract from normal brains. $S_{20}$ for the antigen = 18.1.

Fig. 3. Electron micrograph of the largest antigen partially purified by enzyme treatment, exclusion chromatography and centrifugation. Negative staining with 0.02 M-Na$_2$HPO$_4$ and sodium tungstate. The photograph is a negative.

PLATE 2

Fig. 4. Electron micrograph of the largest antigen purified by enzyme treatment, sedimentation, two cycles of exclusion chromatography on agarose pearls and density-gradient centrifugation. Negatively stained with 0.02 M-Na$_2$HPO$_4$ and sodium tungstate. Photograph is a positive.

Fig. 5. The centrifugation of the antigen in CsCl of density 1.308 g/cm$^3$ in the fixed partition cell. Schlieren pattern after 361 min. at 50,740 rev./min. The meniscus is on the left.

Fig. 6. Composite photograph of agarose electrophoretogram of extracts of normal and infected mouse brains purified as described in the text with rabbit serum for comparison, and an immunoelectrophoretogram of the extract of infected brains. The five samples were submitted to electrophoresis simultaneously in one sheet of agarose which was divided for separate development of the electrophoretograms and the immunoelectrophoretogram and reassembled for photography. KPn; extract of normal brains. LPn; extract of infected brains. RS; rabbit serum.
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