CHANGES IN SOME BIOPHYSICAL CHARACTERISTICS OF AFRICAN HORSESICKNESS VIRUS (NO. 3922) DURING ATTENUATION.

by

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CONTENTS.

SUMMARY.

AFRICAN HORSESICKNESS VIRUS (No.3922, Type 7), attenuated for the horse by serial passage in suckling mouse brain, was studied at various passage levels to determine whether any change in the biophysical characters of the infectious particles had occurred during the process of attenuation. Such changes were indeed observed.

Propagation of the virus in tissue culture was accomplished only after the modification of standard culture media by the addition of egg white, a complex substance containing a number of proteins including the enzyme lysozyme. Some evidence is presented to show that the presence of egg white materially assisted in the successful cultivation of horsesickness virus, as well as in the formation of plaques in monolayers of cultured cells. Electron micrographs of horsesickness virus obtained from these cultured cells, and the results of a study of the fine structure of infected mouse nervous tissue, are presented.

A remarkable change in the buoyant density of the infectious particles of this horsesickness virus was found to occur during attenuation. The 'wild' ⁄ or virulent strain was found to consist mainly of particles of density

⁄ 'wild' strain refers to virus at the lowest passage level studied, i.e. passage 5.

1.26 gm/ml. The attenuated strain however proved to be composed of particles with densities quite different from that of the wild strain, predominantly 1.21 and 1.34 gm/ml. This alteration of the buoyant density appeared to be directly related to the degree of attenuation.

Studies in electrophoresis using a newly designed apparatus showed that the wild strain of horsesickness virus is homogeneous in its migration in an electric field. The attenuated strain showed a changed electrophoretic pattern indicating the presence of particles of different mobilities. As in density gradient analysis, electrophoresis showed a fundamental difference between the wild and attenuated strains of this virus. It was possible also to show a correlation between the slowly migrating component of the attenuated strain and the fraction of higher density.

The sedimentation coefficient of the infectious particles of the No.3922 strain of horsesickness virus was studied at various stages of attenuation and the particle size at three passage levels was calculated. The particle size and other characteristics determined in this way were compared with the results of measurements obtained from ultrafiltration of the virus through collodion membranes. It was found that the diameter of the infectious particles of the attenuated strain is greater than that of the wild strain.

This study shows that physical measurements may be used to give much additional information about the degree of attenuation of a strain of horsesickness virus, and together with the results from tissue culture and electron-microscopic studies, may lead to a better understanding of the process of attenuation of viruses. It is felt that the physical parameters might find some useful application in the field of vaccine development and control of African horsesickness.

INTRODUCTION.

The search for effective chemotherapeutic substances for the control and cure of virus infections in animals and man has only just begun. Immunisation procedures remain the most effective means available of preventing virus diseases. Smallpox vaccination was undoubtedly practised in ancient China (Parish, 1965) but it was only late in the 18th century that Jenner (1798) made his classical observation that the causal agent of cowpox could provide protection against smallpox in man. The origin of cowpox virus is not known but in the light of recent knowledge it seems probable that it was formed by the natural infection of bovines by smallpox virus. Propagation in this animal may then have resulted in the attenuated form known as cowpox.

Research based on the hypothesis that a virulent virus may be attenuated by propagation in a different animal species has resulted in the preparation of a number of vaccines. Following upon the observation (Theiler, 1930) that the mouse was susceptible to the virus of yellow fever when inoculated by the intracerebral route two types of yellow fever vaccine were prepared. French workers developed a neurotropic strain by prolonged passage of Theiler's mouse-adapted strain in the brains of mice,

while Theiler further modified the neurotropic and viscerotropic properties of the virus by prolonged passage in chick-embryo cell cultures. These vaccines are prepared today for distribution as dried infected mouse-brain tissue for cutaneous scarification and the 17D strain in the form of dried infected chick embryo tissue for subcutaneous injection.

The use of yellow fever vaccines has greatly reduced the prevalence of the disease in endemic zones. This success has led to much emphasis on the use of living attenuated viruses as immunising agents against both human and animal diseases, based on the assumption that the modified virus in such vaccines would be sufficiently stable not to revert or back-mutate to the earlier state of virulence under conditions of routine use. In both the veterinary and human medical fields there have been many outstandingly successful achievements and many live virus vaccines are now available for protection against various communicable diseases.

In man, the introduction of living attenuated poliovirus vaccine (Sabin, 1959) and living attenuated measles vaccine (Enders et al., 1960) has been proclaimed one of the greatest successes in the history of preventive medicine. In the veterinary field, where the demand for protective vaccines has been influenced more by economic than emotional

considerations, many successes just as notable have been achieved over the years by using vaccines of living attenuated strains in domestic animals. In the Republic of South Africa for instance the Onderstepoort Veterinary Research Institute has manufactured large quantities of vaccines for such diseases as bluetongue in sheep, lumpy skin disease in bovines and African horsesickness, to mention only three.

In all these endeavours the degree of success of the vaccine depends on many factors such as the number of serologically distinct types of the infective agent capable of causing the disease, the isolation and ease of propagation of the responsible virus, and the 'attenuation' or 'modification' of the virus to a form that will multiply in the tissues of the host and stimulate a protective level of immunity without at the same time endangering the life of the host or causing the ill effects of the 'wild' form of the virus.

In the search for suitable strains from the three types of poliovirus, Sabin (1959) cloned selected variants from plaques in tissue culture after varying numbers of passages. Some variants were avirulent in the sense of being incapable of causing paralysis in man, yet they were fully capable of establishing an intestinal infection and inducing a high grade of protective immunity to a challenge

infection. It was found possible with these non-paralytogenic variants to identify in them a variety of genetic markers which differentiate them with considerable precision.

With the animal viruses of bluetongue, lumpy skin disease and African horsesickness it has not yet been possible to develop such a detailed study of the individual characteristics of the attenuated, serologically distinct types of virus which have proved to be so effective in the polyvalent vaccines widely used in the Republic of South Africa and certain other areas.

The fact remains that the well tried and fruitful procedure of repeated passage of the responsible virus in some unnatural cell system such as mouse brain, chick embryo or line of cultured cells, has yielded 'attenuated' strains suitable for use as vaccines. Observations at frequent intervals during this process indicate the degree and extent of the attenuation of the strain for the original host. What in fact determines the characteristics of the strains most suited for use as vaccines can at present only be expressed in terms of their specificity, their antigenic effectiveness, their relative avirulence for the original host and their stability under defined conditions.

Very little research has been undertaken to discover what alteration of physical properties may accompany the

attenuation process of viruses. At present no physical parameters are known which could be used to measure or define the attenuated state.

This thesis is an account of some biophysical and biochemical changes observed in the No.3922 strain of African horsesickness virus Type 7 during 103 passages in mouse brains.

CHAPTER I.

HORSESICKNESS VIRUS.

Origin.

The virus investigated in this work, designated African horsesickness virus Type 7, No.3922, (HSV), was obtained from the Director of Veterinary Services, Onderstepoort, South Africa. The original isolate had been passaged intracerebrally through suckling mice to obtain the attenuated* strain. During this process samples were taken at every tenth passage level starting at the fourth and ending at the hundredth. The samples were freeze-dried and made available to the author.

Stocks of virus from various passage levels were prepared by resuspending the freeze-dried material in egg-white medium (see below) and infecting 4-day-old suckling mice by intracerebral inoculation. When the typical paralytic symptoms produced by this virus were observed, the sucklings were killed by ether vapour and the brain removed aseptically. The brains were stored at -5° until required.

* The process of attenuation of the horsesickness virus types, as carried out at Onderstepoort, usually results in a strain non-pathogenic for horses before the hundredth passage.

Suspension medium.

It is important when dealing quantitatively with viruses that little or no infectivity be lost due to circumstances beyond the limits of an experiment. Most viruses require the stabilising effect of some protein, usually an albumin, and the pH value and temperature may be important. It was found by Polson and van Rooy (1953) that egg white effectively stabilised all the strains of horsesickness virus then available. A solution based on this substance was found to be an excellent medium for the strain used in the present work.

Egg white medium.

Normal saline (NaCl 0.85% w/v in distilled water)	93 ml
Egg white	5 ml
Triton X (0.1% v/v in distilled water)	1 ml
Antibiotics (see below)	1 ml

It was found that this solution was well buffered (pH 8.0) due to the presence of the egg white. This is a satisfactory pH value for use with horsesickness virus which is sensitive to acid solutions (Alexander, 1935).

Triton X 100 (octyl-phenoxy polyethoxy ethanol, Shell Chemicals) was added to the solution to prevent

surface denaturation of the ovalbumin present in egg white. This protein tends to denature easily, especially if the solution is shaken. It was feared that virus particles might adhere to any insoluble protein so formed. This would lead to erroneous results especially in preparing dilutions for titrations. The addition of Triton X effectively overcame this problem.

Egg white was taken aseptically from hens' eggs obtained commercially. Filtration through surgical gauze yielded an easily soluble fraction which was stored (-20^{o}) until used.

Antibiotics. Penicillin (final concentration 100 units/ml) and Streptomycin (final concentration 100 microgram/ml) were added to the solution to control the growth of possible bacterial contaminants.

For some experiments the egg-white medium could not be used (see Chapter IV). Fowl serum (5% v/v) in phosphate buffer (pH 8.0) was found to be a satisfactory substitute.

When a virus suspension was required for an experiment, infected mouse brains were removed from the refrigerator, allowed to thaw at room temperature, and triturated in egg-white medium. The suspension was then clarified by centrifuging at 10,000 rev/min for 10 min in a No.40 Spinco rotor and the supernatant fluid used.

Titration.

The various samples obtained from experiments were titrated for infectivity by intracerebral injection in 3-week-old white mice. Ten-fold dilutions of the samples were made in egg-white medium (0.2 ml sample plus 1.8 ml diluent) and 0.03 ml injected into each of 6 mice for every dilution. The mice were observed daily until no more deaths occurred (21 days). The incubation period for virus at different passage levels varied considerably. For passage 5 virus onset of the paralytic signs appeared between 7 to 14 days but with the attenuated virus the period was 3 to 7 days depending on the amount of virus injected. The Reed and Muench (1938) method was usually applied for calculating the 50% end points. Use was made of the Kärber method (Parker, 1961) in those instances where the Reed and Muench method fails, for example in titrations where there are less than 6 positives in an undiluted sample. For the sake of uniformity the results of all titrations were converted to the number of infectious particles per ml using the fact that at the 50% end point there are, statistically, 0.69 units in the volume injected (Parker, 1961).

Special care was given to the syringes and needles used for injection as serious errors may result unless

certain precautions are taken. It is most important that each mouse should receive exactly the same injection volume (0.03 ml) and that the animal suffers the least possible trauma. This may be achieved by ensuring that there is no air bubble between the bottom of the plunger in the syringe and the injection fluid, and that the hypodermic needles used are sharp.

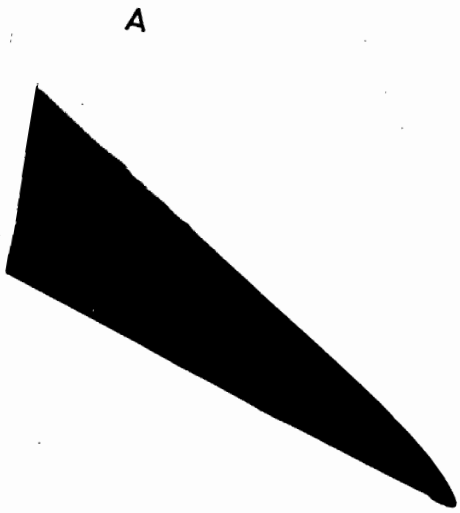
Syringes and needles were prepared in the following manner before every titration. All the needles (27 x ½ inch), new or used, were sharpened by hand on the inner surface of a B24 Quickfit glass-ground joint. The object was not only to sharpen the needles but also to change the angle of the tip to approximately 60 degrees. (Fig.1) Needles so sharpened are more suitable for the penetration of bony tissue as the point is stronger and less liable to blunt or bend. The sharpened needles and the syringes were thoroughly cleaned and the plungers lubricated with a little silicone grease. Distilled water was introduced into each syringe and all air bubbles removed. A sharpened needle was fitted and the unit autoclaved with the needle submerged in distilled water. This procedure ensured a sterile syringe with the whole space below the plunger filled with water. When used for injecting, the excess water was expelled and the syringe filled with the sample without introducing a bubble of air. The syringes used

Figure 1. Photomicrographs of hypodermic needles (27 gauge x ¼ inch long) used for intracerebral injection of mice.
Magnification x 150.

A New unused hypodermic needle. The tip, although not perfectly shaped, is sharp but is liable to damage.

B New unused hypodermic needle. Many instances of such damaged or imperfectly sharpened needles were found. In this condition they are unsatisfactory for the intracerebral injection of mice.

C Hypodermic needle after sharpening. The point has been ground to an angle of approximately 60 degrees and is sharp. This is typical of all needles used in the intracerebral injection of 3-week-old mice for the titration of horsesickness virus.



A

B

C

were all of 0.25 ml capacity and when prepared as described were satisfactory for the accurate intracerebral injection of 0.03 ml samples in 3-week-old mice. These precautions ensured that no more than 1% of mice died as a result of the injection. Accurate end points could thus be calculated in all cases.

The possibility of viral contamination of the horsesickness strain investigated was checked periodically during the course of the present work. Neutralisation of stock virus (No.3922) by specific immune serum (supplied by The Onderstepoort Veterinary Research Institute) in all cases, showed that the horsesickness virus used in this investigation was not contaminated by other viruses pathogenic for mice by intracerebral inoculation.

CHAPTER II.

TISSUE CULTURE OF HORSESICKNESS VIRUS.

Introduction.

The technique of tissue and cell culture has proved a powerful tool in the study of viruses. By observing the development of nerve fibres in synthetic media Harrison (1907) originated a method which has since been developed and refined by many workers.

Tissue culture methods were used in the present study to propagate horsesickness virus and to demonstrate its presence by the formation of plaques. A biological difference between the wild and attenuated strains of this virus became apparent soon after this work was started. Attenuated virus could not be adapted to any of the cell systems investigated. Initial attempts to propagate even the low passage virus were unsuccessful. A medium was eventually evolved, however, which was satisfactory for the propagation of passage 5 virus and which could be used to demonstrate plaque production. Virus propagated in tissue culture was also used for electron microscopy.

Materials.

Attempts to propagate the No.3922 strain of horsesickness virus in tissue culture using conventional media failed. A different type of system had to be found. It has been noted that egg white acted as an excellent stabilising agent for many of the strains of this virus (Polson and van Rooy, 1953) and that it has been found satisfactory for the No.3922 strain. Another application of egg white was found by White (1937), who discovered that a bacteriophage with specific affinity for the polysaccharides of Vibrio cholerae, but which ordinarily had little or no lytic action, attacked these bacteria vigorously in the presence of egg white. White attributed the success of egg white in this system to the lysozyme it contained thinking that polysaccharides on the surface of the bacteria were digested by this enzyme allowing the easy penetration of the bacteriophage into the host.

Considering these factors it was felt that if egg white was added to the tissue culture medium it might influence the system in such a way as to allow the propagation of horsesickness virus. A tissue culture medium containing egg white was prepared as follows:-

Hanks' inorganic salt solution plus lactalbumin hydrolysate (5% w/v)	87.5 ml
Egg white	5 ml
Penicillin (4 million units/100 ml Hanks' solution)	0.25 ml
Streptomycin (4.0 gm/100 ml Hanks' solution)	0.25 ml
Fowl serum	5 ml
Triton X (0.1%) (0.10 ml/100 ml Hanks' solution)	1 ml
Sodium bicarbonate (2.5 gm/100 ml distilled water)	1 ml
Phenol red	0.002 gm

Final pH (7.5)

The Hanks' solution was prepared (Parker, 1961) and sterilised by autoclaving.

Egg white was obtained from fresh hen's eggs by sterilising the outside of the shell with Dakin's solution (see below), breaking the shell and removing the white using a Pasteur pipette. Because much of the egg white so obtained is more or less insoluble in water it was filtered through four layers of surgical gauze. The very viscous insoluble fraction was retained on the filter. The filtrate so obtained dissolved easily in water. The egg white was stored at -20°.

Triton X was added to prevent the denaturation of

ovalbumin (Chapter I).

Fowl serum was obtained from the clotted blood of adult cocks. The red cells were removed by centrifuging and the serum sterilised by Seitz filtration.

Baby Hamster kidney (BHK) cells and mouse liver fibroblast (LF) cells, both permanent cell strains, were used. These cells had been cultured for many generations in a medium similar to that detailed above but containing no egg white or Triton X.

Virus. Horsesickness virus No.3922 passage 5 was used. Infected suckling mouse brains were triturated in egg-white medium and the suspension centrifuged at 10,000 rev/min for 10 min in sterile centrifuge tubes to sediment tissue debris and any bacteria which may have been present. The clear supernatant fluid was used to infect tissue culture cells.

Dakin's solution (as discussed by Parker, 1961) was found to be a most useful sterilising agent. It was prepared as follows:-

Sodium hypochlorite (10% solution)	108 ml
Hydrochloric acid (1 N)	12 ml
Sodium bicarbonate	16 gm
Distilled water, to make	2 litres

Final pH 8.0

Neutral red. A 0.02% (w/v) solution of this vital stain in Hanks' medium at a final pH of 6.5 was found most suitable for staining cells to reveal the presence of plaques.

Agarose was prepared by the method of Russell et al. (1964) described in Chapter VIII.

Trypsin. 0.25 gm trypsin (Difco, $\frac{1}{250}$) was dissolved in 100 ml calcium - and magnesium - free Hanks' solution (pH 7.8) and sterilised by Seitz filtration.

Methods.

Both types of cells employed were of the continuously growing, cell line, variety. Initially only BHK cells were used. Cells were removed from bottles by digestion with trypsin. Low speed centrifugation (600 x g) sedimented the dispersed cells which were washed with growth medium and used to seed new bottles; 0.5 million cells per 4 oz bottle in 10 ml medium. After two days incubation (37°) the cells had adhered to the surface of the bottle and begun to multiply. Virus suspension (LD_{50}: 500/ml) (0.5 ml) was then added to three bottles and three were kept as controls, uninoculated. At the same time 0.5 ml virus suspension was added to 10 ml medium and titrated in mice. Inoculated cells were incubated for 7 days or

until a cytopathic effect was observed. The supernatant fluid was then removed and inoculated into fresh bottles and also titrated in mice.

Virus that had been passaged 8 times in tissue culture was used to inoculate BHK cells grown in a large bottle (20 oz). After gentle mixing, 0.2 ml liquid was removed and titrated in mice. A sample (0.2 ml) was thereafter taken from the bottle every 24 hr for 7 days and titrated in mice. In this way the development of the virus in tissue culture cells was measured.

Using the method of Porterfield (1960) it was possible to obtain plaques with tissue culture passage 8 horsesickness virus. BHK cells and later also LF cells were grown in bottles as described to form a confluent sheet. The medium was then discarded and the cells inoculated with virus at different concentrations. After allowing 30 min absorption time at 37° the cells were covered to a depth of 5 mm with growth medium containing 0.7% (w/v) agarose. When the agarose had gelled the bottles were incubated for 4 days at 37°. Uninoculated bottles were treated in the same manner and kept as controls. After incubation 10 ml of neutral red solution was added to each bottle. This vital stain reacts only with living cells. Any discrete necrotic areas

present in the cell sheet are thus revealed as plaques.

Results.

Initial attempts to grow horsesickness virus No.3922 in tissue culture using BHK cells and a variety of conventional media failed. Virus remained viable in the bottle for several days but was unable to develop and eventually disappeared. When egg white was incorporated in the medium an immediate response was obtained. It was noted that cells grew well in the egg-white medium (Fig. 2A), perhaps better than in ordinary medium. When inoculated with virus a cytopathic effect was observed after 4 to 6 days (Fig. 2B). Cells tended to round up, the cytoplasm becoming granular and opaque. Many cells eventually came free from the glass either individually or as a large sheet. A few apparently unaffected cells were left firmly attached to the glass.

Results of the experiment to determine the growth characteristics of horsesickness virus in tissue culture are presented in Table I and Fig. 3. Relating the virus titre to time yields a growth curve that is typical of many viruses (Luria, 1953). The number of infectious virus particles inoculated (325 per ml as titrated in mice) dropped during the first 24 hr to nil. After

Figure 2.

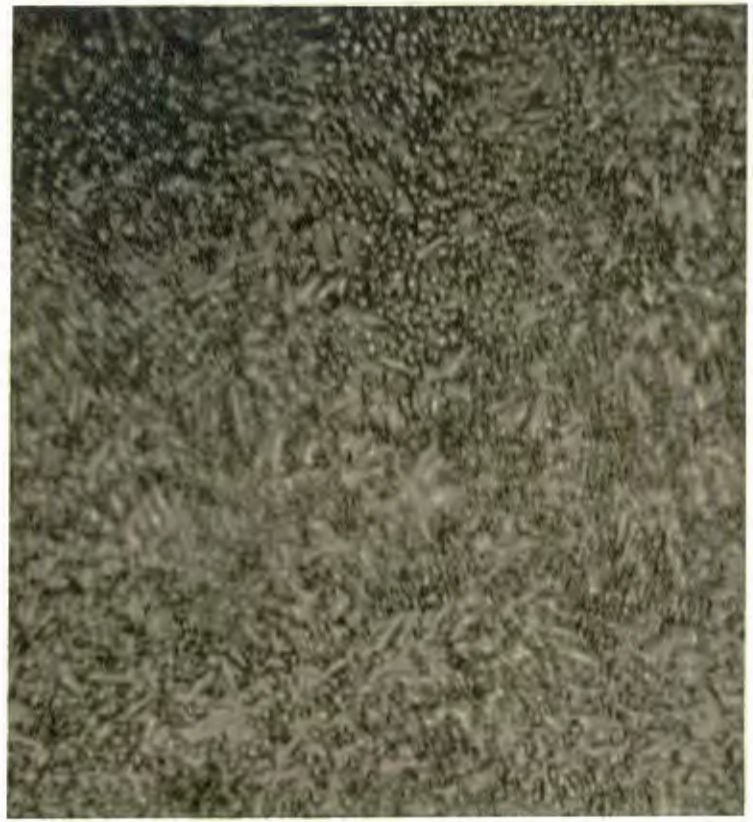
A Photomicrograph (x 150) of normal BHK cells growing in egg-white medium.

B Photomicrograph (x 150) of BHK cells four days after inoculation with horsesickness virus. The cytopathic effect observed was a general granulation and rounding up of the cells.

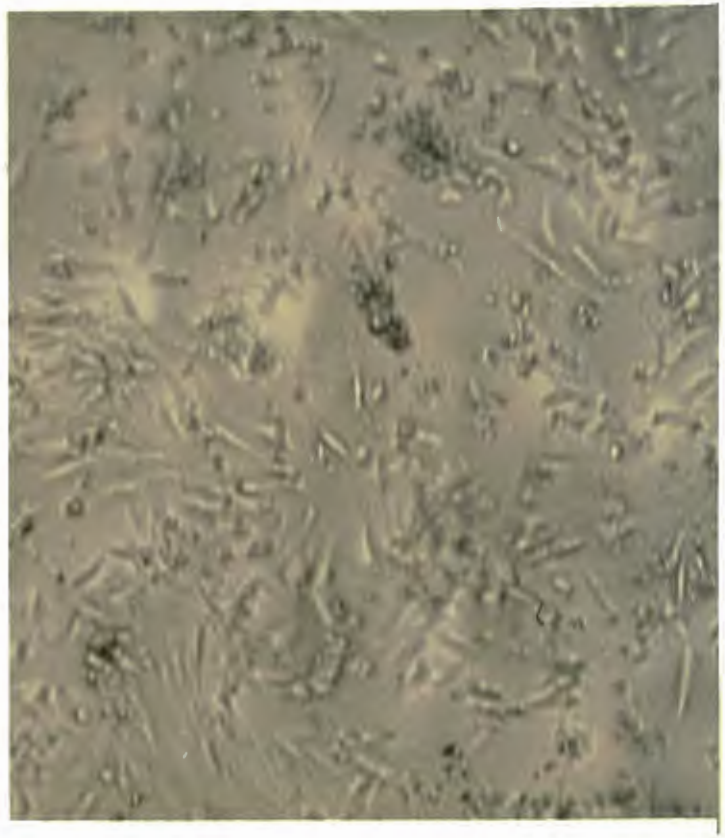
C Photograph (x 2) of plaques formed in a monolayer of LF cells by horsesickness virus.

This bottle was inoculated with approximately 100 mouse infective doses per ml.

C



B

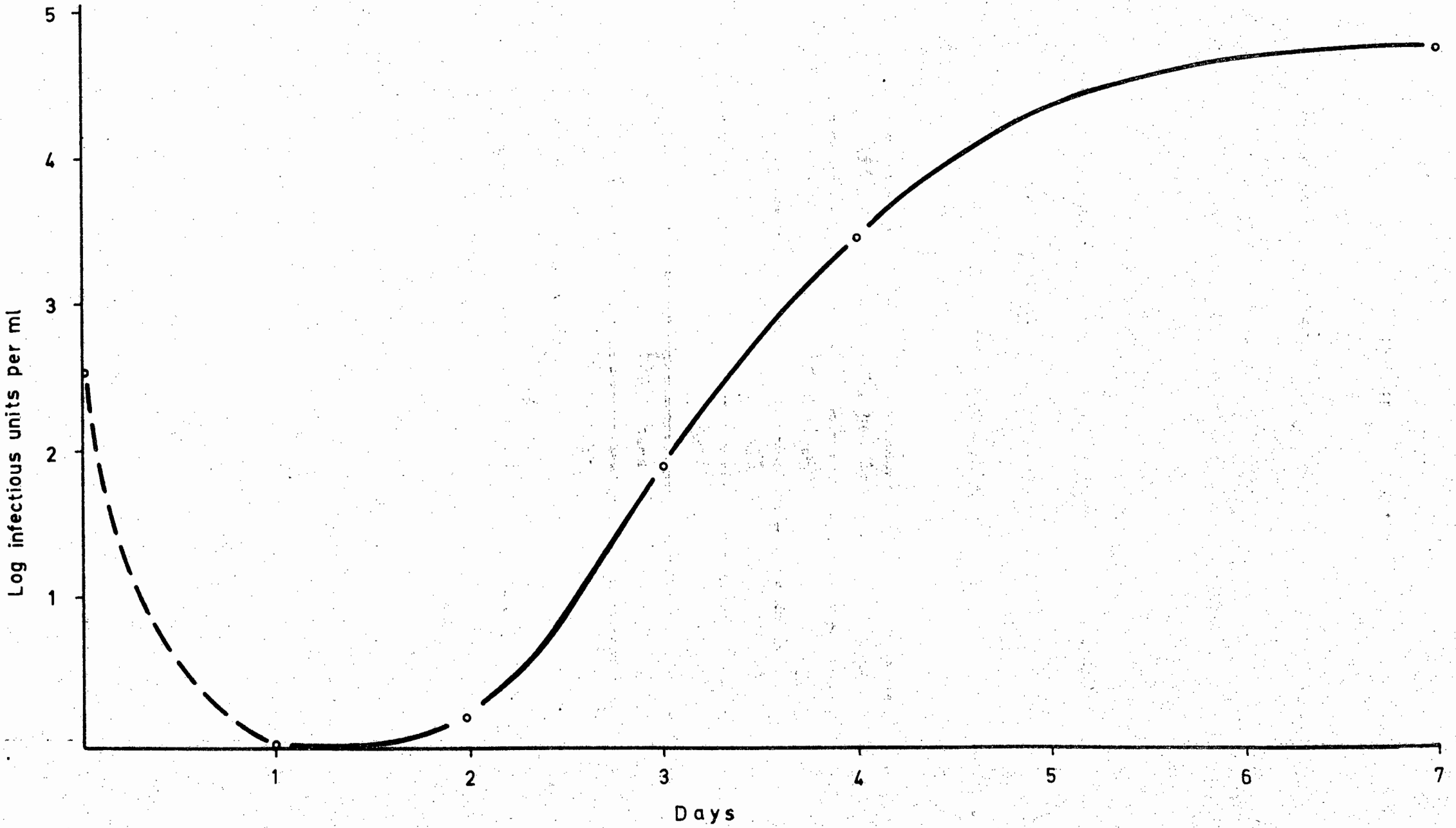


A

Figure 3. Growth curve of horsesickness virus (passage 5) in tissue culture.

Table 1. Propagation of horsesickness virus (passage 5) in tissue culture. (Samples titrated in mice.)

Days	Number of infectious units (per ml)
0	3.25×10^2
1	Nil
2	15.6
3	9.16×10^1
4	2.90×10^3
7	5.78×10^4


Log infectious units per ml
5
4
3
2
1
1
2
3
4
5
6
7
Days

this eclipse period the number of viruses present increased logarithmically for 2 days. Virus continued to be produced but at a slower rate until the 7th day when the experiment was terminated as all the cells had been destroyed.

Plaques of horsesickness virus were obtained using either BHK or LF cells under agarose overlay in bottles (Fig. 2C). Two sizes of plaques were observed. The majority were approximately 0.5 mm in diameter while some were 2 to 3 mm in diameter.

The plaques were somewhat irregular in shape with ill-defined borders. The impression gained was that some cells were resistant to infection by the virus with the result that clearly circumscribed round plaques were not formed.

Discussion.

Initial attempts to grow the Type 7 strain of horsesickness virus (No.3922) in tissue culture were unsuccessful. By noting the conclusions of White (1937) and Polson et al. (1953) and incorporating egg white in the medium success was immediately achieved. While it is realised that tissue culture is by no means an exact technique, and that even different batches of serum may

drastically influence the results, it is nevertheless considered that the addition of egg white to the medium was the main factor in establishing the propagation of horsesickness virus in tissue culture. The mode of action of the egg white is thought to be that of assisting the penetration of virus into the cell. The fact that the virus has now been propagated in both BHK and LF cells means that these cells have always been potentially able to sustain the growth of the virus. Although the addition of egg white may have changed the metabolism of the cells in some way it is thought more likely that it is the penetration of the cell wall by the virus that is facilitated by the egg white. Egg white contains approximately 2.6% of the enzyme lysozyme (Fevold, 1951). This enzyme may have digested mucopolysaccharide either on the cell wall or adsorbed to the virus particle and so assisted in the establishment of infection of the cell.

An added advantage of having egg white in the medium is that it contains a trypsin inhibitor (Fevold, 1951) which effectively neutralises any of this enzyme that may remain in solution after washing the cells.

In connection with the possible rôle of lysozyme in assisting in the infection of tissue culture cells by horsesickness virus an interesting report (Romeo and

de Bernard, 1966) has been noted. These authors find that lysozyme can be included in, and masked by, an artificial mixture of phospholipids and proteins thus effectively preventing the enzyme from acting on any substrate. Further, the enzyme is completely unmasked on the addition of Triton X to the solution. It is certain that the tissue culture medium used in the present study contained both phospholipid and protein as these substances are present in serum. It is therefore possible that the lysozyme may have been masked had it not been for the presence of Triton X. It has been explained that Triton X was included in the tissue culture medium to reduce the denaturation of ovalbumin but, if the finding of Romeo and de Bernard apply to the present system, it may also have helped to free the enzyme from the masking action of the phospholipids and proteins.

The adaptation of horsesickness virus to tissue culture has been reported by Mirchamsy and Taslimi (1963). These authors were able to propagate a virus strain (No.28) in primary hamster kidney cells. The virus had undergone 4 intracerebral passages in mice and, in tissue culture caused a pronounced cytopathic effect. These workers used an enriched normal medium (CSV 6), Cooper et al. (1959) containing 20% calf serum.

When the present investigation was started a method of producing plaques with horsesickness virus had not been discovered. Mirchamsy and Taslimi (1966) showed how this may be achieved using monkey cells and methyl cellulose and agar as overlay material. The plaques obtained using this method were of two sizes namely, less than 1 mm (small) and from 2 to 5 mm (large), similar to those found in the present work.

The development of tissue culture methods for propagating horsesickness viruses opens a new field of investigation. Differences in the physical characteristics of virus particles obtained from tissue culture and mouse brain may be found, and analysis of the results could lead to a better understanding of the details of virus replication. The development of strains suitable for preparing live virus vaccines may be accomplished with greater ease using tissue culture methods than is possible with mice. In particular, propagation of the virus in tissue culture at temperatures lower than the body temperature of the homoiothermic host may result in rapid attenuation. This technique has proved rewarding in attempts to attenuate poliovirus (Sabin and Lwoff, 1959). Also, it is known that some Arboviruses when taken from their Arthropod host in winter are often only weakly pathogenic for the warm-blooded host and may

therefore be partly attenuated (Mussgay, 1964). Application of techniques developed in the study of insect viruses (Smith, 1967) may help to improve the present tissue culture methods for viruses like horsesickness virus, which have arthropod vectors (du Toit, 1944). For example, the virus may be propagated in larval tissue.

CHAPTER III.

ELECTRON MICROSCOPY OF HORSESICKNESS VIRUS.

Introduction.

An attempt was made to obtain electron micrographs of horsesickness virus (No.3922) by the method of thin sectioning. Previous experience had shown that adequate purification presents the main difficulty in obtaining good electron micrographs of this virus obtained from suckling mouse brains. The starting material is a complicated mixture of macromolecules and the separation of virus in a state pure enough for electron microscopy is a difficult procedure. Polson and Deeks (1963) achieved a separation of the A 501 strain of horsesickness by first precipitating with polyethylene glycol. The partly purified virus so obtained was further separated from contaminating material by zone electrophoresis. In the present work two different methods were employed. Firstly, thin sections of infected mouse brain and spinal cord tissue were prepared and studied by electron microscopy. The electron micrographs so obtained yielded much information regarding the subcellular structure of the infected nervous tissue but no virus particles were detected. Secondly, electron micrographs

were made of infected tissue culture cells that had been disrupted on electron microscope grids. These revealed the virus particles of horsesickness.

THIN SECTIONS.

Materials and apparatus.

Virus. Infected brains were taken from 3-day-old suckling mice that had received intracerebral injection with horsesickness virus No.3922 (passage level 103). The tissue was harvested after 3 days when the animals showed definite signs of illness. For comparison, brain material was also taken from normal healthy mice. Virus from other passage levels was also investigated but efforts were concentrated on the fully adapted strain as this virus is capable of reaching a higher concentration in mouse brains.

Initially, tissue was taken at random from the brain. Later, specific parts were sectioned, especially the cerebellum, olfactory lobe, medulla oblongata and meninges at both the ventral and dorsal surfaces. The meninges was investigated because it was considered that, like poliovirus in humans, horsesickness virus may cause a meningitis in mice.

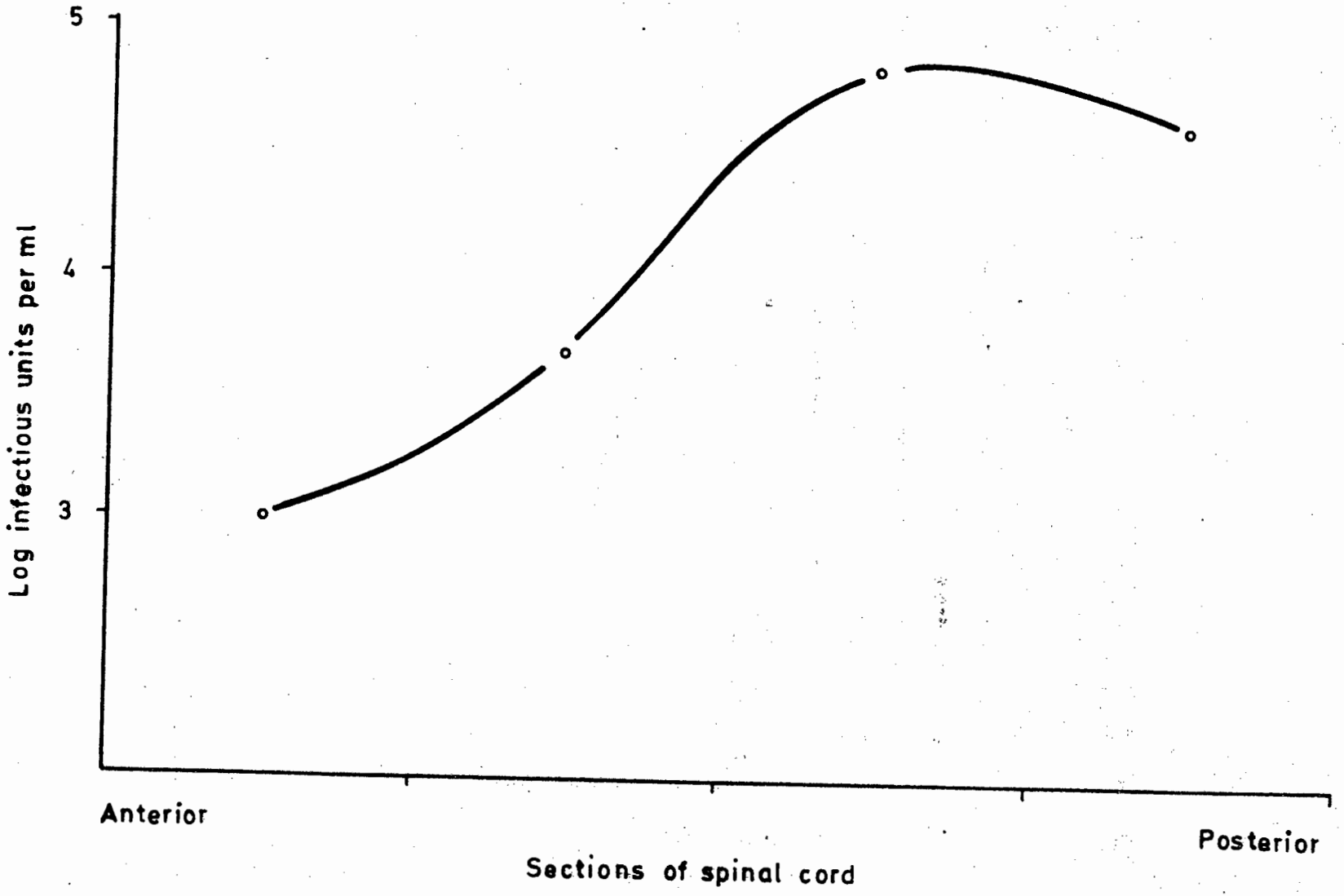
Spinal cord tissue was taken from infected and healthy 3-week-old mice. The spinal cord was sectioned at a point approximately two-thirds of its length from the brain, in the lumbar region. This position was chosen because it was found that more virus was present here than in any other part of the spinal cord. This was shown by dissecting out the spinal cord from three infected mice, washing in sterile egg-white buffer, and cutting into four equal lengths. Each section was triturated with sterile ground glass and the virus so obtained was suspended in egg-white buffer and titrated. From the result of a typical titration (Fig. 4, Table 2), it may be seen that most virus was found in the lumbar region of the spinal cord. It is not known why more virus should be found in one part of the spinal cord than another or if the distribution as shown in the figure changes during the course of the disease. It has been observed however, that the hind limbs of sick mice often become paralysed. This may be due to damage caused by horsesickness virus to the nerve cells of the lumbar region of the spinal cord.

Fixative. Millonig's buffered osmium tetroxide fixative, as detailed by Pease (1964) was prepared, except that the quantities of the components were changed so that exactly 0.50 gm O_s O_4 could be used. The

Table 2. Titration results of spinal cord of 3-week-old mouse infected with horsesickness virus No.3922 (passage 103).

Part titrated	Number of infectious units/ml.
Anterior quarter	2.3×10^4
2nd quarter	7.27×10^4
3rd quarter	1.15×10^6
Posterior quarter	7.27×10^5

Fig. 4. HSV in mouse spinal cord



$O_s O_4$ (0.50 gm) was supplied by British Drug Houses Ltd., England, in sealed ampoules.

Millonig's buffer was prepared as follows:-

$NaH_2 PO_4 H_2O$ (2.26 % w/v solution in distilled water)	13.83 ml
NaOH (2.52% w/v solution in distilled water)	2.83 ml
Water (distilled)	1.53 ml

To this solution was added:-

Glucose	0.09 gm
$O_s O_4$	0.50 gm

Final pH of the fixative was 7.3.

Embedding material (Pease, 1964). The epoxy resin and necessary additives were mixed as follows:-

Epoxy resin (Epon No.812)	8.1 ml
Curing agents. Dodecenyl succinic anhydride	5.0 ml
Methyl nadic anhydride	4.45 ml
Accelerator (246, tri dimethyl aminomethyl phenol)	0.35 ml

The solvent for this material is 1:2 Epoxy propane (AR).

Two important considerations for successful embedding are (i) the volume of all components used must be accurately measured and (ii) all components must be

free of water. The following procedures were adopted:-

(i) As most of the liquids used are too viscous to pipette, and as weighing was considered too slow a process because water may be absorbed from the atmosphere, syringes were used to measure the required volume of embedding materials. Four accurately calibrated glass syringes of suitable volume were chosen, one for each component. The Luer-Lok metal fittings were sawn off and discarded. The clean, dry syringes could now be filled with liquid and accurate volumes delivered into a mixing vessel.

(ii) Farquar (1956) suggested filtering the embedding material through Drierite ($CaSO_4$) to remove water. The technique used here was to add dry silica gel to each of the substances used. The liquids were contained in glass bottles supplied with plastic stoppers and were left in contact with the silica gel for at least two weeks before use. With these precautions, blocks which had satisfactory characteristics for thin sectioning could be prepared. No trouble was experienced with bubble formation which is usually attributed to the presence of moisture.

Stain. Reynolds lead citrate stain (Reynolds, 1963) was prepared as follows:-

Lead nitrate	1.33 gm
Sodium citrate	1.76 gm
Water	30 ml
When dissolved add:-	
Sodium hydroxide (1 N)	8 ml
Triton X (0.01%)	0.1 ml
Water, to make	50 ml

All liquids used were free of carbonates. The pH value of the final solution was 12.0.

The presence of carbon dioxide in solution in this stain may lead to the formation of lead carbonate which will spoil the electron micrograph. This was prevented by using CO_2-free distilled water. The distilled water was obtained from an all-glass still designed by the author. The reservoir of the still was fitted with a CO_2 trap of soda lime. Atmospheric carbon dioxide was prevented from dissolving in the solution during the staining process by using a simple apparatus (Fig. 5). This was constructed from a Pyrex boiling tube (diameter 2.5 cm) cut to a length of 10 cm and supplied with a rubber stopper. On the circumference of a smaller tube (1 x 7 cm), four prongs were blown to hold it central in the outer tube. The tubes were secured at an angle of approximately 45 degrees to the horizontal. Five ml of

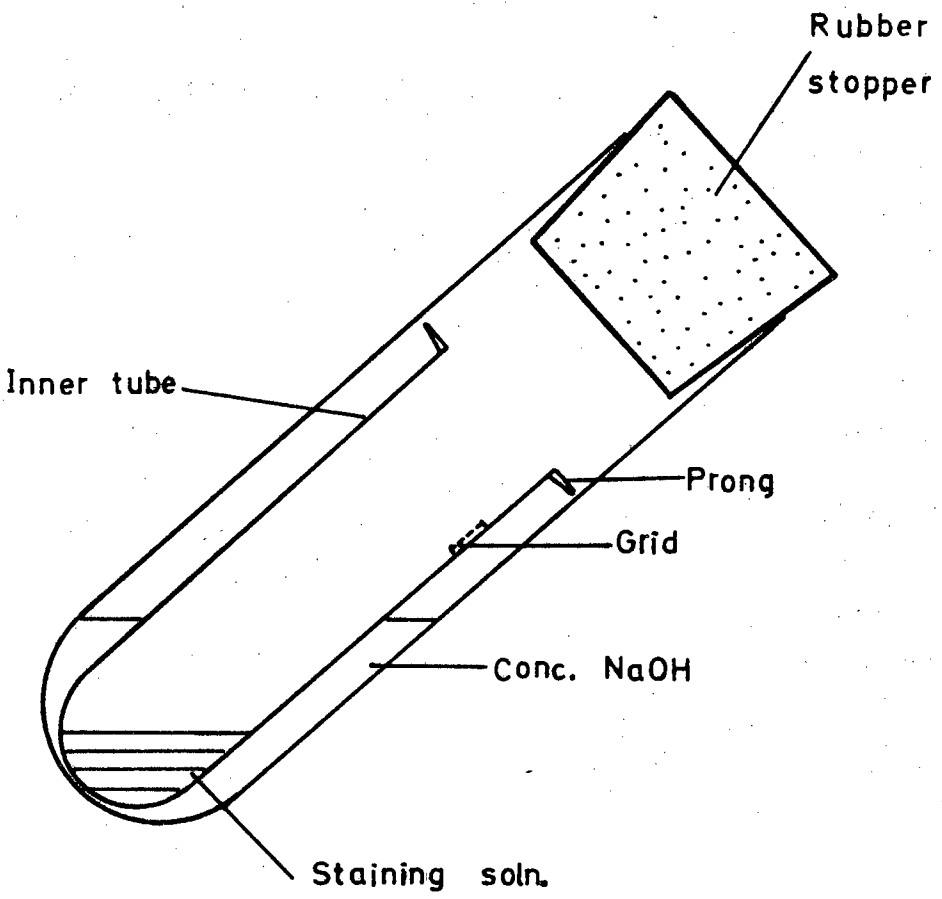


Fig. 5

Apparatus used for staining sections

a concentrated solution of NaOH were placed in the large tube and one ml of stain solution in the inner tube. Also in the inner tube, on the dry inside surface near the top, was placed an electron microscope grid carrying the sections. The apparatus was sealed with a rubber stopper and allowed to stand for 15 min so that any CO_2 present could dissolve in the NaOH solution. The apparatus was then placed in the vertical position so that the grid would slide into the staining solution. After staining for the required length of time the grid was removed.

The Reynolds stain had to be modified by the addition of a small amount of Triton X. This was necessary as it was found that the grid was held by surface tension on the surface of the staining liquid. If the grid floated with the sections uppermost satisfactory staining did not occur. By adding Triton X the surface tension of the staining solution was reduced, so that the grid always sank through the liquid, thus completely immersing the sections and allowing the stain to act.

Ultramicrotome. The instrument used was manufactured by Ivan Sorvall (USA) and was based on the single pass, non lubricated design of Porter and Blum (1953). Glass knives, as first suggested by Latta and Hartman (1950), were prepared form ¼ inch plate glass by the

'free break' method. Each knife was provided with a reservoir by fixing adhesive plaster around the cutting edge. The reservoir was filled with distilled water. The knife was clamped at an angle of 3 degrees for sectioning.

Electron microscope. The instrument used was a Metropolitan Vickers Model EM3A.

Method.

The same procedure was followed for both virus-infected tissue and non-infected tissue. First a small piece of brain or spinal cord was taken from ether killed mice. This was placed directly in buffered osmium tetroxide fixative and cut with a stainless steel blade into fragments not larger than 1 mm cube. Fixation was allowed to proceed for 60 min at room temperature. The fixed tissue was washed in distilled water (three changes) and dehydrated in absolute ethanol (six changes). The alcohol was removed by suspending the tissue in epoxy propane (six changes). The tissue was infiltrated with epoxy resin by placing it in a mixture of one part epoxy propane and one part embedding medium, (30 min) then in one part epoxy propane and two parts embedding medium (30 min) and finally in undiluted embedding medium (60 min).

Use was made of a mechanical shaker during these steps to keep the solutions well mixed. The tissue was finally placed in fresh embedding material contained in No.00 gelatin capsules, (previously dried at 100°). Polymerisation was carried out in an incubator (60°) for 72 hr. When the block was hard, the end containing the tissue was trimmed to the shape of a truncated pyramid. The block was then clamped in the ultramicrotome and sections were cut approximately 100 mμ in thickness as determined by their silver-gold interferance colour (Peachey, 1958). The sections were transferred to a clean carbon-coated electron microscope grid and air dried. They were then stained, washed in water, dried and observed in the electron microscope.

Results.

In Fig. 6 is presented a selection of electron micrographs of ultrathin sections of brain and spinal cord of infected and non-infected mice. Although some particles were seen on electron micrographs of infected tissue which were virus-like, their structure was never so definite nor their numbers so great that they could be identified as viruses.

Fig. 6A is an electron micrograph of uninfected

Figure 6.

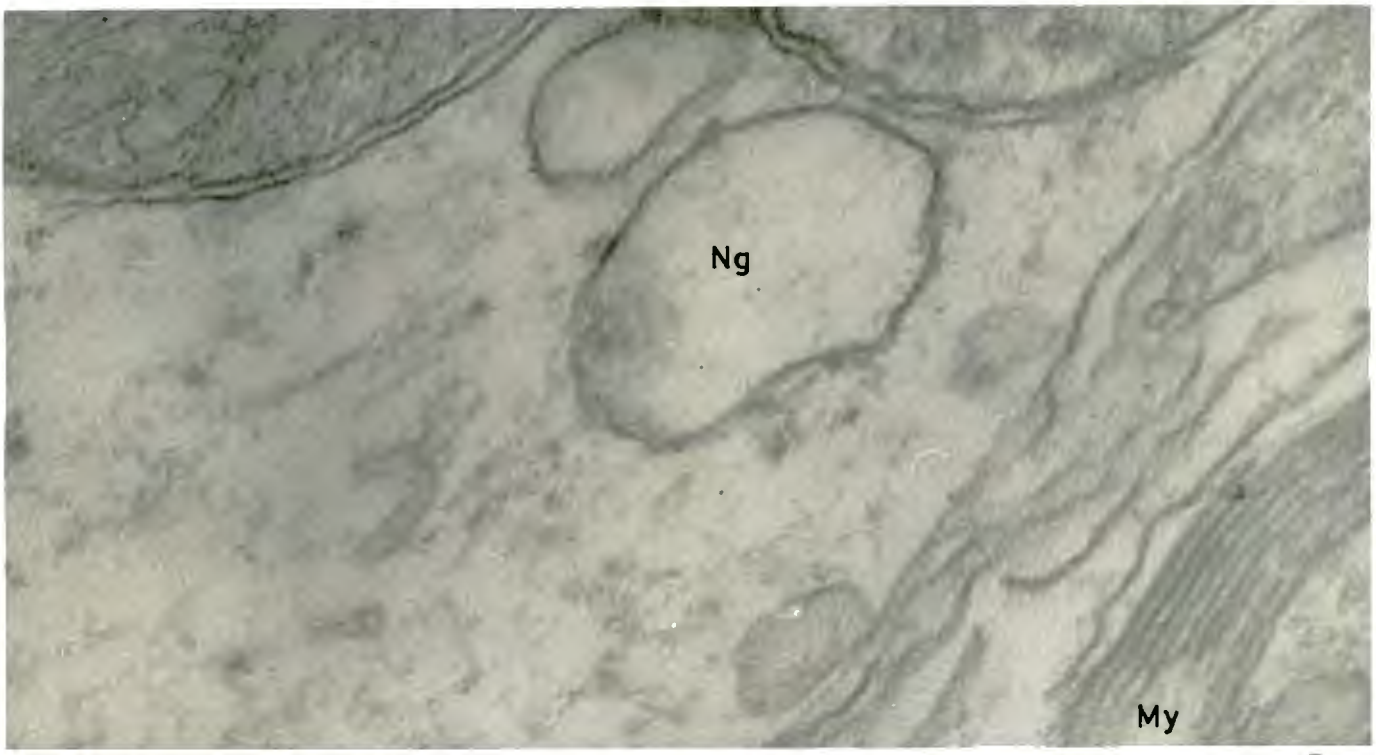
A. Electron micrograph of a section of normal (uninfected) mouse brain from the ventral surface of the cerebellum. Granular endoplasmic reticulum is clearly visible. (Mag. 26,160)

B. Electron micrograph of section of infected spinal cord. No virus could be detected. This section shows the typical degeneration of nerve cells observed in infected tissue. The cell cytoplasm appears to become electron dense and uniform. (Mag. 30,000)

C. Electron micrograph of section of infected spinal cord again showing a fairly uniform cell matrix containing vesicles, a mitachondrion and an axon surrounded by myelin lamellae. (Mag. 100,000)

D. Electron micrograph showing long filamentous structures sometimes associated with infected cells but not observed in normal cells. (Mag. 60,000)

A



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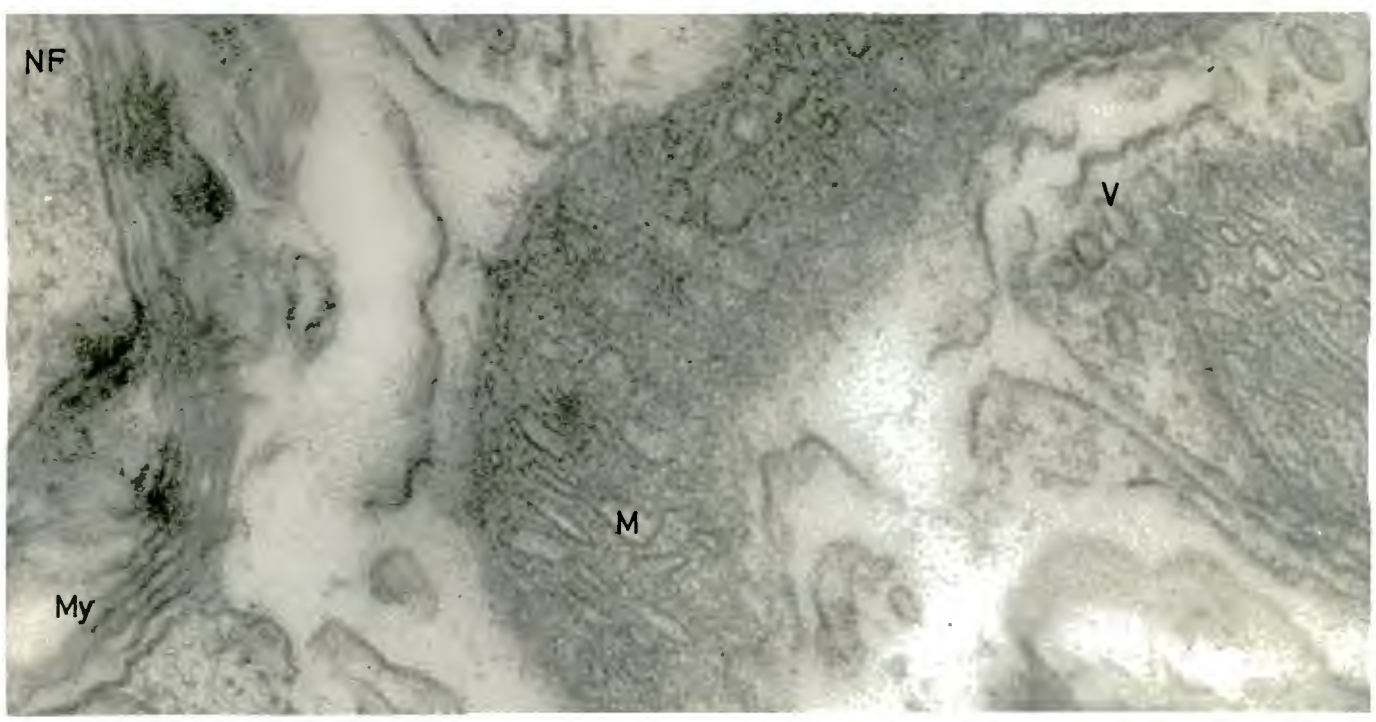


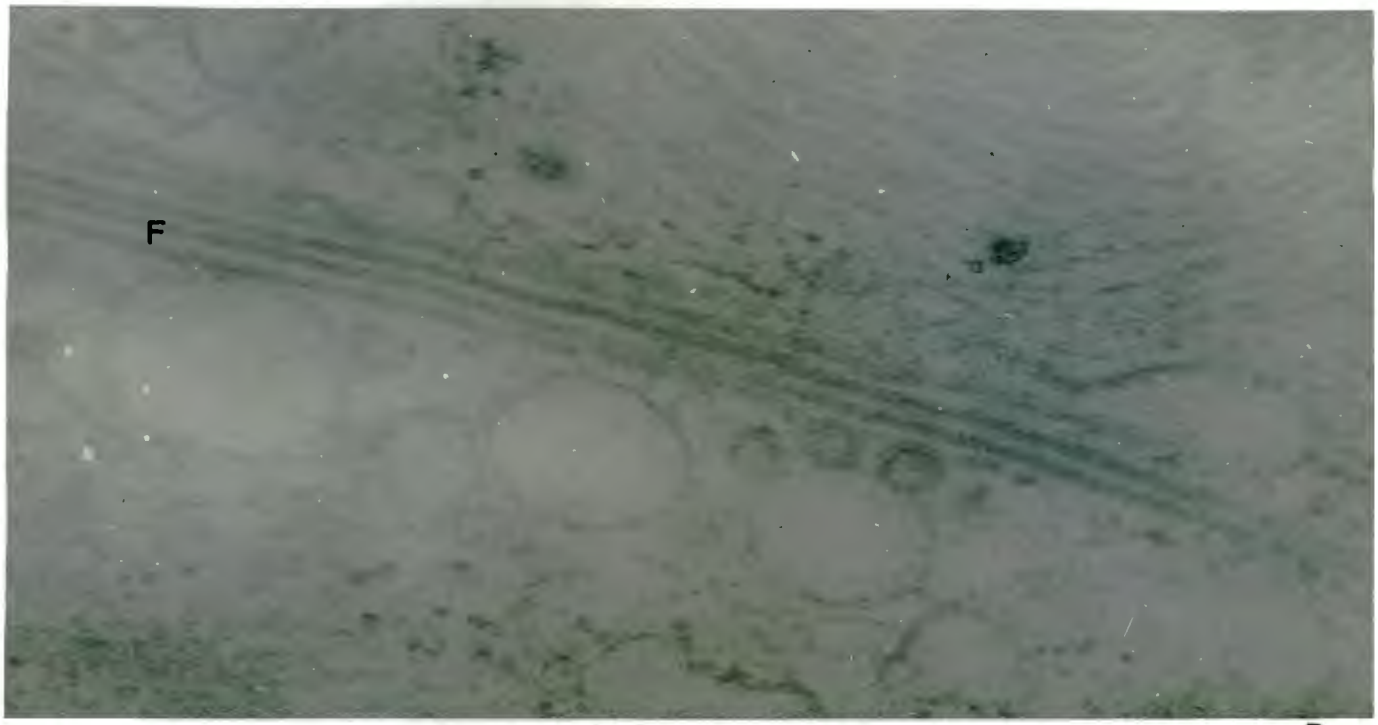
C

Figure 6 (Contd.):

E. Survey electron micrograph of a section of mouse brain infected with horsesickness virus. (Mag. 12,000)

F. Electron micrograph of infected mouse brain showing breakdown of cell organelles. (Mag. 20,900)

G	Granular endoplasmic reticulum
M	Mitochondrion
NF	Nerve fibre
Nf	Neurofilament
R	Ribosomes (free)
Go	Golgi complex
N	Nissl body (Ergastoplasm)
V	Synaptic vesicles
Ng	Neuroglial cells
My	Myelin sheath
F	Filamentous structure
Nu	Nucleus
C	Connective tissue



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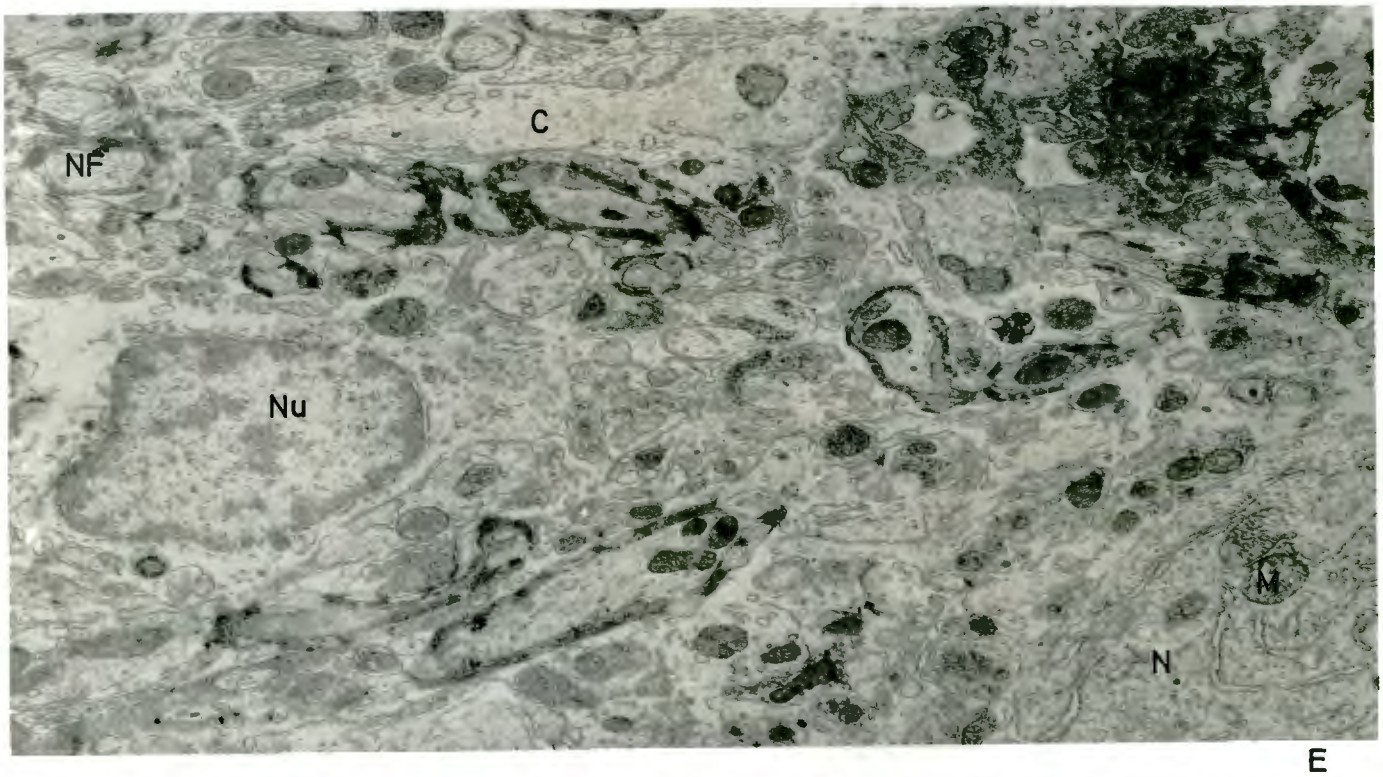

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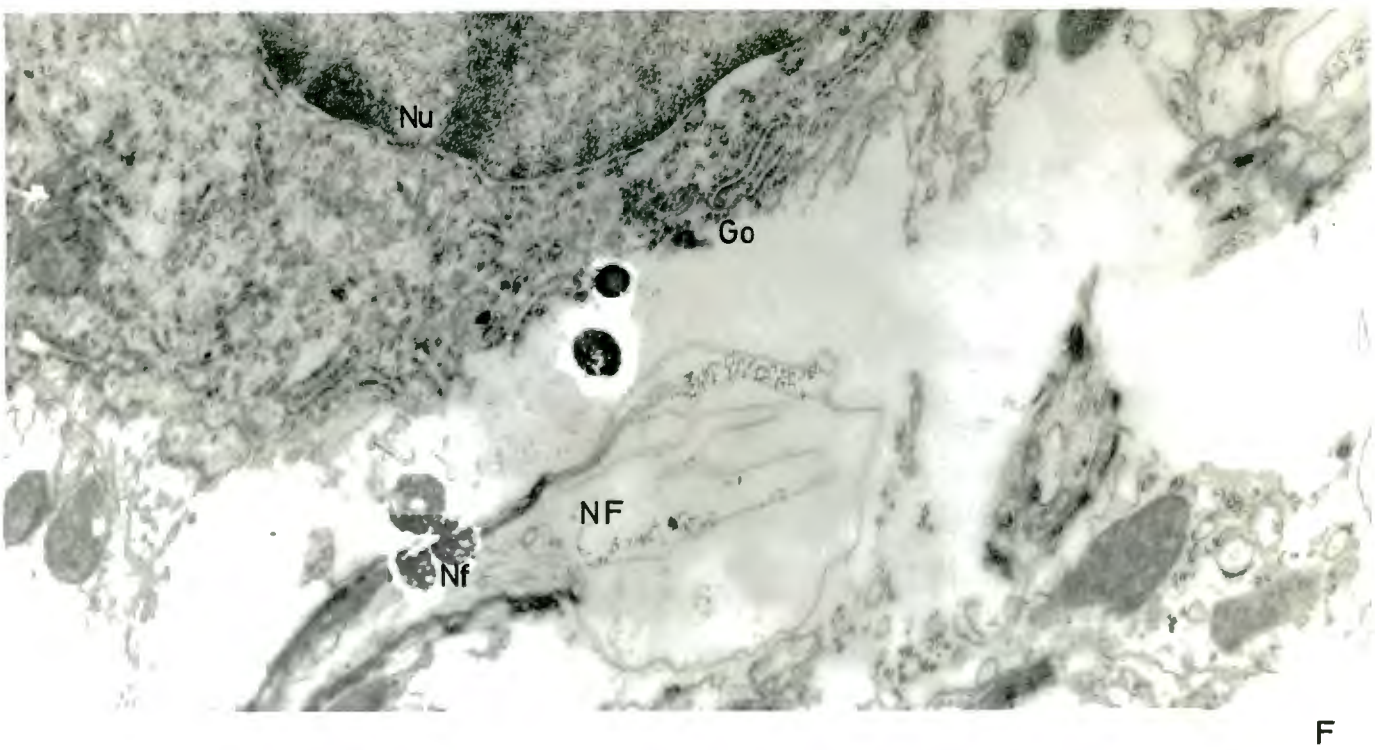


F

brain cell. Typical of normal cells is the compact structure of the sub-cellular components with no 'free spaces' present. Large numbers of ribosomes are found in the cytoplasm, some free and some attached to elements of the endoplasmic reticulum. The nerve fibres may be naked or myelinated and are sometimes seen to be surrounded by neuroglial cells which are thought to support and nourish the nerve cells (Porter and Bonneville, 1964). Many mitochondria are present.

In contrast to the ordered arrangement of organelles found in healthy cells Fig. 6B shows how, in tissue infected with horsesickness virus, much of the cell matrix tends towards an almost homogeneous form. This is thought to be due to the breakdown and dissolution of cell components as a result of viral activity. The identification of many structures is uncertain in diseased cells. It was observed however that the nerve fibres were less affected than other cell components. It may be that nerve fibres are unable to produce viral components because of their specialised structure and therefore remain intact while other organelles lose their integrity. The site of formation of viral nucleic acid and proteins is possibly in the nucleus ^ or cytoplasm of the more generalised neurologlial and Schwann cells. (Mirchamsy and Taslimi, 1964)

In Fig. 6B may also be seen a section of an infected

nerve cell from the spinal cord of a mouse showing a nerve fibre surrounded by a myelin sheath (laid down by a Schwann cell: Geren, 1954). Part of what may be a neuroglial cell may also be seen but other structures are too ill-defined to identify in this damaged cell.

Fig. 6C is similar to the one just described and shows a mitochondrion which appears to be breaking down, open spaces devoid of any cell components and synaptic vesicles.

Filamentous objects sometimes observed in infected cells (Fig. 6D) are of unknown origin. They are thought to be indicative of some cellular reaction to virus infection as they were not seen in normal cells. They are considered in more detail in the discussion.

Fig. 6E shows a general view of a section of infected mouse brain tissue at low magnification (12,000 x). Some breakdown of cellular organelles may have occurred near the nucleus but generally the section has the appearance of that of a normal cell and was probably not seriously infected. The elongated, semi-homogeneous structure is probably connective tissue. Apart from the many nerve fibres and mitochondria, this section includes an area consisting of ribosomes, both free and attached to the endoplasmic reticulum, the latter being partly in the cysternal phase. This area is part

of the ergastoplasm or Nissl granule.

Fig. 6F is an electron micrograph of a section of infected brain tissue where the cell has almost entirely disintegrated. The nucleus is however intact but there is no evidence of virus multiplication. Part of the Golgi complex may be seen and also a longitudinal section of a myelinated nerve fibre containing neurofilaments. A section of this fibre has entirely disintegrated. Large areas of the cell are devoid of structure indicating an advanced state of degeneration.

DIRECT OBSERVATION OF CELL CONTENTS BY ELECTRON MICROSCOPY.

Introduction.

With the development of a technique for propagating horsesickness virus in tissue culture, a further attempt at electron microscopy was undertaken. The method used was based on the work of Zwillenberg and Burki,(1966) who reported that electron micrographs of viruses may be obtained by suspending infected cells in ammonium acetate solution, adding a suitable stain and allowing the suspension to dry on an electron microscope grid. On drying, the cells burst open and

the contents, not greatly disorganised, are deposited on the grid in a thin layer. The ammonium acetate, being volatile, sublimes because of the vacuum in the electron microscope. This technique proved to be both simple and rewarding.

Materials.

Virus. The virus used for electron microscopy was taken from the 8th tissue culture passage. LF cells (Chapter II) growing in egg-white medium had been inoculated 4 days previously with tissue culture passage 7 horsesickness virus. Uninfected cells were taken as controls.

Stain. Sodium silicotungstate (Valentine and Pereira, 1965) was prepared by dissolving 4 gm in distilled water (100 ml).

Ammonium acetate. A 5% (w/v) solution of ammonium acetate in distilled water was prepared. Bovine plasma albumin (0.01% w/v final concentration) was added to the solution to assist in the spreading of liquid on the grid.

Method.

Cells infected with horsesickness virus which were

just beginning to show signs of a cytopathic effect were scraped from the tissue culture bottle using a rubber spatula and centrifuged at 500 x g for 2 min. The supernatant fluid was removed with a fine-drawn pipette and the cells resuspended in Millonig's buffer and re-centrifuged. After removing as much as possible of the supernatant fluid, so that salt crystals should not obscure the electron micrographs, the cells were resuspended in ammonium acetate solution. Two drops of this cell suspension were mixed with two drops of stain and a small quantity placed on a carbon-coated electron microscope grid and allowed to dry. The preparation was then viewed in the electron microscope.

Results.

In Fig. 7A may be seen a group of particles which appear to be enclosed in a vesicle-like structure composed of elements of the endoplasmic reticulum. The average diameter of these particles is 40 mμ. This is somewhat smaller than that calculated from sedimentation and density data for the No.3922 strain of horsesickness virus (Chapter VII). These virus-like particles were not observed in control uninfected cells.

Another group of virus particles is seen in Fig.7B.

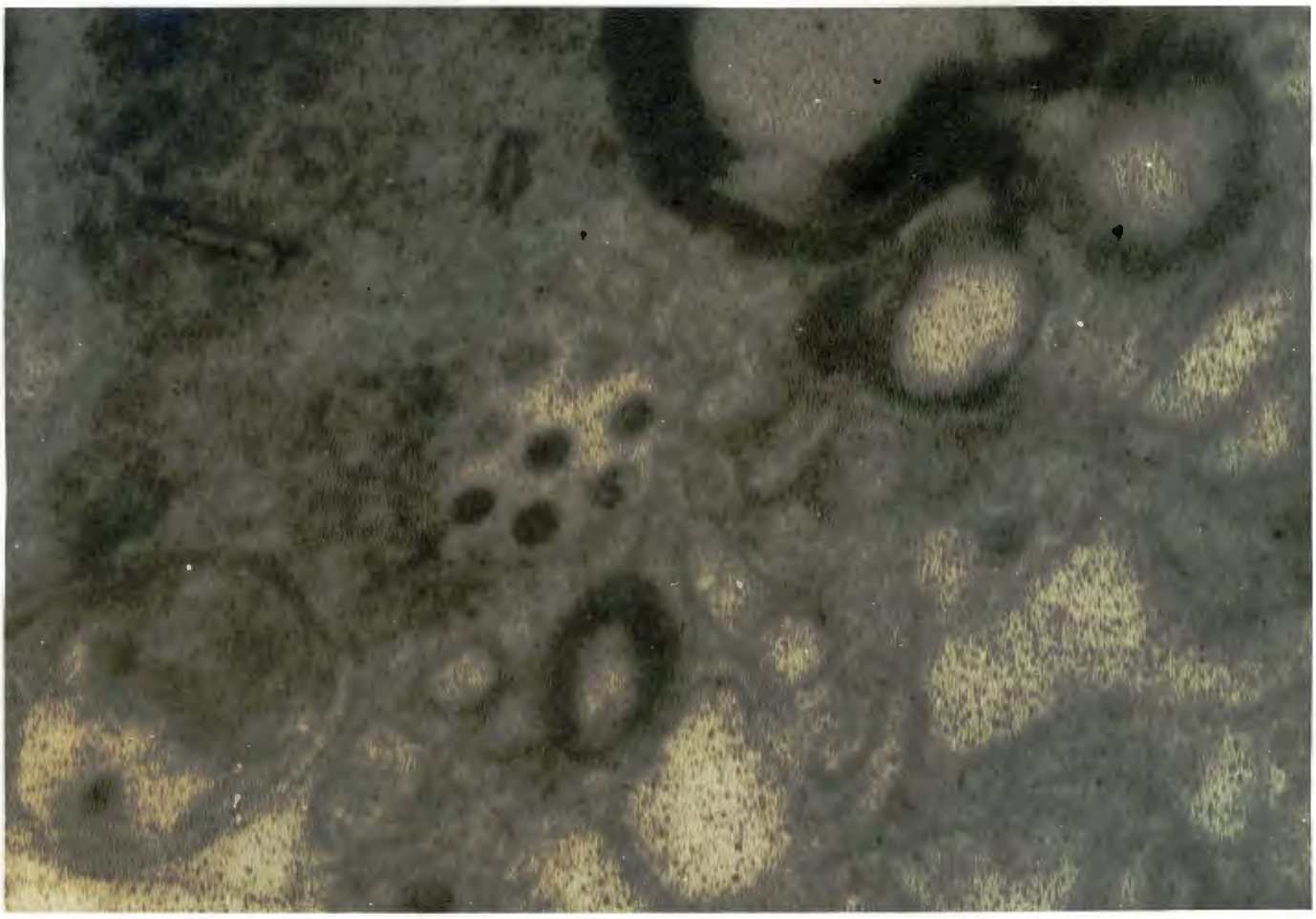
Figure 7.

Electron micrograph of horsesickness virus propagated in LF cells and negatively stained with sodium silicotungstate.

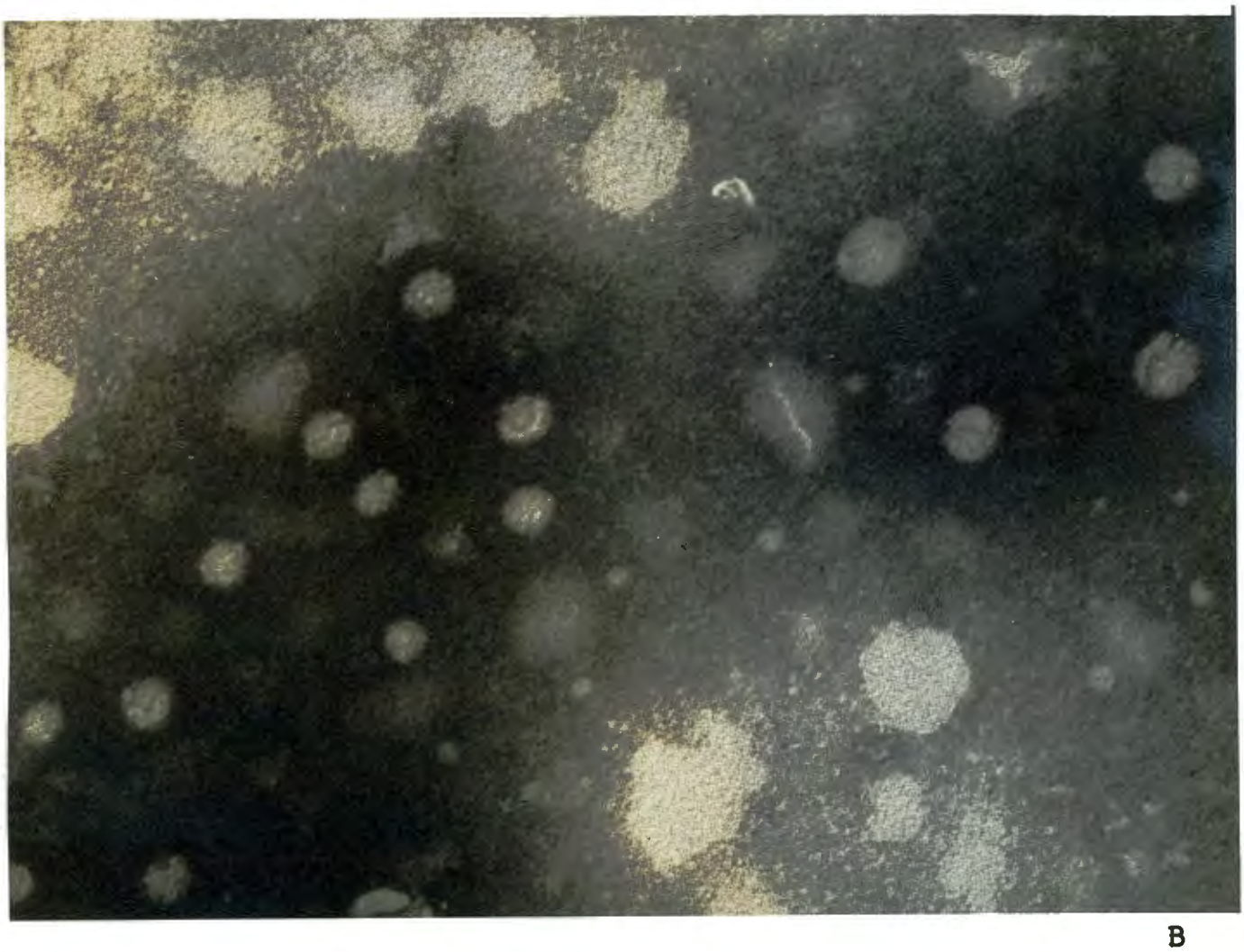
A. Virus particles contained in a vesicle-like structure. (Mag. 184,000)

B. Separate virus-like particles.

Inspection of a number of electron micrographs led to the conclusion that these particles are hexagonal in shape. A number of smaller particles which may be of viral origin are present (Mag. 300,000)



A



B

This is not the same preparation used to obtain the micrograph of Fig. 7A but the size of the particles was again found to be approximately 40 mμ. The resolution of this micrograph is such that a greater degree of photographic enlargement was possible (total magnification: 300,000). Some structural details of the particles are now revealed, especially the six-sided shape and the presence of distinct capsomeres. The geometrical shape of many of the particles is typical of that of an icosahedron (Almeida and Ham, 1965). Although the resolution of this micrograph is satisfactory, the so-called haemagglutinin that is thought to be associated with many horsesickness viruses and which was demonstrated for the A 501 strain by Polson and Deeks (1963) is not revealed. It is possible that the discrepancy between the size of the particles obtained from electron micrograph measurements and sedimentation studies (Chapter VII) may be due to the fact that the particles photographed were not fully mature and had yet to receive a final coat of viral or cellular material.

Included in the micrographs are many small particles that could not be identified as normal cell components and may be unassembled virus components.

Discussion.

In attempting to locate horsesickness virus in the nervous tissue of infected mouse brains and spinal cord it was assumed that this would be the most likely, or even the only possible site of multiplication for the neurotropic, attenuated strain used. Evidence for this assumption is the paralytic symptoms the virus produces in mice, and the results (Alexander, 1935) found for other horsesickness strains. Indeed it was the infected brain that was the source of all virus used in this work. It is therefore certain that virus is present in the brain of infected mice and from the present results (Fig. 4) also in the spinal cord. No virus was detected by the method used although many different preparations were made. The average number of virus particles present in the mouse brain was calculated to be approximately 3×10^8 per ml. This may be too small a number to detect easily by thin sectioning. Much of the virus may be localised in a part of the brain not yet investigated. It is possible that horsesickness virus is produced in only a special area of the brain as is the case of measles virus (Matumoto et al., 1964). These investigators, studying the effects of measles virus on suckling mouse brains, found histological changes in the cerebrum only

and not in the cerebellum, spinal cord or the visceral organs. Special attention was paid to those micrographs showing a section of the cell nucleus because of the observation of Mirchamsy and Taslimi (1964) that thie may be the site of multiplication of some horsesickness viruses. No evidence of multiplication of the No.3922 strain in the nucleus could be found.

Filamentous structures were observed in some infected cells. The origin and importance of these forms is not known but similar objects have been noticed (Hirumi et al., 1967) in nervous tissue of insects infected by wound tumour virus.

The comparatively simple technique of viewing the burst-cell contents was successful in that virus particles were identified with reasonable certainty. These particles always appeared to be situated in the cytoplasm of the cell and were similar to those found by Polson and Deeks (1963). There is some evidence that the No.3922 strain of horsesickness virus may also be composed of identical subunits as suggested by the hypothesis of Crick and Watson (1956). If this is the case the virions of the No.3922 strain are probably icosahedral in form with 92 capsomeres in the capsid according to the method of calculation of Horne and Wildy (1961). Although an accurate count of the number of capsomeres was not

possible it was observed that many of the particles were six-sided (Fig. 7B). The diameter of the particles found by electron microscopy in the present work (40 mμ) is smaller than those of the A 501 strain of horsesickness virus found by Polson and Deeks (1963) (70 to 80 mμ) which had been propagated in suckling mouse brains.

CHAPTER IV.

ULTRAFILTRATION OF HORSESICKNESS VIRUS.

Introduction.

The size of a particle may be measured by finding the diameter of an aperture through which it will just pass. If a filter with apertures of such unique diameter is not available others that have greater and smaller pore sizes may be used. By finding the number of particles passing through these different apertures under standard conditions a very good estimation of the particle size may be obtained by extrapolation. This is the principle underlying those sieving or filtration methods designed to measure particle size.

To apply this method it is essential to have a series of filters each with uniform pores of known diameter. Methods for preparing and calibrating filters suitable for the determination of particles of colloidal size were developed by Elford (1931), and later by Bauer and Hughes (1935).

These methods were followed in this work and used to determine the particle size of horsesickness virus. The results are compared with those obtained by ultracentrifugation and some conclusions regarding particle shape of the virus are made.

PREPARATION OF MEMBRANES.

Materials and apparatus.

It is stressed by Elford that all organic solvents used in the preparation of membranes must be pure and free of water. This is important as the proportion of solvents present and the amount of water added greatly affects the pore size of the final membrane. If it is desired to prepare membranes of a particular pore size and not rely on trial and error methods, exact quantities of pure substances must be used.

To obtain pure anhydrous solvents the methods of Bauer and Hughes (1935) were followed. In all cases analytical grade chemicals were used. Ether (diethyl ether) was dehydrated over sodium, acetone over anhydrous K_2CO_3 and ethyl alcohol over CaO. After storage in the dark for an appropriate time each of these chemicals was filtered and redistilled. Primary amyl alcohol does not require purification. Pure acetic acid was obtained by freezing (16^{o}) analytical grade reagent and decanting the liquid.

The cellulose nitrate used was obtained from Mallinckrodt Chemical Works USA. (Trade name Parlodion), and was used without further purification. Although the

material used to prepare membranes bore the trade name 'Parlodion' the better known term 'Collodion' has been retained in this discussion.

Tray. Collodion solutions were allowed to evaporate from a glass tray (diameter 40 cm. : depth 9 mm.). Care was taken to place the tray in an exactly horizontal position so that membranes should be of uniform thickness.

Temperature and humidity control. Use was made of a room in which the temperature and humidity of the air was kept constant (20°, and 70% humidity).

Method.

Principles. Elford discovered that amyl alcohol and acetone are mutually 'antagonistic' in their ability to dissolve cellulose nitrate. Either of these solvents together with ethyl alcohol and ether, dissolves cellulose nitrate but if both are present in certain ratios the cellulose nitrate coagulates. A mixture containing all four solvents may be prepared which dissolves the cellulose nitrate completely. If this solution is placed in an open basin or tray the more volatile solvents evaporate fastest and so the ratio of acetone to amyl alcohol is changed and the cellulose nitrate coagulates

in the form of an 'ultra-gel' structure. This results in the formation of membranes of suitable physical characteristics for use as ultra filters with a range of permeability from approximately 10 mμ to 3μ. The principles underlying the formation of the filters are embodied in Elford's term 'gradocol membranes'; graded coagulation of collodion. Elford also found that the pore size of membranes could easily be controlled by adding to the collodion solution various quantities of acetic acid and water. The addition of water, a non-solvent, up to 5% by volume produces a gradual increase in the permeability of the membranes, while increasing the amount of acetic acid results in membranes with smaller pore diameter.

Permeability is also affected by the temperature and humidity of the atmosphere while evaporation is taking place. If evaporation is allowed to proceed quickly at a high temperature membranes of small pore size will be formed.

Procedure. Parlodion (75 gm) and ethyl alcohol (125 gm) were placed in a brown, 2.5 litre plastic-stoppered bottle and allowed to stand at room temperature for 24 hr. During this time the Parlodion absorbs alcohol and swells. Ether (375 gm) was added and the mixture shaken to dissolve the Parlodion. Acetone (575 gm) was

then added and shaking continued for 2 hr. The addition of amyl alcohol (287 ml) completes the mixture which was stored in the dark for 3 weeks. Before use this stock solution (1018 ml) was diluted with ethyl alcohol (93 ml) and ether (925 ml).

Membranes prepared from this solution will have a pore size of approximately 600 mμ. The size may be adjusted between the limits of 450 mμ to 24 mμ by adding to 200 ml of stock solution 0.5 ml to 2.5 ml of acetic acid. When the final solution was prepared, a 200 ml portion was brought to the same temperature at which evaporation was to take place and then poured into a level circular glass well. Evaporation was allowed to continue at a temperature of 20° and a relative humidity of 70% for 90 min. In order to obtain uniform evaporation of solvents from the surface of the liquid a circular cardboard cylinder was placed 4 cm above the well using four cork stoppers. The dense vapour evaporating from the liquid moved out from under the cardboard shield and was replaced by air from above. The slow circulation so caused ensured uniform evaporation and resulted in membranes of uniform thickness and pore size. After evaporation had taken place for the prescribed time the membrane was flooded with distilled water. Membranes so obtained must be washed to remove all traces of solvents. This may best be accomplished by washing the membranes twice a day for 7 days with a solution of

ethanol (10% v/v) in distilled water. Pure distilled water is then used for a further 7 days.

Washed membranes were cut with a punch to a suitable size, making allowance for slight shrinkage when boiled. Only those parts of a membrane that had uniform light transmission and were therefore of uniform thickness (Polson and Madsen, 1953) were used. They were then stored in a solution of Methiolate (0.01% w/v) in water to prevent the growth of micro-organisms.

Theory.

In order to derive a formula for calculating the pore size of collodion membranes the following four assumptions must be made:-

(i) The pores are cylindrical and of uniform bore.

(ii) The cylinders are at right angles to the surface.

(iii) The total volume of all the cylinders is equal to the water content of the membrane as found by measuring the 'wet' weight of the membrane and the weight after drying at 100°.

(iv) Poiseuille's law applies.

The Poiseuille relation (Equation No.5), Chapter V,

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

describes the viscous flow of liquid through a capillary. The volume is related to the radius and length of the capillary, the pressure, the period of time that the pressure is applied and the viscosity of the liquid. As the pressure, time and volume may be measured and the length of the capillaries is equal to the thickness of the membrane (assumption (ii) above), the diameter of the pores may be computed.

The relation as stated is for a single capillary. For a membrane of n capillaries

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

As the capillaries are assumed to be cylindrical in shape the volume of all the capillaries is $n \pi r^2 l$ ml. This may be taken as equal to the total weight of water contained in the membrane. So $n \pi r^2 l = w$

and $n = \frac{w}{\pi r^2 l}$

Substituting for n

$$V = \frac{\pi p r^4 t}{8 l \eta} \times \frac{w}{\pi r^2 l}$$

Therefore

$$V = \frac{p w t r^2}{8 l^2 \eta}$$

and rearranging

$$r = 2l \sqrt{\frac{2 V \eta}{p t w}} \qquad [8 = 4 \times \sqrt{4}]$$

The radius or diameter of the pores may then be determined. In the above relations:-

V = volume of liquid (water) flowing per sq cm in time t (ml)

t = time (secs)

p = pressure (dynes)

r = radius of pores (cms)

l = length of pores (cms)

η = viscosity of water at temperature of experiment (poise)

n = number of pores in membrane

w = weight of water per sq cm of membrane (gm).

Calibration of membranes.

The pore diameter of a membrane is related to the volume of water that will pass through it under controlled conditions. The apparatus (Fig. 8) used was similar to that of Elford's but was modified for the following reasons. Difficulties arise because a water column is connected to the membrane cell. The hydrodynamic pressure so obtained forces water through the membrane. If a vertical column is used with rubber connections, the pressure will expand the rubber to a degree depending on the height of the column. The change in height of the column is being measured as the volume of water

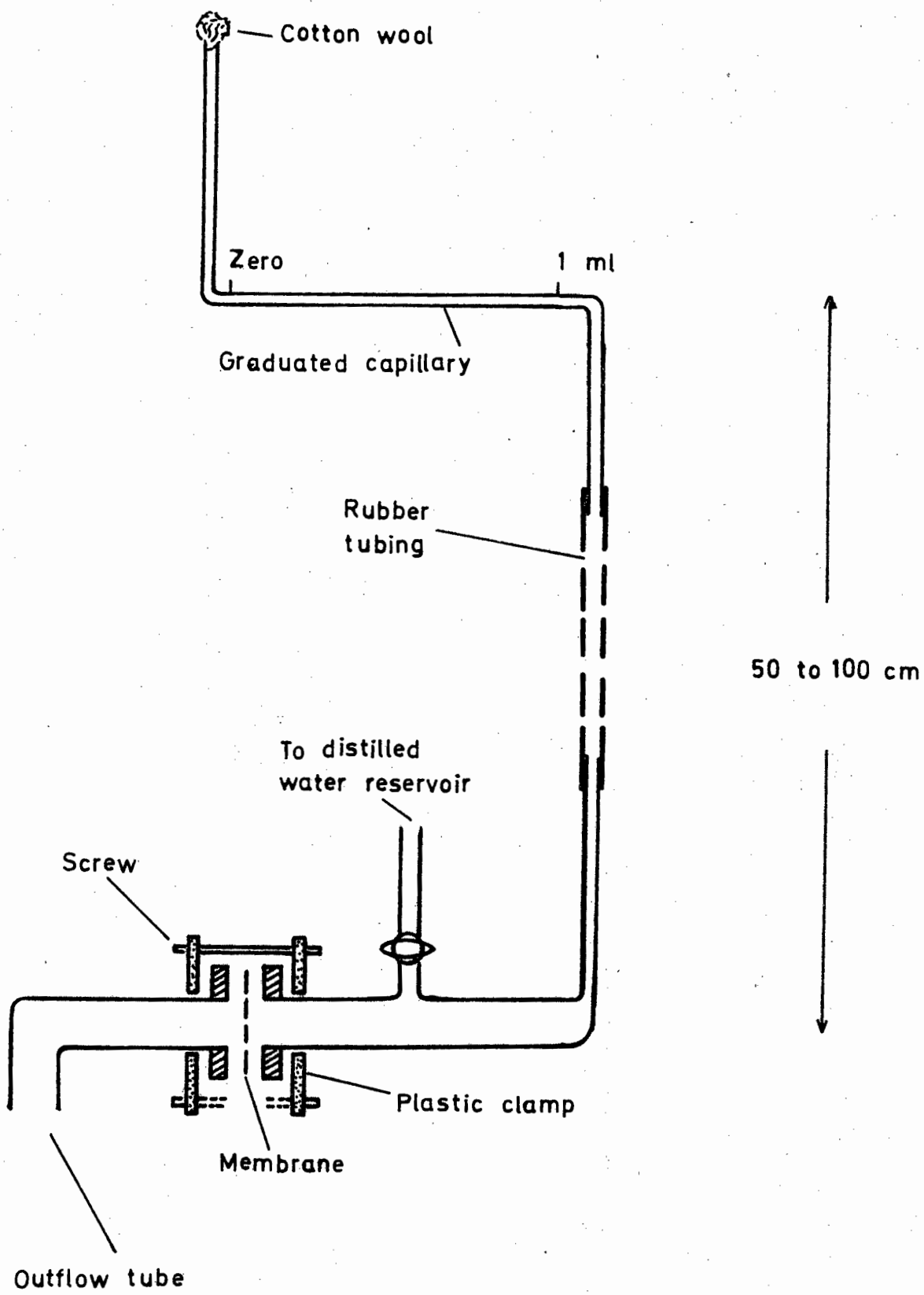


Fig. 8 Apparatus for determining pore size of membranes

passing through the membrane and an erroneous reading will result due to the contraction of the rubber as the pressure changes. Even the membrane itself may have sufficient elasticity to affect this reading. The problem may be overcome by bending an accurately graduated pipette so that the graduated section lies in a horizontal position. If the meniscus is brought to the zero mark at the beginning of a determination the flow rate may be measured at constant pressure as there is no change in the total height of the column. Evaporation of water and the ingress of dust is prevented by having a long side arm to the pipette, the open end of which is lightly plugged with cotton wool.

The rest of the apparatus consisted of two glass tubes with flattened ends ground smooth. The inside diameter of the outlet tube (1.02 cm) was slightly smaller than that of the tube to which it was clamped (1.10 cm). This ensured that the total area available for the passage of water through the membrane was always constant because the tubes could be secured so that the larger aperture never overlapped the smaller aperture. The tubes were clamped together with a membrane between them by means of two plastic plates with four screws. A perfectly water-tight joint was always achieved without the aid of grease. A side arm with tap was incorporated

in the apparatus to facilitate filling from a distilled-water reservoir.

The pore size of a membrane may be determined by clamping it in the flow cell and filling the apparatus with pure water. The purity of the water used is of the greatest importance as any small particulate matter present may enter the pores and so change the rate of flow through them. From the Poiseuille equation it may be seen that the volume of liquid passing through a capillary is proportional to the 4th power of the radius. Small particles will thus have a large effect on the flow rate so precautions must be taken to keep the system free of all particulate matter. The height of the water column was adjusted so that approximately 0.1 ml water passed through the membrane in 1 to 3 min. Water was allowed to filter through the membrane until the flow rate was constant. The time taken for a given volume to flow through the membrane was then measured. The mean of at least three measurements was taken. The temperature of the water and the height of the water column were also measured.

The thickness of a second membrane, cut from the same sheet as that used in the flow cell, was measured using a micrometer screw. To prevent squeezing the membrane between the jaws of the micrometer two glass

cover slips were used, the total thickness of these being subtracted from the final reading. This measured (l), the length of the capillaries.

The weight of water per square cm of the filter must also be estimated. An exactly circular membrane of known diameter was weighed wet, after blotting off excess moisture. After drying to constant weight at 110° the weight was again measured. The difference between these weights, divided by the area of the membrane gives the volume of water per sq cm.

A table of the relevant data may be constructed as follows:-

Table 3.

Data required for calculating the average pore diameter of Collodion membranes.

Flow cell	Diameter		1.02	cm
	Area		0.817	sq cm
Water	Temperature		20.2	°
	Viscosity	(η)	0.01	poise
	Volume passing through membrane		0.10	ml
	Volume passing through one sq cm of membrane	(V)	0.1225	ml
	Time	(t)	189	sec
Volume water contained in one sq cm membrane		(ω)	0.01676	gm

Thickness of membrane	(l)	0.020	cm
Pressure. Column ht (cm) x 980.6	(p)	49.030	dynes

The average pore diameter (APD) of the membrane may now be calculated.

$$APD = 4l \sqrt{\frac{2V\eta}{pt\omega}}$$

$$101 \text{ m}\mu$$

Precautions.

The size of particles determined with collodion membranes prepared and calibrated as described above cannot be accepted as exactly correct. There are certain shortcomings of this method which must be considered.

(i) In the derivation of the formulae for calculating pore size certain assumptions are made which must be, in some degree, incorrect. For example it is unlikely that all the pores are exactly circular and of the same diameter and length. Also the water content of a filter will probably not all reside in the pores only, so that a measure of the water content no more than approximates to the true total pore volume.

(ii) Various surface phenomena may occur in the pores of a membrane. The adsorption of relatively large colloids

like proteins and viruses will restrict the movement of other particles entering the pores. Errors from this source may be reduced by "saturating" the membrane with amino-acids before it is used in an experiment. The filtering properties of membranes of smaller pore size may be altered by the adsorption of water molecules.

(iii) Electrical kinetic phenomena (White et al., 1935) occurring at the pore surface may also alter the filtering characteristics of a collodion membrane. When charged particles are forced through the pores a streaming potential is developed. The free flow of colloidal particles is then opposed by electroendosmosis. This problem may be overcome by ensuring that the ionic strength of the solution is high enough to "discharge" the particles and so neutralise the effect.

(iv) A partial blocking of a pore may occur if two particles enter simultaneously. Other smaller particles may still be able to pass through.

(v) Membranes are calibrated by measuring the rate of flow of water. This lays emphasis on the larger pores and the calculated average pore size is larger than is in fact the case.

These errors combine and result in an estimated pore size that is too large. The limiting (extrapolated) diameter of a particle, experimentally determined by ultra-

filtration must therefore be reduced. It has been found (Elford, 1931 and Black, 1958) that the magnitude of the correction varies. It is greatest for the smaller pore sizes and approaches zero as the pore size increases. A factor of 0.68 (Polson, Personal communication) has been used in this work. This figure was educed by comparing ultrafiltration results with the diameter obtained from electron microscopy, ultracentrifugation and other physical measurements of a number of viruses and other colloidal particles investigated in this laboratory.

ULTRAFILTRATION OF HORSESICKNESS VIRUS

Materials and apparatus.

It has been explained that solutions containing egg white have been used as a virus suspending medium whenever possible. This solution could not be used in ultrafiltration experiments. It was found that whenever a solution containing egg white was passed through an ultrafilter complete blockage occurred almost immediately. This could not have been caused by denatured ovalbumin. Although easily denatured, its structure is apparently not changed by passage through an ultrafilter as this

method has been used to determine its size (Elford, 1931). A possible cause may have been the adsorption of the basic lysozyme onto the negatively charged collodion membrane.

The solutions used in the determination of the particle size of horsesickness virus by ultrafiltration were:-

Phosphate buffer (0.067 M). The buffer was prepared by dissolving 11.85 gm $Na_2HPO_4 \cdot 2H_2O$ and 9.13 gm KH_2PO_4 in a total of 1 litre distilled water (pH 7.5).

Virus suspending medium. Fowl serum (5% v/v) was added to phosphate buffer. This solution had a pH of 7.5. When suspended in this medium there was no loss of infectivity of the virus in the time required for ultrafiltration experiments.

Virus diluent for titration. Egg-white medium (Chapter 1) was used.

Broth for pretreating collodion membranes. Panmede broth, a solution of amino acids (Paines and Byrne Ltd., England) (5% w/v) was prepared in phosphate buffer (pH 7.5). All solutions were centrifuged to remove particulate material

Filter holder. A diagram of the type of filter holder used is shown in Fig. 9. In principle the device

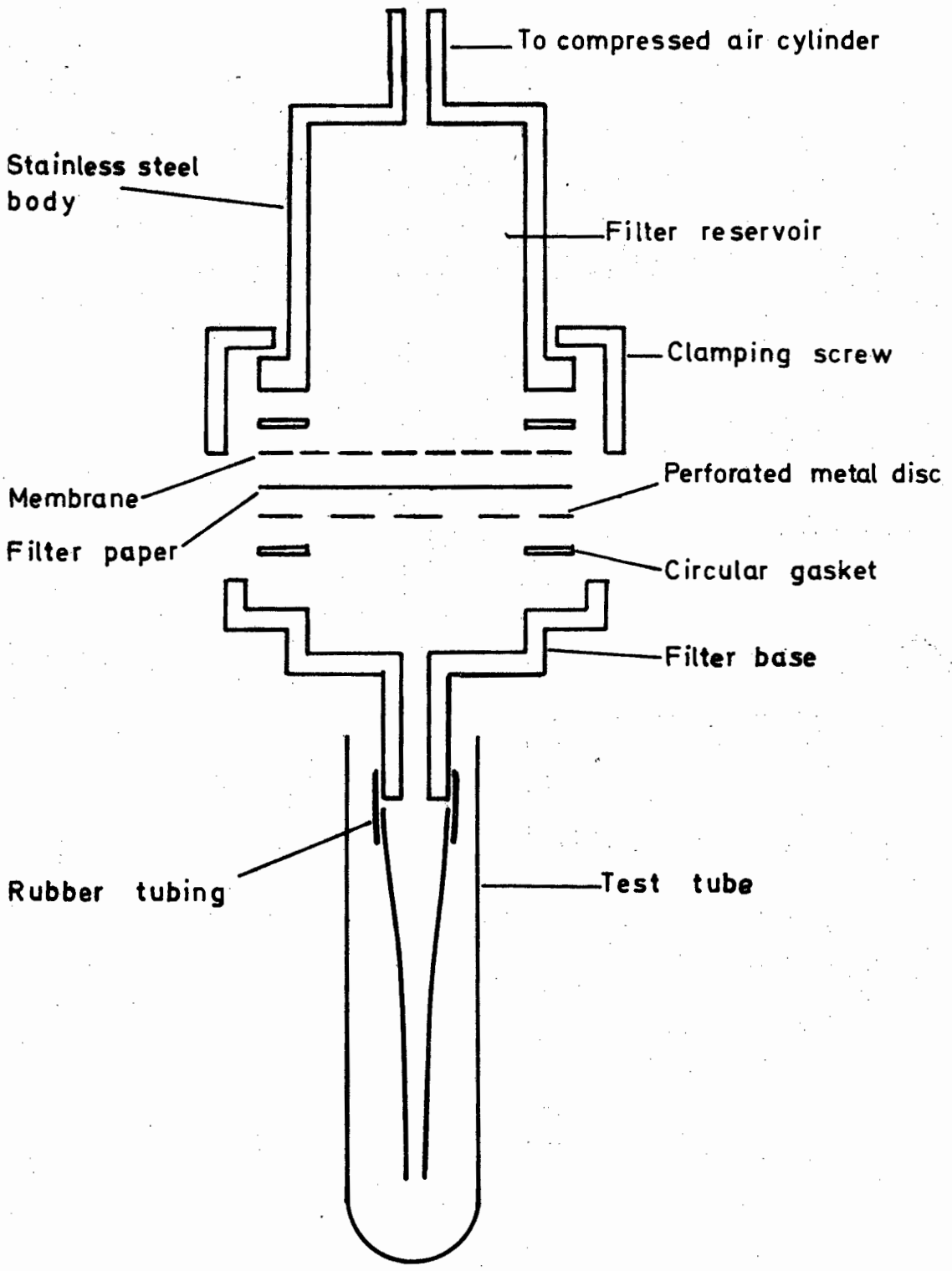


Fig. 9 Diagram of filter holder

is designed to clamp and support a membrane so that liquid may be filtered under pressure. The perforated metal disc supplies the necessary support for the membrane. Above this disc is placed a circular piece of hardened filter paper (Whatman No.542). The purpose of this paper is to ensure that the whole of the centre section of the membrane acts as a filter. If the paper is omitted the membrane would be pressed against the perforated metal disc. Filtration could then not take place effectively in those areas where the collodion membrane contacted the solid parts of the disc. The filter paper also helps to equalise the pressure on the membrane. The annular rubber gaskets ensure a pressure-tight seal between the filter and the upper and lower sections of the filter holder when these are screwed together. To the lower end of the filter was secured a test tube to receive the filtrate. Before use all parts of the filter were sterilised, the metal sections and test tubes by autoclaving and the collodion membranes, filter paper and gaskets by boiling in distilled water.

Preparation of virus. Infected mouse brains were triturated in phosphate buffer containing fowl serum and centrifuged at 10,000 rev/min for 10 min to remove tissue debris. This clarified virus suspension was then passed through a membrane of APD 470 mμ to remove particulate matter of density less than 1.0 gm/ml which would not have

been pelleted by centrifuging. Fifty ml of material prepared in this manner containing approximately 1×10^5 infectious units per ml was used for each experiment.

Method.

Six or seven filters were prepared for each experiment, each with a membrane of different pore size. The filters were placed in a stand supplied with a manifold from which compressed, filtered air (pressure 1 atmosphere) could be taken. All membranes were pretreated with 5 ml of Panmede broth by placing this volume in the filter reservoir and applying air pressure. When all the liquid had passed into the test tubes it was discarded and the test tubes replaced. Five ml of the virus suspension was then placed in each filter reservoir and the air pressure again applied. The filtrates so obtained were diluted and titrated for virus infectivity.

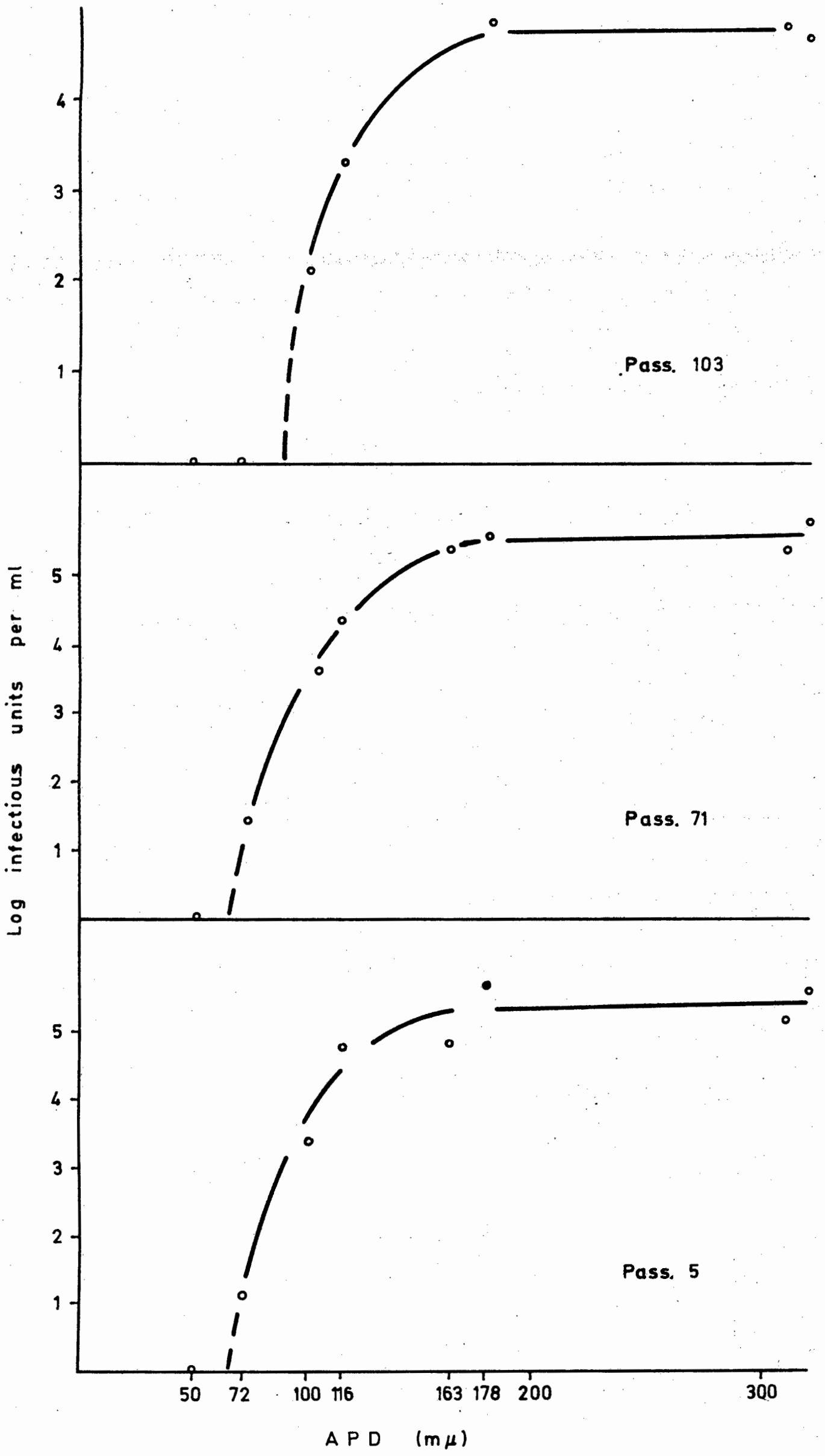
Results.

Titration results (Table 4) and ultrafiltration curves (Fig. 10) summarise the results obtained for horse-sickness virus at three levels of attenuation. Extrapolation of the ultrafiltration curves of passages 5

Table 4. Titration results of ultrafiltration of horsesickness virus No.3922.

	APD of Membrane (mμ)	Number of Infectious units (per ml)
Passage 5	52	Nil
	72	1.45×10^1
	105	7.27×10^3
	116	7.27×10^4
	163	7.27×10^4
	178	5.78×10^5
	310	1.45×10^5
	Original	4.09×10^5
Passage 71	52	Nil
	72	1.45×10^1
	105	4.38×10^3
	116	2.30×10^4
	163	2.30×10^5
	178	4.09×10^5
	310	2.30×10^5
	Original	7.27×10^5
Passage 103	52	Nil
	72	Nil
	105	1.45×10^2
	116	2.30×10^3
	178	9.16×10^4
	310	7.27×10^4
	Original	4.59×10^4

Figure 10. Ultrafiltration curves of horsesickness virus No. 3922.


Log infectious units per ml
Pass. 103
Pass. 71
Pass. 5
1
2
3
4
5
50
72
100
116
163
178
200
300
APD (mμ)

and 71 resulted in almost identical end points, namely 66mμ. On applying the correction factor a particle size of 45mμ was obtained for virus at both these passage levels. The ultrafiltration curve of virus at passage 103 extrapolated to a figure of 90mμ which, when corrected, yielded a particle diameter of 61mμ.

Discussion.

The ultrafiltration results are all lower than the particle size found by ultracentrifugation and density measurements (Chapter VII). This could be due to experimental error or the fact that the correction factor, which is an average figure, is not accurate for this horsesickness virus. The most significant results of the ultrafiltration titrations were those obtained using filters of the smallest pore diameter. It may be seen from Fig.10 that no virus of passage 103 passed through either the 52mμ or the 72mμ filters, indicating that the virus must have a greater diameter than (72 x 0.68) or 48mμ. Some virus of both the less attenuated strains passed through the 72mμ filter but not through the 52mμ filter indicating that the size in these viruses must be less than 48mμ. It might have been expected that at least some of the infectious particles from

passage 5 and 71 strains were smaller than the average. These, passing through the 72mμ membrane, would give the effect of a small size compared with the ultracentrifugation results. The argument cannot be sustained however because, especially in the case of passage 71 virus, ultracentrifugation indicates that such small particles are absent (Fig. 14). A satisfactory explanation of these results cannot at present be given. Ultrafiltration does however indicate a slight increase of particle size with attenuation and thus substantiates the ultracentrifugation results (Chapter VII).

The smooth curves obtained for all the ultrafiltration experiments indicate a logarithmic relation between infectivity and pore size of the membrane. This is typical of the ultrafiltration of spherical particles. This evidence indicates that the infectious particles of this horsesickness virus are spherical and not filamentous.

CHAPTER V.

SEDIMENTATION COEFFICIENT OF HORSESICKNESS VIRUS.

Introduction.

The sedimentation coefficient is a fundamental characteristic of a colloidal particle. It may be used to distinguish and classify different colloids. When combined in suitable equations with density and viscosity measurements, valuable information as to the size, shape, molecular weight and degree of hydration of particles may be deduced. Many techniques for measuring the sedimentation coefficient of colloidal particles are now available and some have been developed especially for virus particles.

In the present work the sedimentation coefficient of horsesickness virus at three different passage levels was determined. Changes of this parameter with the degree of attenuation of the virus were noted and the results were used to calculate the particle size of the virus.

Theory.

In order to understand the details and experimental procedure of many biophysical techniques some knowledge of

the relevant mathematical background is essential. In this and the following chapters the more important formulas used are derived. The derivations are not rigorous in the strict mathematical sense, and the proofs are only developed as far as the scope of this thesis demands, but an attempt has been made to show the basic concepts.

Sedimentation.

The theoretical aspects of centrifugation of colloids was first considered in detail by Svedberg (1925). He found that particles moved at a characteristic velocity when subjected to a centrifugal field, and defined sedimentation (s) as the rate of movement per unit field of force, or

$$s = \frac{\frac{dx}{dt}}{\omega^2 x}$$

x : distance from axis of rotation (cms)

t : time (sec)

ω : Angular velocity (radians/sec)

: (rev/sec x 2π)

There are many ways of deriving the fundamental sedimentation relationships. The following derivation combines the approaches of Svedberg (in, Svedberg and Pedersen, 1940), Bull (1951) and Pollard (1953). The quantities to be considered are the mass of the particle, the densities of the particle and solution, the viscosity of the solution, the angular velocity and the volume of the particle. The force moving the particle outwards is mass x acceleration = $m \times \omega^2 x$. The particle, being suspended in a liquid, is also subjected to a buoyancy effect $V \rho \omega^2 x$.

The net total outward force is thus

$$m \omega^2 x - V \rho \omega^2 x$$

or

$$m \omega^2 x - m \bar{V} \rho \omega^2 x$$

Therefore $m \omega^2 x (1 - \bar{V} \rho)$ ..... 1.

m = molecular weight (gm)

$\bar{V}$ = partial specific volume of particle (ml/gm)

ρ = density of solution (gm/ml)

V = volume of particle (ml)

The force resisting the outward motion is the product of the velocity of the particle and the frictional coefficient (f), that is $f \frac{dx}{dt}$

These two forces may be equated

$$m(1-\bar{V}\rho)\omega^2 x = f\frac{dx}{dt}$$

and rearranging $$\frac{m(1-\bar{V}\rho)}{f} = \frac{\frac{dx}{dt}}{\omega^2 x}$$

Therefore $$\frac{m(1-\bar{V}\rho)}{f} = s \text{ (by definition) ..... 2.}$$

The velocity at which a particle moves per unit centrifugal force (s) is small. By multiplying by the factor 10^{13} a convenient figure is obtained for most proteins and viruses. This unit is termed the svedberg (S).

It is usual to reduce the experimental result to a theoretical value equivalent to the rate that the particle would move through pure water at 20°. The sedimentation coefficient thus reduced is written (s_{20}) and the

dimension, seen from the ratio $\frac{\frac{dx}{dt}}{\omega^2 x}$ is $l\,t^{-1}\,t^2\,l^{-1}$ which simplifies to t (secs).

l = length

t = time

From the Stokes' relation (Chapter VII), the radius (r) of a spherical particle of density (ρ_p) moving through a liquid of density (ρ_l) and viscosity (η) at a velocity (u) due to a centrifugal force is

$$r = \sqrt{\frac{9u\eta}{2(\rho_p-\rho_l)\omega^2 x}}$$

If the particle moves at uniform velocity from x_1 to x_2 in time (t)

$$r = \sqrt{\frac{q\eta \ln \frac{x_2}{x_1}}{2(\rho_p - \rho_l)\omega^2 t}}$$

Elford (1936) has shown that if the concentration of particles contained in a specific section of a centrifuge cell of length ($l = 1$) before centrifuging is (C_o), and after time (t) is (C_t) then, because acceleration changes with the distance from the axis of rotation

$$r = \sqrt{\frac{q\eta \ln \left(\frac{x_1 + l}{x_1 + l\,\frac{C_t}{C_o}}\right)}{2(\rho_p - \rho_l)\,\omega^2 t}}$$

or

$$D = 7.94 \times 10^7 \sqrt{\frac{\eta \log X}{(\rho_p - \rho_l) N^2 t}} \qquad \ldots\ldots\ldots 3.$$

where (D) is the diameter of the particle in $m\mu$,

$$X = \frac{x_1 + l}{x_1 + l\,\frac{C_t}{C_o}}$$

and N is rev/min.

x_1 = Distance from centre of rotation to liquid meniscus. For the SW 39 rotor used = (5.85 cm).

Because of the possibility of side wall effects (Svedberg and Pedersen, 1940) adversely affecting the

sedimentation when the suspension is contained in capillary tubes as used by Elford, Polson and van Regenmortel (1961) adapted the technique for use with the Spinco SW 39 rotor. The necessary relation for calculating the sedimentation coefficient with the new method was obtained by substituting Stokes' formula in equation (3).

Stokes' formula may be written $D = 2\sqrt{\frac{qs\eta}{2\Delta\rho}}$

where s replaces the velocity (u).

Multiplying equation (3) by 10^{-7} to reduce to cm and substituting for D

then $$2\sqrt{\frac{qs\eta}{2\Delta\rho}} = 7{\cdot}94\sqrt{\frac{\eta \log X}{\Delta\rho N^2 t}}$$

where $$\Delta\rho = \rho_p - \rho_l$$

Simplifying $$\frac{4 \times q \times \eta \times s}{2\Delta\rho} = \frac{63 \times \eta \times \log X}{\Delta\rho N^2 t}$$

Therefore $$S = \frac{63 \times \eta \times \log X \times 2 \times \Delta\rho}{4 \times q \times \eta \times \Delta\rho \times N^2 \times t}$$

$$= \frac{3{\cdot}5 \log X}{N^2 t} \qquad \ldots\ldots\ldots\ldots 4.$$

This relation was used to calculate the sedimentation coefficient of horsesickness virus in the present work, taking $\frac{C_t}{C_o}$ in the X term as 0.1. This means a reduction

of 90% in the number of virus particles, that is, a difference of one logarithmic unit.

Viscosity.

In order that sedimentation coefficients may be compared they are reduced to a standard state, that is the rate at which the particle would sediment in some standard liquid. Water is convenient to use as a standard because at 20° the density is nearly 1 gm/ml (easily ascertained from tables) and the viscosity is nearly 1 centipoise. Unlike diffusion, temperature plays no part in sedimentation work other than the effect it has on the viscosity of the medium. The only correction to be made, therefore, involves viscosities, which in the case of water may be found from tables (Hodgman Ed., 1947) and for the medium used must be determined.

In the derivation of his viscosity equation Poiseuille showed that when a liquid flows, a certain shearing force occurs. The layer of liquid at the wall of a containing vessel is stationary and adjacent layers move with increasing velocity. If the velocity (u) increases from (u) to (du) through a distance (dx) there is a velocity gradient ($\frac{du}{dx}$) and a shearing stress proportional to this gradient. For any layer, the shearing stress is a force (F) per unit

area producing a drag on the faster moving layer and an equal pull on the slower moving layer. The ratio between this tangential stress and the velocity gradient is the coefficient of viscosity (η), that is

$$\eta = \frac{F}{\frac{du}{dx}}$$

The poise is the tangential force over 1 square cm of interface between two layers of liquid 1 cm apart when the velocity gradient is 1 cm/sec^2.

For a liquid flowing through a capillary tube:

$$\eta = \frac{\pi p r^4 t}{8 l V} \qquad \ldots\ldots\ldots\ldots\ldots 5.$$

p = pressure (dynes)

r = radius of tube (cm)

V = volume of liquid (ml)

t = time (sec)

l = length of capillary (cm)

η = viscosity of liquid (poise)

The absolute viscosity of a liquid may be determined using this relation but it is usual to compare viscosities with a standard substance, water. It may be seen from equation (5) that, if the same instrument is used to measure the flow time of both water and the unknown liquid, then all other factors are constant. A quantity,

the relative viscosity, may therefore be defined as the ratio of the flow times of the solution to that of water. The ratio is dimensionless. This procedure would be incorrect if the density of the liquids differed greatly because the hydrostatic pressure (density x height), forcing the liquid through the capillary, would not be equal in both cases. As the densities of the solutions used were very nearly identical with that of water, relative viscosity, as defined above, could be used to reduce sedimentation coefficients to the standard state.

It may be noted that the viscosity adjustment made is one of simple proportion. This is acceptable because proteins suffer no structural change due to temperature in the range 0° to 25°. Unlike water, which forms a lattice structure to an increasing degree as the freezing point is approached, with a corresponding increase in viscosity, the contribution of proteins to the total viscosity remains constant.

Viscosity measurements.

An Ostwald's glass viscometer (Fig. 11) was used to determine the viscosity of the suspension medium used in sedimentation studies. The instrument was manufactured by Technico (Gallenkamp, London) to British Standard No.188

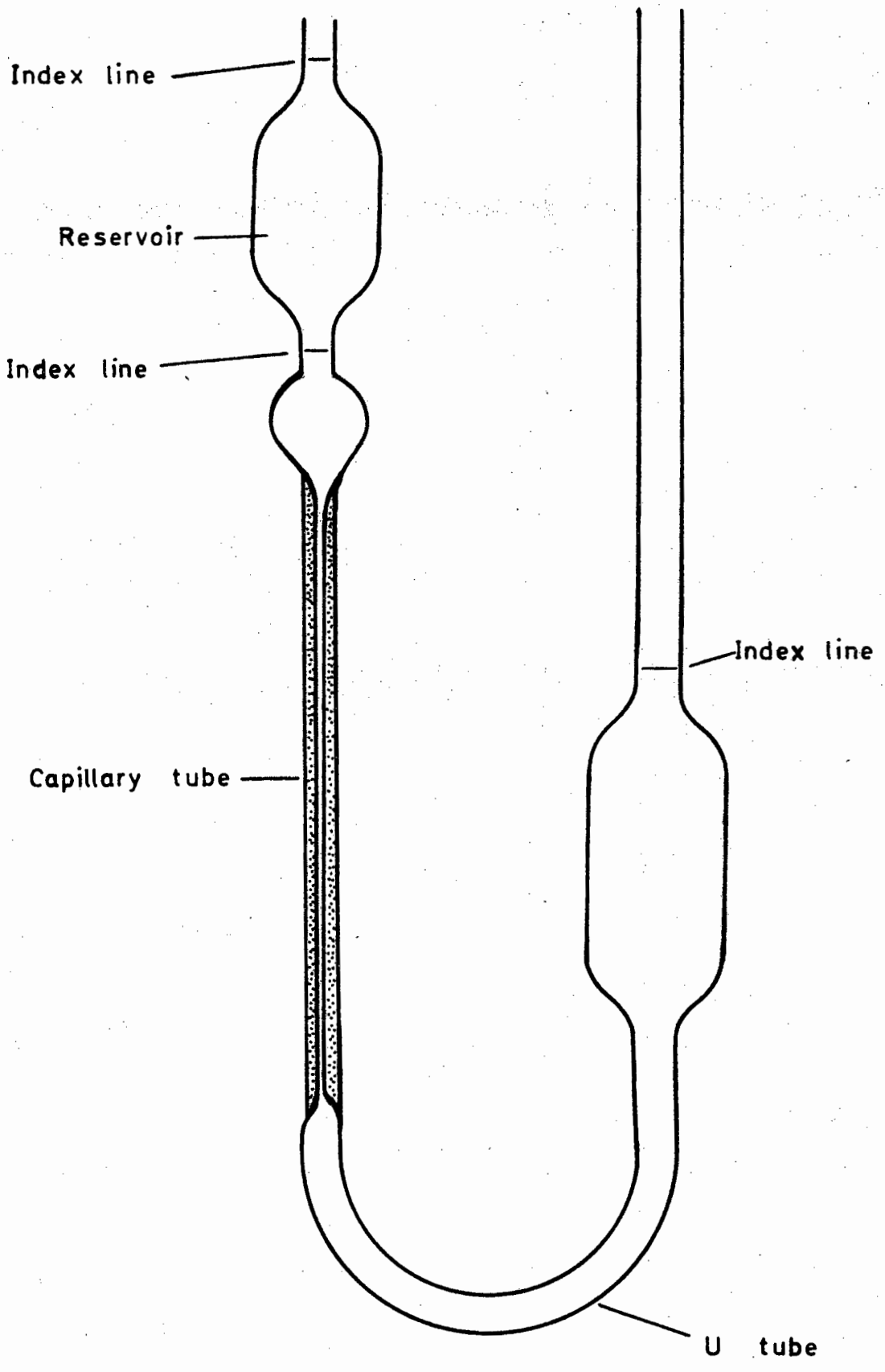


Fig.11 Ostwald viscometer

(Alexander and Johnson,1949). It consisted of a capillary tube of radius 0.06 cm and length 12 cm connected above to a reservoir and below to a wide diameter (0.5 cm) 'U'-tube. A mark inscribed on the 'U'-tube indicates the level to which the viscometer must be filled. Two other marks inscribed above and below the reservoir are used when timing the flow of liquid through the capillary.

Cleanliness is of the utmost importance in viscometry. As may be seen from equation (5) the viscosity is proportional to the fourth power of the radius of the tube through which a liquid is flowing. A small amount of dirt in the capillary of the viscometer will therefore have a great effect on the flow rate and hence on the calculated viscosity. The viscometer was cleaned in chromic acid and well washed with distilled water. The inside surface was never allowed to dry and after every experiment the viscometer was washed, filled with distilled water, and the inlets stoppered. Good quality distilled water was used as a standard and all vessels were rinsed before use to remove dust particles.

Viscosity measurements were made on three different solutions:

(i) Five brains were removed from healthy, normal 5-day-old suckling mice, triturated in egg-white medium

(total 20 ml) and centrifuged twice at 10,000 rev/min for 10 min in the No.40 rotor, and the clear supernatant fluid carefully collected.

(ii) Five brains obtained from 5-day-old sucklings, injected intracerebrally three days previously with horse-sickness virus and all showing typical signs of the disease, were triturated in egg-white medium (total 20 ml) and centrifuged twice at 10,000 rev/min for 10 min and the supernatant kept. This procedure was the same as that used to prepare a virus suspension for the determination of sedimentation coefficients.

(iii) Part of the virus suspension as obtained in (ii) was centrifuged for three hr at 30,000 rev/min in the number 40 rotor and the supernatant fluid kept.

The relative viscosity of these preparations was determined. In each case the clean viscometer was drained and rinsed three times with the solution being measured. After filling with the appropriate solution the viscometer was placed in a water bath provided with glass windows. The temperature of the water bath was kept constant (0.01°) by means of a toluene/mercury thermostat. The water temperature (25.0°) was measured with a standard thermometer (calibration, 0.1°). When temperature equilibrium was attained (approximately 15 min) the level of the solution in the viscometer was adjusted exactly to

the inscribed mark on the 'U'-tube. A rubber tube was attached to the opening of the 'U'-tube and the solution slowly forced up into the reservoir to just above the higher inscribed mark by applying positive pressure with a rubber bulb. The tubing was then removed and with the aid of a light illuminating the apparatus from behind, the time taken for the solution meniscus to move between the scribe marks above and below the reservoir was measured with a stop watch reading to 0.01 min. This procedure was repeated six times for each solution. The average times taken are recorded in Table 5.

Table 5.

Determination of relative viscosity.

Material	Average flow time (min)		η_{rel}
	Water	Soln.	
1. Normal (centrifuged 10,000 rev/min)	3.725	4.065	1.092
2. Infected (ctg. 10,000 rev/min)	3.725	4.350	1.170
3. Infected (ctg. 30,000 rev/min)	3.725	4.010	1.076

The relative viscosity of the solution obtained from infected mouse brains after centrifuging at 30,000 rev/min for 3 hr was used in the determination of sedimentation coefficients (see discussion). This figure (1.076) was multiplied by the viscosity of water at the

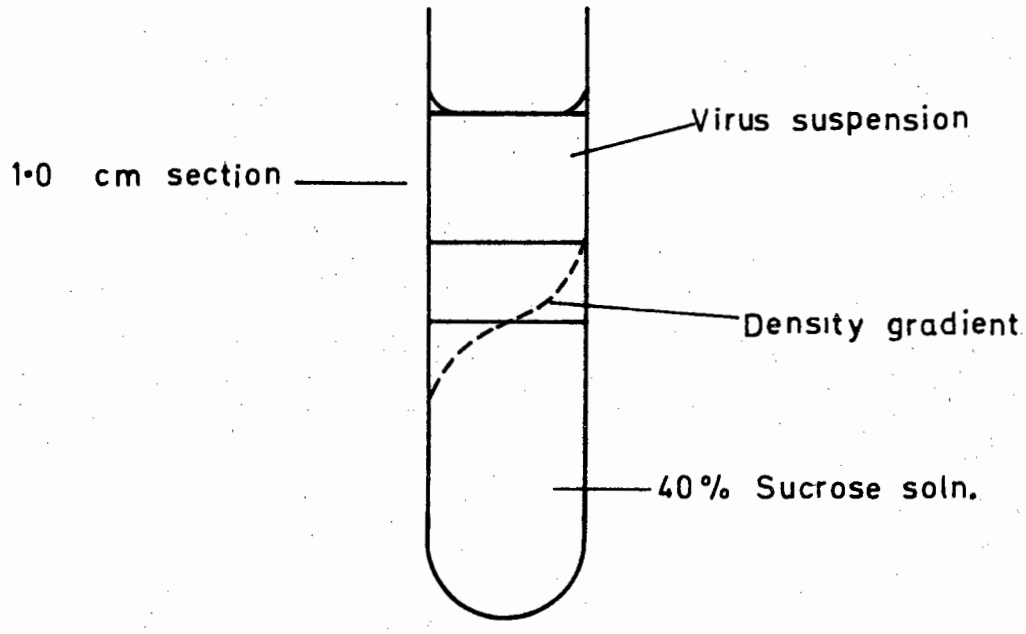


Fig.12 **Marked centrifuge tube**

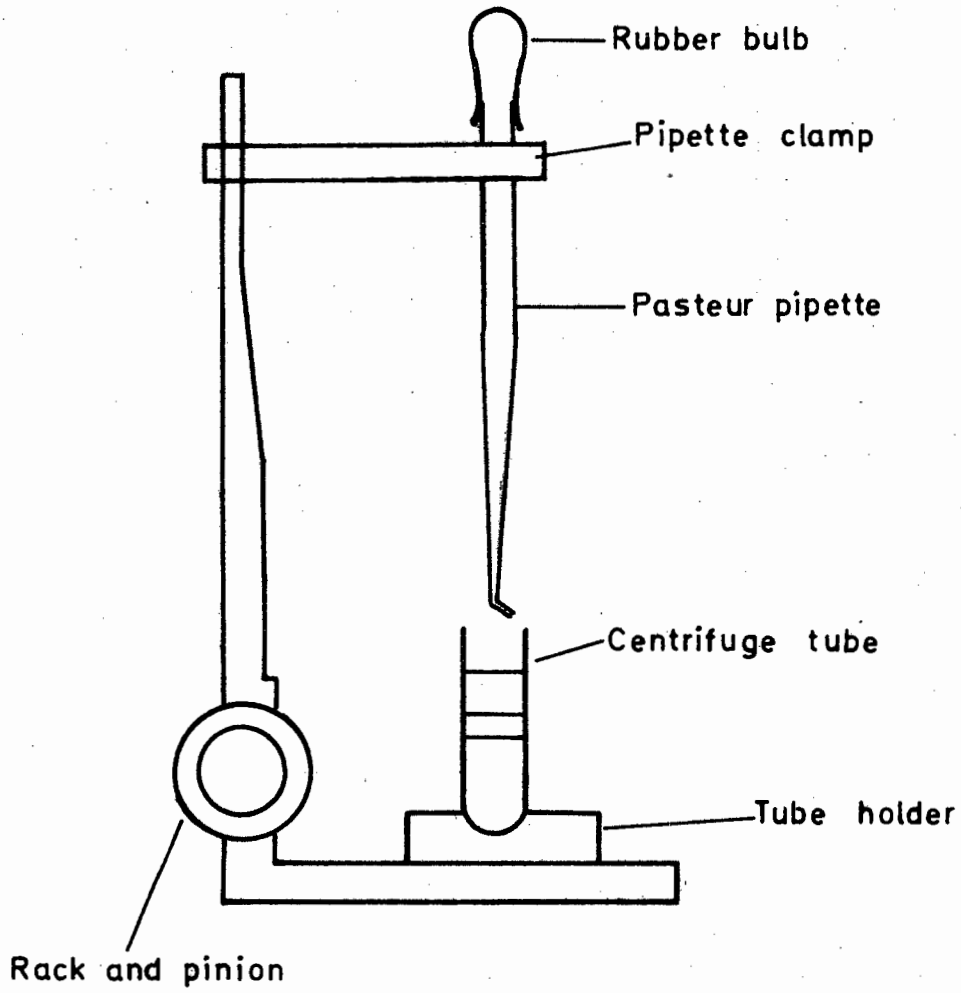


Fig.13 Rack and pinion clamp

temperature of centrifugation to obtain the viscosity of the medium through which the virus particles sedimented. For example at 4.50° the viscosity is 1.076 x 1.55 = 1.667 centipoise.

Materials and apparatus.

The sedimentation coefficient of horsesickness virus (No.3922) at passage levels, 5, 71, and 103 was determined. Virus suspensions were prepared in egg-white medium and centrifuged at 10,000 rev/min for 10 min to clarify.

Sugar solution. A 40% (w/v) solution of sucrose in egg-white medium was prepared.

Centrifuge. A model L Beckman Spinco ultra-centrifuge was used with the SW 39 swinging bucket rotor.

Centrifuge tubes. Cellulose nitrate tubes (0.5 x 2 inches) were marked as shown in Fig. 12.

Sampling device. To facilitate the removal of samples from the tube after centrifugation a pipette clamp mounted on a rack and pinion movement was used (Fig. 13).

Methods.

The method A of Polson and van Regenmortel (1961)

was used to determine the sedimentation coefficient of the virus. In this method the amount of virus in a column of liquid of known height (1 cm) is compared before and after a number of identical samples are centrifuged for a constant time but at different rotor velocities. Depending on the sedimentation coefficient of the particles they will sediment at a faster or slower rate and the rotor velocity needed to reduce the concentration of virus by 90% in a given period of time (30 min) may be determined. During centrifugation at constant temperature the particles may be expected to sediment evenly but when the rotor is stopped and samples taken, convection currents and other disturbances may cause mixing. This is prevented by layering the virus sample over a sugar solution. Diffusion of the sugar into the 0.5 cm section of the tube results in a concentration gradient and therefore a density gradient. This forms an effective trap for viruses that have sedimented into this region.

Centrifugation.

The procedure was to layer the virus suspension, taken from a well mixed stock, over the sugar solution in a centrifuge tube as shown in Fig. 12. The tube was

then allowed to stand for 30 min at 5° for diffusion of the sugar to take place. Centrifugation was then carried out at a temperature between 0° and 5°. The pressure in the rotor chamber was kept as low as possible (20 microns of Hg) to prevent frictional heating.

Each sample was centrifuged for exactly 30 min. The period of centrifugation was timed with a stop watch and the average rotor velocity calculated from a number of odometer readings taken during centrifugation. Six to eight different rotor velocities were selected for each experiment.

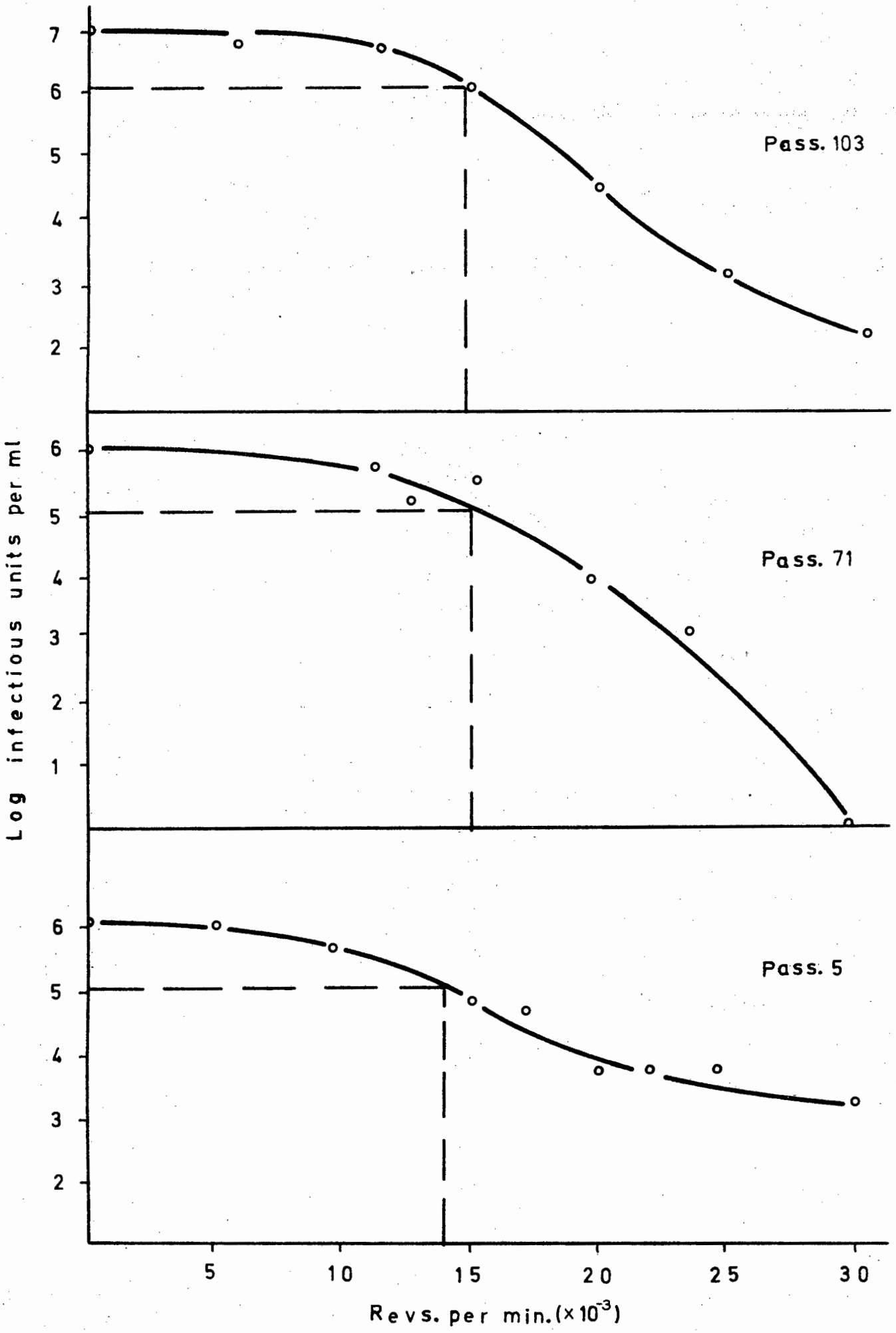
Sampling.

After centrifugation the temperature of liquid in a balance tube was measured. The sample tube was then removed and the liquid contained in the 1 cm section sampled. This must be done very carefully so as not to mix any of the sample with fluid below the 1 cm mark. This was accomplished by using a Pasteur pipette with a fine point drawn down at an angle of 45 degrees. The pipette was secured in the rack and pinion clamp (Fig.13) and slowly lowered as liquid was sucked up from near the meniscus. Virus suspensions that had been centrifuged for 30 min at different rotor velocities were diluted

Table 6. Titration results of sedimentation coefficient experiments of horsesickness virus No.3922.

	Sample Number	Rotor velocity (rev/min)	Temperature (^{o}C)	Number infectious particles/ml
Passage 5	1	9,852	4.5	4.09×10^{5}
	2	20,000	4.5	5.15×10^{3}
	3	15,200	4.5	6.63×10^{4}
	4	5,300	4.5	1.03×10^{6}
	5	17,400	4.5	4.59×10^{4}
	6	30,000	5.0	1.59×10^{3}
	7	24,700	5.5	5.78×10^{3}
	8	22,115	5.0	5.15×10^{3}
		Original		1.15×10^{6}
Passage 71	1	11,400	3.5	5.25×10^{5}
	2	13,000	4.0	1.56×10^{5}
	3	15,340	3.0	3.10×10^{5}
	4	19,670	3.5	7.10×10^{3}
	5	23,800	3.5	1.32×10^{3}
	6	29,600	3.0	Nil
		Original		7.27×10^{5}
Passage 103	1	15,200	4.0	1.004×10^{6}
	2	20,150	3.8	2.90×10^{4}
	3	30,400	5.0	2.25×10^{2}
	4	5,600	3.8	7.27×10^{6}
	5	25,000	3.5	1.52×10^{3}
	6	11,800	3.6	5.39×10^{6}
		Original		1.63×10^{7}

Fig. 14. Sedimentation curves of HSV.



and titrated for infectivity.

Results.

Titration results of the centrifugation of horse-sickness virus at three different passage levels are tabulated (Table 6), and the curves relating infectivity to rotor velocity constructed from these data are presented in Fig. 14. Using the relation (4) and incorporating viscosity corrections, the following sedimentation coefficients were obtained:

Passage 5	568 S
Passage 71	488 S
Passage 103	545 S

Discussion.

No striking change of sedimentation coefficient with degree of attenuation was observed. Virus of passage 103 (545S)has a sedimentation coefficient 4% lower than the unattenuated strain,passage 5 (568S). This however is not far beyond the limit of accuracy of the experiment and may not be significant. The comparatively low figure of 488S for the passage 71 virus

may be of some importance. Either the particles are smaller or they are less dense than those at higher and lower passage levels. Neither of these possibilities explains why all the infectious particles of passage 71 virus should sediment at 30,000 rev/min under the conditions of the experiment (Fig. 14).

It has been noted (Polson and Madsen, 1954) that results of the centrifugation of horsesickness viruses often show a two-step curve with some infectious particles remaining in the supernatant fluid even after prolonged centrifugation. A second fraction of infectious particles of smaller size was shown to be present in these cases. As all the virus of passage 71 was centrifuged down it would seem that only one size of particle is present. Some evidence for the presence of a second, smaller infectious particle was found from the centrifugation curves of virus at passage levels 5 and 103.

In connection with viscosity measurements it is of interest to note that the relative viscosity of the suspension obtained from infected mouse brain is considerably higher (about 7%) than a suspension obtained from normal brains. A second measurement after high speed centrifugation of the infected brain material showed the relative viscosity had dropped to approximately that of normal brain extract. This shows that the increase in

viscosity is probably due to substances formed in the brain cell as a result of the virus infection. These abnormal substances were large enough to be centrifuged out of solution which then had a relative viscosity similar to that of normal brain material.

There is some uncertainty as to which is the proper viscosity to use when correcting sedimentation coefficients to the standard condition. Lauffer (1944) has argued that the viscosity of the solution should be used. This may apply to material like tobacco mosaic virus which has a high intrinsic viscosity and was investigated in solutions of high concentration. The idea was not accepted here. In the present case the particles of horsesickness virus, which are approximately spherical in shape and in low concentration, were considered to sediment through an aqueous medium in which both large and small molecules were dissolved. It is suggested that only those ions and molecules below a certain size, that form a more or less homogeneous continuum in water, can contribute a viscosity effect on the sedimentation of the virus particles (Polson, 1949). Larger particles will have little frictional effect on the virus particles but will contribute greatly towards the viscosity of the solution when this is measured in a capillary viscometer. Further, as pointed out by Cheng and Schachman (1955), the effective viscosity around

a particle must be less than the macroscopic viscosity because of steric exclusion of neighbouring particles. All particles large enough to sediment at 30,000 rev/min in the No.40 rotor in 3 hr were therefore removed and the viscosity of the solution so obtained was used for correcting sedimentation coefficients.

CHAPTER VI.

DENSITY OF HORSESICKNESS VIRUS.

Introduction.

The method of density-gradient centrifugation described by Meselson et al, (1957) has proved of great value in the study of viruses. Very slight density differences are detected by this technique and, as adapted by Polson and Levitt (1963), an experiment may be completed in a few hours.

In the present work the buoyant density of horse-sickness virus (No.3922) at different passage levels was determined in order to discover if any change in density occurred during attenuation. These density data were also needed for the calculation of particle size from sedimentation results.

Theory.

A centrifugal field acting on the mass of a particle results in a force, or weight, which moves the particle relative to the suspending medium. The net force acting on the particle was seen (Equation 1) to be

$$m\left(1-\bar{V}\rho\right)\omega^2 x$$

The partial specific volume ($\bar{V}$) of the particle may be defined as the increase in volume produced in a large volume of solvent on adding 1 gm of solute. The dimensions ml/gm show that the partial specific volume of a particle is numerically equal to the reciprocal of its density. The factor ($1-\bar{V}\rho$) may therefore be written $\left(1-\frac{1}{\rho_{Solute}}\times\rho_{Solution}\right)$ and inspection shows that if the densities are equal the quantity in brackets reduces to zero. Under this condition the centrifugal force acting on the particle and the buoyancy effect are equal and movement of the particle through the liquid ceases. The density of a particle may therefore be determined by centrifuging the substance in a density gradient until sedimentation ceases. The particles will then be at their isopycnic level where the density of the suspending medium is equal to that of the particle. So the density of the particle is automatically discovered by determining the density of the suspension medium at this point. It may also be mentioned that the presence of a density gradient is useful in that it prevents convective mixing.

Materials.

Egg-white medium. Prepared as described in Chapter I.

Caesium chloride. Analytical grade (British Drug Houses) CsCl was used after drying in an oven at 110°. To egg-white medium (approximately 5 ml) was added CsCl (6.0 gm). When the salt had dissolved the volume was made up to 10.0 ml with egg-white medium. The solution was then filtered through No.542 (Whatman) paper to remove particulate contaminants.

Virus. Density determinations were made on horsesickness virus at passage levels 5, 51, 71 and 101. Preparation of the virus was as described in Chapter I.

Method and apparatus.

The original method of density-gradient centrifugation first described by Brakke (1951) and later in more detail by Meselson et al. (1957) required that the substance under investigation be layered on or be mixed with the gradient forming solution. Centrifugation at high speed then automatically resulted in the formation of a density gradient and sedimented the substance, either upwards or downwards, to its iso-pycnic level. The gradient is formed because of the equilibrium established between the centrifugal force, tending to sediment the gradient-forming material, and the diffusive force which tends to redistribute this material. Because the method

required lengthy centrifugation (approximately 24 hr), which could result in the breakdown of labile material, Polson and Levitt (1963) introduced a modification which is suitable for determining the buoyant density of many viruses. The important aspects of the modified technique are that the gradient is preformed and that the virus may be introduced as a narrow band anywhere in the gradient column. Two important advantages are thus attained.

(i) The range of materials which may be used to form the gradient is increased. If the gradient is to be formed by centrifugation only the molecular weight of the substance must be high enough to ensure that sedimentation occurs, with the consequent formation of the density gradient. Also the viscosity of the solution must be low enough to allow the particles to move through a comparatively long distance to reach their iso-pycnic level. The number of gradient forming substances suitable for this technique is very limited. Caesium and Rubidium salts may be used. With the preformed gradient technique many other substances may be employed. The polysucrose Ficol (Oroszlian et al., 1965); silica sol (Juhos, 1966) and Tartrates (McCrea et al., 1961) have been found suitable for virus studies.

(ii) Because of possible toxic and other effects of

inorganic salts on some viruses, substances like Ficol are preferred as gradient forming materials. Solutions of such substances are very viscous at the concentrations needed to obtain suitable densities. In order to sediment particles through such viscous solutions so that they all reach their iso-pycnic level would require a long time if the distance they had to migrate was more than a few millimetres. Using a preformed gradient solves this problem as the substance under investigation may be placed in the gradient at a position near its iso-pycnic level. At most a few trial experiments may be needed to estimate the approximate density of the virus.

In the present work the method of Polson and Levitt (1963) was followed, using a solution of CsCl to prepare a preformed gradient. The virus was found to be insensitive to CsCl at a concentration of 60% for at least eight hours standing in the refrigerator (5^{o}) and during centrifugation at 30,000 rev/min for three hours.

Forming the gradient. The gradient was formed by layering CsCl solutions of decreasing concentration one above the other in a centrifuge tube. A range of solutions of different densities was prepared by mixing 0.1 ml of 60% CsCl with 0.9 ml egg-white buffer; 0.2 ml

of 60% CsCl with 0.8 ml egg-white buffer etc. For the middle fraction the virus suspension replaced the diluent. Starting with egg-white buffer, 0.4 ml amounts of the various CsCl dilutions were drawn into a sterile syringe using the apparatus shown in Fig. 13. The contents of the syringe were then transferred to a SW39 centrifuge tube.

Centrifugation. This was carried out in a Model L Spinco centrifuge using a SW39 rotor. It was found that centrifugation at 30,000 rev/min for three hr was sufficient to move all the virus particles to their isopycnic position. To prevent frictional heating the lowest possible pressure in the centrifuge chamber was secured (20 microns Hg). This is important in density-gradient work as slight differences in the density of the liquid column are magnified by the centrifugal field (de Duve et al., 1959) and could cause mixing by convection.

Sampling. There are some difficulties associated with sampling the contents of density-gradient tubes after centrifugation. These arise mainly from possible contamination of one fraction by another. If the bottom puncture method is used virus particles may adhere to the inner wall of the centrifuge tube, especially near the

outlet, and mix with subsequent fractions as they are withdrawn. If the tube is sampled from the top, residual virus from one sample may slowly run down the wall and contaminate other samples. Slicing the tube into sections using a special machine is possible, but the necessary apparatus was not available. Other methods using bottom puncture and controlled flow rate (Szybalski, 1960) have been described. A compromise was used in the present work by sampling from both the bottom and the top of the tube. Four or five samples of equal volume were taken from the top using a fine-drawn Pasteur pipette with the tip bent downwards at 45 degrees. The clamping device (Fig. 13) was used. The tube was then punctured at the bottom and the remaining liquid sampled. Individual fractions were well mixed and one quarter (approximately 0.1 ml) was removed for refractive index measurements, and the remainder was dialysed.

Dialysis. Before samples taken from a density gradient column could be titrated in mice the CsCl had to be removed. This was accomplished by dialysis against a large volume of 0.06 M phosphate buffer (pH 8.0) for 10 hr at 5°. Because of the different quantities of CsCl in the samples, dialysis resulted in a change in the

Table 7. Data used for constructing the standard density/refractive index curve.

CsCl Solution (%)	Weight of solution (gm)	Density gm/ml	Refractive index
60	3.0409	1.457	1.3782
45	2.8475	1.365	1.3697
36	2.6740	1.281	1.3616
24	2.4837	1.191	1.3532
12	2.2911	1.098	1.3442
Diluent	2.0983	1.006	1.3351

Determination of volume of pycnometer.

weight (full distilled water, 24°)	4.4121 gm
weight (Empty, dry)	2.3325 gm
Weight water	2.0796 gm

Correction for density of water at 24°i.e. 0.99707 gm/ml.

$$\text{Volume pycnometer} = \frac{2.0796}{0.99707} = 2.0855 \text{ ml.}$$

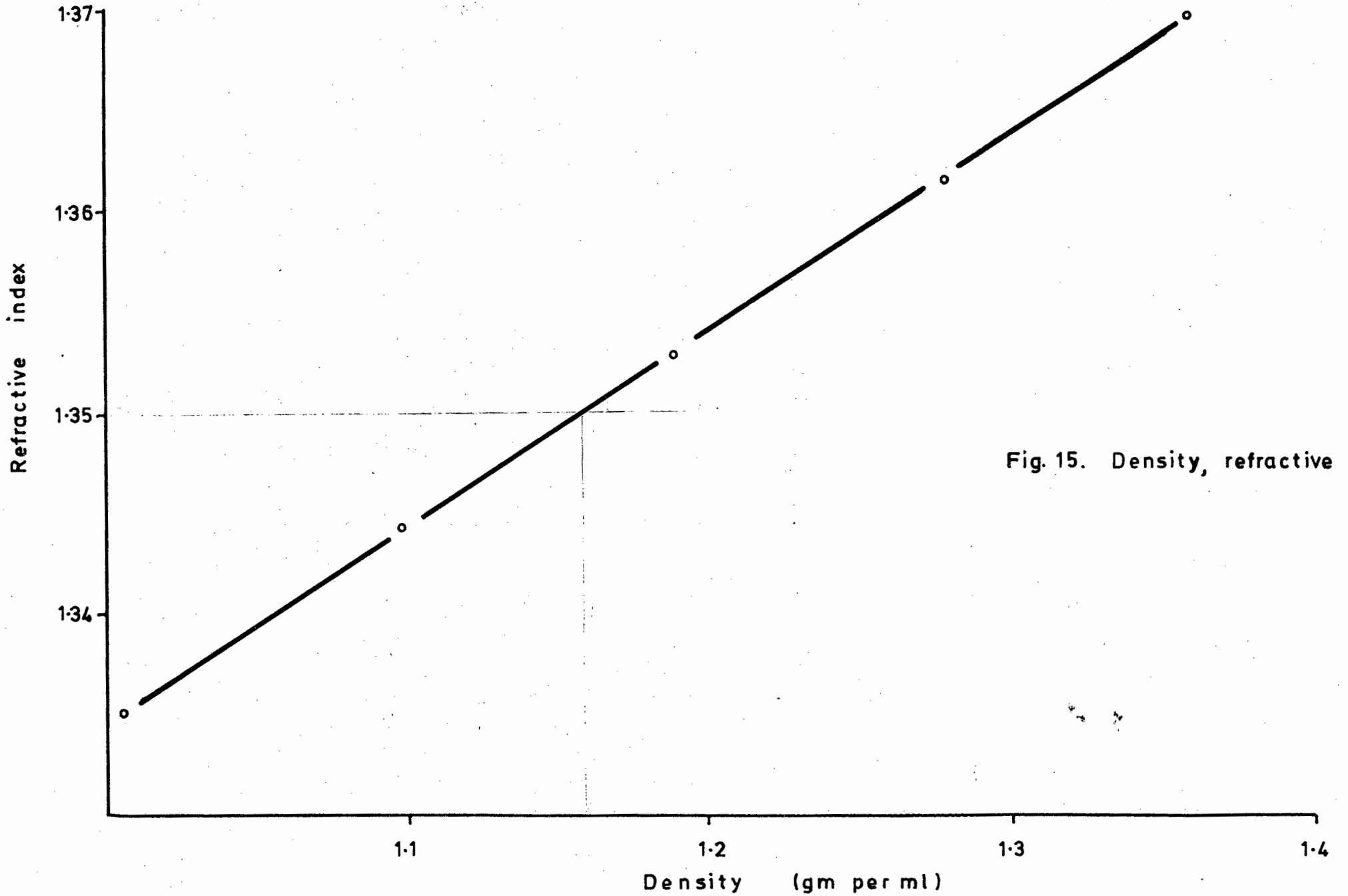


Fig. 15. Density, refractive index curve

volume of the fractions. This was corrected by making the volume of the diffusate of each fraction up to 2 ml with egg-white buffer before dilution and titration.

Standard density, refractive index curve. The average density of each sample was obtained by measuring the refractive index at 25° and finding the corresponding density from a standard curve. A standard curve relating refractive index of CsCl solutions in egg-white buffer to density was constructed. Exact quantities of dried (105°) CsCl salt were weighed and dissolved in egg-white medium. The density of each solution was determined pycnometrically and the refractive index measured at 25°, (Table 7). From these data the standard curve (Fig. 15) was constructed.

Results.

The density of the infectious particles of horse-sickness virus (No.3922) passage 5 was found to vary between 1.1 and 1.4 gm/ml with an infectivity peak at 1.265 gm/ml (Table 8, Fig. 16). At passage level 51 a peak of infectivity was found at 1.21 gm/ml and a shoulder on the curve indicating a slight concentration of virus at a position corresponding to a density of 1.34 gm/ml, was noted. Virus at higher passage levels

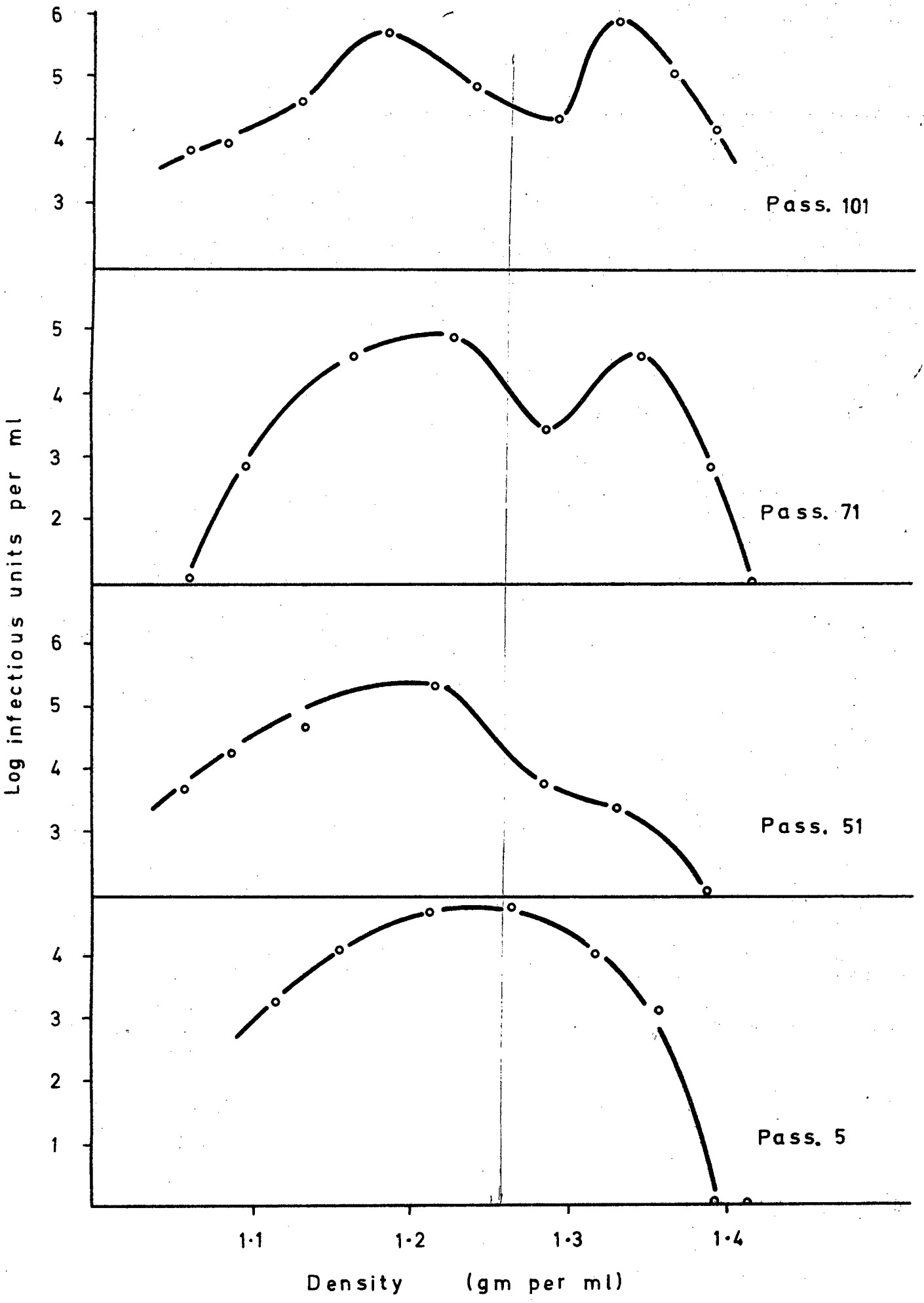
Table 8. Density gradient analysis of horsesickness virus No.3922.

	Sample Number	Refractive index	Density (gm/ml)	Number of Infectious Units/ml
Passage 5	1	1.3753	1.4405	Nil
	2	1.3732	1.4185	Nil
	3	1.3709	1.3945	Nil
	4	1.3676	1.3600	1.45×10^3
	5	1.3634	1.3208	1.45×10^4
	6	1.3592	1.2720	7.27×10^4
	7	1.3540	1.2175	5.78×10^4
	8	1.3487	1.1630	1.45×10^4
	9	1.3448	1.1205	2.3×10^3
Passage 51	1	1.3405	1.060	5.27×10^3
	2	1.3437	1.094	1.59×10^4
	3	1.3480	1.138	3.90×10^4
	4	1.3558	1.222	2.30×10^5
	5	1.3618	1.285	5.39×10^3
	6	1.3669	1.338	2.30×10^3
	7	1.3720	1.391	Nil
	8	1.3750	1.423	Nil
	9	1.3763	1.436	Nil
	10	1.3773	1.447	Nil

Table 8 (Contd.):-

	Sample Number	Refractive index	Density gm/ml	Number of Infectious Units/ml
Passage 71	1	1.3409	1.0650	Nil
	2	1.3450	1.1075	4.09×10^3
	3	1.3511	1.1700	3.99×10^4
	4	1.3574	1.2355	7.27×10^4
	5	1.3627	1.2925	2.30×10^3
	6	1.3677	1.3450	4.09×10^4
	7	1.3722	1.3925	7.27×10^2
	8	1.3746	1.4175	Nil
Passage 101	1	1.3706	1.3920	5.78×10^4
	2	1.3682	1.3663	1.45×10^5
	3	1.3650	1.3325	7.27×10^5
	4	1.3611	1.2920	2.30×10^4
	5	1.3564	1.2430	9.16×10^4
	6	1.3511	1.1862	1.45×10^5
	7	1.3463	1.1365	3.65×10^4
	8	1.3417	1.0883	9.16×10^3
	9	1.3391	1.0610	7.27×10^3

Fig. 16. Density curves of HSV.



(71 and 101) showed an increasing tendency to divide into two fractions of density 1.21 and 1.34 gm/ml. This resulted in a reduction in the amount of virus at a density of 1.26 gm/ml where, at passage level 5, it is maximal.

Discussion.

Studies of the density of horsesickness virus (No.3922) showed a definite, continuous change of this physical property with the degree of attenuation. While most of the passage 5 virus was found to have a density of 1.265 gm/ml progressive attenuation results in a change of density of the infective particles, some becoming more dense (1.34 gm/ml) while most aquire a lower density (1.21 gm/ml). With this knowledge it may be possible to measure the degree of attenuation of the virus by determining the density of the particles. If it can be shown that the density of some other viruses is also related to the degree of attenuation they too may be similarly investigated. An extension of this idea is that over-attenuation of a virus strain may result in a continued change of density of the particles. This may help in the control of vaccine virus strains and perhaps allow some prediction as to their efficiency.

CHAPTER VII.

CALCULATION OF PARTICLE SIZE OF HORSESICKNESS VIRUS.

Introduction.

The diameter of a colloidal particle may be estimated from sedimentation and density data using Stokes' Law. This relation is true for spherical particles only. It is assumed here that the No.3922 strain of horsesickness virus is spherical. Only round particles were seen in electron micrographs of the virus (Chapter III) and the ultrafiltration results (Chapter IV) also indicate spherical particles, so it is likely that this assumption is correct.

Theory.

Stokes' relation may be derived using dimensional equations (Riggs, 1963). If a sphere moves through a liquid under gravity or by a centrifugal field the velocity (u) attained will depend on the magnitude of the force applied (F), the viscosity of the liquid (η) and the surface area of the sphere with radius (r). It is therefore possible to state that velocity $\propto$ force $\times$

viscosity x radius.

Writing dimensions and including the proportionality constant (C);

$$l t^{-1} = C (m l t^{-2})^{\alpha} (m l^{-1} t^{-1})^{\beta} (l)^{\gamma}$$

m : mass

l : length

t : time

According to the laws of dimensional analysis the factors on the right hand side of the equation must equal $l t^{-1}$

Therefore the indices $\alpha - \beta + \gamma = 1$

$$-2\alpha - \beta = -1$$

$$\alpha + \beta = 0$$

and by inspection $\alpha = 1$, $\beta = -1$, $\gamma = -1$

The actual relation must therefore be written.

$$\text{velocity} = \frac{\text{force}}{\text{viscosity} \times \text{radius}}$$

or $$u = C \frac{F}{\eta r}$$

But the force (F) applied to the sphere is acceleration x

effective weight i.e. $F = g \times (\rho_s \times V_s) - (\rho_l \times V_l)$

or $$F = g \times V (\rho_s - \rho_l)$$

because the volume of the sphere (V_s) and the volume of the liquid it displaces (V_l) is equal and $V = \frac{4}{3} \pi r^3$

Therefore $F = g \times r^3 (\rho_s - \rho_l)$ $\left[\frac{4}{3} \pi = \text{constant}\right]$

Therefore $u = \frac{C g r^3 (\rho_s - \rho_l)}{\eta r}$

It was shown by Stokes that $u = \frac{2}{9} \times \frac{g r^2 (\rho_s - \rho_l)}{\eta}$

or $D = 2 \times \sqrt{\frac{9 s \eta}{2 \Delta \rho}}$ ..... 6.

for unit field of force where the sedimentation coefficient (s) replaces the velocity (u).

D : Diameter of particle (cms)

s : Corrected sedimentation coefficient (sec)

η : Viscosity of water at 20^0 (poise)

$\Delta \rho$: Density difference between particle and water (gm/ml)

g : Acceleration due to gravity (980 cm sec^{-2})

ρ_s : Density of sphere (gm/ml)

ρ_l : Density of liquid (gm/ml)

V_s : Volume of sphere (ml)

V_l : Volume of liquid (ml)

Particle size of horsesickness virus.

Using relation(6)the particle diameter of virus from three passage levels of the horsesickness strain was calculated from the relevant density and sedimentation data to be:-

Passage 5	62 mμ
Passage 71	63 mμ
Passage 103	68.4 mμ

Discussion.

The sedimentation coefficient of horsesickness virus No.3922 at the three passage levels studied were similar and did not indicate any progressive change concurrent with attenuation. The surprisingly clear-cut changes in density found were therefore all the more unexpected. On combining these data to calculate the particle size a slight but definite change was found; the size of the virus tends to increase slightly during attenuation. It must be noted that, for the virus at higher passage levels, there are two densities that could be used in the calculation. In each case only the lower density figure was used as this represented the

greater proportion of virus present. It is presumed that the sedimentation coefficient, as determined in Chapter V, referred to the largest fraction of particles because of the great reduction of infectivity, that is, approximately 90%, in each case.

The slight increase of particle size with degree of attenuation confirms the ultrafiltration results where this tendency was first noted.

CHAPTER VIII.

ZONE ELECTROPHORESIS OF HORSESICKNESS VIRUS.

Introduction.

The migration of charged colloidal particles in an electric field was first studied in detail by Tiselius (1937) using the moving boundary technique. Practical difficulties associated with this method led to the development of zone electrophoresis by using Brakke's (1951) principle of establishing a density gradient of a neutral substance to prevent convection (Svensson and Valmet, 1955). Simplification of this apparatus by Polson and Cramer (1958) resulted in a technique with wide applications. Electrophoresis is a gentle method of separating and purifying charged substances and is useful in characterising proteins and viruses.

Electrophoresis may also be adapted to solve special problems. In disc electrophoresis the method of preventing convection with a density gradient cannot be used. Various gels have been substituted with good results. Gels have also been used in the apparatus of Polson and Cramer as well as other solid supporting media (Porath and Hjertén, 1962). Agarose (Araki, 1958) may

be used for this purpose (Hjertén, 1962). Russell et al. (1964) were able to separate viruses of similar electrophoretic mobility but of different size using agarose sieves. Hjertén (1963) has developed an anticonvection medium of an agarose suspension for use in electrophoresis.

An agarose suspension has some advantages over the sucrose density gradient system.

(i) It is not necessary to place the sample in a concentrated sucrose solution, a procedure that may lead to the precipitation of material.

(ii) The medium through which the substance migrates is uniform in all respects.

(iii) It is essential that a concentration gradient be formed across the initially uniform sample layer when a sugar gradient is used. This takes place by diffusion and requires 1 to 3 hr. If an agarose suspension is used this delay is obviated.

(iv) The formation of a density gradient by any of the methods normally used (Svensson, 1960) is another time consuming step which is eliminated if the gel suspension is used.

(v) The apparatus required for electrophoresis using an agarose suspension may be even less complicated than the Polson and Cramer model. Hjertén found it necessary to construct an electrophoresis column with a "shunt" to

overcome the disturbing effects of electroendosmosis. For the present work a new type of apparatus was designed.

Electroendosmosis is a problem that may be serious when using agar gels and must be carefully considered. It arises from the fact that the solid phase, agarose, is not absolutely pure but carries a slight residual negative charge. When an electric current is passed through the gel a flow of water, positively charged with respect to the agarose, may result. This water will move towards the negative electrode. Concurrently the negatively charged agarose is constrained to move towards the positive electrode by electrophoresis. These factors may cause cracks in the column or may result in false migration rates. The magnitude of these two factors was measured using the techniques described below and it was found that they had no adverse effects on the result.

Once the method had been satisfactorily developed it was used to measure the electrophoretic characteristics of horsesickness virus at various levels of attenuation. It was possible to show that a change in this property of the virus took place during attenuation.

Materials and apparatus.

Virus. Suspensions of horsesickness virus No.3922

in egg-white buffer were partly purified by differential centrifugation as described in Chapter I.

Veronal-glycerol buffer (0.05 M). This buffer was based on that of Graber and Williams (1955). Barbitol sodium (5,5-diethyl barbiturate, 15.9 gm) was dissolved in approximately 500 ml distilled water. To this solution was added N-HCl (23 ml) and glycerol ($HOCH_2CHOHCH_2OH$: density 1.255 to 1.260 gm/ml, 100 ml) and the volume made up to 2 litres (pH 8.5).

Veronal buffer (0.05 M). A second buffer was prepared that was exactly the same as the one described above except that the glycerol was omitted. Tests showed that these buffers, with or without agarose, did not in any way harm mice when injected intracerebrally as in the titration of virus.

Agarose. The method of Russell et al. (1964) was used to prepare agarose from Ionagar No.2 (Oxo Ltd., London). One litre of a 4% (w/v) solution of agar in distilled water (80°) and 1 litre of polyethelene glycol (PEG) (40% w/v) in distilled water (80°) were mixed with stirring. Agarose is precipitated by the PEG as a fine flocculate. The mother liquor, which contains most of the charged agaropectin in solution, was removed by filtration through a fine nylon gauze. The precipitate of agarose was then washed with water to remove the PEG

and air dried. Three successive PEG precipitations resulted in an agarose with very little electroendosmosis as measured by disc electrophoresis (Russell et al., 1964).

Agarose suspension. Attempts to prepare a satisfactory agarose suspension as described by Hjertén (1963) were unsuccessful. It was found that the particles of agarose gel were far too large, and that convective mixing occurred in the interspaces. Such a suspension tended to settle under gravity causing an apparent high mobility of substances, and sometimes resulted in cracks forming. Also it was found, as described by Hjertén, that ultraviolet absorption measurements are unreliable due to light scattering by the gel particles. If an agarose suspension is to be satisfactory as an anticonvectant in electrophoresis the particles must be as small as possible, almost molecularly dispersed. A 0.2% (w/v) agarose suspension was prepared as follows. To 2 litres hot (80^{o}) veronal/glycerol buffer was added agarose (4 gm). This mixture was stirred and the temperature increased to boiling. Gentle boiling was continued until the agarose had completely dissolved. The hot solution was then filtered through Whatman No.1 filter paper to remove traces of particulate matter. The filtrate was allowed to stand at room temperature until it had gelled. This gel was broken by shaking and then forced through the fine

nozzle (5μ) of a disintegrator (A. Polson, personal communication) using nitrogen at a pressure of 68 atmospheres. The particles of the 0.2% agarose slurry so obtained were very fine. Gas bubbles trapped in the liquid were removed by subjecting the slurry to a low pressure. A second agarose suspension was prepared in the same manner except that veronal buffer without glycerol was used. Once prepared the agarose suspensions could be kept for at least one month at 5° without any deterioration. This preparation is effective in preventing convective mixing, does not harm the virus or impede its migration and could be used in ultraviolet absorption recording instruments.

Haemoglobin. This protein has proved to be a useful standard in electrophoresis; it is coloured, easily prepared and its electrophoretic mobility is known. It was prepared from citrated rabbit blood. Red cells were washed three times in normal saline and then lysed in distilled water. The solution so obtained was extracted three times with toluol to remove lipid material and stored at -5° until required.

Phenol red was used as a second reference substance. Sufficient of the dry powder was added to the virus suspension to impart a definite red colour.

Egg-white medium (Chapter I) was used as a virus

suspending medium.

Saturated sodium chloride. This solution was prepared by adding approximately 360 gm NaCl to a total of 1 litre veronal buffer.

Zone electrophoresis apparatus. A new type of apparatus based on that of Polson and Cramer (1958) was designed for the zone electrophoresis of horsesickness virus (Fig. 17). The design of this apparatus differs from that of other models in two important respects. Firstly, because an agarose suspension is used to prevent convection a concentration gradient is not used so that all the parts necessary for the formation of a gradient are omitted. Secondly the method of sampling has been changed.

Difficulties are often experienced when taking samples from electrophoresis columns because substances tend to linger in corners where the flow rate is low. This causes a tailing effect and some of the separated substances may be partly remixed. In the new apparatus the bottom of the electrophoresis column is drawn down to form a narrow-bore tube. There are no sharp corners to prevent the uniform flow of liquid. If the contents of the column are run out slowly at a uniform rate, using a peristaltic pump, satisfactory samples are obtained.

The apparatus consists of a vertical electrophoresis

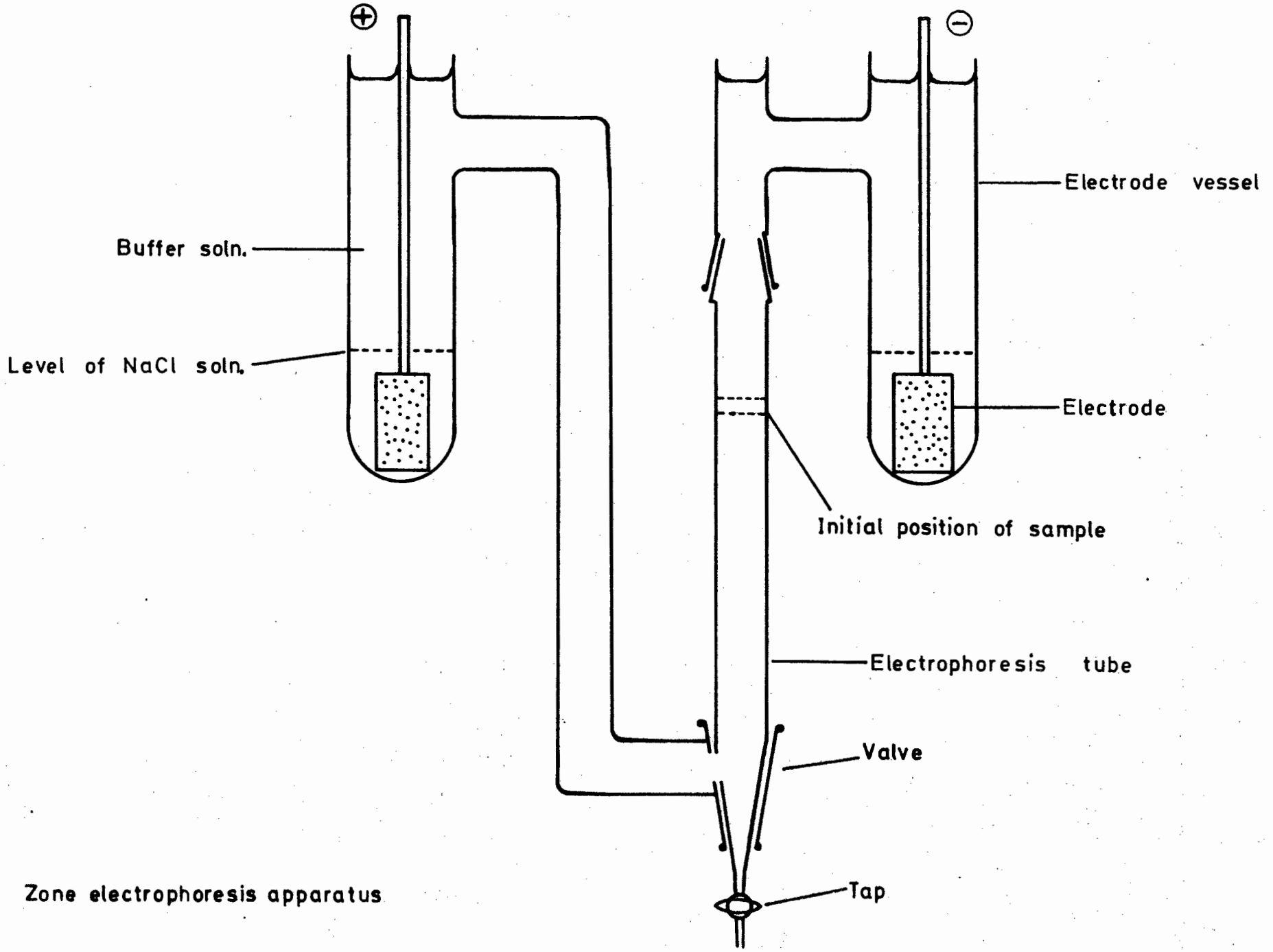


Fig.17 Zone electrophoresis apparatus

column with glass-ground joints at its lower and upper ends. A hole (1.0 cm diameter) was made in the wall of this tube where it fits into the bottom joint, to correspond with the bottom end of a second tube, the two tubes being in the form of a 'U'. By rotating the electrophoresis column in the joints the contents of the two tubes may be brought into contact or sealed from one another. At the upper end of each tube is an electrode vessel with silver/silver chloride reversible electrodes. The top of the electrophoresis column is open.

Pump. A peristaltic pump was used for sampling the column. The pump delivered liquid at a steady rate of 7 ml/hr.

Ultraviolet absorptiometer. To record the position of ultraviolet absorbing material in the electrophoresis column at the completion of an experiment the agarose suspension was passed through a recording absorptiometer (Uvcord, LKB, Sweden).

Measurement of electroendosmosis.

An apparatus was designed and built to measure quantitatively the electroendosmotic flow that results when an electric current is passed through agar gels.

One method has already been referred to, that of plate electrophoresis (Russell et al., 1964) but this is not sensitive enough when agarose gels of low charge are used and cannot be employed for agarose suspensions. The new method has the added advantage that endosmotic flow is measured under the same conditions obtaining in electrophoresis. The apparatus (Fig. 18) is based on well-known principles of charged particles in an electric field (Abramson et al., 1942), and was constructed as follows. A glass tube 7 cm long and 2 cm diameter with eight indentations 0.5 cm deep was fitted with a B 19 glass-ground joint at each end. (The indents were made to hold a gel firmly in position, a solution being allowed to solidify in the tube. In the present work the indents played no part as only experiments on agarose suspensions are described). On each end of the 7 cm tube is fitted a side arm with a right-angled bend. A B 24 joint is used on each side arm to secure tubes supplied with calibrated capillaries. A drilled rubber stopper, fitted into the upper end of each right-angled tube carries a 1 cm diameter glass cylinder 10 cm long. The space between this cylinder and the outer tube is the electrode vessel, the Ag/AgCl electrodes fitting into position as shown in the diagram and covered with a saturated NaCl solution. The inner cylinder is made long (10 cm) to

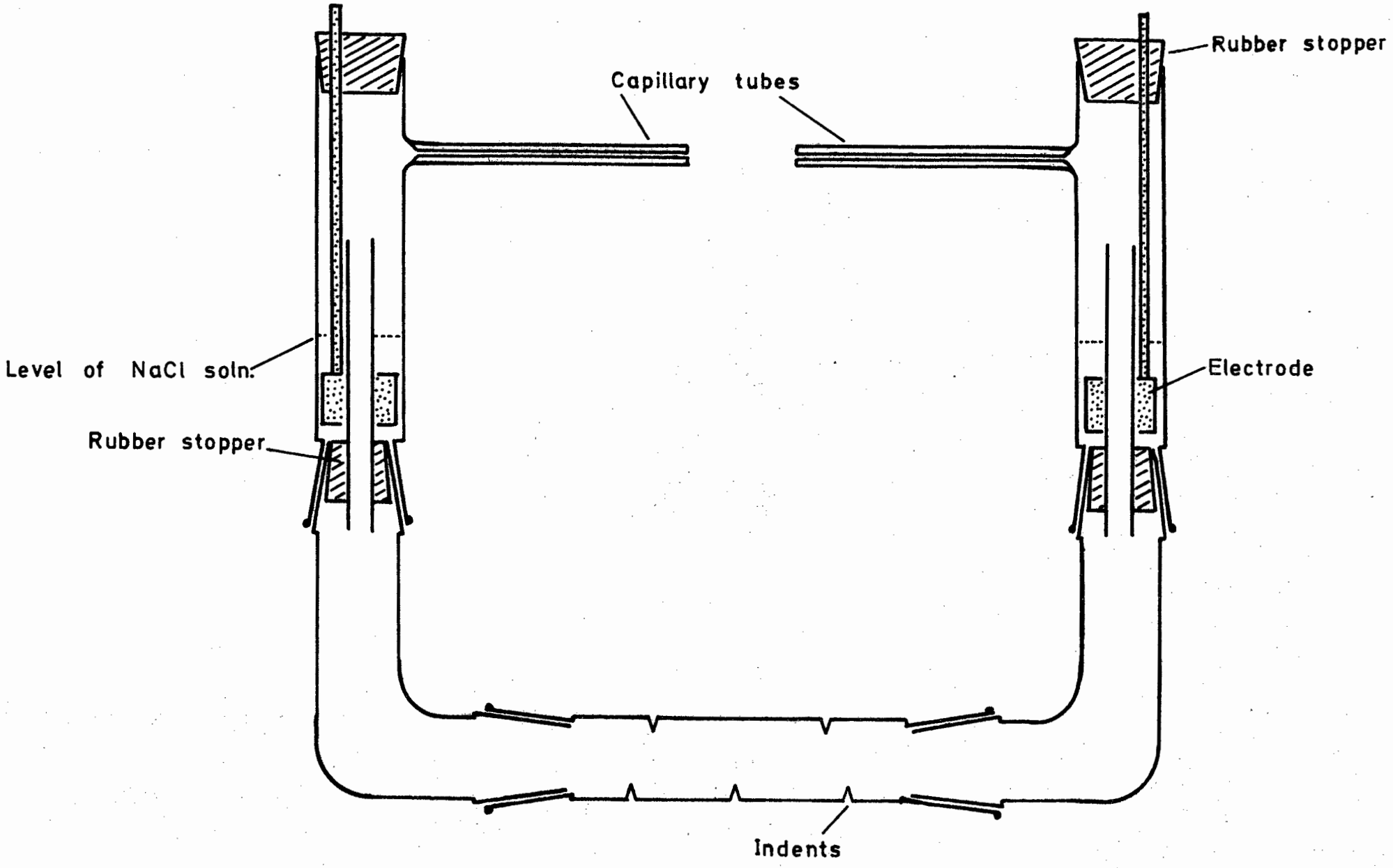


Fig. 18 Apparatus for determining electroendosmotic flow

ensure that NaCl will not diffuse out of the electrode vessel during an experiment. The apparatus, filled with agarose suspension, with all air bubbles removed and supplied with electrodes, was placed in a water bath (20^{o}) and connected to a source of direct current (10mA, 120 volts).

Results of measurements of electroendosmosis of agarose suspensions with this apparatus showed that after an initial increase in volume due to expansion of liquid caused by the heating effect of the current no further change occurred. With an agar gel in the apparatus electroendosmosis could be detected and measured by observing the rate of flow of liquid in the capillary at the negative electrode. This did not happen with agarose suspension in the apparatus and it is concluded that no electroendosmosis occurred.

Method.

The electrophoresis apparatus was assembled and sterilised with boiling water. With the valve open veronal buffer was introduced through the bottom tap so that both limbs were filled without trapping air bubbles on the side walls. In the same manner agarose suspension in veronal/glycerol buffer was added until it had reached

a height of approximately 5 cm in both limbs. The valve was closed by rotating the electrophoresis tube one quarter turn and addition of agarose suspension continued until a height of 15 cm in the electrophoresis column was reached. The tap was then closed. Agarose suspension in veronal buffer without glycerol but containing 5% egg-white was layered to a height of 5 cm on top of the suspension in the electrophoresis column using a Pasteur pipette. The pipette was introduced through the top opening of the column and a sharp interface was formed at the junction between the two suspensions. The electrode vessels were filled with veronal buffer, some egg white being added to the negative electrode vessel. The electrodes were placed in position and covered with saturated NaCl solution. The virus sample with phenol red and haemoglobin added was mixed (1+1) with agarose suspension and inserted in the column at the interface using a finely drawn Pasteur pipette. The levels of liquid in the electrode vessels were made equal, the valve opened and direct current passed for 15 hr at a voltage gradient of 3 volts/cm (15 mA) so as to cause a downward migration of the sample.

After electrophoresis the positions of the reference pigments were noted. The column was sampled by closing the valve, connecting the outlet tap to a peristaltic pump

with capillary tubing, passing the effluent through an ultraviolet absorptiometer and collecting samples (3 ml) equivalent to 1 cm length of the electrophoresis column. The samples were diluted and titrated for infectivity.

Results.

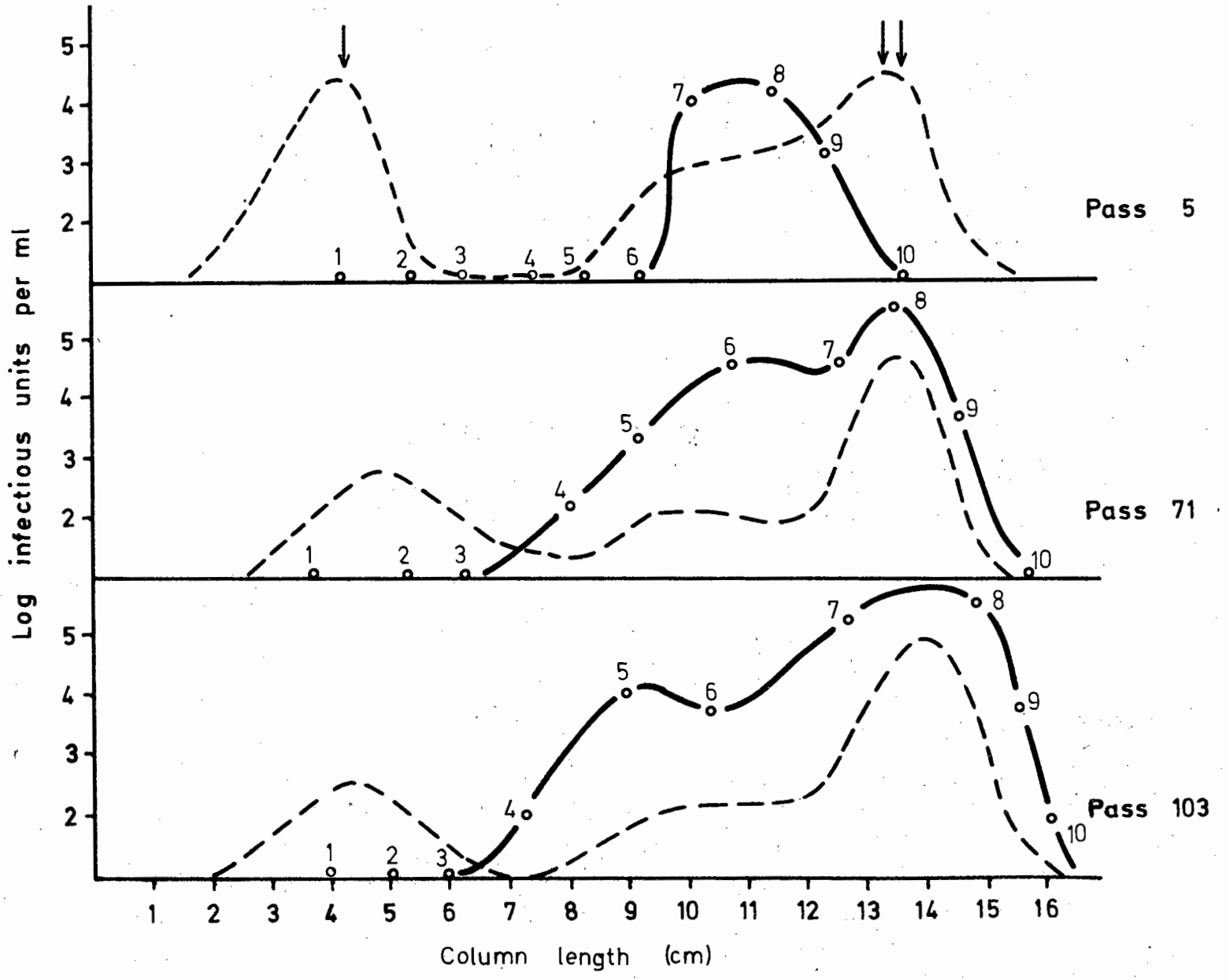
The results of some zone electrophoresis experiments on horsesickness virus No.3922 at different passage levels are summarised (Table 9) and plotted, together with the ultraviolet absorption trace, in Fig. 19. Because all experiments were carried out under similar conditions the amount of migration of the reference substances is almost identical in each case. However, the infectivity curves of the virus from different passage levels are different. Virus of passage 5 was found to be restricted over 3 cm of the column, the infectivity peak having moved 2.0 cm further than the haemoglobin. Virus of passage level 71 appears to consist of two components of different electrophoretic mobility which have been partly separated. The mobility of the main peak of infectivity coincides exactly with that of haemoglobin while the second fraction of infectious particles is electrophoretically inhomogeneous some migrating slightly further than haemoglobin and some nearly as far as phenol red. After 103 passages in mouse

Fig. 19. Infectivity curves (solid lines) of horsesickness virus No.3922 after zone electrophoresis. Ultraviolet absorption trace shown in dashed lines. Direction of migration from right to left. The position of the phenol red is indicated by a single arrow and that of haemoglobin by double arrows. Individual samples are numbered.

Table 9. Zone electrophoresis of horsesickness virus (No.3922) at three different passage levels.

Sample Number	Number of infectious units/ml		
	Passage 5	Passage 71	Passage 103
1	Nil	Nil	Nil
2	Nil	Nil	Nil
3	Nil	Nil	Nil
4	Nil	1.45×10^2	1.45×10^2
5	Nil	2.3×10^3	1.29×10^4
6	Nil	4.49×10^4	7.27×10^3
7	1.15×10^4	3.99×10^4	2.3×10^5
8	1.29×10^4	3.65×10^5	5.78×10^5
9	1.75×10^3	7.27×10^3	8.74×10^3
10	Nil	Nil	1.45×10^2

Fig. 19


Log infectious units per ml
5
4
3
2
1
2
3
4
5
6
7
8
9
10
Pass 5
Pass 71
Pass 103
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Column length (cm)

brains zone electrophoresis of the virus reveals still greater separation into two distinct electrophoretic entities. One fraction retains the same mobility as haemoglobin while the other migrates at a velocity half-way between that of haemoglobin and phenol red. There is little virus at the position where the unattenuated strain was at a maximum.

Density gradient analysis of slowly migrating electrophoretic component.

Considering the results of density and electrophoretic measurements of the attenuated virus it may be seen that both techniques resolved the infectious particles into two main fractions. In order to establish whether one of the electrophoretic components corresponds to one of the density fractions a sample of virus (passage No.103) was fractionated by electrophoresis. Fractionation on a density basis may be expected to result in a sharper differentiation than can be accomplished by electrophoresis, but this method was not chosen because it subjects the virus particles to severe conditions. The milder electrophoretic method was therefore used.

On completion of electrophoresis, carried out as described before, the contents of the column were sampled

and the slowly migrating virus fraction, corresponding to the position of the haemoglobin, was collected, concentrated by pervaporation and analysed by density-gradient centrifugation in a caesium chloride gradient.

The results (Table 10 and Fig. 20) show a peak of high infectivity corresponding to a density of 1.37 gm/ml and, compared to Fig. 16, little other material. It is therefore concluded that the slowly migrating electrophoretic fraction corresponds to the fraction of higher density.

Discussion.

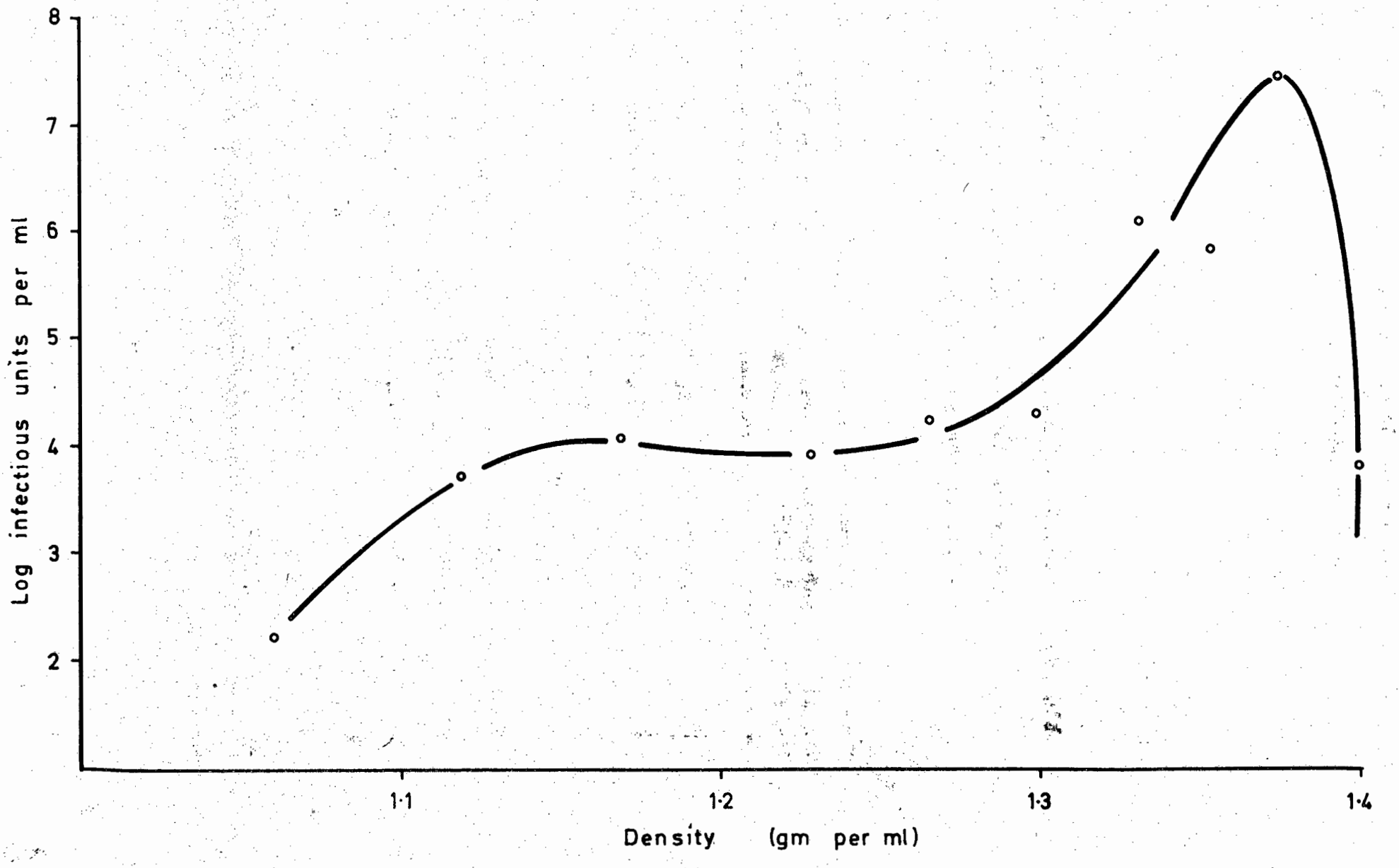
The method of conducting zone electrophoresis of viruses in a dilute agarose suspension using the apparatus described was found satisfactory for comparing the mobility of the different passage levels of horsesickness virus (No.3922). The agarose suspension forms a very powerful anticonvectant and the resolution of substance was good. Ultraviolet absorption methods could be used with the suspension when glycerol was added because of the increased refractive index of the solution, although detailed analysis was not possible. Glycerol also increased the density of the slurry and a density shelf could be formed which facilitated the application of the virus

Fig. 20 Density curve of the slowly migrating infectious particles of horsesickness virus (Passage No.103). Position of maximum density of 1.37 gm/ml.

Table 10. Density-gradient analysis of the slowly migrating infectious particles of horse-sickness virus (Passage No.103).

Sample Number	Refractive Index	Density (gm/ml)	Number of Infectious units/ml
1	1.3732	1.400	9.16×10^3
2	1.3705	1.375	2.9×10^7
3	1.3686	1.360	7.27×10^5
4	1.3622	1.330	1.45×10^6
5	1.3631	1.295	2.3×10^4
6	1.3601	1.265	2.3×10^4
7	1.3565	1.227	9.16×10^3
8	1.3510	1.168	2.3×10^4
9	1.3460	1.120	5.7×10^3
10	1.3412	1.065	1.45×10^2

Fig. 20


Log infectious units per ml
8
7
6
5
4
3
2
1·1
1·2
1·3
1·4
Density (gm per ml)

suspension to the column. A further advantage of using a suspension of agarose is that while the agarose shows no tendency to settle under gravity, it may easily be removed by centrifugation, even by a small laboratory centrifuge. The low centrifugal force needed to sediment the agarose makes it possible to obtain a clear virus suspension from zone electrophoresis samples for further investigations.

Critical measurements of the degree of retardation of particles moving through the agarose suspension were not made, but from experience of zone electrophoresis using a sucrose gradient, it is considered that neither phenol red not haemoglobin are slowed at all. The larger virus particles may have been retarded slightly although this is doubtful. Hjertén (1963) found that the diffusion coefficient of southern bean mosaic virus in agarose suspension agreed closely with that found in free diffusion.

The main criticism of the method is that some electrophoretic movement of the agarose suspension occurs. During the experiments described it is estimated that the agarose migrated approximately 0.5 cm. This movement, however, was uniform throughout the column and there was no change in the relative position of the migrating substances. Splitting of the column was observed during early experiments with consequent convection and mixing of

material in the agarose-free zone. This was prevented by reducing the length of the agarose column as much as possible and lowering the current to 15 mA. The difficulty did not occur in any of the experiments on horsesickness virus.

The problem of electroendosmosis does not arise when using a suspension of very finely divided agarose. In this connection an interesting phenomenon was noted. If an electric current is passed through a charged gel, for example agar, the solid phase is constrained to move in a certain direction by electrophoresis. This is impossible as the gel is a solid, so the liquid, oppositely charged with respect to the gel, moves by electroendosmosis in the opposite direction. If an electric current is passed through a liquid the charged particles move by electrophoresis and electroendosmosis cannot occur. It was noted that the agarose suspension used has some of the physical properties of both a solid and a liquid. The suspension acts as a slightly charged liquid and the particles move by electrophoresis but it does not possess the properties of a solid to a sufficient degree to cause a backflow of water and sustain the hydrostatic pressure so developed. Electroendosmosis could therefore not occur in this suspension. However, the tendency to create a backflow must be present. It is suggested therefore that

it may be possible to form an agarose suspension in a state intermediate between solid and liquid so that movement by electrophoresis and electroendosmosis neutralise each other. The agarose suspension used appears to have this property to some degree.

As regards the electrophoresis of horsesickness virus an interesting change was seen to have occurred during attenuation. The charge carried by unattenuated virus particles was sufficient to allow migration under conditions of the experiment for 40% of the distance travelled by phenol red. An apparent gradual change took place during attenuation until at passage 103 there are two main fractions of different electrophoretic mobilities. The faster moving component travelled 60% of the distance moved by phenol red, while the slower migrated only 20% relative to phenol red. The adsorption of charged particles on the virus, with a consequent change in the electrophoretic velocity of the infective particles, is always a factor that must be considered. Except for this possibility, the difference in electrophoretic mobility must be due to a change in the surface charge of the infectious particles.

Whatever changes take place to alter the electrophoretic mobility they must be associated with the surface of the virus. It is the surface protein however that

gives the virus its specific immunogenicity and causes the formation of antibodies which protect against invasion by the pathogenic virus strain. The sites on the attenuated virus which elicit the formation of antibodies must therefore be identical to those of the virulent strain. As the horsesickness strain investigated (passage 103) may be used as a vaccine, it must be supposed that any changes which occurred to alter the electric characteristics, involved only non-specific protein and did not affect the immunogenic sites on the surface of the virus. This matter is further discussed in the following chapter.

CHAPTER IX.

CONCLUSIONS.

From a survey of the literature it is apparent that most research on the attenuation of viruses has been directed towards finding methods of producing strains suitable for use in vaccines. A number of methods now exist for attaining this end. Each aims at creating a maximal differential between virulence and antigenecity, that is, emphasising the attributes of safety and potency.

In general these methods make use of either the cloning of mutant virus particles and testing them for changes in virulence and in other properties, or the arbitrary selection of a cell system in which to passage the virus strain repeatedly until observation shows that a fortuitous change has occurred in the viral population which permits its use as a vaccine.

In principle the purpose of vaccination is to induce a state of immunity to disease. Success may be gauged by measuring the level of circulating antibody and, in experimental animals, also by challenge inoculation. It is found that while a wild virus strain may cause a fatal disease in a susceptible animal, inoculation with attenuated virus produces little or no illness, but results in the formation of specific antibodies which provide protection

against challenge by the original virulent strain. This is one important measurable difference between virulent and attenuated strains; other differences such as temperature dependence and characteristic development in tissue culture have been described.

A relation between degree of attenuation and the temperature of cultivation of some viruses has been established. Dubes and Wenner (1957) showed that cold-adapted (37°) poliovirus loses virulence and cannot multiply at the body temperature of the monkey (39°). Lwoff and Lwoff (1959) have found that the attenuated strains of poliovirus are less resistant to heat than the wild forms. The extent of inhibition of poliovirus at elevated temperatures is related to the degree of attenuation (Sabin and Lwoff, 1959) and it is possible to select mutants of an attenuated strain by cultivation at increasingly higher temperatures. The strain was fully virulent in monkeys but repeated passage at 37° yielded virus that was no longer paralytogenic. Conversion of poliovirus to a virulent strain and then back to an attenuated strain is therefore possible.

Gerber and Kirschstein (1960) reported that infective RNA of poliovirus carries the genetic information which determines the degree of neurovirulence of the virus.

Only RNA extracted from virulent strains showed undiminished reproductive capacity at 40°. Kilham (1959) showed that myxoma virus (virulent) is more resistant to high temperatures in tissue culture than the less virulent fibroma virus, and he pointed out that this corresponds to the natural history of these two virus infections since fibroma virus causes local tumours in the cooler parts of the body, such as the skin and testes, while myxoma invades the body of the host and causes widespread infection and death.

The influence of temperature at which the virus is propagated may have been important in the attenuation of horsesickness virus for equines by repeated passage in suckling mouse brains (Alexander, 1935). The average body temperature of the mouse is 36.5° but when reacting to the effects of infection by horsesickness virus the temperature falls to 35°. Most of the virus is therefore produced at this lower temperature which may be contrasted with the average body temperature of the healthy horse which is 37.7° but which rises to 41° (Henning, 1956) when infected with horsesickness virus.

A number of experiments have been described relating plaque characteristics to virus virulence. Thus Vogt et al. (1957) proved that some variants of poliovirus (delayed mutants) form fewer plaques under 'acid' agar overlay than

under a 'basic' overlay. These mutants were less neuropathogenic for the monkey than the original strain. Hardy and Hearn (1961) reported a method of differentiating, on the basis of plaque morphology, between a virulent strain of Venezuelan equine encephalomyelitis virus (VE 1) and a strain attenuated for the mouse (ClLV 8). The size of plaques formed by these strains was VE 1, 3 to 4.5 mm diameter in 48 hr and for ClLV 8 was 0.5 to 1 mm diameter in 78 hr, both in chick fibroblast cells.

A similar relation between plaque size and infectivity occurs with foot-and-mouth-disease virus (Cottral et al., 1966). Both small and large plaques are formed by this virus and infective particles obtained from the larger plaques were significantly more infective for cattle than the small plaque virus.

Tolskaya et al. (1966) have investigated the relation between two wild strains and two attenuated strains of poliovirus and their ability to multiply in cells cultured in hypotonic media. It was demonstrated that the wild type virus could not multiply in hypotonic solutions (30% below isotonicity), although some infective RNA was produced. The attenuated strain was able to multiply under these conditions but at a reduced rate.

Mutants with physical characters different from those of the parent strain have been observed. Goodheart (1965)

noted a variant of encephalomyocarditis virus with particles of density 1.37 gm/ml, the wild type having a modal density of 1.334 gm/ml. Similarly Roizman and Roane (1961) describe particles of herpes simplex virus with different densities namely 1.271 and 1.260 gm/ml. The density mutant strains described in these papers did not however refer to a strain of virus suitable for use as a vaccine.

Indirect measurement of lowered pathogenicity of virus strains usually relies on biological methods. Surprisingly little research has been undertaken in regard to the detailed chemical and physical changes accompanying the attenuation process. Mayr et al., (1961) studying pig paralysis virus found the attenuated strain is less resistant to both heat and formalin than the virulent strain. Also, the sedimentation coefficients of this virus are 156 S (wild type) and 149 S (attenuated type). The authors considered the decrease of resistance to heat and formaldehyde to be due to a reduction of the protective action of the protein coat. The proteins of the attenuated strain are thought to have undergone structural changes because they were formed in a cell which was not the usual host of the virus and therefore not competent to form the precisely correct proteins.

A striking physical change discovered for horse-sickness virus (No. 3922) which seems to run parallel with the degree of attenuation, is density. The particles of the wild strain (passage 5) with an average buoyant density of 1.265 gm/ml appear to differentiate as attenuation proceeds into two fractions of density 1.21 gm/ml and 1.34 gm/ml. The reason for this change of density is not clear. There is little doubt that some cellular material, probably lipoprotein, is incorporated into the virus during the maturation process as in the case of myxoviruses (Kates et al., 1961). Inclusion of such material into or onto the surface of the virus may be partly adventitious but the apparent gradual definite density changes observed indicate some genetic influence. Experiments to resolve this problem are at present being undertaken. These experiments are designed to remove possible external contaminating material from the surface of the virus by ultrasonic irradiation. Density-gradient analysis of ultrasonically treated horsesickness virus (strain A 501) indicated a change in the buoyant density of all the infective particles to a final value of 1.233 gm/ml. It has been suggested (Russell, in press) that the density value so obtained may be thought of as an 'intrinsic' density, that is, a density that reflects a fundamental property of the virus particle. Possible changes of the

intrinsic density with attenuation of the No. 3922 horse-sickness virus are still being investigated.

An interesting possibility of studying the process of attenuation is suggested by the results of density measurements obtained in the present investigation. The attenuated strain is composed of a mixture of particles of different densities while the density of particles of the wild strain show a fairly wide scatter about a single peak. Infective particles of unattenuated virus at the lowest and highest density range of this scatter have the same densities as the principle components of attenuated virus. It is suggested that repeated separation of the wild strain into fractions of highest and lowest density and passage of this material may result in a more expeditious formation and selection of a vaccine strain.

It is generally accepted that attenuated virus results in one of two ways. Either there is a selection of suitable particles, initially present in the population, by a particular environment; or a gradual change takes place in all the virus particles initially present, due to some mutagenic property of the system, as they are passaged from one generation to another. Some information regarding these possibilities may be found if density is a true measure of attenuation. If attenuation is speeded by selecting a particular density fraction as suggested above

it would mean that some attenuated virus was present initially. On the other hand if density fractionation did not result in the rapid formation of a vaccine strain it would have to be concluded that a gradual change in all the virus particles was occurring. It may thus be possible to decide between the selection and mutation theories for this virus.

Density gradient analysis of the No.3922 strain indicates a change of buoyant density of the infectious particles with attenuation. Information as to when the attenuated state of this strain was attained is not available but from a study of the curves in Fig.16 and Fig 19, it may be assumed that a change occurred after approximately fifty passages in mouse brains. This may be compared with the blind passage of infectious bovine rhinotracheitis virus in tissue culture by Schwarz et al. (1957) who found that this virus was attenuated after 40 passages, and the production of a vaccine strain of horsesickness virus (strain S 28) by Mirchamsy and Taslimi (1964) after 65 intracerebral passages in mouse brains. The number of passages necessary to bring about attenuation will however differ depending on the virus strain used and the method applied. For example by treating poliovirus at 50° with aluminium chloride, Wallis and Melnick (1963) were able to attenuate the virus

after only 3 passages in tissue culture.

The density determination of the slowly migrating electrophoretic component with the result of 1.37 gm/ml substantiates the higher density found for the unfractionated virus at passage 101 (1.34 gm/ml) and also correlates these two fractions. It may be assumed that the faster migrating electrophoretic component is composed of particles of lower density.

Consequent to the present findings relating the density of horsesickness virus particles with degree of attenuation, Wesselsbron virus (Weis et al., 1956) has been similarly analysed (M. Smith, personal communication). Although these viruses are unrelated it was found that the density of the infectious particles of Wesselsbron virus changed with the degree of attenuation in much the same way as does horsesickness virus. By density-gradient analysis the pathogenic strain (tissue culture passage 10) was shown to have a single broad infectivity peak (1.22 gm/ml) while the attenuated strain (tissue culture passage 110) had two infectivity peaks (1.16 and 1.25 gm/ml). These results show that the phenomenon of change of buoyant density with attenuation is not confined to a single virus type and may prove to be a feature of many viruses for which a vaccine strain has been prepared.

Analysis of horsesickness virus No. 3922 by zone electrophoresis revealed another important biophysical difference between the wild strain and that attenuated for the horse. The change in the electrophoretic patterns of virus from different passage levels indicates that a fundamental change occurred at the surface of the virus particles during attenuation. These results are anomalous in this respect. In electrophoresis it is mainly the electrostatic character at the surface of a particle that determines the degree of mobility; size and shape play only a minor role (Stern, 1956). The change in electrophoretic mobility observed therefore reflects a considerable alteration in the charge density of the surface proteins of attenuated virus. Yet the attenuated virus elicits the formation of antibodies effective against the wild strain.

If the change of electrophoretic mobility of the virus represented a considerable change in the chemical nature of the surface proteins one might expect a change in the antigenic nature of the wild virus, but the antibodies produced to the attenuated virus would then not react with the virulent strain. A possible explanation is that although many of the proteins on the outside surface of the attenuated virus particles had in fact changed, antigenic determinants on the inner surface of the capsid may remain identical with those om the surface

of the virulent particles. The formation of antibodies by the attenuated strain, effective against the virulent strain, would thus depend on the introduction into the reticuloendothelial system of subunits of vaccine strain virus. These may arise from the large amount of partly assembled viral components or soluble antigen liberated on the disintegration of infected cells.

For such a mechanism to succeed it is essential that comparatively large sections of viral material should act as antigen. Individual molecules would probably not ilicit antibodies homologous with the complete wild virion because it is not only the amino acid composition of a protein that determined specificity but also the conformation of the molecule (Crumpton, 1966) and the shape may be expected to change when the molecule is assembled to form the capsid. Larger viral fragments may however be antigenically related to the complete virion and so elicit the formation of antibody to the virus. (van Regenmortel, 1966; Levitt and Polson, 1964).

The particles of Wesselsbron virus have also been found to show different electrophoretic mobilities dependant on degree of attenuation. (M. Smith, personal communication). Unlike the No. 3922 horsesickness virus strain studied, the unattenuated Wesselsbron virus (tissue culture passage 10) is electrophoretically inhomogeneous (Parker, 1966) but

exhibits an infectivity peak corresponding to a mobility just less than that of phenol red. After 110 passages in tissue culture however the main infectivity peak lies within the haemoglobin band. This indicates a considerable reduction of mobility with degree of attenuation and in this respect is similar to the changes occurring in the attenuation of horsesickness virus.

The agarose suspension as prepared in the present work for zone electrophoresis has some interesting properties. As an anticonvectant it is very efficient. Convective mixing does not readily occur even when the bottom of a 30 cm column of the agarose suspension is heated to 80° while the temperature at the top is 20°. The slurry appears to have some of the physical properties of both solids and liquids and in this sense is thixotropic (Glasstone, 1953).

The suspension may be regarded as a liquid in that it flows freely but when swirled in a circular container the liquid comes to rest initially and the oscillates 4 or 5 times, behaving as an elastic solid. The electrical properties of the agarose suspension are also interesting. It was suggested that the electrophoretic effects of the suspension, behaving as a liquid, and the electroendosmotic effects, behaving as a solid, could be made to neutralise each other if the correct physical conditions were found.

It appears that some such mechanism actually occurred in the experiments described. Subsequent measurements of the electrophoretic mobility of a sample of the agarose slurry in a sucrose gradient using the same experimental conditions as described showed that the sample migrated 4 cm. In the electrophoresis of horsesickness virus no more than 0.5 cm migration of the agarose slurry occurred. It is possible that by preparing a slurry of slightly larger granules and thereby increasing the solid-like properties a suspension could be formed that showed no movement in an electric field.

From the results of centrifugation and density measurements it may be concluded that the No. 3922 strain of African horsesickness virus has an average sedimentation coefficient of 534 S and a particle diameter of approximately 64mμ, the latter figure being an average of the calculated sizes. This size may be compared with the average figure determined by Polson and Madsen (1954) for the larger particle of a number of horsesickness virus strains namely 50.8mμ.

A slight variation of particle size with the degree of attenuation was observed for the No. 3922 strain of horsesickness virus. Averaging the available results it was found that the wild, pathogenic strain has an approxi-

mate diameter of 47mμ. At the 71st passage level the size is 54mμ and at passage 103 it is 65mμ. Mayr et al., (1961) found a decrease in the sedimentation coefficient of pig paralysis virus with attenuation and predicted that the attenuated strain would be slightly smaller than the pathogenic strain, but no figures were presented. The particle size of the horsesickness virus as determined by ultrafiltration was less than that obtained from sedimentation results. This may be due to inaccuracies in the membranes or the use of a non-ideal correction factor. Another possibility is that material, adventitious or viral, was removed from the virion during passage through the filter. The results do however confirm an increase in the diameter of the infectious particles with attenuation. They also support the view that the particles are approximately spherical.

The ideal of propagating horsesickness virus (No.3922) at different stages of attenuation in tissue culture and comparing growth characteristics and physical properties has not yet been achieved. The work of others in this field indicates that differences may be expected, partly because of changes accompanying attenuation and partly because of the different cell system used to grow the virus. Drake and Lay (1962) found that some virus characteristics,

such as sensitivity to heat, ultraviolet light and pH, may change depending on the type of cell in which the virus is grown. They find for Newcastle disease virus that the phenotype is determined not only by the viral genome but also by the cellular host. The change is not due to selection of pre-existing mutants but to alterations in the somatic portions of the virus not specified completely by the viral RNA.

Following on this work Stenback and Durand (1963) found a change of density of Newcastle disease virus occurred when the virus was grown in chick or hamster cells. Myxoviruses are particularly prone to this type of change as they aquire structural material directly from the host cell (Kates et al., 1961). It is not unlikely that horsesickness virus may be similar in this respect. Density changes would then depend on two factors. Firstly, changes due to attenuation and secondly, changes due to propagation in different host cells.

Another type of change that may occur in tissue culture is the loss of haemagglutinating property of viruses. Haemagglutination of some horsesickness strains has been described by Pavri (1961). Growth in tissue culture may result in the loss of this property as in the case of some ECHO viruses (Maisel et al., 1961), and also Wesselsbron virus (Parker, 1966).

Tissue culture methods have been used to measure another difference between pathogenic and attenuated viruses; that of interferon production. Isaacs et al., (1963) found that while RNA does not induce interferon production in homologus cells viral RNA often does. Sellers (1963) showed for foot-and-mouth disease virus that attenuated strains produce more interferon and are more sensitive to interferon than are virulent strains. This may be one of the reasons why attenuated virus strains do not cause severe illness (Ruiz-Gomez and Isaacs, 1963).

The tissue culture system used in the present work contained egg white and Triton X. While these substances were used because of their individual properties, that is the enzyme action of egg white and the protective action of Triton X, it may be important that they were present together. Egg-white contains ovalbumin, conalbumin, and ovomucoid (Fevold, 1951), and some of these proteins may have been advantageous to the cell system. Lysozyme, molecular weight 17,200 (Martin and Ames, 1961), which is thought to have played an important role in establishing the propagation of horsesickness virus in tissue culture, may well have been effectively excluded from the system by the masking action of phospholipids and proteins (Romeo and de Bernard, 1966) had it not been for the presence of Triton X. If this were the case it was a fortunate

coincidence that both these substances were included in the medium.

The size of horsesickness virus as found by electron microscopy was approximately 40mμ. This is much less than that found by dynamic measurements, namely 62mμ (passage 5). The reason for this discrepancy may depend on many factors. These include an alteration in the size of the particles when subjected to the vacuum in the electron microscope, adsorption of matter onto the virus particles leading to false ultracentrifugation and density results and, perhaps most important, a possible change in size due to adaptation to growth in tissue culture; for example the loss of haemagglutinin from the surface of the virus.

The filamentous structures observed in some infected cells are of some interest. In form they are similar to the lipoprotein (Fawcett, 1966) of the myelin sheath of nerves. This sheath is laid down in vertebrate nervous tissue by Schwann cells (Geren, 1954). It is thought that myelinated nerves do not occur in invertebrates. Hirumi et al. (1967) however show electron micrographs of nerves of an insect which are enclosed in what appears to be a myelin-like structure and described as "a spiral sheath". They show filamentous structures in virus infected cells similar in appearance to the strands found in the present

work and this lends some support to the suggestion that the filamentous structures are derived from the myelin lamellae.

It must be assumed that the physical differences between attenuated and wild virus are the result of a genetic change. It has been shown by Sabin and Lwoff (1959) that the reproductive capacity of poliovirus at high temperatures is influenced by genetic factors and that these factors are closely correlated with neurovirulence. Confirming this report, Gerber and Kirschsten (1960) showed conclusively that it was the infective RNA of poliovirus that carries the genetic information which determines the degree of neurovirulence. If it be accepted that a genetic change is essential in the step from a virulent to an attenuated strain it is not surpsising that various physical differences in form and reactions of the virus should accompany the change. It must now be determined exactly what these chemical and physical changes are, and how they are correlated with virulence and attenuation.

Until quite recently only empirical procedures could be used in attempts to attenuate viruses; the vaccine strains had to be obtained by trial and error. Detailed knowledge of the processes involved is however accumulating.

It is now possible to suggest the possibility of deliberate phenotypic mixing of viruses in order to obtain a non-virulent derivative of a pathogen (Burnet, 1959) without modifying its antigenic character. This and other ideas may one day become practicable and physical measurements of viruses may play a part in determining the degree of attenuation achieved.

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ABBREVIATIONS.

APD	Average pore diameter.
BHK	Baby hamster kidney cells.
cm	Centimetre.
o	Degrees centigrade.
gm	Gram
hr	Hour
HSV	African horsesickness virus No.3922, Type 7.
LF	Mouse liver fibroblast cells.
M	Molar solution (Contains 1 gm molecular weight per litre of solution).
mA	Milliampers.
min	Minutes.
ml	Milliletre.
mm	Millimetre.
mμ	Millimicron = 10^{-7} cm.
μ	Micron = 10^{-4} cm.
N	Normal solution (Contains 1 gm equivalent weight per litre of solution).
oz	Ounce.
PEG	Polyethylene glycol of molecular weight 6,000.
rev/min	Revolutions per minute.
RNA	Ribonucleic acid.
sec	Seconds.
sq	Square.

Symbols

c Concentration (Number of particles per unit volume).

D Diameter (cm).

f Frictional coefficient.

F Force (dynes).

g Gravitational acceleration (cm sec^{-2}).

l Length (cm).

m Mass (gm).

n An unknown number.

N Rev/min.

p Pressure (dynes).

r Radius (cm).

s Sedimentation coefficient (sec).

s_{20} Sedimentation coefficient under standard conditions (water at 20°).

S Sedimentation coefficient (svedbergs).

t Time (sec).

u Velocity (cm/sec).

V Volume (ml).

$\bar{V}$ Partial specific volume (ml/gm).

w Weight (gm).

x Distance from centre of rotation. (cm)

η Viscosity (poise).

ρ Density (gm/ml).

ω Angular velocity (radians/sec).

v/v	Volume by volume.
w/v	Weight by volume.
w/w	Weight by weight.

APPENDIX.

The author has been associated with the research work and the preparation of manuscripts for the following publications:-

Russell, B., Head, T.H., and Polson, A. (1966). A method of preparing agarose. Biochim. biophys. Acta 86, 169.

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