WESSELSBRON VIRUS

A biophysical, biochemical and

serological study.

by

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1. - SUMMARY

The application of a variety of virological techniques to the study of Wesselsbron virus has resulted in the compiling of much worthwhile information on the biophysical, biochemical and serological properties of this virus.

Wesselsbron virus, like most arboviruses, showed marked sensitivity to the action of both ether and sodium deoxycholate.

The presence of a haemagglutinin in suspensions of Wesselsbron virus, propagated in suckling mouse brains, was confirmed, and this was found to react optimally with goose red cells within a pH range 6.1 - 6.3. The haemagglutinin present in the fluid of cultures of virus-infected foetal lamb kidney cells haemagglutinated optimally at pH 5.9. Suspensions of Wesselsbron virus, which had been repeatedly passaged in foetal lamb kidney cells, showed severe limitation of haemagglutinin activity or failed to haemagglutinate at all. The haemagglutination inhibition test established the specificity of the reaction and demonstrated a sharing of antigens between Wesselsbron virus and another Group B arbovirus.

Zone electrophoresis in a sucrose density gradient revealed the presence

of a main infective component of relatively high mobility. The haemagglutinin appeared as two widely separable components, one associated with the whole virus and another of very low electrophoretic mobility.

The buoyant density of the virus particle was determined by density gradient centrifugation and found to be 1.22 gm/cc, a figure lower than that for many other viruses. The density of the haemagglutinin proved to be of the same order lying between 1.189 and 1.327 gm/cc.

Ultracentrifugation studies indicated a sedimentation coefficient of 144.05 S for the infective particle and 72.11 S for the haemagglutinin. The diameter of the infective particle was calculated from these density and sedimentation coefficient data to be 34.3 mμ.

Support for this particle size estimation was derived from ultrafiltration experiments in which the infectious particle behaved as one having a diameter of 33.3 mμ and it was held back by a membrane with an APD of 62 mμ. The vast majority of the haemagglutinin was held back by membranes with an average pore diameter of 222 mμ.

The attempts to isolate infectious nucleic acid from the whole virus

particles were successful. The fact that this infectious material was completely inactivated by ribonuclease established Wesselsbron virus as one of the RNA viruses. This assumption was supported by the early appearance and development of Wesselsbron virus antigens in the cytoplasm of the infected cells demonstrated by the fluorescent antibody technique.

Studies of Wesselsbron virus infected foetal lamb kidney cells by electron microscopy, emphasised the cytoplasm as the site of virus synthesis. Remarkable and apparently unique orderly patterned inclusions were demonstrated in the cytoplasm of infected cells. Whole virus particles measuring approximately 34 mμ in diameter were observed scattered or in clusters in cisterns of the endoplasmic reticulum.

II - INTRODUCTION

In late summer 1954/55, a hitherto unknown virus was isolated in the Wesselsbron area of the Orange Free State, in the Republic of South Africa, following an outbreak of disease in a flock of sheep (Weiss, Haig and Alexander 1956). The clinical symptoms resembled those of Rift Valley Fever, but the infected sheep had been immunized against this disease two weeks previously. The virus was isolated from the brain of an eight day old lamb that had succumbed during the outbreak, and was subsequently called "Wesselsbron" virus, strain van Tonder, but now internationally known as Wesselsbron virus.

Weiss et al (1956) described a typical clinical picture of a febrile reaction in adult sheep accompanied by a low mortality rate, but a 100% death rate among foetuses carried to full term and in new born lambs.

Belonje (1958) however, in describing an outbreak of Wesselsbron virus disease in the Middelburg area (Cape), maintained that sheep of all ages were susceptible, and that the mortality rate even in the subacute form of the disease was 75%.

It was found in the laboratory, that infant and adult mice were susceptible to virus infection by the intracerebral route while mice less than

ten days old were also susceptible to intraperitoneal injection. Reports indicate that guinea-pigs, rabbits, cattle, horses, pigs and embryonated hens' eggs may be successfully infected with Wesselsbron virus (Weiss *et al* 1956).

The virus affects man causing an influenza-like illness characterized by fever, headache, severe muscular pain and prostration (Weiss *et al* 1956). A case of a naturally infected human being was reported by Smithburn, Kokernot, Weinbren and De Meillon (1957). Two additional cases were reported by Heymann, Kokernot and De Meillon (1958) among personnel undertaking field investigations during the Middelburg outbreak in 1957. Serological tests confirmed Wesselsbron virus to be the causative agent.

Antibodies to Wesselsbron virus have been found in the sera of human beings (Smithburn *et al* 1959), and in the sera of domestic quadrupeds (Kokernot, Smithburn and Kluge 1961) in Northern Natal. Kokernot, Szlamp, Levitt and McIntosh (1965) conducted a serological study amongst humans in the Caprivi Strip and Bechuanaland Protectorate in 1959, and found that 40% of the sera tested had neutralizing antibodies to Wesselsbron virus.

Wesselsbron virus is transmitted principally by *Aedes (Banksinella) circumluteolus* (Theo.) mosquitoes (Muspratt, Smithburn, Paterson and Kokernot 1957), and by *Aedes (Ochlerotatus) caballus* (Theo.) (Kokernot, Paterson and De Meillon 1958). Vectors of minor importance are *Aedes (Neomelaniconion)* spp., *Mansonia uniformis* and *Culex univittatus* (Kokernot, Smithburn, Paterson and De Meillon 1960).

Wesselsbron disease may be controlled by the immunization of sheep (Neitz 1965) with live virus vaccine attenuated by repeated passage in foetal lamb kidney cells.

Apart from the isolation of Wesselsbron virus and subsequent epidemiological studies in Southern Africa, little work on this virus has been reported.

Polson (quoted by Weiss et al 1956) determined the size of Wesselsbron virus to be of the order of 30 mμ.

Wesselsbron virus has been classified in Group B of the arthropod-borne group of animal viruses on the basis of its serological interaction detected specifically by the haemagglutination inhibition test (Casals 1957).

Wesselsbron virus was found to propagate readily in lamb kidney tissue cultures (Weiss 1957), and Porterfield (1959) successfully produced plaques in monolayers of chick embryo fibroblast cells infected with Wesselsbron virus.

This investigation was undertaken in order to determine some of the biophysical, biochemical and serological properties of Wesselsbron virus as part of a contribution to our knowledge of the properties of the African arboviruses.

III - WESSELSBRON VIRUS

A. INTRODUCTION

An arthropod-borne animal virus is defined as one which, in nature, is able to infect haemaphagous arthropods by their ingestion of infected vertebrate blood; it multiplies in their tissues and is transmitted by bite to susceptible vertebrates (World Health Organ. Tech. Rep. Ser., Arthropod-borne Viruses 1961).

The arboviruses as a group do, however, possess several common physicochemical properties.

It is well known to virologists that arboviruses are generally very unstable in aqueous tissue suspensions, and a protein such as serum or bovine plasma albumin must be added to the diluent in order to maintain maximum infectivity. If they are to be preserved for any length of time, the tissue suspensions should be lyophilized or stored at -70°C. Infected brain material, as such, may be preserved at -20°C for several months. The viruses in this group are most stable at pH 7-9 (Rivers and Horsfall 1959).

Arboviruses are readily inactivated by ether (Andrewes and Horstmann 1949) and bile salts e.g. sodium deoxycholate (Theiler 1957), suggesting that lipids play a role in maintaining the structural integrity of arbovirus particles (Mussgay 1964).

In this chapter, the preparation of Wesselsbron virus stock material and Wesselsbron virus antiserum and the methods of infectivity titrations in mice and tissue cultures are described. An account is presented of some of the physico-chemical and serological properties of Wesselsbron virus.

B. MATERIALS AND METHODS

1. Buffers and diluents

The following buffers and diluents were used throughout the investigation of Wesselsbron virus. They are listed in alphabetical order.

(i) Acetate buffer - pH 5.3

NaOH	20 gm
CH_3COOH (glacial)	36 ml

Made up to 10 litres with distilled water.

(ii) Antibiotic solution

Penicillin	1,000,000 units/ml
Streptomycin	1,000,000 μgm/ml
Normal saline	50 ml

filtered and stored at -20°C.

One ml of the antibiotic solution contains 20,000 units penicillin and 20,000 μgm streptomycin.

(iii) Bovine plasma albumin (Bpa)

Bovine albumin powder - fraction V from Bovine plasma manufactured by

Armour Pharmaceutical Co. Ltd. (England).

(iv) Borate buffered saline (BBS) pH 8.6

0.035 M H_3BO_3 / 0.0175 N NaOH

0.0075 N HCl / 0.073 M NaCl.

Borate stock

H_3BO_3	62 gm
NaOH	20 gm

made up to 5 litres with distilled water.

Hydrochloric Acid - 0.1 N

HCl (density = 1.186)	45.4 ml

made up to 5 litres with distilled water.

Borate buffered saline (BBS) for use

Borate stock	350 ml
Hydrochloric acid - 0.1 N	150 ml
Normal saline	500 ml
Distilled water	1,000 ml

(v) "Deinhibitorized" rabbit serum (DRS) pH 7.0

Normal rabbit serum was dialyzed against acetate buffer pH 5.3 for 48 hours at 4°C to remove a well-known inhibitor (Turner, Kipps and Polson 1961). The precipitate was removed by centrifugation at 1,200 rpm for 10 minutes in the MSE refrigerated centrifuge at 4°C. The pH of the supernatant was adjusted to 7.0 with sodium phosphate (Na_2HPO_4).

(vi) Distilled water

Glass distilled water.

(vii) Normal 0.85% (physiological) saline

0.85% (w/v) NaCl was prepared in distilled water.

(viii) Phosphate buffers 0.2 M and 0.02 M pH 7.0

Disodium hydrogen phosphate (Na_2HPO_4) 0.2 M.

Na_2HPO_4	28.4 gm

made up to 1 litre with distilled water.

Potassium dihydrogen phosphate (KH_2PO_4) 0.2 M.

KH_2PO_4	27.2180 gm

made up to one litre with distilled water.

Phosphate buffer 0.2 M pH 7.0 for use

2 vols 0.2 M Na_2HPO_4 + 1 vol 0.2 M KH_2PO_4

Phosphate buffer 0.02 M pH 7.0 for use

2 vols 0.2 M Na_2HPO_4 + 1 vol 0.2 M KH_2PO_4

+ 27 vols distilled water.

(ix) Phosphate buffered saline (PBS) pH 7.0

2 vols 0.2 M Na_2HPO_4 + 1 vol 0.2 M KH_2PO_4

+ 27 vols normal saline.

(x) Serum saline (SS)

Normal saline	95 ml
"Deinhibitorized" rabbit serum	5 ml
Antibiotic solution	0.5 ml

This diluent was used throughout for the preparation of suspensions of Wesselsbron virus unless otherwise stated.

2. Wesselsbron virus preparations

Two strains of Wesselsbron virus have been used in this investigation.

Throughout this study, unless otherwise stated, centrifugations of Wesselsbron virus suspensions, at speeds varying between 8,000 and 34,000 rpm, were carried out in the Spinco model L preparative ultracentrifuge using the Spinco rotor no 40.

Low passage strain of Wesselsbron virus

A comprehensive biological study of the low passage strain of Wesselsbron virus was undertaken. The virus was at its 10th passage level in tissue culture.

Lyophilized stock virus: The contents of an ampoule of freeze-dried Wesselsbron virus infected tissue culture fluid from the 10th passage in foetal lamb kidney cells (obtained from the Veterinary Research Institute, Onderstepoort, Transvaal) were suspended in 2 ml of serum saline, and eight 3-4 day old suckling mice were injected with 0.01 ml aliquots intracerebrally.

Approximately 94 hours after injection, the sucklings showed signs of paralysis and after 98 hours were moribund. They were sacrificed by ether anaesthesia and their brains were removed aseptically and stored at -20°C (SMB_1).

Three of these suckling mouse brains were triturated in 12 ml serum saline. This preparation represented a 10^{-1} suspension of Wesselsbron virus infected suckling mouse brains. This suspension was clarified by centrifugation at 10,000 rpm for 10 minutes. Each of ninety 3-4 day old suckling mice were injected intracerebrally with 0.01 ml of the clear supernatant fluid.

Approximately 98 hours after inoculation the brains of these ninety suckling mice were harvested. The brains were ground in a chilled mortar with a little serum saline and sterile ground glass. This suspension was clarified by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then centrifuged at 30,000 rpm for 60 minutes, and the pellet, resuspended in 42 ml serum saline, represented an undiluted virus suspension (10^0). This suspension was dispensed in 1 ml amounts and lyophilized (SMB_2).

Infected brain material: The contents of one ampoule of the above freeze dried material was reconstituted in 4 ml serum saline and 0.01 ml was injected intracerebrally into each of eight 3-4 day old suckling mice. With these brains (SMB_3) a 10^{-1} suspension of Wesselsbron virus was prepared in serum saline and injected into a further 60-90 suckling mice. The brains from these

mice were harvested on the fourth day after infection and stored at -20°C. At this stage the low passage strain of Wesselsbron virus had undergone four suckling mouse brain passages in this laboratory (SMB_4). Fresh infected brain material was prepared every three months from an ampoule of the lyophilized stock virus described above.

Virus suspensions: The working samples for all experiments were 10^{-1} suspensions of Wesselsbron virus infected suckling mouse brains (SMB_4) prepared by triturating one virus infected brain in 4 ml serum saline (unless otherwise stated) and clarifying the suspension by centrifugation at 10,000 rpm for 10 minutes. These virus preparations will henceforth be referred to as 10^{-1} Wesselsbron virus.

High passage strain of Wesselsbron virus

The high passage strain of Wesselsbron virus had undergone 110 passages in tissue culture, and was studied in far less detail in this investigation.

Infected brain material: The contents of an ampoule of freeze-dried Wesselsbron virus infected culture fluid from the 110th passage in foetal lamb kidney cells (obtained from the Veterinary Research Institute, Onderstepoort) was reconstituted in 2 ml of serum saline. This high passage strain was

subjected to two passages in suckling mouse brains in this laboratory and the infected suckling mouse brains from the 2nd passage were stored at -20°C for periods not longer than 3 months before use.

Only the haemagglutinating activity of suspensions of suckling mouse brains infected with this high passage strain, and its intracellular development as observed in thin sections of virus infected foetal lamb kidney cells, were investigated. This strain is referred to as Wesselsbron virus (high passage strain).

3. Methods of titration

The infectivity of Wesselsbron virus was titrated in 3-4 week old mice or in cultures of foetal lamb kidney cells in roller tubes.

When mice were used, serial tenfold dilutions of the material to be titrated were prepared in serum saline; groups of 4-6 mice were injected with 0.04 ml aliquots of each dilution intracerebrally.

When cultures in roller tubes were employed, tenfold dilutions were made in tissue culture medium (Hanks' LA maintenance medium - see Appendix I); each of 4-6 tubes per dilution were infected with 0.1 ml of the virus suspension.

The 50% end points were determined by the method of Reed and Muench (1938).

4. Preparation of antiserum

One hundred adult mice were injected intraperitoneally at twice weekly intervals for 4 weeks with 0.5 ml of a 20% suspension by weight of Wesselsbron virus prepared in 5% normal mouse serum in saline.

Two weeks after the last injection, the mice were bled by heart puncture. The serum was clarified by centrifugation at 10,000 rpm for 30 minutes and stored in 1 ml ampoules at -20°C.

5. Neutralization test

Antigen - Tenfold serial dilutions of 10^{-1} Wesselsbron virus were prepared in serum saline.

Sera - A 10^{-1} dilution of each of the following sera was prepared in saline and inactivated at 56°C for 30 minutes.

(i) Wesselsbron virus antiserum prepared in mice

(ii) Wesselsbron virus antiserum prepared in sheep (supplied by the Veterinary Research Institute, Onderstepoort).

(iii) Normal mouse serum

(iv) Normal sheep serum.

The mixtures of virus and various sera were held at room temperature for $1^1/_2$ hours prior to injection in 3-4 week old mice.

6. Stability of Wesselsbron virus in various diluents

The stability of Wesselsbron virus was tested in the following diluents over a period of 4 days.

(i)	Normal saline	+ 0.4%	Bpa	pH 5.17
(ii)	0.02 M phosphate buffer	+ 0.4%	Bpa	pH 7.09
(iii)	BBS	+ 0.4%	Bpa	pH 8.53
(iv)	Normal saline	+ 5%	DRS	pH 6.80
(v)	0.02 M phosphate buffer	+ 5%	DRS	pH 7.16
(vi)	BBS	+ 5%	DRS	pH 8.60

10^{-1} suspensions of Wesselsbron virus were prepared in each of the above diluents. The samples were stored at 4°C, and titrated daily in cultures of foetal lamb kidney cells in roller tubes.

7. Stability of Wesselsbron virus at different temperatures

A 10^{-1} suspension of Wesselsbron virus was prepared in 0.02 M phosphate buffer containing 5% "deinhibitorized" rabbit serum (DRS).

Samples were stored at -20°C, 4°C, 22°C and 37°C and titrated daily in cultures of FLK cells in roller tubes.

The sample stored at -20°C was divided into 4 separate aliquots to avoid daily freezing and thawing which might affect the virus adversely.

8. Sensitivity of Wesselsbron virus to diethyl ether

Diethyl ether was added to 10^{-1} Wesselsbron virus so that the final concentration of ether was 20%. The sample was stored for 18 hours at 4°C and shaken occasionally. A control tube, which contained an aliquot of the virus suspension, but to which no ether had been added, was handled in a similar manner.

9. Sensitivity of Wesselsbron virus to sodium deoxycholate

Equal volumes of 10^{-1} Wesselsbron virus and sodium deoxycholate, at concentration of 10^{-3} in 0.02 M phosphate buffer pH 7.0, were held at 22°C for 30 minutes. The control tube, which contained equal parts of virus suspension and serum saline diluent, was similarly incubated and titrated.

C. RESULTS

The results presented below all refer to the low passage strain of Wesselsbron virus.

Infectivity titres of Wesselsbron virus during passage in suckling mouse brains

The results of the infectivity titrations of Wesselsbron virus infected suckling mouse brain suspensions, after each passage of the virus in suckling mouse brains (SMB), are listed in Table 1. The titrations were performed on 10^{-1} suspensions of the virus-infected suckling mouse brains, which were

serially diluted and injected into 3-4 week old mice by the intracerebral route.

Table 1. Infectivity titres of Wesselsbron virus during passage in suckling mouse brains (SMB).

No. of passages in suckling mouse brains (SMB)	LD_{50}/0.04 ml
SMB_1	7.24
SMB_2 before lyophilization	8.24
SMB_2 after lyophilization	6.75
SMB_4	8.00
SMB_4	7.33
SMB_4	7.50

It will be seen from Table 1 that there was a 30-fold drop in the infectivity of Wesselsbron virus after lyophilization. After the virus had been further passaged in suckling mouse brains to the 4th passage level (SMB_4), the Wesselsbron virus infected brain material contained 7.33 - 8.00 LD_{50}/0.04 ml.

In Table 2, the infectivity of Wesselsbron virus was titrated in 3-4

week old mice and in FLK cells in roller tubes to compare the two titration techniques.

Table 2. Comparison of infectivity titres of Wesselsbron virus determined in mice and in tissue culture.

Experiment no.	LD_{50} /0.04 ml	$TCID_{50}$ /0.1 ml
1	7.50	7.33
2	7.66	7.00
3	7.40	6.84

As shown in Table 2, the infectivity of virus infected brain material as determined in 3-4 week old mice (LD_{50}) and in tissue culture ($TCID_{50}$) were found to be similar despite the difference in the volume of the inocula.

Neutralization test results

The outcome of the neutralization of Wesselsbron virus by various sera appears in Table 3.

Table 3. Neutralization of Wesselsbron virus by various sera.

Sera tested (10^{-1} dilution)	LD_{50} /0.04 ml
Wesselsbron virus antiserum - prepared in mice	2.00
Wesselsbron virus antiserum - prepared in sheep	4.66
Normal mouse serum	7.66
Normal sheep serum	7.50
Control virus (without serum)	7.50

Both stock antisera produced a marked reduction in the infectious titre of Wesselsbron virus in suckling mouse brain preparations. The sheep antiserum, from an experimental infection, caused a 1,000-fold reduction in infectivity, while the mouse-immune serum produced a dramatic reduction of 300,000-fold.

Stability of Wesselsbron virus in various diluents

Figures la and lb illustrate the stability of Wesselsbron virus in normal saline, phosphate buffer and borate buffered saline (BBS), which contained either bovine plasma albumin (Bpa) or "deinhibitorized" rabbit serum (DRS).

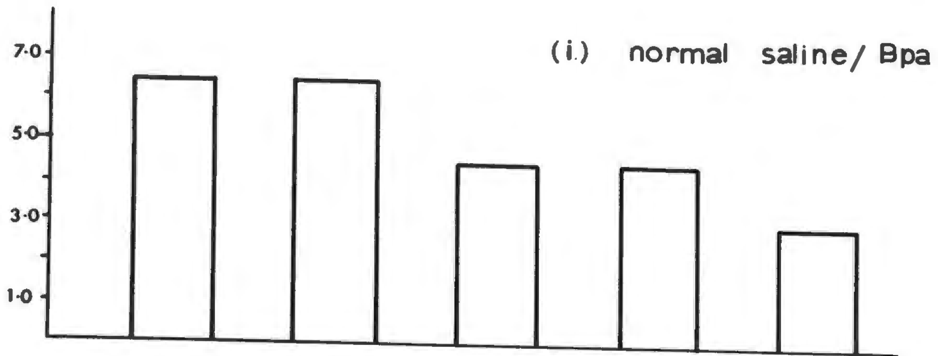
These figures show that no difference was evident in the titres of Wesselsbron virus infected mouse brain material suspended in the different

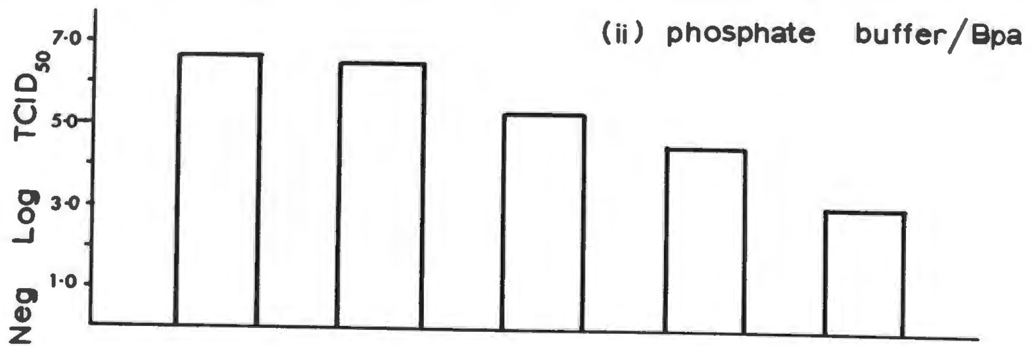
Fig. 1 (a).

Stability of Wesselsbron virus in:

(i) normal saline;
(ii) phosphate buffer;
(iii) borate buffered saline

each containing 0.25% bovine plasma albumin


7·0
5·0
3·0
1·0
(i.) normal saline/ Bpa


Neg Log TCID50
7·0
5·0
3·0
1·0
(ii) phosphate buffer/Bpa

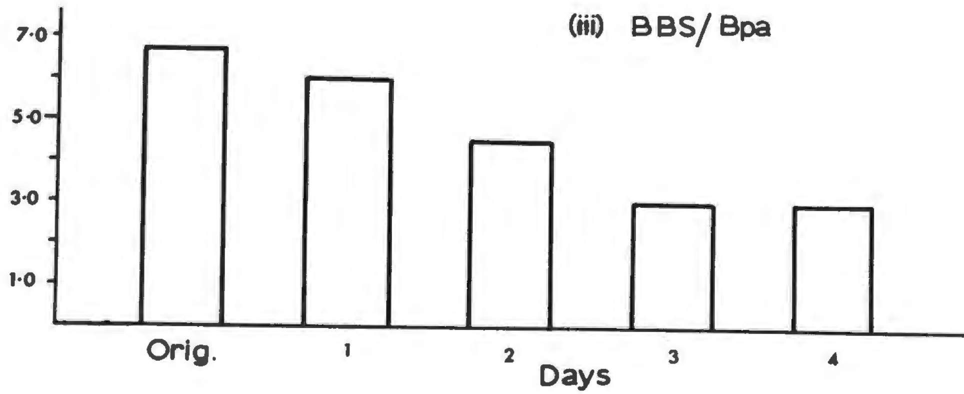
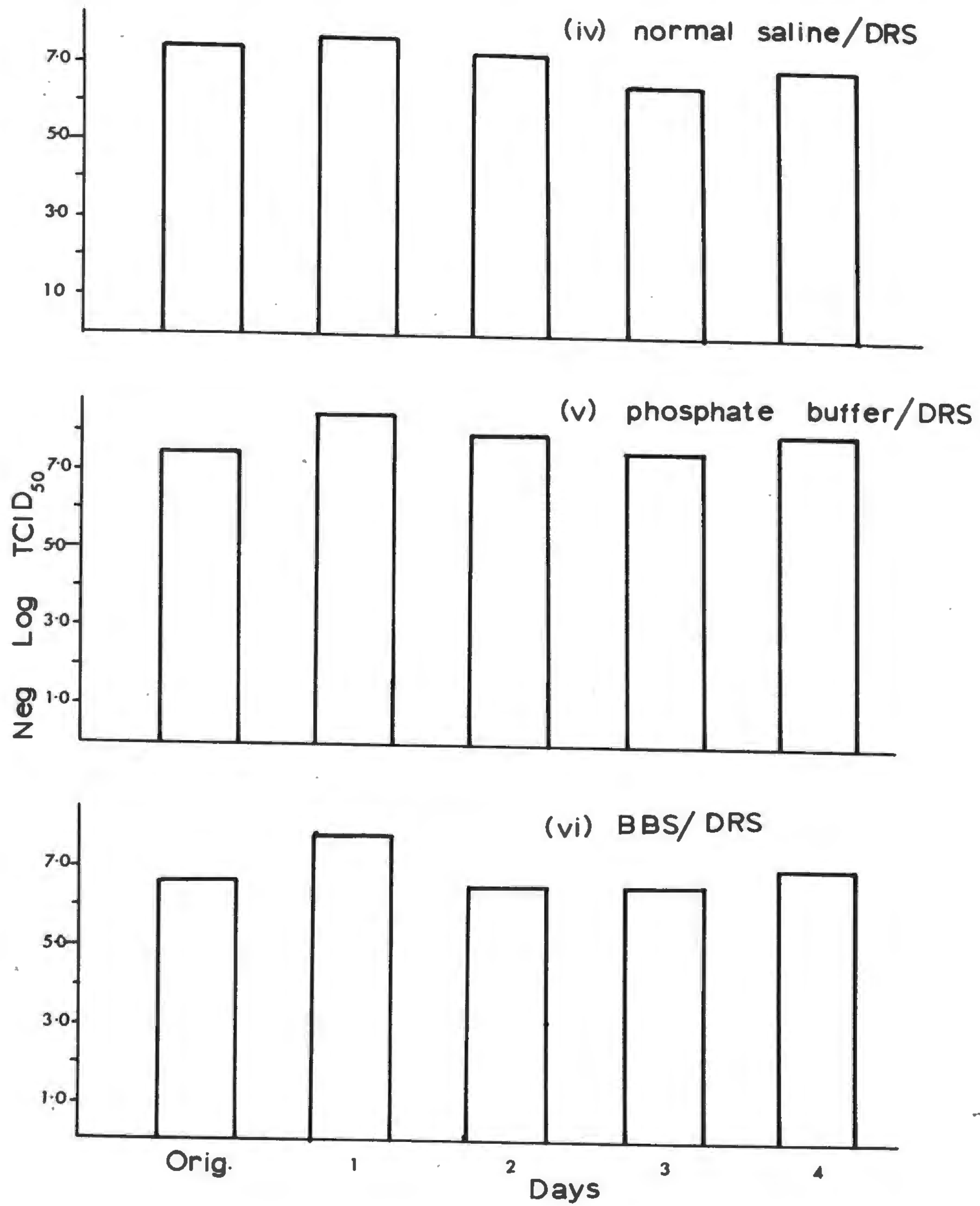

7·0
5·0
3·0
1·0
(iii) BBS/Bpa
Orig.
1
2
3
4
Days

Fig. 1 (b).

Stability of Wesselsbron virus in:

(i) normal saline;
(ii) phosphate buffer;
(iii) borate buffered saline.

each containing 5% "deinhibiterized" rabbit serum.



(iv) normal saline/DRS
7·0
5·0
3·0
1·0
(v) phosphate buffer/DRS
Neg Log TCID50
7·0
5·0
3·0
1·0
(vi) BBS/ DRS
7·0
5·0
3·0
1·0
Orig.
1
2
3
4
Days

diluents which contained either Bpa or DRS after 24 hours storage at 4°C. However over the full period of 4 days, Wesselsbron virus was appreciably more stable when suspended in the diluents containing DRS than when suspended in those containing Bpa.

The three diluents themselves appeared to be equally suitable.

Stability of Wesselsbron virus at different temperatures

The viability of Wesselsbron virus suspensions prepared in 0.02 M phosphate buffer containing 5% DRS and stored at -20°C, 4°C, 22°C or 37°C for periods up to 4 days, was determined by daily titrations in FLK cells in roller tubes. (Table 4).

Table 4. Stability of Wesselsbron virus at different temperatures.

Day	$TCID_{50}$ /0.1 ml			
	-20°C	4°C	22°C	37°C
0	6.66	6.66	6.66	6.66
1	7.00	7.50	7.33	7.50
2	6.50	7.33	7.00	6.66
3	7.33	7.45	>6.50	3.50
4	4.66	7.33	>6.50	3.00

The results listed in Table 4 show that the Wesselsbron virus suspensions

stored at 4°C and 22°C were stable over the period tested, whereas there was a 100-fold reduction by the 4th day in the material stored at -20°C, and more than a 1,000-fold reduction in infectivity of the material stored at 37°C.

Sensitivity of Wesselsbron virus to diethyl ether and sodium deoxycholate

The infectivity of the Wesselsbron virus suspensions treated with ether and sodium deoxycholate was determined by titration in FLK cells in roller tubes and the results are seen in Table 5.

Table 5. Effect of ether and sodium deoxycholate on Wesselsbron virus

$TCID_{50}$/0.1 ml		
Original virus suspension	+ 20% diethyl ether	10^{-3} sodium deoxycholate
6.50	2.00	< 2.00

Table 5 shows that Wesselsbron virus is markedly inactivated by both 20% diethyl ether and 10^{-3} sodium deoxycholate.

D. DISCUSSION

In this chapter some of the fundamental properties of the low passage strain of Wesselsbron virus have been investigated.

It is evident that the lyophilized stock virus must be passaged in suckling mouse brains before use as there is a considerable loss in infectivity during dehydration. After a further two passages in suckling mouse brains the titre of Wesselsbron virus was $10^{7.0} - 10^{8.0}$ LD_{50}.

The infectivity of Wesselsbron virus was effectively neutralized by homologous antisera, prepared in mice or sheep.

It is apparent that bovine plasma albumin (Bpa) is not as effective as "deinhibitorized" rabbit serum (DRS) for maintaining infectivity of Wesselsbron virus for periods longer than 24 hours. No appreciable difference in titre was found when Wesselsbron virus was suspended in the different diluents containing DRS. Either 0.02 M phosphate buffer at pH 7.0 or normal saline, both containing 5% DRS were subsequently used for the preparation of Wesselsbron virus suspensions. However, in all experiments in which the haemagglutinating activity of Wesselsbron virus was investigated, bovine plasma albumin was used to stabilize the virus as it was found that DRS agglutinated goose cells. These experiments were all completed within 24 hours of preparation of the virus suspensions in order that the infectivity of the suspension would remain maximal. The most commonly used diluting fluid to which the bovine plasma albumin was added was 0.02 M phosphate buffer at pH 7.0 and not normal saline as it was found that bovine plasma albumin lowered the pH of saline to 5.17.

It is evident that Wesselsbron virus is stable in a diluent of 0.02 M

phosphate buffer, pH 7.0 (containing 5% DRS) up to a period of 4 days when stored at 4°C and 22°C but not at 37°C. The storage temperature of choice was therefore 4°C for Wesselsbron virus suspensions.

The sensitivity of the virus to the action of ether and bile salts agrees with the finding that arboviruses are inactivated by these agents (Rhodes and van Rooyen 1962) and suggests that there is an essential lipid component in the viral structure.

IV - TISSUE CULTURE

A. INTRODUCTION

The work of Harrison (1907) on the developing nerve fibre is considered the true beginning of tissue culture.

During the subsequent twenty years, Burrows (1911), Carrel (1912), Steinhardt, Israeli and Lambert (1913), Parker and Nye (1925), Strangeways and Fell (1926), Maitland and Maitland (1928) and others, were responsible for improvements in methods and techniques of culturing cells, tissues and organs in vitro.

The present importance of tissue culture in virology is accredited to Enders, Weller and Robbins (1949) who observed that Poliovirus would grow in human tissues of non-nervous origin in tissue culture.

Another significant advance was made by Mascona (1952). He found that the elimination of calcium and magnesium from the dispersing medium enhanced the dissociation of cells of early embryonic rudiments treated with trypsin. Rous and Jones (1916) had previously shown that trypsin dispersed the cells of tissue culture.

Balanced salt solutions are employed in tissue culture to maintain the pH and osmotic pressure in the medium, and also to provide an adequate concentration of essential inorganic ions. When supplemented with whole serum or protein hydrolysates, these solutions are capable of supporting the growth of living cells. The solution of Hanks and Wallace (1949) is one of the

commonly used balanced salt solutions.

Trypsin-dispersed cell suspensions may be prepared from numerous types of freshly explanted tissues.

The infectivity of the virus suspensions in many of the experiments with Wesselsbron virus was titrated in cultures of foetal lamb kidney cells in roller tubes.

The possibility of producing plaques in monolayer cultures of chick embryo cells (Dulbecco 1952, Porterfield 1959, 1960) and in agar suspensions of chick embryo cells (Cooper 1955) infected with Wesselsbron virus (low passage strain), was investigated.

B. MATERIALS AND METHODS

1. Diluents and media

The diluents and media used in the preparation and for the maintenance of foetal lamb kidney cells and chick embryo tissue cultures listed below are fully described in Appendix I.

Agar (1.2%)

Cooper galactose medium

Gey's balanced salt solution and Standard Gey's

Hanks' balanced salt solution (Hanks' BSS)

Hanks' balanced salt solution containing lactalbumin hydrolysate (Hanks' LA)

Hanks' LA containing 10% calf serum (Hanks' LA growth medium)

Hanks' LA containing 2% calf serum (Hanks' LA maintenance medium)

Tris 0.05 M pH 7.6

Tris - Gey's medium

Tris - growth medium

Tris - overlay medium

Trypsin base

Trypsin solution

Trypsin / versenate solution.

2. Cultures of foetal lamb kidney (FLK) cells

(a) Preparation of FLK cell cultures

The kidneys of a foetal lamb were removed aseptically. The capsules were removed and the kidneys were cut up into small pieces in a sterile beaker, then washed in approximately 100 ml of trypsin base. The fluid was decanted and 100 ml of 0.25% trypsin solution were added. A sterile magnet was inserted, and the beaker placed in a water bath over a magnetic stirrer. The treatment with trypsin was allowed to proceed for $1^1/_4$ hours at 37°C.

The cell suspension was filtered through sterile gauze and the filtrate centrifuged at 1,000 rpm for 5 minutes. The cells were washed in approximately 20 ml of Hanks' LA growth medium and centrifuged at 1,000 rpm for 5 minutes.

The cells were resuspended in Hanks' LA growth medium, and the packed cell volume was determined. The cells were diluted with Hanks' LA growth medium so that the cell concentration was 1 ml packed cells to 500 ml growth medium.

The diluted cell suspension was dispensed in 50 ml amounts into 20 oz medical flat bottles and incubated at 37°C for 3 days in the horizontal position. On the third day, the fluid in each bottle was replaced with 50 ml. of Hanks' LA maintenance medium. These primary cultures were incubated for a further 2 days.

The medium was then discarded, and 20 ml trypsin/versenate solution was added to each bottle to facilitate the suspension of the cells. After 15 minutes incubation at 37°C, the cells were suspended by shaking and the suspension centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded, the cells washed in Hanks' LA growth medium, centrifuged again at 1,000 rpm for 5 minutes and suspended in the same medium at concentration 150,000 cells/ml.

The cell suspension was dispensed into test tubes in 1 ml amounts per tube. The tubes were incubated at 37°C in an almost horizontal position for 2 days. The fluid was then replaced with 1 ml Hanks' LA mainten ance medium and these tubes, which contained secondary cultures of FLK cells, were rotated. To prepare secondary FLK cultures in 2 oz medical flat bottles; 7 ml of the cell suspension was dispensed per bottle.

Cultures of FLK cells in roller tubes and bottles were used up to a period of 4 days after preparation. The maintenance medium was changed daily until the cultures were required for use.

(b) <u>Titration of Wesselsbron virus in FLK cells in roller tubes</u>

Serial tenfold dilutions of 10^{-1} Wesselsbron virus were prepared in Hanks' LA maintenance medium and inoculated into a series of FLK cultures in roller tubes. Four to six tubes were infected with 0.1 ml aliquots of each virus dilution and the cells were examined daily for cytopathic effect.

The end points were determined 5 days after infection and titres expressed as negative logarithms of the dilution of virus suspension causing cytopathic changes in 50% of the infected cultures ($TCID_{50}$).

(c) <u>Propagation of Wesselsbron virus in FLK cell cultures</u>

For the propagation of Wesselsbron virus in foetal lamb kidney cells, secondary cultures of FLK cells were grown in 2 oz medical flat bottles in Hanks' LA growth medium. After 3 days, the cell sheets were confluent.

The medium was discarded and the bottles were infected with 0.5 ml of 10^{-6} dilution of 10^{-1} Wesselsbron virus (prepared in Hanks' LA maintenance medium) and placed in the incubator at 37°C for one hour. The inoculum was then removed, the cells washed and overlaid with 7 ml of Hanks' LA maintenance medium. The fluid was titrated for infectivity in FLK roller tube

cultures at daily intervals.

3. Monolayer cultures of chick embryo (CE) cells for plaque production (Porterfield's technique)

(a) Preparation of CE cell monolayer cultures

Step I. 12 day old chick embryos were harvested. The viscera were removed and the embryos were chopped up in a sterile beaker and washed once in Hanks' balanced salt solution (Hanks' BSS). The Hanks' BSS was discarded, 0.25% trypsin solution added, and the embryos were trypsinized for 30 minutes at 37°C over a magnetic stirrer.

The cell suspension was filtered through sterile gauze and the filtrate was centrifuged at 1,000 rpm for 5 minutes. The cells were washed in Hanks' LA containing 5% calf serum, and centrifuged again at 1,000 rpm for 5 minutes.

Step II. The cells were resuspended in tris-growth medium, filtered through a coarse glass filter, and counted in a Spencer bright line haemocytometer. The cells were diluted in tris-growth medium to give a final concentration of 5×10^6 cells/ml.

The final cell suspension was distributed into petri dishes in 5 ml amounts and incubated at 37°C in a humidified incubator for 2 days, by which time the cell sheet was confluent.

(b) Titration of Wesselsbron virus in CE cell monolayer cultures

Serial tenfold dilutions of 10^{-1} Wesselsbron virus were prepared in tris-Geys medium.

The growth medium was removed from the monolayer cultures. The cells were washed once with tris-Geys and 0.5 ml of each virus dilution was pipetted on to two plates. The inoculated cultures were incubated at 37°C for 1-3 hours. The remainder of the inoculum was then removed, and a mixture of 3 ml agar (1.2% melted) and 3 ml tris-overlay medium was added to each plate. The agar overlay was allowed to set at room temperature and cultures were incubated for 3-4 days at 37°C then they were stained with $^{1}/_{10,000}$ neutral red diluted in tris-overlay medium.

4. Suspensions of chick embryo (CE) cells in agar (Cooper's technique)

(a) Preparation of CE cell suspensions

Step I. This is identical with that for monolayer cultures.

Step II. Cells were dispersed in Cooper galactose medium (single strength) containing 5% fowl serum, filtered through a coarse glass filter and counted. The cells were diluted to 2.0×10^{7} per ml.

This chick suspension was used immediately in virus infected agar suspensions.

(b) Titration of Wesselsbron virus in suspensions of CE cells in agar

Serial tenfold dilutions of Wesselsbron virus were prepared in Cooper galactose medium (single strength) containing 5% fowl serum.

A mixture of

Ionagar (1.2% melted)	2 ml
Cooper galactose (double strength) + 10% fowl serum	2 ml
Chick cell suspension (2.0×10^7 cells/ml)	1.0 ml
Virus dilution	0.1 ml

was poured on to each plate and allowed to set at room temperature. The cultures were incubated for 4 days and then stained with a solution of $^1/_{2,500}$ neutral red diluted in phosphate buffered saline containing 1.5% glucose.

C. RESULTS

Titration of infectivity of Wesselsbron virus in FLK cells in roller tubes

The cultures in roller tubes were examined daily. Wesselsbron virus produces a cytopathic effect in FLK cell cultures 3–4 days after infection, typified by the separation of the spindle shaped cells into groups, followed by the rounding of the cells and complete destruction of the cell sheet.

Propagation of Wesselsbron virus in FLK cell cultures

The infectivity of the culture fluid from Wesselsbron virus infected FLK cell cultures was determined by daily titrations of the fluid in FLK cells in roller tubes and the results are listed in Table 6.

Table 6. Propagation of Wesselsbron virus in FLK cells

Period after infection (hours)	$TCID_{50}$ /0.5 ml	Cytopathic effect
4	3.00	-
21	3.00	-
45	3.66	-
69	6.00	+
93	6.50	++

- = no cytepathic effect visible

+ = cytopathic effect noticeable

++ = complete cytopathic effect

It will be seen from Table 6 that the infectivity titre of the supernatant fluid reached a maximum when the cell sheet was completely destroyed and this occured approximately 93 hours after the FLK cells were infected.

Titration of Wesselsbron virus in CE cell monolayers (Porterfield's technique)

Table 7 shows the results of four attempts to produce plaques on CE

cell monolayer cultures infected with Wesselsbron virus. The plaques were counted on the 4th day after infection.

Table 7. Plaque production on chick embryo cell monolayer cultures.

Experiment No.	Plaque forming units/ml
1	8.0×10^8
2	Nil
3	Nil
4	17×10^8

Suspensions of Wesselsbron virus from infected mouse brains were found to contain 8 to 17×10^8 plaque forming units per ml in two experiments. The plaques were easily seen, regular in outline and measured 2-3 mm in diameter.

No plaques were obtained in the other two experiments despite the fact that the conditions of the experiments were identical with those in which plaque production was successful. No explanation was found for these discrepant results.

Titration of Wesselsbron virus in suspensions of CE cells in agar (Cooper's technique)

No plaques were obtained in agar suspensions of chick embryo cells infected with Wesselsbron virus.

D. DISCUSSION

The cytopathic effect produced by Wesselsbron virus in FLK cell cultures is easily observed when these cultures are examined microscopically and thus the presence of the virus can be detected. After approximately four days incubation, the virus titre reached a level of $10^{6.5}$ $TCID_{50}$/0.5 ml at a time when the cell sheet was showing rapid degeneration to a point of almost complete destruction.

The titration of Wesselsbron virus by plaque production in chick embryo cell monolayers did not give reproducable results, it was therefore found necessary to use cultures of foetal lamb kidney cells in roller tubes for the titration of Wesselsbron virus.

Numerous unsuccessful attempts at producing plaques in Wesselsbron virus infected agar suspension of chick embryo cells were made.

The reasons for the poor results using these two plaque techniques have not been investigated.

In 1959, Porterfield successfully obtained plaques on chick embryo cell monolayers infected with Wesselsbron virus and other group B arboviruses. Henderson and Taylor (1960), however, found that Group B arboviruses did not produce plaques on similar monolayers.

Schulze and Schlesinger (1963) suggested that a sulphated polysaccharide in aqueous agar extracts has an inhibiting effect on Dengue and other arboviruses in Group B and recommend the use of a methylcellulose overlay medium. The effect of this overlay medium is being investigated.

V - HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION

A. INTRODUCTION

The phenomenon of haemagglutination was discovered independently by Hirst (1941) and by McClelland and Hare (1941).

The first demonstration of an arbovirus haemagglutinin was that of Japanese B Encephalitis (Sabin and Buescher 1950). They demonstrated haemagglutinin activity in a saline extract of infected suckling mouse brains that had been centrifuged at 13,000 rpm for 60 minutes. Other methods of extraction of haemagglutinin from mouse brains include extraction with acetone and ether (Casals and Brown 1953); treatment with BBS followed by centrifugation at 13,000 rpm for 60 minutes (Chanock and Sabin 1953); sucrose-acetone extraction and protamine sulphate treatment (Clarke and Casals 1958). Many of the group B arboviruses have been found to show haemagglutinin activity in 10-20% alkaline suspensions after moderate centrifugation (Clarke and Casals 1958); and Porterfield and Rowe (1960) prepared haemagglutinins by extraction with fluorocarbon.

Erythrocytes of chicks less than 24 hours old (Sabin and Buescher 1950), of pigeons (MacDonald 1952) and of geese (Porterfield 1957) have been found suitable for haemagglutination of arboviruses.

Sabin (1951) recorded the presence of a lipid or lipoprotein inhibitor

in normal sera and Porterfield and Rowe (1960) have suggested that this inhibitory activity against Group B arboviruses is associated with certain phospholipids. These non-specific inhibitors present in normal and immune sera may be removed with lipoid solvents such as ether, chloroform or toluene (Sabin 1951) by acetone extraction (Sweet, Chanock and Sabin 1953) or by adsorption with bentonite or kaolin (Clarke and Casals 1955, 1958).

The loss of haemagglutinating ability by arboviruses that have undergone more than a hundred mouse passages has been described by Sabin (1951) and by Casals and Brown (1953).

Casals and Brown (1953, 1954) classified a number of arboviruses into two groups on basis of serologic relationships using the haemagglutination and haemagglutination inhibition techniques. Group A viruses showed optimal haemagglutination at 37°C and pH 5.6 - 6.4, and group B haemagglutinins were most effective at 4° - 20°C and pH 6.0 - 7.4.

The present classification of the arbovirus group is based on the study of the serological interactions between 47 distinct arboviruses by Casals (1957).

The techniques of haemagglutination and haemagglutination inhibition of Clarke and Casals (1958) which have been used extensively in the identification of arboviruses, were used, with some modifications, in the study of Wesselsbron virus.

B. MATERIALS AND METHODS

1. Diluents

(i) Borate buffered saline containing 0.4% bovine plasma albumin, pH 8.6 (BBS/Bpa) was used throughout as the diluent for the haemagglutinin and for the antisera, and not borate buffered saline at pH 9.0 as used by Clarke and Casals (1958).

(ii) Various adjusting diluents were used for resuspending the goose cells in order to detect haemagglutination at different hydrogen ion concentrations.

I. Basic salt solution : 0.15 M NaCl / 0.2 M Na_2HPO_4

NaCl	8.77 gm
Na_2HPO_4	28.39 gm

made up to 1 litre with distilled water.

II. Acidic salt solution : 0.15 M NaCl / 0.2 M $NaH_2PO_4.2H_2O$

NaCl	8.77 gm
$NaH_2PO_4.2H_2O$	31.21 gm

made up to 1 litre with distilled water.

The basic and acidic salt solutions were mixed in varying proportions to give adjusting diluents (Table 8), which when mixed with equal volumes of BBS/Bpa (pH 8.6) gave the desired hydrogen ion concentrations (Clarke and Casals 1958).

Table 8. Composition of the various adjusting diluents, which when mixed with an equal volume of BBS/Bpa (pH 8.6), yielded the pH's given in the third column.

Basic salt soln. I. ml	Acidic salt soln. II ml	Final pH
1.50	48.50	5.60
6.25	43.75	5.93
11.00	39.00	6.11
16.00	34.00	6.31
22.50	27.50	6.58
27.50	22.50	6.76
32.00	18.00	6.97
39.50	10.50	7.36

2. Preparation of haemagglutinin from infected brain material

A 10^{-1} suspension of Wesselsbron virus (low passage strain) was prepared in BBS/Bpa. The suspension was clarified by centrifugation at 10,000 rpm for 10 minutes and the supernatant used as haemagglutinin in the haemagglutination (HA) and haemagglutination inhibition (HI) tests.

Similar haemagglutinin preparations were also made from the high passage strain of Wesselsbron virus.

3. <u>Preparation of haemagglutinin from infected cultures of FLK cells</u>

Two ounce medical flat bottles containing secondary foetal lamb kidney cells were infected with 0.5 ml of a 10^{-6} dilution of Wesselsbron virus (low passage strain). The cells showed complete cytopathic effect by the 4th day after infection. The culture fluid was harvested and tested for the presence of haemagglutinin.

4. <u>Preparation of goose red cell suspensions</u>

A goose was bled from the wing with a 20 ml syringe containing 10 ml of chilled normal saline. The contents of the syringe were immediately added to 100 ml of chilled saline in a 250 ml centrifuge tube. The cells were washed three times in normal saline by centrifugation and stored as a 10% suspension at 4°C.

The cells were suspended in the various adjusting diluents immediately before use to give a concentration of 0.25%. The optical densities of these cell suspensions were measured in a Unicam colorimeter fitted with an Ilford blue filter no. 303 and the suspensions were adjusted, when necessary, to an optical density of 0.78.

5. <u>Preparation of antisera for HI test</u>

Wesselsbron virus (low passage strain) antisera, prepared in mice and sheep, were treated with kaolin to remove certain non specific inhibitors of haemagglutination and adsorbed with goose cells to remove goose cell agglutinins before being used in the HI tests.

A 25% suspension of acid-washed kaolin was prepared in BBS. One part of serum and four parts of BBS were treated with five parts of 25% kaolin suspension for 20 minutes at 22°C. After centrifugation at 2,500 rpm for 30 minutes, 2.0 ml of the supernatant fluid were adsorbed with 0.04 ml packed washed goose cells at 4°C for 20 minutes. The mixture was shaken at intervals. The goose cells and agglutinins were removed by centrifugation at 1,500 rpm for 10 minutes at 4°C. The supernatant, at pH 8.6, was considered to represent a $^{1}/_{10}$ dilution of antiserum.

Mouse antisera to West Nile virus, Rift Valley Fever virus (neurotropic strain) and African Horsesickness virus (A 501) were treated in the same manner and were used as controls.

6. Technique for haemagglutination (HA)

Doubling dilutions from $^{1}/_{20}$ to $^{1}/_{10,240}$ were made in BBS/Bpa of the haemagglutinin preparations from both the low passage and the high passage strains. A volume of 0.4 ml of each haemagglutinin dilution was delivered into each of the 8 cups of standard perspex plates in the vertical rows.

Goose cells in various adjusting diluents (covering pH range 5.60 - 7.36, see Table 8) were added to each horizontal row of cups in the standard perspex plates.

The HA tests on the low passage and high passage strains of Wesselsbron virus were carried out in duplicate - one plate being incubated at 37°C and the other at 22°C.

The haemagglutinin of the fluid from Wesselsbron virus (low passage strain) infected FLK cells was tested on the 4th day after infection. The culture fluid was diluted $^1/_5$ with BBS/Bpa and tested at final hydrogen ion concentrations 5.64, 5.70, 5.94, 6.17 and 6.35 at 22°C.

Results were read two hours after addition of the goose red cells. Haemagglutinin titres were expressed as the reciprocal of the highest dilution showing complete haemagglutination. The "endpoint" of complete haemagglutination contained one haemagglutinating dose (1 HAD) in 0.4 ml.

7. Technique for haemagglutination inhibition (HI).

The haemagglutinin preparation from brain material infected with Wesselsbron virus (low passage strain) was diluted in BBS/Bpa to give an estimated 4-8 HAD'S in 0.2 ml.

Doubling dilutions of antisera, from $^1/_{20}$ to $^1/_{10,240}$ were made in BBS/Bpa in the horizontal cups in a standard perspex plate in 0.2 ml volumes. Amounts of 0.2 ml of Wesselsbron virus haemagglutinin, containing 4-8 HAD's, were added to all the cups containing the antisera dilutions. Haemagglutinin alone and the antisera alone were included as controls.

The test plates were incubated overnight at 4°C. The following day, 0.4 ml of goose cell suspension in the adjusting diluent which gave a final pH 6.1 - 6.2 (the optimal pH as determined in the preceding haemagglutination test) was added and the test plates incubated at room

temperature for two hours.

The HI titres of the antisera were expressed as the reciprocal of the highest dilution of each serum which gave complete inhibition of the reaction. The test was valid only if the control haemagglutinin titration showed presence of 4-8 HAD's and the antisera and goose red cell controls were negative.

C. RESULTS

The optimal conditions for haemagglutination of goose cells by

(i) Wesselsbron virus infected suckling mouse brain suspensions (low and high passage strains), and

(ii) the culture fluid harvested from Wesselsbron virus (low passage strain) infected FLK cell cultures were determined, since it was known that arboviruses varied markedly in their pH and temperature requirements for the demonstration of haemagglutinin activity.

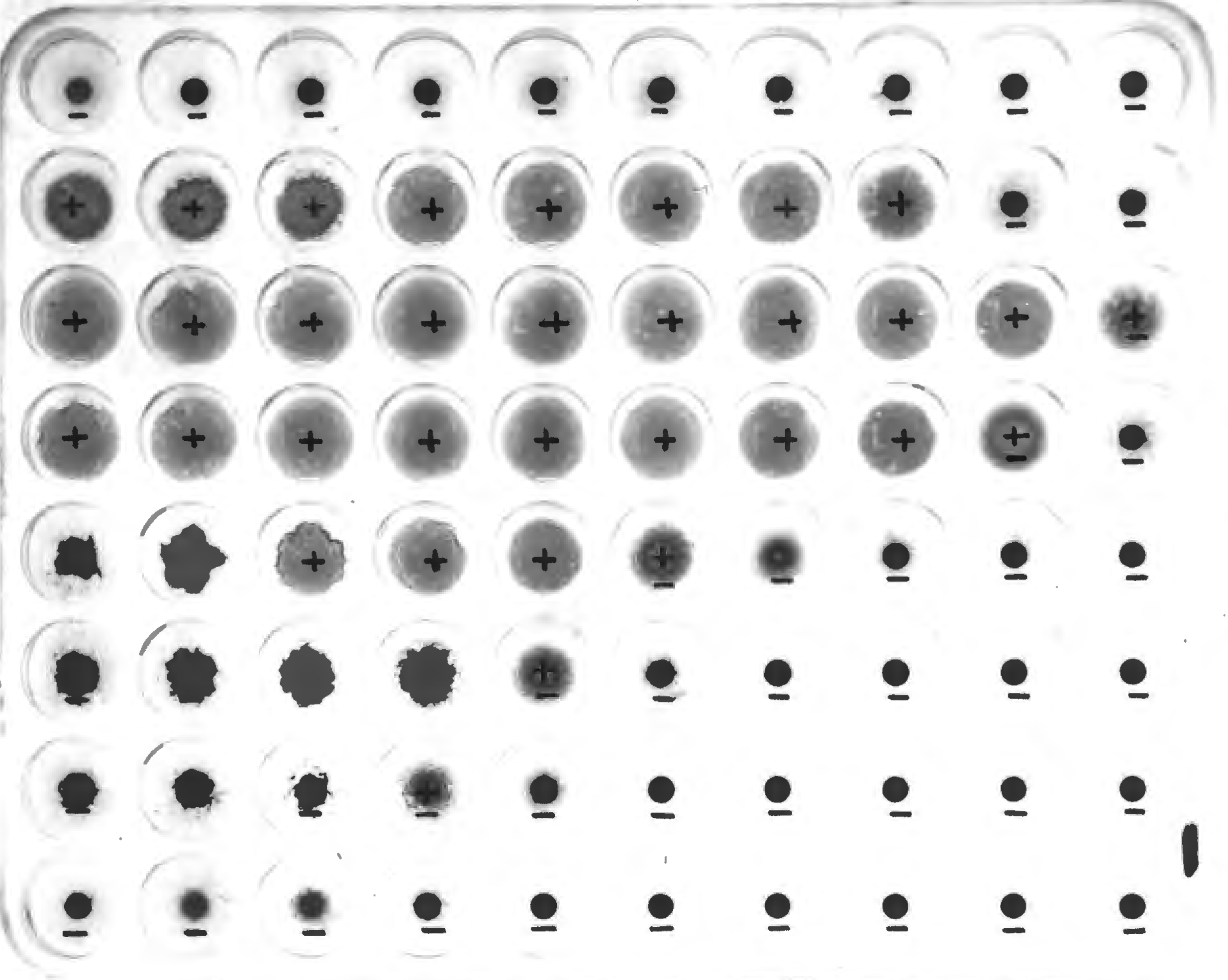
From the outset it was evident that goose cells, as recommended by Porterfield (1957) were very suitable for these procedures.

Haemagglutination with infected suckling mouse brain preparations

Table 9 reflects the results of haemagglutination, at different hydrogen ion concentrations, by BBS/Bpa extracts of suckling mouse brains infected with

Table 9

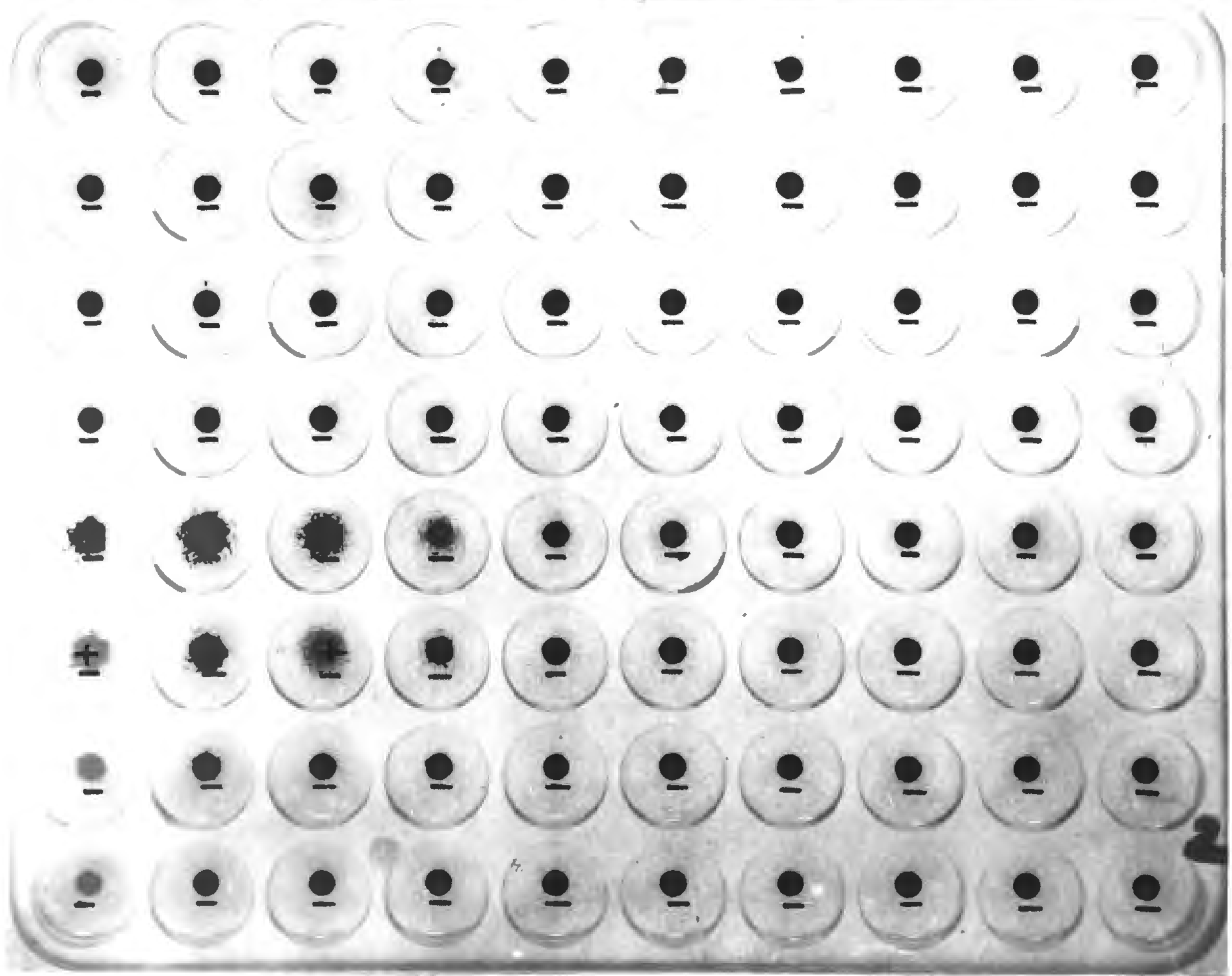
Haemagglutination, at different hydrogen ion concentrations, of Wesselsbron virus (low passage strain) extracted from suckling mouse brains.



1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
pH
5.60
5.93
6.11
6.31
6.58
6.76
6.97
7.36

Table 10

Haemagglutination, at different hydrogen ion concentrations, of Wesselsbron virus (high passage strain) extracted from suckling mouse brains.


1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
pH
5·60
5·93
6·11
6·31
6·58
6·76
6·97
7·36
2

the low passage strain of Wesselsbron virus and Table 10 records the results with similar preparations from brains infected with the high passage strain.

In both tables the findings are those of experiments carried out at room temperature (22°C), since comparable tests performed at 37°C showed exactly similar results, and since the phenomenon of elution was not encountered.

It is apparent from these results that the low passage strain causes haemagglutination of goose cells over a wide range of pH values and that the highest dilution of the haemagglutinin giving a complete (+) reaction is $^{1}/_{5120}$ at pH 6.11.

In striking contrast with this, the high passage strain showed practically no haemagglutination under the same conditions, other than a partial or uncertain reaction in the pH range 6.58 - 6.76 to a titre of only 80.

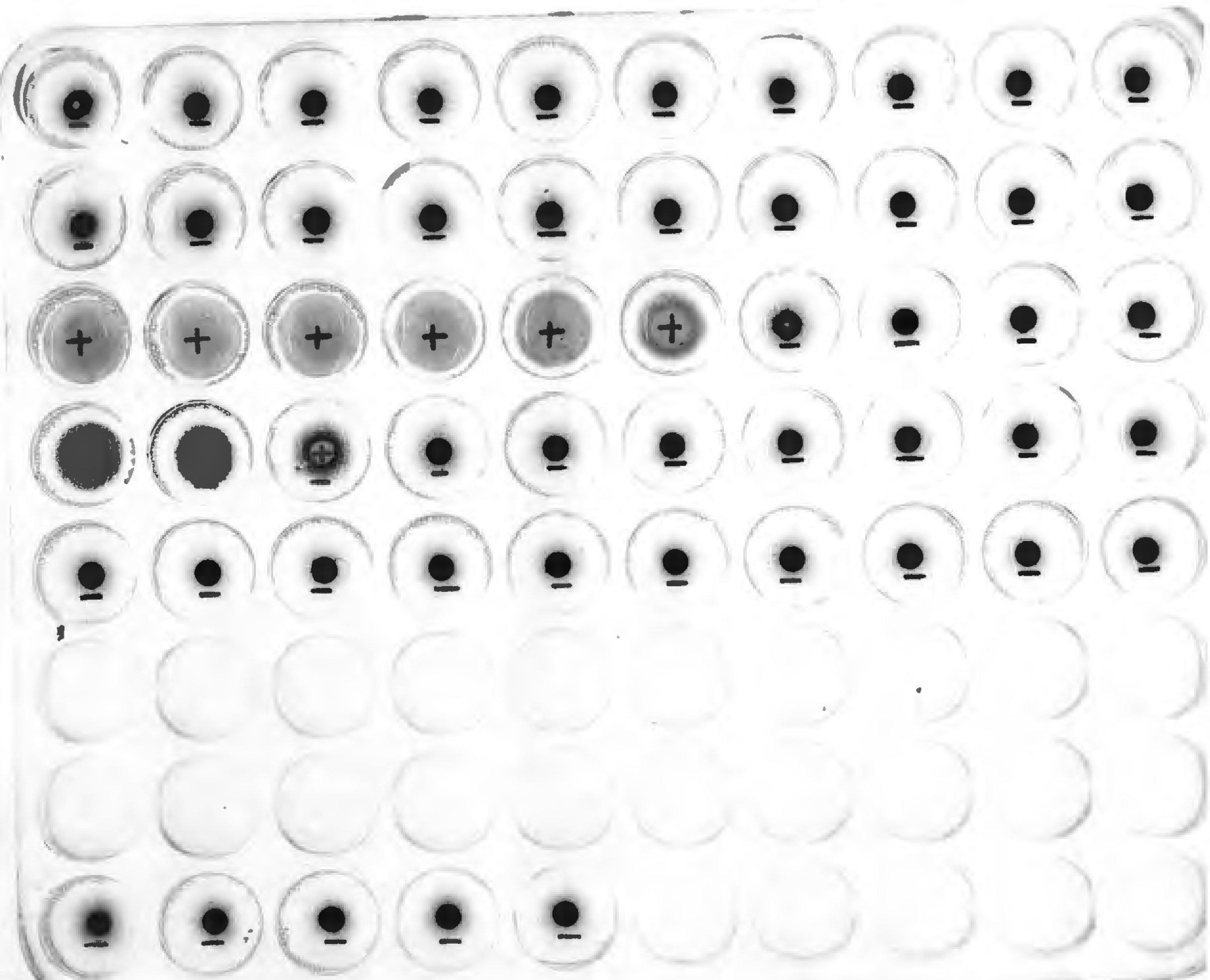
Haemagglutination with infected FLK cell culture preparations

Haemagglutination of goose cells by fluids harvested from Wesselsbron virus (low passage strain) infected FLK cell cultures is presented in Table 11. The pH range for optimal reaction was consistently lower and narrower and the haemagglutination titre much lower than that obtained with Wesselsbron virus infected suckling mouse brain haemagglutinin preparations. In the experiment recorded here, a haemagglutinin titre of 320 was obtained at pH 5.94.

This result was obtained from cultures infected with low passage strain

Table 11.

Haemagglutination, at different hydrogen ion concentrations, of Wesselsbron virus (low passage strain) harvested from infected FLK cell cultures.



1/10
1/20
1/40
1/80
1/60
1/320
1/640
1/1280
1/2560
1/5120
pH 5·60
5·70
5·94
6·17
6·35
RBC CONTROLS

of Wesselsbron virus. Although the high passage strain showed cytopathic effects in FLK cell cultures, the presence of haemagglutinin in these cultures was not confirmed.

Haemagglutination inhibition tests

The phenomenon of haemagglutination inhibition was demonstrated by mixing Wesselsbron virus (low passage strain) infected suckling mouse brain haemagglutinin preparations with homologous antisera (Table 12).

The HI titre of the antiserum prepared in mice was found to be 10,240 and that of the antiserum prepared in sheep, 5,120.

The results of the HI tests using heterologous as well as homologous antisera are presented in Table 13.

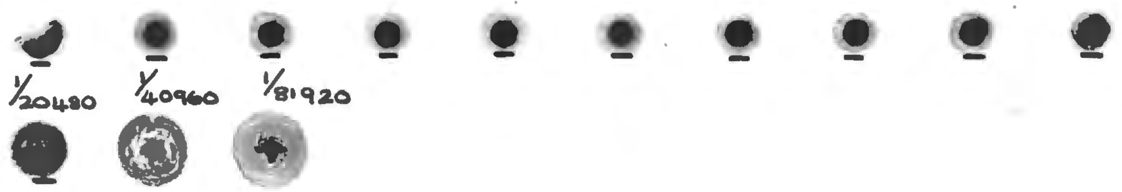
The Wesselsbron antisera prepared in mice inhibited haemagglutination by 4-8 HAD's to a titre of 10,240.

Antisera to the viruses of Rift Valley Fever and African Horsesickness similarly prepared in mice showed no inhibition of haemagglutination by Wesselsbron virus over the range of dilutions tested, starting at $^{1}/_{20}$.

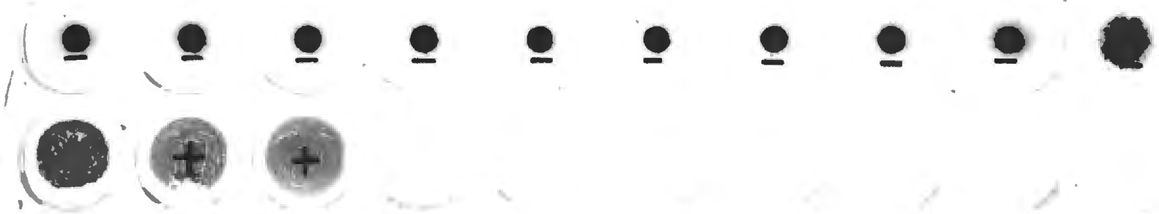
The mouse antiserum to West Nile virus however, caused significant inhibition to a titre of 320 suggesting the sharing of antigens between Wesselsbron virus and West Nile viruses. This observation is in keeping with the recommendation that Wesselsbron virus should be placed with the Group B arboviruses.

Table 12

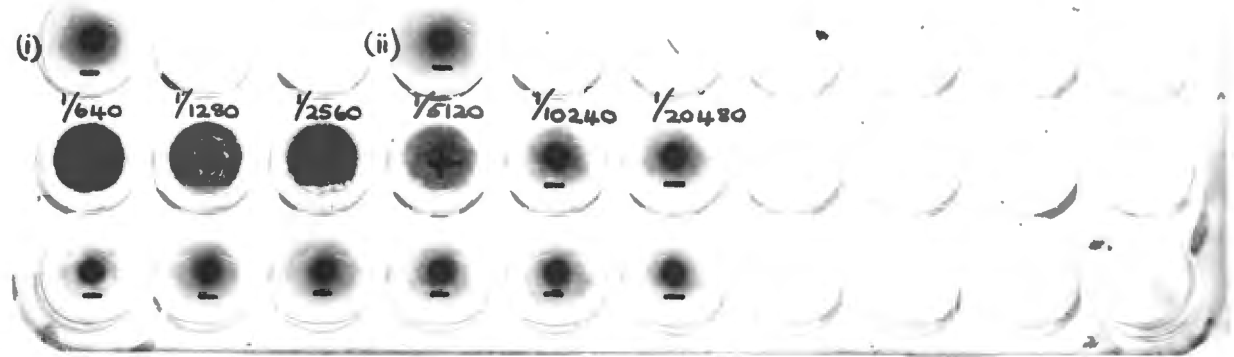
Haemagglutination inhibition of Wesselsbron virus (low passage strain), extracted from suckling mouse brains, by homologous antisera prepared (i) in mice
(ii) in sheep.



1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
1/5120
1/10240
(i)
1/20480
1/40960
1/81920



(ii)



CONTROLS
As
Ag
RBC
(i)
(ii)
1/640
1/1280
1/2560
1/5120
1/10240
1/20480

Table 13

Haemagglutination inhibition of Wesselsbron virus (low passage strain), extracted from suckling mouse brains, by antisera to (i) Wesselsbron virus (prepared in mice)
(ii) West Nile virus
(iii) Rift Valley Fever virus
(iv) African Horsesickness virus.

1/20 1/40 1/80 1/160 1/320 1/640 1/1280 1/2560 1/5120 1/10240

(i)

(ii)

(iii)

(iv)

As

(i) (ii) (iii) (iv)

1/640 1/1280 1/2560 1/5120 1/10240 1/20480 1/40960 1/81920 1/163840 1/327680

Ag

RBC

CONTROLS

D. DISCUSSION

A haemagglutinin for goose cells at pH 6.11 and 22°C has been demonstrated in BBS/Bpa extracts of the low passage strain of Wesselsbron virus from infected suckling mouse brains. A positive haemagglutination result was obtained in a dilution of $^1/_{5120}$ under conditions of optimal pH and temperature.

No significant haemagglutinin activity was shown in similar extracts of the high passage strain of Wesselsbron virus from infected suckling mouse brains.

Sabin (1951) reported that the strains of the four Group B arboviruses, Japanese B Encephalitis, St. Louis Encephalitis, West Nile and Russian Spring-Summer Encephalitis, which had undergone more than 100 mouse passages, failed to yield haemagglutinin.

The optimal pH of the haemagglutinin recovered from the fluid of Wesselsbron virus (low passage strain) infected FLK cell cultures is lower than that for the haemagglutinin prepared from suckling mouse brains, little activity was demonstrated at the optimal pH of the latter. The pH range of the FLK cell culture haemagglutinin was found to be narrower, and the HA titre considerably lower.

These observations are similar to those of Kundin and Diercks (1960)

and Diercks, Kundin and Porter (1961) who showed that Japanese B Encephalitis virus haemagglutinins from hamster kidney cell culture fluids had a much lower titre and a narrower pH range than the haemagglutinin extracted from the infected suckling mouse brains.

Non-specific inhibitors in the calf serum added to Hanks' LA medium were thought to be responsible for the low titres of the FLK cell culture haemagglutinins. The calf serum was therefore treated with kaolin before addition to the Hanks' LA medium but no increase in haemagglutinin titre resulted.

Likar, Buckley and Clarke (1962) found that by omitting phenol red from tissue culture fluids, the titre of the haemagglutinins produced in infected cell cultures increased; however, this was not found to be the case in Wesselsbron virus infected tissue cultures.

A recent report by Darwish and Hammon (1966) describes a method for producing a high titred haemagglutinin from hamster kidney cell cultures infected with Japanese B Encephalitis virus. This procedure involves the removal of the serum-containing medium at the first sign of cytopathic effect and the replacement with 2.5 ml serum-free medium 199 at pH 8.0. The fluid was harvested 16 hours later and haemagglutination titres of at least $^1/_{1024}$ were obtained.

The haemagglutination inhibition test showed no sharing of antigens

with the two arboviruses causing Rift Valley Fever and African Horsesickness, but a distinct antigenic cross reaction with one Group B arbovirus, i.e. West Nile virus.

VI - DENSITY GRADIENT ZONE ELECTROPHORESIS

A. INTRODUCTION

Zone electrophoresis in a density gradient may be used for the purification or separation of viruses and for the determination of the homogeneity of virus preparations. It is not suitable however, for the measurement of absolute electrophoretic mobilities.

Density gradient electrophoresis was introduced by Brakke (1953a, 1955) for the separation of plant viruses. The electrophoretic method of Svenson and Valmet (1955) was applied by Cramer, Lerner and Polson (1957) to the study of Mouse Encephalomyelitis virus (FA strain). Polson and Cramer (1958), using a modified Svensson and Valmet apparatus, achieved a satisfactory degree of purification of Type 1 Poliovirus. Rous Sarcoma (Cramer 1959) and Polyoma (Cramer and Stewart 1960) have also been studied by the same method. Polson and Deeks (1962) used density gradient zone electrophoresis for the classification of enteroviruses. Van Regenmortel (1960, 1961 and 1964 a and b), van Regenmortel and Fowle (1962) and van Regenmortel, Nel and Hahn (1964) have successfully applied the method to the study of plant viruses.

The technique of zone electrophoresis in a sucrose density gradient was applied to semipurified suspensions of Wesselsbron virus (low passage strain) in order to determine possible differences in the electrophoretic mobilities of the infective particle and the haemagglutinin.

The apparatus used in the experiments on Wesselsbron virus is a modification of that used by Polson and Cramer (1958). The difference lies in the design of the capillary sampling tube.

B. MATERIALS AND METHODS

1. Preparation of virus suspension.

Ten Wesselsbron virus infected suckling mouse brains were triturated in 10 ml BBS containing 0.4% Bpa and centrifuged at 10,000 rpm for 10 minutes. The virus was then concentrated by centrifuging the supernatant at 30,000 rpm for 60 minutes. The pellet was redispersed in 3 ml of a 35% sucrose solution in BBS +0.25% Bpa and clarified by centrifugation at 8,000 rpm for 10 minutes. To 2 ml of the supernatant, phenol red and haemoglobin were added. The progress of the electrophoresis was gauged by the distance migrated by these two reference substances.

2. Procedure of zone electrophoresis in a density gradient

A 40% (w/v) sucrose solution was prepared in BBS and adjusted to pH 8.6 with NaOH.

The apparatus is shown in Fig 2. The sucrose density gradient is formed in vertical column A through capillary X; C and D are the electrode vessels and the electrodes used were Ag - AgCl in saturated sodium chloride. The apparatus was sterilized in boiling distilled water before use.

With the capillary sampling tube (X) in the lowered position, BBS pH 8.6 was introduced through Y to the level of the tubes connecting the U-tube with the electrode vessels. The 40% sucrose solution containing 0.25% Bpa was introduced through Y and allowed to rise to the top of the funnel (Z) of the capillary sampling tube. The stopcock was then closed and the capillary sampling tube was adjusted to the raised position.

The gradient mixing device is shown in Fig 3. Flask A was filled with 150 ml of BBS and Flask B with 150 ml 40% sucrose. Both flasks contained 0.25% Bpa. Flask A was closed with a rubber stopper and placed over a magnetic stirrer. The mixing device delivers a solution with a steadily increasing sucrose concentration. The gradient formed is logarithmic. The sugar concentration gradient formed in column A is approximately linear in the range in which the observations were made (Figure 4) (Polson and Deeks 1962).

Two ml of the virus suspension was introduced through X and was followed by 15 ml of 40% sugar (containing 0.25% Bpa) to raise the virus level to a level designated the origin (O). The capillary sampling tube was then adjusted to the lowered position.

Fig. 2.

Zone electrophoresis apparatus.

(i) capillary sampling tube (X) in raised position
(ii) capillary sampling tube (X) in lowered position

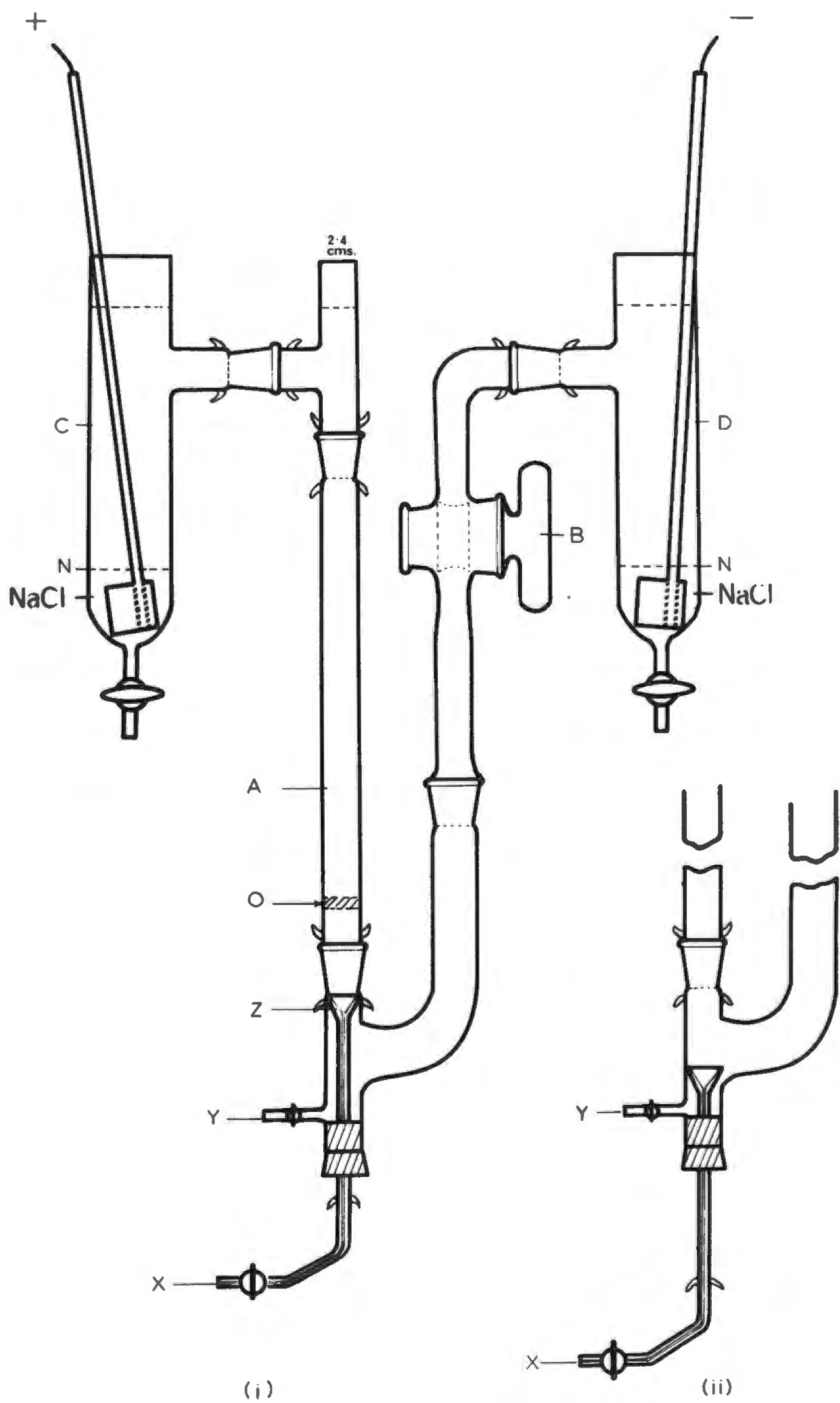

+
−
2·4 cms.
C
D
B
N
N
NaCl
NaCl
A
O
Z
Y
X
Y
X
(i)
(ii)

Fig. 3.

Gradient mixing device.

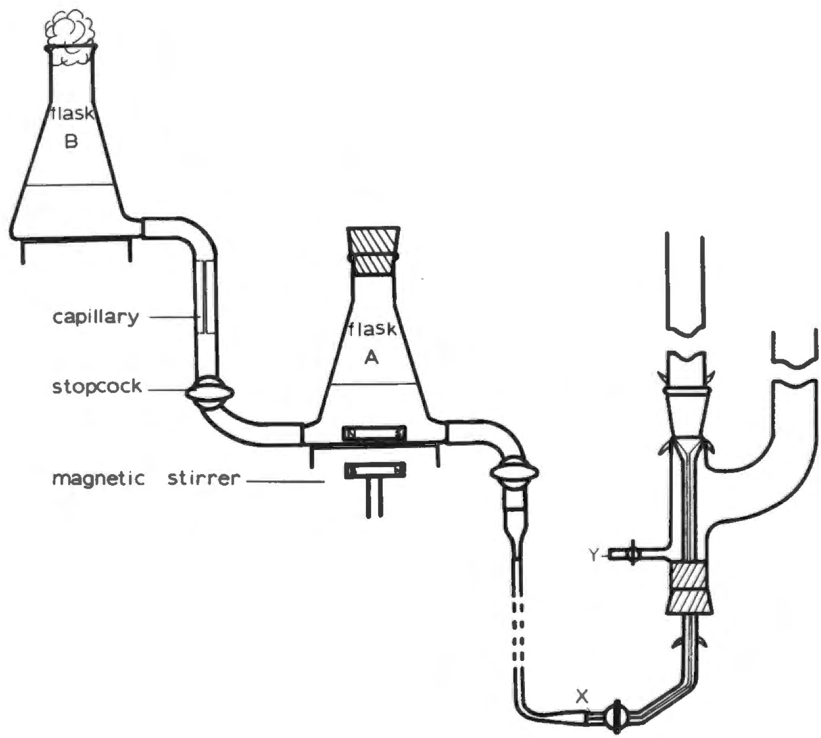
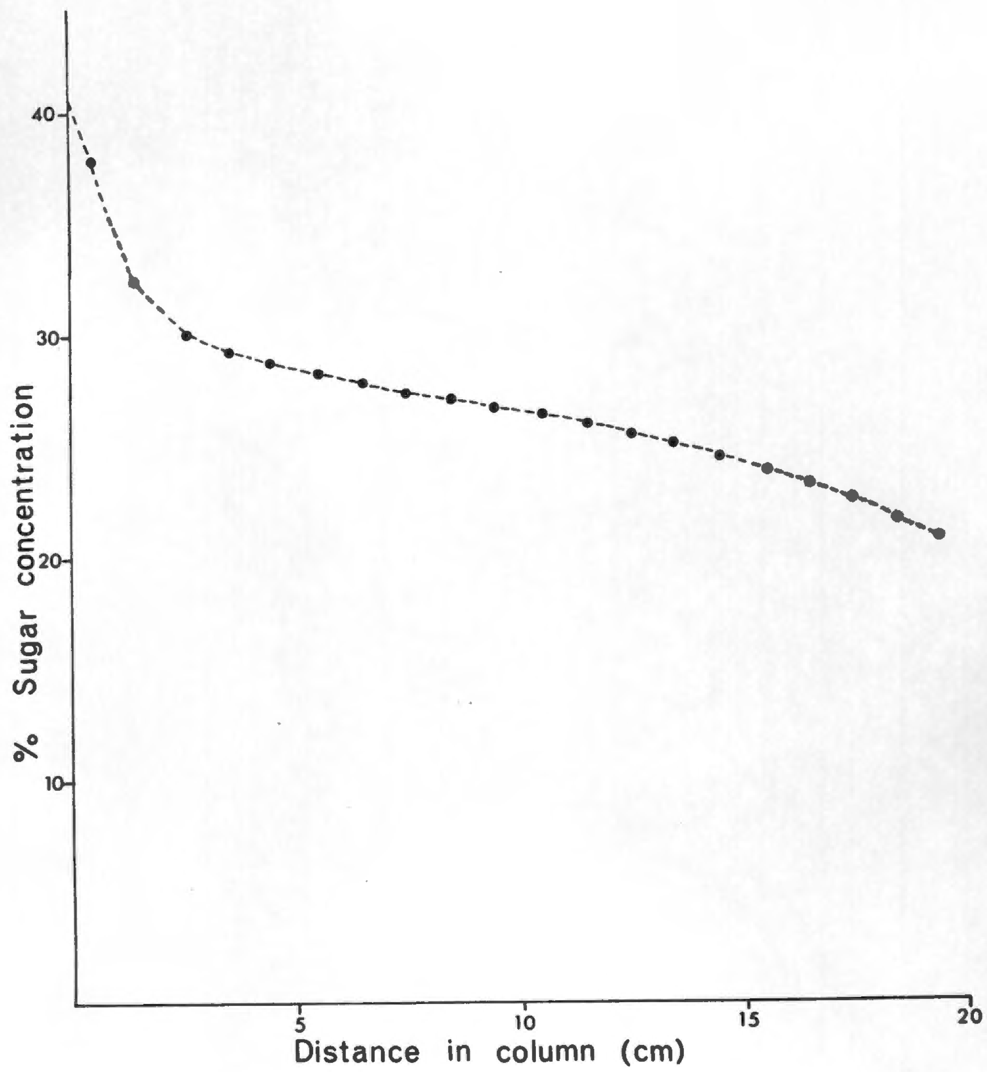

flask
B
capillary
stopcock
flask
A
magnetic stirrer
Y
X

Fig. 4.

Sugar concentration gradient formed in column A of the electrophoresis apparatus.


40
30
20
10
% Sugar concentration
5
10
15
20
Distance in column (cm)

A saturated solution of NaCl was added to each of the electrode vessels to the levels N; Ag - AgCl electrodes were inserted and the levels of the buffer in the vessels were equalized. Stopcock B was then opened cautiously.

A time lapse of at least 20 minutes was necessary for a concentration gradient to form in the virus band at O. In this way, the undesirable effect of convection was avoided.

Electrophoresis was allowed to proceed for 22-23 hours at 4°C with a current of 22 mA. In this time the phenol red migrated approximately 13 cm from the origin O. The stopcock was closed and the column examined with an argon mercury fluorescent lamp (Fig 5) for regions of opalescence; such regions were recorded photographically.

Capillary X was returned to the raised position and 15 ml of sucrose were run out and discarded. Samples of 1 cm column length (approximately 3 ml) were subsequently collected and the fractions titrated for infectivity in FLK cells in roller tubes and tested for haemagglutinating activity against goose cells at pH 6.11 at 22°C.

C. RESULTS

Photographs obtained from two typical zone electrophoresis experiments on Wesselsbron virus are shown in Fig 6. The reference substances, haemoglobin

Fig. 5.

Argon mercury fluorescent lamp.

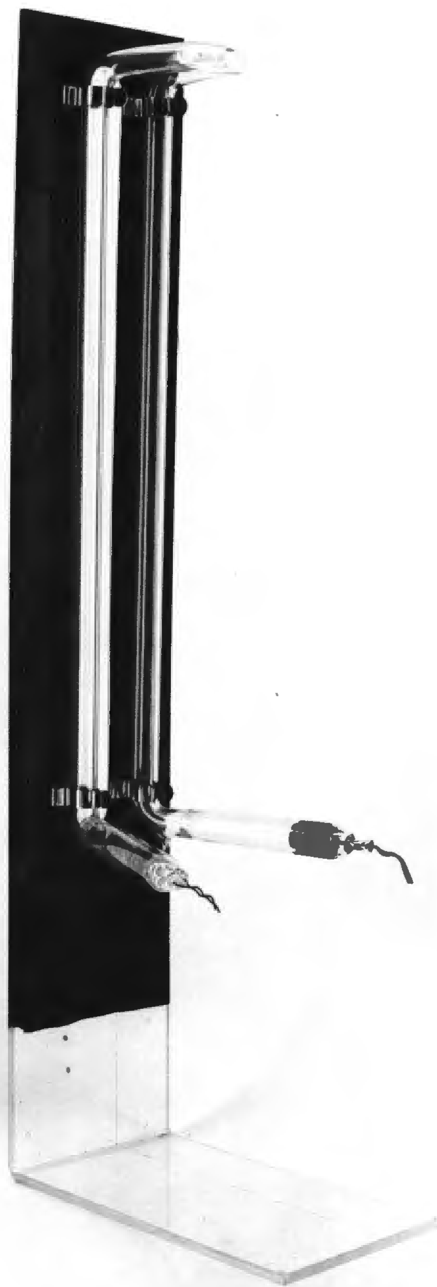
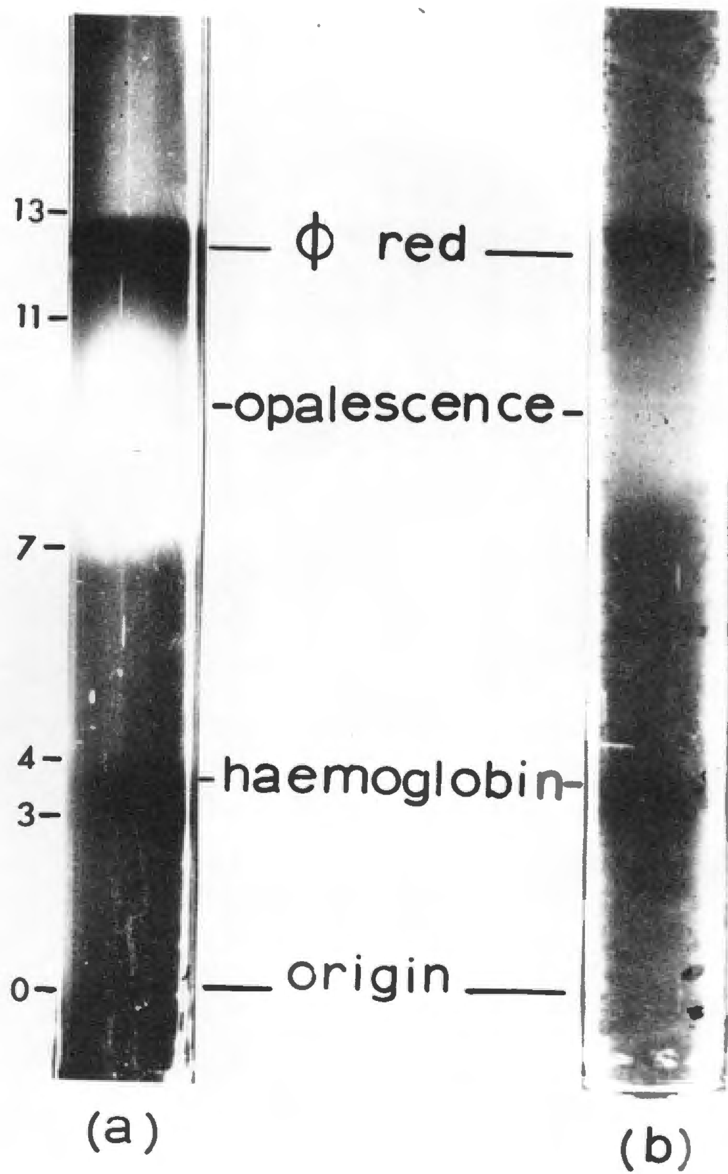


Fig. 6.

Photographs of the columns from two zone electrophoresis experiments on Wesselsbron virus.


13
11
7
4
3
0
ϕ red
opalescence
haemoglobin
origin
(a)
(b)

and phenol red, are evident at positions 3-4 cm and 11-13 cm from the origin O respectively. A zone of opalescence is visible at a position 7-10 cm from the origin. The density regions in the photographic negatives from the same two experiments were measured with a Beckman analytrol (densitometer) and are presented graphically in Fig 7.

The infectivity and haemagglutination titrations of the individual samples collected from the column after the electrophoresis of the virus suspension for 22 hours are recorded in Table 14 and Fig 8.

It is evident from Table 14 and Fig 8 that there is a main infective component 9-12 cm from the origin and it has a slightly slower migration rate than the phenol red. This component corresponds with one of the lesser peaks, beyond the region of greatest density, shown in the graphical presentation of the densitometric recordings of the same experiment in Fig 7b.

It is suggested from these results that the zone of light scattering in the electrophoresis column may be separated from the zone of highest virus concentration by prolonging the period of electrophoresis beyond the 22 hour period of this experiment.

Fig. 7.

Densitometric recordings obtained from the photographs of the two zone electrophoresis experiments (see Fig.6).

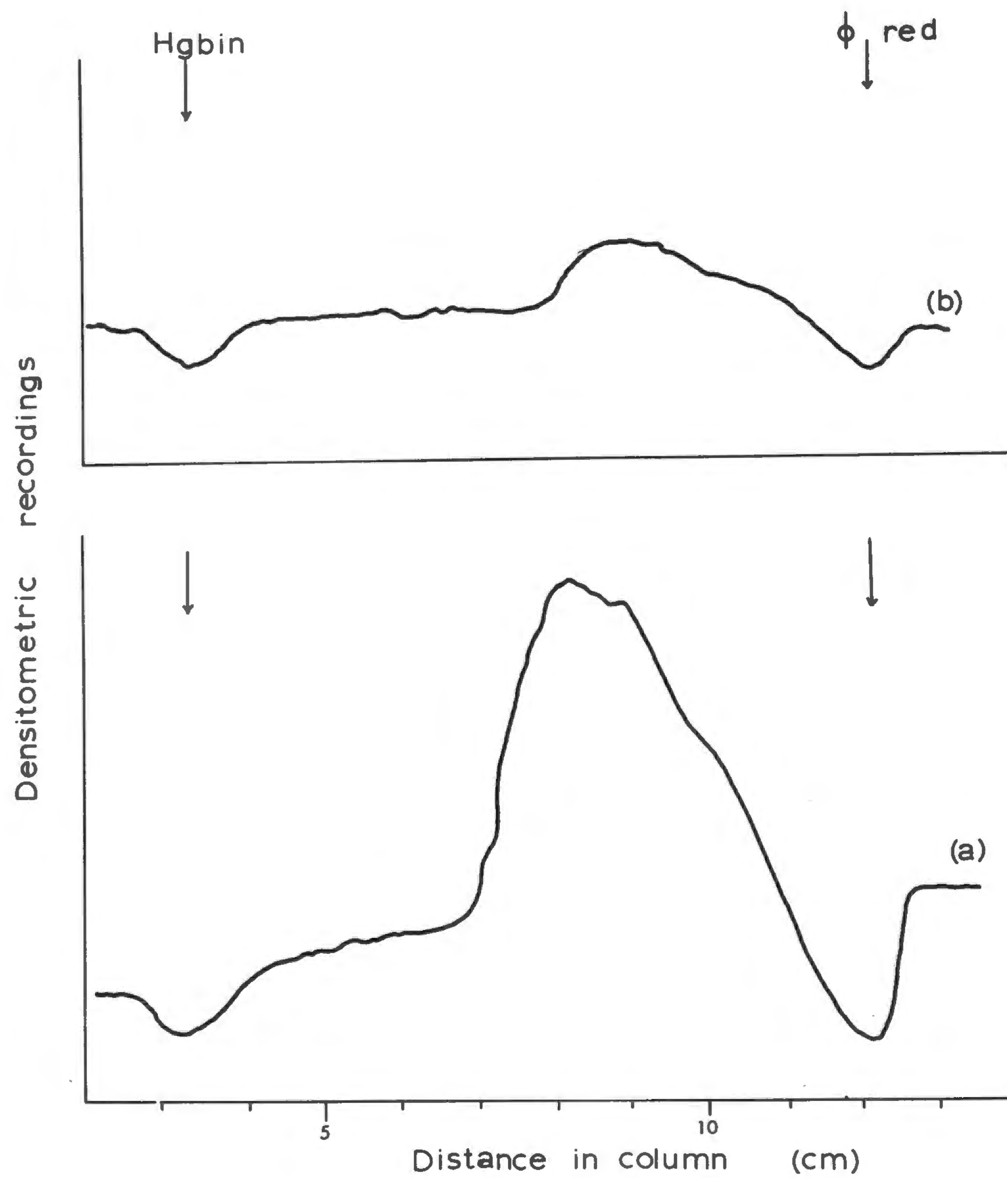

Hgbin
ϕ red
(b)
(a)
Densitometric recordings
5
10
Distance in column (cm)

Table 14. Infectivity and haemagglutination titrations of samples collected after zone electrophoresis of Wesselsbron virus for 22 hours at 4°C.

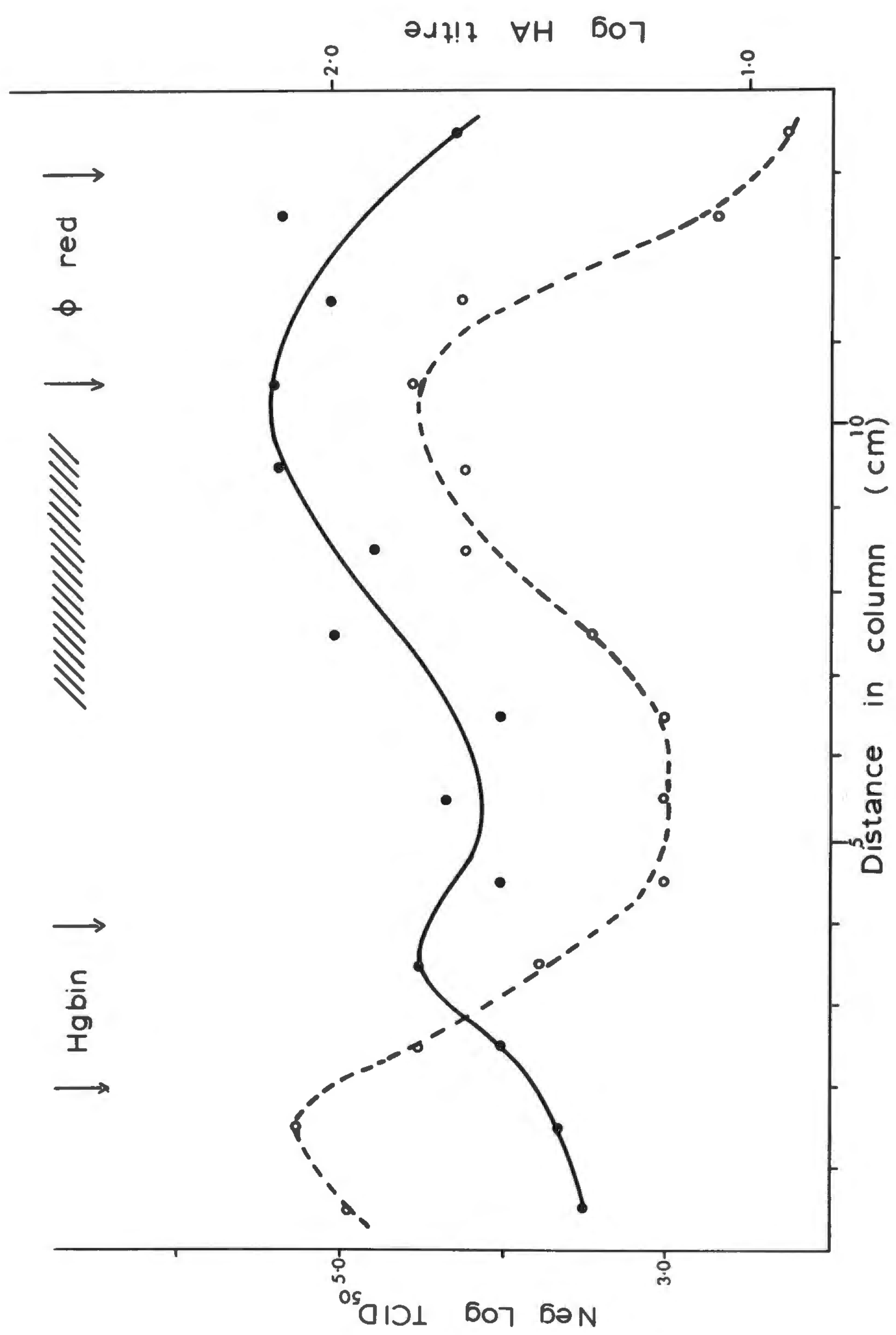
Distance in column (cm from origin)	Infectivity $TCID_{50}$/0.1 ml	Haemagglutination HA titre/0.4 ml
1	3.50	128
2	3.66	192
3	4.00	64
4	4.50	32
5	4.00	16
6	4.33	16
7	4.00	24
8	5.00	24
9	4.75	32
10	5.33	32
11	5.33	48
12	5.00	32
13	4.75	8
14	4.25	6

Fig. 8.

Infectivity and haemagglutination titration results of the individual samples collected after zone electrophoresis of Wesselsbron virus for 22 hours at 4°C.

———— = infectivity

-------- = haemagglutination



Hgbin
φ red
Log HA titre
2·0
1·0
Neg Log TCID50
5·0
3·0
Distance in column (cm)
5
10

D. DISCUSSION

It is apparent that there are two distinct haemagglutinating components, one of which is associated with the infective particles. The non infectious haemagglutinin component of low mobility consists either of products of disintegration of the viral coat or of incomplete virus particles which are still capable of demonstrating haemagglutinating activity and which carry little or no nett charge.

It is also evident that the infective particles are spread over a wide range of fractions and therefore are electrophoretically inhomogeneous. The significance of the minor infectivity peak consisting of particles of comparatively low mobility has not yet been assessed. The major infective component with its accompanying haemagglutinin has a mobility only slightly less than that of the phenol red reference substance and has a migration rate similar to that of the arbovirus, African Horsesickness virus (pantropic strain) (Polson and Russell 1965).

These findings suggest that zone electrophoresis in a density gradient may be an effective method for the purification of Wesselsbron virus.

VII - DENSITY GRADIENT CENTRIFUGATION

A. INTRODUCTION

Density gradient centrifugation has been used for the isolation, separation and purification of macromolecules and Brakke (1951, 1953b) has shown it to be an effective method for the separation of plant viruses.

In the basic method of equilibrium sedimentation in caesium chloride (CsCl) density gradients (Meselson, Stahl and Vinograd 1957), the CsCl solution is allowed to equilibrate under the influence of a centrifugal field. The macromolecules are driven by the centrifugal force to a region where the difference between the density of a given molecule and that of the suspending medium is zero, that is they reach their isodensity level.

The technique described by Polson and Levitt (1963), in which a preformed gradient of CsCl is prepared, and the material under investigation inserted at a position in the gradient close to its expected isodensity level, considerably reduces the equilibration time.

The buoyant densities of the arboviruses, Rift Valley Fever (Polson and Levitt 1963), Sindbis (Mussgay and Rott 1964), Dengue Type 2 (Stevens and Schlesinger 1965) and African Horsesickness, strain A501 (Russell 1965) have been determined.

Using the method of Polson and Levitt (1963) the buoyant densities of the infective particle and of the haemagglutinin of Wesselsbron virus (low passage strain) have been determined.

B. MATERIALS AND METHODS

1. Preparation of virus suspension

Two Wesselsbron virus infected suckling mouse brains were triturated in 8 ml of 0.02 M phosphate buffer, at pH 7.0, containing 0.4% bovine plasma albumin (phosphate buffer/Bpa). The suspension was clarified by centrifugation at 10,000 rpm for 10 minutes. The supernatant was centrifuged at 30,000 rpm for 60 minutes and the resulting pellet resuspended in 4 ml of phosphate buffer/Bpa. After further clarification at 10,000 rpm for 10 minutes, 0.5 ml of this virus suspension was mixed with 0.5 ml of the 60% CsCl solution. This mixture, containing a final CsCl concentration of 30%, was inserted in the preformed density gradient in the relevant position.

Twelve brains instead of two were used for the preparation of a Wesselsbron virus suspension containing a HIGH concentration of virus. The suspension was treated in the same way as the above LOW concentration virus preparation.

2. Preparation of Caesium chloride gradient

A 60% (w/v) solution of CsCl was prepared in phosphate buffer/ Bpa.

Solutions containing 6 to 54% of CsCl were prepared by mixing the original CsCl solution (60%) with phosphate/buffer/Bpa in different proportions as shown in Table 15.

Table 15. CsCl solutions for the formation of the density gradient.

60% CsCl solution (ml)	phosphate buffer/ Bpa (ml)	Final CsCl concentration (%)
0.1	0.9	6
0.2	0.8	12
0.3	0.7	18
0.4	0.6	24
0.6	0.4	36
0.7	0.3	42
0.8	0.2	48
0.9	0.1	54

The densities of two of these solutions were determined with the aid of a pyknometer and their refractive indices were measured using an Abbe refractometer. The values obtained were checked for accuracy with

the standard refractometric density curve prepared in this laboratory.

The density gradient was formed in a syringe, supported in the apparatus as shown in Fig 9, by slowly withdrawing 0.4 ml of each of the CsCl solutions (Table 15), starting with the least dense. The virus suspension (0.4 ml) was inserted at the 30% CsCl level. The contents of the syringe were then carefully transferred to a 12 x 50 mm cellulose nitrate centrifuge tube; the apparatus being raised during the introduction of the gradient into the tube in order to keep the tip of the needle just below the meniscus.

3. <u>Centrifugation procedure</u>

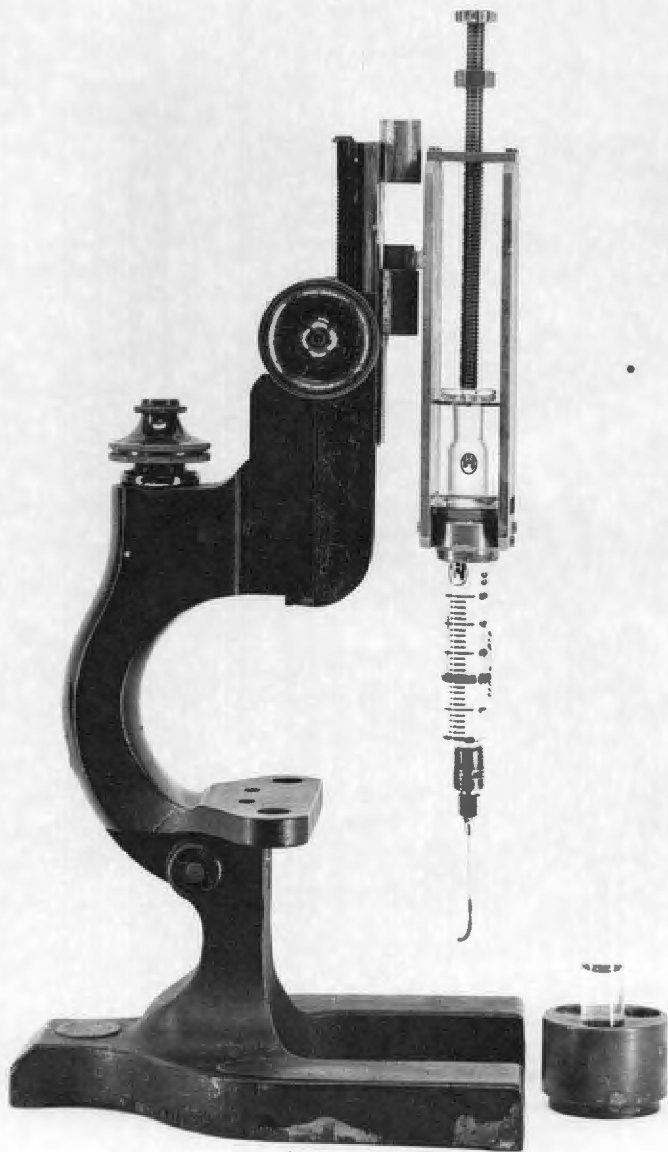
The centrifugation was carried out in the swinging bucket SW-39 rotor in the Spinco model L ultracentrifuge at 30,000 rpm for 3 hours at 0-4°C.

After removal from the rotor, the bottom of the tube was pierced with a sterile pin and eleven consecutive samples, consisting of 10 drops each, were collected in sterile test tubes. Using a known volume of each fraction, the refractive indices were determined.

After dialysis against one litre of 0.02 M phosphate buffer, at pH 7.0, for 2 hours at 4°C with continual stirring, serial tenfold dilutions of the samples were prepared in serum saline and titrated for infectivity in mice.

Fig. 9.

Apparatus used for the formation of the CsCl density gradient.



Twofold dilutions were used in the haemagglutination titration.

C. RESULTS

The standard refractometric density curve of the CsCl solutions is presented in Fig 10, and it is routinely used in all density gradient centrifugation experiments. In order to ensure that the conditions of the present experiment fell within these standards, the densities and the refractive indices of two of the samples from one experiment were determined and were found to fall exactly on the standard curve.

Density gradient centrifugation using a LOW concentration of Wesselsbron virus

The results of the infectivity titrations of the eleven samples from one experiment after 3 hours centrifugation at 30,000 rpm are shown in Fig 11. A sharp peak of infectivity was obtained at density of 1.22 gm/cc. The slight increase in titre in the least dense fraction (1.024 gm/cc) may be due to the association of some of the infective particles with light lipoidal matter from the mouse brains which rise up the gradient.

No haemagglutinin activity was exhibited by these samples.

Fig. 10.

Standard refractometric density curve of the CsCl solutions.

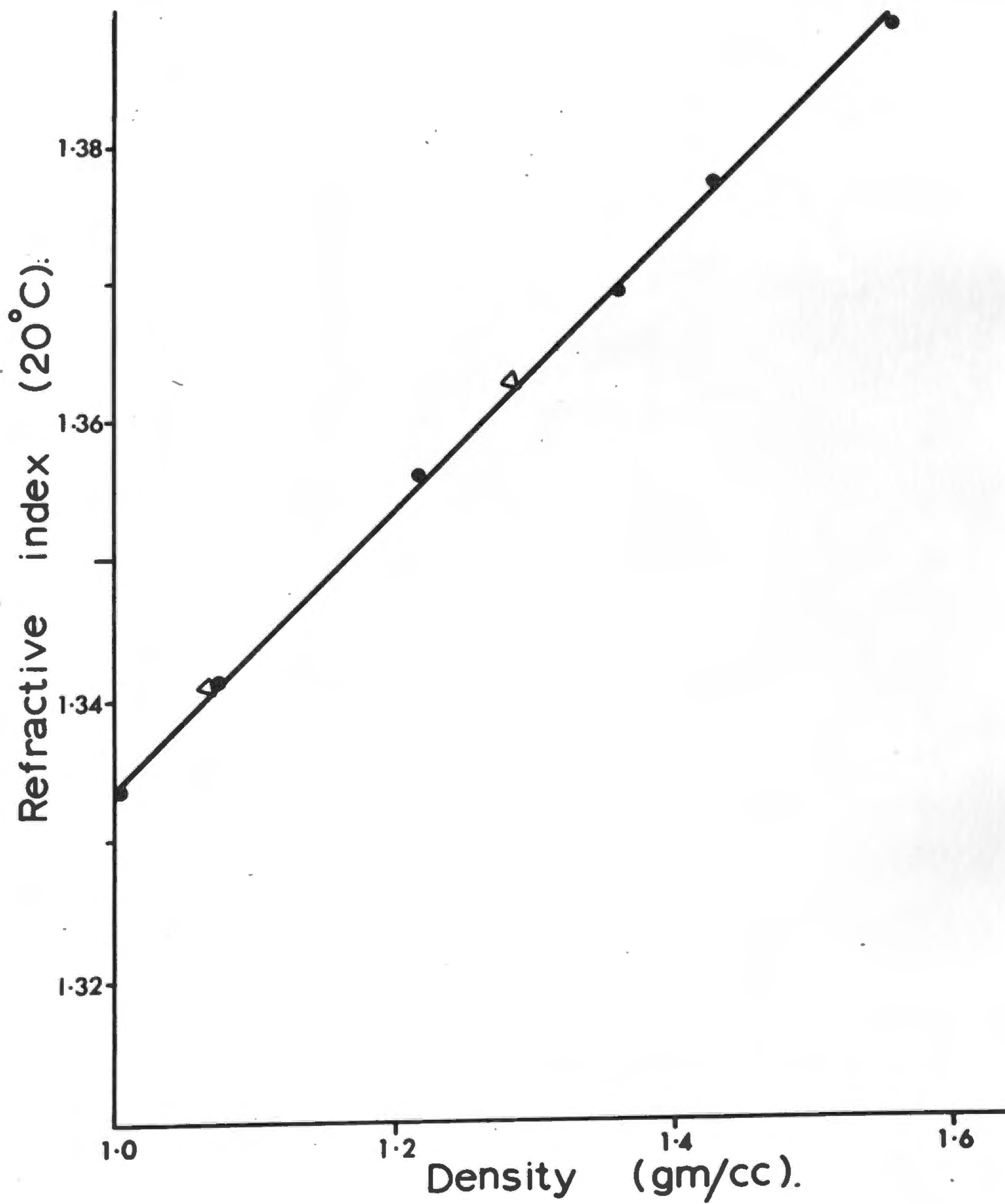
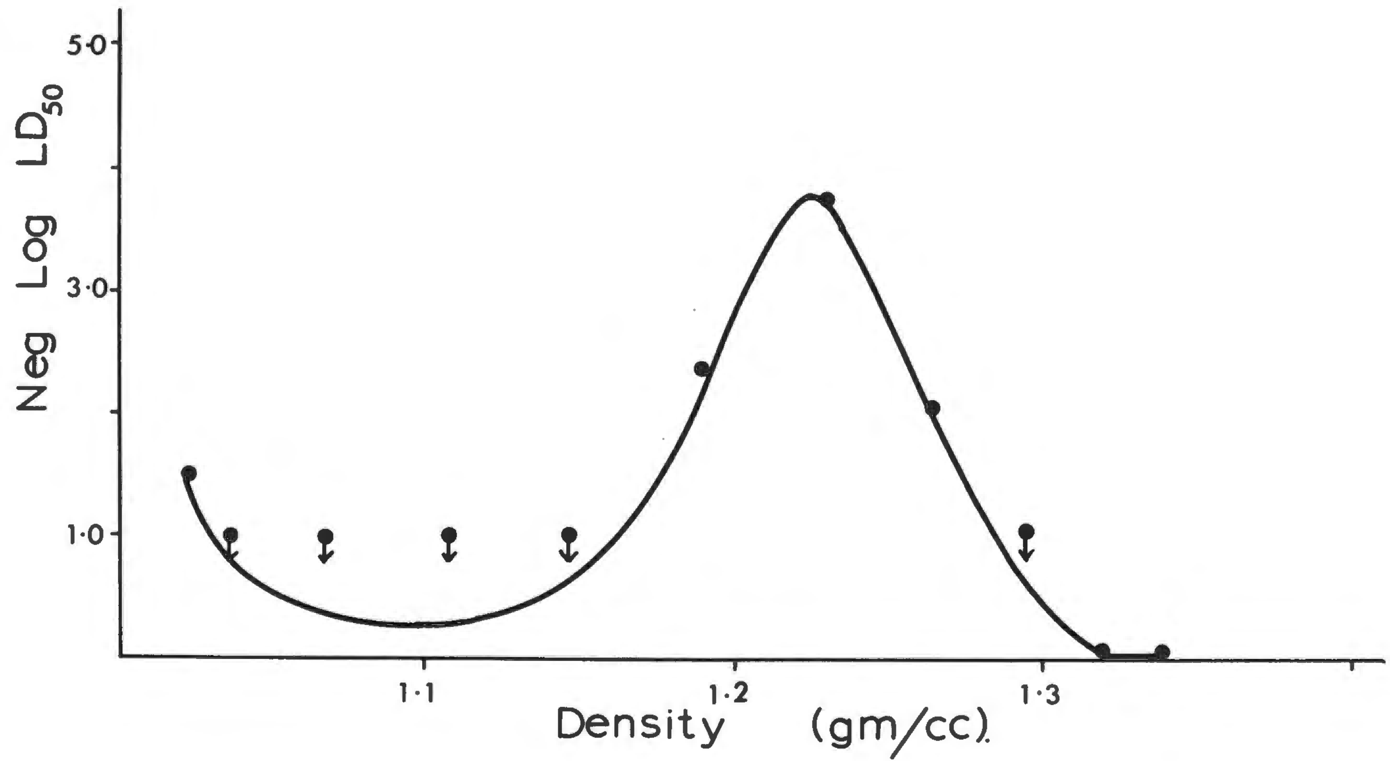

1·38
1·36
1·34
1·32
Refractive index (20°C).
1·0
1·2
1·4
1·6
Density (gm/cc).

Fig. 11.

Infectivity titration results after CsCl density gradient centrifugation of Wesselsbron virus using a LOW concentration of virus.


Neg Log LD50
5·0
3·0
1·0
1·1
1·2
1·3
Density (gm/cc).

Density gradient centrifugation using a HIGH concentration of Wesselsbron virus.

The results of the infectivity titrations of the eleven samples from one of the experiments in which a HIGH concentration of virus was used, are given in Fig 12. A density of 1.22 gm/cc was again obtained for the main infective particle but the infectivity in the gradient was more widely dispersed and a considerable amount of infective material had risen to the top of the tube.

In this experiment, haemagglutination was detectable and the haemagglutinin was distributed widely between densities 1.189 and 1.327 gm/cc with two apparent maxima at 1.189 and 1.268 gm/cc (Table 16).

D. DISCUSSION

The infective virus particles appear to be fairly homogeneous with respect to density, and their distribution in the gradient was found to be Gaussian (Meselson, Stahl and Vinograd 1957). The buoyant density of Wesselsbron virus was found in both experiments to be 1.22 gm/cc. This was a somewhat surprising result since virus particles of this order of size might have been expected to have a density of 1.33 gm/cc. The lesser density in this instance may be accounted for by the incorporation of a lipid component in the Wesselsbron virus particle, a suggestion which finds some

Fig. 12.

Infectivity titration results after CsCl density gradient centrifugation of Wesselsbron virus using a HIGH concentration of virus.

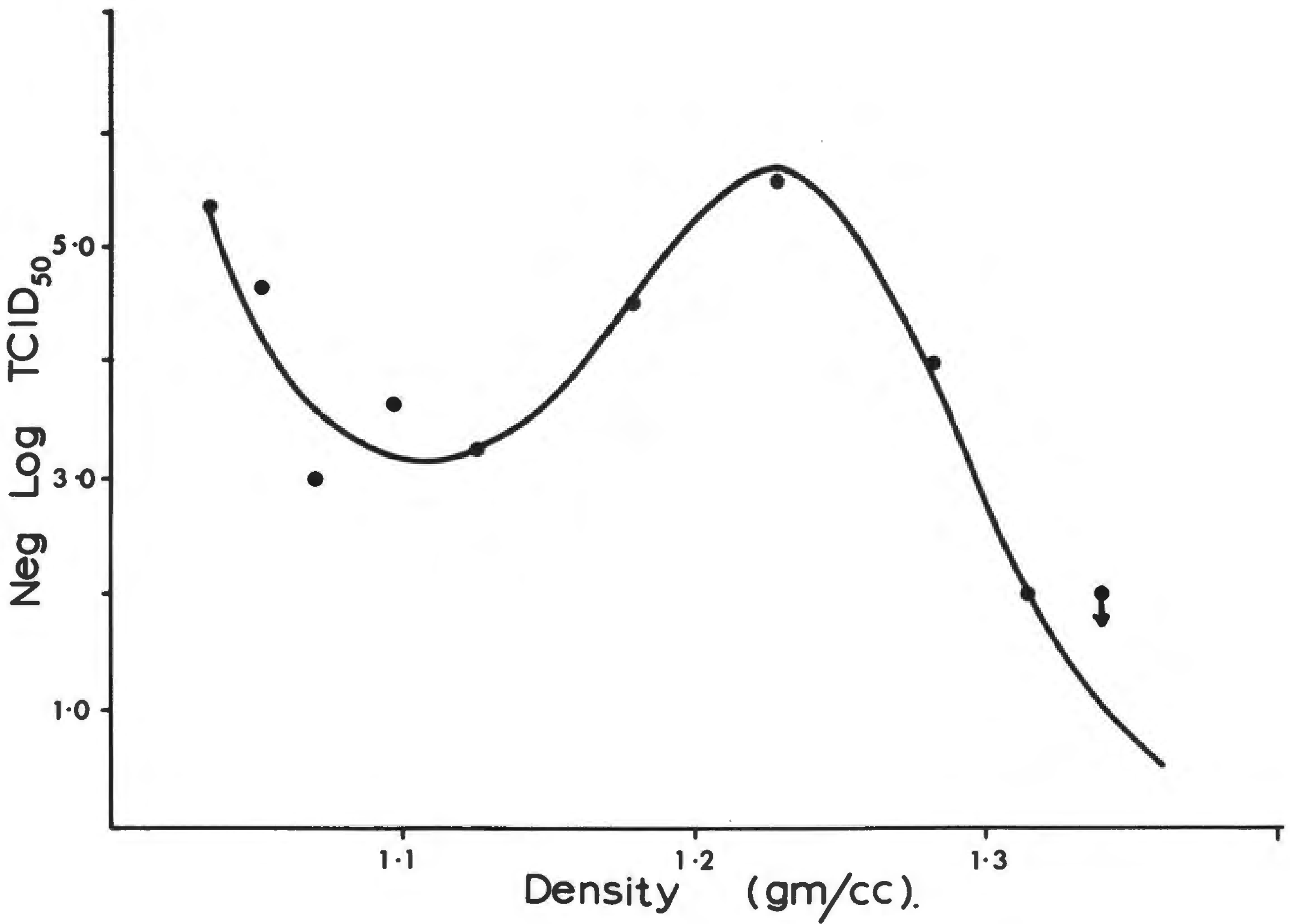
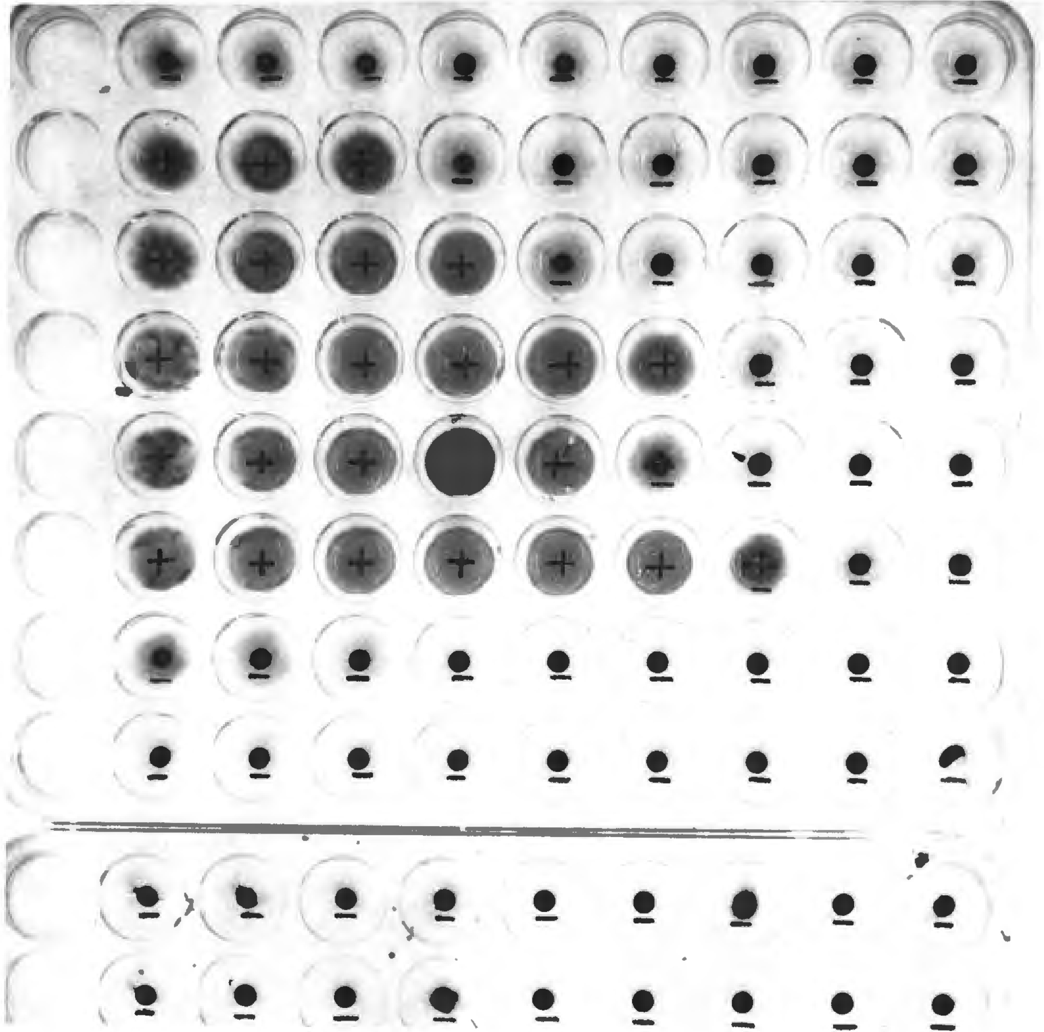

Neg Log TCID50
5·0
3·0
1·0
1·1
1·2
1·3
Density (gm/cc).

Table 16.

Haemagglutination titration results after CsCl density gradient centrifugation of Wesselsbron virus using a HIGH concentration of virus.



1/10
1/20
1/40
1/80
1/160
1/320
1/640
1/1280
1/2560
(gm/cc)
1·345
1·327
1·306
1·268
1·233
1·189
1·142
1·099
1·070
1·039

support from the extreme sensitivity of the virus to ether. The density of 1.22 gm/cc is similar to that found by Polson and Levitt (1963) for Rift Valley Fever virus particles which had densities which varied from 1.23 to 1.1 gm/cc. Russell (1965) reported that the infective particles of African Horse-sickness virus (strain A501) had densities of 1.18 gm/cc and 1.36 gm/cc.

Brakke (1964) found that the virus zone in density gradient centrifugation widened as the amount of virus floated on the gradient column increased; this finding was confirmed when a higher concentration of Wesselsbron virus was inserted in the density gradient. Zone spreading was evident and furthermore, a higher proportion of infective material was found in the least dense fractions viz. 1.031 - 1.051 gm/cc. The larger amount of brain material including lipid, is probably responsible for the presence of this heterogeneity.

The haemagglutinin components appeared heterogeneous with respect to density as no sharp peak was evident. The haemagglutinin was present in samples of densities 1.189 - 1.327 gm/cc with maximal titres at densities 1.189 and 1.268 gm/cc.

Mussgay and Rott (1964) reported two haemagglutinins of Sindbis virus with densities 1.19 and 1.24 gm/cc respectively. Two haemagglutinins of similar densities were reported in an investigation of Dengue Type 2 virus (Stevens and Schlesinger 1965). In both cases the infective component was found at a density of 1.24 gm/cc.

VIII - DETERMINATION OF SEDIMENTATION COEFFICIENT

A. INTRODUCTION

The sedimentation coefficient is a characteristic constant for a given molecular species in a given solvent at a given temperature (Svedberg and Pedersen 1940). It is the distance that a particle will sediment per second per dyne unit force and is measured in Svedberg units (S) one S being 10^{-13} cm/sec/dyne.

The determination of the sedimentation coefficients of pure substances in solution is usually performed in an analytical ultracentrifuge, in which the movement of sedimenting boundaries is followed by light absorption or light refraction methods.

When dealing with unpurified virus suspensions, the Spinco model L or LH ultracentrifuge is used and the rate of sedimentation of the infective particles is found by determining the boundary position after centrifugation, by infectivity titration or serological measurements. For such impure preparations, the techniques of Pickels (1943), Polson and Linder (1953), Polson and Madsen (1954) and Polson and van Regenmortel (1961) were designed to determine sedimentation coefficients.

If the virus particle, for which the sedimentation coefficient has been determined, is assumed to be spherical and the densities of the virus and the

suspending medium are known, the particle size may be calculated. (Pollard 1953).

Method A of the two ultracentrifugation techniques developed by Polson and van Regenmortel (1961) was used for the determination of the sedimentation coefficients of the infective particle and the haemagglutinin of Wesselsbron virus (low passage strain)

The formula of Polson and van Regenmortel, derived for the determination of $s_{20^\circ w}$ is based on the formula developed by Elford (1936) for determining the size of a virus particle from rate of sedimentation in inverted capillaries, viz.

$$d = 7.94 \times 10^{7} \sqrt{\frac{\eta_0 \log X}{(\rho_p - \rho_m) N^2 t}} \quad \ldots \; 1$$

where d = particle diameter (mμ)

η_0 = viscosity of medium at temperature of experiment

$X = \dfrac{x_1 + l}{x_1 + l\, c_t/c_0}$

x_1 = distance from axis of rotation (cm)

l = length of cell (cm)

c_t/c_0 = ratio of average virus concentration in effective column after and before centrifugation.

ρ_p = density of particle (gm/cc)

ρ_m = density of medium (gm/cc)

N = average effective velocity (rpm)

t = time of centrifugation (minutes)

When the above formula is coupled with the Stokes-Einstein equation

viz.

$$r^2 = \frac{9}{2} \frac{s_{20°w} \, \eta_{20}}{(\rho_p - \rho_m)} \quad \ldots\ldots\ldots\ldots 2$$

where r = radius of particle (mμ)

η_{20} = viscosity of water at 20°C

the following equation for the sedimentation coefficient is obtained

$$s_{20°w} = 3{\cdot}50 \; \frac{\eta_0 \, \log X}{\eta_{20} \, N^2 t} \quad \ldots\ldots 3$$

From the curve which relates the titre to the speed of centrifugation, the average effective velocity (N) at the position of <u>one logarithm drop</u> in infectivity i.e. a 90% reduction of titre, is ascertained. Therefore the ratio of the virus concentration in the effective column (1 cm) after and before centrifugation (c_t/c_0) is 0.1. If less than a one log drop in titre is used as the critical value it is likely that the reading would fall within the confidence limits of the titration.

B. MATERIALS AND METHODS

1. Preparation of virus suspension

A 10^{-1} suspension of Wesselsbron virus was prepared by triturating five infected suckling mouse brains in 20 ml of 0.02 M phosphate buffer

containing 0.4% bovine plasma albumin (phosphate buffer/Bpa) and clarified by centrifugation at 10,000 rpm for 10 minutes.

2. Method of determination of sedimentation coefficient

A 40% (w/v) solution of sucrose was prepared in phosphate buffer/Bpa.

Cellulose nitrate centrifuge tubes (12 x 50 mm) were graduated in a manner illustrated in Fig 13 (i). The distance between a and b measures 1.0 cm, b to c measures 0.5 cm and the distance below c, 2.5 cm. The virus suspension was introduced carefully to the bottom of one of the tubes till the meniscus reached level d (Fig 13 (ii)) just below the graduation marked c. With a Pasteur pipette, 40% sucrose solution was introduced below the virus suspension to raise the sugar virus interface to c and the virus meniscus above a. The excess virus suspension above a was removed to bring the virus meniscus to the graduation marked a. (Fig 13 (iii)).

Each tube was placed in the refrigerator (4°C) for approximately 20 minutes before centrifugation to allow diffusion of the sucrose into the virus suspension, thus creating a concentration gradient between b and c. (Fig 13(iv)).

In each experiment a total of six experimental tubes, prepared in this way, were centrifuged at rotor velocities 10,000, 15,000, 20,000, 25,000, 30,000 and 32,000 rpm for a standard time of 45 minutes in the swinging bucket SW-39 rotor in the Spinco model LH preparative ultracentrifuge

Fig. 13.

Preparation of tubes for determination of sedimentation coefficient

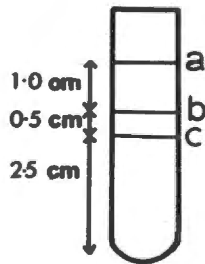
(i) graduated centrifuge tube

(ii) virus suspension introduced to level a

(iii) sucrose introduced below virus suspension

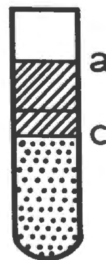
(iv) formation of concentration gradient between b and c

virus suspension
40 % sucrose

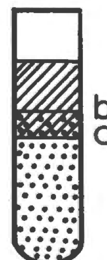


(i)

(ii)



(iii)



(iv)

at 0–4°C.

The average effective rotor velocity during the period of centrifugation was determined by a method similar to that described by Bradish, Brooksby, Dillon and Norambuena (1952). The rotor velocities were carefully noted at frequent intervals during acceleration, during the run and during the deceleration period. The square of the actual rotor velocities was plotted against the time of centrifugation and the area included by the curve was measured with a planimeter. The average effective velocity was calculated according to the formula:

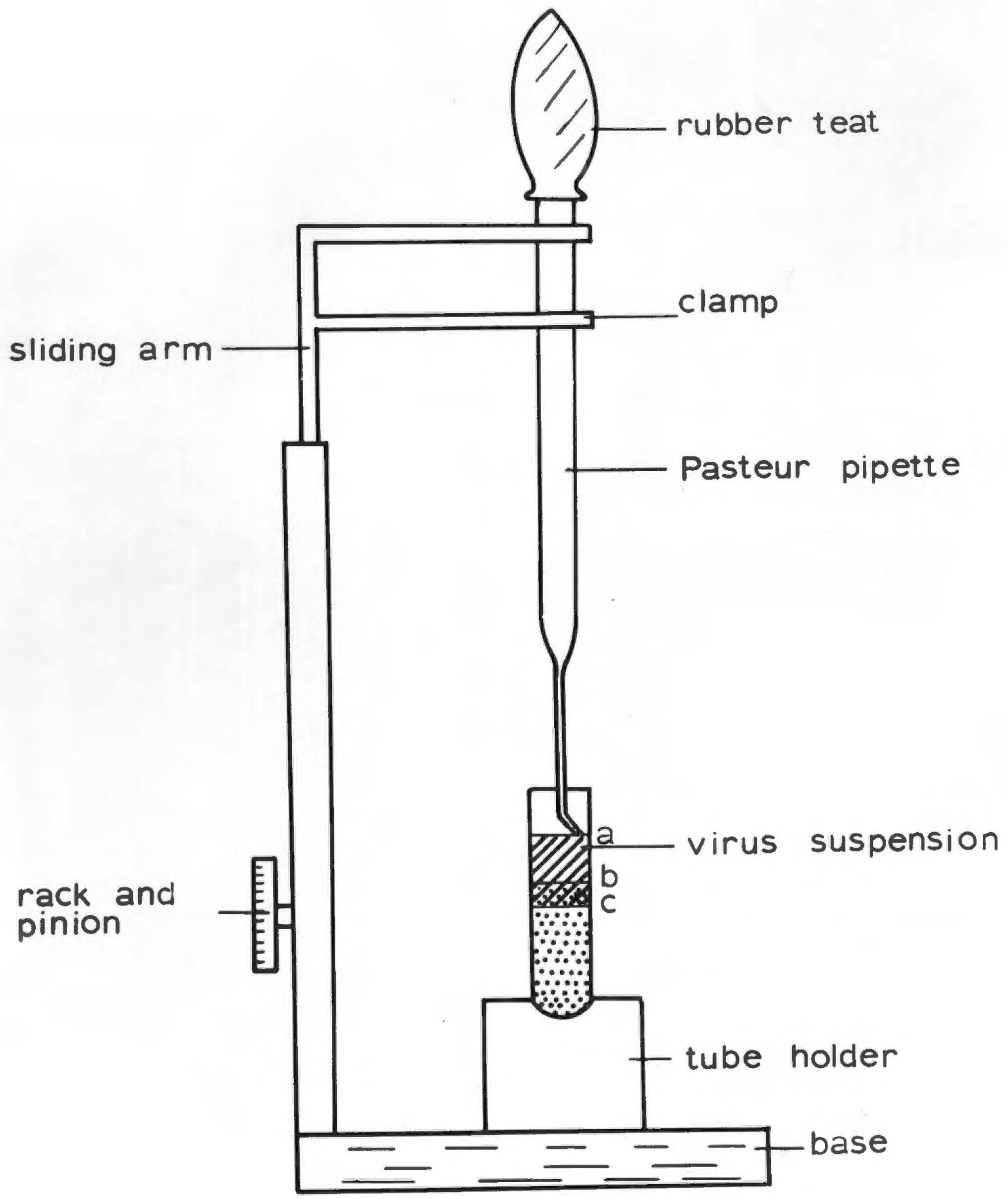
$$\text{Average effective velocity} = \sqrt{\frac{\text{area included by curve}}{\text{time}}}$$

After each centrifugation at a particular rotor velocity, the virus suspension between levels a and b in the experimental tube was removed using a finely drawn out Pasteur pipette with a bent tip supported in the apparatus as in Fig 14. The temperature of one of the balancing tubes was recorded immediately after each centrifugation.

At the completion of each experiment the six virus samples and original uncentrifuged virus suspension were titrated for infectivity in FLK cells in roller tubes and tested for haemagglutinating activity.

Fig. 14.

Apparatus used for removing the virus suspension from the centrifuge tubes after ultracentrifugation.


rubber teat
clamp
sliding arm
Pasteur pipette
a
virus suspension
b
c
rack and
pinion
tube holder
base

C. RESULTS

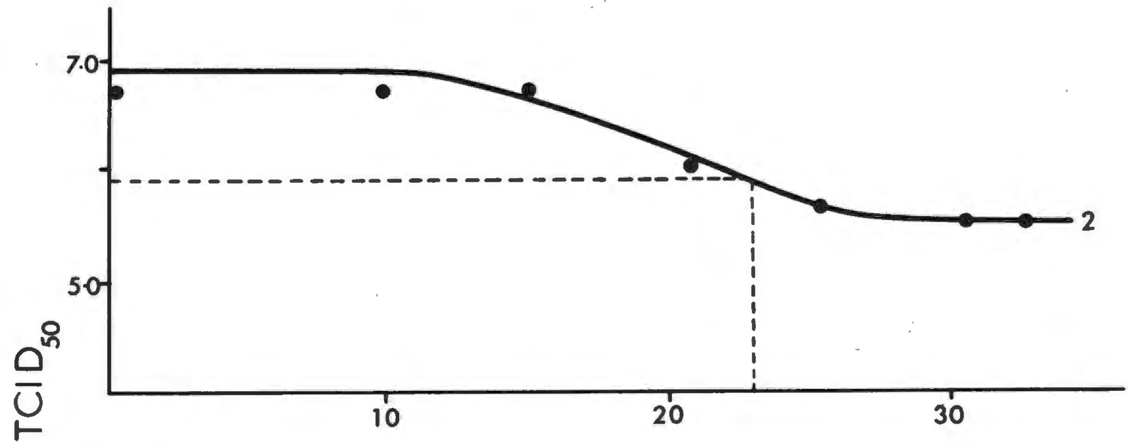
The results of two centrifugation experiments are recorded in Fig 15. The infectivity titres of the six fractions sampled from each experiment were plotted against the average effective velocities at which they were centrifuged. The titres of the control uncentrifuged virus suspensions were plotted on the ordinates. From the curves, the average effective rotor velocity at the position of one logarithm drop in titre, was ascertained in each experiment. The sedimentation coefficient($s_{20^\circ w}$) of Wesselsbron virus was calculated by inserting in equation 3 the values listed in Table 17.

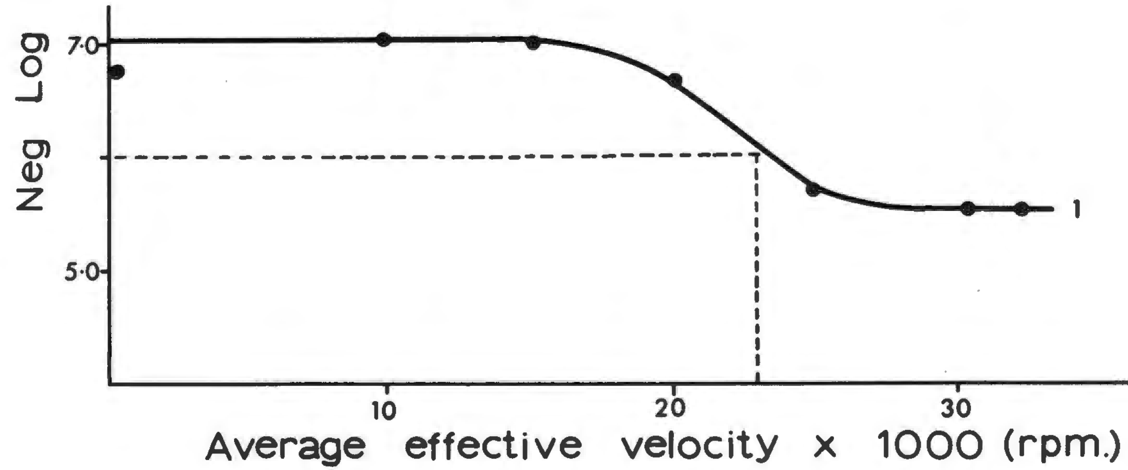
Table 17. Data for calculation of the sedimentation coefficient of the infective particle of Wesselsbron virus.

	Experiment 1	Experiment 2
η_0 (centipoise)	1.6376	1.6686
x_1 (cm)	5.85	5.85
l (cm)	1.00	1.00
c_t/c_0	0.1	0.1
η_{20} (centipoise)	1.00	1.00
N (rpm)	23,000	23,000
t (minutes)	46.52	46.47
Average temperature of experiment	3.5°C	3.0°C

Fig. 15.

Sedimentation diagrams of the infective particles of Wesselsbron virus (experiments 1 & 2).


7·0
5·0
10
20
30
2
TCID50


Neg Log
7·0
5·0
10
20
30
1
Average effective velocity x 1000 (rpm.)

The sedimentation coefficient of Wesselsbron virus was calculated to be 142.6 S in the first experiment and 145.5 S in the second. The mean value for the sedimentation coefficient of the infective particle of Wesselsbron virus is therefore 144.05 S.

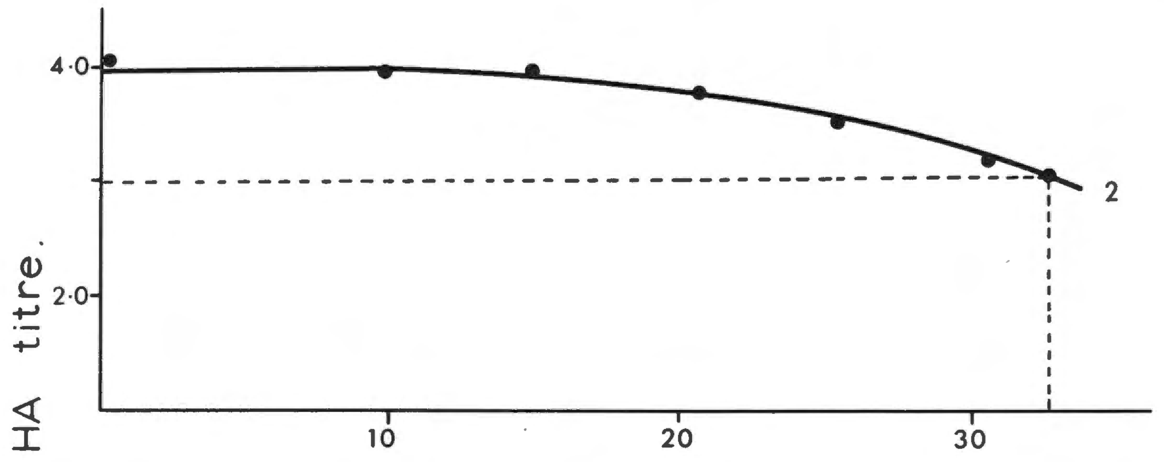
The logarithms of the haemagglutination titres of the six fractions sampled in each of the two experiments, were plotted against the average effective velocities at which the samples were centrifuged (Fig 16), and the average effective velocity at the position of one logarithm drop in haemagglutinin titre was ascertained in each experiment. The values listed in Table 18 were inserted in equation 3 and the sedimentation coefficient of the haemagglutinin of Wesselsbron virus was determined.

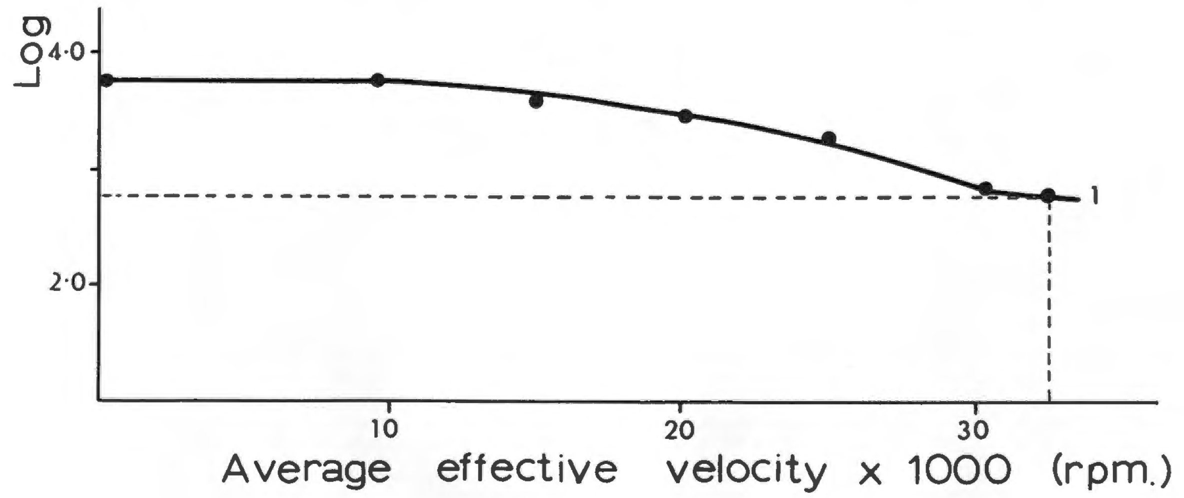
Table 18: Data for calculation of the sedimentation coefficient of the haemagglutinin of Wesselsbron virus.

	Experiment 1	Experiment 2
η_0 (centipoise)	1.6376	1.6686
x_1 (cm)	5.85	5.85
l (cm)	1.00	1.00
c_t/c_0	0.1	0.1
η_{20} (centipoise)	1.00	1.00
N (rpm)	32,400	32,600
t (minutes)	46.52	46.47
Average temperature of experiment	3.5°C	3.0°C

Fig. 16.

Sedimentation diagrams of the haemagglutinin of Wesselsbron virus (experiments 1 & 2).


4·0
2·0
10
20
30
2
HA titre.


Log
4·0
2·0
10
20
30
1
Average effective velocity x 1000 (rpm.)

In these two experiments, the sedimentation coefficient of the haemagglutinin was calculated to be 71.86 S and 72.36 S respectively. The average value for the sedimentation coefficient of the haemagglutinin of Wesselsbron virus was thus found to be 72.11 S.

D. DISCUSSION

The sedimentation coefficient of the infective particle of Wesselsbron virus was determined to be 144.05 S by the method of Polson and van Regenmortel (1961-method A).

By the same method, the sedimentation coefficient of the haemagglutinin of Wesselsbron virus was calculated to be 72.11 S.

It is of interest, that after centrifugation at 32,000 rpm, the infectivity and haemagglutinin titres of the virus samples showed only 50-fold and 10-fold reductions respectively. It is possible that another infective fraction, and perhaps another haemagglutinating fraction, having lower sedimentation coefficients will be found if higher rotor velocities and more extended times of centrifugations are employed.

Earlier sedimentation experiments carried out on Wesselsbron virus

suspended in a saline medium, containing 0.4% Bpa, yielded a value of 265 S for the sedimentation coefficient of the infective particle and 210 S for the haemagglutinin. The pH of the saline suspending medium was subsequently found to be low (5.1 - 5.3), due to the acid albumin. It is thought that there was a considerable amount of aggregation of the virus particles in these preparations resulting in elevated values for the sedimentation coefficients.

Polson (1954) determined the sedimentation coefficient of Yellow Fever virus to be 170 S and Hampton (1958) reported two infective components of West Nile virus one of which had a sedimentation coefficient of 164.9 S. The value of 144.05 S obtained for the sedimentation coefficient of Wesselsbron virus is of the same order as these two Group B arboviruses.

When the value for the density of Wesselsbron virus, 1.22 gm/cc, and the value of the sedimentation coefficient, 144.05 S were substituted into the Stokes-Einstein equation (equation 2), the diameter of the infective particle of Wesselsbron virus was calculated to be 34.3 mμ.

Assuming the haemagglutinin to be spherical, its diameter is calculated to be between 19.9 mμ and 26.2 mμ when the values 1.189 - 1.327 gm/cc for the density and 72.11 S for the sedimentation coefficient are inserted in the Stokes-Einstein equation. This assumption would be valid only if similar particle size results were obtained by ultrafiltration or diffusion experiments.

IX - ULTRAFILTRATION

A. INTRODUCTION

Ultrafiltration, as a method of estimating the size of microscopic and submicroscopic dispersed particles, has been used in biological studies for the past sixty years.

Bechold (1907, 1908 a, b) impregnated filter paper with solutions of nitrocellulose in glacial acetic acid in an attempt to prepare filters of sufficiently small pore size. These filters, as Elford (1931) discovered, had great variability in pore size, due mainly to the distorting influences of the cellulose fibres of the filter paper.

Various techniques for producing membranes of different pore sizes have been developed. Using an ether-alcohol solution of collodion, Bigelow (1907), Bartell and Carpenter (1923) and Pierce (1927) varied the time allowed for the evaporation of the solution. Others treated air-dried collodion membranes with different concentrations of alcohol in water (Brown 1915, Nelson and Morgan 1923). Non-volatile agents have been used; Pierce (1927) used ethylene glycol and Schoep (1911) added glycerol to ether-alcohol solutions of collodion.

Asheshov (1925) was the first to use volatile reagents to grade membrane permeability. He added varying amounts of acetone to increase, or amyl

alcohol to decrease membrane permeability.

Elford (1931) used the same reagents as Asheshov. He utilized the antagonistic solvent action between amyl alcohol and acetone to bring about a progressive aggregation of nitrocellulose particles during evaporation. The addition of glacial acetic acid to the solution of nitrocellulose in ether and alcohol to which certain fixed proportions of amyl alcohol and acetone had been added, resulted in a decrease in the permeability of the membrane, whereas the addition of water increased the permeability.

Bauer and Hughes (1935) used Elford's method but substituted parlodion (Mallinckrodt Chemical Works) for Du Ponts parlodion because the ratio of the tensile strength of the membrane to thickness was poor when the latter was used. Polson (1941) confirmed Bauer and Hughes' finding, and with their method, obtained constant and reproducible results.

The passage of broth through the membranes prior to filtration of a virus suspension was recommended by Ward and Tang (1929) and found by Elford (1933) to be essential to reduce the adsorption of the virus particles to the membrane.

The filterability of virus particles is dependent on the viral concentration, as gradacol collodion membranes have the capacity of adsorbing the virus and only when this has been satisfied will the virus pass freely through the membrane (Elford and Andrewes 1932).

The adsorption capacity of the membrane for proteins has been found

to be greatest near the isoelectric points of the protein. Therefore the ultrafiltration of viruses is normally carried out in the pH range 7.0 - 9.0 which is beyond the isoelectric point of most viruses and a range within which most viruses are stable.

Numerous viruses have been studied by ultrafiltration through gradacol membranes. The results obtained have given some indication of the size of the infective particles and have been compared with the results obtained for particle sizes calculated from sedimentation and density data or measurements by electron microscopy.

A gradacol membrane does not behave as an ordinary sieve. Elford (1933) investigated effects of applied pressure, concentration of the virus, membrane thickness and adsorption phenomena and established the following relationship between particle size and the limiting pore size:

Correction factor	For membranes with APD mμ (limiting pore size)
0.33 - 0.50	10 - 100
0.50 - 0.75	100 - 500
0.75 - 1.00	500 - 1000

Lea (1947) suggested a factor of 0.83 and Black (1958) proposed that the factor 0.64 be used irrespective of particle size. A factor of 0.68

was calculated from the data in Table 19 (Polson 1966 - personal communication) and applied to ultrafiltration studies of a variety of African animal viruses.

This correction factor (0.68) was used in the investigations on Wesselsbron virus.

B. MATERIALS AND METHODS

1. Preparation and calibration of gradacol membranes

The method used was that of Bauer and Hughes (1935).

In order that the water content of the membranes might be accurately controlled, it was necessary to dry and redistil the solvents before use. Ether (BDH) was dried over metallic sodium; acetone (BDH) over anhydrous potassium carbonate; and absolute alcohol (BDH) over calcium oxide. Primary amyl alcohol (BDH) was used as supplied by the manufacturers.

Parlodion (75 gm) (Mallinckrodt Chemical Works) and 125 gm of absolute alcohol were mixed in a 2.5 litre dark glass bottle fitted with a plastic screw cap. The parlodion was left to swell for 24 hours at room temperature; 375 gm of ether was added and the mixture was shaken periodically until all the parlodion dissolved. After addition of 575 gm of acetone the mixture was agitated in a mechanical shaker for 2 hours. Primary amyl alcohol (287 ml) was added and the stock collodion solution was then

Table 19. Relationship between size of virus particles as measured by electron microscopy and the ultrafiltration end-point.

Virus	Size of virus (by electron microscopy)(mμ)	Reference	Filtration end-point (mμ)	Reference	Ratio E-m/ F. end-pt
Influenza	100 - 123	Rhodes & van Rooyen (1962)	160	Elford, Andrewes & Tang (1936)	0.55 - 0.70
Horse-sickness	70	Polson & Deeks (1963)	100	Polson (1941)	0.70
Rift Valley Fever	70	Levitt, Naudé & Polson (1963)	100	Polson & Levitt (1963)	0.70
Rabbit Papilloma	45	Williams (1955)	67	Schlesinger & Andrewes (1957)	0.67
Poliovirus II	27	Schwerdt & Schaffer (1955)	38	Melnick (1955)	0.71
Tobacco Ringspot	25	Williams (1955)	38	Stanley (1939)	0.66
CBO	25	Polson (1966)	33	Polson (1966)	0.76
				Average =	0.68

shaken for a further 2 hours and stored for 2-3 weeks in a dark cupboard.

The glass cell (Fig 17) in which the membranes were prepared consisted of two pieces of plate glass 7 mm thick and 50 cm square. A hole, 40 cm in diameter, was cut in the one square of glass and the two pieces of glass were cemented together with egg white to make a well.

The preparation of the collodion membranes was carried out in a room with constant temperature (20°C) and constant humidity (70%).

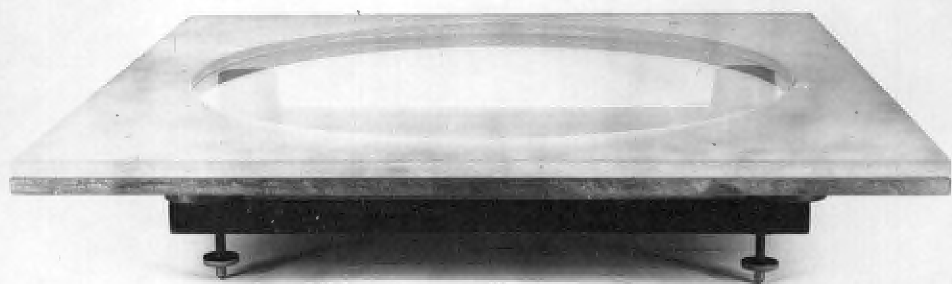
The cell was raised above a levelling table on four rubber stoppers to ensure even distribution of temperature about the cell. A paper cylinder, 45 cm in diameter and 20 cm high, supported on 3 corks was placed round the concavity of the cell to avoid extraneous draughts.

The stock collodion solution was diluted with an equal volume of a mixture containing one part by weight of ethyl alcohol to nine parts by weight of ether. The final mixture used in the preparation of the membranes consisted of 93 ml alcohol, 925 ml ether and 1018 ml of stock collodion solution which was divided into 200 ml amounts for each membrane. Acetic acid which had been frozen at 10°C and thawed twice to remove traces of water was added in varying amounts from 0.5 - 2.5 ml to give membranes of average pore diameter (APD) 450 - 24 mμ.

The 200 ml of diluted solution to which the required amount of acetic acid had been added was mixed well and placed in the constant temperature and humidity room for at least one hour prior to use. The solution

Fig. 17.

Glass cell for preparation of gradacol membranes.



was then poured into the middle of the cell and allowed to remain for 90 minutes.

Distilled water was poured immediately into the cell at the edge and after 15 minutes the membrane separated from the glass and floated free.

All the membranes were washed for one week in daily changes of 5% alcohol, to which 1/10,000 merthiolate had been added and for one week in daily changes of distilled water.

From the area of uniform opacity, membranes 3.5 cm. were cut with a steel punch. They were sterilized by steaming at atmospheric pressure for 30 minutes and stored under water in sealed glass jars.

The APD of the membranes was calculated from the rate of flow of water and the determination of water content of each particular membrane. The formula used is based upon the assumption that Poiseuille's law

$$V = \frac{\pi p r^4}{8 l \eta}$$

governing the passage of water through a capillary tube, governs the rate of flow of water through the pores of the membrane. Thus taking the number of pores into account, the radius is calculated from formula

$$r = 2l\sqrt{\frac{2V\eta}{p\omega}}$$

where r = radius of pore (cm)

l = thickness of membrane i.e. length of pores (cm)

V = rate of flow (ml/sec)

η = specific viscosity (CGS units)

ω = water content of one sq cm of membrane

p = pressure producing flow (dynes/sq cm)

The thickness of each membrane was measured between two microscope cover slips using a micrometer screw; the rate of flow was determined using a flow cell (Fig 18) and the water content was obtained by weighing the membranes before and after drying at 90°C for 24 hours.

2. Preparation of virus suspension

Twenty infected suckling mouse brains were triturated in 80 ml 0.02 M phosphate buffer at pH 7.0 which contained 0.4% bovine plasma albumin (Bpa). The suspension was clarified by centrifugation at 10,000 rpm for 10 minutes in the Spinco model L ultracentrifuge. The supernatant fluid was stored at 4°C.

3. Method of filtration

The filtrations were carried out under positive air pressure of 15 lbs/ sq inch using a pump connected to a manifold and the metal filters as shown in Fig 19.

The assembly of the filters is shown in Fig 20 and consists of a perforated metal disc, which supports a Whatman hardened filter paper No.541 and the gradacol membrane; all three are held between two rubber washers

Fig. 18.

Flow cell for determination of the rate of flow of water through the gradacol membranes.

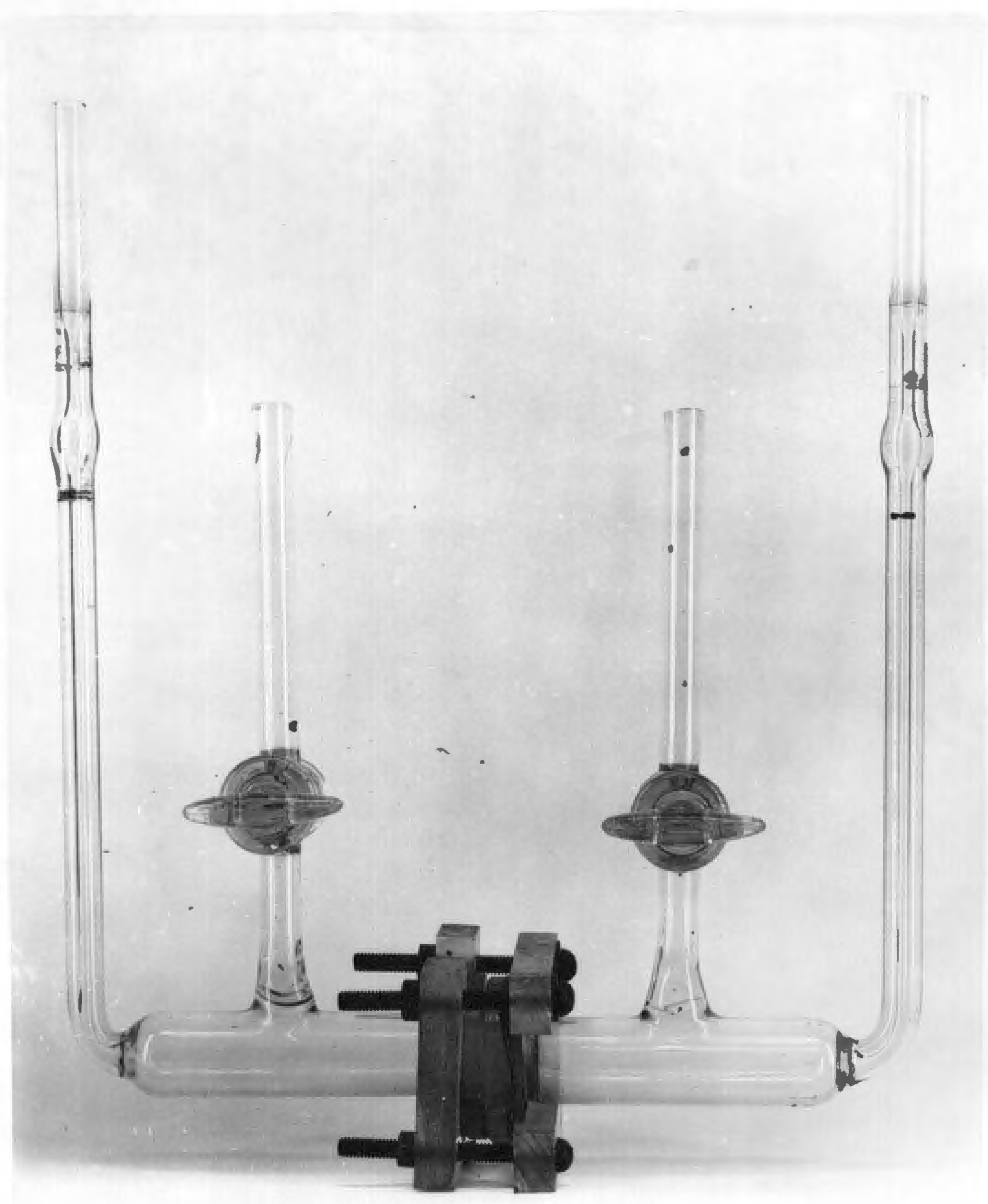


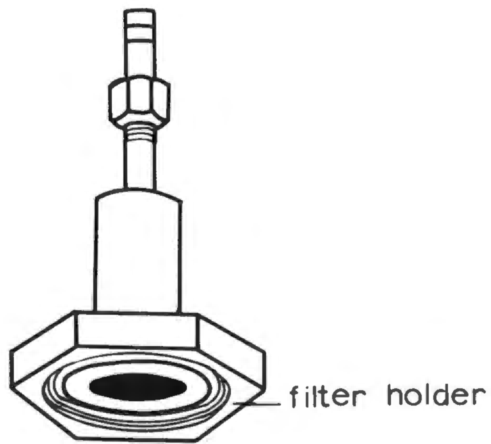
Fig. 19.

Arrangement of apparatus used in ultrafiltration.

10
20
30
40
50
60

Fig. 20.

A filter in detail.

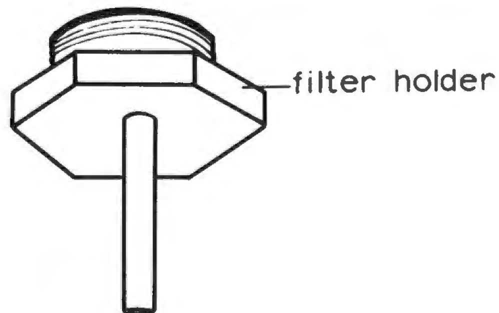

filter holder

rubber washer

gradacol membrane

filter paper

perforated disc

rubber washer

filter holder

in the brass nickel-coated filters.

The entire units were autoclaved except for the filter paper disc and gradacol membrane, which were boiled in distilled water before the filters were assembled. Membranes of APD's 450, 334, 310, 245, 222, 178, 160, 150, 119, 77, 72, 63, 40 and 24 mμ were used in three ultrafiltration experiments.

Panmede nutrient broth (manufactured by Paines and Byrne Ltd., England) was filtered through the 334 mμ membrane in the first two experiments and through the 450 mμ membrane in the third, and subsequently 5 ml of the filtrate was passed under pressure through each of the other membranes. The virus suspension, after filtration through the 334 mμ or 450 mμ gradacol membranes was also filtered in 5 ml amounts through the membranes with smaller pore size.

The filtrates that had passed through membranes of APD 450, 334, 245, 160, 150, 119, 77, 72, 63, 40 and 24 mμ were titrated for infectivity in 3-4 week old mice, whereas the filtrates through membranes of APD 450, 334, 310, 245, 222, 178, 160, 150, 119, 77, 72, 63, 40 and 24 mμ were tested for haemagglutinin activity.

C. RESULTS

The results of the infectivity titrations of the filtrates from three

ultrafiltration experiments are given in Table 20.

Table 20. Ultrafiltration of the infective particle of Wesselsbron virus.

APD of membrane (mμ)	Experiment 1 $TCID_{50}$/0.1 ml	Experiment 2 $TCID_{50}$/0.1 ml	Experiment 3 LD_{50}/0.04 ml
450	-	-	7.50
334	5.50	4.66	-
245	-	-	6.66
160	-	-	4.55
150	4.66	2.00	-
119	2.66	1.50	3.66
77	1.50	-	-
772	-	1.00	1.75
63	< 1.00	-	< 1.00
40	< 1.00	< 1.00	< 1.00
24	-	< 1.00	-

- = not done

It is evident from Table 20 that the infective particle of Wesselsbron virus does not pass through membranes with APD of 63 mμ or less. The infectivity titres of the filtrates were plotted against the average pore diameters of the membranes through which they had passed (Fig 21) and the titration

Fig. 21.

Infectivity titration results from three ultrafiltration experiments plotted against the APD's of the membranes. The titres in experiments 1 & 2 are expressed as neg Log $TCID_{50}$, whereas in experiment 3 the titres are expressed as neg Log LD_{50}.

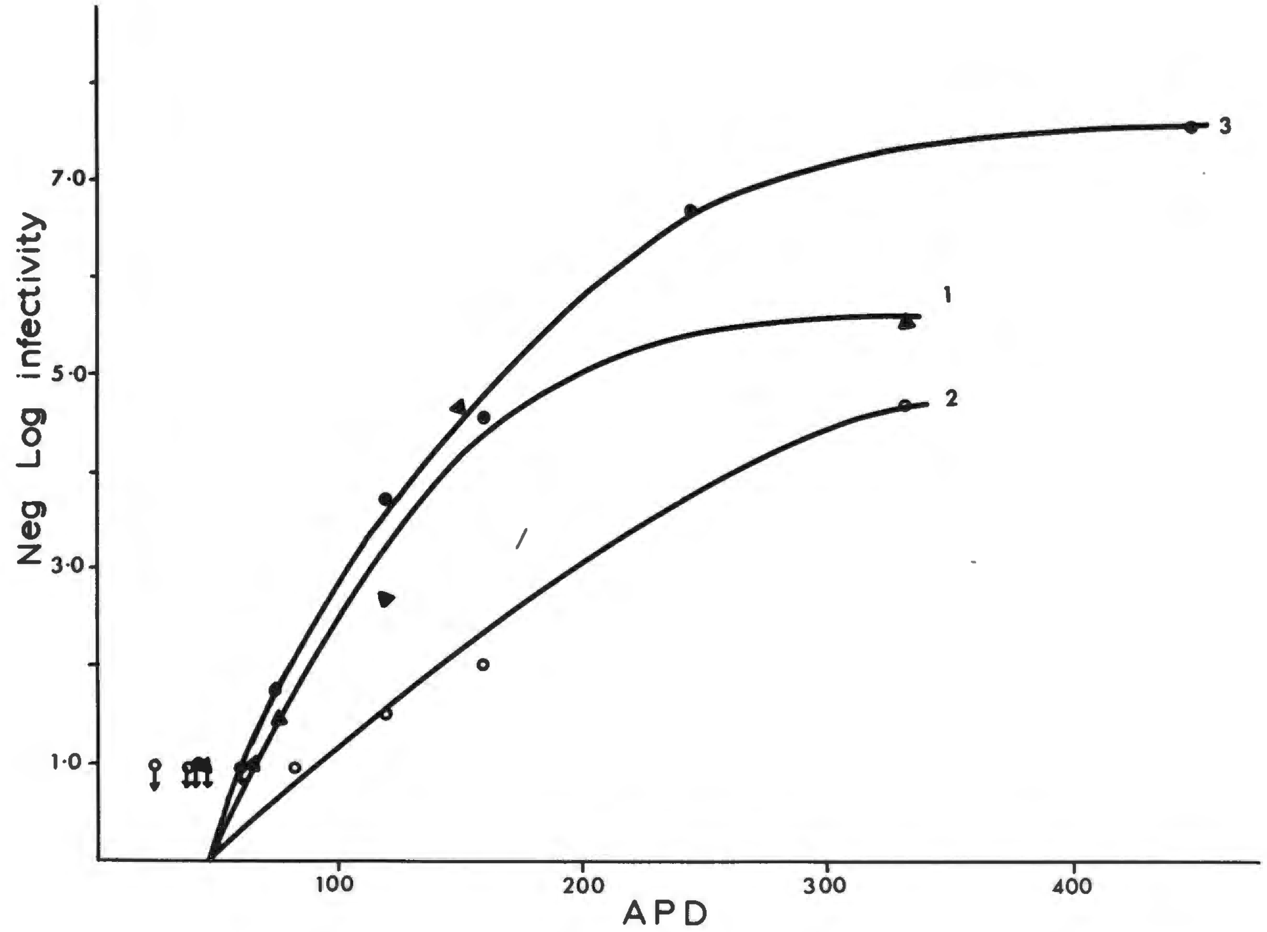
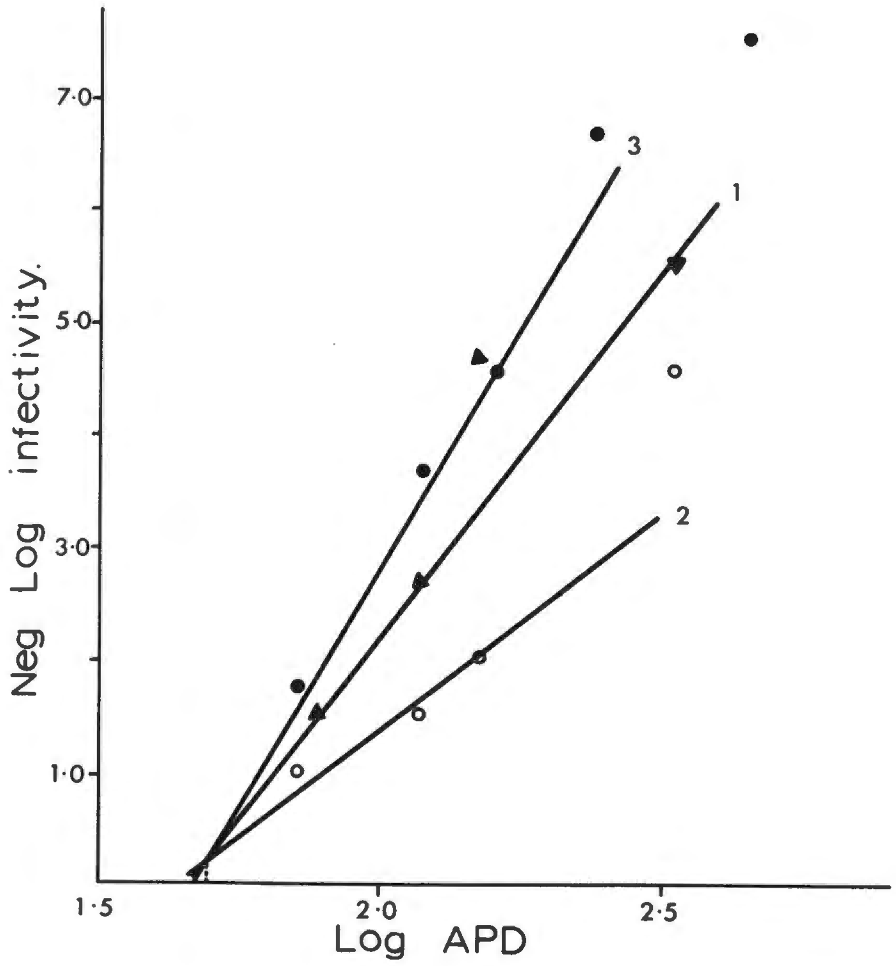

Neg Log infectivity
7·0
5·0
3·0
1·0
100
200
300
400
APD
3
1
2

Fig. 22.

Infectivity titration results from three ultrafiltration experiments plotted against the logarithms of the APD's of the membranes. The titres in experiments 1 & 2 are expressed as neg Log $TCID_{50}$, whereas in experiment 3 the titres are expressed as neg Log LD_{50}.


Neg Log infectivity.
7·0
5·0
3·0
1·0
1·5
2·0
2·5
Log APD
1
2
3

results were plotted against the logarithms of the average pore diameters of the membranes (Fig 22). In the latter figure the results fall on straight lines and the point at which the three lines intersect is taken as the filtration end-point. A value of 45 mμ is thus obtained and when multiplied by the factor 0.68, a corrected value of 33.3 mμ is obtained for the diameter of the infective particle of Wesselsbron virus.

The haemagglutination titration results from three experiments are given in Table 21.

Table 21. Ultrafiltration of the haemagglutinin of Wesselsbron virus.

APD of membrane (mμ)	Experiment 1 HA titre/0.4 ml	Experiment 2 HA titre/0.4 ml	Experiment 3 HA titre/0.4 ml
450	-	-	2560
334	1280	5120	2560
310	-	-	2560
245	-	-	2560
222	-	-	80
178	-	-	40
160	-	-	< 20
150	< 20	< 20	-
119	< 20	< 20	-
77	< 20	-	-
72	-	< 20	-
63	< 20	-	-
40	< 20	< 20	-
24	-	< 20	-

It will be seen from these results that a large proportion of the haemagglutinin of Wesselsbron virus is held back by membranes of APD 222 mμ or less. No haemagglutinin was detected in the filtrates that had passed through membranes of APD 160 mμ or less. It was not possible to obtain straight line graphs when the haemagglutinin titres (Experiment 3) were plotted against the logarithms of the APD's of the membranes, so it has not been possible to estimate the size of the haemagglutinin by ultrafiltration.

D. DISCUSSION

Ultrafiltration is not simple filtration through a sieve and surface phenomena play an important part in the ultrafiltration process.

The size of the infective particle of Wesselsbron virus has been estimated by ultrafiltration to be 33.3 mμ. The results of the three experiments were in good agreement and the method of a wholly logarithmic plot of the results permitted an accurate extrapolation to the filtration end-point.

The size of the haemagglutinin is more difficult to estimate by ultrafiltration. There appears to be one haemagglutinin or possibly two, of much larger size than the infective particle. It is known that when any

Wesselsbron virus preparation contains less than 3.50 LD_{50}/0.04 ml, haemagglutinin is not detectable. The virus suspension which had passed through the membrane of APD 160 mμ showed no haemagglutinin activity, but contained 4.55 LD_{50}/0.04 ml. This suggests that the infective particle and the haemagglutinin are loosely associated. The haemagglutinin might have been stripped from the outside of the virus particle when the virus suspension was filtered through membranes of APD 160 mμ or less and it is possible that free haemagglutinin is too small to prevent the red cells from settling out in the haemagglutination test.

Somewhat similar findings were obtained by Sabin and Buescher (1950) who reported that Japanese B Encephalitis virus haemagglutinin did not pass through a gradacol membrane of APD 200 mμ or less despite the fact that the infective particle diameter is 20-30 mμ.

At least one of the possible explanations of these curious results is that the haemagglutinin strips from the virus particle in the form of filaments or sheets and would therefore not obey the laws governing the passage of spherical particles through gradacol membranes.

The preparation and calibration of the gradacol membranes is long and tedious and perhaps these are the reasons why ultrafiltration is so infrequently used in virus studies.

X - INFECTIOUS RIBONUCLEIC ACID

A. INTRODUCTION

The isolation of infectious ribonucleic acid (RNA) from Poliovirus, West Nile, and Mengo viruses by Colter, Bird and Brown (1957) and Colter, Bird, Moyer and Brown (1957) were the first demonstrations that infectious macromolecules could be isolated from animal viruses.

The cold phenol method of Gierer and Schramm (1956) was used for the isolation of infectious RNA from numerous arboviruses - from Eastern Equine Encephalomyelitis virus (Wecker and Schäfer 1957), Semliki Forest virus (Cheng 1958), Murray Valley Encephalomyelitis virus (Ada and Anderson 1959 a), Dengue viruses I and II (Ada and Andersen 1959 b), Japanese B Encephalomyelitis virus (Nakamura 1961) and Yellow Fever virus (Nielsen and Marquardt 1963).

Other methods used for the isolation of infectious RNA from virus suspensions, include treatment with hot phenol, sodium deoxycholate, guanidine hydrochloride hot $M/_1$ NaCl, heat alone (61°C, 85°C), and sodium dodecylsulphate (Duponol).

It was necessary in all cases to demonstrate that the infectivity of the RNA preparations was not due to residual virus. This was shown by the fact that RNA was sensitive to ribonuclease; it was precipitated with alcohol

or with M NaCl at 4°C; it was not neutralized by specific antiviral gammaglobulin; it showed characteristic ultra-violet spectra with a peak at 260 mμ.

The infectious RNA may be assayed in vivo or in vitro. Direct plating on cell monolayers (Alexander, Koch, Mountain and von Damme 1958) or the infectious centres method (Ellem and Colter 1960) are most commonly used for the titration of RNA isolated from arboviruses. The former method has the disadvantage that the cell monolayers may be damaged by the hypertonic solutions used for suspending viral RNA, which have been found to be necessary for maximal RNA adsorption to the cells.

The infectivity of the infectious RNA has been found, in most cases, to be very low compared with that of the intact virus from which the RNA was isolated. It was thought that the low infectivity titres were due to poor plating efficiency and to overcome this, the RNA dilutions were made in hypertonic solutions (Alexander et al 1958), and the host cells were treated with hypertonic saline at high pH prior to inoculation of the RNA (Koch, Koenig and Alexander 1960). Ribonucleases in the preparation destroy RNA and Fraenkel-Conrat, Singer and Tsugita (1961) suggested the addition of bentonite to adsorb any nucleases present, and to stabilize the infectious RNA. However, Nakamura and Ueno (1964) suggest that it is the heavy metal contamination in the RNA preparation and not the nucleases which are responsible for the low titres and have suspended and stored Japanese B

Encephalitis RNA in 0.01% sodium versenate. It is apparent that the optimal conditions for maximal infectivity of infectious RNA preparations is a characteristic of the particular viral RNA and the tissue culture system in which it is assayed.

The method used for the isolation of infectious RNA from crude suspensions of Wesselsbron virus infected suckling mouse brains was that of Gierer and Schramm (1958) as modified by Naudé (1965). Cold phenol was used for the dissociation of the nucleoprotein and bentonite was added to the brain suspension to inactivate ribonucleases.

B. MATERIALS AND METHODS

1. Preparation of bentonite suspension

0.1 M Sodium Versenate pH 7.0

Na versenate 37.225 gm

Distilled water 500 ml

NaOH (10 N) to bring pH to 7.0

made up to final volume of one litre with distilled water.

0.01 M Acetate pH 6.0

CH_3COONa 0.2051 gm

Adjusted to pH 6.0 with 0.01 M acetic acid.

made up to final volume of 250 ml with distilled water.

Bentonite (45 gm) was stirred mechanically in 900 ml of distilled water. The suspension was centrifuged at 2,500 rpm, in the MSE centrifuge, for 15 minutes and the supernatant was centrifuged at 8,500 rpm (Spinco rotor No. 30) for 20 minutes in the Spinco model L centrifuge. The pellet was resuspended in 0.1 M sodium versenate and left at room temperature for 48 hours. The bentonite was washed twice and on each occasion the pellet resuspended in 0.01 M acetate pH 6.0.

The bentonite content of the final suspension was calculated by centrifuging a known aliquot, resuspending the pellet in water and weighing after drying in a 37°C incubator.

2. Isolation of Wesselsbron - RNA

(i) Phenol - (Analar Grade) - freshly distilled "in vacuo" and saturated with 0.02 M phosphate buffer at pH 7.0. Stored at 4°C.

(ii) Ether - (Anaesthetic grade - Natal Cane By Products Ltd.) - freshly distilled and saturated with 0.02 M phosphate buffer at pH 7.0. Stored at 4°C.

(iii) 0.0005 M Versenate in 0.02 M phosphate buffer at pH 7.0

Sodium versenate 0.93062 gm

made up to 500 ml with 0.02 M phosphate buffer pH 7.0.

Stored at 4°C.

Diluted 1 : 10 with distilled water for use.

The centrifugations of the samples were carried out in the swinging bucket rotor SW-25 in the Spinco Model L preparative ultracentrifuge at 0-4°C.

Ninety Wesselsbron virus infected suckling mouse brains were ground in a chilled mortar with 32 ml aliquots of phenol and 0.0005 M versenate in 0.02 M phosphate buffer (pH 7.0) and 450 mg bentonite. The suspension was transferred to a sterile 4 oz ground glass stoppered bottle standing in ice and shaken vigorously for 5 minutes. After centrifugation at 10,000 rpm for 30 minutes at 4°C, the aqueous layer was removed and treated with 450 mg bentonite and 32 ml of phenol, shaken for 5 minutes and centrifuged at 10,000 rpm for 2 minutes at 4°C. The phenol treatment was repeated twice, the final centrifugation being for 20 minutes to sediment the bentonite.

The supernatant was transferred to a chilled ground glass stoppered bottle and shaken five times with 30 ml aliquots of cold ether to remove traces of phenol and finally the traces of ether were removed from the aqueous layer by placing the RNA preparation in a beaker in a dessicator under negative pressure for 30 minutes.

The final RNA preparation (approximately 28 ml) was stored at -70°C.

3. <u>Identification of Wesselsbron - RNA</u>

A solution of crystalline ribonuclease (Seravac Laboratories) con-

taining 1 mg/ml was prepared in PBS.

Infectious RNA and the ribonuclease solution were mixed in equal volumes and incubated at room temperature for 15 minutes. As a control, infectious RNA and PBS were mixed and incubated as above. Tenfold dilutions of both the mixtures were prepared in PBS and titrated in mice

4. Infectivity of Wesselsbron virus and Wesselsbron-RNA

One Wesselsbron virus infected suckling mouse brain was triturated in 4 ml serumsaline and the suspension was clarified by centrifugation at 10,000 rpm for 10 minutes. Tenfold dilutions of the supernatant were prepared in serum saline and titrated in 3-4 week old mice.

Tenfold dilutions of Wesselsbron-RNA were prepared in PBS and titrated without prior incubation in 3-4 week old mice.

6. RESULTS

Infectious RNA, isolated from crude suspensions of Wesselsbron virus infected suckling mouse brains, was identified as such by its sensitivity to the action of ribonuclease and by its ability to produce the characteristic symptoms of Wesselsbron virus infection in 3-4 week old mice when injected by the intracerebral route.

The results of three Wesselsbron-RNA isolations are listed in Table 22,

and the protocols of one of these is presented in full in Table 23.

Table 22. Infectivity titres of Wesselsbron virus and Wesselsbron-RNA preparations.

	$LD_{50}/0.04$ ml			
	Without incubation		Incubated 22°C / 15 mins	
	Original Wesselsbron virus	Wesselsbron-RNA	Wesselsbron-RNA + Ribonuclease	Wesselsbron-RNA + PBS (control)
Experiment 1	8.31	3.50	< 0.50	3.00
Experiment 2	8.76	3.63	< 0.50	3.83
Experiment 3	8.00	3.33	< 0.50	3.50

It is evident from Table 22 that the Wesselsbron-RNA preparations are inactivated by ribonuclease and that the infectivity titres of the RNA preparations represent only small fractions of the infectivity of the intact virus in the original untreated virus suspensions. The results of the control titration (column 4 - Table 22) prove that the reduction in the titre of the RNA preparations by the addition of ribonuclease is not due to incubation at 22°C.

Many attempts were made to titrate Wesselsbron-RNA by plaque counting techniques in monolayers of chick embryo cells, according to the standard techniques of Alexander et al (1958) and Ellem and Colter (1960) as modified by Naudé (1965). Despite the precautions of suspending the

Table 23. Results of Experiment 2 in detail.

	Dilution	Accumulative totals of deaths — Days after injection: 1	2	3	4	5	6	7	8	9	10	11	12	Total no. of mice injected.
Original virus preparation	-5	.	.	.	.	.	.	6	6	6	6	6	6	6
	-6	.	.	.	.	.	.	6	6	6	6	6	6	6
	-7	.	.	.	.	.	.	5	5	5	5	5	5	5
	-8	.	.	.	.	.	.	.	2	4	6	6	6	6
	-9	.	.	.	.	.	.	1	2	2	2	2	2	6
Wesselsbron-RNA	0	.	.	.	.	.	.	5	5	5	5	5	5	5
	-1	.	.	.	.	.	.	6	6	6	6	6	6	6
	-2	.	.	.	.	.	.	3	5	5	5	5	5	5
	-3	.	.	.	.	.	.	3	4	4	4	4	4	5
	-4	.	.	.	.	.	.	.	2	2	2	2	2	6
Wesselsbron-RNA + ribonuclease	-0.5	.	.	.	.	.	.	.	.	.	.	.	.	5
	-1	.	.	.	.	.	.	.	.	.	.	.	.	6
	-2	.	.	.	.	.	.	.	.	.	.	.	.	6
	-3	.	.	.	.	.	.	.	.	.	.	.	.	6
	-4	.	.	.	.	.	.	.	.	.	.	.	.	6
Wesselsbron-RNA + PBS (control)	-0.5	.	.	.	.	.	.	4	6	6	6	6	6	6
	-1	.	.	.	.	.	.	6	6	6	6	6	6	6
	-2	.	.	.	.	.	.	6	6	6	6	6	6	6
	-3	.	.	.	.	.	.	6	6	6	6	6	6	6
	-4	.	.	.	.	.	.	2	2	2	2	2	2	6

infectious RNA in a wide range of saline solutions of different molarities (0.14 M- 0.66 M) no plaques were ever seen using either the direct plating method or the technique of infectious centres.

D. DISCUSSION

By a variety of procedures, infectious RNA has been isolated by many workers from the small ether resistant viruses and from numerous arboviruses. In this work, infectious RNA was successfully isolated from Wesselsbron virus for the first time.

The infectivity of Wesselsbron-RNA, compared with the infectivity of the intact virus from which it was isolated, represents a yield of only 0.001%.

The efficiency of assay of infectious RNA has been reported to be as high as 1% of the starting virus activity in some virus systems (Schaffer 1962), but Naudé (1965) working with another arbovirus (West Nile virus) was able to achieve a yield of no more than 0.1% of the original infectivity after taking into account the optimal conditions of pH, molarity, temperature and time.

Many other workers in this field have not been rewarded with such success but it is presumed that further investigations of the optimal requirements for plating efficiency will produce a considerable improvement in the final

yield of Wesselsbron-RNA.

It was thought that cytological studies on infected cells would assist in the identification of the sites of Wesselsbron-RNA synthesis. The next section shows the results of these investigations.

XI - LIGHT MICROSCOPY

A. INTRODUCTION

Light microscopy is widely used in the examination of cell cultures and fixed tissue preparations in the study of the cytopathology of infected cell cultures and for the presence of inclusion bodies.

Fluorescent antibody studies, initiated by Coons and Kaplan (1950) permit investigations into production of viral antigen in infected cells.

Cover slip preparations of the Wesselsbron virus infected FLK cells, fixed in Bouin's fluid and stained with haematoxylin and eosin were studied by light microscopy for the presence of inclusion bodies, Nucleic acid changes were investigated by the acridine orange staining method of Armstrong (1956); the fluorescent labelled antibody technique (Coons 1958) was used to observe specific viral antigen.

B. MATERIALS AND METHODS

1. Preparation of Wesselsbron virus infected FLK coverslip cultures

Roller tube cultures of FLK cells containing coverslips were infected with 0.1 ml aliquots of 10^{-6} suspension of Wesselsbron virus infected suckling mouse brains and examined over a period of 4 days by the following

techniques.

2. Haematoxylin and eosin staining procedure

Bouin's Fixative

Picric Acid (saturated aqueous solution)	7.5 ml.
Formalin (40% formaldehyde)	2.5 ml
Glacial Acetic acid	0.5 ml

1% Eosin phloxine stain

Eosin solution (1%)	70 ml
Phloxine solution (1%)	30 ml

At daily intervals, coverslip cultures of infected and control uninfected FLK cells were fixed in fres hly made up Bouin's fixative for 1-24 hours. The preparations were rinsed in absolute alcohol for 5 minutes and stored at room temperature in 70% alcohol until stained.

The cultures were washed in tap water to remove the fixative and stained with 0.4% haematoxylin for 5-10 minutes. After rinsing in water for 30 seconds, the cells were differentiated in 0.5% acid alcohol (HCl in 70% alcohol), washed in running water for 15-20 minutes and counter-stained with 1% eosin phloxine stain for 2-3 minutes. The cells were passed through several changes of 96% alcohol, absolute alcohol, two changes of xylol and mounted on microscope slides in Depex.

3. Acridine Orange staining procedure

A solution of acridine orange ($^{1}/_{200}$) was prepared in 0.2 M acetate buffer at pH 4.7 and stored at 4°C. Before use, this stain was diluted $^{1}/_{100}$ in acetate buffer.

The coverslip cultures of infected and control uninfected FLK cells were rinsed with acetate buffer, fixed in absolute alcohol for 10 minutes, and stored in 70% alcohol at 4°C.

The cells were then hydrated through 50% alcohol, three changes of distilled water and one of acetate buffer to remove all traces of alcohol. After staining in acridine orange for 30 minutes, the excess stain was removed from the coverslip cultures by washing in seven changes of buffer. The cells were mounted in buffer on microscope slides.

4. Indirect fluorescent antibody staining technique

Wesselsbron virus antiserum (prepared in mice) was inactivated at 56°C for 30 minutes and diluted $^{1}/_{10}$ with PBS.

Fluorescein isothiocyanate labelled anti-mouse serum was supplied by Antibiotics Inc. California U.S.A. This was previously tested and found to stain optimally when diluted $^{1}/_{20}$ with PBS.

Coverslip cultures of infected and control uninfected FLK cells were washed in two changes of PBS, one change of absolute alcohol and

stored in absolute alcohol at -70°C for 1-4 days.

The alcohol was allowed to evaporate from the coverslip cultures and the cells were moistened with PBS. A few drops of Wesselsbron virus antiserum ($^1/_{10}$) was added and the cells were incubated at 37°C for 30 minutes.

The coverslips were placed in a petri dish containing wet filter paper to avoid drying during incubation.

After the cells had been washed gently three times in PBS, 2-3 drops of fluorescent antiserum ($^1/_{20}$) was added, and the cells incubated at 37°C for 30 minutes as above.

The cells were washed well and mounted in buffered glycerol on microscope slides. The preparations were stored at 4°C.

C. RESULTS

The FLK cultures were examined histologically by taking 'floating' coverslip preparations from the infected cell cultures and from the control uninfected cultures each day.

Preparations stained with haematoxylin and eosin confirmed the appearance and the progression of the changes associated with the cytopathic degeneration of the cell sheets. The earliest lesions were the development of 'holes' or defects in the otherwise intact sheet of cells, associated with a peripheral clumping of the cells which became rounded with more deeply staining cytoplasm. Some cells became grossly elongated and tautly stretched as thin filaments of cytoplasm across the open spaces in the cell sheet. Similar stretched cells were also occasionally encountered in the

control cultures, but it was their number that appeared important in the infected cultures.

In the final stages the infected cells all became rounded, shrunken, with deeply eosinophilic staining cytoplasm and with coarse granular disintegration of the nuclei.

In none of these preparations of foetal lamb kidney cells were structures encountered which could be designated as inclusion bodies in either nucleus or cytoplasm. The nuclear chromatin remained normally distributed up to the time of rapid degeneration of the nucleus. Cells showing mitotic figures were very numerous in the control cultures and were encountered occasionally in the infected cell cultures up to the third day, suggesting that a small proportion of the cells were not infected even at this stage.

The absence of any hint as to the site of virus synthesis in the cell from these histological investigations indicated the necessity of study by other techniques to establish this point.

In the preparations stained with acridine orange in attempts to detect increased synthesis of DNA or RNA, all the cells in the infected and uninfected cultures stained with equal colour intensity for nuclear DNA. There was no suggestion of inclusion body formation in the nucleus. By contrast the greater amount of flame red coloured staining of the cytoplasm of the cells infected with virus pointed strongly to enhanced RNA synthesis in these cells compared with the uninfected control cells. Despite this change in staining properties there was no evidence of a viral inclusion body.

The technique making use of fluorescein-tagged anti-mouse gamma-globulin proved eminently successful in demonstrating the early appearance of viral antigen in the cytoplasm of the infected FLK cells. Specific viral antigen was detected at 44 hours in the perinuclear or juxtanuclear position in the cytoplasm in a small proportion of the cells at the periphery of the "holes" in the cell sheet (Fig. 23). By 72 hours, 90% of the cells in the infected cultures displayed bright fluorescence in the cytoplasm. Later preparations (96 hours) showed increasing degeneration of the cells and gave no further information about the viral synthesis.

D. DISCUSSION

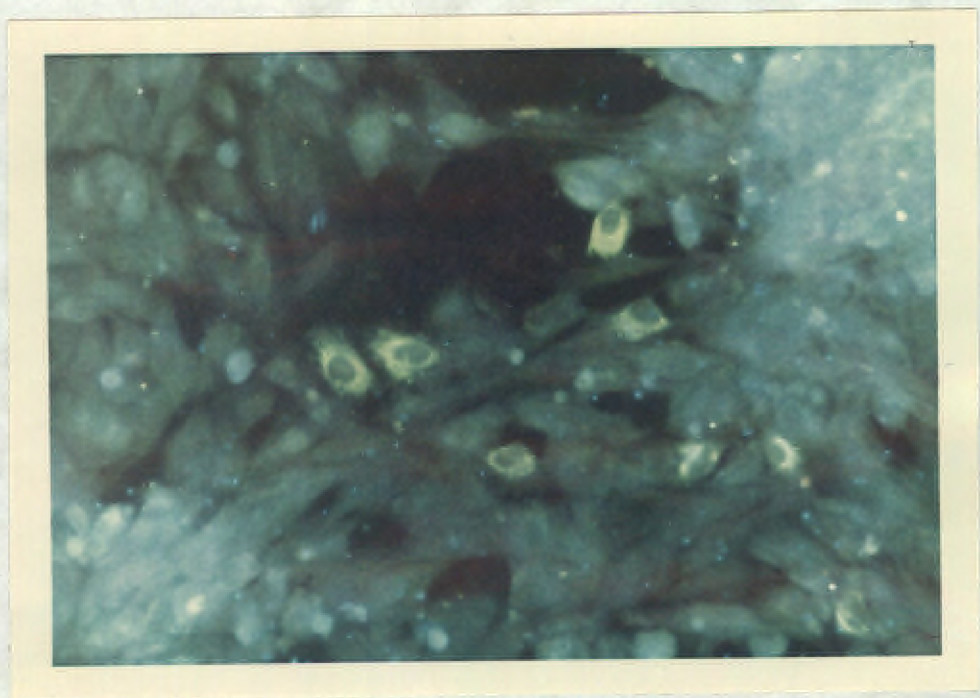
Three staining procedures provided valuable information regarding the site of synthetic activity in Wesselsbron virus infected cells.

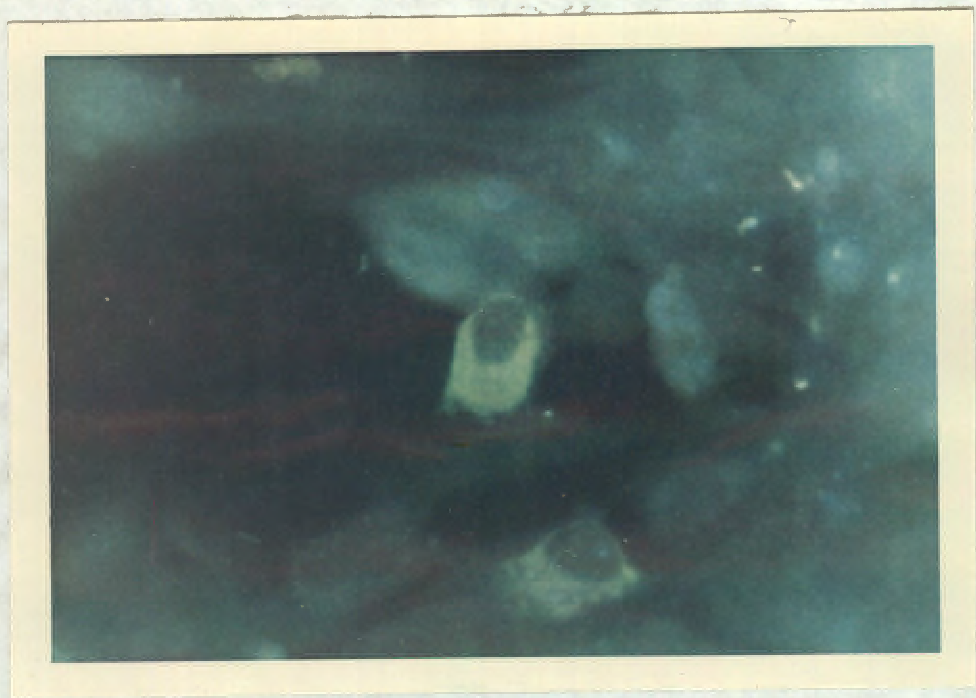
Although viral inclusion bodies were not demonstrated in either cytoplasm or nucleus, the acridine orange staining procedure and the fluorescent antibody staining technique both pointed strongly to the cytoplasm as the site of greatest synthetic activity. These observations were thought to be in keeping with the fact that Wesselsbron virus was shown to be an RNA virus by the successful isolation of infectious Wesselsbron-RNA.

In order to gather more information on these changes taking place in the cytoplasm of infected cells it was decided to examine them under the electron microscop

Fig. 23.

Viral antigen in the cytoplasm of infected FLK cells demonstrated by the indirect fluorescent antibody staining technique.





XII - ELECTRON MICROSCOPY

A. INTRODUCTION

The electron microscope is one of the most useful aids in the study of viruses.

Since the invention of the first Porter-Blum microtome in 1953 it has been possible to cut sections of tissues of the required thinness for observation in the electron microscope and from these sections the virologists has gained much knowledge of viruses in their intracellular environments.

With some viruses, it has been possible to determine the sites of replication and to observe the method of their release from the cells.

Methods of fixing, embedding and sectioning tissues have been adequately described by Pease (1964). Osmium tetroxide (OsO_4) has been used for many years for fixation of tissues and it is well known that it has a staining affinity for lipids and proteins rendering them more visible in the electron microscope. The most widely used fixative is that of Palade (1952), consisting of 1% osmium tetroxide buffered to the alkaline side of neutrality with veronal buffer.

Caulfield (1957) suggested the addition of a small amount of sucrose to Palade's fixative in order to increase the tonicity and also suggested lowering the salt concentration.

Zetterqvist (1956) recommended the addition of balanced salts to Palade's fixative. Millonig (1961) in contrast to Palade, recommended a phosphate buffered osmium tetroxide fixative and obtained excellent results. Other fixatives include aldehyde and permanganate (Luft 1956).

It appears that the optimal temperature for fixation is 0°C, and it is necessary to chill the tissue and fixative before fixation. A satisfactory state of preservation is achieved in 30–60 minutes.

Tissues are dehydrated by submersion in a graded series of ethyl alcohol watery solutions before being embedded in either one of the thermo-setting plastics such as butyl methacrylate, or in one of the cross-linked epoxy resins e.g. epon or araldite.

Butyl methacrylate was introduced by Newman, Borysko and Swerdlow in 1949. It has the highly desirable qualities of (a) partially volatilizing in the electron beam, thus removing background material, (b) it gives a reasonable specimen contrast and (c) it is easy to cut. But it has the undesirable quality of decomposing in the electron beam resulting in collapse of fine structures during observation. Polymerization damage is also observed in sections embedded in methacrylate and this is due to the uneven polymerization of the methacrylate producing variable shrinkage with consequent distortion of the tissue.

By using partially prepolymerized methacrylate (Borysko 1956) damage to specimens may be minimized.

Morgan, Rose and Moore (1957), Pease (1964) and others have described some adverse effects of methacrylate embedding on tissues.

In this investigation the thin sections of FLK cells infected with Wesselsbron virus were embedded in methacrylate and satisfactory results were obtained. Normal uninfected tissues were well preserved, the double nuclear membrane was easily observed. The mitochondria were not swollen and the cristae were clearly seen. The endoplasmic reticulum did not appear irregular or swollen.

Maaløe and Birch-Andersen (1956) introduced epoxy-resins as embedding medium for tissues. These resins do not decompose in the electron beam. They do, however, result in sections of very low contrast, but suitable stains have been developed to overcome this.

Epon appears to be the embedding medium of choice at present and it is currently being used for embedding Wesselsbron virus infected FLK tissues in order to compare the results with those obtained with methacrylate.

Ultramicrotomes of various designs, fitted with glass or diamond knives are capable of producing extremely thin sections (20-50 mμ). Glass knives are made from plate, mirror or crystal glass and are only used at one cutting session.

A trough is built up around the fracture edge of a triangular piece of glass, and filled with dilute acetone so that sections may be floated away from the knife edge.

Sections are mounted on carbon coated grids and stained. A lead hydroxide stain (Watson 1958) or a lead citrate stain (Reynolds 1963) are most commonly used.

2. MATERIALS AND METHODS

1. Preparation of fixative

Osmium tetroxide (2%) - 1 gm ampoule of osmium tetroxide, from which the label had been removed and the outside washed well, was placed in a 100 ml wide necked glass stoppered bottle and 25 ml of 3 x distilled water was added. The ampoule was smashed by vigorous shaking and a further 25 ml distilled water was added. The flask containing the osmium tetroxide was left at room temperature for a few hours and stored 4°C overnight to allow the OsO_4 to dissolve completely.

Stock buffer solution - was prepared as follows:

Sodium veronal	14.7 gm
Sodium acetate $3H_2O$	9.7 gm

made up to 500 ml with distilled water. The buffer was stored at 4°C.

Hydrochloric acid (0.1 N) - 8.6 ml of concentrated HCl (36% 11.6 M) was dissolved in distilled water to make one litre.

1% OsO_4 Fixative (pH 7.4)

OsO_4 2%	10 ml

Stock buffer solution	4 ml
HCl 0.1 N	4 ml
Distilled water	2 ml
Sucrose	0.9 gm

2. Preparation of embedding medium

The hydroquinone inhibitor in the methacrylate monomer was removed by shaking 200 ml n-butyl methacrylate and 50 ml methyl methacrylate with 100 ml 2% NaOH in a separating funnel. This was repeated once, followed by three washings with water. The methacrylate monomer was dried by adding anhydrous sodium sulphate and stored at 4°C.

To prepare prepolymerized monomer, 0.5 gm benzoyl peroxide was added to 25 ml methacrylate monomer. When dissolved, this mixture was dispensed in 5 ml amounts in stoppered tubes and placed in an oven at 50-60°C for approximately 2 hours with frequent agitation. When the viscosity of the liquid approximated that of glycerol, the tubes were removed and stored at 4°C.

3. Preparation of lead citrate stain

Lead nitrate	1.33 gm
Sodium citrate	1.76 gm
Distilled water	± 30 ml
NaOH $N/_1$	8 ml

Distilled water added to final volume of 50 ml.

4. Preparation of infected FLK cells

Secondary cultures of foetal lamb kidney cells were grown in 2 oz medical flat bottles in Hanks' LA growth medium. When the cell sheets were confluent the growth medium was poured off and each of four bottles were infected with 0.5 ml of a 10^{-1} suspension of the low passage strain of Wesselsbron virus. This inoculum contained 2.5×10^7 $TCID_{50}$. The infected bottles were incubated in the horizontal position at 37°C for one hour. The inoculum was then removed with a pasteur pipette, the cells washed twice and overlaid with 7.0 ml of Hanks' LA maintenance medium.

Four bottles of secondary FLK cells were treated the same way as above except that they were not infected with virus. These served as controls.

The infected and control uninfected FLK cell cultures were incubated at 37°C. The cells from one of the infected cultures and from one of the controls were removed each day, washed twice in Hanks' LA (without serum) and fixed for 30 minutes in 1% OsO_4 at 4°C. The cells were dehydrated in two changes of 75% ethyl alcohol, each of 6 minutes duration and three changes of 100% ethyl alcohol, 10 minutes in each. After the cells had been immersed in the methacrylate monomer for 60 minutes they were placed in the bottom of a gelatin capsule (size 00) and prepolymerized methacrylate was poured into the capsule. The methacrylate filled capsules were left at

room temperature for 30 minutes and then placed in an oven at 50°C - 60°C for two days.

A Porter-Blum microtome, fitted with a glass knife was used for cutting the sections.

Thin sections of tissues were stained with lead citrate and examined in a Siemens Elmiskop I electron microscope.

Preliminary investigations of the intracellular development of the <u>high passage</u> strain of Wesselsbron virus were made using the same technique as above. Secondary foetal lamb kidney cell cultures in 2 oz medical flat bottles were infected with 0.5 ml of a 10^{-1} suspension of this strain of the virus. The electron microscopic study of the infected cells obtained from these cultures was not extended beyond 48 hours after infection.

C. RESULTS

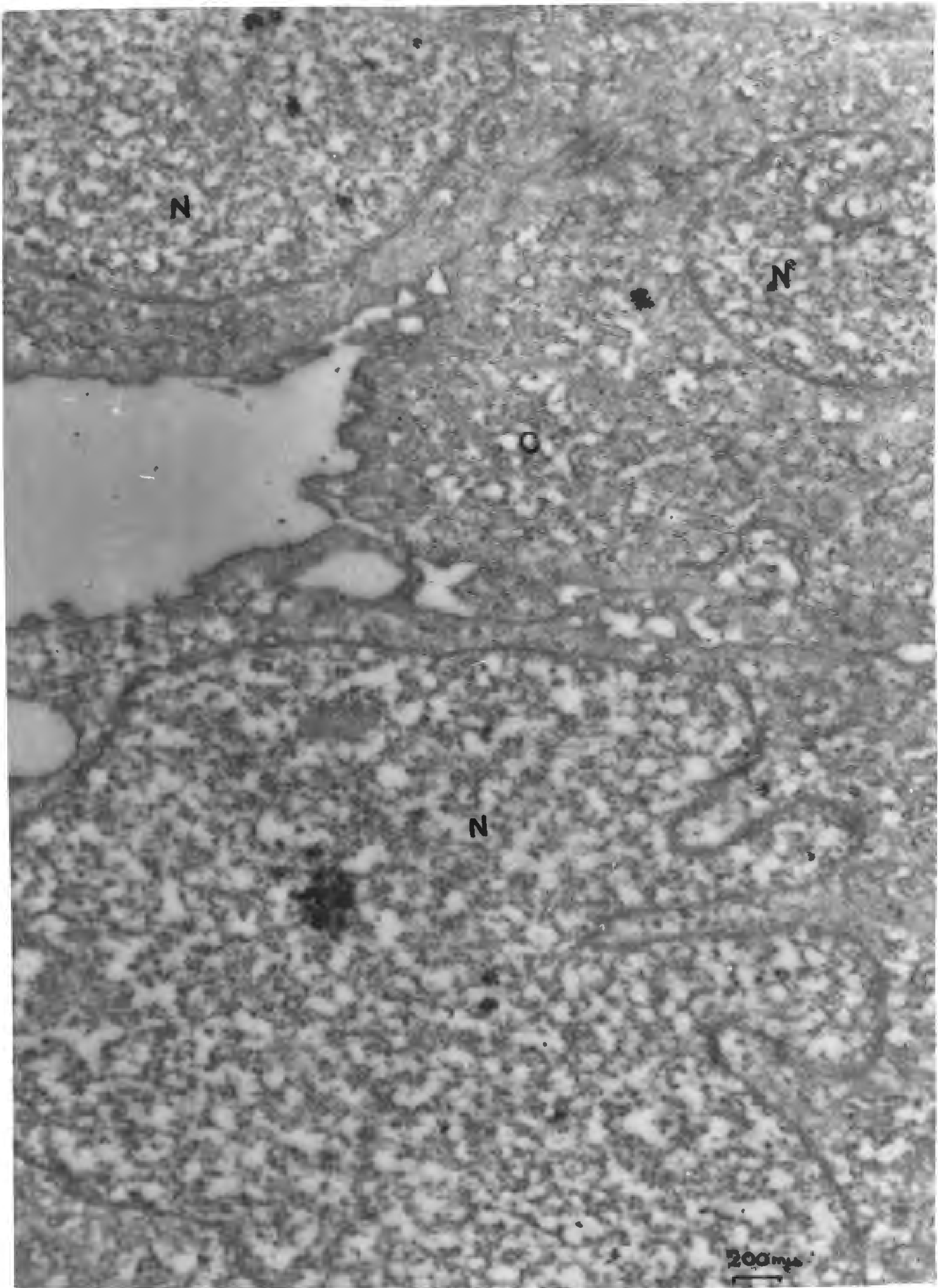
Thin sections of foetal lamb kidney cells infected with the <u>low passage</u> strain of Wesselsbron virus were prepared from cells 24, 48, 72 and 96 hours after infection. Sections were also prepared of the control uninfected cultures.

An electron micrograph of control uninfected FLK cells appears in Fig 24. The intracellular organelles are clearly identified and regular in appearance. The nuclear chromatin is evenly distributed and the cytoplasm

Fig. 24.

Electron micrograph of uninfected FLK cells (33,000X)

N = nucleus
C = cytoplasm


N
N
C
N
200 mμ

is only slightly vacuolated.

Wesselsbron virus infected cells did not show any distinct changes until 48 hours after infection, when the nuclei appeared closely packed, and the chromatin marginated. The most striking observation was the appearance of many unusual orderly-patterned inclusions in "crystalline-like" array in the cytoplasm of a very high proportion of the cells. A low power electron micrograph (Fig 25) shows this regularity and the three dimensional nature of the inclusions evidenced by the frequent directional changes in the cross-hatching.

The varying organization within the inclusions is clearly visible in Figs 26 and 27. Sections through different planes show areas of hexagonal units in honey comb formation (A) and four sided units in reticulate array (B). This may suggest an arrangement of short hexagonal cylinders rather than aggregates of polyhedral shells.

Similarly organized structures were never observed in uninfected foetal lamb kidney cells cultured under the same conditions, indicating that this effect is not likely to have resulted from damage to the cells during preparation for electron microscopy.

These inclusions appeared to be intimately associated with the endoplasmic reticulum which showed gross vesiculation in the vicinity of the arrays (Figs 26 and 27).

Many electron dense granules, not associated with the endoplasmic reticulum were visible in the cytoplasm (Figs 26 and 27) resembling free

Fig. 25.

Electron micrograph of orderly patterned inclusion in cytoplasm of infected FLK cells 48 hours after infection (31,300X)

I = orderly patterned inclusion

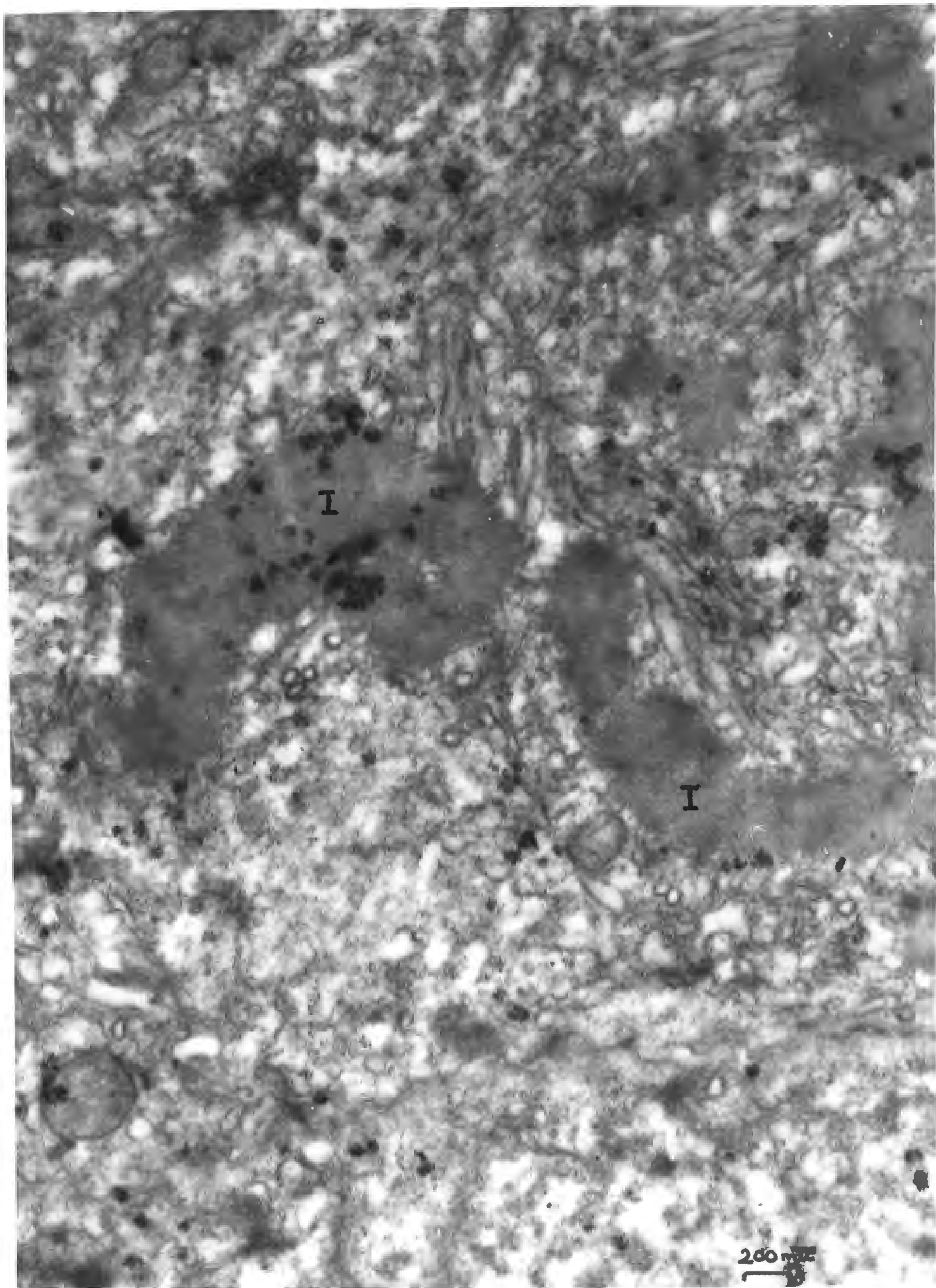

I
I

Fig. 26.

Electron micrograph of orderly patterned inclusion
48 hours after infection (133,000X).

A = area of hexagonal units in honey comb formation
B = four sided units in reticulate array

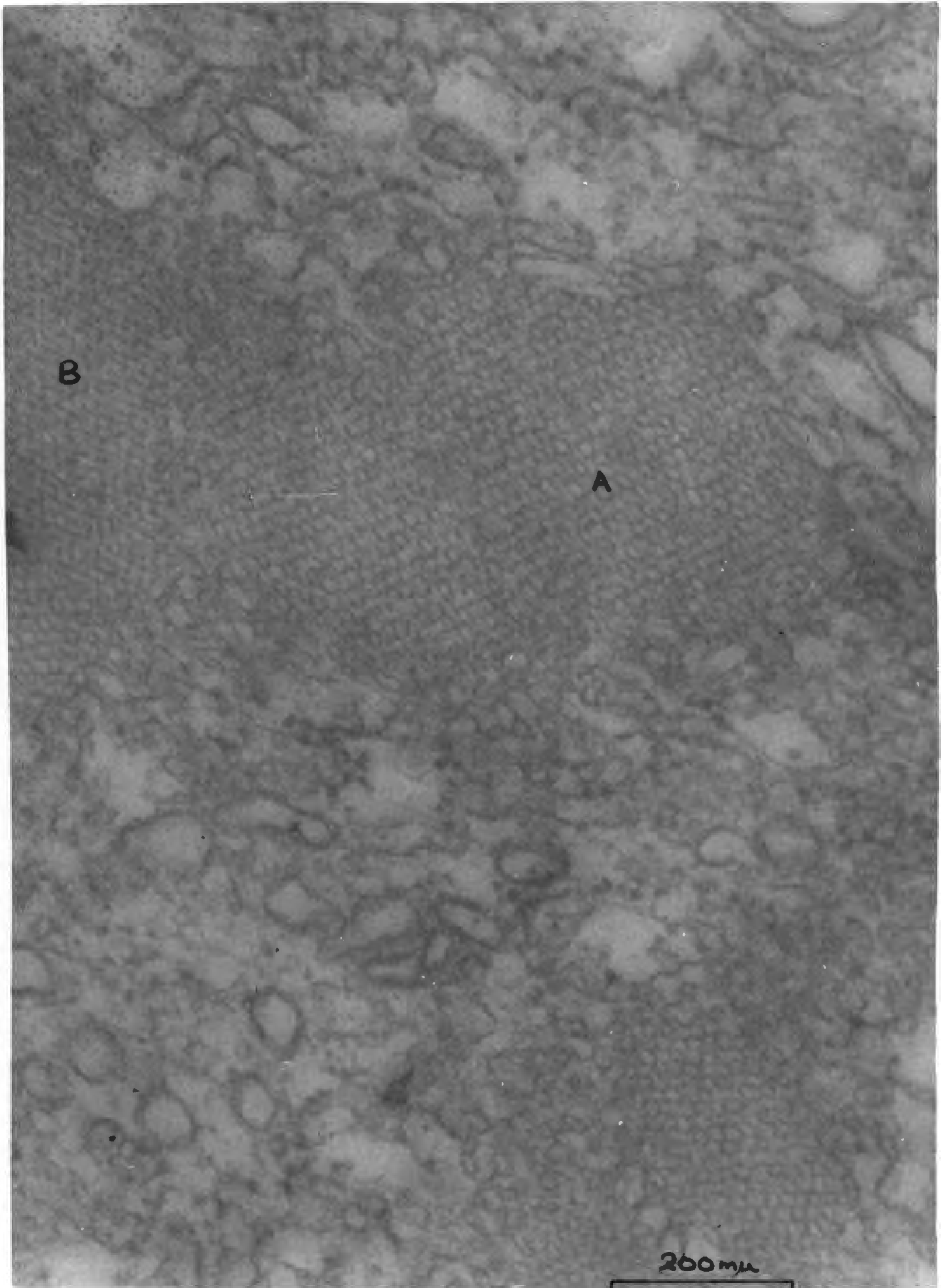
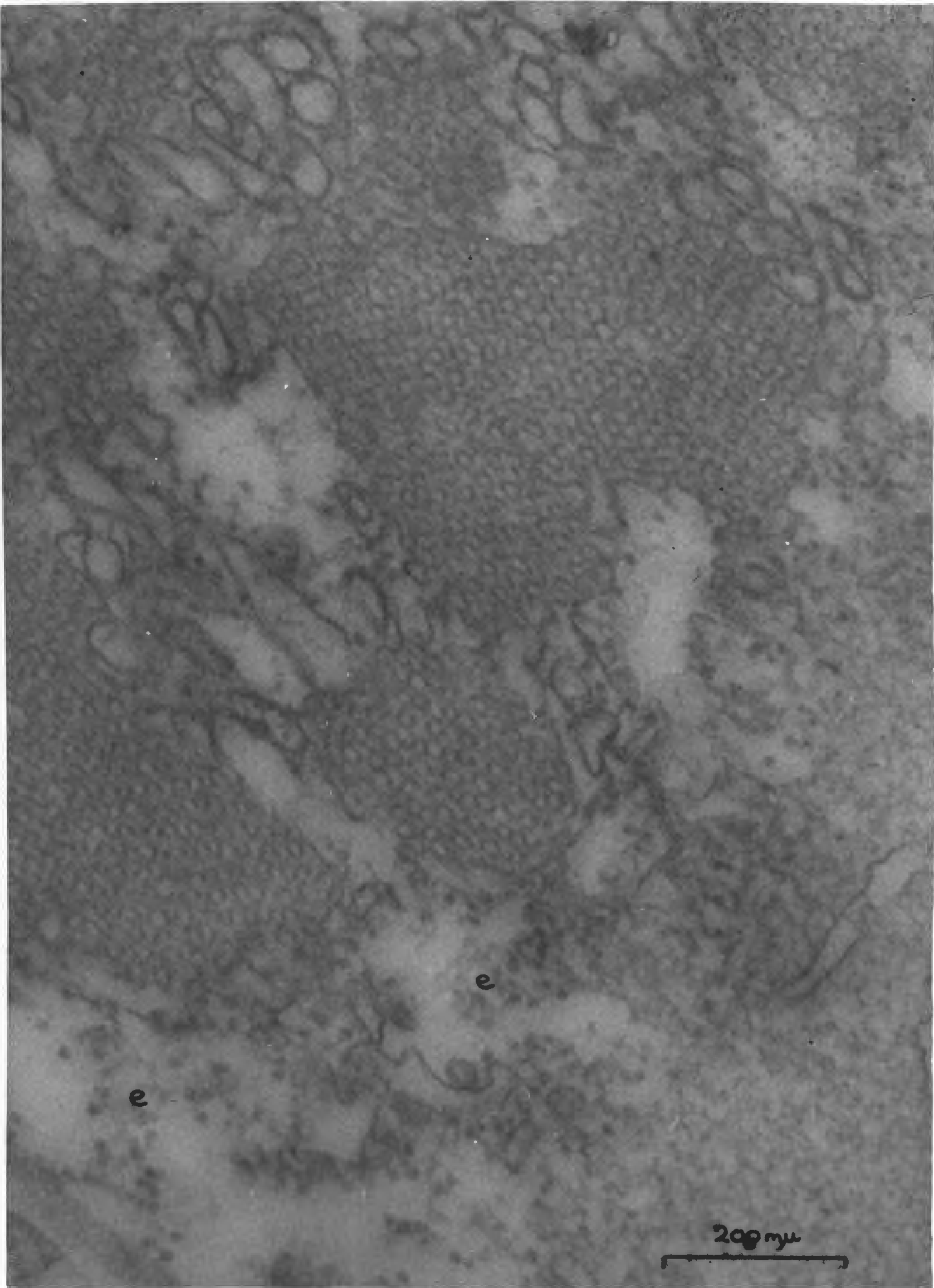

B
A
200mμ

Fig. 27.

Electron micrograph of orderly patterned inclusion 48 hours after infection showing the association of the inclusion with the endoplasmic reticulum (133,000X)

e = electron dense granules in cytoplasm.


e
e
200 mμ

ribosomes.

In some instances, particles 15 mμ in diameter were seen grouped together in projections from the edge of the inclusions (Figs 28 and 29). These resemble those seen in HEp-2 cells infected with West Nile virus (Southam _et al_ 1964), but in this instance the particles were complete virus particles 35 mμ in diameter.

Occasionally "virus-like" particles measuring approximately 34 mμ were seen in the cisterns of the endoplasmic reticulum (Fig 30). These particles possessed an inner dense "core" surrounded by a less dense "coat" giving confidence to the suggestion that they were virus particles.

After 96 hours, the "crystalline-like" inclusions were no longer evident in the infected cells, but characteristic areas of dense aggregates (Fig 31) were visible in the cytoplasm in comparable numbers and in situations similar to those of the ordered arrays from which they are presumed to have originated. In Fig 31 three mitochondria are visible and two "virus-like" particles measuring approximately 34 mμ (arrowed).

Ultrathin secti ons of foetal lamb kidney cells infected with a significantly lower concentration of Wesselsbron virus, i.e. 0.5 ml of 10^{-6} dilution of the virus suspension, representing 2.5×10^2 $TCID_{50}$, did not show the orderly patterned inclusions until 96 hours after infection and the inclusions did not appear in large numbers.

Fig. 28.

Electron micrograph of an inclusion showing projections at the edge 48 hours after infection (66,600X)

p = projections

BOND

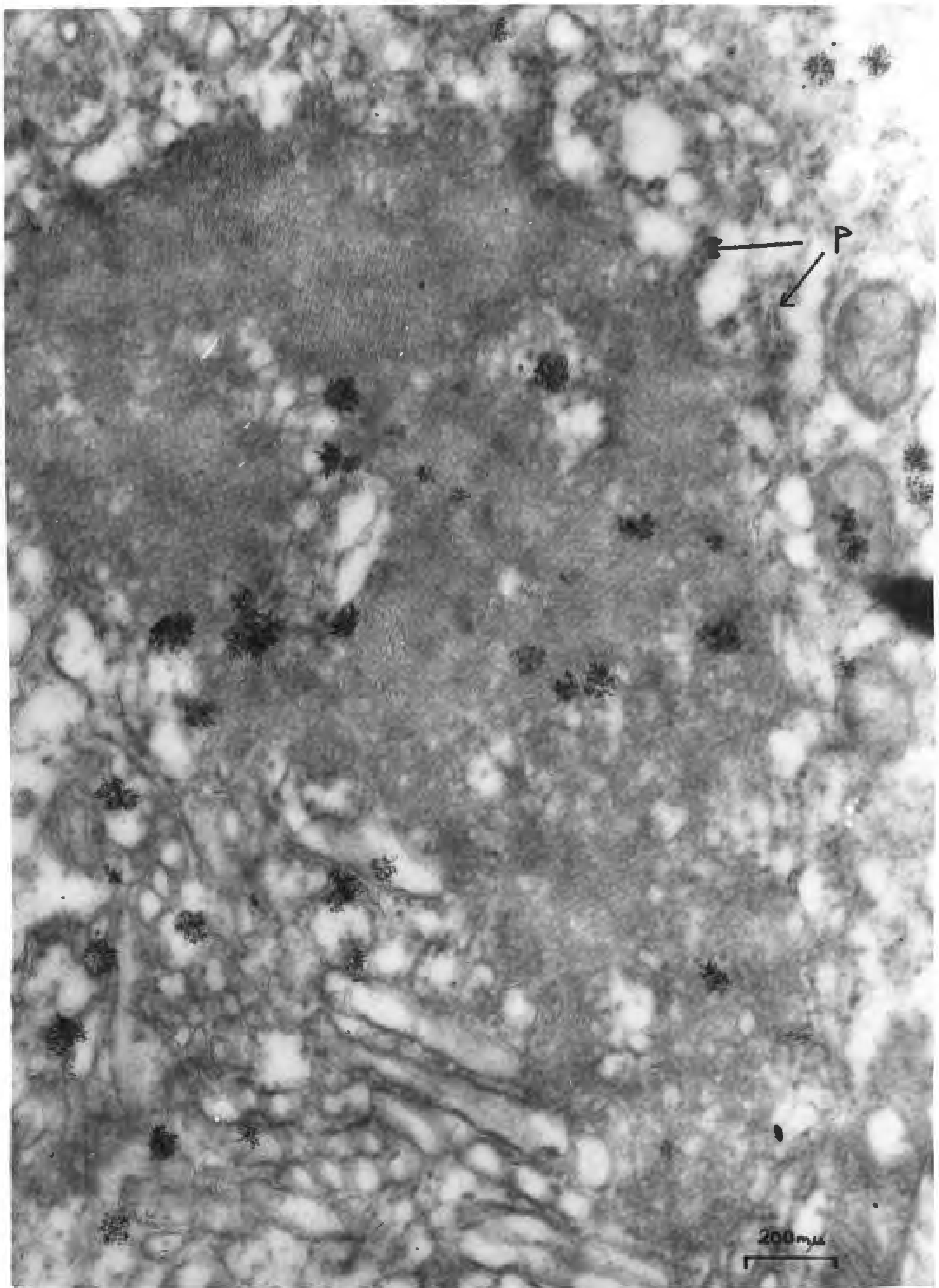

P
200mμ

Fig. 29.

Electron micrograph of the projections at the edge of an inclusion containing 15 mμ particles 48 hours after infection (140,000X).

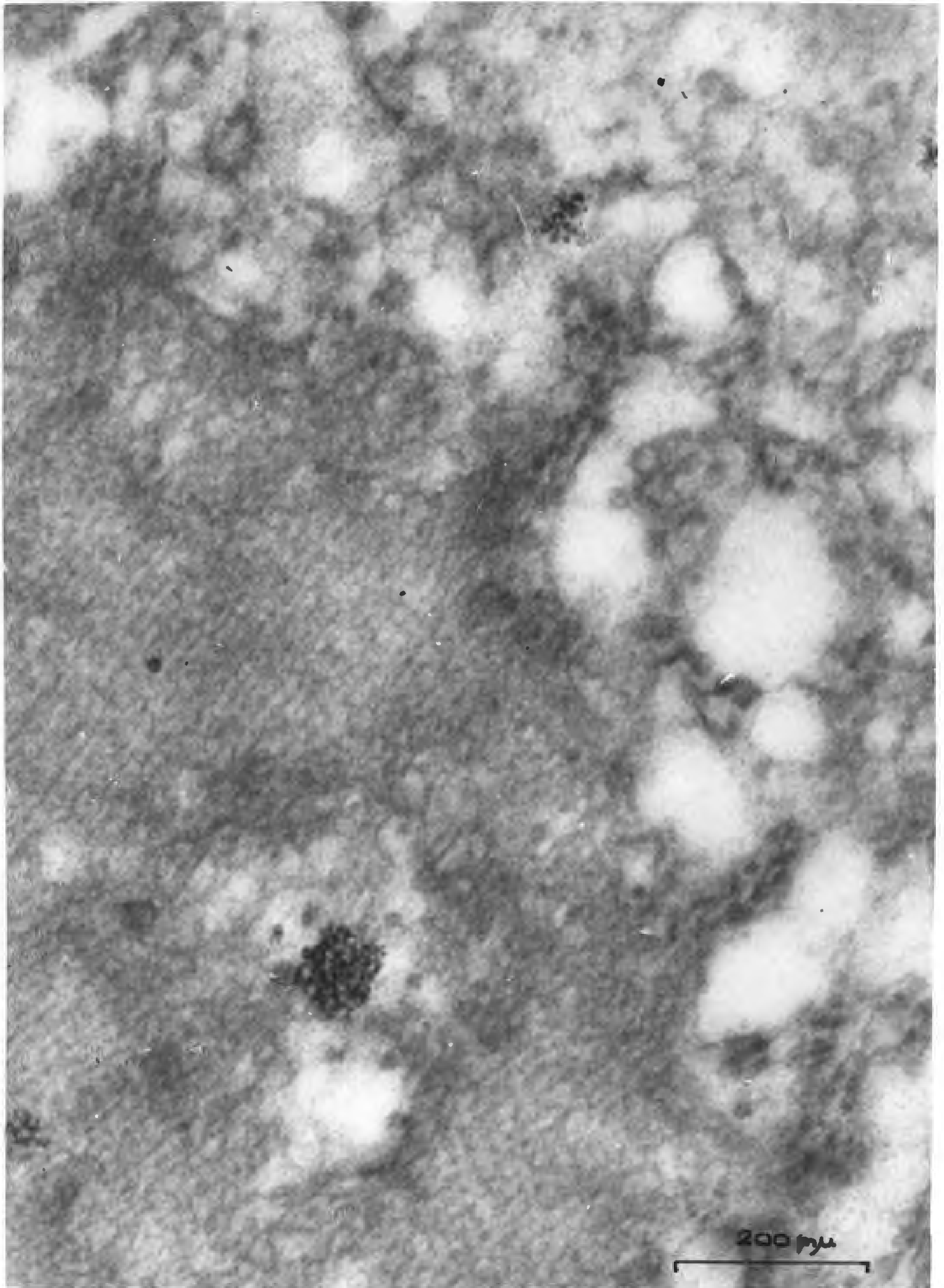

200 mµ

Fig. 30.

Electron micrograph of "virus-like" particles in cistern of the endoplasmic reticulum 48 hours after infection (140,000X)

V = "virus-like" particles.

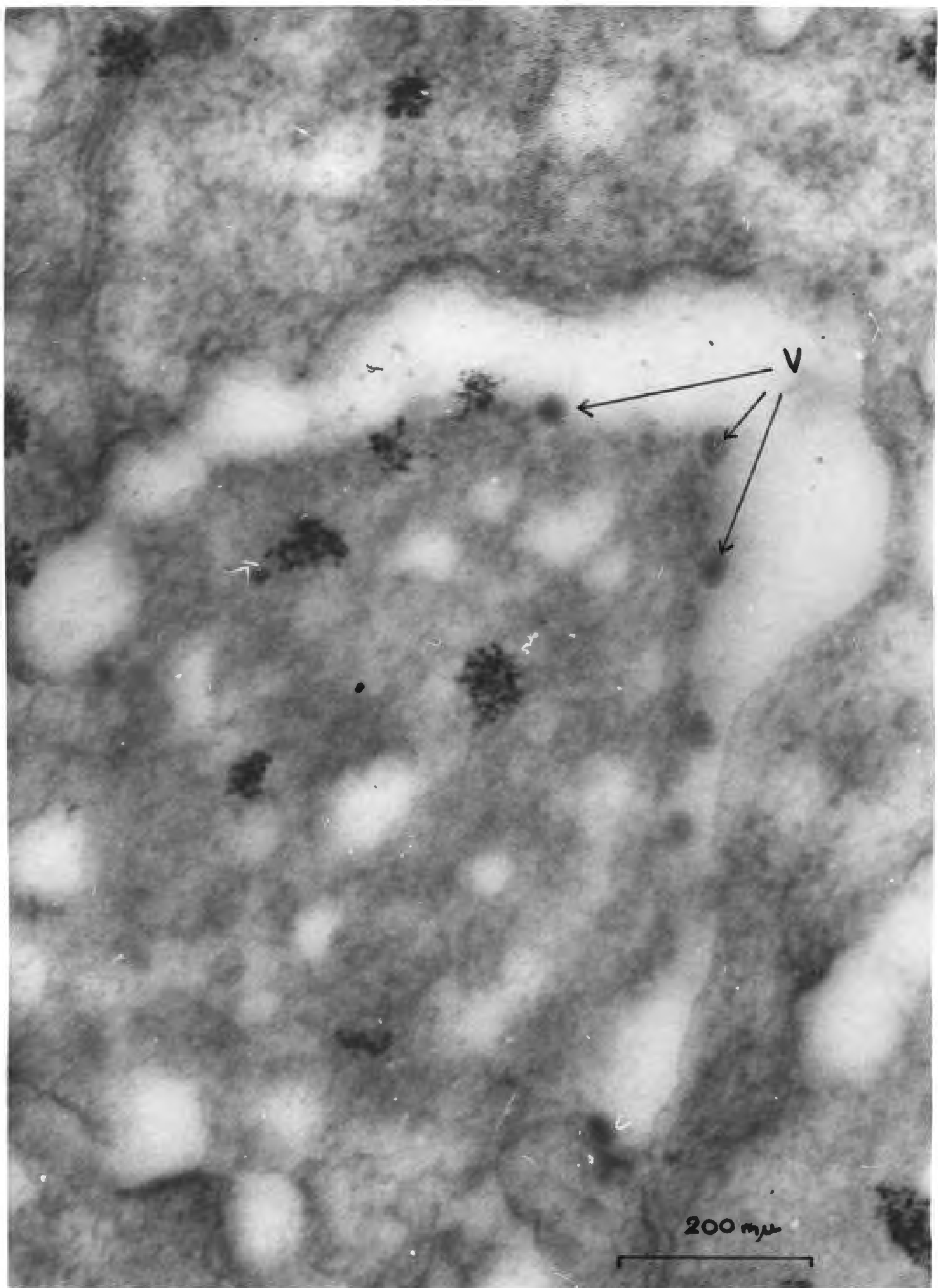
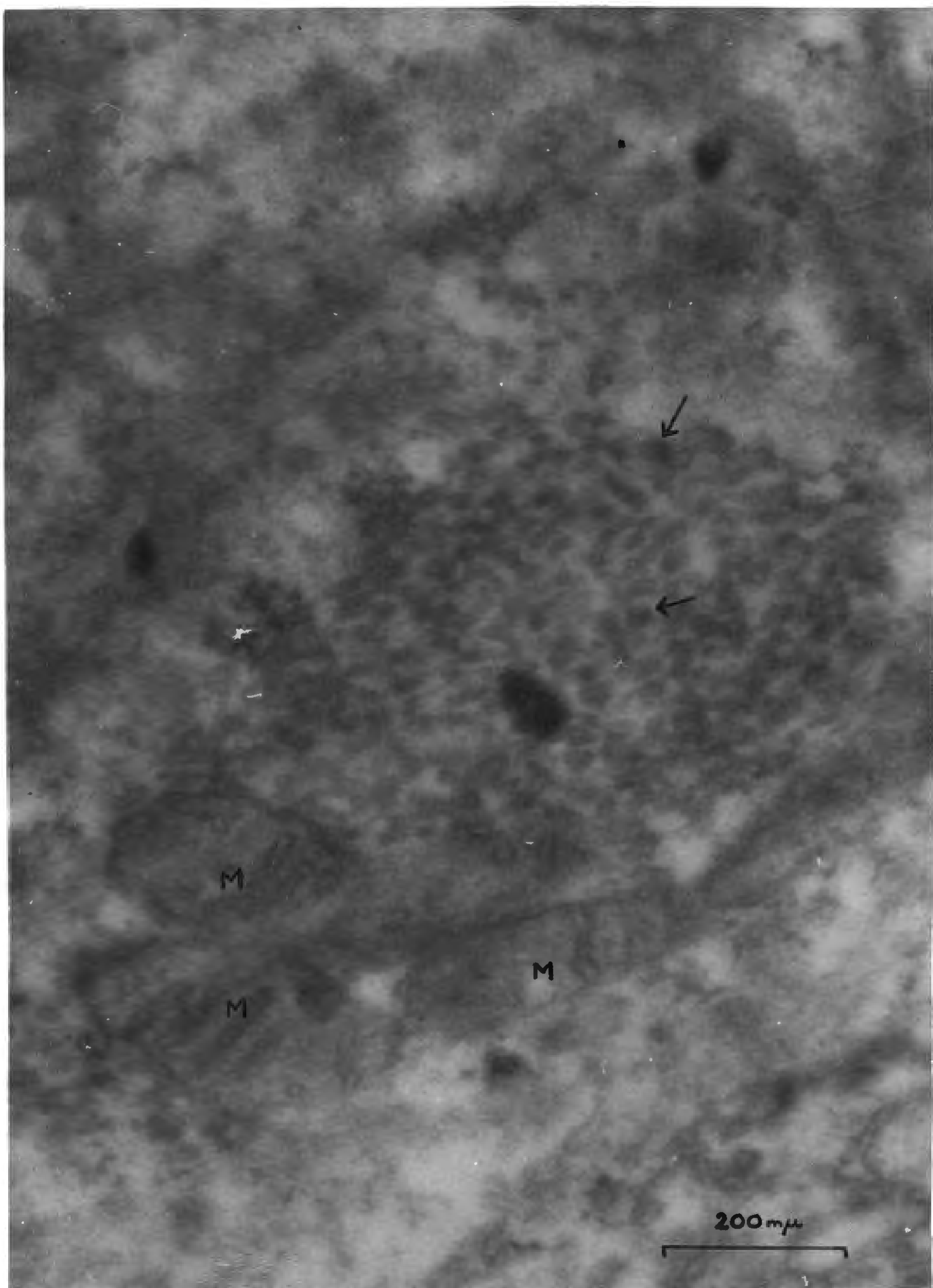

V
200 mμ

Fig. 31.

Electron micrograph of dense aggregate present in cytoplasm 96 hours after infection. Two "virus-like" particles are arrowed (133,000X)

M = mitochondria.


M
M
M
200 mμ

Sections of cells infected with a high concentration of the high passage strain of Wesselsbron virus, also showed inclusions approximately 48 hours after infection. These inclusions, however, appeared to have lost the orderly patterned structure described above (Fig 32), although they still possess an irregular pattern of basically similar structure. Their association with the endoplasmic reticulum is clearly evident. Within some of the dilated cisterns a few groups of virus particles were observed (Fig 33) measuring 30 mμ in diameter with an electron dense core measuring approximately 20 mμ.

D. DISCUSSION

Intracytoplasmic inclusions with "crystalline-like" structure have been described in many animal virus growth studies : Adenovirus; Morgan, Howe, Rose and Moore (1956): Herpes virus; Morgan, Jones, Holden and Rose (1958): Cossackie virus; Morgan, Howe and Rose (1959): Poliovirus; Stuart and Fogh (1959): Measles virus; Kallman, Adams, Williams and Imagawa (1959): ECHO virus; Stuart, Fogh and Plager (1960): Mengovirus; Dales and Franklin (1962): EMC virus, Hinz, Barski and Bernhard (1962): "K" virus; Dalton, Kilham and Zeigel (1963): SV 40 virus; Granboulan, Tournier, Wicker and Bernhard (1963): SV 4 virus; Sattar and Rozee (1965). But in nearly every instance the crystalline arrays consisted of virus particles. Similar crystalline inclusions have also been described in some virus infected

Fig. 32.

Electron micrograph of inclusions found in the cytoplasm of FLK cells 48 hours after infection with the high passage strain of Wesselsbron virus (66,600X).

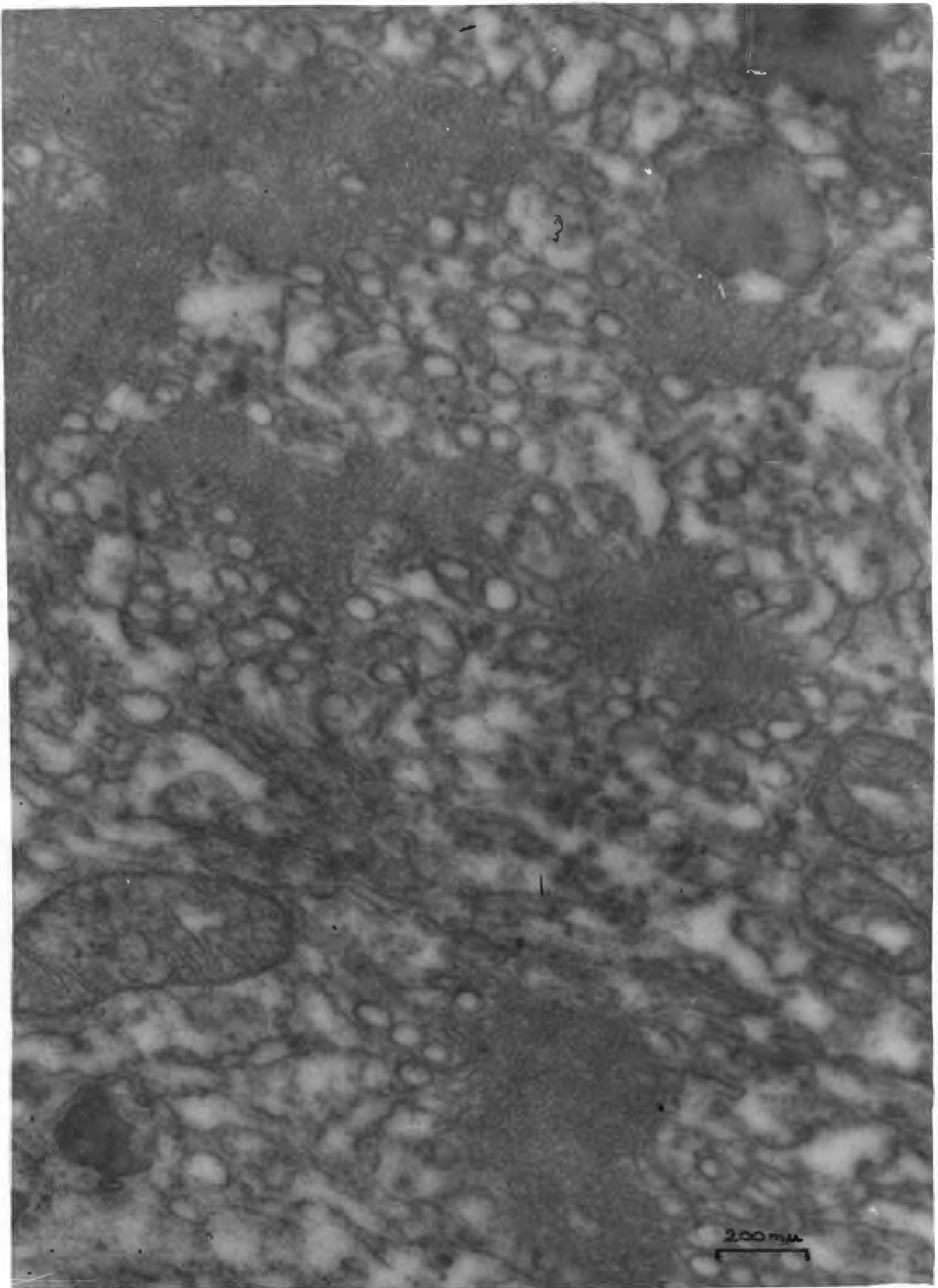
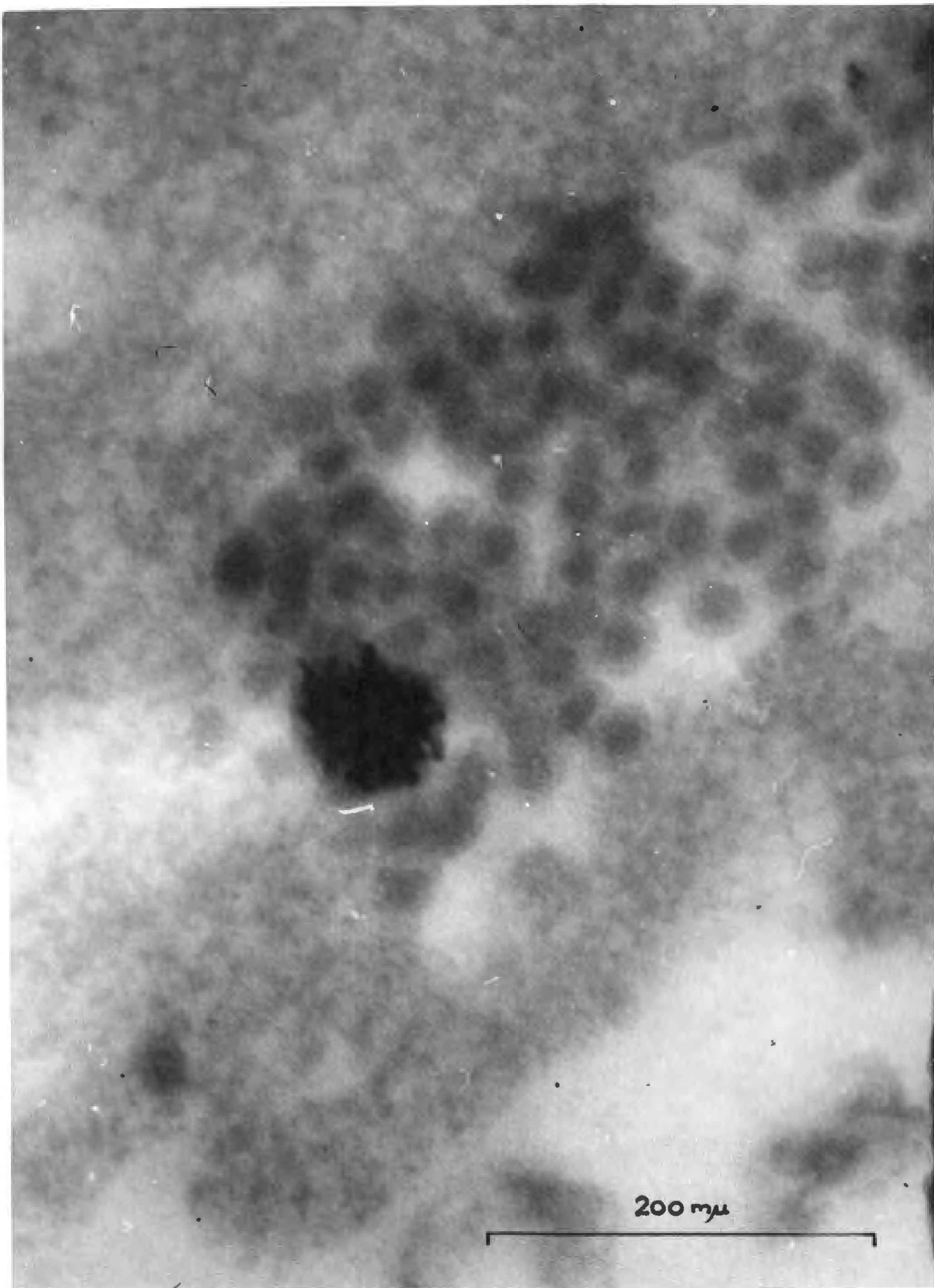

200 mμ

Fig. 33.

Electron micrograph of group of virus particles in the cytoplasm of FLK cells 48 hours after infection with the high passage strain of Wesselsbron virus (280,000X).


200 mμ

plants and insects.

In contrast to the findings of all the workers in which the crystalline array consists of electron dense particles, the "crystalline-like" inclusions found in Wesselsbron virus infected FLK cells consist of electron translucent subunits. Except for a phage-infected Group C streptococcus (Cole 1965), which displays regular structures most closely simulating those seen in Wesselsbron virus infected foetal lamb kidney cells, it is believed that this type of inclusion is unique.

From electron microscopic studies of thin sections of other arbovirus infected tissue culture cells, it appears that viruses in this group, such as Venzuelan Equine Encephalitis and Japanese B Encephalitis (Mussgay and Weibel 1962; Ota 1965) mature characteristically at the membranes of the cytoplasmic vacuoles. No evidence to support these observations was found in Wesselsbron virus infected cells. Dense precursor particles are not uncommon in the cytoplasm. "Crystalline-like" inclusions have not been reported in other arbovirus infected tissue culture cells, although the granular foci in West Nile virus infected cells described by Southam et al (1964) appear to have vaguely similar regular structure.

It is suggested that the intracytoplasmic inclusions observed in Wesselsbron virus-infected FLK cells are associated with viral multiplication and represent structures involved in, or required for, the assembly of virus particles which may first appear in incomplete form in masses at the periphery

of the inclusions, and later in complete form in the cisterns of the endoplasmic reticulum.

Franklin (1958) stated that lipid containing viruses obtain their lipid from host cell as intact lipid material, whereas nucleic acid is newly synthesized. This hypothesis has been supported by Kates, Allison, Tyrrell and James (1961) with their work on influenza virus and by Defendi (1962) who stated that the reproduction of all viruses containing lipids is intimately related to the membranes of the nucleus, endoplasmic reticulum or cell surface, i.e. to preformed cellular lipid. The cytoplasmic inclusions observed in Wesselsbron virus-infected FLK cells are intimately associated with the endoplasmic reticulum and therefore it is highly likely that the inclusions represent a stage in the assembly of viral lipid.

It is disappointing that so few virus particles were observed in FLK cells infected with the low passage strain of Wesselsbron virus but as there was a time interval of 24 hours between each culture studied, it is quite possible that the stage at which a large number of virus particles appeared in the cytoplasm (cf. West Nile, Southam et al 1964) was missed.

The fact that the characteristic inclusions are only evident 96 hours after infection with a low concentration inoculum of Wesselsbron virus and then only in comparatively few numbers compared with their abundance in cells infected with a high concentration of virus after 48 hours, indicates that "crystallization" occurs only when the virus concentration is great.

Cytoplasmic inclusions were observed in FLK cells infected with the high passage strain of Wesselsbron virus 48 hours after infection and numerous virus particles were visible in groups in the cytoplasm.

Stereo electron micrographs of the "crystalline-like" arrays were attempted but the results were disappointing, probably due to the thinness of the sections.

XIII - CONCLUSIONS

The grouping of the arboviruses is dependent on the fact that they multiply in arthropods and are subsequently transmitted by bite to susceptible vertebrates. The fact that they have certain properties and structural features in common has become obvious in recent years.

It may be stated that the members of this group possess a virus specific haemagglutinin; that lipids are essential for their structural integrity; that they are RNA viruses; that they are spherical and have an internal core surrounded by a membrane (Mussgay 1964).

Wesselsbron virus was found to possess these characteristics. Abundant haemagglutinin was detected in infected suckling mouse brain suspensions causing haemagglutination of goose red cells at pH 6.1 - 6.3 at room temperature; the presence of a lipid component in the structure of Wesselsbron virus was demonstrated by the fact that the infectious virus was inactivated by both ether and a bile salt and was supported by the finding of a low buoyant density for the infectious particle; the nature of the nucleic acid was confirmed by the successful isolation of infectious RNA; the spherical configuration is confidently proposed from the evidence that the estimated particle size derived from sedimentation and density data agrees with that from ultrafiltration experiments, and electron micrographs confirmed the presence of virus particles with an electron dense core and a less dense coat.

This study highlights once again the important considerations of the relationship of the haemagglutinin to the whole infectious virus particle.

The apparently confusing results which have a bearing on this relationship have been reported on by many other workers investigating the nature of arboviruses. Anderson (1959) regards the virus particle and the haemagglutinating particle as identical. Sabin and Buescher (1950) assumed the haemagglutinin of Japanese B Encephalitis virus to be associated with the virus particle since they found that centrifugation at 13,000 rpm for one hour did not reduce the activity of the haemagglutinin preparation whereas after centrifugation at 31,440 rpm for one hour, all the haemagglutinin was deposited with the infectious virus. On the basis of these results the haemagglutinin appeared to have a macromolecular structure at least the size of the infectious particle (viz. 15-22 mμ by ultrafiltration). Yet by ultrafiltration the haemagglutinin was retained by membranes with average pore diameter of 200 mμ. They therefore suggested that much of the haemagglutinin of Japanese B Encephalitis virus exists in an aggregated or polymerized form which is larger than the infectious particle. Cheng (1961), working on Semliki Forest virus produced evidence supporting the theory that the infectious particles were also the haemagglutinating particles; he showed that the ratio of erythrocyte dimer forming units to plaque forming units was 1:1. Conflicting results were obtained by Kitaoka and Nishimura (1963) who sedimented Japanese B Encephalitis virus in a caesium chloride gradient

and observed the various fractions under the electron microscope. They found the particles in the infectious fraction to be 50 mμ in diameter whereas the particles in the haemagglutinating component had diameters of 10 mμ. Thus a separation of the haemagglutinating and virus particles was achieved. Mussgay and Rott (1960) similarly separated the infectious particles and the haemagglutinin of Sindbis virus by caesium chloride density gradient centrifugation. Smith and Holt (1961), by chromotography on calcium phosphate columns showed that the arboviruses of Groups A and B had two haemagglutinins, one of which, in both groups, was the virus particle.

The results of the investigation of the haemagglutinin of Wesselsbron virus may be summarized thus:-

(i) by zone electrophoresis the virus suspension revealed two haemagglutinating components, one having the same mobility and migrating with the main infectious virus fraction and the other of very low mobility and widely separated from the former.

(ii) from density gradient centrifugation studies the buoyant density of the haemagglutinin of Wesselsbron virus was found to be of the same order as that of the infectious particle.

(iii) In estimation of the sedimentation coefficients, the figure for the haemagglutinin was found to be half that for the whole infectious virus particle.

(iv) by ultrafiltration through gradocol membranes the infectious particles

were found to pass through membranes with APD 72 mμ but retained by membranes with APD 63 mμ; in striking contrast with this, the haemagglutinin passed freely through membranes of APD 245 mμ but was held back by membranes with APD 222 mμ.

It is not possible to be certain that the virus particle is ever completely free of haemagglutinin since the tests for haemagglutinin are not as sensitive as those for infectious virus units. But in the ultrafiltration experiments the concentration of infectious virus which passed the membranes of 160 mμ was at least 10-fold greater than the minimum infectious virus detectable by haemagglutination, yet no haemagglutinin activity was demonstrated.

These findings suggest that the bulk of the haemagglutinin may be separated from the infectious particle without loss of infectivity and that the physical state of the haemagglutinin does not conform with that of a spherical particle.

It is difficult to explain these observations by any way other than that the haemagglutinin forms only a very loose association with the infectious virus particle and that when it splits from the latter it assumes a filamentous or sheet-like configuration.

Whatever the true nature of the structure of Wesselsbron virus, the theoretical considerations arising from this study prove to be similar to those

which have occupied the minds of other research workers dealing with arbo-

viruses.

XIV - APPENDIX I.

DILUENTS AND MEDIA

The following are the media used in the preparation and for the maintenance of foetal lamb kidney and chick embryo tissue cultures. They are listed in alphabetical order.

1. Agar

Oxoid agar was washed ten times in tap water, ten times in distilled water and three times in acetone before being dried at 37°C.

1.2% agar solution was prepared in distilled water, dispensed in 9 ml amounts in McCartney bottles and autoclaved at pressure of 15 lbs/sq in for 20 minutes.

2. Chick embryo extract (CEE)

Ten day old chick embryos, after limbs, viscera, beaks and eyes had been removed, were homogenized with Standard Gey's (1 ml/embryo). The homogenate was centrifuged at 2,000 rpm for 30 minutes at 4°C. Penicillin (100 units/ml) and Streptomycin (100 μgm/ml) were added to the supernatant. The CEE was stored at -20°C.

3. Cooper galactose medium (double strength) - CGx2

NaCl	16.0 gm
KCl	1.0 gm
Galactose	12.0 gm
NH_4Cl	0.1 gm
$MgCl_2.6H_2O$	10.15 gm
Lactalbumin hydrolysate	10.0 gm
$CaCl_2$	0.5 gm
$NaH_2PO_4.H_2O$	0.25 gm
Inositol	0.20 gm
Glutamine	0.20 gm
Glutamic acid	0.60 gm
Methionine	0.20 gm
Arginine hydrochloride	0.50 gm
Biotin	0.002 gm
Folic acid	0.002 gm
Nicotinamide	0.002 gm
Calcium pantothenate	0.002 gm
Pyridoxine hydrochloride	0.002 gm
Thiamine hydrochloride	0.002 gm
Riboflavine	0.002 gm

final pH adjusted to 7.2 - 7.5 with $^N/_3$ NaOH and the solution made up to 1 litre with distilled water.

4. Gey's balanced salt solution

Gey's A (10 x concentrated)

NaCl	70.0	gm
KCl	3.7	gm
$Na_2HPO_4.12H_2O$	3.01	gm
KH_2PO_4	0.237	gm
Glucose	10.0	gm

made up to 1 litre with distilled water and filtered through a fine glass filter no. 5/3.

Stored 4°C.

Gey's B

$MgCl_2.6H_2O$	0.42	gm
$MgSO_4.7H_2O$	0.14	gm
$CaCl_2$	0.34	gm

made up to 100 ml with distilled water and filtered through a fine glass filter no. 5/3. Stored 4°C.

Gey's C

$NaHCO_3$	2.25	gm

made up to 100 ml with distilled water and pressure filtered.

Stored 4°C.

Standard Gey's

Gey's A (diluted 1:10)	90	ml
Gey's B	5	ml
Gey's C	5	ml

5. <u>Hanks' balanced salt solution (Hanks' BSS)</u>

Hanks' stock A

NaCl	160.0	gm
KCl	8.0	gm
$MgSO_4 . 7H_2O$	4.0	gm
$CaCl_2$	3.8	gm

made up to 1 litre with distilled water and filtered through two layers of filter paper. Two ml $CHCl_3$ added.

Stored at room temperature.

Hanks' stock B

$Na_2HPO_4.2H_2O$	1.52	gm
KH_2PO_4	1.20	gm
Glucose	20.0	gm
Phenol red (0.4%)	100	ml

made up to 1 litre with distilled water and filtered through two layers of filter paper. Two ml $CHCl_3$ added.

Stored at room temperature.

Hanks' balanced salt solution for use

Hanks' stock A	25	ml
Hanks' stock B	25	ml
Distilled Water	450	ml

Solution autoclaved at pressure of 10 lbs/sq in for 10 minutes.

Stored at 37°C.

6. Hanks' lactalbumin hydrolysate medium (Hanks' LA)

Hanks' BSS	500	ml
Lactalbumin (5%)	50	ml
Antibiotic solution	2.5	ml

Stored 37°C.

7. Hanks' LA growth medium

Hanks' LA	500	ml
$NaHCO_3$ (5%)	3.5	ml
Calf serum	50	ml

8. Hanks' LA maintenance medium

Hanks' LA	500	ml
$NaHCO_3$ (5%)	13.5	ml
Calf serum	10	ml

9. Lactalbumin hydrolysate 5.0% (LA)

5% lactalbumin hydrolysate (w/v) made up in distilled water and autoclaved at pressure 10 lbs/sq in for 10 minutes.

10. Sera

Calf and fowl sera were seitz filtered and inactivated at 56°C for 30 minutes.

Stored -20°C.

11. Sodium bicarbonate 5% ($NaHCO_3$).

5% $NaHCO_3$ (w/v) made up in distilled water and pressure filtered.

Stored 4°C.

12. Tris (tris-hydroxymethyl aminomethane) 0.05 M pH 7.6

Tris	2.42	gm
Gey's A	90	ml
Gey's B	5	ml
Distilled water	5	ml
HCl (0.2 M)	76.8	ml

made up to 400 ml with a solution of Gey's A & B and H_2O

-18 vol : 1 vol: 1 vol.

13. Tris Gey's medium

Gey's A (diluted 1:10)	90	ml
Gey's B	5	ml
Tris 0.05 M	5	ml

Filtered through fine glass filter no. $^5/_3$

14. Tris growth medium

(i)	Gey's A (diluted 1:10)	90	ml
	Gey's B	5	ml
	Gey's C	1	ml
	Tris 0.05 M	4	ml

Filtered through fine glass filter no. $^5/_3$

To 89 ml of this solution (i)

LA (0.5%)	5	ml
Calf serum	5	ml
CEE	1	ml
Antibiotic solution	0.5	ml

was added.

Stored 4°C.

15. Tris overlay medium

Gey's A (10 x conc)	20	ml
Gey's B	10	ml
Tris 0.05 M	10	ml
LA 0.5%	10	ml
Distilled water	40	ml

Filtered through fine glass filter no. 5/3

Calf serum	10	ml
CEE	1	ml
Antibiotic solution	0.5	ml

Stored 4°C.

16. Trypsin base

Trypsin stock A

NaCl	80.0	gm
KCl	4.0	gm

made up to 500 ml with distilled water and filtered through two layers of filter paper. One ml $CHCl_3$ added.

Stored at room temperature.

Trypsin stock B

$Na_2HPO_4.2H_2O$	0.76	gm
KH_2PO_4	0.60	gm
Glucose	10.0	gm

made up to 500 ml with distilled water and filtered through two layers of filter paper. One ml $CHCl_3$ added.

Stored at room temperature.

Trypsin base

Trypsin stock A	100	ml
Trypsin stock B	100	ml
Distilled water	1800	ml

Autoclaved at 10 lbs/sq in for 10 minutes.

Stored 4°C.

17. Trypsin solution

Trypsin stock 5%

5% trypsin (1:250) (w/v) solution made up in trypsin base and seitz filtered.

Stored -20°C.

Trypsin solution for use (.25%)

Trypsin base	95	ml
Trypsin stock 5%	5	ml
$NaHCO_3$ 5%	2	ml
Antibiotic solution	0.5	ml

18. **Trypsin/Versenate solution**

Stock trypsin/versenate solution

NaCl	40.0	gm
KCl	2.0	gm
Glucose	5.0	gm
$NaHCO_3$	2.9	gm
Trypsin	2.5	gm
Sodium versenate	1.0	gm
Phenol red (0.2%)	5.0	ml

made up to 500 ml with glass distilled water.

Stock ATV was pressure filtered and 2.5 ml antibiotic solution added.

The solution was stored at -20°C.

Trypsin/versenate solution for use

Stock trypsin/versenate	11	ml
Distilled water	100	ml

XV - APPENDIX II

APD	=	average pore diameter
BBS	=	borate buffered saline
BSS	=	balanced salt solution
Bpa	=	bovine plasma albumin
CE	=	chick embryo
CsCl	=	caesium chloride
DRS	=	"deinhibitorized" rabbit serum
FLK	=	foetal lamb kidney
gm	=	gram
HA	=	haemagglutination
HI	=	haemagglutination inhibition
M	=	molar
mA	=	milliampere
ml	=	millilitre
mm	=	millimetre
$m\mu$	=	millimicron
PBS	=	phosphate buffered saline
PFU	=	plaque forming units
rpm	=	revolutions per minute
S	=	Svedberg unit
$s_{20^\circ w}$	=	sedimentation coefficient
SMB	=	suckling mouse brain.
w/v	=	weight per volume

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