Some Properties of Fixed Rabies Virus

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(Accepted 19 June 1967)

SUMMARY

The Pasteur strain of fixed rabies virus was inactivated by most common organic solvents, but survived a single treatment with trifluorotrichloroethane. The virus was stable at pH values between 5·0 and 10·0. Inactivation by heat was similar to that of other viruses. The virus was destroyed by trypsin and damaged by phospholipase C but not by ribonuclease; enzyme inhibitors did not preserve the infectivity of mouse-brain suspensions. Viral infectivity was diminished only after repeated freezing and thawing or long exposure to ultrasonic vibration. Equilibrium centrifugation in sucrose solutions gave two peaks of infectivity corresponding to densities of 1·13 g./cm.³ and 1·19 g./cm.³. Chromatographic purification on ECTEOLA-cellulose was unsuccessful. Partially purified virus was readily inactivated by the photodynamic effect of methylene blue. Potent suspensions of virus did not agglutinate red cells of several mammalian and avian species under different conditions of pH and temperature.

INTRODUCTION

Despite the attempts to prepare rabies vaccine in tissue culture (Fenje, 1960; Ott & Heyke, 1961; Wiktor, Fernandes & Koprowski, 1964), infected brain from either immature (Fuenzalida, 1964; Gispen, Schmittman & Saathof, 1965; Svet-Moldavsky, Svet-Moldavskaya & Kiseleva, 1960) or adult animals is still the most abundant source of virus. Vaccines prepared from adult brain material carry with them the risk of damage to the central nervous system of the recipient. However, recent work on the characterization of the factor in neural tissue causing experimental allergic encephalomyelitis (Kies et al. 1962; Lakao & Roboz-Einstein 1965; Laatsch et al. 1962; Robertson, Blight & Lumsden, 1962) suggested a re-examination of some of the physicochemical properties of rabies virus from brain. Many of the properties attributed to one of the oldest known viruses are almost folklore and have not been examined for many years. Recently Habel (1964) stressed the paucity of information on physical and chemical properties of the virus. The present work records observations on some of these properties with a classical strain of 'fixed' rabies virus grown in adult mouse brain.

METHODS

Virus

The Pasteur strain of fixed rabies virus was used for all experiments. Stock virus was stored below −10° as whole infected guinea pig brain in 50% glycerol saline. Virus for experimental use was grown in mouse brain. Stock guinea pig brain was homogenized to give a 10% (w/v) suspension in 0·015M-NaCl containing 2% (v/v) calf serum.
Volumes of 0.02 ml of a suitable dilution were inoculated intracerebrally into 10 to 21 g. mice anaesthetized with ether. Mice were usually moribund 7 to 9 days after inoculation, when they were killed and their brains collected aseptically and stored at $-70^\circ$. Suspensions (10%, w/v) were prepared from brains in convenient amounts by homogenization in a Servall Omnimix cooled to $0^\circ$ in an ice bath. The suspending medium was usually a tenfold dilution of phosphate buffered saline (PBS) in distilled water. Gross debris was removed by centrifugation at 2000 g for 10 min. at $0^\circ$. After experimental manipulation 2% calf serum was added to samples stored overnight at $4^\circ$ or for longer periods at $-70^\circ$. The identity of the virus in experimental material was established in neutralization tests with standard rabies antiserum. The absence of lymphocytic choriomeningitis virus in the seed was confirmed by serological tests.

**Virus assay**

Samples were diluted in PBS containing 2% calf serum (serum saline). The volume inoculated was 0.02 ml. Each dilution was tested intracerebrally in 5 mice. Mice weighed 18 to 21 g. at inoculation and were observed for 21 days. Deaths were recorded daily; any occurring within 48 hr of inoculation were regarded as non-specific and the animals excluded from the calculation of the end point. Fifty per cent end-point titres were calculated by the method of Reed & Muench (1938) and virus concentrations expressed as LD 50/ml.

**Treatment with solvents**

The effects of solvents on viral infectivity were tested in two ways. In the first, solvents chilled to between $0^\circ$ and $-10^\circ$ were added in suitable amounts to 10% suspensions of infected mouse brain at $0^\circ$. After vigorous mixing for 1 min. in a Servall Omnimix, the samples were centrifuged at 1500 g for 10 min. Supernatant or aqueous phases were sampled, diluted 100-fold to decrease the concentration of miscible solvent and titrated. In some cases interfacial or sedimented precipitates were dissolved or eluted in volumes of serum saline equal to the volume of starting material and also titrated. In the second method 1 g. amounts of whole unsuspended infected brain were treated for 3 min. in the Omnimix with 15 ml. of various solvent mixtures at $0^\circ$ to $-10^\circ$. All were centrifuged, the deposits extracted with 10 ml. of serum saline and titrated together with untreated controls.

**pH stability**

Infected mouse brain was homogenized in distilled water to produce a 20% (w/v) suspension. After centrifugation at 2000 g for 15 min. at $0^\circ$ the supernatant fluid was treated with 10% Arcton 113 (Trifluorotrichloroethane, Imperial Chemical Industries), followed by centrifugation at 2000 g for 15 min. Arcton treatment and the use of high titre virus allowed extensive initial dilution and eliminated the possible protective effects of extraneous protein. Three ml. volumes of virus suspensions were added to 3 ml. volumes of McIlvaine's buffer mixtures at pH values from 3.0 to 8.0. McIlvaine's phosphate solution was adjusted with 0.01 M-NaOH to give pH values of 9.0 and 10.0. Similar volumes mixed with serum saline were used as controls. Mixtures were kept at $0^\circ$ during manipulation and held for 18 hr at $0^\circ$; pH values were checked before and after exposure. Samples were restored to neutrality by tenfold dilution in 2% calf
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Serum in McIlvaine's buffer (pH 7.0) and titrated for infectivity. Buffers of other composition and molarity were also tested. Acetate buffer was used for pH values of 3.0, 4.0 and 5.0; Clark and Lubs borate + KCl + NaOH mixtures for pH values of 8.0, 9.0 and 10.0; and tris buffer for pH values 9.0 and 10.0. All the buffers were 0.025M in the final virus + buffer mixture, except for pH 12.0 for which 0.01M-NaOH was used.

Heat inactivation

Ten per cent suspensions of infected brains in dilute PBS were treated with 10% (v/v) Arcton and centrifuged at 2000 g for 15 min. The supernatant fluid was distributed in 0.25 ml. volumes in 2 ml. ampoules, sealed and kept in an ice bath until used. The ampoules were heated by submersion in a water bath for various times at various temperatures ± 0.2°C. Heating was arrested by dropping the ampoules into an ice bath, after which they were snap frozen and stored at -70°C until titrated.

Buoyant density

Sucrose solutions of known specific gravity were prepared in 0.015M-NaCl, pH 7.0, from the tables in The Handbook of Chemistry and Physics (Chemical Rubber Co., 30th edition, 1947). Concentrations of 10, 19, 27 and 35% were placed in separate centrifuge tubes calibrated in cm. from the bottom of the tube to 5 cm. One ml. of the sucrose solution was removed and replaced with 1.0 ml. of virus suspension partially purified by treatment with Arcton. The tubes were centrifuged at 69,000 g for 2 hr at 0 to 4°C in the 20 ml. swingout head of the MSE Superspeed 50 centrifuge. Buffer controls were included in the runs. Samples for titration were taken from the tubes at the top and bottom centimetres of the liquid columns.

Density gradient centrifugation

In tubes graduated as for buoyant density experiments, density gradients were prepared from 50% sucrose to 20% in 5% steps to give a column height of 5 cm. Virus suspensions were prepared from infected mouse brain and partially purified by treatment with Arcton followed by centrifugation for 10 min. at 10,000 rev./min. or by centrifugation through 30% sucrose. This material was mixed with an equal volume of 30% sucrose in buffer, and calf serum added to 2%. Two ml. volumes were applied to the gradients and the tubes centrifuged at 0-4°C at 69,000 g for 2 hr or at 96,000 g for 48 hr. Samples were taken at 0.5 cm. intervals from the bottom of the tube with an MSE tube piercing device (MSE Technical Publication no 33). Part of each sample was kept for sucrose estimation and part was immediately diluted in serum saline for titration. Controls consisting of the starting virus suspension were held in contact with the highest and lowest sucrose concentrations for 2 hr at 0 to 4°C without centrifugation. Sucrose was estimated either chemically or with a refractometer.

Purification on cellulose

The method of Thomas et al. (1965) was meticulously followed. Two samples of ECTEOLA-cellulose were used; Whatman E.T. 11, and Schleicher & Schuell, standard ECTEOLA no. 73 as specified by Thomas et al.
Freezing, thawing, and ultrasonic treatment

One ml. samples of 10% (w/v) suspension, partially purified with Arcton, in 20 ml. hard glass ampoules were snap frozen by immersion in a mixture of solid CO₂ + acetone followed by thawing at 20° to 30°. Similar tubes were exposed to ultrasonic vibration at 20 kcyc./sec. for 15 min. at 4 to 5A with a Dawe Soniprobe (Dawe Instruments Limited, Western Avenue, London, W. 3).

Enzyme inhibitors

Inhibitors were added to Arcton-purified suspension of rabies virus in the following concentrations: Bentonite, 1 mg./ml.; heparin, 500 i.u./ml.; sodium azide, 0.025% (w/v); p-chloromercuribenzoate, 0.5 mm. These and untreated controls were incubated for 18 hr at 37° before titration.

Enzymes

Crystalline trypsin (Seravac Laboratories Ltd, Maidenhead, Berkshire, England) was added to Arcton-purified suspensions of rabies virus to give a final concentration of 1 mg./ml., and the mixtures were buffered with phosphate to pH 8.0 and a final molarity of 0.02. Crystalline ribonuclease (Seravac Laboratories Ltd) was similarly used at 1 mg./ml. but buffered at pH 7.4; and phospholipase C (Koch-Light and Co., Colnbrook, Buckinghamshire, England) at 1 mg./ml. and pH 7.4. Controls consisted of virus+enzymes previously heated at 100° for 30 min. In some experiments the enzyme in the trypsin control was neutralized by soya bean trypsin inhibitor (Seravac Laboratories Ltd) and the ribonuclease controls by Mg²⁺ and heparin. In virus controls, the enzyme protein was replaced by bovine crystalline albumin (Armour Fraction V), 1 mg./ml. All samples were incubated at 37° for 2 hr, and diluted with an equal volume of 4% calf serum saline for titration.

Photoinactivation

The procedure described for the photoinactivation of vaccinia virus by Turner & Kaplan (1965) was used. Ten per cent suspensions of infected mouse brain were prepared in pH 7.0 PBS, and partially purified with Arcton. Methylene blue was added to a concentration of 3 x 10⁻⁵M. Ten ml. volumes of the mixture were illuminated at an intensity of 50 foot-candles in 9 cm. Petri dishes on a reflecting surface of aluminium foil. After illumination for suitable times each 10 ml. sample of dye+virus mixture was shaken with 1:0 ml. of packed ion-exchange resin (Dowex AG 50w- × 8 Na⁺ form) to remove free dye. Two per cent calf serum was added before titration. Unilluminated dye+virus mixtures (dark controls) and virus illuminated in the absence of dye (light controls) were included. The effect of the ion exchanger on rabies virus was controlled by preparing dye+virus mixtures and shaking them immediately with resin. All manipulations in the presence of dye were made under a safelight and at room temperature, approximately 22°.

Haemagglutination

Blood was drawn from various species into Alsever's solution and the red cells washed 3 times in 0.15M-NaCl. Cells were suspended in 200 times their packed cell volume of buffered saline to give a 0.5% suspension. The saline used for suspending
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cells and for diluting virus suspensions contained CaCl₂ and MgCl₂ both 0.00005M and was buffered with 0.066M-Sorenson's phosphate buffer to pH values of 6.0, 7.0, 8.0, and 9.0. Most of the erythrocytes, particularly those of the dog, were too fragile for use above or below this pH range. Virus was prepared from Arcton-treated infected mouse brain and contained 10⁻⁶ LD₅₀/ml. Uninfected mouse brain controls were prepared in the same way. Virus and control dilutions in 0.5 ml volumes were set out in triplicate in standard perspex plates at the various pH values. One set was held at 0 to 4°C, one at room temperature (20 to 25°C) and the third at 37°C. Tests for haemagglutination were made with the erythrocytes of guinea pig, dog, mouse, rabbit, hamster, fowl and goose, and read after 2 to 5 hr. Blood from geese and dogs was supplied through the courtesy of the M.R.C. Laboratories, Mill Hill.

RESULTS

Solvent extraction

Rabies virus was inactivated by most common organic solvents (Table 1). Virus survived treatment with Arcton, which also reduced the protein content of the suspensions three- to fourfold. Some virus survived treatment with methanol and acetone.
exposure, to pH 8.2 and of the pH 10.0 mixtures to pH 9.0; decreases of the same order
occurred with the other alkaline buffers. The mean of the starting and finishing values
of these was used in the construction of the figure. Turbidity appeared in mixtures
held at pH 4.0 and pH 5.0. Where pH showed marked effects on infectivity, buffers of

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**Fig. 1.** pH stability of rabies virus. Arcton-treated virus with an initial titre of $10^8$ LD$_{50}$/ml.
exposed for 18 hr at 0 to 4° to different H ion concentrations. △, tris buffer 0.025M; ■,
borate buffer 0.25M; ○, acetate buffer 0.025M; ○, McIlvaine's buffer 0.2M; □, NaOH
0.01M.

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**Fig. 2.** Fast and slow components in the thermal inactivation of rabies virus.

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other composition and molarity were used to confirm results and ensure that the observed effects were due to H⁺ concentration and not to the constitution of the buffers.

**Thermal inactivation**

Partially purified rabies virus suspensions were exposed to different temperatures for different periods. The virus was stable at 37° for up to 2 hr, lost 90% of its infectivity after 4 to 6 hr and > 99.9% after 24 hr. A trace of viable virus was found after 5 min. at 60° but inactivation was complete after 10 min. Exposure to temperatures between 45° and 60° resulted in a series of two-component curves (Fig. 2). Inactivation rates calculated from the fast components (Kaplan, 1958) of these curves were inversely proportional to the absolute temperature when plotted according to the Arrhenius equation (Fig. 3).

**Enzyme inhibitors**

Loss of infectivity after 18 hr at 37° was increased by the known protease inhibitors, sodium azide 0.025% and the thiol reagent p-chloromercuribenzoate 0.05 mM. Of the nuclease inhibitors bentonite and heparin (Philipson & Kaufman, 1964; Roth, 1953) used at 1 mg./ml. and 500 i.u. respectively, only heparin protected slightly.
Buoyant density

At 69,000 g rabies virus was sedimented within 2 hr through sucrose solutions of 10, 19 and 27 %. Practically no virus was deposited through 35 % sucrose (Table 2).

![Sucrose density gradient centrifugation of rabies virus.](image)

Virus was centrifuged for 20 hr at 69,000 g through a sucrose gradient between 10 and 35 %. Infective virus was recovered from a fairly broad range of samples approximating to 90 % of the virus applied to the gradient (Fig. 4). This suggested that rabies virus is heterogeneous with respect to particle density, size or state of aggregation. Approximate sedimentation coefficients calculated from the data by the method of Martin & Ames (1961) gave maximum and minimum $S_{20}$ values of 480 and 230 respectively. With centrifugation to equilibrium at 96,000 g for 48 hr on a similar gradient, the little virus that was recovered appeared in two peaks of infectivity with buoyant densities of 1.13 g./cm.$^3$ and 1.19 g./cm.$^3$ (Fig. 5). In all gradient experiments a pigmented, lipid-containing layer remained practically stationary at the top of the tube.

Table 2. Centrifugation through sucrose

<table>
<thead>
<tr>
<th>Sucrose concentration (g./100 g.)</th>
<th>Infectivity recovered log LD$_{50}$/ml.</th>
<th>Top cm.</th>
<th>Bottom cm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (buffer)</td>
<td>&lt; 4.0</td>
<td>6.1</td>
<td>7.9</td>
</tr>
<tr>
<td>10</td>
<td>&lt; 4.0</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>19</td>
<td>&lt; 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>&lt; 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>&lt; 4.0</td>
<td></td>
<td>&lt; 4.0</td>
</tr>
</tbody>
</table>
**Effect of enzymes**

Rabies virus was highly sensitive to trypsin, moderately so to phospholipase C and virtually insensitive to ribonuclease (Table 3).

**Chromatography on ECTEOLA-cellulose**

Suspensions of rabies-infected mouse brain were partially purified by centrifugation at 10,000 g for 10 min. and applied to columns of ECTEOLA-cellulose (Thomas et al. 1965; Sikes et al. 1966). With one batch of cellulose (Whatman E.T. II) total nitrogen was decreased by 90% in the eluates but there was a loss of more than $10^4$ LD$_{50}$ of virus. Adsorption and elution occurred with a different brand of ECTEOLA (Schleicher & Schuell) (Table 4); the recovery, however, was much less than that reported by Thomas et al. (1965) and in terms of specific activity (LD$_{50}$/mg. N$_{a}$) no significant purification was accomplished.

![Equilibrium centrifugation of rabies virus through sucrose gradient.](image)

**Table 3. Exposure of rabies virus to enzymes**

<table>
<thead>
<tr>
<th>Enzyme (1 mg./ml.)</th>
<th>Residual infectivity after 2 hr at 37°C (log LD$_{50}$/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test</td>
</tr>
<tr>
<td>Trypsin</td>
<td>&lt; 1.7</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>4.5</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>4.3</td>
</tr>
<tr>
<td>Bovine albumin</td>
<td>--</td>
</tr>
</tbody>
</table>
Freezing and thawing and exposure to ultrasonic vibration

Infected mouse brain suspensions, partially purified by Arcton and containing $10^6.5 \text{LD}_{50}/\text{ml}$, lost little infectivity after one cycle of freezing and thawing. After ten cycles, however, the loss was 90%. Vigorous ultrasonic vibration for 15 min. reduced infectivity $>1000$-fold.

Table 4. Chromatography on ECTEOLA-cellulose

<table>
<thead>
<tr>
<th>Applied to column</th>
<th>Log LD$_{50}$</th>
<th>Total N$_2$ ($\mu$g.)</th>
<th>% Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column washings</td>
<td>$&lt;2.5$</td>
<td>960</td>
<td>$&lt;0.00005$</td>
</tr>
<tr>
<td>0.3M-KCl eluate</td>
<td>7.6</td>
<td>550</td>
<td>30</td>
</tr>
</tbody>
</table>

* Corrected for dilution.

Photoinactivation

The kinetics of photodynamic inactivation of rabies virus by methylene blue were similar to those of many other viruses (Fig. 6). However, inactivation was slower than with vaccinia virus (Turner & Kaplan, 1965). Under conditions which inactivated vaccinia virus in 10 to 15 min., rabies virus suspensions containing $10^{6.7} \text{LD}_{50}/\text{ml}$. 

Fig. 6. Photoinactivation of rabies virus.
required 60 min. for complete inactivation. No inactivation occurred in unilluminated
dye-virus mixtures. Shaking with ion-exchange resin was also without effect on
virus infectivity. Illumination in the absence of methylene blue resulted in a tenfold
drop in titre. This suggests that partially purified mouse-brain suspension contains
some endogenous photosensitizer or that rabies virus is inherently photosensitive.

**Haemagglutination**

Rabies virus suspensions containing $10^{7.5} \text{LD}_{50}/\text{ml.}$ did not agglutinate the red
cells of guinea pig, dog, mouse, rabbit, hamster, fowl or goose over the pH range
6.0 to 9.0 and at temperatures of 0 to 4°, 20 to 25° and 37°.

**DISCUSSION**

The results of ether treatment of rabies virus are confused (van Rooyen & Rhodes,
1948). Pinteric, Fenje & Almeida (1963) suggested that rabies virus had the character-
istics of the ether-sensitive myxoviruses, although Andrewes & Horstmann (1949)
classified rabies as ether resistant. Depoux (1965) found that the Pasteur strain was
ether sensitive, as we and Tagaya, Ozawa & Kondo (1953) did. Crandell & Hillis
(1963), using four strains of street and one of fixed virus, found that 20% ether for
24 hr decreased infectivity 1.5 to 2.5 logs. The ionic strength and pH of the suspending
medium and the initial protein concentration of virus suspensions are critical in
determining ether sensitivity (Turner, unpublished results). Unless these factors are
controlled ether sensitivity may not have the taxonomic value that is often attributed
to it. Certainly ether extraction does not appear to be a reliable method for the purifica-
tion of rabies virus from infected neural tissue. The technique of cold methanol pre-
cipitation (Cox et al. 1947) was applied to the purification of rabies virus by Tagaya
et al. (1943) and Sawai & Makino (1954). Neither their results nor ours justify the
adoption of the method for purification.

The other solvents were tested for their effect on rabies virus largely because they
have been used, either singly or in combination, as extractives for the allergic en-
cephalomyelitic (AE) factor present in the neural tissue of all the adult animals used
for producing rabies vaccine. Habel, Bell & Wright (1949) and Bell, Wright & Habel
(1949) described a potent AE-free vaccine prepared from neural tissue extracted with
benzine + ether, but there is no record of its use. Our results show that with the excep-
tion of the fluorocarbon Arcon 113 (Gessler, Bender & Parkinson, 1956) the organic
solvents tested either singly or in combination drastically reduced the infectivity of
mouse brain suspensions. In experiments to be reported elsewhere we have shown that
the bulk of the encephalitogenic substance is associated with the Arcon phase when
uninfected brain is extracted.

There are few direct references to systematic experiments on the effect of H+ con-
centrations on rabies virus. It might be expected that the range of stability observed
by us would narrow with increased time and temperature of exposure. Hamparian
(1963) reported rabies virus to be unstable at pH 3.0 and Bailly (1945) that it remained
viable for 24 hr at 10° in solutions buffered with sodium carbonate at pH values of
10, 11 and 12. Inactivation by unbuffered solutions of tetracyclines is solely attribut-
able to exposure at pH values between 3.0 and 4.0 (Kitselman, 1958; Lepine, Pavlanis
Purification involving acid precipitation was described by Behrens et al. (1939) and Tagaya et al. (1953). Removal of protein, however, was accompanied by loss of virus, which was unstable below pH 6.0 and lost infectivity rapidly at pH 5.0. Because rabies virus is unstable below pH 5.0, it is improbable that acid or isoelectric precipitation would be feasible for purification. Stability at the alkaline end of the pH range, however, may be of value since the AE factor in neural tissue is said to become unstable and dialysable at pH 9.0 (Robertson et al. 1962; Caspary & Field, 1965). A knowledge of the range of pH stability may be useful in chromatographic methods of purification.

The pattern of heat inactivation of rabies virus is similar to that of many other animal viruses (Woese, 1960). Its high thermolability, nevertheless, presents a practical problem (van Rooyen & Rhodes, 1948; Mobest, 1958; Okada, 1953), and it is common practice to maintain virus between 0° and 4° during manipulation and to incorporate serum or other protective colloid in suspending media (Mobest, 1959). Brain proteinases were described by Marks & Lajtha (1965) and suspensions of infected brain probably contain tissue enzymes. The loss of infectivity at 37° prevented by normal serum suggested that protection might be due to the presence of enzyme inhibitors in serum. Since no protection of virus was afforded by known enzyme inhibitors it seems unlikely that infectivity is reduced by the action of endogenous enzymes.

Jonnesco (1936) and Bailly (1945) recorded that human and dog pancreatic juice inactivated rabies virus, and attributed inactivation to the trypsin in their extracts. Hirano (1934) found rabies relatively resistant to trypsin but susceptible to lipase, a conclusion also reached by van Rooyen & Rhodes (1948) in reviewing the literature. In our hands, however, trypsin rapidly inactivated purified rabies virus. The moderate inactivation by ribonuclease suggests that little, if any, of the infectivity of virus suspensions exists as free RNA. The effects of phospholipase C suggest that some essential component(s) of the virus structure may be susceptible to its action. Myxoviruses are highly sensitive to inactivation by phospholipase C, but their susceptibility is decreased by host cell components incorporated into the viral envelope; the addition of sphingomyelin increases such resistance (Simpson & Hauser, 1966). Our finding only a moderate effect with phospholipase C could well have been due to such substances derived from brain.

Neurath et al. (1966) published density gradient centrifugation studies on rabies virus while this work was in progress. Our results substantially confirm theirs, although our estimates of sedimentation constant and buoyant density are a little lower. It is interesting to note that myelin-containing material does not readily sediment through sucrose concentrations (Laatsch et al. 1962) that permit the passage of rabies virus.

Chromatography on ECTEOLA-cellulose was clearly less effective in purification and in recovery of infectivity in our hands than in those of Thomas et al. (1965) and Sikes et al. (1966). This discrepancy may be due to our using a different batch of ECTEOLA-cellulose with an exchange capacity (0.35 m-equiv./g.) slightly different from that used by Sikes et al. (1966) (0.32 m-equiv./g.).

The susceptibility of suitably prepared suspensions of fixed rabies virus to dye-sensitized photodynamic action was reported by Shortt & Brooks (1934) and Galloway (1934). Galloway found that virus in unfiltered suspensions containing methylene blue was insusceptible. Photoinactivation by methylene blue may be substantially inhibited by nucleic acid in solution and to a lesser extent by protein (Turner &
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Kaplan, 1965) so that failure to inactivate may not be attributable only to the presence of particulate impurity. Our results confirm that partially purified material is readily inactivated and that the relation of inactivation to time and illumination intensity is similar to that with other viruses.

Almeida & Waterson (1966) stated that rabies virus has not been shown to be capable of haemagglutination and is unlikely to contain neuraminidase. We are not aware of any reports of systematic attempts to demonstrate haemagglutination with rabies virus. Our results, with red cells from several species of animal, tested over a range of pH values and temperature and with a buffer composition that should have provided optimum conditions for agglutination, were negative. Donald & Isaacs (1954) found a ratio of infective dose to haemagglutinin of \(10^6:1\) with the myxoviruses; on this basis our material should have been sufficiently potent. Those structures of rabies virus that readily disintegrate, evident in preparations under the electron microscope, may be those containing a haemagglutinin. Since our experiments with ribonuclease suggest that free RNA is a negligible source of rabies infectivity, it is probable that our highly infective extracts of mouse brain contained considerable amounts of intact rabies virus. Accordingly it may be assumed that our preparations did not agglutinate red cells because they are inherently unable to, rather than for any other reason.

The skilful technical assistance of Miss D. Roberts and Miss J. K. Moore is gratefully acknowledged.

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(Received 15 May 1967)