EXPERIMENTS WITH THE SOLUBLE ANTIGEN OF RABIES IN SUCKLING MOUSE BRAINS

By the late M. van den Ende, A. Polson and G. S. Turner

The C.S.I.R. and U.C.T. Virus Research Unit, Department of Pathology, University of Cape Town (South Africa)

INTRODUCTION

Rabies virus has been successfully adapted to the brains of suckling mice in a number of laboratories. The method has made available material with a high content not only of virus but also of antigen reactive in complement-fixation tests. A non-infective particle which remains in the supernatant fluid of a suspension, from which the virus has been removed as completely as possible by centrifugation, retains a large proportion of the complement-fixing power of the original suspension. This small particle or soluble antigen has been found to be approximately 12 mμ. in diameter (Polson & Wessels, 1953). Suckling mouse brains yield high concentrations of the soluble antigen. The concentration in which it can be obtained, together with its uniformity of particle size, appeared to us to make the rabies soluble antigen a particularly useful one to study with a view to gaining an insight into its nature and significance.

It appears to be important to determine the part which is played by soluble antigen in the immunization of animals against rabies. Experiments have therefore also been undertaken to determine the relative immunizing values of the soluble antigen of virus fractions. The results of preliminary immunological experiments are reported in this paper.

MATERIALS AND METHODS

Virus

The Flury strain of rabies virus adapted to the egg and as used in the so-called avianized virus vaccine was supplied to us by Dr R. A. Alexander of the Onderstepoort Veterinary Research Laboratories. The egg material received had a titre of 10^2 when tested by intracerebral inoculation in doses of 0·03 ml. into 3–4 weeks old mice. Injected intracerebrally into 3–7 days old sucklings it caused death or illness in all the mice in 6 days. It was adapted to suckling mice by serial intracerebral passage. The virus content of suckling brain emulsions, as determined by intracerebral titrations in 3–4 weeks old mice, increased steadily until, after the sixth passage in sucklings, titres as high as 10^8 or more were obtained. The virus content of eggs infected with the egg-adapted strain was significantly lower and in our hands the titre seldom exceeded 10^4 for 3–4 weeks old mice. Titres were calculated according to the method of Reed & Muench (1938).

https://doi.org/10.1017/S002217240003727X Published online by Cambridge University Press

Preparation of sera

Mice. Groups of fifty or more adult mice aged 8 weeks or more were used for the preparation of each serum. For the stock immune serum each mouse received five intraperitoneal injections each of 0·5 ml. of a 10 % emulsion in saline of freshly collected rabies-infected suckling mouse brain. The immunized mice were bled from the heart 10 days after the last injection and the serum removed from the clot of the pooled blood. The serum was inactivated at 56° C. for 36 min. and stored at −20° C.

Soluble antigen and virus fractions for immunization were prepared by differential centrifugation and then heated to 56° C. for 30 min., or inactivated by phenol or formalin in a final concentration of 0·5 or 0·35 %, respectively. The antigens were tested for the absence of active virus by the intracerebral inoculation of the material into groups of six or more 3–4 weeks old mice. They were then incorporated with adjuvant consisting of lanolin, liquid paraffin and *Mycobacterium phlei* similar to that used by Ada, Perry & Pye (1953).

Mice were immunized either by a single subcutaneous injection of 0·25 ml. of the antigen incorporated in adjuvant or by multiple injections. Those receiving multiple doses were given first 0·25 ml. of antigen in adjuvant subcutaneously, followed 10 days later by three intraperitoneal injections of the heated antigen without adjuvant at 3–4 day intervals, and finally another subcutaneous dose of antigen in adjuvant. All mice were bled 10–12 days after the last injection and the serum collected and stored in the usual way.

Rabbits. Each rabbit received 1·0 ml. of the antigen in adjuvant subcutaneously into each flank. The injections of antigen in adjuvant were repeated at monthly intervals until a total of three doses had been given. The rabbits were bled 10–12 days after each immunizing injection.

Preparation of soluble antigen

Groups of fifty to several hundred suckling mice aged 3 days were used. Each mouse received 0·02 ml. of a rabies virus-infected brain suspension intracerebrally. The mice were almost invariably dead or moribund on the third day after injection. The survivors were killed in ether vapour. The brains from the mice which died on the second and third days as well as those killed on the third day were collected aseptically and used immediately or stored until used at −70° C.

The brains were emulsified in M/7·5 phosphate buffer at pH 8·2 by trituration or in a Waring blendor using 1·0 ml. of buffer per brain. This gives an approximately 20 % emulsion (wt./vol.). The emulsion was centrifuged at 2000 r.p.m. for 30 min. to remove gross particulate matter. The supernatant (SNF) was subjected to ultra-centrifugation sufficient to deposit the virus (usually 15,000 to 20,000 r.p.m. for 1 hr. in the Spinco Model L centrifuge).

In most experiments the SNF after ultra-centrifugation, i.e. crude soluble antigen, was subjected to precipitation at pH 4·3. For this purpose the crude soluble antigen was dialysed overnight in a large excess of pH 4·3 citrate buffer at 0–4° C. The precipitate which formed on dialysis was separated by centrifugation

and suspended in the required volume of phosphate buffer at pH 8·2. The undissolved portion of the precipitate was removed by centrifugation at approximately 2000 r.p.m. for 30 min. or at 10,000 r.p.m. for 10 min. or longer.

Complement-fixing antigens were also prepared by saline extraction of brains previously treated with acetone and ether according to the method described by Casals (1949).

Complement-fixation tests

These were carried out by the method described by Casals & Olitsky (1950). The sera were heated at 56° C. for 30 min. before use. Some of the mouse sera required a second heating and sometimes centrifugation at 2000 r.p.m. for 1 hr. to remove their anticomplementary effect. When several samples were compared for the amount of complement-fixing antigen present, they were all tested at the same time with a single dilution of immune serum (usually 1/20).

Titres are recorded as the highest dilution to give approximately 50 % fixation as determined by the degree of haemolysis present in the tubes.

Neutralization tests

Serial dilutions of the serum to be tested were made and to unit volumes of each dilution an equal volume of virus suspension containing 20 or 200 MLD per 0·03 ml. was added. The mixtures were allowed to stand for 30 min. at room temperature and 30 min. at 0–4° C. before each was tested intracerebrally in 0·03 ml. doses in groups of six mice aged 3–4 weeks. The mice were observed daily for 3 weeks for characteristic symptoms and death.

Enzymes

Crystalline trypsin, ribonuclease and deoxyribonuclease were prepared in this laboratory (Polson, 1956). Their enzymic activity was tested on appropriate substrates prepared in the case of ribonuclease (RNAse) and deoxyribonuclease (DNAse) from yeast and thymus, respectively.

Determination of pH stability of soluble antigen

Three sets of tubes were prepared. The tubes of the series each contained 2·0 ml. amounts of McIlvain's buffers of pH 2·0–8·0 or for pH 1·0 a 2·0 ml. volume of 0·1 N-HCl, for pH 10 a 0·2 M-solution of Na_2HPO_4 and for pH 12 a 0·2 M-solution of NaOH. To each tube was added 0·5 ml. of soluble antigen. One series was then held at 0–4° C., another at room temperature and the third at 37° C. for 20 hr. The final pH in each tube was then determined, the solution brought to neutrality by addition of acid or alkali and the volume made up to a total of 5 ml. This corresponds to a dilution of 1/10 of the soluble antigen added. Each sample was then tested for its complement-fixing power in the presence of 1/20 mouse immune serum.

Ultrasonic vibration and freezing and thawing

Material for exposure to ultrasonic vibration was placed in a sac of thin rubber cooled in an ice bath and subjected to vibration at 870–1000 kc./sec. for periods varying from 6 to 36 min.

Alternate freezing and thawing was brought about by the successive immersion of material in a Pyrex tube into a CO_2-alcohol mixture at approximately $-70°$ C. and water at 37° C.

Electrophoresis experiments

Soluble antigen in the form of supernatant fluids separated from suspensions of rabies-infected suckling mouse brains by ultra-centrifugation was dialysed against phosphate buffer of pH 8·2 and ionic strength of 0·1. The suspension was then subjected to electrophoresis and samples were taken from the contents of the electrophoresis tube according to the technique of Polson, Joubert & Haig (1946). Each sample was tested by complement fixation for its soluble antigen content.

RESULTS

The finding that brains of suckling mice infected with the Flury strain of rabies virus contain complement-fixing antigen of such small particle size that it is not deposited by ultra-centrifugation which will remove the virus has been confirmed.

Table 1. *Results of complement-fixation tests with crude rabies-infected brain emulsion, and the fractions separated by centrifugation*

	Volume in relation to original	CF titre	*CF titre* corrected for volume	Virus titre log LD_{50}
(1) Original emulsion	1	400	400	4
(2) SNF 1	1	240	240	1
(3) Pellet 1	0·2	640 (70)	128 (14)	5·1
(4) SNF 2	0·2	280	56	—
(5) Pellet 2 resuspended	0·2	320 (100)	64 (20)	—
(6) SNF 3	0·2	60	12	—
(7) Pellet 3 resuspended	0·2	320 (80)	64 (16)	—
(8) SNF 4	0·2	20	4	1
(9) Pellet 4 resuspended	0·2	280 (100)	56 (20)	5·6
(10) Pellet 4 resuspended and respun at 10,000 r.p.m. for 10 min.	0·2	60 (10)	12 (2)	—

The deposit (Pellet 1) of the first centrifugation was resuspended in one-fifth the original volume, and recentrifuged to give pellet 2 and SNF 2. This process was repeated four times. CF titres are expressed as reciprocals of highest dilution giving approximately 50 % fixation in the presence of 1/20 mouse immune serum. Figures in brackets express the highest dilution of antigen giving 50 % fixation without serum (anti-complementary effect).

The results in Table 1 show that the heavy particles which include practically all the infective virus can be almost entirely separated from the 'soluble antigen' by repeated washing in phosphate buffer. The washed heavy particle fraction, though containing most of the infective virus, appears to have but little specific complement-fixing power.

The virus-containing 'heavy' fraction from which soluble antigen has been almost completely removed by repeated washing was exposed to repeated freezing and thawing or to ultrasonic vibration in attempts to break up the virus. The results recorded in Table 2 show that there was some release of soluble antigen by these methods, but the amount was not such as could have been anticipated if

soluble antigen was a constituent of the virus released in significant amount by its disruption. The small amount of soluble antigen released is more likely to have been derived from tissue components rather than virus in the bulky deposits obtained by centrifugation. There was some evidence that prolonged ultrasonic vibration did reduce the specific fixation by the 'virus' fraction without at the same time releasing significant amounts of soluble antigen into the supernatant fluids.

Table 2. *Results of complement-fixation tests with virus-containing deposit from rabies-infected newborn mouse brains subjected to ultrasonic vibration for 6 or 36 min., or to alternate freezing and thawing*

	Volume in relation to original	CF titre	CF titre corrected for volume
(1) Original	1	1120*	1120*
(2) Soluble antigen	1	480	480
(3) Fifth wash of pellet	1	7	7
(4) Final pellet resuspended†	0·33	70	23
(5) Sample 4. Frozen, thawed and recentrifuged. SNF	0·33	20	7
(6) Sample 4. Frozen, thawed and recentrifuged. Resuspended deposit†	0·33	50	17
(7) Sample 4. U. Vib. and recentrifuged. SNF	0·33	60 (15)‡	20 (5)
(8) Sample 4. U. Vib. and recentrifuged. Resuspended deposit†	0·33	50 (20)	17 (7)

After treatment the suspensions were recentrifuged at 20,000 r.p.m. for 30 min. to separate 'soluble' from virus antigen and the supernatants and deposits tested separately.

* Antigen anticomplementary to dilution of 1/80.

† The resuspended deposits were centrifuged at 10,000 r.p.m. for 10 min. in an attempt to reduce their anticomplementary power.

‡ Figures in brackets obtained after 36 min. exposure to ultrasonic vibration.

U. Vib., subjected to ultrasonic vibration.

Titrations of the infectivity and specific complement-fixing power of ultracentrifuged samples of brain suspensions from groups of suckling mice killed at different times after a single intracerebral injection of the virus have revealed that the rise in infectivity precedes the first appearance of soluble antigen. Soluble antigen, however, continues to increase after the maximum titre of infectivity has been reached (Table 3). The differences in the rate of production may depend simply on the relative differences in the sensitivity of the test methods, but they nevertheless appear to contrast with those obtained with influenza virus-infected tissues in which detectable soluble antigen appears before haemagglutinin or infective virus.

Investigations undertaken to elucidate the properties of soluble antigen have revealed that it is fairly stable at pH 6·0–10·0 (Table 4). When dialysed against buffer at pH 4·3 it is completely precipitated, but only one-fifth to one-half of the original soluble antigen can be recovered from the precipitate (Table 5). Repeated

Table 3. *Showing the increase in infective virus and complement-fixing antigen in the brains of suckling mice infected intracerebrally*

Time (hr.)	Virus titre	Soluble antigen
Zero	3·2	< 5
14	1·1	< 5
24	3·0	< 5
39	3·5	7
48	5·0	60
62	5·9	120
72	6·1	240

Soluble antigen: reciprocal of highest dilution of supernatant fluid of brain suspension centrifuged at 20,000 r.p.m. for 1 hr. which shows 50 % fixation in the presence of 1/20 mouse immune serum.

Virus titre: titre of 20 % brain suspension in log LD_{50} tested in 3–4 weeks old mice.

The brains of ten sucklings were used for each sample.

Table 4. *Showing the stability of rabies soluble antigen when exposed for* 20 *hr. to different pH's at different temperatures*

pH	0–4° C.	R.T.	37° C.
1·0	< 10	< 10	< 10
2·0	80 AC	35 AC	20 AC
6·0	240	280	240
8·0	320	320	280
10·0	280	240	120
12·0	40 AC	20 AC	15 AC
Control	320		

Titres are expressed as reciprocals of highest dilutions giving approximately 50 % fixation in the presence of 1/20 mouse immune serum.

AC, anticomplementary.

Table 5. *Showing the result of complement-fixation tests on soluble antigen separated by centrifugation and subjected to precipitation at* pH 4·3 *and dialysis*

	Volume relative to original	CF titre	CF titre corrected for volume	Virus titre
(1) Original emulsion	1	2560	2560	7·3
(2) SNF 1	1	800	800	3·4
(3) SNF 2 (SNF 1 recentrifuged)	1	640	640	2·0
(4) SNF 2, precipitated at pH 4·3 and redissolved	0·25	850	212	—
(5) SNF from pH 4·3 precipitation	1	0	0	—
(6) Sample 4, ether-extracted	0·25	320	80	—
(7) Sample 6, dialysed	0·25	320	80	—

Virus titre expressed as log LD_{50} for 3–4 weeks old mice.

https://doi.org/10.1017/S002217240003727X Published online by Cambridge University Press

extraction with ether, as in the experiment from which the result is recorded, usually resulted in a significant loss of soluble antigen.

Repeated extraction of soluble antigen in phosphate buffer at pH 8·2 by chloroform results in complete removal of protein and of soluble antigen detectable by complement fixation.

The action of crystalline trypsin, ribonuclease and deoxyribonuclease all resulted, during a period of 1–4 hr. incubation, in reduction of complement-fixing power of the antigen to approximately two-thirds or half of the original. A typical result, such as recorded in table 6, shows that tryptic activity under the conditions

Table 6. *The effect of tryptic digestion on soluble antigen*

	C.F.T.*	Protein† mg. %
Soluble antigen – control	240	225
Soluble antigen + trypsin 0 min. + trypsin inhibitor	240	500
Soluble antigen + trypsin for 30 min. + trypsin inhibitor	200	350
Soluble antigen + trypsin for 1 hr. + trypsin inhibitor	140	350
Soluble antigen + trypsin for 2 hr. + trypsin inhibitor	140	225
Soluble antigen + trypsin for 4 hr. + trypsin inhibitor	140	225
Soluble antigen + trypsin for 6 hr. + trypsin inhibitor	140	225

The soluble antigen was prepared by centrifugation, precipitation at pH 4·3 and re-solution in pH 8·2 buffer. It was treated with 2 mg. crystalline trypsin per ml. for ½–6 hr. after which soya bean trypsin inhibitor was added to all tubes.

* C.F.T., complement-fixing titre as reciprocal of the highest dilution giving approximately 50 % fixation in the presence of 1/20 mouse immune serum.

† Protein determinations made by turbidimetric method.

of the experiment was complete in 2 hr., and reduction in complement-fixing power occurred in the same period. A sample of soluble antigen treated successively with ribonuclease overnight and trypsin for 2 hr. was compared for particle size with an untreated sample by ultra-centrifugal analysis. The enzyme action had reduced its complement-fixing power by 75 %, but the residue appeared to be of the same particle size as the original antigen.

The addition of 66 % ammonium sulphate to material which had been treated with ribonuclease for 18 hr., trypsin for 2 hr. and dialysis against distilled water in the cold for 2 days, resulted in a precipitate which contained the soluble antigen. Treatment of the precipitate with phosphate buffer at pH 8·2 resulted in the recovery of approximately one-half of the soluble antigen, but the material was slightly anticomplementary.

The soluble antigen withstands heating at 56° C. for 30 min. without loss of *in vitro* serological activity. It is partially inactivated at 65° C. for 30 min. and completely inactivated when held at 80° C. for 30 min. It maintains its activity in the frozen state (– 20° C.) for 1 month or longer, but gradually deteriorates on storage at 0–4° C. It is not significantly affected by 0·35 % formaldehyde, 0·5 % phenol or 1/10,000 merthiolate. By electrophoretic analysis the soluble antigen appears, under conditions of our experiments, to be a single component with a mobility, at pH 8·2, equal to that of haemoglobin.

Table 7. *Results of complement-fixation and neutralization tests with sera of rabbits immunized against various antigens prepared from rabies-infected infant mouse brains*

		Single immunizing dose			Multiple doses of antigen		
Rabbit no.	Antigen used for immunization	C.F.T. versus rabies soluble antigen	C.F.T. versus normal brain antigen	Neutralization titre	C.F.T. versus rabies soluble antigen	C.F.T. versus normal brain antigen	Neutralization titre
640	Heated rabies soluble antigen	30	30	<3·2	1920	480	<3·2
669		50	30		960	240	
681		60	30		960	240	
666	Heated rabies 'virus'	30	40	<3·2	640	400	<3·2
618		40	40		480	400	
659		60	60		*	*	
663	Normal brain antigen	30	40	<3·2	60	60	—
604		15	30		100	100	—
680		40	60		240	240	—
53	Living virus	—	—	—	70–120	50–130	±3000
57							
58							
59	Heated rabies soluble antigen (without adjuvant)	—	—	—	120–200	120–240	<3·2
72							
73							
Stock mouse serum†		—	—	—	480	<5	>320 (±5000–10,000‡)

* Not available as rabbit died from intercurrent disease.

† Stock mouse serum: serum from mice immunized by repeated intraperitoneal injection of fresh rabies-infected suckling mouse brains containing living virus.

‡ Titre obtained in subsequent tests to determine approximate end point.

C.F.T., reciprocals of highest dilution of serum giving approximately 50 % fixation in the presence of a single concentration of antigen.

Neutralization titre: reciprocal of highest dilution of serum protecting against 10 LD_{50} of virus injected intracerebrally into 3–4 weeks old mice.

The results of experiments to determine the antigenic potency of 'soluble antigen' and 'virus' fractions in rabbits and mice are summarized in Tables 7, 8 and 9. The soluble antigen for immunization of the rabbits was prepared by centrifugation

Table 8. *Results of complement-fixation and neutralization tests with sera of mice immunized against heated or phenolized rabies antigens from suckling mouse brains*

	Complement fixation with		
Antisera prepared against	Rabies soluble antigen	'Normal' soluble antigen	Neutralization titre
Rabies soluble antigen phenolized	160	—	> 320
Rabies 'virus' phenolized	320	—	320
Rabies soluble antigen—heated	160 (15)*	< 5	< 3·2
Rabies 'virus'—heated	240 (< 10)	< 5	6
Antigen from normal brains	< 5	< 5	< 3·2
Stock mouse serum†	800	< 5	> 320

* Figures in brackets are values obtained in mice which had received a single immunizing injection in adjuvant.

† Stock mouse serum: serum from mice immunized by repeated intraperitoneal injections of fresh rabies-infected suckling mouse brains.

Rabies 'virus': fraction separated by ultra-centrifugation, and repeatedly washed in phosphate buffer.

Rabies soluble antigen: supernatant fluid from emulsion of rabies-infected suckling mouse brains.

Antigen from normal brains: supernatant fluid from emulsion of uninfected normal suckling mouse brains.

Neutralization titre: reciprocal of highest dilution of serum giving approximately 50 % mortality in the presence of 100 LD_{50} of virus injected intracerebrally into 3–4 weeks old mice.

Complement-fixation titres expressed as reciprocals of the highest dilutions giving approximately 50 % fixation in the presence of a single concentration of antigen.

Table 9. *Results of complement-fixation and neutralization tests with sera of mice immunized against antigens prepared from rabies soluble antigens*

Antisera prepared against	C.F.T.	Neutralization titre
Rabies soluble antigen phenolized	60	50
Rabies soluble antigen heated	120	32
Rabies soluble antigen formalinized	60	32
Rabies soluble antigen untreated	80	120
Stock mouse serum	960	± 6000

C.F.T.: reciprocal of highest dilution giving approximately 50 % fixation in the presence of a single concentration of antigen.

Neutralization titre: reciprocal of highest dilution of serum protecting against 20 LD_{50} of virus injected intracerebrally into 3–4 weeks old mice.

and precipitation at pH 4·3 from an emulsion of suckling mouse brains in pH 8·2 buffer. The material fixed complement to a dilution of 1/2560 in the presence of 1/20 mouse immune serum. As attempts to free the material of virus particles by filtration without greatly reducing the soluble antigen content failed, the small

amount of residual virus present was destroyed by heating at 56° C. for 36 min. or by treatment with formaldehyde or phenol. A similar extract was prepared from the brains of normal suckling mice of similar age to those used for the preparation of the rabies soluble antigen. A suspension containing predominantly virus particles was obtained from rabies-infected suckling mouse brains by centrifugation and the repeated washing of the deposit in phosphate buffer. The suspension immediately after preparation had an infective titre of $10^{5\cdot2}$ when titrated in 3–4 weeks old mice. The suspension was heated at 56° C. for 30 min. and tested for viable virus in 3–4 weeks old mice. No virus appeared to be present although death resulted within 48 hr. of about 50 % of the mice receiving undiluted material intracerebrally. This short incubation and the symptoms shown were not typical of those brought about by the strain of rabies virus used. A ten-fold dilution of the suspension elicited no symptoms. The method of immunization employed has already been outlined.

The results of complement-fixation tests with the different bleedings cannot be compared on any exact quantitative basis, as tests carried out at different times using different reagents (complement, soluble antigen, etc.) show unavoidable variations. They can, however, be compared with the stock immune mouse serum which was used as a reference.

The results recorded in Table 7 do show that in rabbits heated soluble antigen and virus are each capable of stimulating the formation of complement-fixing antibody, and that a significant part of the antibodies are specific for the soluble antigen as distinct from species-specific brain antigens. The soluble antigen fraction appears to elicit more complement-fixing antibody than the preparation containing predominantly virus. The complement-fixing antibodies elicited by the heated antigens, are, however, devoid of *in vivo* neutralizing power. In a separate experiment the sera of the rabbits immunized by the injection of multiple doses of unheated soluble antigen containing a detectable amount of residual live virus showed the presence both of complement-fixing and of neutralizing antibody.

Fresh preparations of soluble antigen and washed 'virus' antigen were used for the immunization of mice. The results obtained with the sera from groups of mice immunized against a number of differently prepared antigens show considerable variation. As in rabbits the heated antigens stimulate the formation of complement-fixing antibody. In the experiment of which the results are recorded in Table 8 antigens were prepared from a brain suspension with an original infective titre of $10^{6\cdot2}$, whilst the soluble antigen fraction had an infective titre of $10^{1\cdot0}$ before treatment by heat or phenol and fixed complement to a dilution of 1/1600. Phenol appeared in this experiment to preserve the ability to evoke neutralizing antibody, whilst heating seriously reduced this property of the antigen. Further experiments have, however, shown that the antibody response in mice to antigens containing no detectable living virus, and in particular the amount of neutralizing antibody elicited, may be scanty. Thus in experiments in which soluble antigens treated by heat, 0·5 % phenol or 0·35 % formaldehyde were used all three groups developed comparable, but relatively low titres of both complement-fixing and neutralizing antibody (Table 9). The soluble antigen content of the material used

for immunization was, as judged by complement fixation with stock immune mouse serum, comparable to that used in previous experiments.

Several of the immune sera were tested for the presence of precipitins by Oakley & Fulthorpe's (1953) modification of Oudin's method. The sera of three rabbits immunized against rabies soluble antigen each gave rise to three or more rings when tested with a rabies soluble antigen preparation. The sera of rabbits immunized with an extract from normal suckling mouse brains prepared in identical manner to the soluble antigen of rabies gave fewer and feebler rings with rabies soluble antigen. None of the rings with the latter series of sera was as marked as the densest ring with the first sera. None of the mouse sera, including that from animals which had received multiple doses of living virus intraperitoneally, and which contained abundant complement-fixing, as well as neutralizing, antibody showed the presence of precipitin when tested with the soluble antigen preparations as used in these experiments.

In a separate investigation Mead (1956) has obtained soluble antigen of greater concentration and purity which gives a single specific zone of precipitation with mouse immune serum.

Attempts have been made to demonstrate anaphylaxis *in vitro* with uterine muscle from guinea-pigs passively sensitized with the serum from rabbits immunized against heated soluble antigen. These, though suggesting the presence of a specific antigen in extracts of rabies-infected brains other than those present in similar extracts from normal brains, have not been decisive because of the difficulty of removing substances from the brain extracts which themselves have a smooth-muscle stimulating action.

DISCUSSION

The complement-fixing power of the soluble antigen of rabies, present in brains of suckling mice infected with this virus, resists heating at 56° C. for 30 min. and it is not completely destroyed by trypsin, RNAse or DNAse. It is relatively stable over a pH range of 6–10. The fact that it can be precipitated at pH 4·3 offers a useful method for its concentration, even though only part of the soluble antigen can be recovered from the precipitate. Its rate of production in brain appears to correspond to the production of virus. In this respect it appears to differ from the soluble antigen of influenza which appears before haemagglutinin or infective virus. This difference may, however, only depend on differences in the amounts of soluble antigen produced relative to virus, and on the relative insensitivity of the tests we have employed.

The significance of the soluble antigen remains in doubt. It does not increase significantly in suspensions of virus subjected to ultrasonic vibration or repeated freezing and thawing. This suggests, though it does not prove, that it is not an essential constituent of the virus. It may also mean that soluble antigen is present in infected brains in amounts far greater than is required for virus synthesis.

Attempts to obtain higher concentrations of soluble antigen by preliminary treatment of brain suspensions by methods designed to concentrate the inclusion

bodies present (Moulton, 1954) and subsequent extraction with dilute alkalis have failed. We have also not succeeded in determining the intracellular position of soluble antigen by the use of fluorescent antibodies.

Although in our experiments it has not been possible to separate completely the soluble antigen from virus particles, the results of immunization experiments with partially purified antigen suggest that soluble antigen is itself antigenic. Thus soluble antigen preparations, in which small amounts of residual live virus have been killed by heated or phenol, elicit complement-fixing, but not neutralizing, antibodies in rabbits. Similar antigens in mice have also evoked complement-fixing antibody, but the results in the latter species differ significantly from those in the former in that mice can also produce neutralizing antibodies under the conditions of the experiments. It appears not unlikely that the material used for immunization contained traces of virus, which though modified and not detectable by ordinary tests for infectivity, are still capable of limited multiplication in the more susceptible of the two species of animal employed. It is, therefore, unlikely that the soluble antigen in spite of its antigenicity will be of significance in protection against infection. Preparations from virus-infected brain material, freed as effectively as possible from soluble antigen, but containing most of the virus originally present, were after heat or antiseptic treatment no more effective in stimulating the formation of neutralizing antibody than the soluble antigen fractions. This may simply mean that an insufficient concentration and purification of virus has been achieved to act after inactivation as an effective antigenic stimulus. Our results do confirm in a very striking manner that inapparent infection, such as follows the intraperitoneal infection of mice, is a very effective stimulus for the production of antibodies detectable by complement fixation and neutralization tests.

Ozawa (1954) has undertaken immunization experiments with merthiolated antigens prepared from rabies-infected guinea-pig or mouse brains. His soluble antigen appeared to stimulate little formation of either complement-fixing or neutralizing antibody. The soluble antigen content of his preparations appeared, however, to be significantly lower than those of the majority of ours and our results are therefore probably not comparable.

Further efforts at the identification of soluble antigen and the elucidation of its significance must await improved methods for its concentration and purification.

SUMMARY

A study has been made of the properties of soluble antigen in the brains of infant mice infected intracerebrally with the Flury strain of rabies virus.

Soluble antigen is produced at the same time as infective virus, and reaches a high concentration in a period of 2–3 days.

It can be partially purified by precipitation at pH 4·3. It is partially resistant to the action of trypsin, RNAse and DNAse. It is relatively stable at pH 6–10.

Experimental results suggest that the soluble antigen remains antigenically active after heating at 56° C. and treatment with 0·5% phenol or 0·35% formal-

dehyde, but that such heating markedly reduces the ability to stimulate formation of neutralizing antibody.

Rabbits and mice appear to differ in the production of neutralizing antibody following immunization against soluble antigen in which residual live virus was inactivated by heat, phenol or formaldehyde.

It is suggested that this difference may depend on the different susceptibility to traces of incompletely inactivated virus remaining in the immunizing antigens.

The authors are grateful to Miss T. Madsen for her assistance in some aspects of this work. Dr N. Sapeika kindly made available facilities for the *in vitro* anaphylaxis experiments.

Financial assistance was received from the Nkana-Kitwe and Chingola Poliomyelitis Research Funds.

REFERENCES

Ada, G. L., Perry, B. T. & Pye, J. (1953). *Aust. J. exp. Biol. med. Sci.* **31**, 391–404.
Casals, J. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 339.
Casals, J. & Olitsky, P. K. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 315.
Mead, T. H. (1956). Personal communication.
Moulton, J. E. (1954). *Amer. J. Path.* **30**, 533.
Oakley, C. L. & Fulthorpe, A. J. (1953). *J. Path. Bact.* **65**, 49.
Polson, A. (1956). *Biochim. biophys. Acta*, **22**, 61.
Polson, A., Joubert, J. J. & Haig, D. A. (1946). *Biochem. J.* **40**, 265.
Polson, A. & Wessels, P. (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 317–20.
Reed, L. J. & Muench, H. (1938). *Amer. J. Hyg.* **27**, 493.
Ozawa, Y. (1954). Personal communication.

(*MS. received for publication* 13. xii. 56)