4. Grid titration

The best conditions for each virus-antiserum system must be determined experimentally by varying the concentrations of reactants. The chequer board system used to determine these conditions is best presented diagramatically.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

lines 2,3,4 : use 10 µg/ml coating IgG
lines 5,6,7 : use 1 µg/ml coating IgG
lines 8,9,10: use 0,1 µg/ml coating IgG

line A : use concentrated sap or 1/10 pure virus (1 mg/ml)
line B : use 1/10 sap or 1/100 pure virus
line C : use 1/100 sap or 1/1000 pure virus
line D : use 1/1000 sap or 1/10000 pure virus

lines 2,5,8 :use 1/200 dilution of conjugate
lines 3,6,9 : use 1/800 dilution of conjugate
lines 4,7,10: use 1/3200 dilution of conjugate

lines E and F: are used as controls for healthy sap diluted 1/10 and 1/100 respectively in PBS-Tween 20

lines G and H: are used as controls of PBS-Tween 20 only

5. Ouchterlony plates (Ziemiecki and Wood, 1975; Purcifull and Batchelor, 1977; Whitlock (pers. comm.)).

3% SDS in M/15 phosphate buffered saline, pH 7,2

buffer - M/15 phosphate buffered saline

agar - Ionagar 2 or agarose (0,22g) in 13 mls of distilled water and 13 mls of phosphate buffered saline, pH 7,2

Appendix IV

Electrophoresis

1. Immunoelectrophoresis (Hudson and Hay, 1980).

a) Materials:

1. Barbitone buffer pH 8,2
   Dissolve 12 g sodium barbitol in 800 mls gd H_2O
   Dissolve 4, 4 g barbitone in 150 mls gd H_2O then heat to 95°C
   Mix the two solutions and adjust the pH with 5 N NaOH. Add 0,15 g sodium azide and adjust the final volume to 1 litre

2. Precoated slides:
   Clean several slides with methanol. Dissolve 0,5 g of Oxoid # 1 agar in 100 mls of gd H_2O, Heat to boiling. Smear on slides with a paint brush to form a thin coat and allow to dry.

3. 2% agarose in barbitone buffer:
   Dissolve 2g of agarose in 50 mls of gd H_2O
   Add 50 mls of hot barbitone buffer. Mix well.
   Pipette 3-4 mls of hot agar onto each slide.

b) Methods:

Wells and troughs were cut into the agar but only the agar from the wells is initially removed. A few drops of purified IgG at a concentration of 1 mg/ml, was mixed with a drop of Bromophenol blue tracking dye. Electrophoresis is carried out at 5 milliamps per gel for 1/2 an hour or until the dye reaches the end of the slide. The agar from the troughs was then removed with a pasteur pipette and concentrated goat anti rabbit serum or IgG only was placed in the trough. Slides were left in the humidity chamber overnight to precipitate. Slides were washed in physiological saline for 2 hours, then left in

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glass distilled water overnight. They were stained in 0,2 % Coomassie blue for 2 minutes and destained in a mixture of 45 % methanol 1 % glacial acetic acid and 45 % water until the agarose background became clear.

An ultrasensitive staining method for proteins in polyacylamide gels was also attempted (Merril et al., 1981).

2. Protein gel electrophoresis

1. Gels:

	8 %	7 %
Acrylamide: Bis (30,0g : 0,8 g)	28,0 ml	34,0 ml
1 M Tris, pH 8,8	45,0	60,0
10 % SDS in gd H_2O	1,2	1,6
gd H_2O	39,7	33,0
Degas		
Then add		
1,5 % A.P.	6,0 ml	8,0 ml
TEMED	30,0 ul	40,0 ul
Total volume	120,0 ml	160,0 ml

Gels were pre-electrophoresed overnight at 20 milliamps per gel.

2. Splitting solution:

8 M Urea	4,8 g
2 % SDS	0,1 g
2 % 2-mercaptoethanol	0,2 ml
Distilled water	10,0 ml

A few drops were added to purified virus samples. In the case of marker protein, 100 ul was added per vial of powder. Only 12 ul however need be applied to the gel. All samples must be heated in a boiling water bath before being applied to the gel.

3. Bath buffer:

Tris	15,15 g
Glycine	72,03 g
SDS	5,0 g

dissolved in 5 litres of distilled water

4. Fixative:

Use 15 % TCA in water. Fix overnight.

Proteins on gels were also fixed in a solution of 50 % methanol plus 12 % acetic acid for at least 20 minutes. Excess lauryl sulphate was removed by rinsing the gels in three 200 ml 10 minute washes in a solution containing 10% ethanol and 5% acetic acid (Merril et al., 1981.

5. Stain:

Gels were stained overnight at 37°C with continuous swirling.

45 % methanol
10 % glacial acetic acid
0,25 % Coomassie or Kenacid blue

The dye was dissolved in a small quantity of methanol then filtered. The remaining methanol, water and acetic acid was then added. Gels were also stained using the following method:
soaked for 5 minutes in 0,0034 M potassium dichromate and 0,0032 N nitric acid, washed 4 times in deionized water then placed in 0,012 M silver nitrate for 30 minutes. Gels were rinsed with image developer solution containing 0,28 M sodium carbonate and 0,5 ml of formalin per litre. The gels were agitated until the image had reached the desired intensity. Development was stopped by discarding the developer and adding 1 % acetic acid. Gels were washed in distilled water before being stored.

6. Destaining of gels:
3 litres of 45 % methanol and 7 % acetic acid in water were used to destain gels over a period of 1½ hours.

3. RNA gel electrophoresis

1. Preparation of discontinuous gels (Petri, 1972):

Two stock aqueous acrylamide solutions are made:

(w/v) Solution A: 15 % acrylamide
0,75 % bisacrylamide

(w/v) Solution B: 15 % acrylamide
0,35 % bisacrylamide

Each solution must be filtered and is kept at 4°C
Gels were made in 8 cms x 8 cms cassettes with well formers placed at the top. The discontinuous gel sections were made by mixing the following components:

	6,8% lower gel	2,4% upper gel
Stock acrylamide sol.	11,4 (B) ml	4,8 (A) ml
5 x conc. E. buffer	5,0	6,0
Deionized water	8,6	18,2
TEMED	10,0 ul	12,0 ul
10 % fresh A.P.	250,0 ul	300,0 ul
	25,260 ml	30,312 ml

The first three components were mixed and heated to 25°C in a water bath then degased under vacuum. Immediately before using the gel, the final two components were added. The solutions were mixed with the minimum of aeration before being poured into the cassette moulds.
The upper gel was made first and the cassette was filled to a height of 4 cms. The gel was polymerized completely before the addition of the 6,8 % lower

gel. After polymerization, water was placed on the gel until used. Gels were pre-electrophoresed for 4 hours before sample application.

2. Running buffer:

1 x E buffer containing 0,2% SDS.

4. RNA extraction (Perry et al., 1972).

a) Materials:

All glassware must be rinsed with D.P. and autoclaved, all buffers must contain 0,2% D.P. and be autoclaved before use.

1. Resuspension buffer for virus: NETS buffer

0,1 M NaCl
10 mM Tris, pH 7,4
1 mM EDTA
0,2% SDS

2. Phenol : chloroform cocktail:

To redistill phenol, heat to melting before adding an equal volume of chloroform i.e. use 50mls phenol + 50 mls chloroform. Add an equal volume of NAE buffer (100 mls). Mix with a magnetic stirrer and leave to settle out into the two phases: Never mix the cocktail and aspirate only the lower phase for RNA extraction.

NAE buffer:
10 mM NaAc
0,1 M NaCl
1 mM EDTA

3. E Buffer:

   36 mM Tris
   30 mM NaH_2PO_4
   1 mM EDTA (disodium salt)
   pH 7,7 to 7,8 at room temperature

4. RNA resuspension buffer:

   E buffer containing 0,2 % SDS and 6 % sucrose

5. Escherichia coli commercial preparation:

   2 ul stock E. coli purified prep.
   5 ul P.P. plus gd water
   2 ul 1 x E buffer and sucrose
   1 ul dye

6. Ethedium bromide stain:

   Make up a stock 5 mg/ml water solution. Dilute the stock 0,1 ml/litre of water for staining of gels.

b) Methods:

1. Preparation of Escherichia coli standard

   Inoculate 250 mls of nutrient broth with E. coli; leave for 48 hours at 37°C, then centrifuge at 3,000 RPM for 15 minutes to precipitate the bacteria. Pipette the precipitate into a glass homogenizer and 0,5 mls of resuspension buffer. E. coli was disrupted by 20 strokes of the plunger. RNA from the resultant mixture was separated using the same procedure as for virus.

2. RNA extraction - Method A.

   The procedure is best represented by the following flow diagram -

resuspend virus or E. coli in 200 µl of NETS buffer

add an equal volume of phenol:chloroform cocktail

vortex, then allow to settle on ice for 10 - 15 minutes

centrifuge 10,000 RPM for 5 mins at 2°C

remove the top aqueous phase. To the remaining organic phase and the interface add 200 µl of NETS buffer again. Re-extract RNA

vortex, allow to settle on ice again and centrifuge

combine both aqueous phases and precipitate out the RNA

At this stage the preparation can be scanned for the presence of RNA to determine yield

before the precipitation of RNA, the remaining phenol must be extracted with ether

use 2-3 times volume of pure diethyl ether

invert the eppindorf tube 7 times exactly

centrifuge the emulsion at 10,000 RPM for 2-3 minutes. Keep the precipitate

repeat the procedure at least 5 times until the suspension is clear of phenol

RNA is precipitated by adding first 1/10 volume 3 M NaAc then 2 volumes of freezing (-70°C) absolute ethanol

Invert the eppindorf tube 7 times, freeze at -70°C until solid

centrifuge 2-3 minutes at 8,000 RPM

Wash the precipitate with freezing ethanol and shake the contents of the tube. Do not mix

centrifuge at 8-10,000 RPM

dissolve the RNA precipitate in E buffer with SDS and 6 % sucrose.

3. RNA extraction - Method B

The rapid microextraction procedure of Peacock and Bunting (1971) was followed.

a) Materials:

i) 90 % water saturated phenol:

Melt 25 g crystals in a beaker at 60°C. Weigh 22,5 g of this hot phenol into a measuring cylinder and make up to 100 mls with glass-distilled water.

ii) Peacock's buffer pH 8,3 (10 times concentrated)

1,08 g TRIS
0,55 g Boric acid
0,093 Tritriplex
10 mls gd H_3O

mix and filter before use

iii) 2 % SDS

iv) 0,5 % SDS in Peacocks' buffer
1,25 ml aqueous SDS
0,5 mls of 10 times concentrated Peacocks' buffer
3,25 mls gd H_2O

mix and chill at 4°C before use

b) Methods:

Virus or Escherichia coli was pelleted and resuspended in SDS/Peacocks' buffer. (At this stage E. coli cells are homogenized on ice). An equal volume of water saturated phenol is then added and the components were mixed for 45 seconds. Centrifuge at 10,000 RPM for 10 minutes. The aqueous phase

was ether treated without centrifugation but precipitation with alcohol and acetate was carried out as for Method A. The method is supposed to reduce RNA breakage hence denaturation, thereby providing a greater yield of RNA.

Further methods for RNA extraction were tested such as mixing equal volumes of virus and a solution of 10 percent sucrose and 1 percent SDS (dissolved in 100 mls water) then heating the sample at 70°C for 6 minutes.

4. Visualization of RNA: preparation of RNA for transmission electron microscopy (TEM) (Evenson, 1974).

a) Materials:

i) Hypophase:

1 M TRIS pH 8,5	1 ml
0,1 M EDTA pH 8,5	1 ml
Formamide	10 ml
distilled water	88 ml

ii) Spreading medium for RNA:

Formamide	25 ul
1 M TRIS pH 8,5	5 ul
0,1 M EDTA pH 8,5	5 ul
RNA pure prep.	5 ul
Cytochrome C (1 ug/ml)	5 ul

Prepare immediately before use.

b) Methods:

Pour the hypophase into a black trough forming a good meniscus. Clean the surface with a teflon bar. Rinse a very clean slide with glass distilled water and a little pypophase and place as a ramp in the trough. Scatter talcum powder on the surface of the hypophase. If it is clean, the talcum powder should spread out into a thin film. Combine the

components for spreading the RNA. Slowly pipette a drop of this mixture onto the slide, a few millimetres above the hypophase surface. The drop must run down the slide onto the hypophase surface and spread out, displacing the powder. Touch a formvar coated grid in a central undisturbed area of the drop. Dry the grid before rotary shadowing.