STUDIES ON THE STRUCTURE AND REPLICATION OF CANINE DISTEMPER VIRUS.

by

AILEEN M.T. ARMITAGE,
B.V.M.S., M.R.C.V.S.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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Summary.

The principal aim of the investigation was to gain detailed information on the structure and replication of canine distemper virus.

The first part of the thesis was intended to provide information on the growth of the virus in tissue culture so that a method could be established for the production of the large quantities of virus required for structural and biochemical studies. The growth of the virus in secondary dog kidney cultures, primary alveolar macrophage cultures, a canine embryonic cell strain, and the GH continuous dog kidney cell-line was compared. Secondary dog kidney cultures were the most susceptible to the virus but the highest yields were obtained from the GH cells. By comparing different methods of infecting and maintaining the cells, it was found that large quantities of virus could best be obtained by infecting GH cells in monolayer and collecting the medium two or three times per day, once a clearly recognisable cytopathic effect was present. At this stage, virus accumulated rapidly in the medium, maximum titres being reached within a few hours of a change of medium; its short half-life at 37°C – 47 minutes – precluded any further increase in its titre.

The second part of the thesis describes an ultrastructural study of CDV replication, an attempt being made to infect cells under, or nearly under, one-step conditions. The earliest evidence of infection was the appearance of foci of cytoplasmic nucleocapsid 12 hours after infection. Virus maturation occurred by a budding process which commenced at around 18 hours, and continued at maximum rate over the 24-72 hour period. A prominent feature of maturation was alignment of nucleocapsid beneath the plasma membrane, a fine fringe of surface projections being present on the outer surface of the membranes opposite the nucleocapsid. Inclusion bodies occurred between 24 and 48 hours post inoculation, and consisted of foci of nucleocapsid with a heavy deposition of fine granular material.

Part Three was concerned with the development of a suitable method for the extraction and identification of cytoplasmic nucleocapsid. Nucleocapsid extracted with sodium deoxycholate and banded in density gradients of caesium chloride was found, by electron microscopy, to occur in relatively short, rigid pieces, whereas that extracted with the non-ionic detergents Sterox SL, Nonidet P-40, and Triton X-100, and centrifuged through caesium chloride, more closely resembled the nucleocapsid extracted from cells by osmotic shock and examined without further treatment. Based on electron microscopy, Sterox SL appeared to be the most satisfactory of the detergents tested for extracting the nucleocapsid, especially when used in

conjunction with bovine serum albumin which seemed to stabilise the nucleocapsid. RNase treatment of infected cells labelled with tritiated uridine destroyed most of the cell RNA and permitted detection of nucleocapsid by radioassay. By this means, the buoyant density of the nucleocapsid in caesium chloride was found to be 1.28-1.30 g/cc.

The aim of Part Four was to obtain a method of purifying the virus by equilibrium density gradient centrifugation. The distribution of infectivity, radioactivity and virus particles in density gradients of potassium tartrate, sucrose, and caesium chloride was compared. The sharpest banding was found in potassium tartrate. The virus had a buoyant density of 1.20 g/cc in potassium tartrate, and in sucrose, and 1.23 g/cc in caesium chloride.

Highly concentrated and purified virus was then used for the studies of the viral polypeptides (Part Five), and the sedimentation coefficient of the viral nucleocapsid and RNA (Part Six). At least six polypeptides were present in the virus particle, but four of them were derived from larger protein molecules. Two polypeptides were found to be associated with the nucleocapsid. Rate zonal sedimentation of the nucleocapsid and viral RNA labelled with tritiated uridine, indicated that it occurred in subgenomic lengths. This finding was unexpected since

electron microscopy of nucleocapsid extracted by osmotic shock and examined without further treatment indicated that it existed predominately in unit lengths of approximately 1.1 μ which is regarded as the length containing the full genome of paramyxoviruses.

INTRODUCTION.

Since first described by Jenner in 1809, canine distemper has been recognised as one of the most important diseases of the dog. It is a highly contagious acute or subacute febrile condition characterised by nasal and ocular discharge, gastrointestinal and respiratory signs and leukopaenia. Hyperkeratosis of the foot pads is seen in some dogs. A proportion of affected animals develop nervous manifestations and these may occur either during the acute phase of the disease or several weeks or even months later. Due to the intractable character of the encephalitis, many affected dogs have to be destroyed and euthanasia accounts for a high proportion of the mortality. Other species in the Order Carnivora are also susceptible to canine distemper and the mortality rate varies greatly between species.

In 1905, Carré reported that canine distemper was caused by a virus, but it was not until 1926, when Dunkin and Laidlaw carried out their classical studies, that this was confirmed. Between 1926 and 1948, little further research was carried out but since then, many notable contributions to our knowledge of canine distemper have emerged. These have recently been reviewed in detail by

Appel and Gillespie (1972). However, relatively little work has been done on the replication of the virus and on its macromolecular structure.

Knowledge of the mechanisms involved in canine distemper virus (CDV) replication should benefit research on one of the most important aspects of distemper, namely that of persistent virus infection of the central nervous system. In man, it has been shown that measles virus can persist in the brain in a non-infectious form for a considerable number of years (Hamilton et al., 1973) but this defective infection eventually results in a fatal neurological disease known as subacute sclerosing panencephalitis (SSPE). Since CDV is closely related to measles virus, antigenically as well as structurally (Warren, 1960; Waterson et al., 1963; Breese and de Boer, 1973), it might be expected that it too would be capable of persisting in the brain in a defective form. Evidence that this does indeed occur has been provided by Lincoln et al. (1971;1973) in studies of "old-dog-encephalitis". Virtually nothing is known about the mechanisms by which the production of complete virus is prevented in these animals. Before such mechanisms can be elucidated, a fuller knowledge of normal productive CDV infection is clearly required. This can be obtained most readily from the study of viral replication in cell-culture.

Also, before the study of viral replication can be extended to the molecular level, it is obvious that the macromolecular structure of the virus must first be

established. For example, if the proteins could be extracted from purified virus and their individual structural roles identified, then it might be possible to determine their sequence and duration of synthesis within infected cells. Thus, if such data were established for productive infection, it should be possible to identify any abnormality in virus protein synthesis occurring in defective infection.

The aims of the investigation under report were concerned with providing further information on both of those subjects. The work involved, firstly, an ultrastructural examination of CDV replication in cells infected under one-step conditions and, secondly, the characterisation of the viral proteins and RNA. The latter study, of necessity, called for the development of methods for (1) the growth of the large amounts of virus required for biochemical study, (2) the purification of the virus and (3) the extraction of nucleocapsid from infected cells and intact virus particles.

A major part of this thesis is therefore concerned with the solution of these problems, and it is hoped that the information thus accrued will provide the necessary technical background for future investigations of CDV structure and replication.

The thesis is divided into six parts, viz.

1) Development of a cell-culture system for the growth of CDV.
2) Ultrastructural studies of CDV infection in GH cells.
3) Extraction and isolation of nucleocapsid from infected cells.
4) Purification of the virus.
5) Study of the polypeptides of the virus.
6) Study of the size of the nucleocapsid and RNA of the virus.

PART ONE.

INTRODUCTION AND REVIEW OF THE LITERATURE.

In the study of virus replication, it is obviously desirable that the cell-culture system employed should be highly susceptible to, and capable of supporting the growth of, the virus to a high titre. But if this is to throw any light on the mechanisms of infection *in vivo*, it is important that the system used should be as similar as possible to that occurring *in vivo* i.e., the cell-cultures should be derived from the natural host and the virus strain employed one which has not been adapted to laboratory animals, embryonated eggs or cell-cultures derived from species which are not naturally susceptible. In the case of CDV, there is no cell-virus system which fulfills both of these criteria.

The Onderstepoort and Lederle strains of CDV, both of which are highly egg-adapted, will grow in cultures of chich embryo fibroblasts. Irrespective of the composition of the medium employed, the former strain produces a cytopathogenic effect (CPE) characterised by rounding up and fragmentation of the cells but the Lederle strain will only produce this effect if the cells are maintained in

equal volumes of bovine amniotic fluid and Earle's saline containing 2% calf serum (Karzon and Bussell, 1959). Maximum titres of virus range from 10^6PFU per ml with the Onderstepoort strain (Bussell and Karzon, 1962) to $10^{4.5}EID_{50}$ per ml with the Lederle strain (Cabasso et al., 1959). Both can be adapted to the Vero line of African green monkey kidney cells (Shishido et al., 1967), virus titres reaching around 10^5TCID_{50} per ml, but neither can be serially propagated in dog cell-cultures (Bussell and Karzon, 1965a).

A remarkable variant of the Onderstepoort strain was described by Bussell and Karzon (1965b). This arose during the first passage in AV-3 cells (continuous human amnion) of virus which had been passaged 64 times in chick embryo fibroblasts and 13 times in ferret kidney cultures. Titres of 6.0 - 6.2$\log_{10}TCID_{50}$ per ml were obtained in AV-3 cell-cultures and the virus proved highly cytopathogenic for both primary rhesus monkey kidney cells and continuous green monkey kidney cells. It also was cytopathic for the Fieldsteel line of continuous dog kidney, which was shown to be refractory to all other Onderstepoort variants tested. Subsequent work (Rankin et al., 1972) showed that titres of up to $10^{7.5}TCID_{50}$ per ml were obtainable in AV-3 cells infected with this variant. Hep-2 cells were also found to be highly susceptible to it (Phillips and Bussell, 1973) and provided higher yields of virus than did Vero cells (Appel and Robson, 1973).

It would seem that the Bussell variant of the Onderstepoort strain represents a rare mutant. Thus the parent virus passaged 64 times in chick embryo fibroblasts could not be continuously cultivated in AV-3 cells and, after 13 passages in ferret kidney cultures, failed to grow in the Fieldsteel line of continuous dog kidney cells and grew to a titre of only $10^{3.5}TCID_{50}$ per ml in the WS line of continuous human amnion (Bussell and Karzon, 1965b). Its importance lies in its suitability for neutralisation tests (Appel and Robson, 1973) and certain biochemical studies (Waters and Bussell, 1974) though, for the reasons already given, its passage history makes it less than ideal for studies on the natural cell-virus interaction.

Simian cells have been used for the growth of two other strains of CDV. Harrison et al. (1968) first adapted the CSL (Commonwealth Serum Laboratories) strain to ferret kidney cultures and from there passaged it 37 times in the diploid cell-strain BS-C-1 derived from African green monkey kidney cultures. This was done as a means of attenuating the virus for vaccine production. Yields of 10^{5}-$10^{6}TCID_{50}$ per ml were obtained but not before the ninth day post-inoculation, when large syncytia occupied the whole of the cell-sheet. Lavender and Bewsey (1973) adapted an attenuated variant of the Snyder Hill strain to the LLC-MK_2 line of continuous rhesus monkey kidney, again for vaccine production. Titres mostly

ranged from $10^{3.7}$ to $10^{4.7}$ EID_{50} per ml and since a CPE was never seen in this system, it was possible to make multiple harvests of infected culture medium. Other than this, neither of these systems seems to have any special advantage over that of dog cell-cultures infected with a strain of virus adapted directly to them.

Isolation of virulent CDV in dog kidney (DK) cultures was first reported by Rockborn (1958). Forty-two days after the inoculation of primary monolayers with serum from an infected dog, he observed generalised degeneration and vacuolation of cells. During subsequent passage, the time taken for the production of a CPE dropped rapidly until, by the eighth passage, only two days were required. By the fourth day, numerous syncytia were scattered throughout the cell-sheet. Bittle et al. (1961) isolated virus by inoculating DK cultures with a suspension prepared from the spleen of an infected dog. The time required for this strain to produce a CPE was gradually reduced from 18 days in the initial passage to nine days by the 21st passage, after which no further reduction in time occurred. In un-stained preparations, the CPE was characterised by vacuolation which resulted in the development of lace-like islands throughout the monolayer. In stained preparations, these islands were seen to be syncytia containing up to 60 nuclei. Serial studies, however, showed that syncytium-formation was preceded by

the appearance of acidophilic cytoplasmic inclusion bodies, these being found up to three days before syncytia were observable. Acidophilic intranuclear inclusions were produced at about the same time as the syncytia but occurred in single cells as well as in the latter. Infectivity titres of the 15th, 16th, 20th and 21st passages ranged from only $10^{1.7}$ to $10^{2.7}$ $TCID_{50}$ per 0.2 ml.

As Harrison et al. (1968) stated, virulent CDV is not easy to grow in cell-culture. Despite repeated attempts, Cabasso et al. (1959) were unable to isolate the virus by the inoculation of cultures of dog and ferret tissues with suspensions of organs from infected animals. Hopper (1959) inoculated DK cultures with virulent virus but when the third blind passage was tested for infectivity by ferret inoculation, it proved negative. Virus survived for four blind passages in ferret kidney cultures and for two blind passages in ferret tracheal cultures. These results suggested that ferret cultures might be more susceptible than dog cultures and further support for this hypothesis came from the work of Vantsis (1959) who found that ferret kidney cultures were more susceptible than monolayers of dog lung which, in turn, were slightly more sensitive than DK cultures.

Bussell and Karzon (1965a) attempted to isolate the Snyder Hill strain of virus by the inoculation of a suspension of infected spleen into both dog and ferret kidney cultures but, despite two blind passages at 26 day

intervals in the DK cultures and six passages in the ferret cultures, no CPE was seen. Failure also attended three attempts to isolate virus from natural cases of distemper by the inoculation of DK and ferret kidney cultures with suspensions of spleen. A much greater measure of success was achieved by Vantsis (1959) who reported the isolation of virus from numerous cases of distemper by the inoculation of healthy cultures with material from those animals. However, although cultures inoculated with blood or organ suspensions from cases of early acute distemper showed a CPE within as little as three to four days, four to five weeks were required when the material was taken from advanced cases of the disease, this variation being considered due to differences in the amounts of virus present in the inoculum.

In an attempt to find a more sensitive method of virus isolation, Vantsis (1959) prepared monolayers from the kidneys of experimentally infected ferrets and from the lungs and kidneys of naturally-infected dogs. In this way, virus was isolated from one or both organs of 12 dogs and seven ferrets, a CPE being observable as early as six or seven days following seeding of the cells. The same method was employed by Cornwell *et al.* (1965) in the adaptation to tissue-culture of the Glasgow 841 strain and by Harrison *et al.* (1968) in the adaptation of the CSL strain. Kasza (1968), Thiel *et al.* (1968) and Smith *et al.* (1970) also used this technique successfully.

Bussell and Karzon (1965a), on the other hand, succeeded in only one of five attempts to isolate virus by the direct growth of kidney cells from naturally-infected dogs. In the one successful attempt, a few small cytoplasmic inclusion bodies were seen on the 16th day post seeding, but syncytia were not found until the 34th day. The yield of virus on day 30 was only $10^{1.2}$-$10^{1.5}TCID_{50}$ per ml.

A more sensitive method of isolation was described by Appel and Jones (1967). These workers prepared cultures of alveolar macrophages from SPF dogs and inoculated them with virulent virus. In all cases, syncytia were produced within two to five days of inoculation and virus replication was shown by serial passage. Syncytia were also found when alveolar macrophage cultures were prepared from dogs with acute distemper. Similar results were obtained by Wright et al. (1974) and by Poste (1971). The latter prepared cultures of alveolar, hepatic, splenic and peritoneal macrophages from neonatal dogs and ferrets. Two virulent and one attenuated strain of virus were found to grow in each type of macrophage culture, titres ranging from $10^{2.7}$ to $10^{3.4}TCID_{50}$ per ml for the attenuated virus and $10^{2.7}$ to $10^{4.5}TCID_{50}$ per ml for the virulent virus. Syncytium-formation with cytoplasmic and intranuclear inclusions was found in all virus-cell combinations, but marked nuclear abnormalities were detected only in

alveolar macrophages infected with virulent virus. Poste (1970) also found that suspension cultures of dog lymphocytes supported the growth of virulent virus. However, in none of these systems did the titres produced exceed those obtained by Imagawa et al. (1963) and Cornwell et al. (1970) from MDCK cells (Madin and Darby continuous canine kidney) infected with the Rockborn and Glasgow 841 strains of virus respectively; Imagawa et al. (1963) reported that titres of up to $10^{4.5}$ $TCID_{50}$ per ml could be obtained for at least 15 weeks from these cells. Furthermore, by passaging the virus four times, each time with a greater number of cells and more virus than on the previous occasion, Cornwell et al. (1971) managed to obtain titres of $10^{5.5}$ to $10^{6.5}$ $TCID_{50}$ per ml, sufficient when concentrated by ultracentrifugation to permit the infection of cells under or nearly under one-step conditions. It therefore seemed that use of a dog kidney cell-line might be the most suitable cell-system for the author's proposed studies.

The aim of this part of the investigation was to find a system for producing the quantities of CDV required for the study of virus structure and replication. This work commenced with a brief investigation of the quantal assay system in general use for the titration of dog-kidney adapted strains of virus (Experiments 1.1 and 1.2). This system was then applied to a comparison of virus production in four types of canine cell-culture, as

described in Experiments 1.3 and 1.4. A study was then made of some variables governing virus production and a method of producing large amounts of virus was evolved (Experiments 1.5-1.8).

MATERIALS AND METHODS.

(1) Primary and secondary dog kidney cultures. Cultures of dog kidney cells were prepared by a modification of the method described by Vantsis (1959). Donor animals were mostly "strays" awaiting destruction and consequently varied widely in age and breed. Kidneys were removed aseptically and the cortices were finely minced. The tissue fragments were washed with phosphate buffered saline (PBS) and then transferred to a flask containing 0.25% trypsin in Earle's balanced salt solution (BSS). This was placed on a magnetic stirrer and trypsinisation allowed to proceed at room temperature for 1½ hours. The cloudy suspension thus produced was discarded and replaced with fresh trypsin solution. Digestion was continued over-night at 4°C, again with the aid of a magnetic stirrer. The resultant cell suspension was filtered through a double layer of gauze and cells sedimented by centrifugation at 500 rpm for 20 minutes in an MSE Major centrifuge. The supernatant was discarded and the packed cells were resuspended in Earle's BSS containing 5% calf serum before being transferred to several graduated centrifuge tubes and deposited by centrifugation at 800 rpm for 10 minutes in a MSE Minor bench centrifuge. Following removal of the supernatant, the packed cell volume was read and the cells were resuspended in 100

times their own volume of Earle's BSS containing 0.25% lactalbumin hydrolysate (LH) and 10% calf serum. The cell suspension was then inoculated either into roller tubes (1 ml each) which were incubated at 37°C as stationary cultures or into 8oz. prescription bottles (20 ml each). Complete monolayers were generally formed in five days.

Secondary cultures were prepared by trypsinising monolayers grown in 8oz. prescription bottles. The cell sheets were washed twice with 20 ml of pre-warmed (37°C) PBS free of calcium and magnesium ions but containing 0.02% sodium versenate. An equal volume of the same solution, but this time also containing 0.125% trypsin, was then inoculated into each bottle and the latter were maintained under microscopic examination in a "hot room" at 37°C. When approximately 50% of the cells had rounded up, the trypsin-versene mixture was discarded and the cells were allowed to detach into the residual fluid in the bottle. They were then resuspended in Earle's BSS containing 0.25% LH and 10% foetal bovine serum (FBS) and dispensed into 30 mm or 50 mm petri dishes or roller tubes according to requirements. The cells from one 8oz. bottle provided monolayers within two days in eight 30 mm dishes or five 50 mm dishes or 20 roller tubes.

(2) GH cell cultures. The GH cell-line was derived by serial cultivation of a secondary greyhound kidney culture

in which foci of tightly-packed, polyhedral, epithelial cells had spontaneously developed. The cells were morphologically indistinguishable from those of the Madin and Darby dog kidney (MDCK) cell line but differed from them in karyotype and in their greater resistance to canine herpes virus (Cornwell, unpublished work). Stock cultures were grown and maintained in 8oz. prescription bottles. For serial cultivation, monolayers were disaggregated by the same method as was used for the production of secondary dog kidney cultures. The split ratio was normally one to four and when 20 ml of cell suspension was seeded into an 8oz. bottle and incubation carried out at 37°C, monolayers were formed in three days. Growth medium consisted of Earle's BSS containing 0.25% LH and 10% FBS. For the maintenance of established monolayers the above medium, but containing only 2% FBS, was employed. The medium was changed every three to four days.

(3) Whole canine embryo cultures. The foetuses were removed aseptically from donor bitches and were finely minced. The tissue fragments were washed in PBS and then treated with 0.25% trypsin in Earle's BSS for 45 minutes at room temperature on a magnetic stirrer. The resultant suspension was discarded and the tissue fragments were re-exposed to fresh trypsin solution for 30 minutes under the same conditions as before. The cell suspension thus obtained was filtered through gauze and the cells were

sedimented by 10 minutes centrifugation at 800 rpm in an MSE Minor bench centrifuge. The supernatant was poured off and the deposit resuspended in a small volume of Earle's BSS containing 10% FBS before centrifugation in graduated tubes at 500 rpm for 5 minutes for determination of the packed cell volume. Following removal of the supernatant, the cells were resuspended in 100 times their own volume in Earle's BSS containing 0.25% LH and 10% FBS and inoculated into 8oz. prescription bottles, 20 ml per bottle. Monolayers formed within 24 hours of incubation at 37°C and, from these, secondary cultures were prepared in 30 mm and 50 mm petri dishes and in roller tubes by the same method as was employed for the DK cells. Several further sub-cultures were made employing a split ratio of one to two; under these conditions, it was found that 20-30 tubes or 8-10 30 mm plates could be produced from one 8oz. bottle, monolayers forming in 48 to 72 hours.

(4) Canine alveolar macrophage cultures. The larynx, trachea and lungs were removed immediately after euthanasia, care being taken that no incision of the lung tissue was inadvertently made. The larynx and the first two inches of the trachea were removed and a filter funnel inserted into the remaining portion of the trachea, the stem of the funnel being of sufficient diameter to ensure a reasonably tight fit. Earle's BSS was then poured into the funnel until the diaphragmatic and cardiac lobes of the lungs

appeared fully distended with fluid. In the case of larger breeds such as the Alsatian, almost two litres of medium were required for complete distension. Following gentle massage of the lungs, the contents were emptied into 250 ml centrifuge bottles which were spun at 1500 rpm for 15 minutes. The sedimented cells were resuspended in a small volume of Earle's BSS and centrifuged at 1500 rpm for 15 minutes in graduated tubes to measure their packed volume and were then resuspended in 50 times their own volume of Earle's BSS containing 0.25% LH and 10% FBS. The cell suspension was inoculated into roller tubes or petri dishes, and, after four hours incubation at 37°C, the fluid was removed and replaced with fresh medium of the same composition. The medium was changed again the following day, by which time a considerable number of cells had detached from the substrate, but thereafter, further detachment was slow. At first, the cultures were composed entirely of round, refractile cells but fibroblasts generally appeared after seven to 10 days and rapidly formed dense monolayers, though some round cells remained recognisable within the monolayer for several days more.

The number of macrophages obtained by the above technique varied widely from dog to dog. While large numbers of cells could readily be obtained from dogs with inflammatory changes in their lungs, relatively few tube or plate cultures could be obtained from the lungs of

normal animals and the proportion of cells attaching to the substrate or surviving the first 24 hours of incubation was often disappointingly low.

(5) Virus strains. The virus used was of the Rockborn strain, obtained in the form of the commercial freeze-dried vaccines "Caninovac" (Hoescht Pharmaceuticals) and "Vaxitas" (Tasman Vaccine Laboratory (U.K.) Limited). The infectivity titres of three batches of vaccine were measured by the method described below and found to vary from $10^{2.7}$TCID$_{50}$ per ampoule to $10^{3.7}$TCID$_{50}$ per ampoule. The CPE produced by the vaccine virus in primary or secondary dog kidney cultures consisted predominantly of strand-formation.

Most of the work was carried out with a line of virus passaged 13 times in primary or secondary dog kidney cultures and then adapted to GH cells as described below. The initial passage was made by infecting a 4oz. bottle of secondary dog kidney cells with three ampoules of vaccine. Ten days incubation was required for the development of an extensive CPE but, on subsequent passages, most of which were made with a one in two dilution of the previous passage (whole-culture frozen and thawed once), strand-formation appeared within 48 hours of inoculation and involved almost the whole of the cell-sheet by the end of the fourth day. Culture medium collected from the third passage on the third day after inoculation was

found to contain $10^{5.5}TCID_{50}$ per ml and, although the infectivity titres of subsequent passages were not measured, there was no lessening in the speed with which the CPE developed; thus, even at the 20th passage in DK cells, an extensive CPE was produced by the third day post inoculation. It would seem, therefore, that prolonged passage at a one in two dilution had no adverse effect on virus replication. The significance of this will be discussed in Part Six of this thesis.

Two 1oz. bottles of secondary DK cultures infected with the 12th passage of the above line of virus were harvested by freezing and thawing, mixed with an equal volume of fresh maintenance medium and inoculated into a 4oz. bottle of freshly-monolayered GH cells. Cell growth continued over the succeeding four days, at the end of which the monolayer consisted of a mass of small, tightly packed cells. As there was no evidence of cytopathic changes, the cells were removed from the glass with trypsin-versene and inoculated into an 8oz. prescription bottle. Although a new monolayer formed within 36 hours, syncytia also appeared during this time and, by the end of another two days, the CPE had become extensive. Further passages were made by infecting GH cells in suspension, the inocula consisting of a one in two dilution of frozen and thawed cultures from the previous passage. Under these conditions, syncytia usually appeared within four days of infection (range

two to six days) and spread sufficiently rapidly to allow harvesting by about the eighth day post infection. A large pool of culture fluid was prepared from the second to the seventh passages in GH cells (mostly from the sixth and seventh passages). This was designated VP1 (Virus Pool 1), divided into a number of aliquots and stored at -75°C.

Other lines of virus were prepared by adapting the vaccine directly to GH cells. In these instances, adaptation was a much slower process than that described above, as the following account will demonstrate. Two 4oz. bottles of freshly monolayered GH cells were each infected with four ampoules of vaccine. There being no CPE at the end of the second week post inoculation, the cells were removed from the glass in one bottle and divided between two 8oz. bottles. New monolayers formed within two days of reseeding and a few syncytia were noted after a further two days. However, it was not until about eight or nine days after reseeding that syncytia were at all numerous. Moreover, the cells in the original 4oz. bottle which had not been reseeded remained normal until finally discarded 34 days after inoculation.

(6) Assay of viral infectivity. Infectivity titrations were performed by the quantal method of assay in stationary roller-tube cultures of primary or secondary

dog kidney cells. Ten-fold dilutions of virus were prepared in Earle's BSS containing 0.25% lactalbumin hydrolysate and 5% calf serum. Each dilution was inoculated into five tubes, 1 ml per tube, and five hours allowed for virus adsorption before removal of the inoculum. Medium was changed twice weekly and the end-points read on the 13th day post infection. Infectivity titres were calculated by the method of Kärber (1931).

(7) Staining procedures for light microscopy. Where appropriate, monolayers of cells were grown in 50 mm plastic petri dishes containing glass coverslips. At intervals after infection, coverslips were removed and the cells stained with haematoxylin and eosin following fixation for three hours in mercuric chloride-formol.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 1.1

The Susceptibility of Four Different Types of Canine Cell Culture.

The principal object was to determine whether or not a more susceptible culture system than that provided by the DK cultures could be found. For this purpose, stationary roller tube cultures of GH cells, canine alveolar macrophages, secondary DK cells and secondary whole dog embryo cells were infected with 10-fold dilutions of stock virus grown in GH cells, five or six tubes being used per dilution, and examined microscopically at daily intervals from the seventh to the 21st day post inoculation. The infectivity titres were calculated from the 50% end-points read on the 17th day post inoculation, by which time the end-points had become fixed.

In GH and DK cultures inoculated with a 10^{-1} dilution of the virus, syncytia were found on the third day post inoculation and increased rapidly in number over the succeeding few days. At lower virus input, however, major differences in the scale and distribution of the CPE arose between the two types of culture. Thus, whereas, by

the ninth day post inoculation, tubes of DK cells inoculated with a 10^{-3} dilution of virus contained large numbers of syncytia which were randomly distributed throughout the cell sheet, tubes of GH cells contained very few syncytia, these being found almost entirely in the sparser regions at the periphery of the monolayer. Since large, granular, sometimes bi- or tri-nucleate cells were always present at the periphery of normal GH monolayers, detection of the CPE in GH cultures inoculated with high dilutions of virus was difficult unless the cells were maintained for at least 17 days post inoculation. In DK cultures, on the other hand, limiting dilutions always produced a clearly recognisable CPE within 12 days.

Cytopathic changes developed more slowly in CE monolayers than in cultures of DK cells though, as with the latter, syncytia were distributed randomly throughout individual monolayers. Limiting dilutions of virus produced a CPE within 16 days of inoculation. Obvious syncytia were not seen in cultures of alveolar macrophages but there was a steady loss of cells from those cultures from about six days post seeding onwards. The infectivity titres of the virus suspension in various cultures were as follows:

Cell type	Titre of virus, $\log_{10}TCID_{50}$ per ml.
Secondary DK cells	5.5
GH cells	4.3
Secondary CE cells	3.7
Alveolar macrophages	$\leqslant 0.5$ +

+ the lowest dilution used was 10^{-1}

These results indicated that the DK cells were approximately 15 times more sensitive than the GH cells and about 60 times more susceptible than the CE cells.

EXPERIMENT 1.2

Some Factors Governing the Reproducibility of the Results Obtained from the Assay System.

Before the decision was made to adopt DK cultures for all subsequent titrations, it was felt that a brief investigation should be made of several factors which might possibly influence the susceptibility of the cells and hence the sensitivity of the assay. The first of these was the age and breed of the donor animal and, since the amount of time which could be allotted to this investigation was limited, it was decided to compare the susceptibility of cells derived from two animals of widely dissimilar age and breed. For this purpose, 10-fold dilutions of stock virus were prepared and each dilution was inoculated into DK cultures derived from (a) an eight week old unvaccinated collie and (b) an eight year old greyhound which had received distemper vaccine at two-yearly intervals throughout its life. Seven tube cultures from each animal were used for each dilution of virus, the results being as follows:-

Donor	Titre of virus, $\log_{10}TCID_{50}$ per ml.
Eight week old collie	4.17
Eight year old greyhound	4.24

From this, it appeared that the age, breed and immune status of the donor animal was unlikely to have any marked effect on the sensitivity of the assay.

The next parameter examined was the age of the monolayer. Ten-fold dilutions of stock virus, each sufficient for the inoculation of 15 tubes, were made up in Earle's BSS, 0.25% lactalbumin hydrolysate and 10% calf serum and stored at -65°C. After one hour, 5 ml of each dilution was thawed and inoculated into tubes of primary DK cells seeded four days previously, 1 ml into each of five tubes. At this stage, the cultures were not quite confluent, about 90% of the glass being covered. At nine and 17 days after seeding of the cells, further inoculations were made with the stored virus dilutions. The results were as follows:-

Age of Monolayer in days	Titre of virus, $\log_{10}TCID_{50}$ per ml.
4	4.1
9	4.1
17	4.3

From these results, it appeared that the age of the monolayer was unlikely to have any marked effect on the susceptibility of the cells.

Comparison was also made of the relative susceptibility of primary and secondary DK cultures derived from the same animal, five tube-cultures of each type being used per 10-fold dilution of virus. The results were as follows:-

Virus stock	Titre of virus, $\log_{10}TCID_{50}$ per ml.	
	Primary Culture	Secondary Culture
1	6.1	6.1
2	5.1	5.1

From these, it seemed that the two types of culture were of comparable sensitivity; choice between the two was therefore solely a matter of convenience.

The last parameter briefly investigated was the variation in plating efficiency with different volumes of inoculum. Ten-fold dilutions of a virus suspension were prepared and each inoculated into 10 tubes of primary DK cells, five of these receiving an inoculum of 0.1 ml, and the remainder the standard 1 ml. After five hours at 37°C, the inocula were replaced with fresh maintenance medium and incubation continued. The results were as follows:-

Volume of inoculum	Titre of virus, $\log_{10}TCID_{50}$ per ml.
0.1 ml.	1.3
1.0 ml	2.7

Plating efficiency therefore seemed to be higher with the larger inoculum. This may have been due to "loss" of virus from the smaller inoculum by adsorption onto the glass above the monolayer. Accordingly, it was decided to standardise on an inoculum of 1 ml in all subsequent titrations.

In order to estimate the likely experimental error when five tubes were used per dilution, duplicate titrations of three different batches of virus were carried out in primary DK cultures, the first using 1.0 $\log_{10}$ dilution steps and the remainder 0.5$\log_{10}$ dilution steps, two separate series of dilutions being made up for each virus suspension. As the following figures indicate, there was a difference of only one positive tube within each pair of titrations.

Virus suspension	Dilution steps	Titre of virus, $\log_{10}$ $TCID_{50}$ per ml.	
		First titration	Second titration
1	1.0 $\log_{10}$	2.50	2.70
2	0.5 $\log_{10}$	3.65	3.75
3	0.5 $\log_{10}$	2.60	2.70

On the basis of all these results, it was concluded that the standard method of assay employed provided a valid means of comparing the infectivity titres of different suspensions of virus, even when individual titrations were performed with monolayers of different age and derived from different animals, though variations of 0.2$\log_{10}$ could be accounted for by experimental error and therefore regarded as statistically insignificant.

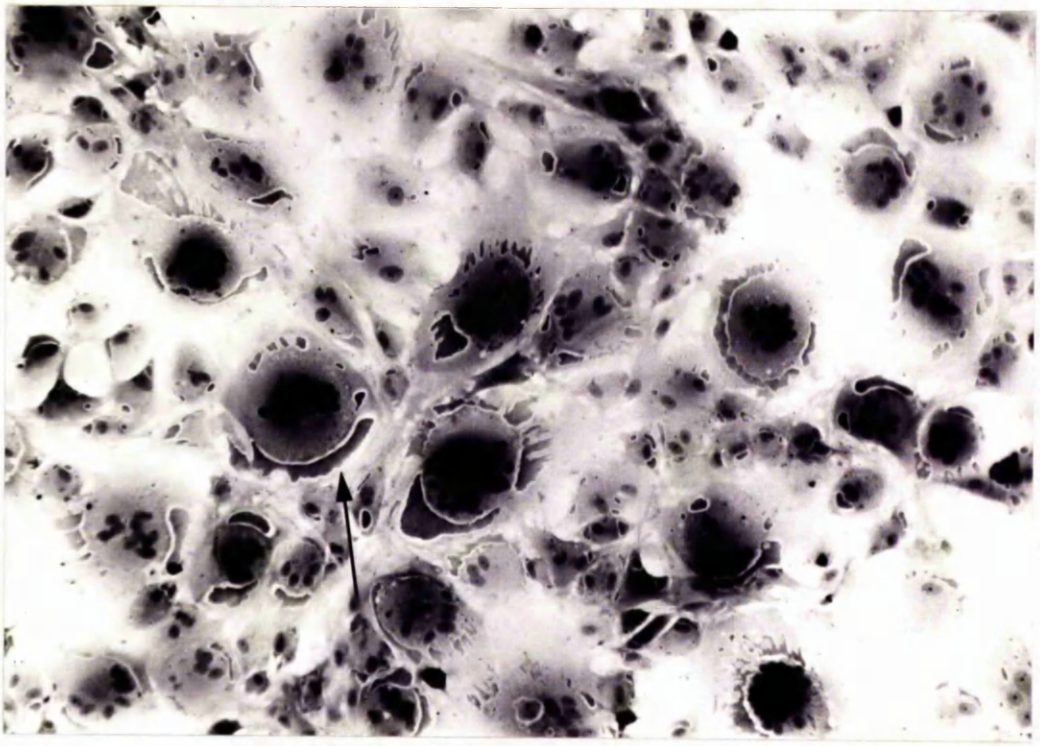
EXPERIMENT 1.3

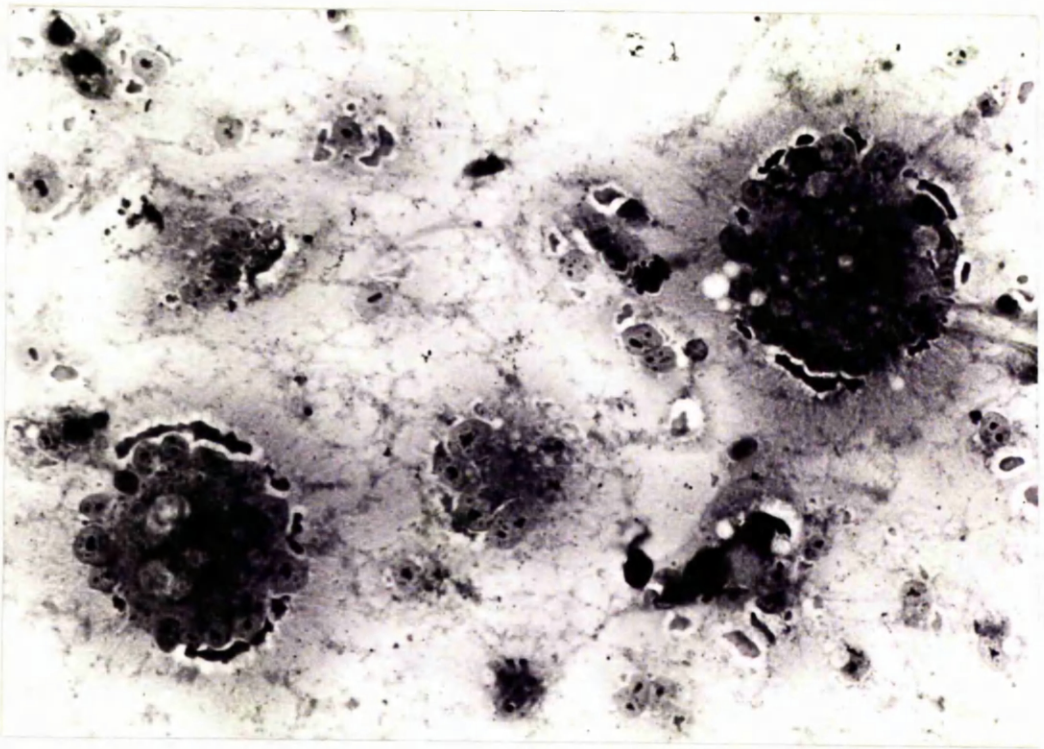
The Growth and Cytopathogenicity of the Virus in GH Cells Secondary Dog Kidney Cells and in a Strain of Canine Whole Embryo Cells.

Although the DK cells had been found to be more susceptible than the other cells tested, it could not be assumed that they would support the growth of the virus to a higher titre. In order, therefore, to determine the most suitable cell system for the growth of new virus stocks, 30 mm petri plates of GH, secondary DK and secondary CE cells were infected with the same stock virus as was used in Experiment 1.1, 2 ml of undilute virus per plate. In addition, three 50 mm plates containing coverslip cultures of the three cell types were each infected with 4 ml of the same undilute virus. Virus adsorption was allowed to proceed overnight, following which the inoculum was removed and replaced with fresh maintenance medium. At daily intervals from the second day post infection (PI) onwards, the fluid and cells were harvested separately from one plate of each type, the cells being removed from the plastic substrate by a silicone rubber policeman, and stored at -75°C pending assay of viral infectivity. The plates were examined by phase contrast microscopy at daily intervals and coverslips removed every second day, fixed in corrosive formol and stained with haematoxylin and eosin for the demonstration of inclusion bodies. The

Figure 1. Syncytia in a secondary DK culture stained with haematoxylin and eosin. Note the random arrangement of the nuclei and the ring of inclusions (arrow). X250

Figure 2. Syncytia in a culture of GH cells, stained with haematoxylin and eosin. The nuclei are arranged in a ring. X400



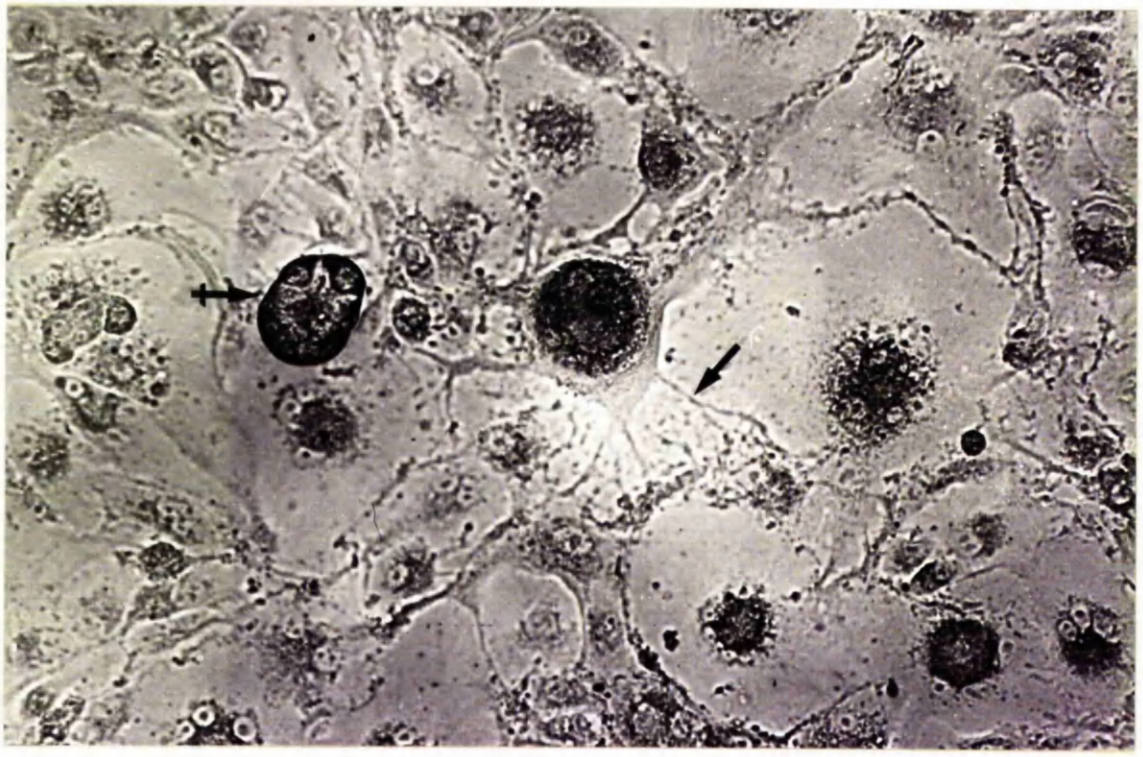


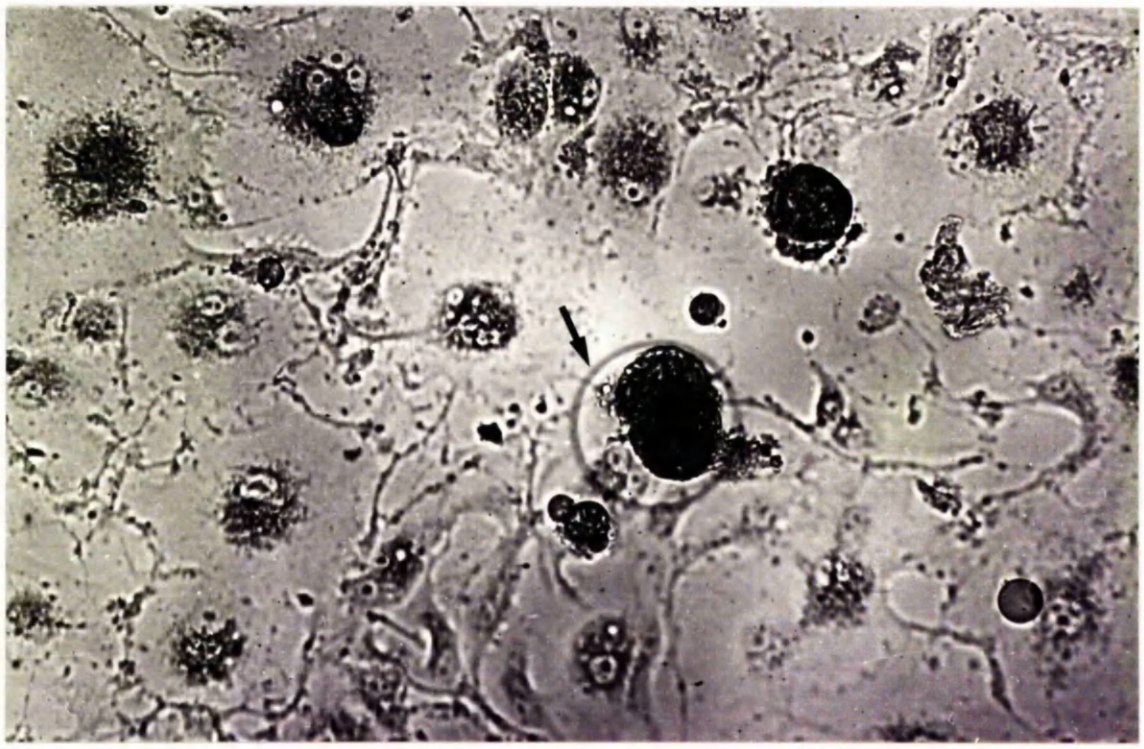
medium in the remaining plates was replaced on the fourth, seventh and 10th days post inoculation.

Cytopathic changes spread most rapidly in the cultures of DK cells. On the second day post inoculation, the cytoplasmic inclusion bodies were found in a small number of mononucleated cells and in cells with up to five nuclei though such polykaryons were rare. By the fourth day, syncytia were fairly numerous and were of characteristic appearance, with the nuclei, usually 5-15 in number, arranged randomly within a rather large mass of granular cytoplasm and with the inclusion bodies forming a ring just inside the periphery of the cell (Figure 1). The number of cells with inclusions continued to increase until, by the eighth day PI, virtually every cell contained one. At this stage, approximately half the nuclei present were incorporated within syncytia but, by the tenth day PI, very few mononucleated cells remained and retraction of the cytoplasm during fusion had resulted in the formation of many irregular spaces. Some syncytia contained as many as 50 nuclei but most possessed between eight and 20. From the sixth day PI onwards, retraction and detachment of the syncytia was observed and, as each began to retract, it adopted a more three-dimensional configuration and became highly refractile. Retraction did not always occur uniformly over the whole surface in contact with the substrate; certain areas of the plasma membrane seemed to remain firmly adherent to the

Figure 3. Syncytia in a culture of GH cells, unstained. The syncytium in the centre of the picture is rounding up. Uneven retraction of the cytoplasm has resulted in the formation of "branches" (arrow). To the left is a syncytium in which the process of retraction has been completed (crossed arrow). X300

Figure 4. Retracted syncytium with a large vesicle appended (arrow), in a culture of GH cells, unstained. X300





substrate so that when the retraction of the remainder took place, the polykaryon adopted a crab-like appearance.

In the GH cultures, the CPE spread much more slowly. Evidence of infection was first seen on the second day PI when moderate numbers of small syncytia were present. These possessed 4-6 nuclei which were arranged in a circle below the cell surface and enclosed an intensely eosinophilic area of cytoplasm. Inclusions were rare in these cells and were found only in a very small number of mononucleated cells. By the fourth day PI, however, many of the syncytia contained inclusions sandwiched between the cell surface and the ring of nuclei beneath it (Figure 2). At this stage, few syncytia contained more than 6-8 nuclei, though, by the sixth day PI, syncytia with up to 20 nuclei were common and inclusions were more numerous in mononucleated cells. It was between the seventh and the ninth days PI, however, that involvement of the cell sheet became extensive; by the latter time, some 70-80% of the nuclei were incorporated within syncytia, up to 30 being present in one cell. Also conspicuous at this time was the retraction of syncytia to form bright, refractile, balloon-like or crab-like structures (Figure 3). Serial observation showed that the latter progress occupied 30 to 60 minutes but seldom preceded detachment of the syncytium by less than two or three hours. Large numbers of refractile syncytia were found floating in the culture medium and, from many of these, a large,

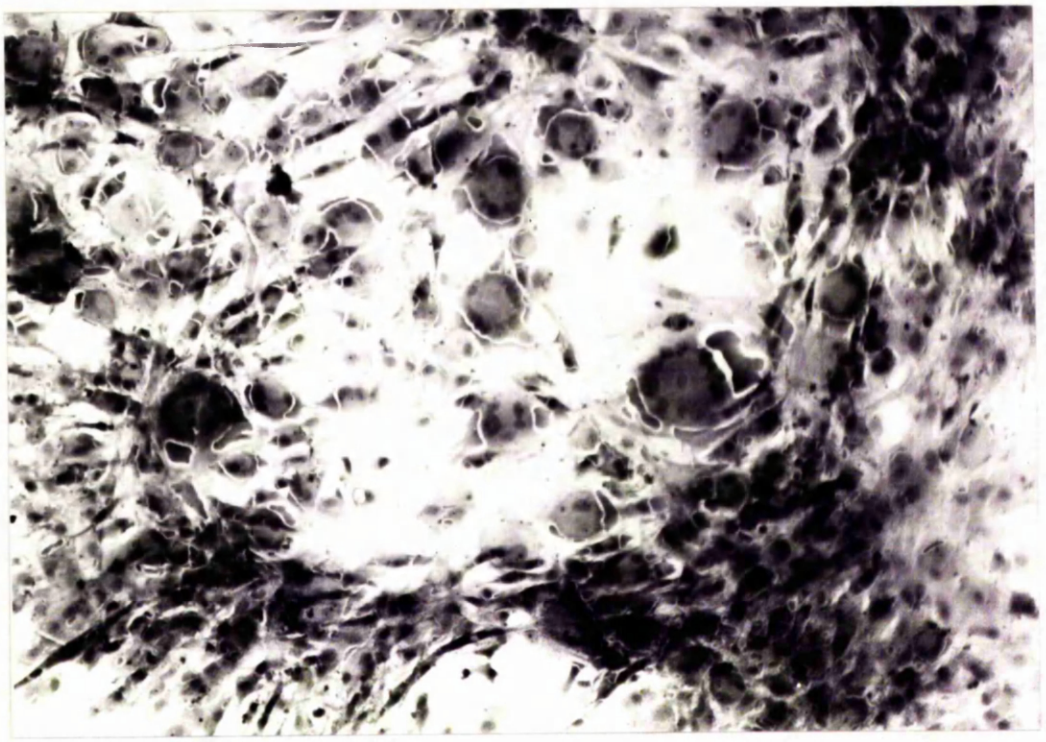


Figure 5. Syncytia in a culture of whole canine embryo cells, stained with haematoxylin and eosin. Note the large number of cytoplasmic inclusions. X250.

translucent, perfectly circular vesicle, presumably containing fluid and often as large as the syncytium itself, was appended (Figure 4). Despite the detachment of so many syncytia, the monolayer did not appear disrupted and few spaces were found on the 11th day PI when observation was terminated.

Cytopathic changes developed even more slowly in cultures of CE cells than in those of the GH cells. On the fourth day PI, inclusions were seen in the mononucleated cells but these were rare and syncytia were totally absent. By the sixth day, inclusions were common in mononucleated cells but only very few syncytia, generally with 8-10 nuclei, were found. It was not until the eighth day that syncytia were at all numerous. These generally contained 10-15 nuclei, often arranged in a circle near the periphery of the cell and with the inclusions wedged between them and the cell surface (Figure 5). By the 10th day PI, syncytia were numerous but the vast majority of the cells remained mononucleated though virtually all of them contained inclusions.

The growth curves are shown in Figure 6, from which it will be seen that the greatest yields of virus were obtained from the GH cells. Despite the rapid involvement of the whole monolayer, DK cultures produced 0.4 to $0.8 \log_{10}$ less virus than the GH cells during the first seven days and 1.0 to $2.2 \log_{10}$ less during the succeeding

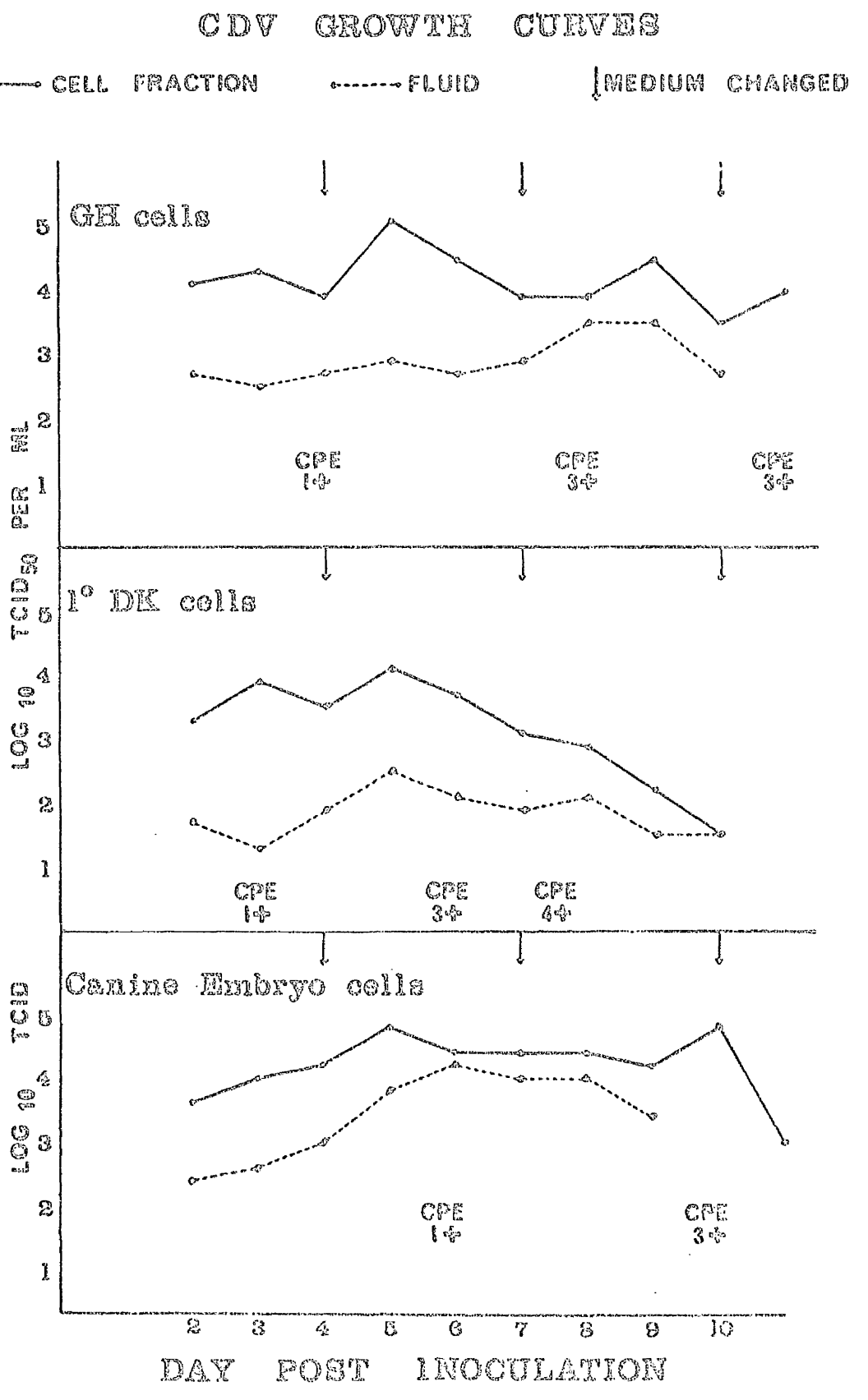


Figure 6. Growth of the virus in three different cell types.

few days when detachment of syncytia was taking place. The CE cultures produced about the same amount of virus as the DK cells during the first three or four days PI but, thereafter, the growth curve more closely resembled that seen with the GH cultures, virus production being held constant over the six to 10 day period instead of declining rapidly as it did in the DK cultures.

In all three types of culture, cell-associated virus generally exceeded extracellular virus by 1.5 to 2.0 $\log_{10}$ $TCID_{50}$ per ml (limits 1.0 to 2.6$\log_{10}$) during the first 5-7 days PI. However, by the time the CPE had become extensive, with few apparently normal cells remaining, the difference in titre between the two fractions had diminished to around 0.8$\log_{10}TCID_{50}$ per ml (limits 0.0 to 1.2$\log_{10}$). In the case of the DK cultures, this was largely due to a fall in the cell-associated virus. In the GH cultures, on the other hand, the extracellular virus titre rose as the proportion of infected cells approached unity.

Except in the case of the DK cultures, where virus production gradually fell away as disruption of the cell sheet took place, the growth curve was characterised by periodic fluctuations, possibly related to the time elapsing since changes of medium were made. For example, except in the case of the CE cultures the yields were always lower on the fourth day PI than on the third day.

Moreover, titres of extracellular virus generally rose by 0.6 to 1.1$\log_{10}$TCID$_{50}$ within 24 hours of a change of medium. Only in CE cultures did yields remain relatively stationary.

Table 1 Comparison of Virus Yield in GH Cells and Alveolar Macrophages.

Type of cell	Cells per plate	Infectivity titre of culture (cell fraction only) $\log_{10}TCID_{50}$ per ml Day post infection				
		1	2	3	4	5
GH	3.5×10^6	NT	3.8	4.0	4.0	4.5
Alveolar macrophages (Alsatian)	7×10^5	0.5	2.8	2.3	2.0	NT
Alveolar macrophages (Mastiff)	5×10^6	1.8	3.1	3.3	3.5	1.0

NT = Not tested.

EXPERIMENT 1.4

The Growth and Cytopathogenicity of the Virus in GH Cells and Canine Alveolar Macrophages.

The aim of the present experiment was to compare the yield of virus obtained from monolayers of GH cells with that produced by cultures of canine alveolar macrophages. But since the proportion of macrophages attaching to the substrate was known to vary markedly from animal to animal, and the influence of cell number on the yield of virus was unknown, two lots of macrophage cultures, both derived from different animals and varying considerably in cell-number, were used. All cultures were maintained in 50 mm plastic petri plates and cell counts were performed immediately prior to inoculation. The remaining plates were each inoculated with 4 ml of VP1 and samples were harvested at daily intervals for titration. The GH cultures remained normal in appearance during the five days following inoculation but, although no syncytia were seen, there was a loss of macrophages from the fourth day onwards and the experiment therefore was terminated after harvests had been made on the fifth day. The infectivity titres are recorded in Table 1 from which it will be seen that, for approximately the same number of cells, the GH cultures produced 3-5 times as much virus as the macrophage cultures over the two to four day period. Thereafter the difference increased substantially as loss

of macrophages occurred. It therefore seemed that, of the four types of culture tested, the GH system was the most likely to provide the large amounts of virus required for study. Accordingly, all future stocks of virus were produced in this system. In the following experiments, optimal conditions for virus production were examined.

EXPERIMENT 1.5

The Plating Efficiency and Virus Yield Obtained by Infecting Cells in Monolayer and in Suspension.

The previous studies showed that when monolayers of GH cells were infected at an input multiplicity of approximately 1.0, four or five days incubation were necessary before a CPE appeared and the maximum titre of extracellular virus reached only $10^{3.5}TCID_{50}$ per ml. Syncytia were most prominent near the periphery of the plates where the cells were less tightly packed than in the centre. During adaption of the virus to GH cells, however, syncytia were often seen within two days if the cells were infected in suspension; the CPE then advanced rapidly and evenly throughout the monolayer and titres of up to $10^{5.5}TCID_{50}$ per ml (e.g. VP1) were obtained. These observations suggested that adsorption of virus to monolayers of GH cells might be a rather inefficient process and that an appreciably higher infection rate probably occurred when the cells were infected in suspension. Since the viral yields were presumably related to the number of infected cells, the yields obtained by each method of infection were compared. For this purpose, 30 mm plates of GH cells were prepared and designated "Group A". When monolayers were complete, the cells were removed from one plate with trypsin-versene and counted (result: 1.12×10^6 cells per plate).

Each of the remaining plates was then inoculated with 2 ml of virus. At the same time, a suspension of stock GH cells was prepared and the cell-concentration was adjusted so that, following infection of the cells in suspension and their subsequent inoculation into 30 mm plates, each of the latter (designated "Group B") contained the same number of cells and the same amount of virus as the plates of Group A. This was achieved by adding 8 ml of virus to 1.8 ml of a cell-suspension containing 2.5×10^6 cells per ml and, after thorough mixing, distributing this between four plates, 2.45 ml per plate. Each of the latter also received 0.25 ml of FBS to promote attachment and growth of the cells. Both groups of plates were incubated at 37°C and the medium was replaced after 24 hours and subsequently at three-daily intervals. Cell-fractions were harvested by scraping at 3,5,7 and 9 days PI and the yields of virus titrated by the standard method. The results were as follows:-

Group	Infectivity Titre $\log_{10}TCID_{50}$ per ml			
	Days.			
	3	5	7	9
A	4.5	4.5	4.7	4.9
B	5.3	5.5	4.5	3.7

The plating efficiency was much higher when the cells were infected in suspension. The CPE was detectable three days PI and spread with such rapidity that, by seven days PI, the cell sheet was broken up by large clear spaces from which the syncytia had detached. Virus

production therefore reached a peak around the fifth day PI and then declined, the titre falling approximately 60-fold over the ensuing four days. By contrast, syncytia were not seen in the Group A plates until the seventh day PI, when they were present in small numbers towards the periphery of the very tightly-packed monolayers, and it was not until the ninth day that they were at all numerous. In accordance with this, the viral titres increased only slightly over the 3-9 day period. However, although the peak titre in Group B exceeded that in Group A by a factor of approximately four-fold, the short duration of the period over which it occurred suggested that, in the long run, more virus might be obtained by infecting monolayers than by infecting cells in suspension; the lower plating efficiency in the former system permitted multiplication of those cells which escaped infection at the time of inoculation, the increased cell population in turn allowing a greater amount of virus to be produced.

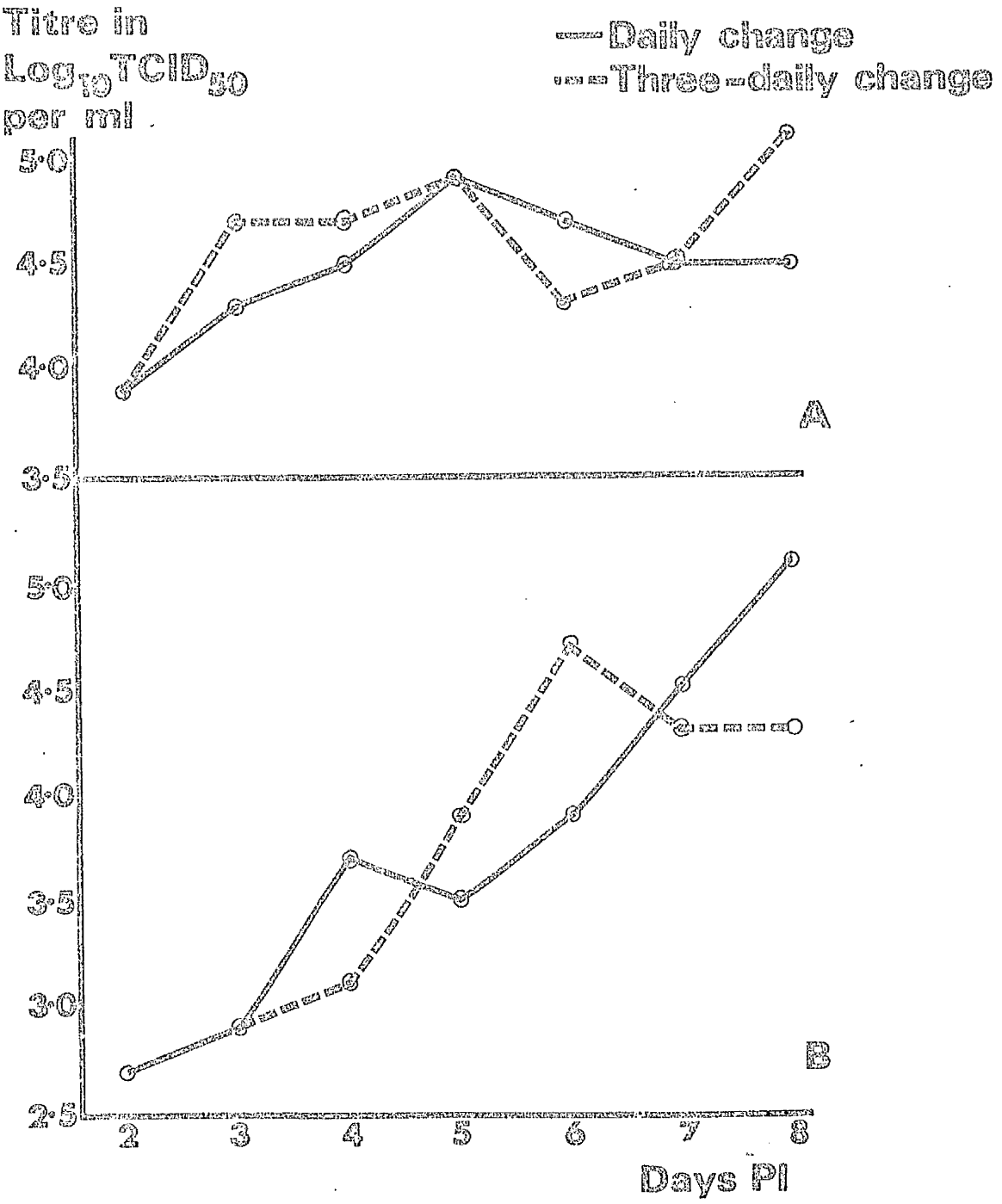


Figure 7. Growth of CDV in cultures changed at daily and three-daily intervals. A - Cell fractions. B - Fluid fractions.

EXPERIMENT 1.6

The Relationship Between Virus Production and the Frequency of Change of Culture-medium.

From the study of the growth curve of the virus in GH cells (Figure 6), it was found that, on two occasions out of three, there was a marked rise in cell-associated virus within one day of a change of medium. Conversely, titres were always lower on the third day after a change of medium than on the second. These findings suggested that renewal of medium might potentiate virus production so that daily changes of medium might have a cumulative effect on the titres obtained. To investigate this, 30 mm plates of GH cells were each infected with 2 ml of VP1 and divided into two groups. In Group A, the medium was changed at daily intervals after infection, whereas in Group B, it was changed only on the third and sixth days after infection. Fluid and cells were harvested individually from both groups at daily intervals and their infectivity titres assayed in the usual manner. Syncytia were first seen on the sixth day PI and were numerous by the eighth day PI, particularly in Group A. The results of the infectivity titrations are recorded in Figure 7 from which it will be seen that, contrary to expectations, there was no overall difference between the groups. Thus on days 2, 4, 5 and 7 the titre of cell-associated virus from Group A differed from that of Group B by $0.2\log_{10}$ or less and the same applied to the titres

of extracellular virus on days, 2, 3 and 7. On the eighth day PI the titre of cell-associated virus in Group A was $0.6\log_{10}$ lower than in Group B but the titre of extracellular virus was $0.8\log_{10}$ higher. It therefore seemed that there was no appreciable advantage in changing the medium at daily intervals.

EXPERIMENT 1.7

Virus Production in GH Cells at the Time of Maximum CPE.

The results of Experiment 1.6 confirmed the earlier observation that once the CPE had become extensive, the titre of extracellular virus closely approached that of cell-associated virus. Indeed, on two occasions, it exceeded the latter. These findings suggested that the best method of obtaining a large volume of high titre virus, relatively free of cell-debris, might be to collect the culture-fluid at daily intervals once the CPE had become extensive. To investigate this possibility, 30 mm plates of GH cells were infected with VP1 and maintained until the 11th day PI, by which time syncytia were present in large numbers.

The cell and fluid fractions were harvested individually on days 11, 13, 15 and 17 and their infectivity titres determined in the usual manner. The results were as follows :-

	Infectivity titre, $\log_{10}TCID_{50}$ per ml			
	Day			
	11	13	15	17
Cell-associated virus	5.3	6.1	5.7	5.1
Extracellular virus	4.7	5.7	5.1	5.1

Thus, although the cell-associated virus exceeded the extracellular virus by a factor of 2.5 to 4-fold for most

of the time, there was a period of at least five days during which the fluid contained over $10^5 TCID_{50}$ of virus per ml. This meant that titres of that order could readily be obtained by collecting the culture fluid over this period; there was no need to subject the cells to ultrasonic waves or to cycles of rapid freezing-and-thawing, procedures which might conceivably damage some of the virus already released.

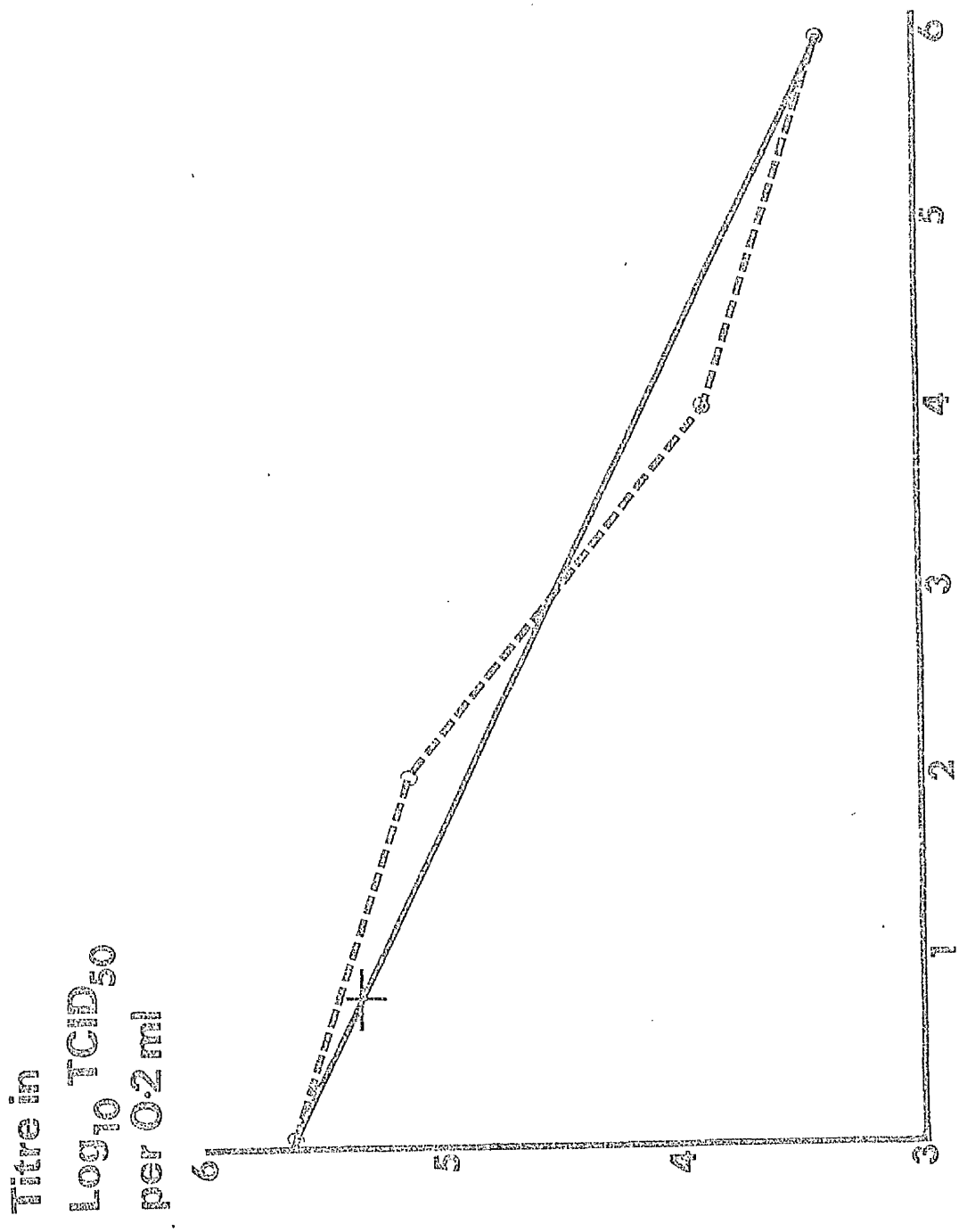


Figure 8. Thermal inactivation of CDV. ⊢ marks the point where the titre has fallen to half that originally present.

EXPERIMENT 1.8

Rate of Virus Accumulation in the Culture Medium.

The next point considered was the rate at which extracellular virus accumulated following a change of medium. If a period of approximately 24 hours was required for the titre to regain the level which it had attained prior to the change, then clearly it would be impossible to harvest at less than daily intervals. If, on the other hand, the titre regained its original level within a few hours, it would be possible to harvest two or three times daily. The time required was obviously dependent upon several factors, e.g., the number of cells still actively producing virus, the intrinsic rate of virus maturation and release and the rate of thermal inactivation of extracellular virus. If inactivation occurred very rapidly, equilibrium was likely to be reached quite soon after a change of medium. The first point investigated, therefore, was the rate of inactivation at 37°C. Stock virus was divided into four aliquots, one of which was titrated immediately and the remainder after two, four and six hours immersion in a water-bath at 37°C. In each titration, eight tubes were used per 10-fold dilution of virus. As will be seen from Figure 8, the inactivation curve obtained was slightly sigmoid in shape, the minor shoulder near the origin being matched by a segment of similar slope over the 4-6 hour period. Taken overall,

however, the half-life was approximately 47 minutes (possible range: 45 to 50 minutes). With such a rapid rate of inactivation, it seemed unlikely that infectivity would rise for long following a change of medium.

To investigate the latter point, the medium was removed from a 4oz. bottle of cells showing an advanced CPE, and its infectivity titre was determined. Fresh medium was added and small volumes of this removed for titration after 1, 2, 4 and 22 hours re-incubation. In calculating the titres, allowance was made for the reduction in the volume of fluid following sampling. The results were as follows:-

Sample Time	Infectivity titre $\log_{10}{}^{TCID}{}_{50}$ per ml	Percentage original infectivity present
Before change	5.0	100
1 hour	4.33	21.4
2 hours	4.58	38.0
4 hours	4.83	67.6
22 hours	4.54	34.7

From these figures, it will be seen that although the original titre was not completely regained in any of the samples titrated, only four hours incubation were required for the titre to reach 68% of that originally present. On this basis, it seemed that fluid could be collected twice or even three times daily without significant loss of titre.

DISCUSSION.

Prior to the commencement of the above experiments, the inability of CDV to grow to a high titre in dog cell-cultures meant that it was very difficult, if not impossible, to obtain the quantities of virus required for biochemical investigation. However, by adapting the virus to GH cells, which were shown to be capable of supporting viral replication for much longer than primary DK cultures, and by making daily or twice-daily collections of the culture medium, it was possible to obtain large volumes of virus having an infectivity titre of approximately $10^5 TCID_{50}$ per ml. The production of a large volume was necessary because the titre of cell-associated virus rarely exceeded $10^{5.5} TCID_{50}$ per ml; the rapid release of virus into the culture-medium meant that more virus could be obtained by collecting this over a period than from the single harvest of the cell-associated virus. This large volume of virus could then be concentrated to provide the quantity of virus required for biochemical studies. Thus, as shown in Part Five, it was possible to produce a concentrated virus suspension containing as much as 200 µg of protein per ml, a quantity sufficient for the demonstration of the individual polypeptides. With the exception of the Onderstepoort -- AV-3 system of Bussell and Karzon,

(1965b), no system has been shown to be more productive than that described here.

When primary DK cells were infected with the virus, syncytia were formed and when these detached from the glass, the spaces thus produced were not readily recolonised by the division or movement of adjacent cells. This may have reflected the limited ability of these cells to divide but it may also have been due to the fact that they were generally less tightly packed than the GH cells. Disintegration of the monolayer therefore occurred fairly rapidly, so that once this stage had been reached, the yield of virus rapidly fell to a low level. This meant that these cultures were ideal for assay but unsatisfactory for virus production.

When GH cells were infected with the virus, detachment of infected cells was followed by multiplication or spreading of adjacent cells so that spaces were not formed until most of the cells were infected. With high input multiplicities, this occurred within a week but, with low multiplicities, it took much longer, e.g., four weeks or more. Moreover, after infection at low multiplicity, cell division continued, even when the serum concentration was as low as 2%, so that the cells became increasingly tightly packed. Under these conditions it was extremely difficult to detect the CPE.

From the results of Experiment 1.5, it would seem it is relatively difficult to infect GH cells when they are in a tightly-packed monolayer. For these reasons, GH cultures were unsuitable for assay but were markedly superior to primary DK cultures for the production of a large quantity of virus. Similar conclusions were reached by Cornwell et al. (1970) who compared the value of MDCK cells with that of primary DK cells for growing and titrating the Glasgow 841 strain of CDV.

The earliest evidence of CPE was generally provided by large syncytia, of globoid, refractile, surface but granular centre, floating in the culture medium. From many of these, a large, translucent, circular vesicle protruded. Later, similar structures but with less protrusive vesicles were seen in gradually increasing numbers in the cell-sheet. These sometimes possessed several refractile branches and, on occasion, transitional forms composed partly of flat, non-refractile areas of cytoplasm and partly of swollen, refractile zones, were observed. These structures were clearly derived from the large, flat, multinucleated masses formed by cell fusion. A similar conclusion was reached by Shaver et al. (1964) who compared the proportions of morphologically different types of syncytia in ferret kidney cultures infected with the 4856 strain of virus.

The results of Experiment 1.3 showed that the CE

cells were unsuitable for assay and had no advantages over GH cells for virus production. As far as the author is aware, there are no published reports of the comparative sensitivity of epithelial cells and fibroblasts to CDV. Relatively low yields of virus were obtained in cultures of alveolar macrophages and obvious syncytia were not observed. The absence of cytopathogenicity may have been related to the attenuation of the virus since virulent virus is known to produce large syncytia in these cultures (Appel and Jones, 1967; Wright et al., 1974). On the other hand, Poste (1971) found that alveolar macrophages were as capable of supporting the growth of the attenuated BW strain as they were of supporting that of two virulent strains. Thus, the BW strain was found to grow to a titre of $10^{3.4}TCID_{50}$ per ml in these cells whereas the two virulent strains grew to titres of $10^{3.8}$ and $10^{2.7}TCID_{50}$ per ml respectively. However, from the results obtained in the present investigation, alveolar macrophages were evidently unsuitable for either production or assay of the Rockborn strain of virus.

The yield of virus seemed to be related to the number of infected cells in the culture. Thus, even in the absence of a CPE (because the proportion of infected cells was low), the titres were relatively high if the absolute number of infected cells was correspondingly high. For example, a titre of $10^{4.5}TCID_{50}$ per ml was found in the cell fraction on the fifth day PI

(Experiment 1.4) though a CPE was not detected. In Experiment 1.5, a much higher infection-rate was obtained by infecting cells in suspension and this resulted in higher yields during the first few days PI: Cells infected in suspension produced $10^{5.3}$ $TCID_{50}$ per ml on the third day, whereas the same number of cells infected as a monolayer with the same amount of virus produced only $10^{4.5}$ $TCID_{50}$ per ml at this time. However, on the ninth day PI, the amount of virus produced by the cells infected in monolayer was greater than that produced by those infected in suspension ($10^{4.9}$ cf. $10^{3.7}$ $TCID_{50}$ per ml) and it is obvious that if the experiment had been continued for a few more days, the total amount of virus produced by the former would have been greater. This was presumably due to the greater number of cells escaping infection at the start of the experiment and therefore capable of division with resultant increase in the cell population and in the total number of cells able to produce virus during the course of the infection.

The shape of the growth curves obtained in Experiment 1.3, suggested that a change of medium stimulated the production of virus. Harrison et al. (1968) found that the titre of CDV grown in ferret kidney cultures was greater, and the CPE advanced more rapidly, when the cultures were changed five times per week rather than three times per week. Better results were likewise obtained by three changes per week than by no changes at

all. In the present investigation, however, it was found that a daily change of medium did not produce the expected advantage. In this general context, it should be noted that although Bussell and Karzon (1965a) reported that the use of enriched medium potentiated the CPE, they also stated that it did not significantly alter the virus yields.

The half-life of the virus at 37°C was found to be approximately 47 minutes (Experiment 1.8). This compares with figures of 38 minutes (Palm and Black, 1961) and one hour (Bussell and Karzon, 1962) for CDV, 38 minutes (Palm and Black, 1961), 42 minutes (Yeh and Iwasaki, 1972), 1-2 hours (Cascardo and Karzon, 1965) and approximately two hours (Black, 1959) for measles virus and 165 minutes for rinderpest virus (Plowright et al., 1969). The shortness of the half-life at 37°C partly accounts for the levelling of extracellular virus titres within several hours of a change of medium and indicates that little is probably gained by prolonging the interval between successive collections of fluid to more than four or five hours. Working with rinderpest virus, Plowright et al. (1969) found that, in most instances, virus was rapidly released after a change of medium and, within 10 minutes, the titre of extracellular virus had reached a level only $10^{0.5}$ $TCID_{50}$ per ml less than that present before the change. Over the subsequent 50 minutes the titre continued to increase slowly so that by the end

of one hour it was the same as that present before the change of medium.

In certain respects, the multistep growth curve produced with the Rockborn strain of CDV in GH cells was similar to the curves published by Shishido et al. (1967) for the Lederle strain in Vero cells, and by Confer et al. (1975a) for the Snyder-Hill (SH), Onderstepoort and R252 strains, also grown in Vero cells. In each case, the titres of both cell-associated and extracellular virus rose sharply within the first two days and, except with the Onderstepoort strain, the titres of extracellular virus were always lower than those of cell-associated virus. (With the Onderstepoort strain there was a loss of cells by the fourth day PI and consequently the extracellular virus titres were greater than the cell-associated titres from then until the termination of the experiment on the seventh day). Variations were seen, however, in the highest titre of virus obtained from each system, and in the time and duration of peak production, e.g. with the R252 and SH strains, cell-associated virus reached a maximum titre of approximately $10^{4.5}$ and $10^{5.3}$ $TCID_{50}$ per ml respectively on the fifth day PI and these titres were maintained until the termination of the experiment on the seventh day. On the other hand, with the Lederle strain, cell-associated virus reached a maximum titre of $10^{5.3}TCID_{50}$ per ml by the fourth day PI and thereafter immediately decreased so that by the sixth

day, it was only $10^{3.5}TCID_{50}$ per ml. In all systems except the SH-Vero cell system, the total amount of virus produced was obviously less than that obtained in the Rockborn-GH system. Comparison with the SH-Vero cell system is impossible since the experiment involving the latter system was terminated prematurely.

PART TWO.

INTRODUCTION AND REVIEW OF THE LITERATURE.

Before commencing an investigation of the components of CDV, a detailed study of the growth and morphogenesis of the virus under one-step conditions was undertaken. By so doing, it was hoped that the principal components of the virus could be identified within the cell, their period of production relative to the growth curve determined and a clearer understanding of particle structure obtained.

The basic structure of the virion was first investigated by Cruickshank et al. (1962) and Norrby et al. (1963) who examined negatively-stained suspensions of the virus in the electron microscope. The virions were found to be very pleomorphic though many were almost spherical. The latter varied in diameter from 110 to 550 nm although most fell into the 150-300 nm range. Intact particles possessed a conspicuous outer membrane about 5 - 8.5 nm in thickness and from this arose a fringe of fine projections 9 - 13 nm in length. Where the phosphotungstate had penetrated partially disrupted particles, a helical nucleocapsid was revealed. The

latter, which was also seen free of particles, had a width of 15 - 18 nm with a central hollow axis approximately 5nm in diameter. Its helical structure was revealed by its serrated outline which had a periodicity of 5 - 6 nm. In size and structure, therefore, the virus closely resembled the paramyxoviruses.

The earliest reported ultrastructural studies of CDV-infected cells were those of Lawn (1970) who examined the chorioallantoic membrane of chick embryos six days after inoculation with egg-adapted virus and Koestner and Long (1970) who described three partially overlapping phases of infection in explant cultures of canine cerebellum. Since the titre of virus used by the latter workers was only $10^3 TCID_{50}$ per ml, multiple cycles of virus replication must have occurred. The times at which the various ultrastructural changes were first detected - the seventh day for cytoplasmic aggregates of virus nucleocapsid and the 14th day for budding virus particles - therefore provide no information as to the times at which these events occur in the individual infected cell. The same criticism can be made of the work of Confer et al. (1975b) who studied Vero cells infected at a multiplicity of 0.03 with three different strains of CDV. Clearly, cells must be infected at or near one-step conditions if a proper sequential study is to be made. This principle is not easily followed, since it is very

difficult to obtain the necessary concentrations of virus. Cornwell et al. (1971), however, managed to obtain an infection-rate of 80% with the Glasgow 841 strain of virus in cultures of MDCK by employing an input multiplicity of 1.9. Tajima et al. (1971) studied the morphogenesis of an avianised strain of CDV in chick embryo fibroblasts and Vero cells but did not take advantage of the high input multiplicity used (up to 10) to relate the ultrastructural changes observed to the stage of the growth curve.

In the work of Cornwell et al. (1971), electron microscopic examination of cells harvested between 24 and 96 hours PI showed that virus maturation occurred throughout the whole of this period though infectivity titrations proved the level of virus production to be significantly greater ($1.0-1.7 \log_{10}$) at 24 hours PI than at subsequent sampling times. Although well-developed foci of nucleocapsid were present in the cytoplasm of most cells by 24 hours PI cytoplasmic inclusions could not be demonstrated by light microscopy at this time. It was not until 48 hours PI that characteristic eosinophilic cytoplasmic inclusions were detected. Ultrastructural examination at this time revealed the presence of many electron-dense bodies containing nucleocapsid apparently enmeshed within a finely granular matrix. In size and shape as well as in time of appearance, these seemed

to correspond to the cytoplasmic inclusions revealed by light microscopy. Since the development of inclusions occurred after the period of maximum virus production. It was suggested that this might reflect an active attempt on the part of the cell to destroy or sequester unutilized nucleocapsid. Another process commencing about 48 hours PI was the accumulation of inclusion body-like material near the cell-surface, particularly within microvilli which increased markedly in number about this time.

No attempt was made in the above study to determine the length of the latent period or to make an ultrastructural study of the events occurring during this period. Furthermore, although the cells were shown to be producing virus as late as 96 hours PI, studies were not extended further and it is possible that changes characteristic of the terminal stages of infection – such as the development of intranuclear inclusion bodies – were missed. The present investigation was therefore designed not only to cover the period studied by Cornwell et al. (1971) but also to encompass the earlier and later stages of infection.

From the Poisson distribution, a multiplicity of infection of 3 is required to ensure that 95% of the cells are infected with at least one virus particle. From this consideration as well as from the necessity of

employing sufficient cells to permit electron microscopic examination at each of the sampling times, it was felt that, rather than attempt a single composite experiment, it would be more convenient to carry out several smaller experiments and from the results of these reconstruct the entire growth cycle.

MATERIALS AND METHODS.

1) Preparation of Virus and Infection of Cells. Virus was prepared by the method described in Part One. Cells were infected in suspension with concentrated virus, as described in detail in the appropriate parts of the Experimental Procedures and Results section.

2) Infectivity Titrations and

3) Staining Procedures for Light Microscopy.

These were carried out as previously described in Part One.

4) Fixation, Embedding and Staining of Cells for Electron Microscopy. Infected cells were grown in 50 mm petri dishes. At selected times, the cells were scraped off the plastic into a small volume of medium by means of a rubber policeman. They were then pelleted by low-speed centrifugation and, after removal of the supernatant, fixed in osmium tetroxide (1% osmic acid -- obtained from BDH Chemicals Ltd. -- in Millonig's phosphate buffer, pH 7.2-7.4) for one hour. The fixed pellet was then dehydrated in an ascending series of 70%, 90% and absolute alcohol, followed by rinsing in propylene oxide. It was then soaked for one hour in a mixture consisting of equal parts of propylene oxide and Araldite and left

overnight in a mixture of 20% propylene oxide and 80% Araldite. It was then embedded in Araldite within a gelatin capsule and the resin was allowed to polymerise at 57°C for 48 hours. Ultrathin sections (approximately 500Å in thickness) were cut on an LKB Mark 3 ultramicrotome, using glass knives, and mounted on uncoated Athene 483 copper specimen grids (obtained from Smethurst High-Light Ltd., Bolton, Lancs.). Sections were stained for 20 minutes with uranyl acetate (as described by Watson, 1958), rinsed first in methanol and then in 50% methyl alcohol in distilled water, and dried on filter paper. They were then stained for 10 minutes with lead citrate (as described by Reynolds, 1963) and finally rinsed with 0.02 N sodium hydroxide in distilled water before drying on filter paper. The sections were examined using an AEI 6B electron microscope at instrumental magnifications of 10,000-40,000.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 2.1

One-step Growth Curve in GH Cells.

The aim of the first experiment was to establish the shape of the growth curve so as to provide a framework on which subsequent ultrastructural studies, perhaps of necessity carried out at a lower multiplicity of infection, might be based. In order to prevent serious distortion of the curve by second and third step infection, it was considered essential to achieve an initial infection-rate of at least 70%. A preliminary attempt to do this using the method described by Cornwell *et al.* (1971) proved unsuccessful, as did two further experiments in which cells were infected with virus concentrated in the ultracentrifuge. However, by further increasing the concentration of virus employed and by reducing the number of cells, a satisfactory infection rate was finally achieved.

Virus was prepared by the method described in the previous section; fluid was collected twice daily from cultures with a good CPE and stored at -70°C. When 1500 ml had been obtained, it was thawed, clarified by low-

speed centrifugation and then spun at 18,500 rpm for 3½ hours in the Type 19 rotor of a Beckman L2 – 65B ultracentrifuge. The resultant pellet was resuspended in 15 ml of medium which had been removed from two Roux flasks of infected cells after five hours of incubation. The final volume of the suspension was 15.2 ml and when 0.2 ml was assayed for infectivity, its titre was found to be $10^{6.9}$ $TCID_{50}$ per ml. The virus was mixed with 1.08×10^7 GH cells in a volume of 3 ml, input multiplicity therefore being approximately 7(6.96). In the preparation of the cell suspension, an effort was made to disaggregate the monolayer more fully than before in order to reduce the size and number of cell-clusters, this being done by increasing the period of exposure to trypsin and by vigorous pipetting. However, when the cells were counted, it was found that many clumps were still present. It therefore seemed probable that, despite the high input multiplicity employed, some cells near the centre of the clumps might escape infection.

The cell-virus suspension was agitated on a magnetic stirrer for 90 minutes at room temperature, following which the cells were sedimented by low-speed centrifugation, resuspended in fresh growth medium and inoculated into 18 tubes – two with coverslips – so that each tube received 6×10^5 cells. The tubes were incubated at 37°C and at 8, 12, 15, 18, 24, 36, 48, 72 and 96 hours PI, two of them (only one at 8 and 12 hours

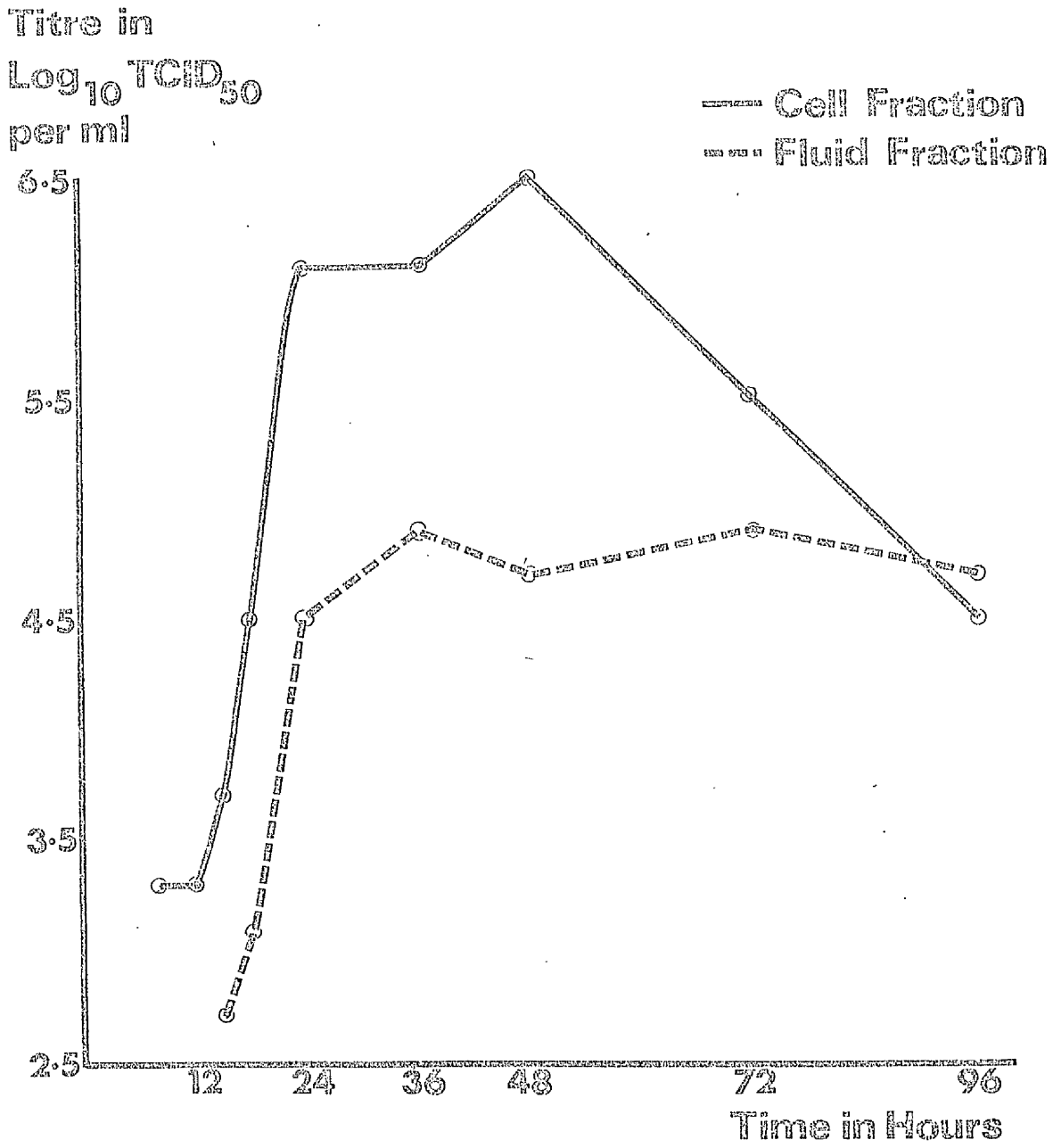


Figure 9. Growth of CDV under one-step, or nearly one-step, conditions.

PI) were harvested for infectivity assay, the fluid being collected separately for titration of the extracellular virus content. At 48 hours PI, the coverslips were stained with haematoxylin and eosin to provide an indication of the infection rate.. Inclusions were found in 72.5% of the cells examined under the x 40 objective (310 of 428 cells examined) but, as some were very small, a further count was carried out with the oil-immersion objective. This showed that approximately 80% of the cells contained inclusions.

The results of the infectivity titrations are shown in Figure 9, from which it will be seen that cell-associated virus first appeared at about 15 hours PI and then rose logarithmically until 24 hours PI, the titre at the latter time being $2.4\log_{10}$ higher than at the former. Virus production continued at a constant rate for the next 12-24 hours though there was a slight increase during the later part of this period, perhaps due to infection of the 20% of cells which had escaped infection at the start of the experiment. Between 48 and 96 hours PI the titre of the cell-associated virus fell by $2.0\log_{10}$, indicating that the end of the cycle was approaching. Nevertheless, although some syncytia were found at 48 hours PI, further advance of cytopathic change was slow and most of the cells still appeared healthy at the termination of the experiment, 96 hours PI. Little virus was released before 18 hours PI but over

the ensuing six hours, the titre of extracellular virus rose by $1.4 \log_{10}$. Release occurred at a fairly uniform rate over the 24-96 hour period though the proportion of virus released was small, cell-associated virus exceeding extracellular virus by $1.2\text{-}1.8 \log_{10}$ over the 24-48 hour period.

EXPERIMENT 2.2.

Ultrastructural Examination of Infected Cells at Different Stages of the Growth Cycle.

The aims of the present experiment were to examine the main ultrastructural events occurring during CDV infection, and to see how these events could be related to the growth curve already established (Experiment 2.1) Since, in Experiment 2.1, the presence of cellular aggregates during infection had apparently limited the infection-rate to around 80%, even when the input multiplicity was as high as 7, it was considered that a similar infection rate could be obtained with an input multiplicity of only 2 - 2.5. Moreover, since the number of cells required for the present experiment was at least several fold greater than that needed for Experiment 2.1, it would have been difficult to carry out the experiment at the higher multiplicity.

By the same methods as were employed in Experiment 2.1, 2.1×10^7 GH cells were infected in suspension with a total of $4 \times 10^7 TCID_{50}$ of virus and, following sedimentation and resuspension in fresh growth medium, were inoculated into seven 50 mm petri plates at a concentration of 3×10^6 cells per plate. After 8, 12, 18, 24, 48, 72 and 96 hours incubation at $37^{\circ}C$, one plate was removed from the incubator and the cells were

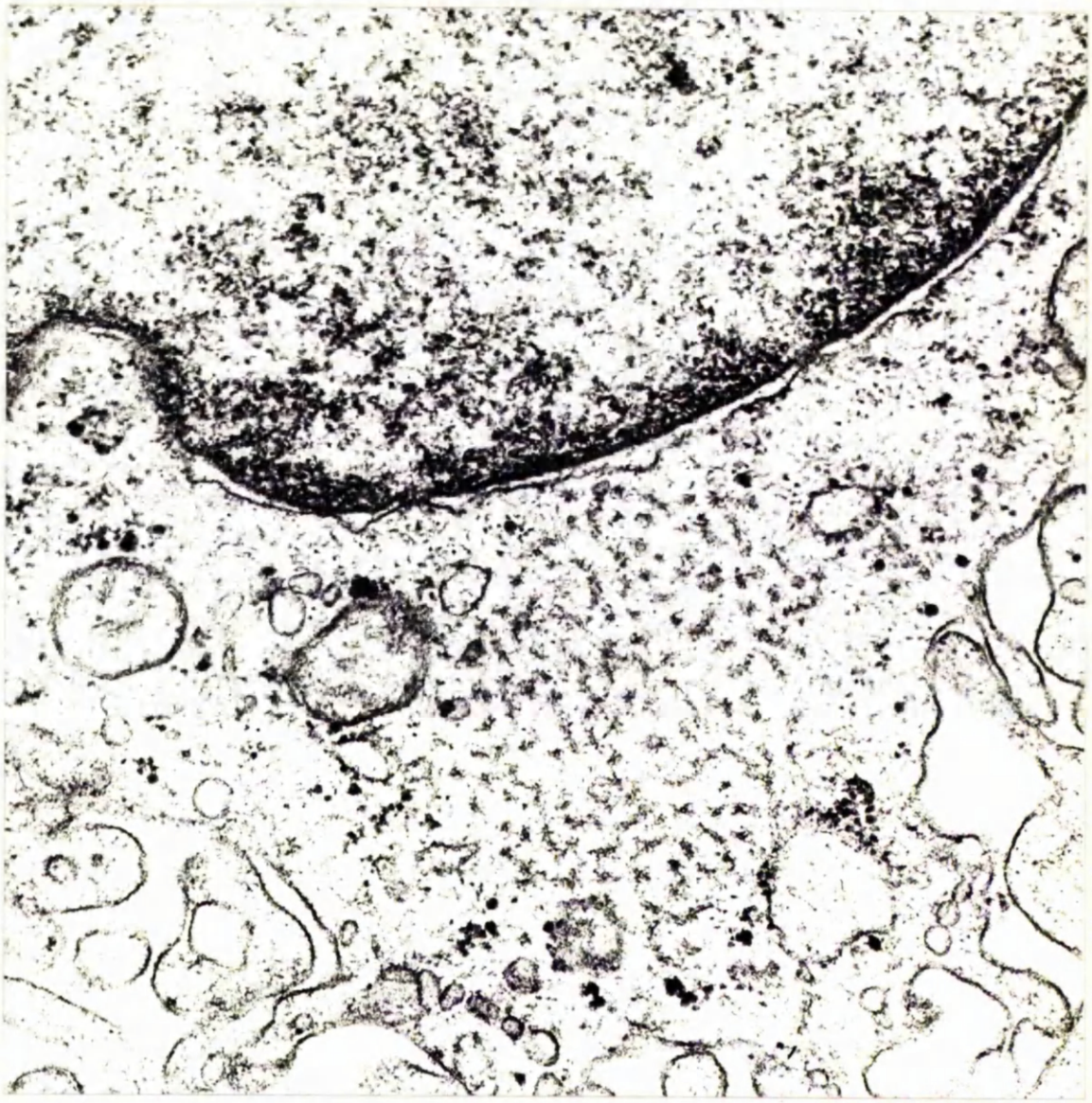


Figure 10. Small focus of nucleocapsid in a perinuclear position. X40,000

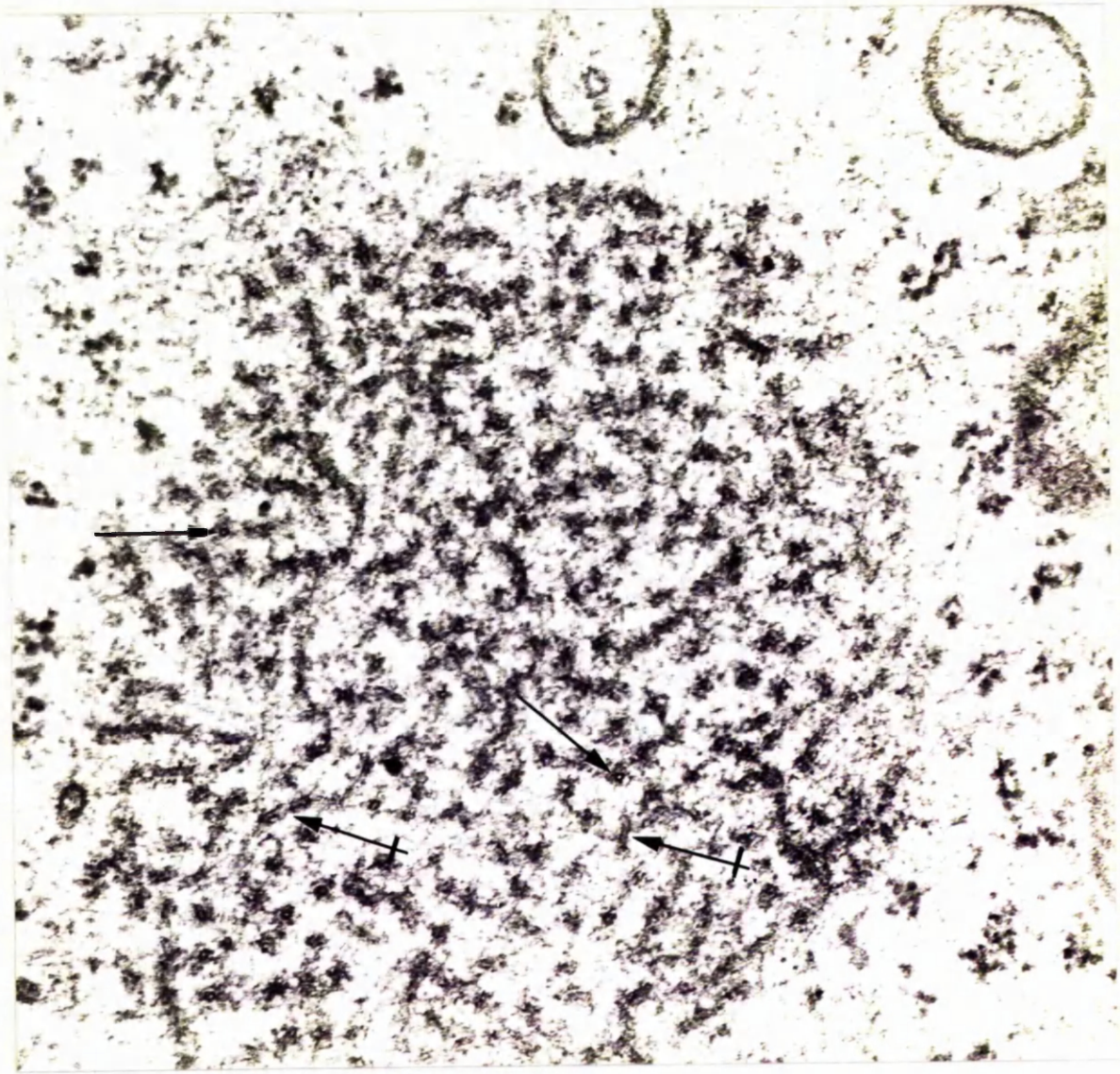


Figure 11. High power photograph of focus of nucleocapsid. Several cross-sections of nucleocapsid are visible (arrows) and short lengths are seen in longitudinal section (crossed arrows). The surface of the nucleocapsid is obscured by granular material. X80,000

harvested for electron microscopy.

No ultrastructural abnormalities were detected at 8 hours PI but, by 12 hours PI, small foci of nucleocapsid, most frequently in a perinuclear position, were readily found (Figure 10). The individual nucleocapsids in each focus appeared as flexible filaments, loosely interwoven so that only short lengths of each were visible in longitudinal section. When cut transversly, they appeared as sharply delineated cylindrical structures composed of an electron-dense membrane approximately 15 nm in diameter surrounding an electron-transparent axial space. Longitudinal and transverse sections merged with each other when the plane of section cut across an abrupt (right angle) change in the orientation of a filament. Despite the loose-knit appearance of the foci, with spaces of between 30 and 70 nm between filaments, the surface of the latter was often obscured by the adherence of fine granular material (Figure 11). No abnormalities were detected at or near the cell-surface.

At 18 hours PI, one or more foci of nucleocapsid was observed in most of the cells examined. Although larger than those present earlier, they appeared otherwise identical to them and again occupied mainly a perinuclear position. A few strands of nucleocapsid were

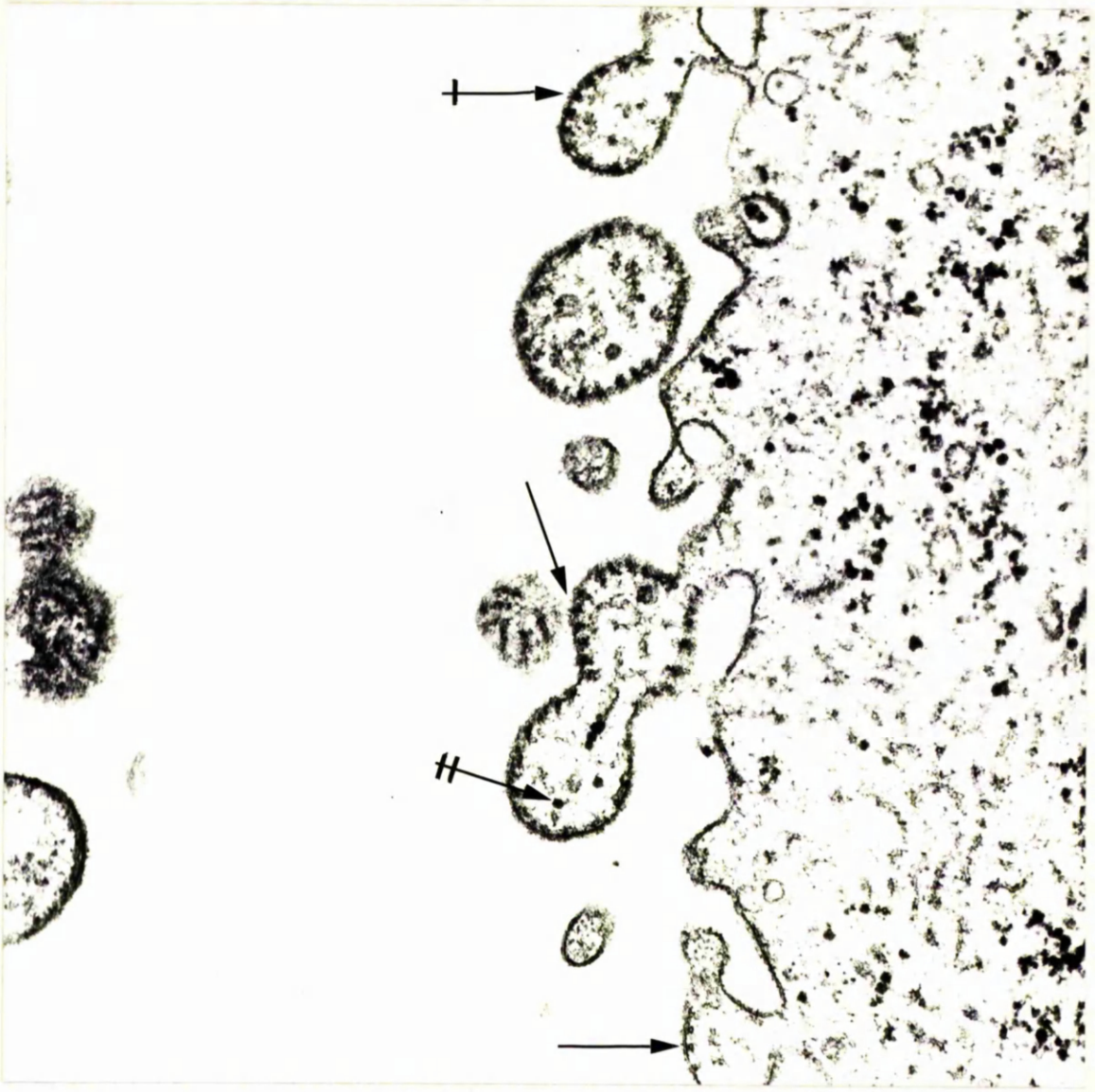


Figure 12. Budding of virus from the cell surface. Cross-sections of nucleocapsid can be seen spaced at regular intervals below the envelope of buds sectioned transversely (arrows). Tufts of fine projections can be seen on the surface of one of the buds (crossed arrow). Ribosome are present in another of the buds (double-crossed arrow). X60,000

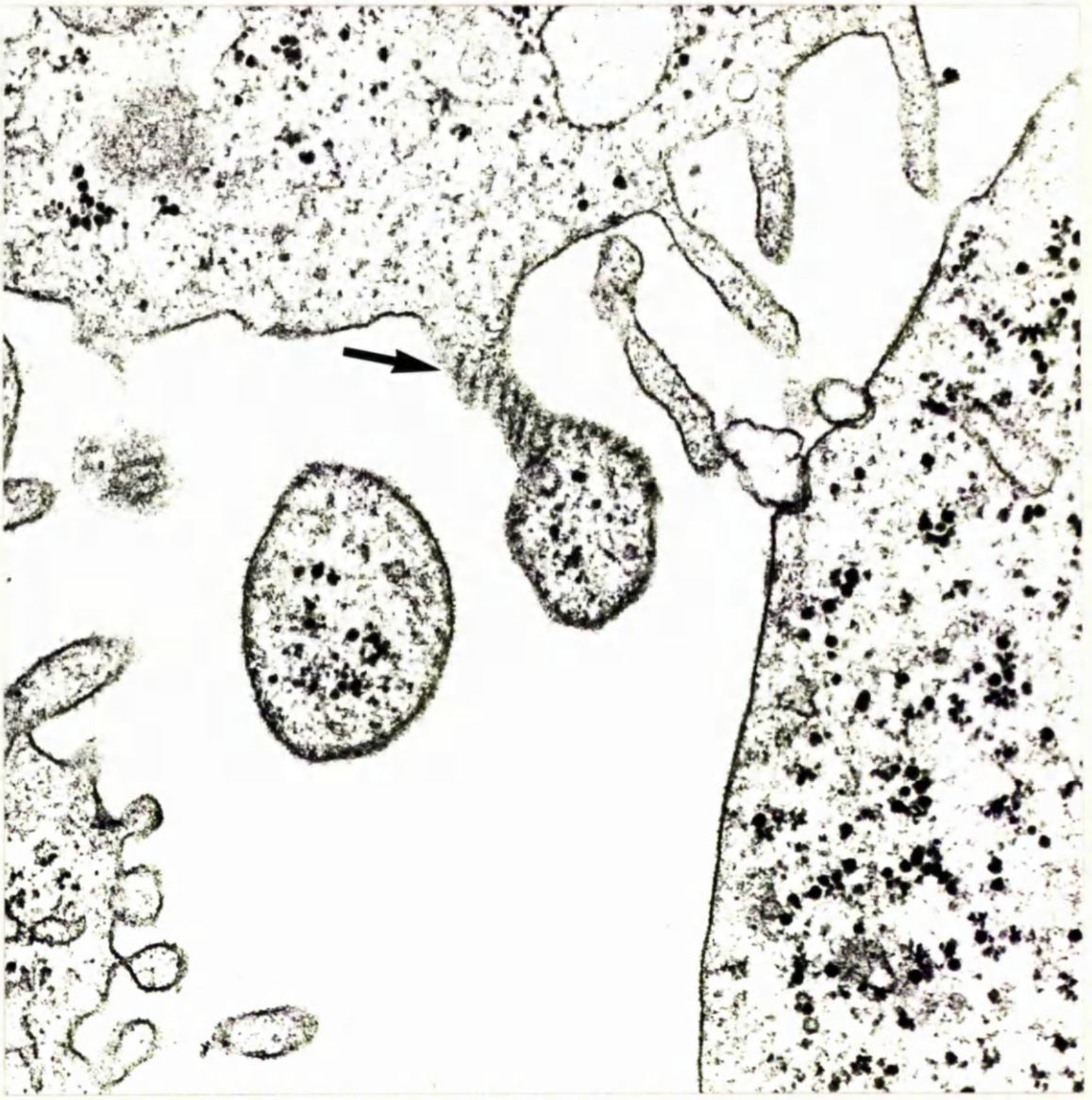


Figure 13. Virus budding from a tangentially-sectioned microvillus. Parallel bands of nucleocapsid can be seen crossing the surface of the microvillus (arrow). Ribosomes are again present in the developing virion. X60,000

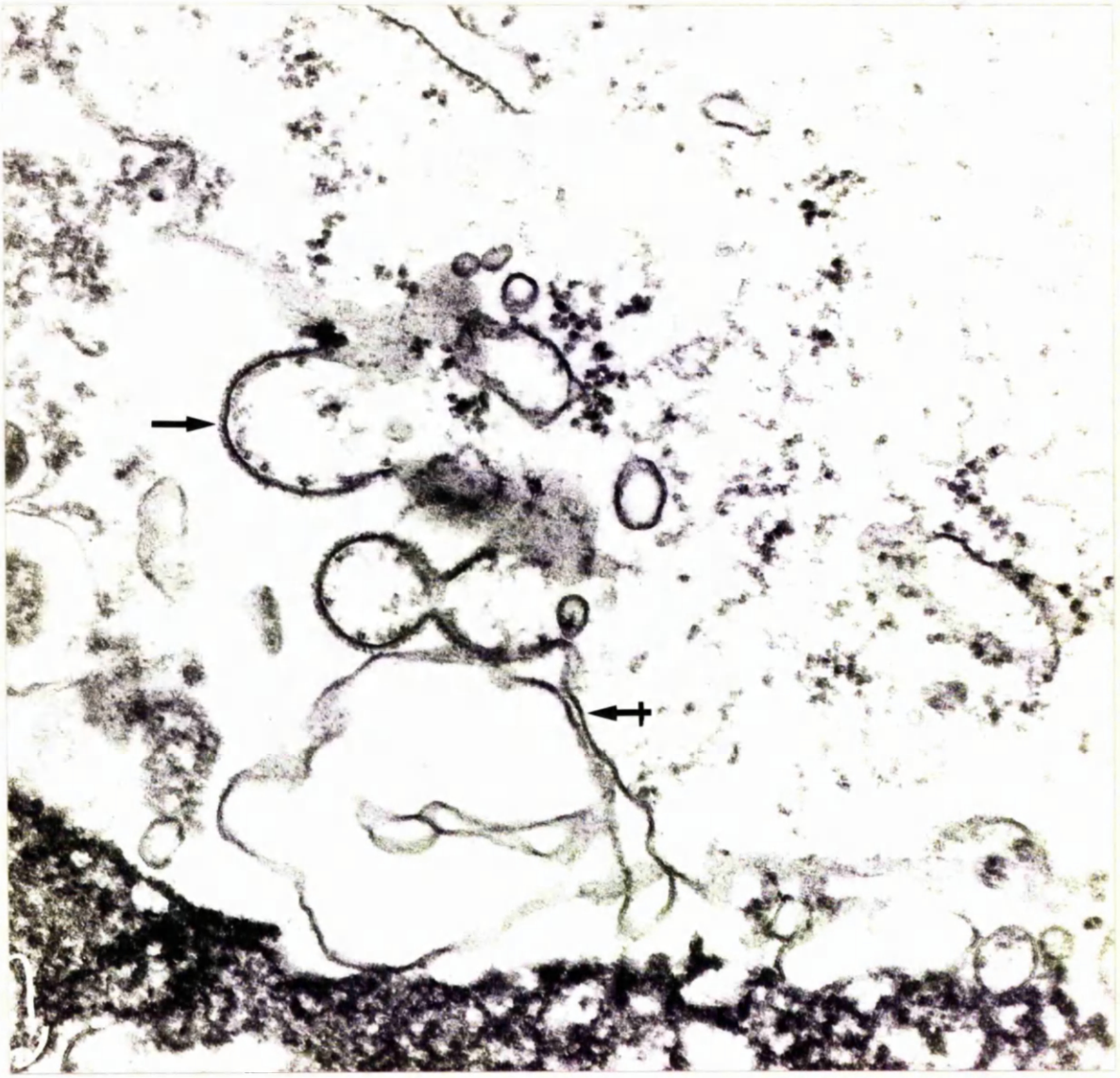


Figure 14. Transversely sectioned viral buds. The nucleocapsid can be seen in cross and longitudinal section and the fringe of surface projections (arrow) contrasts with the "clean" appearance of other parts of the plasma membrane (crossed arrow). X60,000

occasionally found near the cell-surface but the most significant development seen was the formation of virus buds (Figure 12). These were relatively few in number and arose from microvilli at least as frequently as from regions of normal contour. Budding was preceded by the alignment of nucleocapsid below the plasma membrane so as to form a symmetrical quasi-symmetrical configuration. Thus, parallel bands of nucleocapsid, 45-75 nm apart, were observed in tangentially-sectioned microvilli (Figure 13) while a row of cross-sections spaced at the same interval was found immediately beneath portions of transversely-sectioned plasma membrane (Figure 12). Very occasionally, several rows of cross-sections were seen, one below the other and all spaced at the same interval, but generally only a single row was visible. In these areas, a fringe of fine projections, often in tufts and 15 to 20 nm in depth, could be observed on the outer surface of the membrane, directly opposite the nucleocapsid (Figures 12 & 14). Virus maturation occurred by a protrusion and pinching-off of these areas.

At 24 hours PI, most cells examined contained several large foci of cytoplasmic nucleocapsid and virus maturation was considerably more advanced than at 18 hours PI. Numerous virus particles were seen budding from the cell-surface or lying free outside but close to the membrane. Circular, elliptical or dumb-bell like in outline and varying from 180 nm to 380 nm in diameter,

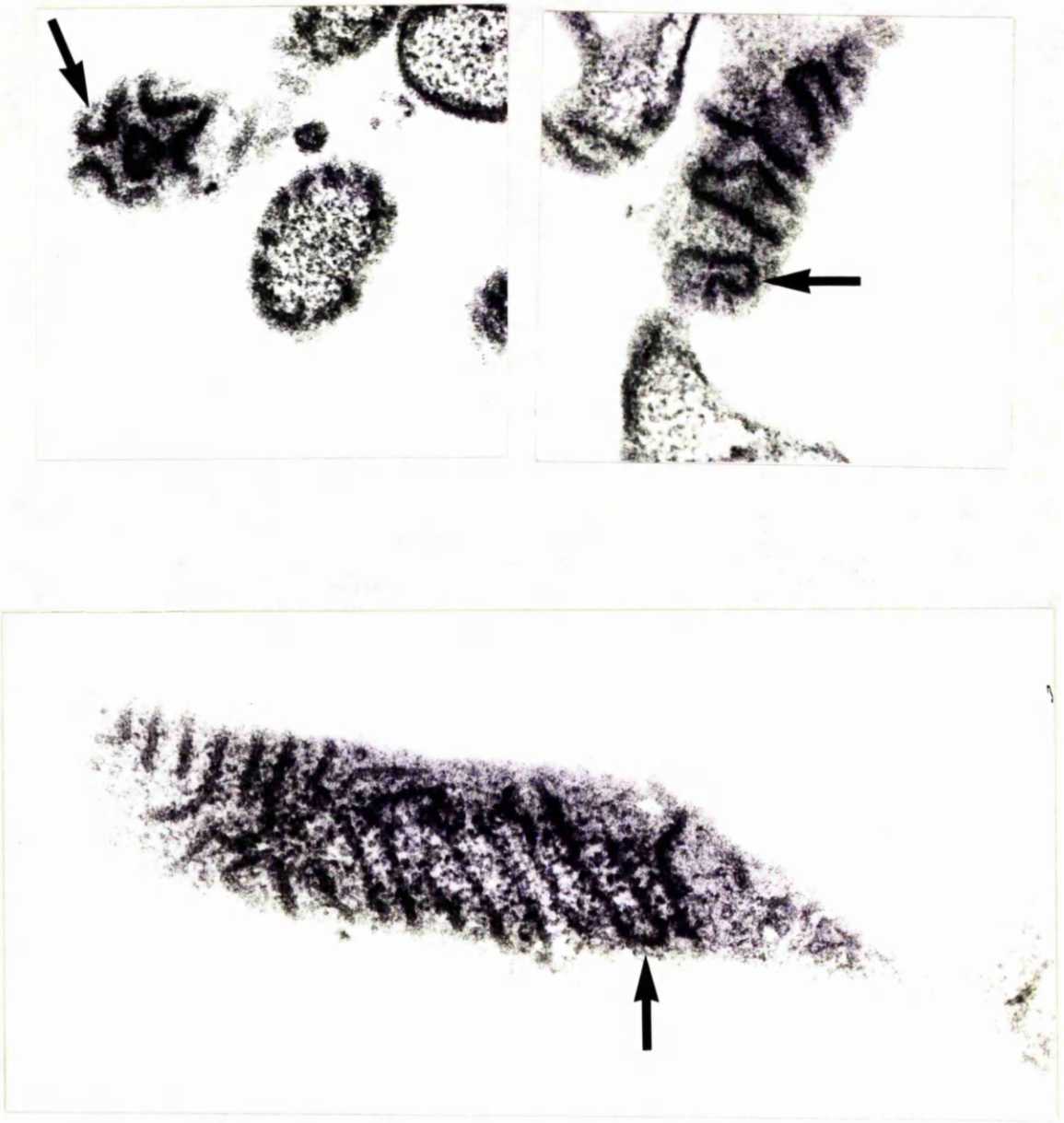


Figure 15. Composite of three tangentially-sectioned virus buds showing reversed loops of nucleocapsid (arrows). X70,000

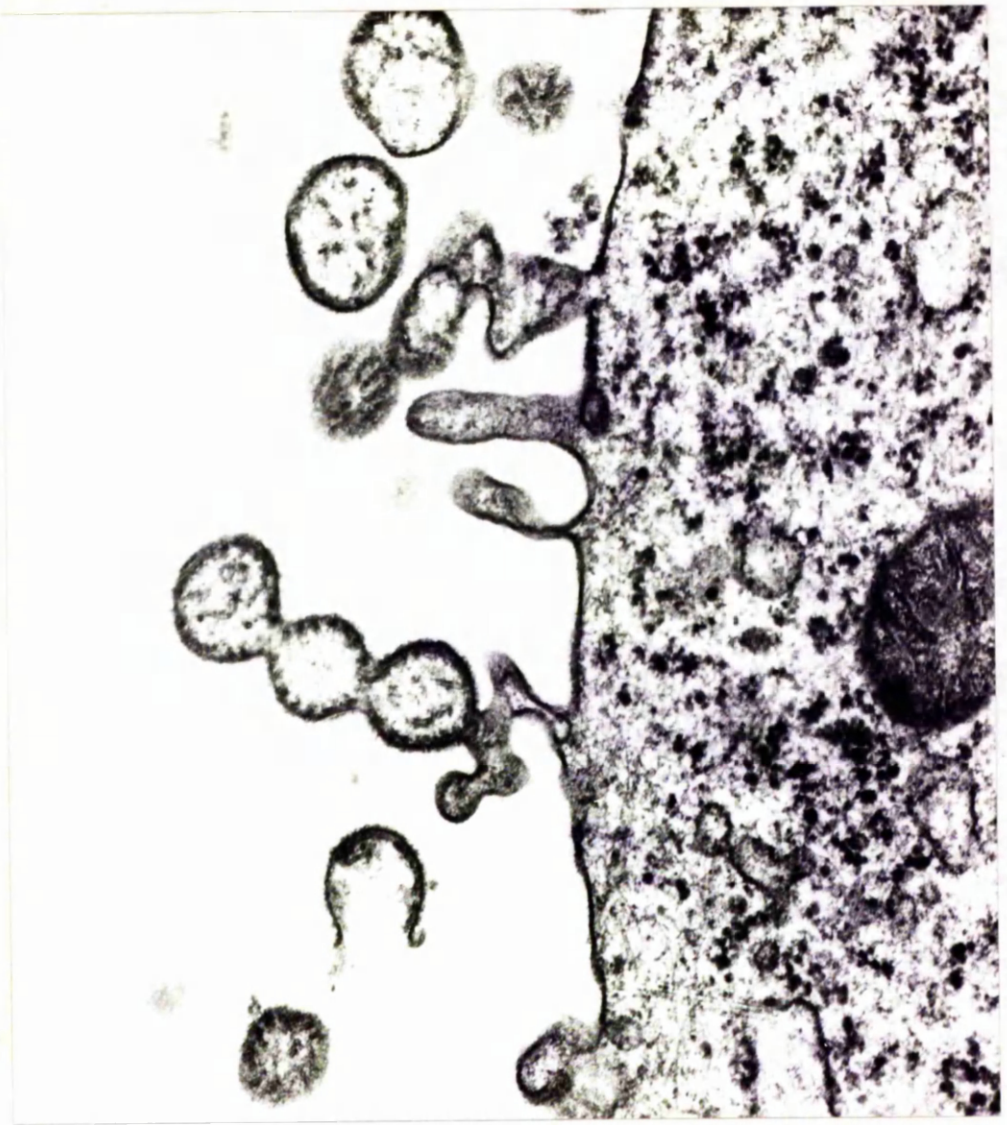


Figure 16. Three virions budding from a single microvillus. X50,000

they could readily be identified by their envelope, with its fringe of fine projections 15 to 20 nm in depth, and by the nucleocapsid arranged in loops round the inner surface of the envelope. Thus, in particles sectioned tangentially, the nucleocapsid appeared as a number of parallel bands crossing the surface of the particle, though, in some particles, the filament was seen to form reverse lopps (Figure 15); those cut transversely had a clock-faced appearance, the circular cross-sections of the nucleocapsid being spaced at regular intervals round the inside of the envelope. In both planes, adjacent filaments were 45 to 75 nm apart. The "core" of the particles consisted of normal cytoplasmic constituents, although strands of nucleocapsid could sometimes be identified therein. Thus, ribosomes and microvesicles were occasionally found inside the virions (Figures 12 & 13).

Virus budding was seen in each of the later samples examined though less was found in the 96 hour sample than in those taken at 24, 48 and 72 hours PI. The process was often limited to certain parts of the cell-surface, particularly those with microvilli, large parts of the remainder apparently being free of viral activity. As many as five virions could occasionally be seen budding from a single microvillus (Figure 16), yet, despite the presence of many buds in one small area, nucleocapsid was often completely absent from the cytoplasm underlying

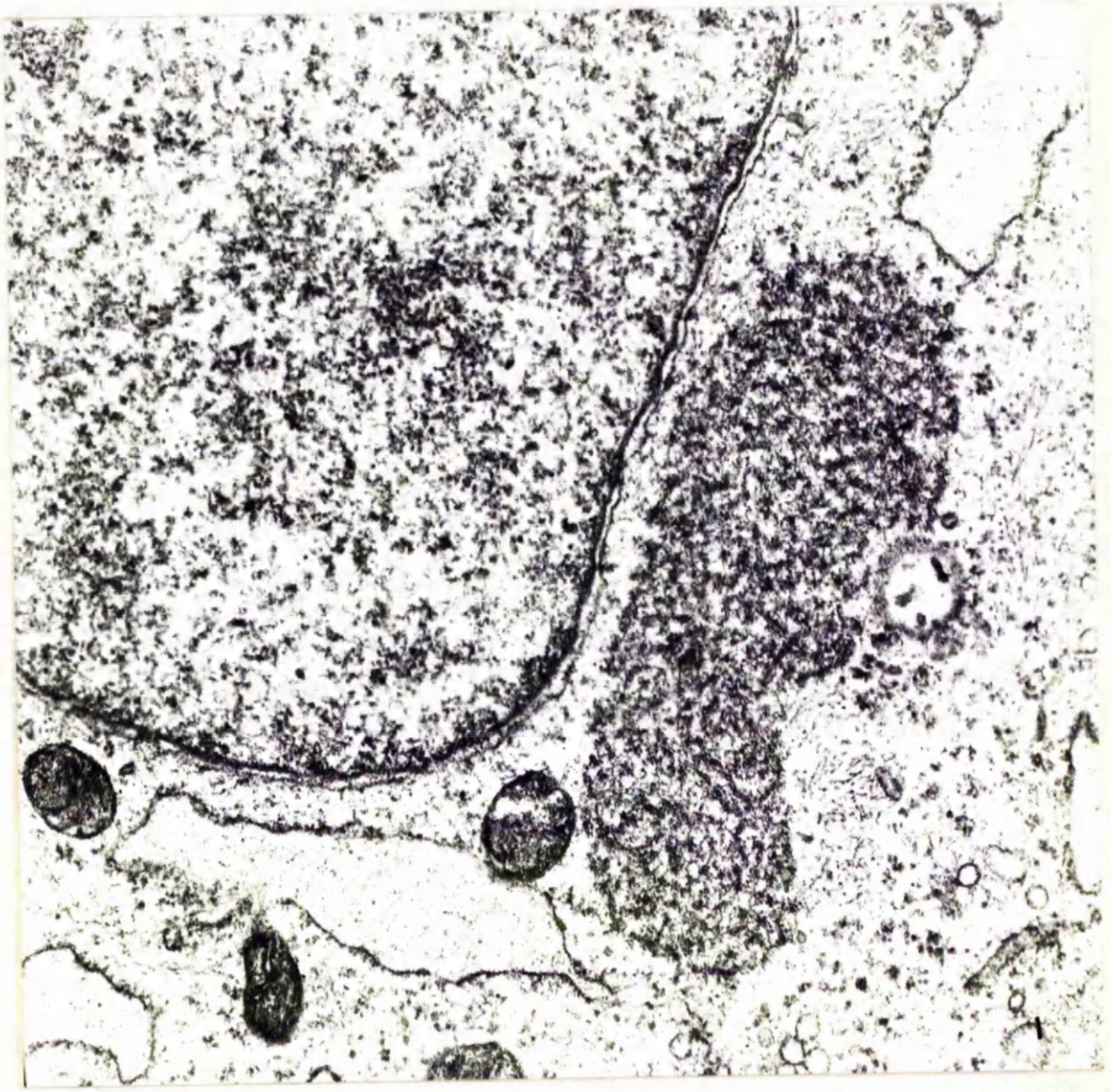


Figure 17. Early stage in the development of the cytoplasmic inclusion body. The aggregate of nucleocapsid appears compact and distinct from the surrounding cytoplasm. X40,000

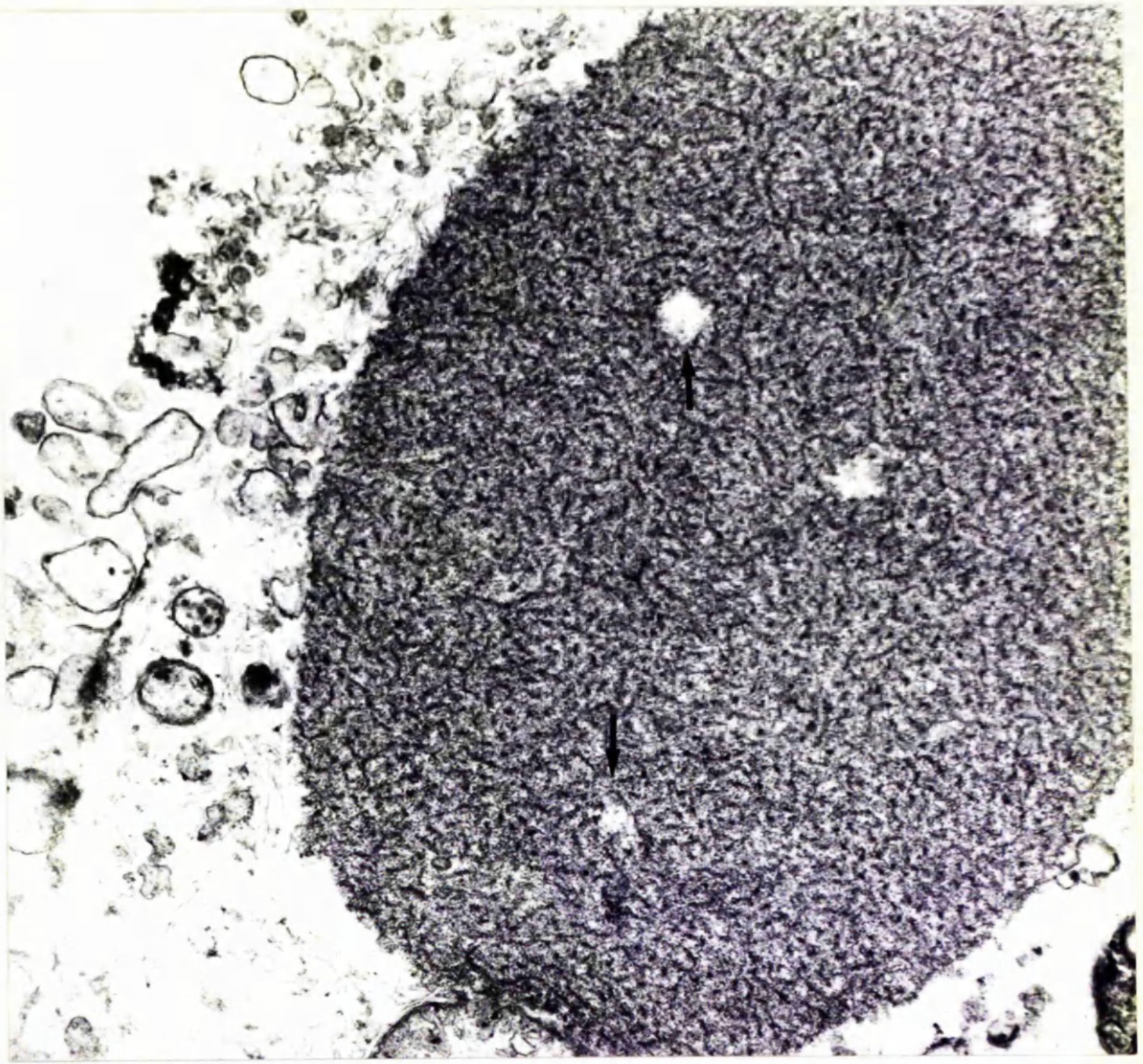


Figure 18. A cytoplasmic inclusion body. Despite the large amount of granular material present, the nucleocapsid is still distinguishable. Several small empty pockets are visible (arrows). X30,000

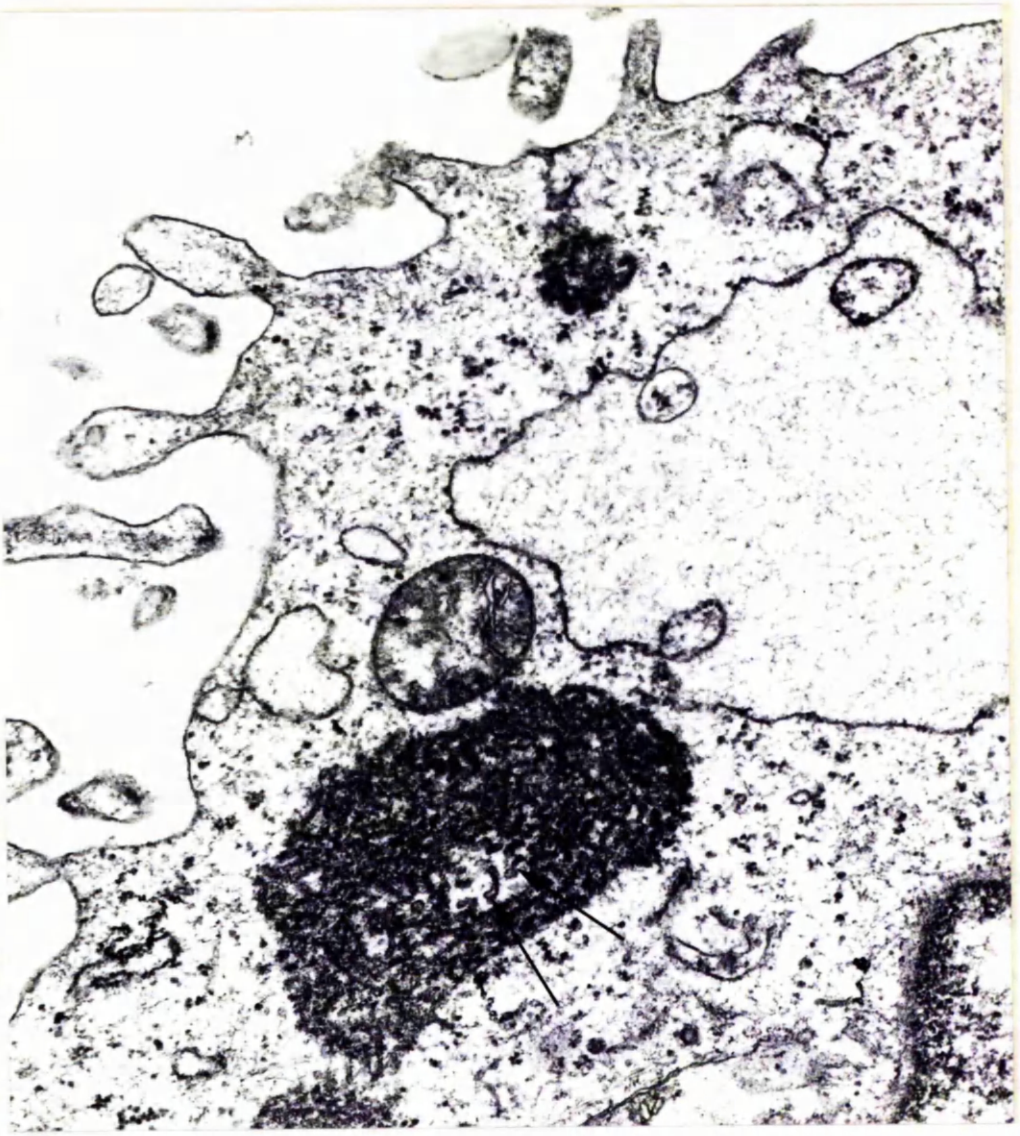


Figure 19. Small cytoplasmic inclusion body. Sacular bodies are present within an electron-lucent pocket, (arrows). X32,000

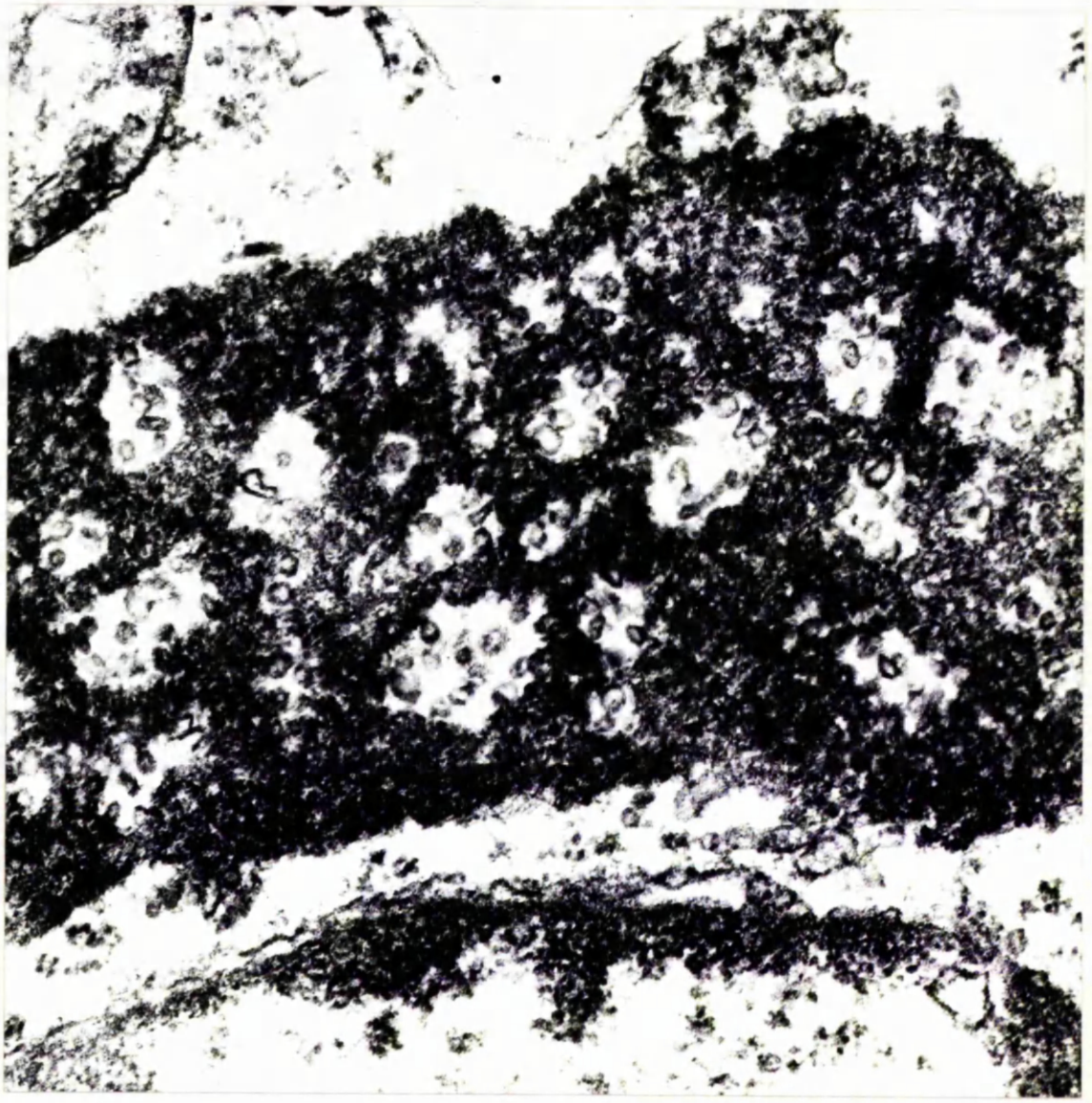


Figure 20. Detail of a cytoplasmic inclusion body showing electron-lucent pockets containing microvesicles (arrows). X60,000

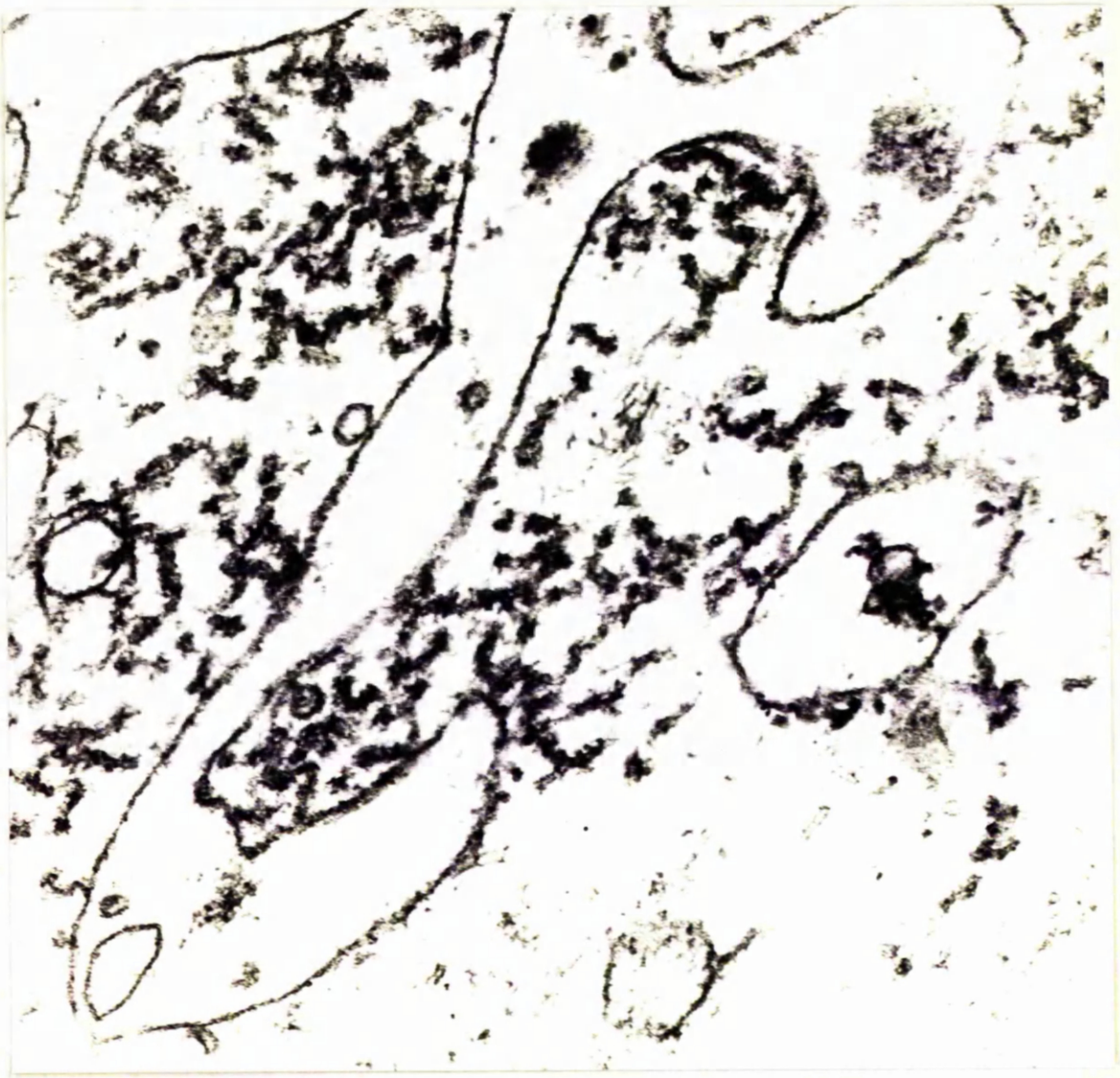


Figure 21. Nucleocapsid-like filaments beneath the cell-surface. The filaments appear coated with electron-dense material. There is no alignment or brush border. X80,000

that area.

At 48 hours PI, the foci of cytoplasmic nucleocapsid were much more compact than those observed earlier and this was at least partly due to a deposition of fine, electron-dense granular material around and between the filaments (Figures 17 & 18). In some instances, the deposit was sufficiently heavy to almost completely occlude the filaments. In many cases, deposition of electron-dense material was accompanied by the appearance of several electron-lucent pockets within the inclusion (Figures 18 & 19). Mainly lying within these pockets but also found round the edge of the inclusion were a number of short tubular or sac-like structures 25 to 40 nm in diameter (Figure 20). Similar inclusions were found at 72 and 96 hours PI, though with increasing time, the density of the granular material tended to become greater.

From 48 hours PI onwards, the surface of the cells became increasingly villous. At the same time, compact masses of electron-dense filaments, approximately 25 nm in diameter, appeared near the cell-surface and within the numerous microvilli present. However, in no instance was a brush border seen on the overlying plasma membrane, nor was there any evidence of alignment (Figure 21). Small knots of filaments containing much electron-dense granular material and surrounded by plasma-membrane were frequently found beyond the cell-surface in these areas

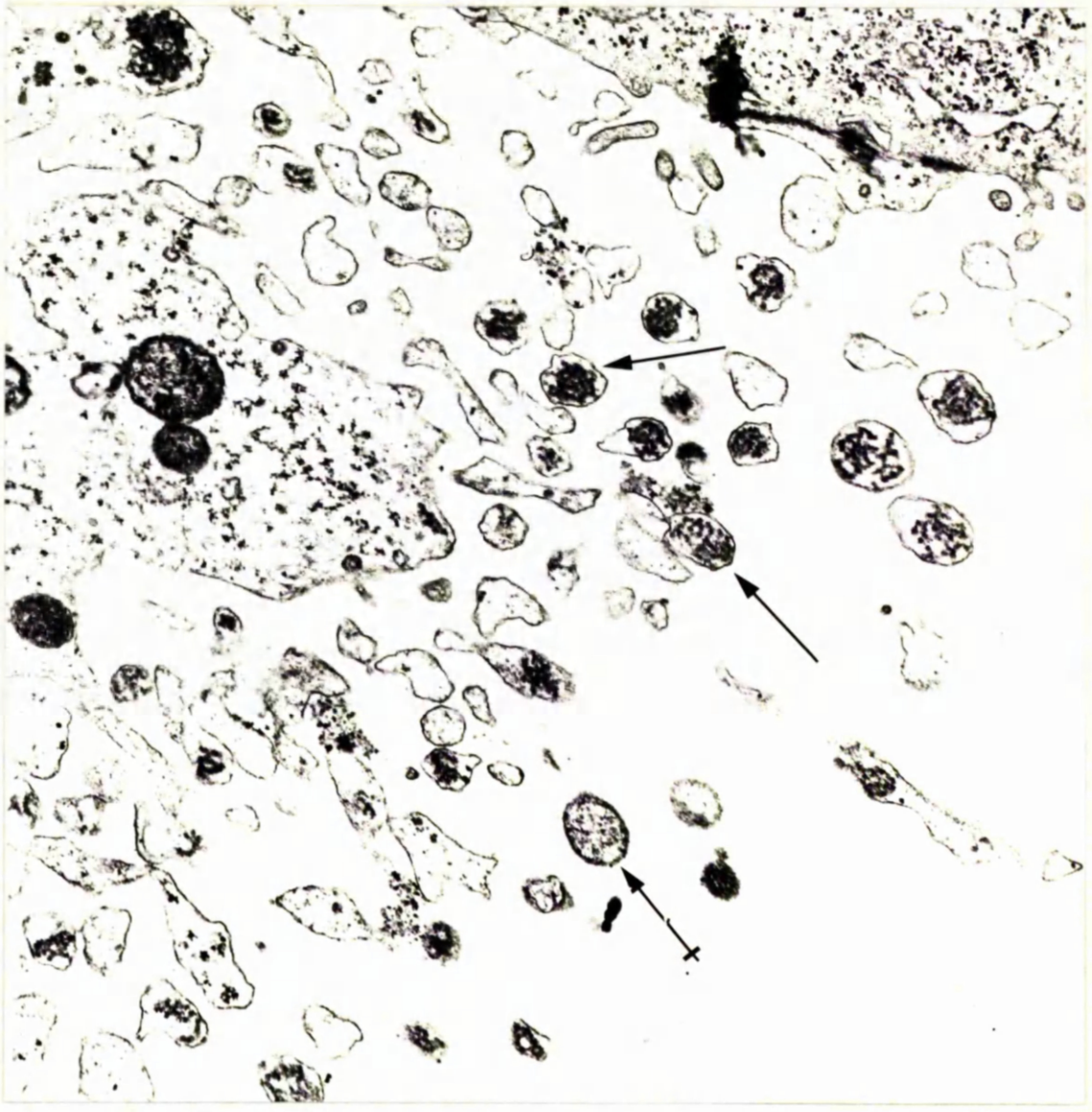


Figure 22. Small knots of nucleocapsid-like filaments within microvilli at the cell-surface (arrows). A single virus particle is also present (crossed arrow). X25,000

(Figure 22) but whether these were attached to the cell beyond the plane of section or actually pinched off from the cell could not be ascertained.

EXPERIMENT 2.3

An Ultrastructural Study of Cells in the Terminal Stages of Infection.

As far as the author is aware, no studies have been published on the ultrastructural appearance of cells in the terminal stages of CDV infection. As described in Part One, older syncytia are recognised by their refractile character, and by their "contracted" or three-dimensional shape, sometimes with branches, and often bearing a large, transparent vesicle. Since these changes occur as a prelude to detachment from the substrate, it was thought that, for study of the terminal stages of infection, sufficient of these degenerate syncytia could be obtained from the fluid of cultures showing an advanced CPE.

Four 8oz. bottles of infected GH cells were prepared. By the sixth day PI, an advanced CPE was present. The medium was discarded, and the cell sheet washed three times with PBS to remove any syncytia already detached. Fresh maintenance medium was added to each of the cultures which were then reincubated for three hours. During this time, a considerable number of syncytia detached. The medium was therefore collected and pooled. By this method, only those syncytia detaching during the previous three hours were harvested for study. The pooled medium was transferred to four centrifuge tubes and the syncytia were pelleted by centrifugation at 1,000 rpm for 5 minutes. One of the four

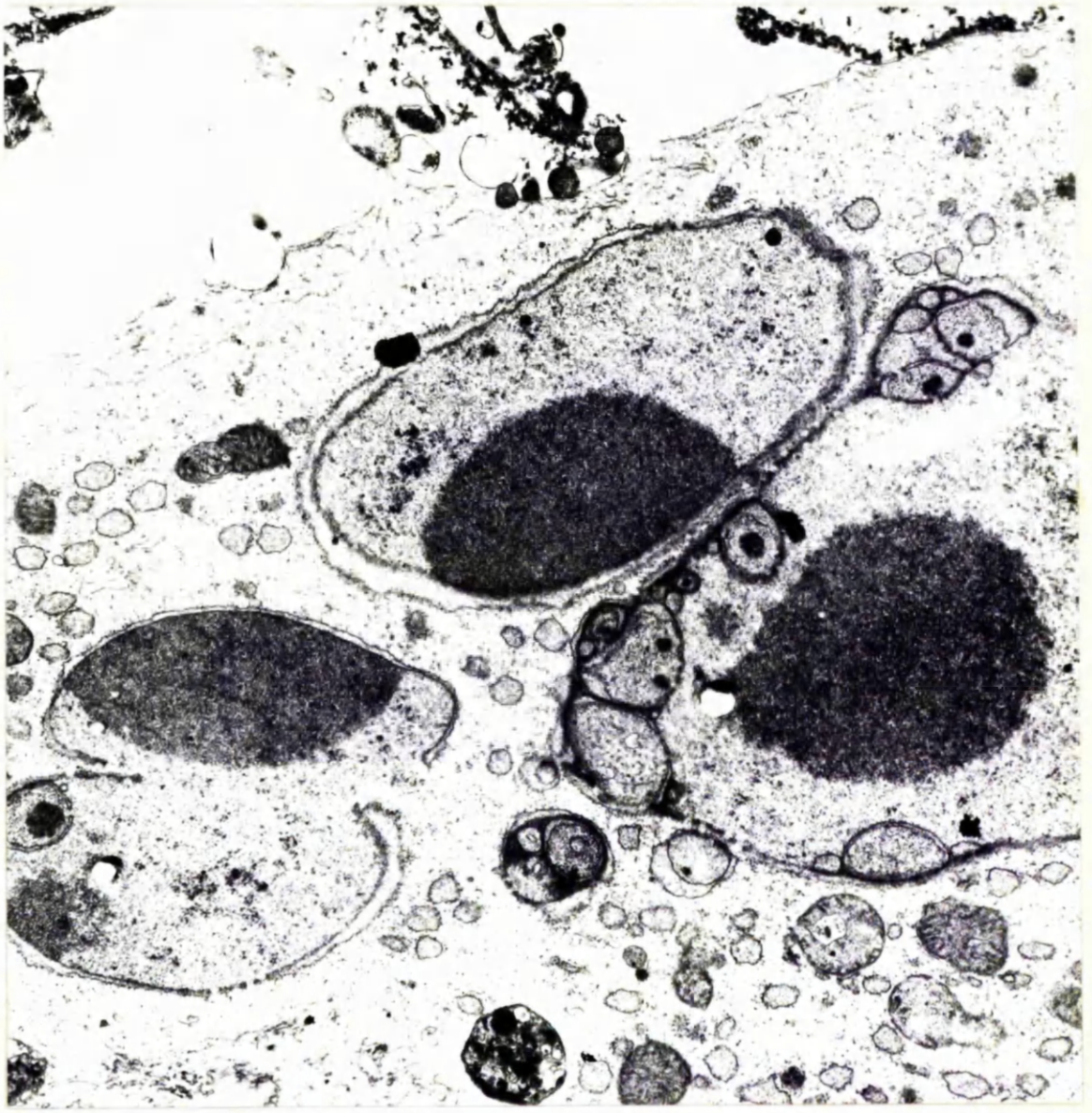


Figure 23. A degenerate syncytium with destruction of the nuclear and cytoplasmic membranes and bulbous ingrowths of the nuclear membranes. The large nuclear "inclusions" seem to be nucleoli. X10,000

pellets was processed for electron microscopy as previously described. The other three were washed once, then resuspended in 15 ml of maintenance medium and divided into three aliquots, each of which was then incubated at 37°C. After 1, 5 and 15 hours, an aliquot was harvested for assay of its infectivity. The most outstanding ultrastructure features of these cells were nuclear damage, intracytoplasmic vacuolation, duplication of cytoplasmic membranes and alignment of nucleocapsid beneath membranes both at the cell-surface and round the intracytoplasmic vesicles.

Considerable variation existed in the severity of the nuclear changes, even between nuclei of the same cell. In some instances, abnormality was limited to a separation of the two layers of the nuclear membrane but, in most cases, the peripheral zone of the nucleus was occupied by a number of sac-like structures apparently formed by infoldings of the nuclear membrane (Figure 23). Large gaps were present in the membrane of some nuclei and in these regions a gradual merger of the cytoplasm and of the more finely granular and markedly denser nucleoplasm could sometimes be seen. In extreme cases, portions of nuclear membrane, identifiable as such by their bulbous ingrowths, appeared free in the cytoplasm. Most nuclei contained an amorphous, finely granular, electron-dense inclusion which often lay against one pole of the nucleus (Figure 23).

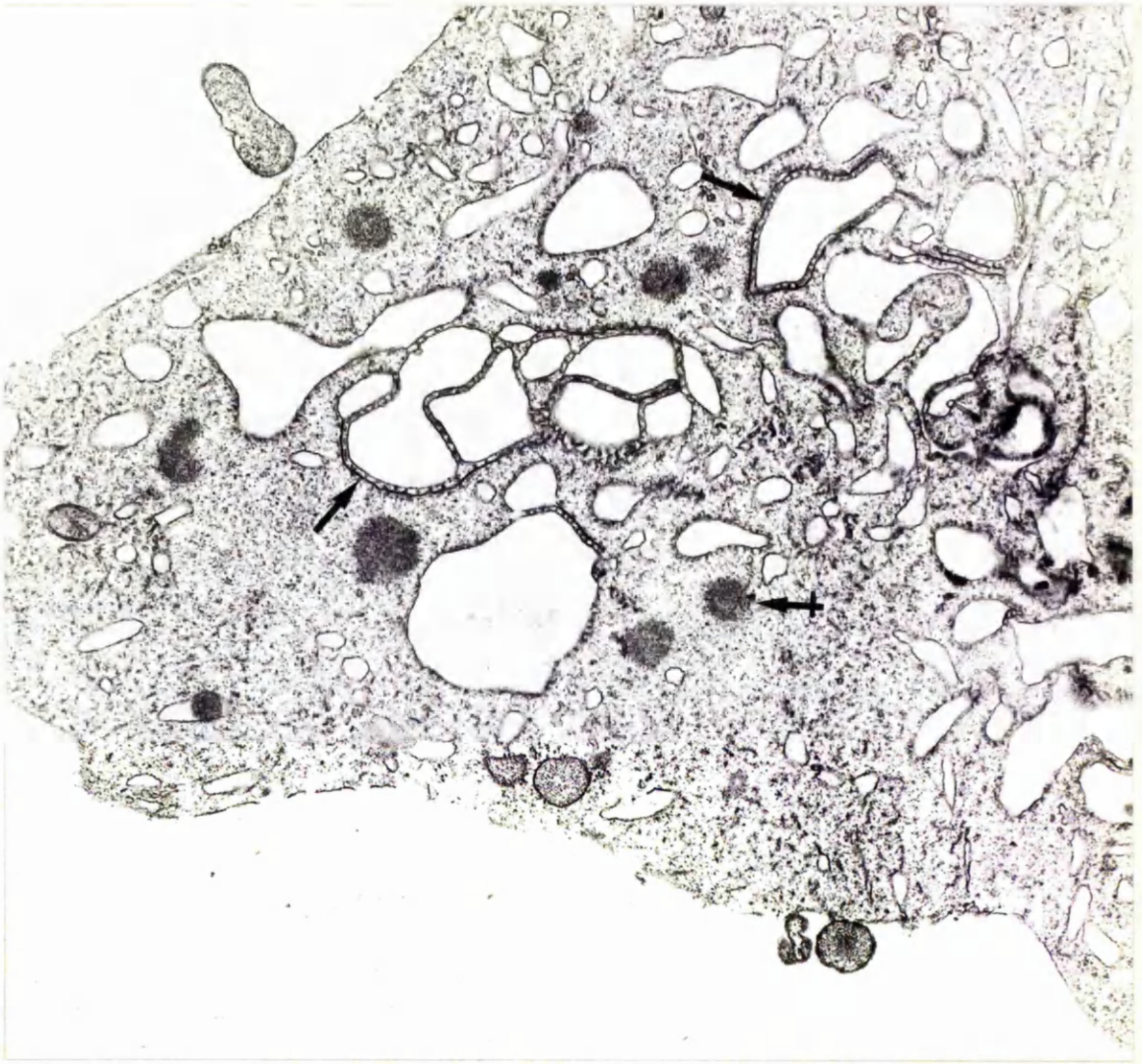


Figure 24. A degenerate syncytium containing many vesicles, some of which are lined with a double membrane (arrows). Cross-sections of nucleocapsid are present between the two layers of double membrane. Numerous electron-dense, granular bodies are also present (crossed arrow). X20,000

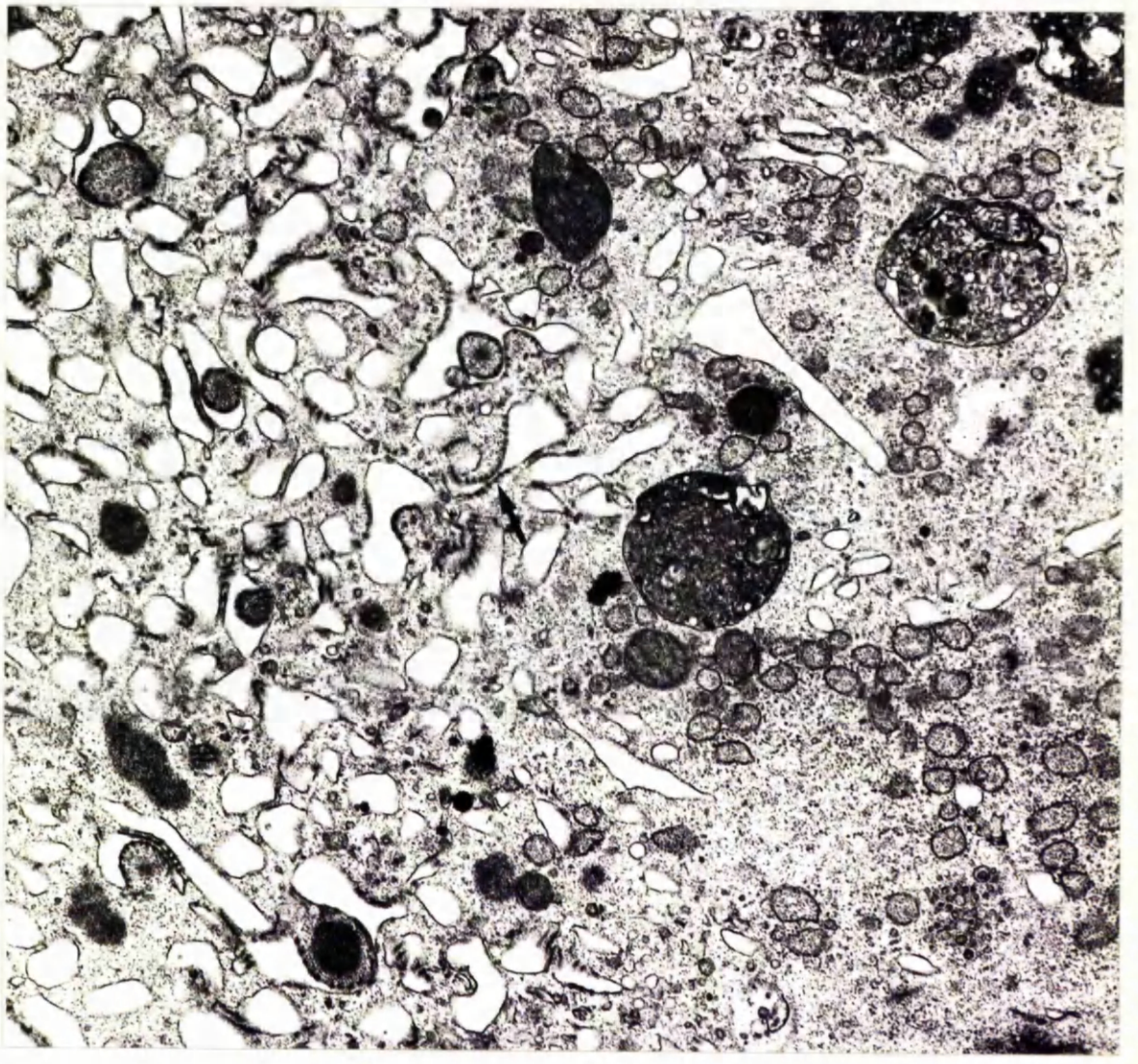


Figure 25. A degenerate syncytium containing many vesicles. Alignment of nucleocapsid can be seen beneath the membranes of tangentially-sectioned vesicles (arrow). Numerous electron-dense granular bodies are present and some of these are being incorporated into virions budding into the vesicles. X15,000

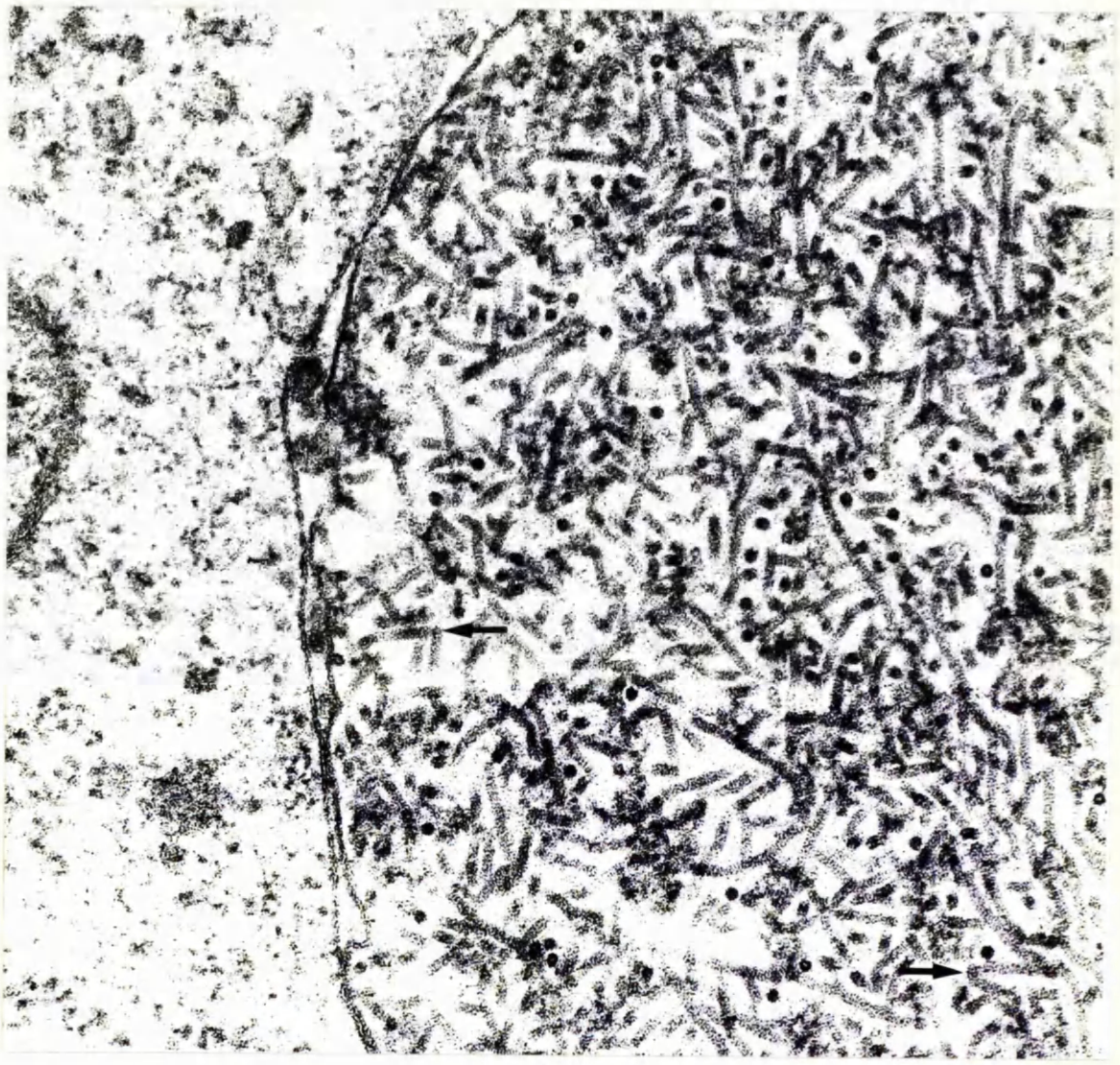


Figure 26. An aggregate of nucleocapsid within a double membrane-lined cavity. The nucleocapsid is not obscured by granular material and appears more rigid than that found free in the cytoplasm. Abrupt changes of orientation are seen (arrows). X80,000

The syncytia were generally very regular in outline with little or no villus formation. In most instances, the density of the cytoplasm, i.e. the concentration of the granular matrix and the spacing of organelles, seemed normal and no structures corresponding to the large vesicles seen with the light microscope were observed. The absence of the latter structures was presumably related to their susceptibility to disruption during centrifugation etc. The most outstanding feature observed at the lower magnifications used was the presence of large numbers of membrane-bound vesicles especially in the more peripheral areas of the cytoplasm, (Figures 24 & 25). These were particularly conspicuous beneath the cell-membrane and were sometimes orientated parallel to it. In some cells, they were bound by double or even triple membranes.

Numerous loose-knit aggregates of nucleocapsid, similar to those described previously, were present throughout the cytoplasm but no evidence was found of cytoplasmic inclusion bodies. However, in a very small proportion of cells, a large aggregate of nucleocapsid was found segregated from the cytoplasm by double membranes (Figure 26). Unlike that previously described, this nucleocapsid consisted of long rigid filaments, whose outline was rendered unusually distinct by virtue of the absence of surrounding granular material.

Alignment of nucleocapsid beneath cytoplasmic membranes was extensive and occurred not only at the outer

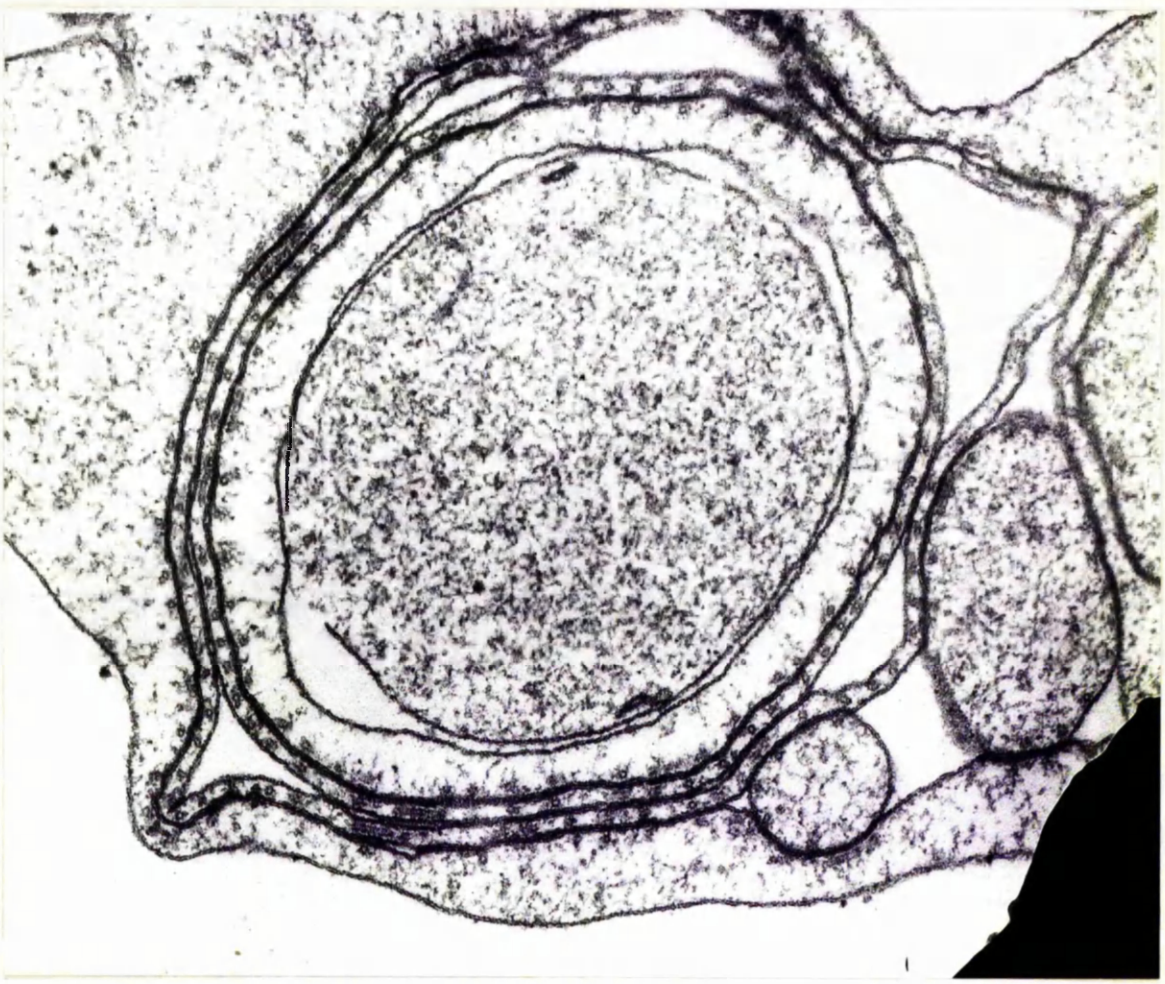


Figure 27. Duplication of cytoplasmic membranes in a degenerate syncytium. Cross-sections of aligned nucleocapsid can be seen between the concentric bi- or tri-laminar membranes but no brush border is visible. X60,000

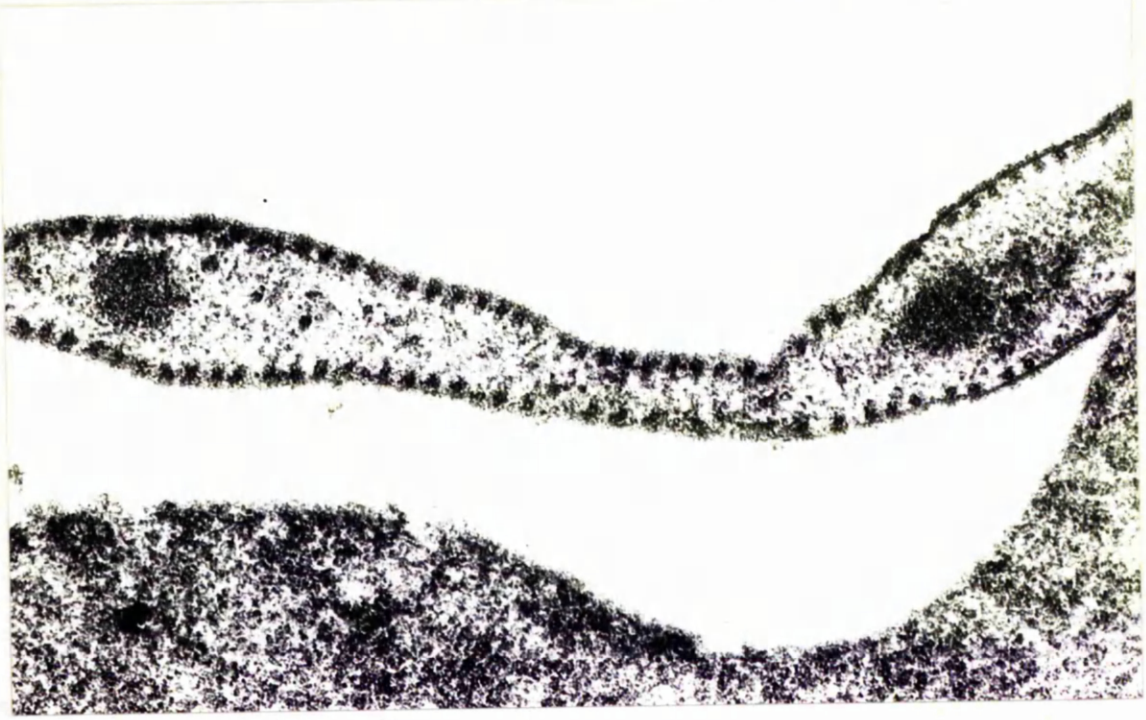


Figure 28. A filamentous viral bud containing two electron-dense granular bodies similar to those in the cytoplasm of degenerate syncytia. X70,000

plasma membrane but also at the membranes lining the intracytoplasmic vesicles. However, the characteristic virus-induced transformation of the membrane, i.e. the formation of a brush border, was generally absent. Figure 27 shows a striking, but by no means unique, example of this. As can be seen, several membranes are present and in many areas, fusion of two adjacent membranes has resulted in the formation of tri-laminar structures. Nucleocapsid has aligned in the cytoplasm enclosed by, and on either side of , these membranes, none of which have a brush border.

Budding of virus was rare in comparison to the degree of alignment present, this observation being supported by the results of the infectivity titrations, shown in the table below.

Sample	Titre in $\log_{10}TCID_{50}$ per ml.
1 hour	2.5
5 hours	2.9
15 hours	0.7

These figures show that only a small amount of virus was produced by these cells, and that this production was only maintained for a short time. With the electron microscope, it was found that some of these virus particles contained a membrane-bound vesicle while others contained a discrete, round, electron-dense granular body (Figure 28). Similar dense bodies were sometimes seen in the cytoplasm of the cells adjacent to the budding virus particles.

DISCUSSION.

Despite the use of an input multiplicity of 7, only 70-80% of the cells were initially infected, as judged by the development of inclusion bodies at 48 hours PI. This may have been because, despite attempts to eliminate them, clumps of cells were present in the cell suspension at the time of infection, the cells in the middle of the clumps being less accessible to the virus. If this was the case, it is clearly impossible to obtain a one-step growth curve in this manner. Infection of cells already in a monolayer might be expected to eliminate this problem, but from the results of Experiment 1.6, cells in a monolayer seem more resistant to infection. The growth curve obtained in the present investigation may therefore have to be accepted as the closest to the one-step curve that can be attained. In any case, had 100% of the cells been infected, the titre at 24 hours PI would have been $\frac{100}{80}$ times higher, i.e. only 0.1 $\log_{10}TCID_{50}$ per ml higher than the titre actually reached. The shape of the curve obtained was therefore very similar to that which would have been produced with an initial infection-rate of 100%.

The results of Experiment 2.1 and 2.2 showed that

the eclipse phase lasted for between 15 and 18 hours. Cell-associated virus detected prior to 15 hours presumably represented virus from the inoculum. Between 15 and 24 hours PI, the cell-associated virus increased approximately 250-fold. The titre remained constant between 24 and 36 hours PI but rose slightly (2.5-fold) between 36 and 48 hours PI. Thereafter, production waned, a 100-fold reduction occurring during the succeeding 48 hours. The shoulder at 48 hours may have been the result of a second cycle of infection occurring in the 20 to 30% of cells which had escaped initial infection. However, since the increase in titre was greater than expected from the number of cells available for a second cycle of infection, it would seem that the cells infected at the outset must still have been producing virus at their maximum rate at 48 hours PI. On this basis it was concluded that virus production occurs at a constant rate between 24 and at least 48 hours PI. The titre of extra-cellular virus increased by only 2.5-fold between 15 and 18 hours PI, but then rose 25-fold during the succeeding six hours, a finding which correlated well with the electron microscopic observations. After a further slight increase (2.5-fold) at 36 hours PI, the titre of extracellular virus remained constant.

Growth curves obtained by infecting cells with CDV under one-step conditions or nearly one-step conditions have been published by Bussell and Karzon (1962) and

Cornwell et al. (1971). That reported by Bussell and Karzon (1962) for the growth of the Onderstepoort strain of virus in chick embryo fibroblast cells had an eclipse phase of only eight to 10 hours and extracellular virus was present from between 10 and 13 hours. The titres then increased until 18 hours PI and then remained constant until 24 hours PI. A further increase produced a peak between 30 and 60 hours PI, probably due to a second cycle of infection. Cell-associated virus exceeded extracellular virus by 1.5 $\log_{10}$PFU per ml at the peak. The eight to 10 hour eclipse phase contrasts with the 15 to 18 hour eclipse phase found in the present investigation. In their studies of the Glasgow 841 strain in MDCK cells, Cornwell et al. (1971) did not determine the length of the eclipse phase but found that the titre of cell-associated virus fell by 1.0 $\log_{10}$ between 24 and 48 hours PI. Extracellular virus reached a maximum titre at 48 hours PI and thereafter closely paralleled the cell-associated virus. In terms of $TCID_{50}$ per infected cell, the yield of cell-associated virus varied from approximately 7 at 24 hours PI to 0.6 at 48 hours PI, whereas, in the present investigation, it ranged from 2.5 to 5.3 over the same period. Since electron microscopy usually revealed more than five virus particles in a single ultrathin section of an infected cell, it is obvious that the actual number of virions at the cell-surface was much higher than the yield of infectious virus might suggest.

In the ultrastructural study (Experiment 2.2), the first evidence of viral infection was the development of aggregates of nucleocapsid in the cytoplasm. These aggregates, which occurred mainly in a perinuclear position, were present by 12 hours PI and increased in number and size during the following 12 hours and persisted throughout the growth cycle. The nucleocapsids in these appeared to be flexible and loosely interwoven and were intermixed with fine granular material which made their outline somewhat indistinct. Due to this latter feature, such nucleocapsids have been termed "fuzzy" nucleocapsids by Dubois-Dalq et al. (1974) as opposed to "smooth" nucleocapsids which lack such surface material.

In the 18 hour sample, alignment of the "fuzzy" nucleocapsid was seen beneath portions of the cell membrane possessing a characteristic brush border of fine surface projections. As the brush border and the aligned nucleocapsid always accompanied each other at this stage, it was impossible to determine whether the aligned nucleocapsid initiated the development of the brush border of vice versa. Virions were formed by protrusion and pinching-off of these areas, the resultant particles consisting of an envelope with fine surface projections and nucleocapsid round the inner surface.

Most of the above ultrastructural features have

been described by several other workers, including Koestner and Long (1970), Lawn (1970), Cornwell et al. (1971), Tajimi et al. (1971) and Confer et al. (1975b) for CDV and by Nakai and Imagawa (1969), Nakai et al. (1969) and Raine et al. (1969) for measles virus infection.

From study of the tangential sections of virus particles (Figure 15), it appeared that the nucleocapsid was arranged not as a true helix but as a series of reverse loops. This would explain why only a single row of nucleocapsid cross-sections was seen aligned below the plasma membrane, i.e. if the nucleocapsid had been wound in a helix, it would have been seen at different levels below the cell surface. Moreover, in the detached syncytia, cross-sections of nucleocapsid were seen aligned between membranes whose close apposition would have precluded the formation of a helix. From examination of transversely-sectioned particles, it was clear that the space internal to the nucleocapsid was composed of material which was very similar to that of the cytoplasm of the adjacent cell, and structures such as ribosomes were sometimes present therein. This was to be expected from the method of virus maturation. In some of the particles budding from detached syncytia, membrane-bound vacuoles were found while others contained electron-dense bodies. These differences in the internal constituents of the virus particles would presumably result in variation in their buoyant density. Also,

as the particles were seen to be very pleomorphic, it was obvious that the ratios of envelope to nucleocapsid and internal material could not have been constant, a variation also likely to affect their density. Thus a degree of heterogeneity with respect to density could be expected in any virus population. The significance of this will become apparent in Part Four. No particles consisting of an outer membrane with the characteristic surface projections but lacking recognisable internal nucleocapsid, were seen in the present investigation. These latter structures have been found in measles virus preparations by several workers including Nakai et al. (1969) and Raine et al. (1969), who considered them to be defective particles.

By 48 hours PI, deposition of more granular material round and between the nucleocapsids in many of the cytoplasmic aggregates had resulted in the formation of foci of increased electron-density. In size, shape, position and time of appearance, these electron-dense bodies seemed to correspond to the inclusion bodies seen in stained preparations examined in the light microscope. Ultrastructurally, so much granular material was present in some of these bodies that the nucleocapsid filaments were almost indistinguishable. Electron-lucent pockets were present in many, and round the edges of the dense area and within the pockets, short tubular or sac-like structures were found. Similar bodies were identified

in the 72 and 96 hour samples. The deposition of the granular material and the development of the sac-like structures round the nucleocapsids may have represented a defence mechanism by which the cell was trying to breakdown or alter the foreign material. This theory was given further support by the finding, from 48 hours onwards, of some of the electron-dense material near the cell-surface and within microvilli which seemed to become more numerous at this stage. This material which appeared to encase filamentous structures, probably nucleocapsid, was also found in "envelopes" of plasma membrane which may either have been detached from the cell or conjoined to the cell beyond the plane of section. Moreover, none of it was seen in the detached syncytia. It therefore seemed as if it was being extruded from the cell. Extrusion of nucleocapsid in a wrapping of plasma membrane might have repercussions in studies of viral buoyant density, dependent on the detection of isotopically labelled virus RNA. A brush border was never seen on the outer surface of pieces of plasma membrane enclosing the electron-dense filamentous material.

Although the author's conclusions were similar to those of Cornwell et al. (1971) in respect of inclusion bodies, other workers have concluded differently. Richter and Moize (1970); Tajima et al. (1971); Watson and Wright (1974) and Confer et al. (1975b) were of the opinion that it was the loose-knit aggregates of

nucleocapsids which represented the inclusion bodies. Kallman et al. (1959) found that the loose-knit aggregates corresponded to cytoplasmic inclusions detected in living cells by phase-contrast microscopy. The compact aggregates containing electron-dense granular material and regarded by the author as the true inclusions have also been described by Raine et al. (1969) and Confer et al. (1975b). The former workers reported that the nucleocapsid frequently became sequestered by smooth endoplasmic reticulum and underwent a structural change leading to disintegration. They suggested that these changes were similar to those occurring at an early stage in autophagic vacuole formation and may have represented a cellular mechanism to isolate and digest the nucleocapsid. Confer et al. (1975b) concluded that the loose-knit aggregates of nucleocapsid represented the earliest ultrastructural counterpart of the cytoplasmic inclusions and that compaction occurring during the subsequent 24 hours led to the formation of the electron-dense, granular inclusions. They suggested that the latter structures may represent the effect of lysosomal enzymes and other cellular digestive processes on the aggregates of nucleocapsid. There is therefore some body of opinion that the cell attempts in some way to eliminate nucleocapsid. Whether or not, the system of vacuoles and tubules which the author found within the inclusions represented the means by which this was executed or was merely part of a normal vacuolar system, such as the

Golgi apparatus which inadvertently had become trapped in the aggregates of nucleocapsid is unknown. Similar structures have been illustrated by Confer *et al.* (1975b) but not mentioned in their text.

The outstanding features of the detached syncytia were the presence of numerous intracytoplasmic vacuoles and membranes, and the extensive alignment of nucleocapsid both at the cell-surface and at the intracytoplasmic membranes, often in the absence of brush borders. These vacuoles and membranes presumably arose at the time of formation of the syncytium from the fusion of the numerous microvilli which are so characteristic of the cells between 48 and 96 hours PI. The alignment of nucleocapsid beneath these membranes is therefore understandable. The lack of brush border may indicate that, in the earlier stages of infection, alignment may precede and be a factor responsible for the formation of the brush border, and that in the detached syncytia some block in the mechanisms involved has prevented the final stage being completed. Dubois-Dalq *et al.* (1974) reported that in Vero cells infected with a latent SSPE-measles virus, viral antigen could be detected over almost the whole of the cell-membrane despite the absence of any ultrastructural changes. It was postulated that this antigen played a part in cell fusion. It is possible, therefore, that a similar antigen may have been present on the plasma membranes of the detached syncytia seen in the present

investigation, despite the absence of the brush border. The tendency of these membranes to fuse and form trilaminar structures would support this. Nucleocapsid cross-sections often appeared directly opposite each other on either side of the membrane. Raine et al. (1969) observed this where the microvilli of non-detached syncytia were in apposition and suggested that it could be accounted for by transfer of nucleoprotein across the membranes.

In a very small number of detached syncytia, "smooth" nucleocapsids were seen. Because they occurred in a double-membrane lined cavity relatively free of granular material, their outlines were very distinct. They also appeared to be less flexible structures than the "fuzzy" nucleocapsids. The double membranes enclosing them were indistinguishable from nuclear membranes and although the diameter of the membrane-bound aggregates was less than that of a nucleus, they may have represented intranuclear inclusions sectioned near one pole of the nucleus.

Wide variations seem to exist in the frequency with which CDV intranuclear inclusions occur in different systems and in their ultrastructural composition. Cornwell et al. (1971) and Watson and Wright (1974) found neither intranuclear inclusions with the light microscope

nor ultrastructural evidence of viral material in the nuclei of infected cells. Poste (1972) and Confer et al. (1975b) found nuclear inclusions in stained preparations but failed to find obvious viral components by electron microscopy and concluded that the stained material was an accumulation of viral protein. Koestner and Long (1970) and Tajima et al. (1971), on the other hand, not only found intranuclear inclusions by staining but also observed aggregates of nucleocapsid in the nuclei. In both of these instances, the nucleocapsid was of the "smooth" variety, paracrystalline arrays being found in the former study. In the case of measles virus infection, the intranuclear inclusions are recognised to consist of "smooth" nucleocapsid, often arranged in paracrystalline arrays (Kallman et al., 1959; Raine et al., 1969). Since these structures mainly occur late on in the infectious cycle, at a time when the cell is producing little infectious virus, it has been suggested that they may provide a mechanism by which the viral genome is maintained in the cell (Raine et al., 1969).

At all stages of infection, the amount of intracytoplasmic nucleocapsid was in excess of that being incorporated into virus particles. Thus, if the intracytoplasmic nucleocapsid could be satisfactorily extracted, the infected cells would provide an excellent source of viral nucleocapsid for detailed study. The

following section compares the results of different methods of extraction, and describes some of the properties of the extracted nucleocapsid.

PART THREE.

INTRODUCTION AND REVIEW OF THE LITERATURE.

The ultrastructural studies just described had shown that the infected cell contained large amounts of nucleocapsid, only part of which became incorporated into new virus particles. On this basis, it seemed probable that the cell would prove to be a richer source of nucleocapsid than the virus itself. Hence, before commencing study of the fine structure of the virus, attempts were made to find a suitable method for the extraction of nucleocapsid from infected cultures and for its isolation from cellular material.

At the start of the present investigation, nothing had been published on the extraction and purification of CDV nucleocapsid, though a number of papers on the extraction of measles and paramyxovirus nucleocapsids had appeared. The earliest work was mainly concerned with determining the buoyant density of the nucleocapsid released from cells by freezing and thawing, determinations being carried out by centrifuging the preparations to equilibrium in density gradients of caesium chloride solution, collecting fractions from these gradients and testing them by complement fixation. The specificity of the

latter test allowed the detection of nucleocapsid in crude cellular extracts, though anomalous results were sometimes obtained. Thus, Norrby (1964) reported that the buoyant density of measles virus nucleocapsid was 1.32 g/cc whereas Numazaki and Karzon (1966) found that it lay between 1.29 and 1.30 g/cc. As suggested by Waterson (1965), such variation may have resulted from the use of different methods of measuring the density of the fractions.

With advances in the technique of radioassay, a more sensitive method of detecting viral components became available. By the addition of the radionucleotide tritiated uridine (^{3}H-uridine) to infected cultures, it was possible to label virus RNA and hence the nucleocapsid of paramyxoviruses. However, since uridine is incorporated into cellular as well as into viral RNA, specificity can only be assured if cellular RNA synthesis is first blocked by the addition of an inhibitor or if the nucleocapsid is separated from cellular RNA by a suitable technique. The inhibitor which is most specifically directed against cellular RNA synthesis is Actinomycin D which prevents transcription from DNA to RNA; since paramyxoviruses do not require DNA for replication, they are not inhibited by this antibiotic. However, although Actinomycin has proved to be of value in the study of Sendai (Blair, 1970; Bukrinskaya, 1973) and measles-SSPE nucleocapsids (Yeh and Iwasaki, 1972), its toxicity generally restricts its use to very short-term experiments. In most instances, therefore, nucleocapsid has been labelled in the absence of

Actinomycin and this has necessitated the use of density gradient centrifugation for its separation from cellular material.

An important advance in the study of paramyxovirus nucleocapsid was the description by Compans and Choppin (1967b) of a technique whereby cells infected with the parainfluenza virus SV5 were disrupted by osmotic shock and the nucleocapsid thus released was purified by three cycles of centrifugation through discontinuous gradients of caesium chloride. Nucleocapsid banded once in this manner showed a length distribution similar to that observed immediately after release from cells, with a sharp peak at around 1 μ, and no contaminating cellular material was found by electron microscopy. However, there is evidence that two or more bandings in caesium chloride solution may result in some degree of fragmentation of paramyxovirus nucleocapsids (Hosaka, 1968; Yeh and Iwasaki, 1972; Waters et al., 1972). Norrby and Hammarskjold (1972) compared the relative efficiency of caesium chloride and sucrose for the purification of measles virus nucleocapsid. They found that fragmentation occurred more readily in caesium chloride but that, due to the dissociating effects of high salt concentration, contamination with viral haemagglutinin was less. However, Yeh and Iwasaki (1972) found that the addition of 1% bovine serum albumin (BSA) to the preparation prior to centrifugation in caesium chloride considerably increased its stability.

In addition to freezing and thawing and osmotic shock, several methods have been used to extract paramyxovirus components from infected cells. These include mechanical homogenisation (Waters et al., 1972; Waters and Bussell, 1974) and the use of ionic (principally sodium deoxycholate) and non-ionic detergents (Blair, 1970; Norrby and Hammarskjold, 1972). Comparison of these for the release of measles virus antigens was made by Norrby and Hammarskjold, (1972). However, although Cutscum and sodium deoxycholate were found to be the most effective for releasing the complement-fixing antigen of the nucleocapsid, this does not necessarily imply efficient release of intact nucleocapsid.

The principal aim of the present section was to find a suitable method for the extraction of CDV nucleocapsid from infected cells and for its identification by radioassay and electron microscopy after centrifugation to equilibrium in density gradients. By so doing, it was hoped that the information obtained would provide a method for the purification of the nucleocapsid for subsequent biochemical analysis.

MATERIALS AND METHODS.

1. Incorporation of radioisotopes. Nucleocapsid was labelled by incorporating ^{3}H-uridine into its RNA. The isotope was fed to infected cultures at a concentration of 20 µCi/ml of tissue culture fluid. In later experiments, an attempt was made to infect the cultures with undilute virus of fairly high titre (10^{5}TCID$_{50}$ per ml) and to incorporate the isotope into the medium within 24 hours of infection. In these instances, therefore, the isotope was in contact with the cells for several days before nucleocapsid was extracted for study. ^{3}H-uridine was obtained from the Radiochemical Centre, Amersham.

In certain experiments, Actinomycin D was fed to the cultures before the addition of the isotope in order to ensure that any labelled RNA was viral and not cellular in origin. It was obtained from Sigma Chemical Co.Ltd., and was used at a concentration of 2 µg/ml.

2. Extraction of nucleocapsid. During the course of the investigation, several methods of disrupting the infected cells were used. These will be described in detail under the appropriate experiment. The employment of the non-ionic detergent Sterox SL was suggested by the work

of Stromberg (1972) who found it to be of special value for the isolation of avian myeloblastosis virus cores. A sample was kindly supplied by the Monsanto Co., St. Louis, Missouri.

3. Density gradient centrifugation. Density gradients were prepared by mixing equal volumes of a concentrated and a dilute solution of the appropriate material in a Buchler gradient mixer coupled to a Buchler polystaltic pump. Centrifugation was carried out in a Beckman L2-65B ultracentrifuge as described in detail under the appropriate experiment. The solutions were made up in Tris saline (TS) (0.1 M NaCl + 0.01 M Tris + 0.001 M EDTA), unless otherwise stated. In most instances, a layer of the dilute solution was applied to the top of the gradient in order to prevent disturbance of the gradient when the sample was added, and this layer is referred to as the buffer layer.

After centrifugation the tubes were transferred to a Buchler universal piercing unit coupled to an LKB peristaltic pump and the gradient was collected dropwise into a series of flat-bottomed vials. The refractive index of alternate fractions was read by means of a Bellingham and Stanley refractometer and for caesium chloride and sucrose gradients, the density of each was determined from published tables (Weast, 1972). Tables

Table 2 Calibration of Potassium Tartrate Solutions.

Concentration	Refractive index	Density. 0.1 ml volumes	Density. 100 ml volumes
10%	1.3458	1.044	1.062
20%	1.3570	1.105	1.120
30%	1.3685	1.167	1.181
40%	1.3803	1.233	1.229
50%	1.3909	1.291	1.280
60%	1.4011	1.346	1.332

of refractive index versus density were not available for potassium tartrate and calibration was carried out by the two methods described below.

1) Six solutions of different concentration, ranging from 10 to 60 %, were prepared and from these 0.1 ml volumes in Lang Levi Auto Zero pipettes were weighed on a Sartorius balance to four decimal places, and the refractive indices measured as above.

2) With the same six solutions, 100 ml volumes in volumetric "A" flasks were weighed on the micro-balance and the refractive indices measured as before.

Both methods of calibration gave essentially the same result, the relevent details being shown in Table 2.

4. Radioassay. To eliminate unincorporated ^{3}H-uridine and other acid-soluble molecules, each fraction was mixed with 0.5 ml of 10% trichloroacetic acid (TCA) and 0.5 ml of bovine serum albumin (BSA). The precipitates were then separated from the acid-soluble material by filtration through Whatman GF/C glass-fibre filter discs inserted into a Millipore 3025 sampling manifold, to which was attached a vacuum pump. Before removal from the manifold, the filters were washed with ethanol to aid drying and, following removal, dried in a hot air oven. Each filter was then transferred to an Intertechnique polyethylene vial containing 5 ml of scintillation fluid, the latter consisting of 0.4% 2,5-diphenyloxazole (PPO) and 0.04% 1,4-di-2-(5phenyloxazoly)-benzene (POPOP) in

sulphur-free toluene. The radioactivity in each was measured in an ABAC SL40 Intertechnique liquid scintillation spectrometer over a period of 10 minutes.

5. Electron microscopy. Electron microscopical examination of negatively stained samples of certain fractions was carried out as described in the subsequent experiments. A collodian coated copper grid was placed on a drop of the appropriate sample and, following absorption of the excess with filter paper, transferred to a drop of 0.15 M ammonium acetate to remove the caesium chloride, potassium tartrate, or sucrose. The excess was then absorbed and the grid placed on top of a drop of 2% phosphotungstic acid (pH 7.2). After 30 seconds, the excess fluid was removed and the grid was examined in an AEI/6B electron microscope at an instrumental magnification of 30,000-40,000.

All electron microscopical examination of samples was carried out on the same day as the gradient was fractionated in order to ensure that falsely negative results, or abnormal morphology due to a time lapse, were not obtained.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 3.1

Electron Microscopical Examination of Nucleocapsid Extracted from Infected Cells by Osmotic Shock.

The purpose of this experiment was to establish the morphology of nucleocapsid extracted from infected cells in such a way as to minimise damage to it. By so doing, it was hoped that a standard could be obtained, against which the effect of various procedures on the morphology of nucleocapsid could be assessed.

Procedure. A 4oz. bottle of GH cells was infected with the virus and the cells subjected to osmotic shock on the fourth day PI when a CPE was present. The cell sheet was washed several times with distilled water (DW) to remove salts, 2 ml of DW was added and the culture refrigerated for 45 minutes. At the end of this time, the cellular and nuclear outlines stood out clearly, as though swollen, but no marked disruption of cells was evident. The DW was removed and applied directly to collodian coated copper grids which were negatively stained, as described above.

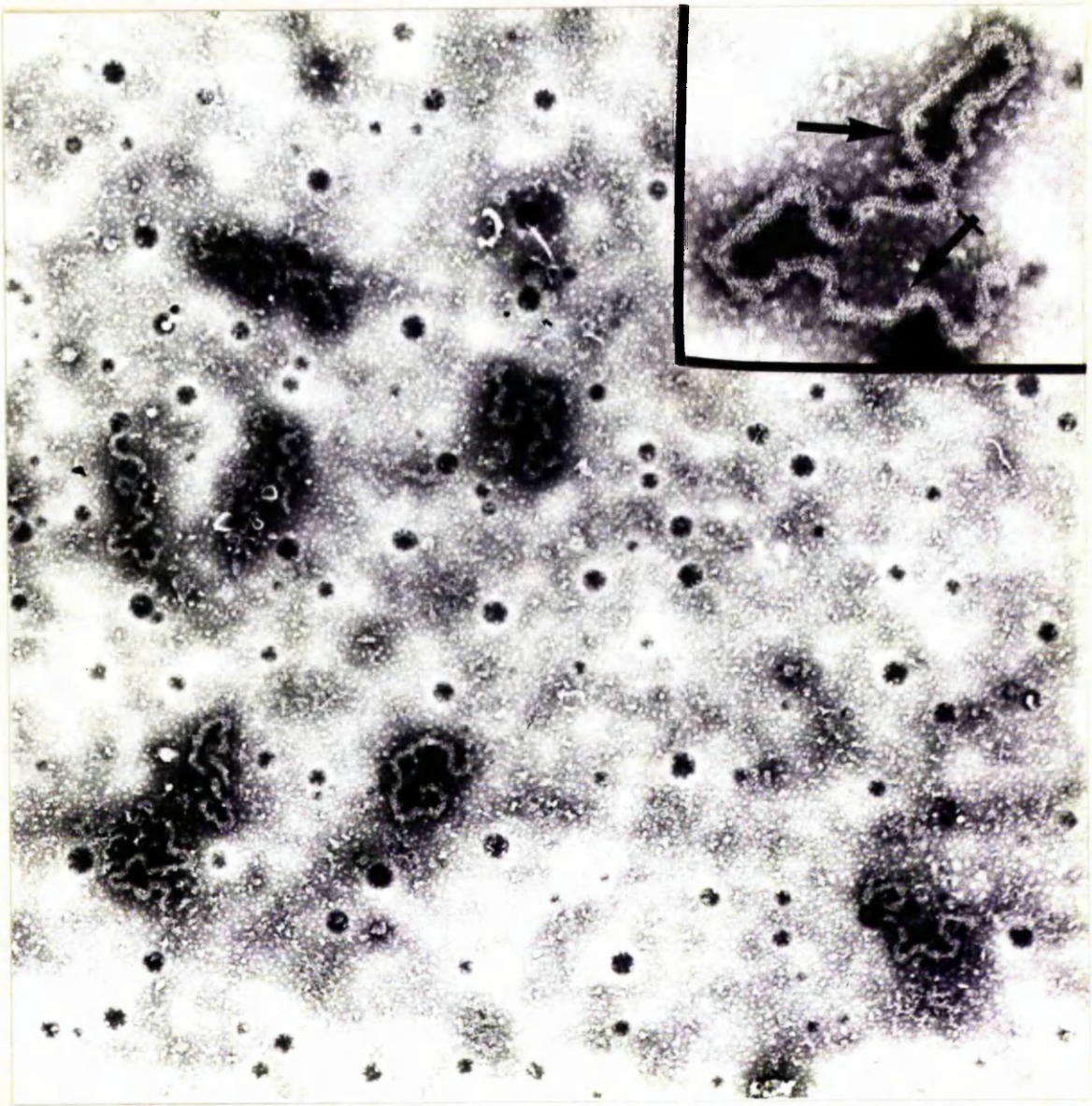


Figure 29. Negatively stained nucleocapsid, extracted by osmotic shock. The insert shows, in detail, a straight section (arrow) and a section which has become rotated on its long axis (crossed arrow). X25,000; insert, X80,000

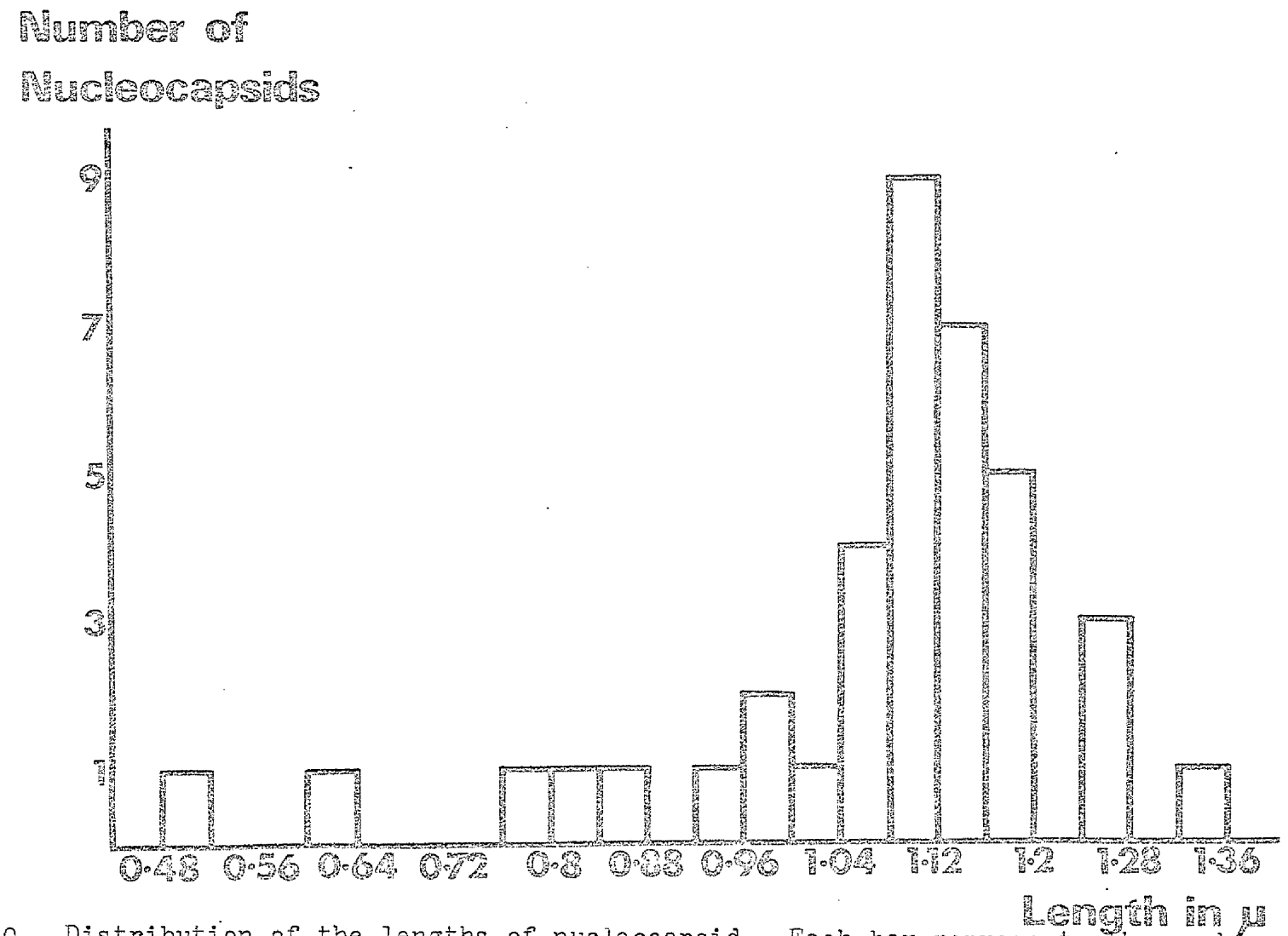


Figure 30. Distribution of the lengths of nucleocapsid. Each bar represents the number of nucleocapsids in each 0.04 μ length range.

Results. Few nucleocapsids were found in the preparation but, as can be seen in Figure 29, those seen had the characteristic herring-bone structure, typical of paramyxovirus nucleocapsid. They appeared to be flexible structures with a diameter of 250 Å, an axial hollow approximately 5 Å in diameter and a subunit periodicity of 7 Å, though, in places, the filament seemed to be twisted by rotation on its long axis (Figure 29, insert). From 38 nucleocapsids measured by means of a map measuring device, the mean length was 1,10 μ. The length distribution is shown in Figure 30.

Conclusions and Discussion. Distilled water treatment of infected cells released nucleocapsid morphologically identical to that described for other paramyxovirus nucleocapsids (Compans and Choppin, 1967a; Kingsbury and Darlington, 1968; Hosaka, 1968; Finch and Gibbs, 1970; Yeh and Iwasaki, 1972; Hall and Martin, 1973; Waters and Bussell, 1974).

Since the characterisation of nucleocapsid by rate zonal centrifugation is dependent upon the maintenance of its complete structure, the recognition of fragmentation is obviously important for it has been shown that in defective measles virus infection, only short lengths of nucleocapsid are produced (Kiley and Payne, 1974; Hall and Martin, 1974a). From the results of the present experiment, it is clear that most, if not all, of the

nucleocapsid produced was complete. The significance of this will be discussed in Part Six.

EXPERIMENT 3.2

To Determine the Buoyant Density of Nucleocapsid in a Sucrose Gradient.

This and the following experiments were designed to determine the buoyant density of the nucleocpasid with a view to using equilibrium density gradient centrifugation as a method of purifying the nucleocapsid. An initial attempt was made to do this by the centrifugation of nucleocapsid labelled with ^{3}H-uridine and extracted by a combination of osmotic shock, freezing and thawing, and mechanical homogenisation of the infected cells. Waters et al. (1972) had sucessfully used this for the extraction of large quantities of CDV nucleocapsid from infected cells but, in the present investigation, little radioactive material was recovered from the density gradients. It was decided therefore to determine whether or not larger amounts of radioactive material could be obtained by disrupting infected cells with sodium deoxycholate (DOC).

Procedure. A 4oz. bottle of infected cells and a 4oz. bottle of normal GH cells were prepared. Twenty-four hours later, they were fed 10 ml of medium containing Actinomycin D. This in turn was replaced after 90 minutes with medium containing Actinomycin and ^{3}H-uridine. Twenty-four hours later, both cultures appeared very degenerate and many cells were floating in the medium;

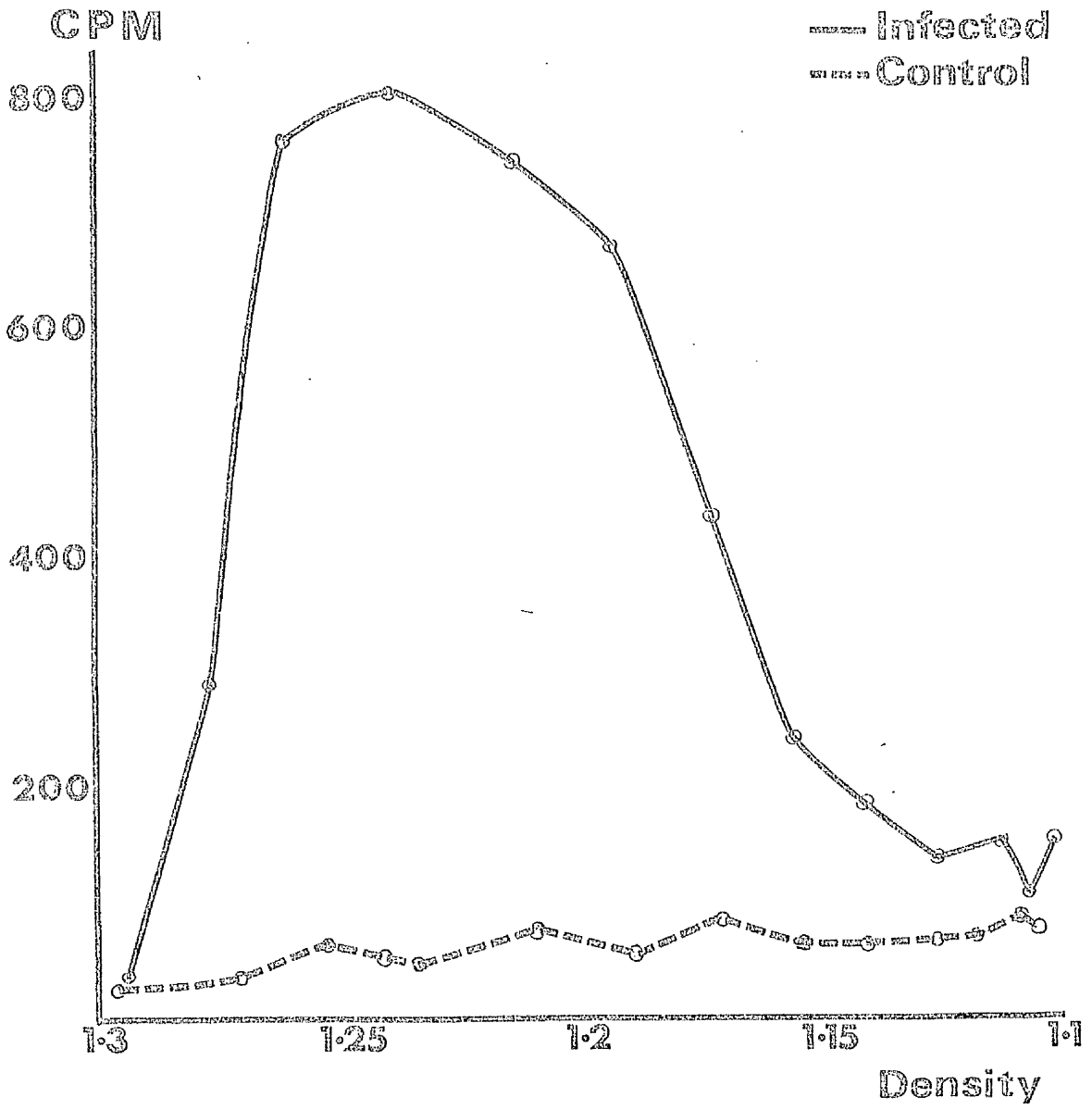


Figure 31. Radioactive profiles obtained by centrifugation of DOC cell-extracts through sucrose density gradients.

this was due to the action of the Actinomycin. Glass beads were added to both bottles and the remaining cells removed from the glass by shaking. Both lots of cells were sedimented from the fluid and each of the pellets was resuspended in 4 ml of 0.2% DOC. The suspensions were left at 4°C for 15 minutes and then clarified by centrifugation at 3,500 rpm for 15 minutes. The supernatants were layered on top of two 7 ml 20-60% sucrose gradients in SW 40 tubes, with a 2 ml buffer layer on top of each gradient, and centrifuged at 30,000 rpm for 20 hours. Twenty twelve-drop fractions were collected from each, and the fractions assayed for radioactivity as described previously.

Results. The distribution of radioactivity in each gradient is shown in Figure 31. As can be seen, a moderate amount of radioactive material was released by the DOC treatment of the infected cells. This was evidently viral, as there was very little radioactivity in the normal cell control.

Conclusion and Discussion. Since more radioactive material was recovered from this than from the previous experiment, it was concluded that DOC was more efficient than the combination of osmotic shock, freezing and thawing and mechanical homogenisation previously employed. However, the amount of radioactivity recovered from the

gradient was still relatively low. This may have been due to the fact that the label was in contact with the cells for only 24 hours. Ideally, label should be fed to the cells soon after infection and maintained there until a CPE is present. Unfortunately, however, this could not be carried out in the presence of Actinomycin, since the high cytotoxicity of the latter produced irreversible damage within about 8 hours. Accordingly, it was decided that, for future work, Actinomycin should be omitted and the label applied for a much longer period of time, the problem of distinguishing viral from cellular RNA being approached in another way.

The heterogeneity of the radioactive material did not permit determination of the buoyant density of the nucleocapsid. This may have been due to the adherence of membranous material to nucleocapsid in varying amounts, as described by Norrby and Hammarskjold, (1972). Since such binding is diminished in high salt concentrations, it was decided to investigate whether or not sharper banding of nucleocapsid could be obtained by using gradients of caesium chloride in place of those of sucrose.

Before continuing with studies of radioactively labelled material, it was thought advisable to examine the effect of extraction with DOC and centrifugation through caesium chloride (CsCl) on the morphology of the nucleocapsid.

Figure 32 (a). DOC-extracted nucleocapsid, negatively stained. Note the comparative shortness, and rigidity of this nucleocapsid compared to the Sterox-extracted nucleocapsid. X60,000

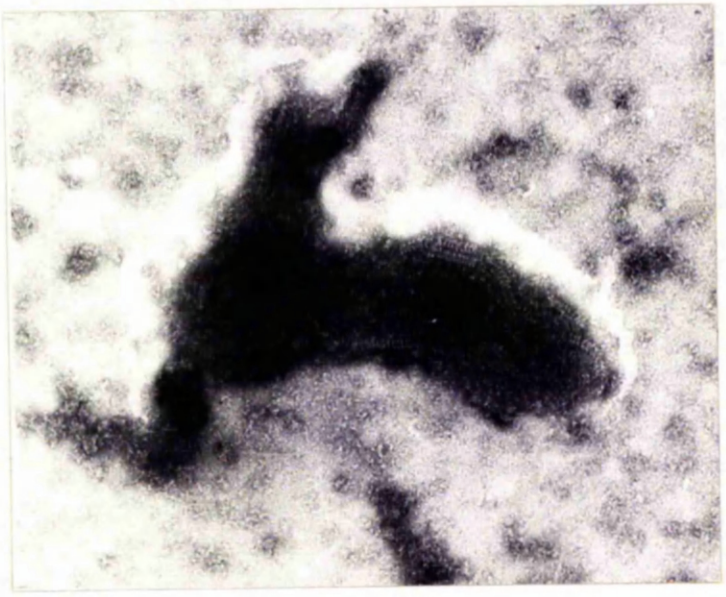


Figure 32 (b). Sterox-extracted nucleocapsid, negatively stained. Due to the flexible nature of the filaments, they are intertwined, and determination of their exact length is therefore impossible. X80,000

EXPERIMENT 3.3

The Morphology of DOC-extracted Nucleocapsid Banded in Caesium Chloride Gradients.

Procedure. An 8oz. bottle of infected cells showing a marked CPE was washed once with PBS and refrigerated at 4°C for 45 minutes following the addition of 5 ml of DOC solution. At the end of this time, no intact cells were recognisable. The DOC solution was then clarified by centrifugation at 2,750 rpm for 15 minutes. Five ml of the supernatant was mixed with sufficient CsCl to provide a final density of 1.3 g/cc. This was then transferred to SW 50.1 tubes and centrifuged at 30,000 rpm for 37 hours. At the end of this time, 10 drop fractions were collected, the refractive indices determined and the density of each fraction calculated from these. Samples from selected fractions were then examined in the electron microscope.

Results. A small number of nucleocapsid were seen in fractions from the density range 1.272 to 1.308 g/cc, most being found in the sample of density 1.281 g/cc. They were relatively short and seemed to be fairly rigid (Figure 32a).

Conclusions and Discussion. The morphology of the DOC-extracted nucleocapsid differed from that of the nucleocapsid obtained by osmotic shock (Experiment 3.1). The presence of relatively short and apparently rigid nucleocapsids indicated that fragmentation had occurred, either on exposure to DOC or as a result of centrifugation through CsCl. It is clear, therefore, that this method of extraction and purification would not be satisfactory for studies requiring maintenance of the complete nucleocapsid. Accordingly, the decision was made to investigate the effect of other detergents on the morphology of the nucleocapsid. If all samples obtained after extraction with different detergents and centrifugation through CsCl contained only short rigid pieces, then it would seem that such alterations in structure occurred as a result of centrifugation through CsCl. It was also decided to investigate the use of preformed CsCl gradients as this would permit reduction in the time required for centrifugation and possibly, therefore, aid the recovery of nucleocapsid. A further improvement was suggested by the work of Yeh and Iwasaki (1972) who found that 1% BSA had a stabilising effect on measles-SSPE nucleocapsid. The next experiment was therefore designed to compare the morphology of nucleocapsid extracted from cells with several non-ionic detergents, with and without BSA, and centrifuged through preformed gradients of CsCl, again with and without BSA.

EXPERIMENT 3.4

The Morphology of Nucleocapsid Extracted by Non-ionic Detergents.

Procedure. 0.5% Nonidet P-40 (NP 40) and 1% Sterox SL were prepared in 1/5th TE buffer (0.05 M Tris, 0.005 M EDTA, HCl to pH 8.0). In addition, 0.5% Triton X-100 was made up without BSA. CsCl solutions of density 1.34 g/cc and 1.25 g/cc were prepared using 1/5th TE buffer, again with and without 1% BSA.

Five 8oz. bottles of infected GH cells with a good CPE were employed. All were washed once with PBS, given 5 ml of the appropriate detergent and left for an hour at room temperature, viz.

Bottle A	NP 40
Bottle B	NP 40 + BSA
Bottle C	Sterox SL
Bottle D	Sterox SL + BSA
Bottle E	Triton X-100

NP 40 appeared to disrupt the cells the most rapidly. Disruption occurred more slowly in the presence of BSA but, at the end of the hour, all of the monolayers had been reduced to a fine suspension in which only nuclei were recognisable. The extracts were then clarified and 4 ml of the supernatant from each was layered on top of an SW 40 tube containing a 7 ml gradient of CsCl, density

1.25 to 1.34 g/cc, with a 1½ ml buffer layer of 1.25 g/cc CsCl. The extracts with BSA were put on top of the gradients which contained BSA and the extracts without BSA were placed on top of gradients without BSA. The tubes were centrifuged at 38,000 rpm for 22 hours. Twelve-drop fractions were collected from each gradient and samples of selected fractions from each were examined in the electron microscope as before.

Results.

1). Sterox SL alone. On examination of a sample from the fraction with density 1.292 g/cc, some clumps of flexible nucleocapsid were seen (Figure 32b). Short pieces and occasionally some longer lengths of nucleocapsid were also seen lying free. The range over which nucleocapsid could be found was not established for this gradient.

2). Sterox SL plus BSA. Nucleocapsid was seen in samples from five fractions within the density range 1.277 to 1.317 g/cc. Most of these five fractions contained a moderate amount of nucleocapsid, most being seen in the sample of density 1.286 g/cc and again a large proportion was in clumps. No nucleocapsid was seen in either of the fractions immediately beyond this density range.

3). NP 40 alone. No nucleocapsid was seen in any fraction from this gradient.

4). NP 40 plus BSA. Nucleocapsid was seen in samples from four fractions, covering the density range 1.273 to 1.31 g/cc. Most was found in the fraction of density 1.296 g/cc.

5). Triton X-100 alone. Small amounts of nucleocapsid were present in two fractions of density 1.286 and 1.292 g/cc respectively.

Since a large proportion of the nucleocapsid seen was present in clumps, its mean length could not be established. Nevertheless, it was obviously larger than that obtained by DOC extraction and appeared much more flexible.

Conclusions and Discussion. The use of non-ionic detergents, particularly Sterox SL, resulted in the release of nucleocapsid similar in flexibility and, perhaps, also in length to that released by osmotic shock. It therefore seemed that the apparent fragmentation seen in the previous experiment was due to the action of DOC.

The use of BSA had a marked effect on the quantity of nucleocapsid recoverable at the end of centrifugation. However, as BSA is used as a spreading agent for negative staining, the apparent difference in the amounts of nucleocapsid surviving centrifugation may merely have

been related to this. With this reservation in mind, the results of the present experiment indicated that the use of BSA was advantageous and that, of the three detergents tested, Sterox SL provided the highest yield of nucleocapsid.

The results of the last two experiments suggested that the buoyant density of CDV nucleocapsid in CsCl gradients lies within the range 1.27 to 1.31 g/cc with a few particles probably lying slightly outside this. Since a better method of extraction than that used earlier was now available it was felt that the radioactive profile obtained from equilibrium density centrifugation of ^{3}H-uridine labelled nucleocapsid might now give a more accurate indication of the density spread. However, with the exclusion of Actinomycin, the problem of identification of viral-specific radioactive material arose. Other paramyxovirus nucleocapsids have been shown to be resistant to the effect of RNase (Compans and Choppin, 1968 ; Hosaka, 1968; Hall and Martin, 1973; Waters and Bussell, 1974) and, before proceeding to label infected cells, it was decided to investigate the possible use of this as a means of eliminating cellular RNA.

EXPERIMENT 3.5

The Radioactive Profile of Cellular RNA, with and without Ribonuclease Treatment, in CsCl Density Gradients.

Procedure. An 8oz. bottle of normal GH cells was prepared. When the cells had monolayered, the growth medium was replaced with maintenance medium containing 20 μCi/ml of ^{3}H-uridine, and the cells were incubated at 37°C.

After five days, the culture was washed once with PBS and 7 ml of Sterox with 1% BSA added. After an hour at room temperature, the extract was removed and clarified. Half of the supernatant was mixed with ribonuclease (RNase) stock solution to give a final concentration of 50 μg/ml of RNase and incubated at room temperature for one hour. This was then layered on top of a 7 ml gradient of CsCl plus BSA, density 1.25 to 1.34 g/cc, with a 1½ ml buffer layer in an SW 40 tube. The other half of the supernatant was layered onto an identical gradient. Both tubes were centrifuged at 38,000 rpm for 20 hours. Seven-drop fractions were collected from each gradient, the refractive indices of alternate fractions measured and radioassay carried out as previously described.

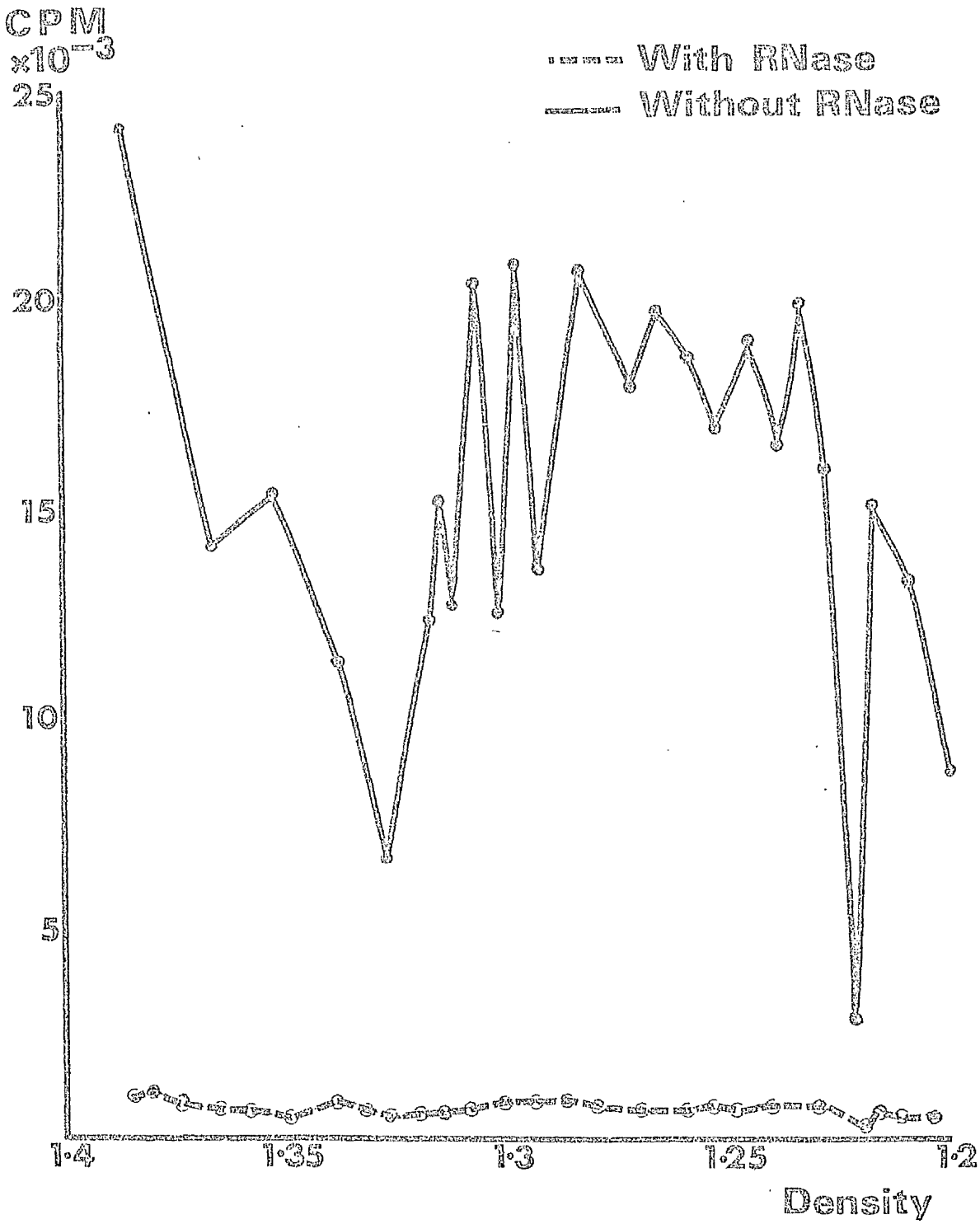


Figure 33. Radioactive profiles from extracts of normal cells, with and without RNase treatment.

Results. As can be seen from Figure 33, the RNase treatment considerably reduced the acid-precipitable radioactivity. In the gradient with the non-treated sample, the total radioactivity over the density range 1.20 - 1.39 was 393,567 CPM whereas, in the other gradient, it was only 20,189 CPM. The profile from the untreated sample was very irregular, while that from the RNase treated sample was almost flat.

Conclusions and Discussion. RNase treatment of cell extracts was successful in breaking up a large proportion of the cellular RNA molecules so as to render them acid soluble. The 'background' radioactivity in the fractions from the RNase treated sample was about 800 CPM. This was obtained from the equivalent of half of an 8oz. bottle which contained many more cells than are present in an 8oz. culture with a marked CPE. The 'background' radioactivity in the latter would therefore be expected to be much lower than 800 CPM. After RNase treatment, there was no obvious peak of radioactivity at a density of around 1.29 g/cc — where previous experiments suggested nucleocapsid to be. It was therefore expected that this method of distinguishing viral RNA from cellular RNA would prove satisfactory.

EXPERIMENT 3.6

To Establish the Buoyant Density of CDV Nucleocapsid by Radioisotopic Labelling and Electron Microscopy.

The present experiment was intended to reveal whether or not RNase treatment reduced the cellular background to a sufficiently low level to permit the detection of banded radioactive nucleocapsid; by so doing it was hoped that the buoyant density of the latter would be determined. The experiment was also intended to correlate the presence of radioactivity with the presence of nucleocapsid, as revealed by electron microscopy, and to examine the effects of the RNase on the morphology of the nucleocapsid.

Procedure. Two 8oz. bottles of infected GH cells were prepared by infecting the cells in suspension. At 24 hours PI maintenance medium containing 20 μCi/ml of ^{3}H-uridine was added to one, and fresh medium without label given to the other. On the fifth day PI when a good CPE was present in both cultures, the medium was removed, both cell sheets were washed once with PBS, and 5 ml of Sterox plus BSA were added to each. The bottles were incubated at room temperature for one hour. The resultant extracts were then clarified, the supernatants treated with RNase and layered on top of gradients identical to those used in the last experiment. The tubes were spun at 37,000 rpm for 16 hours. At the end of this time, there were two bands present in each tube; the lower one consisted of

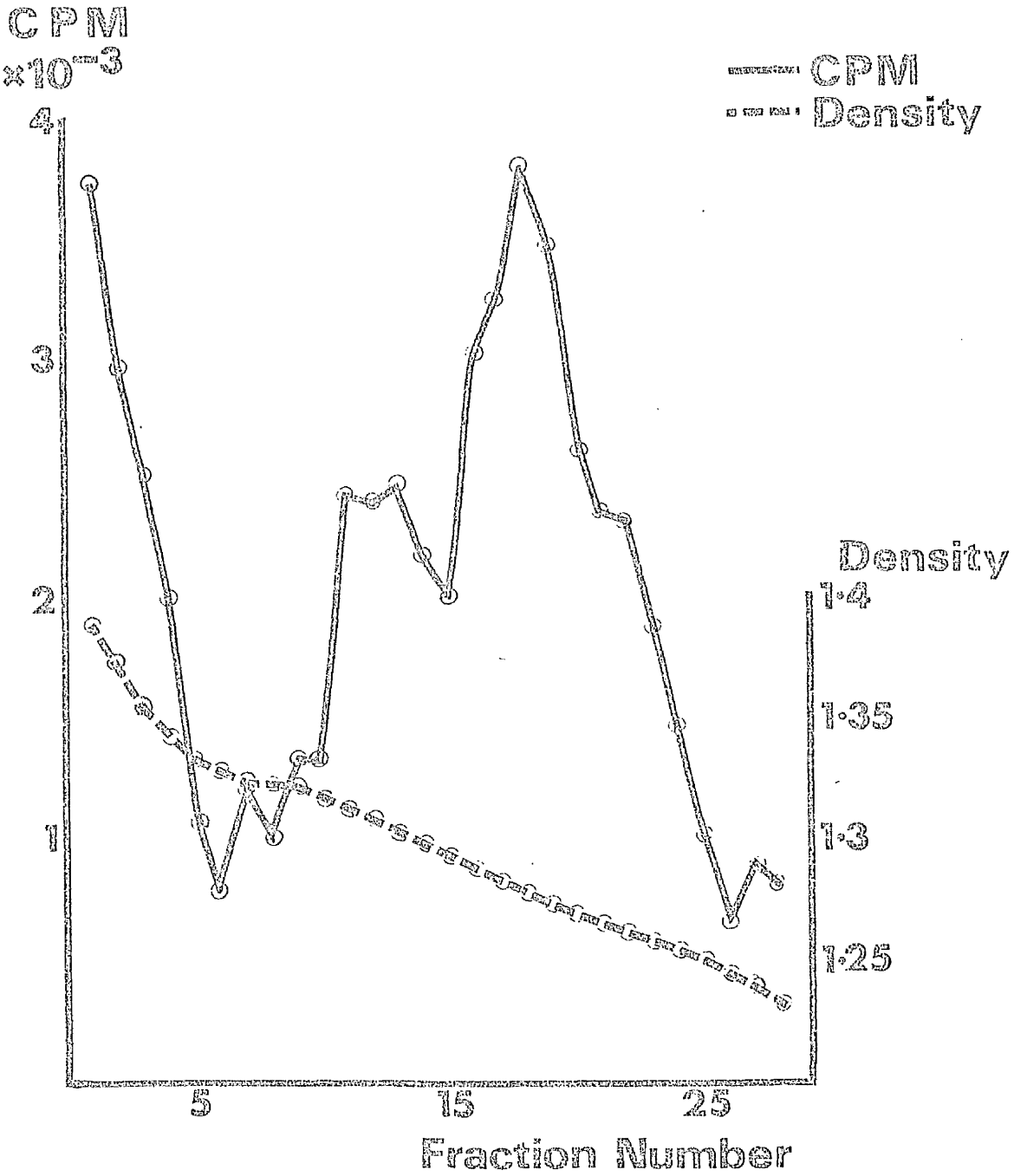


Figure 34. Radioactive profile from an extract of CDV-infected cells, after centrifugation through a CsCl gradient.

white flocculent material, while the upper was finely opalescent. The two bands were separated by a translucent zone about 2 mm in depth. Seven-drop fractions were collected from each gradient and the refractive indices determined as before. The radioactivity of the fractions from the radioactive sample was measured and samples were taken from selected fractions from the other gradient for electron microscopy.

Results. In the radioactive gradient, the band of flocculent material lay in the density range 1.302 to 1.318 g/cc, and the finer opalescent band was of a density around 1.278 g/cc. In the non-radioactive gradient, the flocculent material was in the density range 1.302 to 1.324 g/cc, with the finer material around 1.292 g/cc.

1). Radioactivity. The radioactive profile obtained is shown in Figure 34, from which it will be seen that there was a small, slightly biphasic peak of radioactivity covering the density range 1.295 to 1.315 g/cc. This corresponded roughly with the density range in which the flocculent material was found. A second peak of radioactivity occurred in the density range 1.26 to 1.295 g/cc, with most counts being in the fraction of density 1.278 g/cc, which was the one containing most of the opalescent material.

2). Electron Microscopy. No nucleocapsid was seen in the

fraction of density 1.319 g/cc which was from the middle of the flocculent band. In the fraction of density 1.314 g/cc, only one small piece of nucleocapsid was seen. In fractions of density 1.309 to 1.279 g/cc, the amount of nucleocapsid seen increased with decreasing density. There were many pieces of nucleocapsid free and in clumps, especially in the fraction of density 1.279 g/cc. In fractions of density 1.275 to 1.26 g/cc, the amount of nucleocapsid decreased again, with only a few small clumps being seen in the last one. No nucleocapsid was seen in the fraction of density 1.245 g/cc.

Conclusions and Discussion. The two visible bands found in the gradients following centrifugation of infected cell extracts were not present in gradients through which extracts of normal cells had been centrifuged. It therefore seemed that these bands were viral, not cellular, in origin. Each visible band corresponded to a peak of radioactivity which again was peculiar to the extracts of infected cultures. These peaks of radioactivity therefore would also seem to have been viral, not cellular, in origin. On this basis, it was concluded that the RNase treatment had reduced the cell background sufficiently to reveal the radioactive viral material.

The position of the nucleocapsid in the gradient, as determined by electron microscopy, corresponded to the

position of the second main peak of radioactivity i.e. covering a density range 1.26 to 1.295 g/cc with most at density 1.279 g/cc. This compares with a range of 1.29 - 1.30 g/cc reported by Waters and Bussell (1974). RNase had no effect on the morphology of the nucleocapsid; the pieces seen were flexible and appeared to be of moderate length though, due to aggregation, their precise lengths could not be determined. Unfortunately, due to this aggregation, it seems unlikely that the nucleocapsid in this band could be used for rate sedimentation studies.

It is difficult to determine the nature of the material giving rise to the denser band and peak of radioactivity. It was RNase resistant but no nucleocapsid was seen on electron microscopical examination of fractions from this region. Possible explanations for this will be discussed later.

EXPERIMENT 3.7

Buoyant Density of CDV Nucleocapsid in a Potassium Tartrate Gradient.

Since potassium tartrate has been found by some workers to give sharper banding of virus (McCrea et al., 1961), it was decided to investigate whether or not this would give sharper banding of nucleocapsid, and prevent overlap with the denser radioactive material.

Procedure. Two 8oz. cultures of infected GH cells were prepared. At 24 hours PI fresh growth medium with 20 μCi/ml of ^{3}H-uridine was added to one and fresh medium without label to the other. On the third day PI, when a good CPE was present, the nucleocapsid was extracted with Sterox plus BSA, the extraction process and RNase treatment being carried out as previously described. The extracts were layered on top of 10 ml potassium tartrate (KT) gradients of density 1.2 to 1.35 g/cc.

Since a precipitate appeared when the potassium tartrate was added to the TS containing 1% BSA, the concentration of the BSA was reduced to 0.2% for the 1.2 g/cc solution and omitted altogether from the 1.35 g/cc solution. The samples were centrifuged at 24,000 rpm for 18 hours. At the end of this time, two bands were again present in the gradients. The broader band consisted of

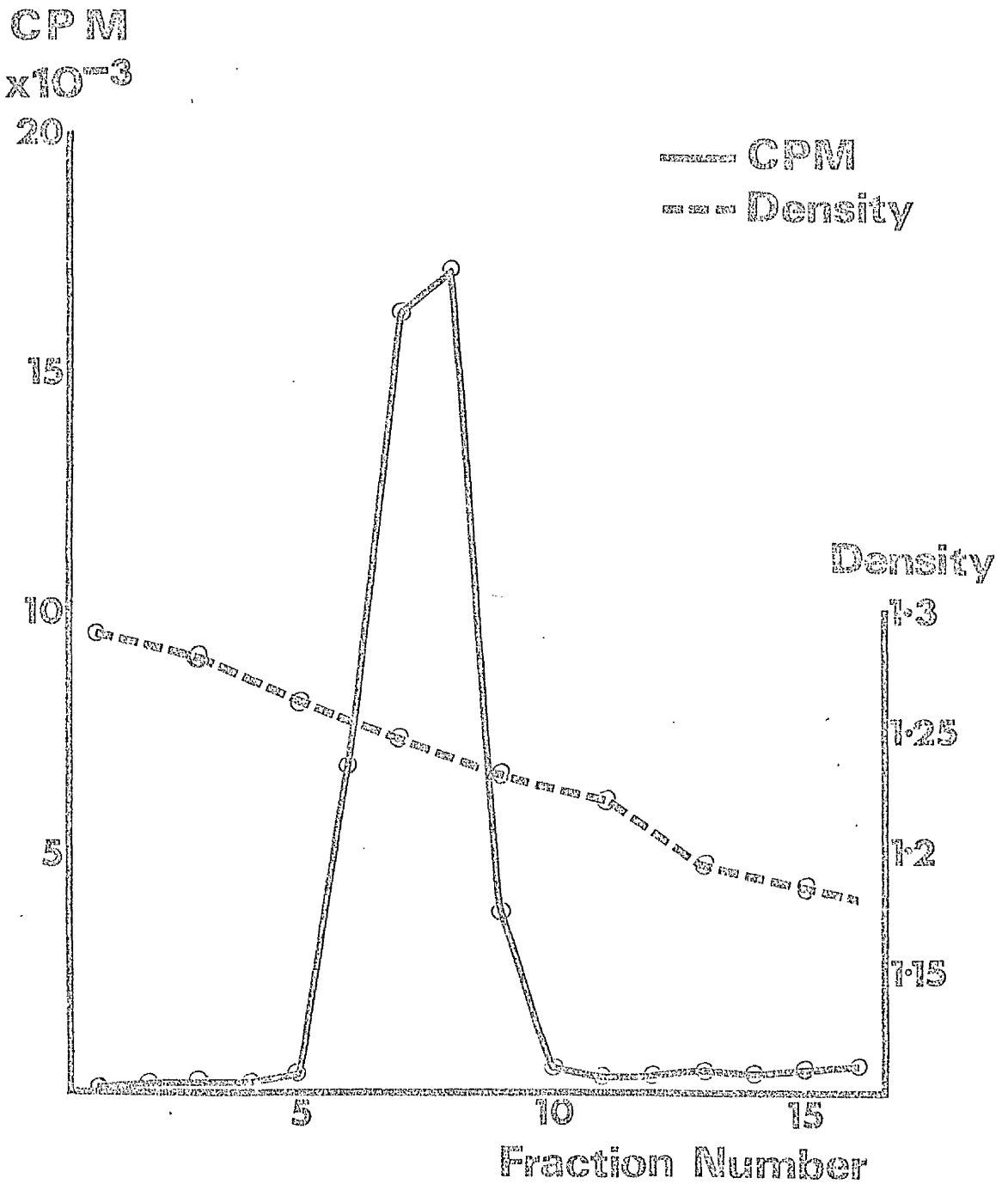


Figure 35. Radioactive profile from an extract of CDV-infected cells, after centrifugation through a potassium tartrate gradient.

finely flocculent material while the less dense band was made up of coarser, flocculent material the two being separated by a very narrow translucent zone about 1 mm in depth. Ten-drop fractions were collected from each gradient and the refractive indices measured. Due to the closeness of the two bands, it was not possible to prevent some mixing during fractionation. In the radioactive gradient, the flocculent material was found to occupy the density range 1.23 to 1.258 g/cc, most being present at a density of 1.245 g/cc. In the other gradient, it occurred in the density range 1.232 to 1.255 g/cc. Radioactive analysis and electron microscopy was carried out as already described.

Results.

1). Radioactivity. As can be seen from Figure 35, a single sharp peak of radioactivity was obtained at a density of 1.245 g/cc. The distribution of radioactivity corresponded to that of the flocculent material.

2). Electron Microscopy. In none of the fractions examined was nucleocapsid identified.

Conclusions and Discussion. With the steepness of gradient used, potassium tartrate failed to resolve the two bands of viral material in the extract. A single sharp peak of radioactivity was obtained because the bands were

sufficiently close together to be distributed between two consecutive fractions.

Though nucleocapsid was not found with the electron microscope, it was presumed that the two bands corresponded to those seen in the CsCl gradients. In the potassium tartrate gradient, however, the region containing the radioactive material was at a much lower density and the overall spread of the latter was less, i.e. the bands were narrower, and the difference between their respective densities was less. It is possible that a shorter, shallower gradient might have aided separation of the two bands.

It is difficult to understand why nucleocapsid was not seen with the electron microscope. It may have been that, with the necessarily low concentration of BSA employed, denaturation of the nucleocapsid had taken place, or that the potassium tartrate salt resulted in poor resolution of particles. To ensure that this result was not merely due to some gross error of preparation, another nucleocapsid extraction, banding in potassium tartrate, and electron microscopical examination was carried out, and the same negative result was obtained.

DISCUSSION.

The experiments just described encompassed several different techniques but collectively provided data on which a regimen for the extraction and purification of nucleocapsid from infected cells could be based.

Electron microscopy of a DW extract of infected cells revealed nucleocapsid having a morphology typical of the paramyxovirus group. Measurement showed that it occurred in unit lengths of approximately 1μ and this was important since it indicated that the virus/cell system employed was capable of producing complete nucleocapsid; it is generally accepted that the full genome of a paramyxovirus is contained within a unit length of 1μ (Hosaka et al., 1966; Compans and Choppin, 1967a; Hosaka and Shimizu, 1968)

The next step was to determine the buoyant density of the extracted CDV nucleocapsid with a view to using this information in the formulation of a method of purifying the nucleocapsid. It was considered that the best way of identifying the latter in the density gradient would be by labelling it with ^{3}H-uridine. Unfortunately, however, it was found that DW extraction was an inefficient process, insufficient nucleocapsid being released to enable it to be identified in the gradient. An attempt was therefore

made to extract it with DOC but, although this procedure released more radioactive viral material, centrifugation of the extract through a sucrose density gradient showed that the material covered a wide density range. This method was therefore of little value for the purification of nucleocapsid. The heterogeneity of the latter in sucrose density gradients was considered as possibly due to the adherence of cellular material to it. It was therefore decided to substitute caesium chloride for the sucrose since high ionic strength is known to facilitate the purification of nucleocapsid (Norrby and Hammarskjold, 1972). However, DOC extracted nucleocapsid centrifuged though caesium chloride was found, by electron microscopy, to be broken up into relatively small pieces. An attempt was therefore made to determine whether this fragmentation was due to the DOC or to the caesium chloride. It was found that nucleocapsid released from cells by non-ionic detergents and centrifuged through caesium chloride did not show the same degree of fragmentation. It therefore seemed that the fragmentation was due to the DOC, though Hall and Martin (1973) and Blair (1970) have found this satisfactory for the extraction of nucleocapsid from measles and Sendai virus particles respectively. As judged by the amount of nucleocapsid observed with the electron microscope, Sterox SL was the most efficient of the three non-ionic detergents tested. This work also showed that CDV nucleocapsid was rather unstable but the addition of BSA both to the detergents and to the gradients increased

the amounts to be seen with the electron microscope.

In order to ensure that the labelled material was viral not cellular in origin, Actinomycin D had been incorporated into the medium in the earlier work. The cytotoxicity of this antibiotic, however, limited the length of labelling to about eight hours and this meant that the total amount of label incorporated into the nucleocapsid was low. In order to increase the amount of radioactivity in the preparation, label had to be left in contact with the cells for a longer period and this precluded further use of Actinomycin. A method of distinguishing viral RNA-containing structures from cellular RNA had therefore to be found and this was successfully accomplished by treating the cellular extract with RNase.

Electron microscopic examination of caesium chloride gradients through which non-ionic detergent extracted material had been centrifuged revealed nucleocapsid in the density region 1.27 to 1.31 g/cc. This was confirmed when radioactively labelled nucleocapsid extracted with Sterox SL was centrifuged to its position of isopycnic density in a preformed caesium chloride gradient. A peak of radioactivity was obtained at density 1.278 g/cc which corresponded very closely to the position of nucleocapsid, as determined by electron microscopy. When gradients of potassium tartrate were substituted for those of caesium.

chloride, a sharp peak of radioactivity occurred at a density of 1.245 g/cc, but no nucleocapsid could be found with the electron microscope when samples of an identical gradient were examined. The same result was obtained when the experiment was repeated. It therefore seemed probable that breakdown of nucleocapsid had occurred in the potassium tartrate. This may have been at least partly due to the necessity of reducing the concentration of BSA to avoid precipitation. De Thé and O'Connor (1966) reported that the buoyant density of NDV nucleocapsid lay in the range 1.24 to 1.26 g/cc, a finding which supports the belief that the peak of radioactivity at 1.245 g/cc was due to CDV nucleocapsid.

From these results, a reasonably efficient method for the extraction and preliminary purification of nucleocapsid was obtained. Time did not permit determination of the degree of purification reached by the one banding in caesium chloride but it is likely that the lability of the nucleocapsid would preclude more than one additional banding. Thus, as judged from the recovery of labelled RNA, Hosaka (1968) found that after two bandings, approximately two-thirds of the nucleocapsid released from the parainfluenza virus HVJ by Emasol-alkali treatment was lost. By means of a dual-labelling technique, i.e., one in which measles-SSPE virus nucleocapsid labelled with ^{3}H was mixed with a normal cell extract labelled with ^{14}C, Yeh and Iwasaki (1972) found that only two bandings were required to attain maximum purification. It is probable,

therefore, that the one banding used in the present investigation produced a substantial degree of purification.

One disappointing feature of the results obtained in the present investigation was the aggregation of nucleocapsid in caesium chloride gradients. This occurred in the presence of BSA although Yeh and Iwasaki (1972) claimed that such aggregation was decreased by the addition of BSA prior to centrifugation. Clumping of NDV, SV5 and measles virus nucleocapsids in caesium chloride was reported by Hosaka and Shimizu (1968), Compans and Choppin (1968) and by Norrby and Hammarskjold (1972) respectively. If most of the nucleocapsid obtained by banding in caesium chloride is present in aggregates, then this method of purification is unsuited to rate zonal studies which depend upon the sedimentation characteristics of individual nucleocapsids. The importance of rate zonal centrifugation lies in its applicability to the study of defective infection in which only short lengths of nucleocapsid are formed (Hall and Martin, 1974a; Kiley and Payne, 1974).

Another problem encountered was the presence of two bands and two peaks of radioactivity in caesium chloride gradients. Two bands have also been found in studies of SV5 (Compans and Choppin, 1967b) and HVJ (Hosaka, 1968) nucleocapsids, but, in both cases, the nucleocapsid in the two bands was morphologically indistinguishable. In the present investigation, however, nucleocapsid was found only in the region of the less dense band. The radioactive

Table 3 Reported Buoyant Densities of Nucleocapsids in CsCl Gradients.

Virus	Buoyant Density	Reference.
Measles	1.286	Waters et al. (1972)
Measles	1.29-1.30	Numazaki and Karzon (1966)
Measles	1.29-1.30	Phillips and Bussell (1973)
Measles	1.30	Hall and Martin (1973)
Measles	1.30	Waters and Bussell (1974)
Measles	1.305	Norrby and Hammarskjold (1972)
Measles	1.32	Norrby (1964)
Measles/SSPE	1.31	Yeh and Iwasaki (1972)
NDV	1.30	Haslam et al. (1969)
SV5	1.297	Compans and Choppin (1967b)
SV5	1.30	Waters and Bussell (1974)
HVJ	1.31	Hosaka (1968)
Sendai	1.31	Bukrinskaya (1973)

material in the other band was RNase resistant and may have been altered nucleocapsid, extracted from the inclusion bodies. Because of the closeness of the two bands and because the density of the nucleocapsid varied slightly from one experiment to another, it was felt that complete separation of the two would be difficult.

From the radioactive profile established in Experiment 3.6 the buoyant density of the nucleocapsid was 1.278 g/cc, but, in some of the earlier electron microscopic studies, it appeared that most of the nucleocapsid was in a region nearer 1.29 g/cc. These results suggested that a degree of heterogeneity exists. Waters and Bussell (1974) reported the buoyant density of the nucleocapsid of the Onderstepoort strain of CDV as being in the density range 1.29 to 1.30 g/cc. Some of the reported buoyant densities of measles and paramyxovirus nucleocapsids are summarised in Table 3.

It was thought that a study of the nucleocapsid from purified virus particles would perhaps help elucidate some of the above problems and might permit estimation of the size of the nucleocapsid. Moreover, purified virus was required before determination of the number and size of the polypeptides of the virion could be undertaken. For these reasons, the next step planned was the formulation of a system for the purification of the virion.

PART FOUR.

INTRODUCTION AND REVIEW OF THE LITERATURE.

A simple method of virus purification is provided by equilibrium density gradient centrifugation. The aim of the following experiments was to establish the buoyant density of CDV in different types of density gradients and, by comparing the tightness of banding and preservation of infectivity in each of these, to determine the optimum centrifugal conditions for purification.

At the commencement of the present investigation, only one publication had appeared on the buoyant density of CDV. This was the report by Elliot and Ryan (1970) that, during continuous flow ultracentrifugation in sucrose gradients, the virus banded in the region of the gradient composed of between 32 and 48% sucrose, the density of which ranged from 1.139 to 1.22 g/cc. In 1973, Phillips and Bussell published data on the buoyant density of Bussell's Onderstepoort variant grown in both avian and human cells. From 10 experiments involving centrifugation of the virus through CsCl gradients, they found that the peak of infectivity lay between 1.226 and 1.242 g/cc, with a mean of 1.231 g/cc. These results were independent of the type of

cell in which the virus was grown. In the only experiment in which potassium tartrate was used, the peak of infectivity occurred at about the same density, namely 1.233 g/cc, with virus found in the density region 1.210 to 1.233 g/cc.

Since the infectivity assay employed in the present investigation is time-consuming and of relatively low precision, it would obviously be advantageous to have a simpler but more precise method of quantitating the virus. With other paramyxoviruses, virions can be detected by reason of their haemagglutinating and haemolytic properties. With CDV, however, no such biological markers have ever been demonstrated. Since radioassay provides a rapid and precise method of comparing the amounts of virus in different fractions, the most obvious course was to attempt to label the virus with radioisotopes and hence obtain a profile of the distribution of radioactivity in the gradient. As far as the author is aware, no such information has yet been published for any paramyxovirus, though profiles of infectivity in CsCl density gradients have been published for measles virus by Norrby (1964) and Norrby et al. (1964) and Phillips and Bussell (1973) and for CDV and respiratory syncytial virus, by Phillips and Bussell (1973), and also for CDV in potassium tartrate gradients by Phillips and Bussell (1973).

Most of the reported buoyant densities of paramyxoviruses are those in density gradients of CsCl. The use of the latter material seems to have followed from the statement of Schluederberg and Roizman (1962) that better separation was achieved in that material than in sucrose. However, from the manner in which that statement is constructed, it is not clear whether it refers to the separation of measles virus components or to the separation of non-viral macromolecules by Meselson et al. (1957) to whom reference is made. McCrea et al. (1961) found that sharp banding of influenza and NDV was obtained in gradients of potassium tartrate, and suggested that this material was highly suited to the purification of animal viruses. The only published direct comparison of the profile of any paramyxovirus in the two types of gradients is that of Phillips and Bussell (1973), described above, which was based on infectivity studies. The two profiles were similar in shape though that in potassium tartrate was more broadly based. However, since more fractions were titrated from the caesium chloride gradient than from the potassium tartrate gradient, exact comparison is difficult.

The buoyant densities reported for measles virus vary from 1.224 g/cc (Phillips and Bussell, 1973) to 1.29 g/cc (Schluederberg and Roizman, 1962). This variation may be due to differences both in the virus preparation and in the cell system employed; Stenback and Durand (1963)

found that NDV grown in avian cells had a density of 1.22 g/cc whereas that prepared in mammalian cell cultures had a density of 1.236 to 1.242 g/cc. In the work of Phillips and Bussell (1973), the density of the infectivity peak varied slightly from one virus preparation to another. In the present investigation, therefore, a single stock of virus was produced for comparison of its profile in sucrose, caesium chloride, and potassium tartrate gradients. In this way, variation resulting from the use of different virus preparations was eliminated.

MATERIALS AND METHODS.

1. Production of radioactively-labelled virus. Unless otherwise stated, radioactive virus was prepared by the following method. Cells were infected in suspension and inoculated into 8oz. bottles or Roux flasks. After 18-24 hours incubation, ^{3}H-uridine was fed to the cells at a concentration of 20 µCi/ml. As soon as syncytia appeared, the tissue culture fluid was harvested and further collections were made twice-daily until major disruption of the cell-sheet had occurred. During this time, the radioiostope was only incorporated into alternate changes of medium.

2. Virus Concentration. Unconcentrated medium from an infected culture was used in Experiment 4.1 but in all subsequent work, concentrated virus was employed.

As far as possible, all steps were carried out at 4°C. Virus-containing tissue culture fluid, collected as described above, was pooled and clarified by low-speed centrifugation. The supernatant was mixed with an equal volume of saturated ammonium sulphate solution (SAS). After one hour, the precipitate which formed was sedimented by centrifugation at 6,000 rpm for 5 minutes in a Beckman J-21B ultracentrifuge. The supernatant was discarded and

the precipitate was redissolved in TS. The volume of TS required was approximately a tenth of the volume of the original pool. The resultant virus suspension was layered on top of discontinuous 20-60% sucrose gradients in SW 27 ultracentrifuge tubes, and spun at 25,000 rpm for 2.5 hours. At the end of this time, an opaque band was present at the interface between the two layers of sucrose and this was collected by the technique of fractionation described in Part Three. A volume of stock RNase solution was added to this banded material to give a final concentration of 100 µg/ml of RNase and the preparation was then dialysed against PBS for 30 minutes. The resultant dialysed sample, which usually had about a sixtieth of the volume of the original pool, was dispensed into aliquots which were stored at -70°C.

3. Density Gradient Centrifugation and
4. Radioassay. These were carried out as previously described under these headings in the Materials and Methods section of Part Three.

5. Infectivity Assays. Following determination of their refractive indices, selected fractions from gradients containing non-radioactive virus were diluted 1 in 10 with 4.5 ml of Earle's solution and then dialysed for one hour against cold Earle's solution. The infectivity titre of each was determined by the method described in Part One.

6. Electron Microscopy. This was carried out as described in Part Three.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 4.1

To Determine the Buoyant Density of CDV Virions.

The aim of the first experiment was to determine whether or not sufficient radioisotope could be incorporated into virus to permit its detection following centrifugation of unconcentrated culture fluid through a sucrose density gradient.

Procedure. An 8oz. bottle of cells infected in suspension with the virus was fed medium containing ^{3}H-uridine at 24 hours PI. Three days later, the medium was collected. Following clarification, 7.5 ml was layered on top of a 10 ml gradient of 20-60% sucrose in an SW 27 tube. The tube was then centrifuged at 25,000 rpm for 17 hours. Ten-drop fractions were collected and radioassay carried out as previously described.

Results. No clear cut peak of radioactivity indicative of banded labelled virus was obtained and the total amount of radioactivity recovered was small.

Conclusions and Discussion. The relatively low counts (maximum of 300 CPM) obtained and the lack of a clear-cut peak suggested that unconcentrated tissue culture fluid does not contain sufficient virus particles to permit resolution of the latter from cellular radioactive material of similar density.

EXPERIMENT 4.2

To Determine the Effect on the Virus Infectivity of Precipitation with Saturated Ammonium Sulphate Solution and Centrifugation through Sucrose.

The results of the previous experiment indicated that it would be necessary to concentrate the virus before attempting to determine its buoyant density. A convenient way of doing this might be to precipitate the virus with saturated ammonium sulphate solution (SAS) and then centrifuge it through 20% sucrose onto a pad of 60% sucrose. The effects on the viral infectivity of the two steps in this procedure were first investigated.

Procedure. A pool of virus was prepared from 272 ml of fluid from infected cultures. Two ml was removed and stored at -70°C for subsequent titration. The remaining 270 ml were clarified, mixed with an equal volume of SAS and left in an ice bath for 1 hour. The precipitate which formed was pelleted by centrifugation at 6,000 rpm for 5 minutes and was redissolved in 28.5 ml of TS. Following the removal of 1 ml for titration, the remaining 27.5 ml were layered on top of a discontinuous gradient of 20-60% sucrose (5 ml of 20% and 3 ml of 60%) in an SW 27 tube. The tube was centrifuged at 24,000 rpm for 2.5 hours. At the end of this time, an opaque band was found at the interface between the 20 and 60% sucrose solutions. This band was collected and dialysed at 4°C against PBS for 45 minutes. The resultant volume was 3.8 ml and this also was stored.

at -70°C for titration. As far as possible, all steps were carried out at 4°C.

Since the above procedures required approximately 6 hours for completion, the stability of the virus at 4°C was investigated. A pool of virus was prepared and divided into five aliquots. One was used immediately for infectivity titration and the remainder stored at 4°C. After 7, 14, 21 and 28 hours at that temperature, one aliquot was removed and its infectivity determined.

Since a common stock of virus was required for subsequent experiments and the latter could not be carried out on the same day, storage of radioactive virus at -70°C was considered essential. The effect on the viral infectivity of one cycle of freezing and thawing was therefore determined. A sample of virus was divided into two aliquots. One was titrated immediately and the other after overnight storage at -70°C.

Results. The results of the above investigations are shown in the tables below, from which it will be seen that the only procedure resulting in loss of infectivity was the centrifugation through sucrose.

a) Concentration Experiment.

Stage	Volume in mls	Concentration	Titre in $\log_{10}$ $TCID_{50}$ per ml	Total infectivity in $\log_{10}$
Initial	270	---	4.1	6.54
SAS ppt	28.5	x9.5	5.1	6.55
Sucrose pad	3.8	x68.4	4.7	5.28

Recovery of infectivity from pad - 5.5%

b) Temperature Sensitivity

Sample	Titre in $\log_{10}TCID_{50}$ per ml
Initial sample	4.7
7 hour	4.7
14 hour	4.9
21 hour	4.7
28 hour	4.9

c) Freezing and thawing.

Sample	Titre in $\log_{10}TCID_{50}$ per ml
Initial sample	4.9
After 1 cycle of freezing and thawing	4.7

Conclusions and Discussion. The use of SAS permitted a concentration of approximately 10-fold without loss of infectivity. By combining this with centrifugation onto a dense sucrose pad, a 68-fold concentration was obtained, though this resulted in a substantial loss of infectivity. However, despite the latter drawback, centrifugation was considered useful, since only fast sedimenting structures

such as medium-sized or large virus particles would be expected to reach the bottom of the 20% sucrose solution during the relatively short period of centrifugation employed. The elimination of cellular material such as ribosomes from the concentrated virus suspension would facilitate the detection of a peak of radioactivity due to banded virus. Also, it was hoped that the number of viral particles might not have been reduced to the same extent as the infectivity.

Storage of virus samples at $4^{o}C$ for 28 hours appears to have had no effect on the titre of the virus and similarly one cycle of freezing and thawing had no deleterious effect.

The method described in this experiment was therefore used to prepare aliquots of concentrated labelled and unlabelled virus for subsequent studies.

EXPERIMENT 4.3

To Determine the Buoyant Density of CDV in Three Types of Density Gradients.

The primary aim of the present experiment was to establish the buoyant density of the virus in density gradients of potassium tartrate, sucrose and CsCl. By comparing the distribution of infectivity and radioactivity in each type of gradient, it was hoped that the most suitable gradient for the purification of the virus would be found.

As it was impossible to centrifuge virus through all three types of gradients at the same time, a pool of virus was prepared and divided into aliquots as already described.

Procedure. A pool of 400 ml of tissue culture fluid containing approximately $10^5 TCID_{50}$ per ml of ^3H-uridine-labelled virus was prepared and clarified. The virus was precipitated with SAS and then centrifuged onto 60% sucrose as described in the previous experiment. The opaque band on top of the 60% sucrose was collected, 300 µg of RNase was added and dialysis was then carried out. The volume at the end of the period of dialysis was 6 ml. This was divided into 4 aliquots which were stored at -70°C.

A pool of non-radioactive virus was prepared and concentrated by the same method as for the radioactive virus.

Table 4. Distribution of Radioactivity in Density Gradients.

	Buoyant Density		Percentage of radioactivity recovered.
Gradients	Range[a]	At Peak	At Peak.
K. Tartrate	1.210-1.185	1.197	27
Sucrose	1.218-1.180	1.206	24
CsCl	1.262-1.229	1.240	13

a - Density of fractions containing 10% or greater of the recovered radioactivity.

Table 5. Distribution of Infectious virus in Density Gradients

Potassium tartrate		Sucrose		Caesium chloride	
Density	$\log_{10}TCID_{50}/ml$	Density	$\log_{10}TCID_{50}/ml$	Density	$\log_{10}TCID_{50}/ml$
				1.250	2.5
1.239	2.9			1.234	4.0
1.225	3.5	1.224	2.7		
				1.218	3.5
1.212	3.9	1.213	3.3		
				1.204	2.7
1.198	5.0	1.200	3.5		
1.184	4.7	1.188	3.3		
1.170	4.1	1.172	3.1		
1.158	2.7	1.155	2.5		

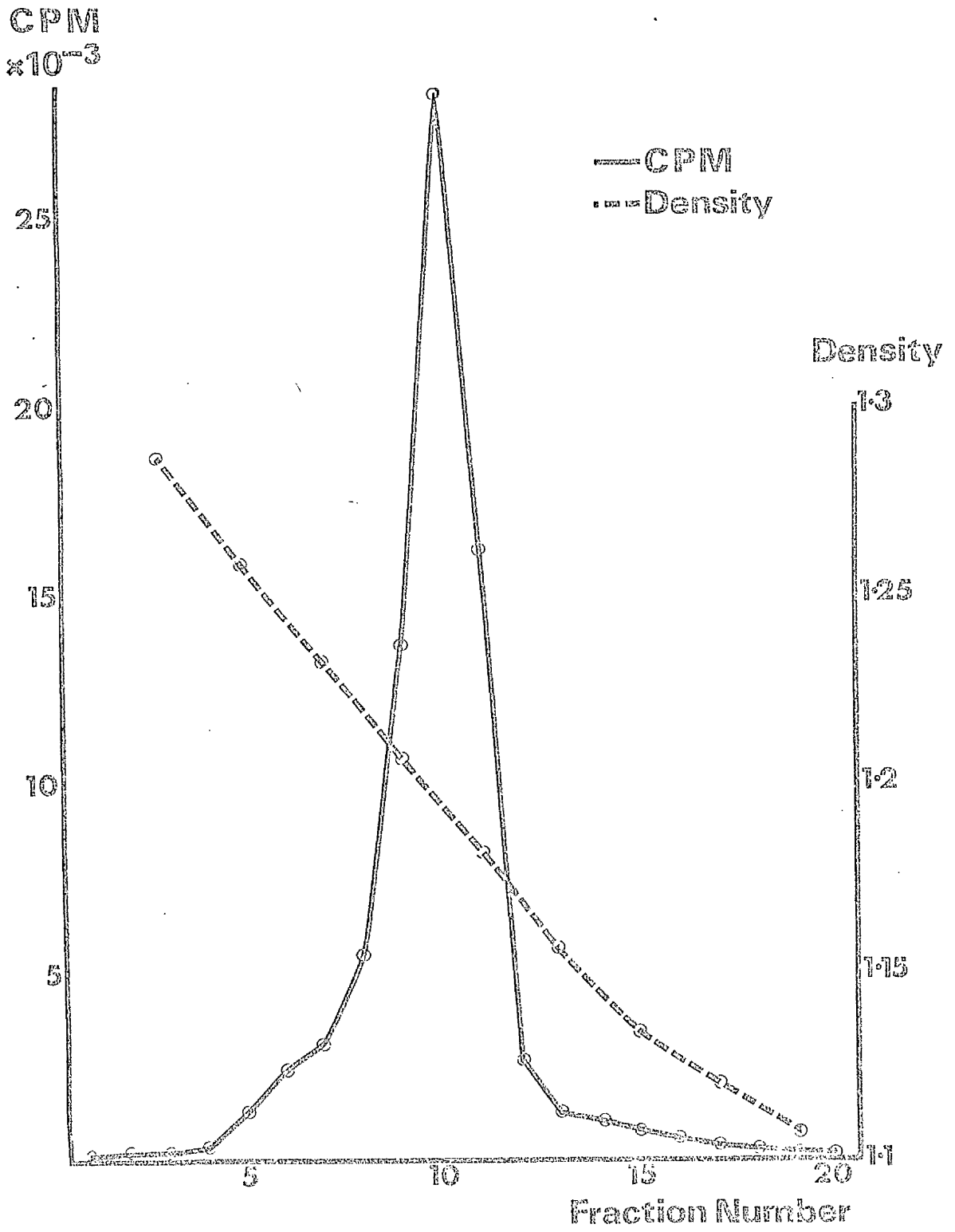


Figure 36. Radioactive profile showing the buoyant density of CDV in a potassium tartrate gradient.

Eight ml gradients of 1.1 to 1.3 g/cc of potassium tartrate, 20-60% sucrose, and 1.1 to 1.3 g/cc of CsCl with a 1.5 ml buffer layer of the appropriate solution, were prepared in SW 40 tubes. An aliquot of radioactive virus was centrifuged through one of each type of gradient to determine the distribution of radioactivity in each. Similarly, aliquots of unlabelled virus were centrifuged through two of each type of gradient to establish the position of visible and infectious virus in the gradients by electron microscopy and infectivity assay respectively. The tubes were centrifuged at 37,000 rpm for 16 hours. Ten-drop fractions were then collected from each, the refractive indices of alternate fractions were measured, and radioassay, infectivity titrations and electron microscopic examination were carried out as previously described.

Results. The results of the radioassay and infectivity assays are shown in Tables 4 and 5. From these it can be seen that, whereas the distribution of radioactivity and infectious virus in the potassium tartrate gradients closely correspond to each other and to those in the sucrose gradients, they differed markedly from the patterns seen in the gradients of caesium chloride.

a) Potassium tartrate. As can be seen from Figure 36, the radioactive profile in gradients of potassium tartrate was

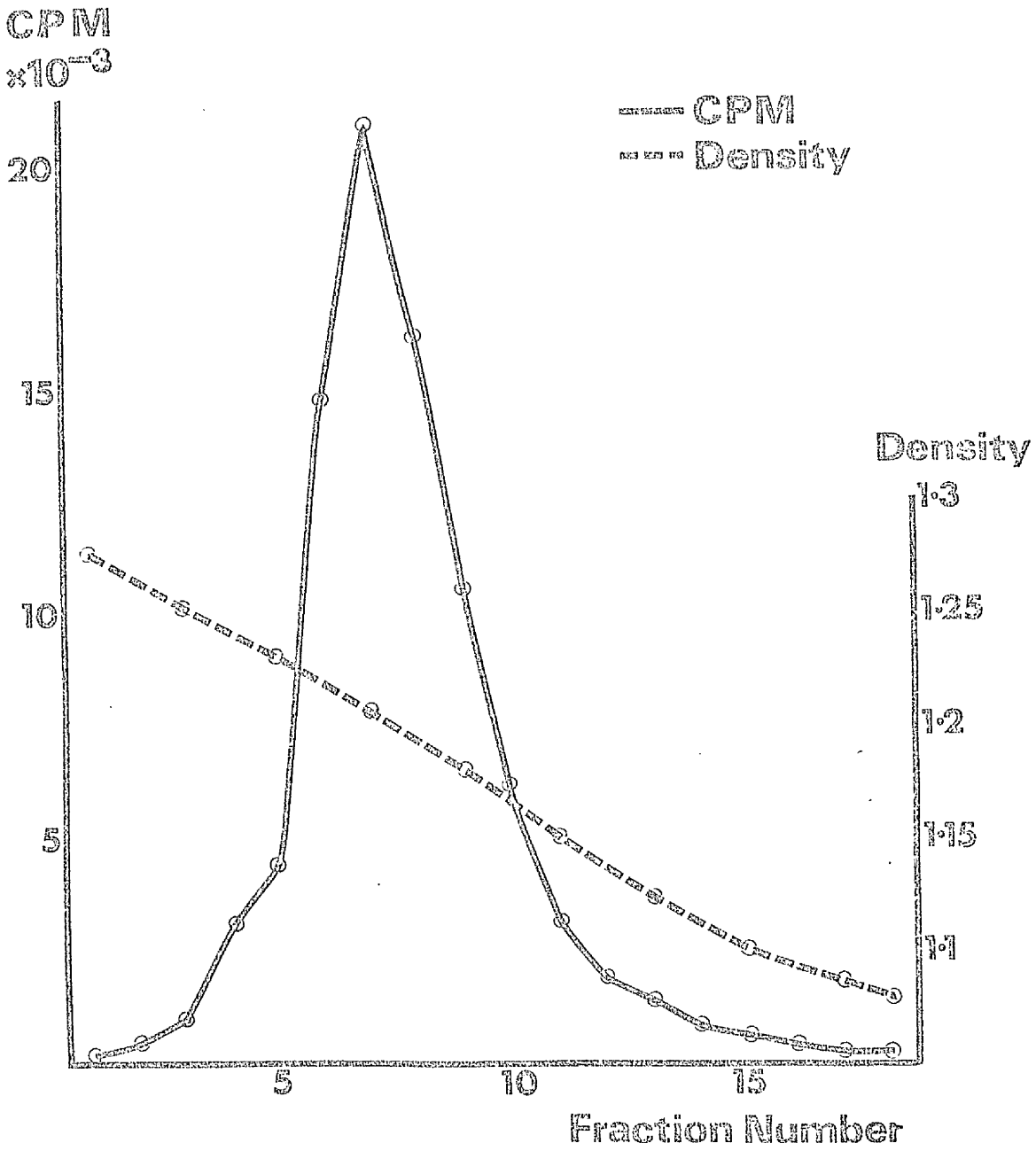


Figure 37. Radioactive profile showing the buoyant density of CDV in a sucrose gradient.

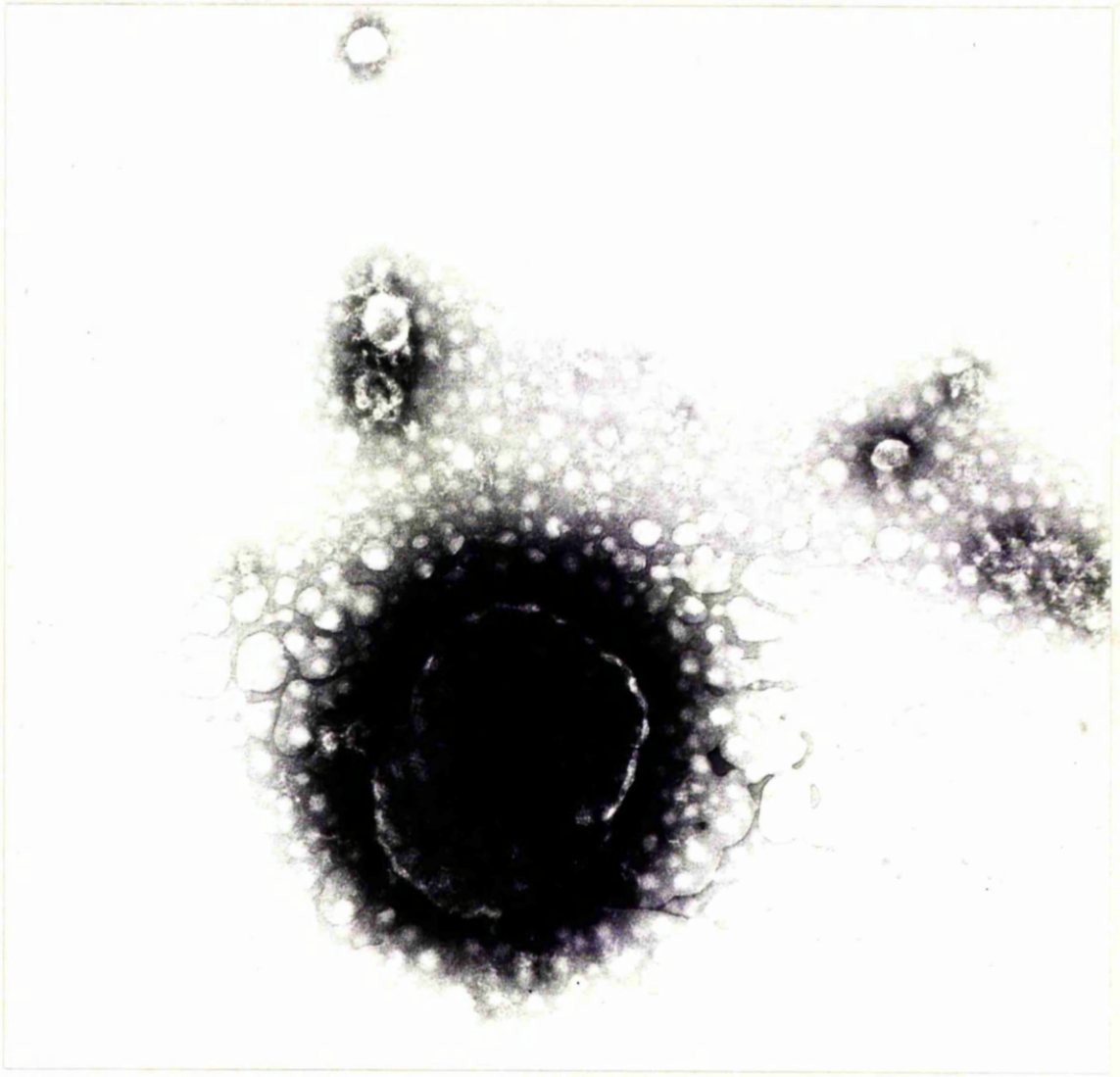


Figure 38. Negatively-stained CDV particle, from a sucrose density gradient. X80,000

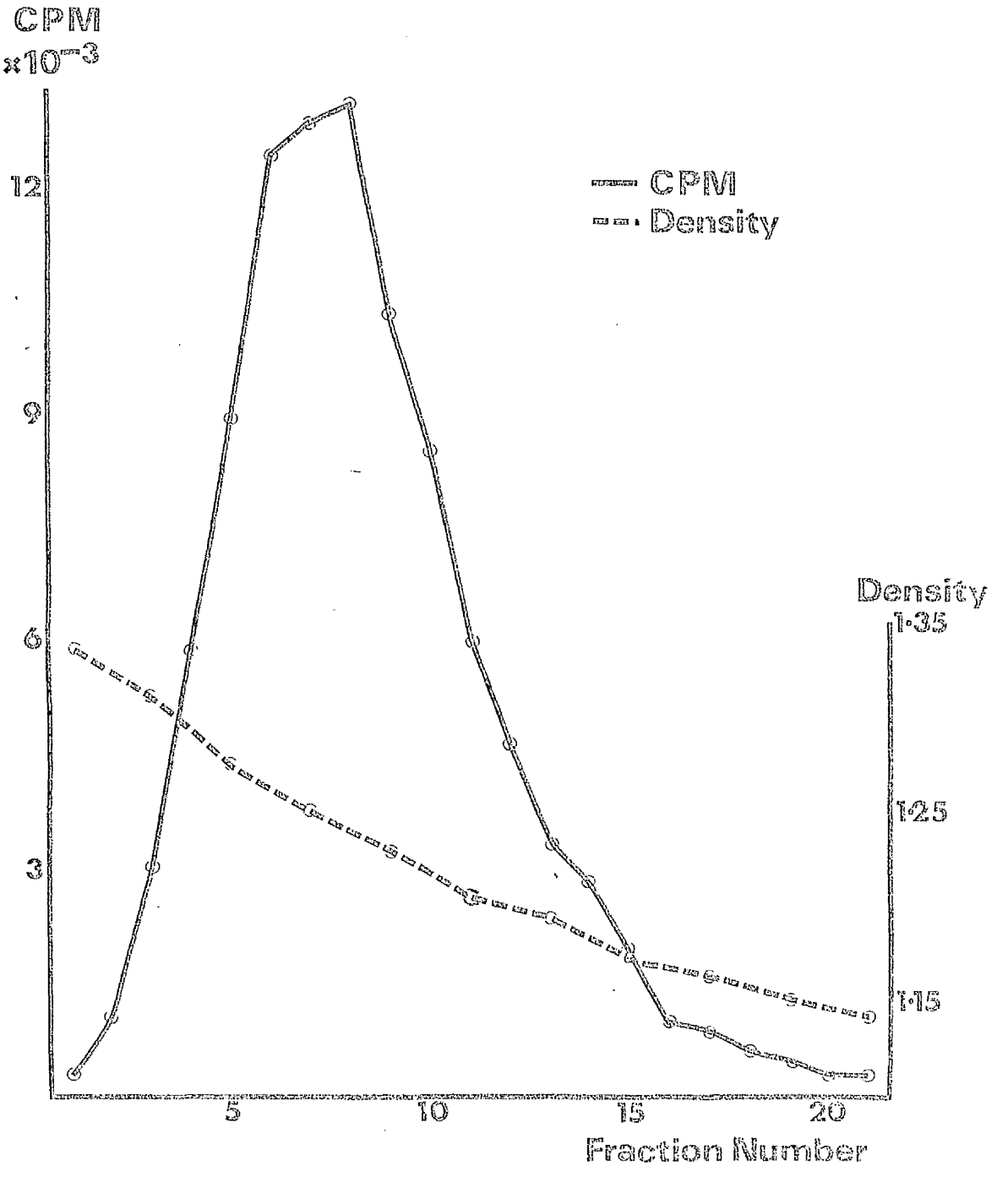


Figure 39. Radioactive profile obtained by centrifugation of CDV in a CsCl gradient.

characterised by a sharp peak at a density of around 1.195 g/cc, the amount of radioactivity present at this point being some 12-fold greater than at the base of the peak. The results of the infectivity titrations are recorded in Table 5. The distribution of infectivity closely matched that of radioactivity. In reading these titrations, a prozone phenomenon was observed, CPE being absent or delayed in tubes inoculated with the lower (10^{-1} to 10^{-2}) dilutions of fractions corresponding in density to those containing the greatest amounts of radioactivity. With the electron microscope, virus particles were observed in fractions having a density range of 1.176 to 1.237 g/cc, the greatest number being found in the range 1.190 to 1.214 g/cc.

b) Sucrose. The radioactive profile in sucrose density gradients (Figure 37) was very similar to that in potassium tartrate, though, as Table 4 reveals, the peak tended to have a broader base. The infectivity titres are recorded in Table 5, which shows that although the peak occurred at the same density as in potassium tartrate, the amount of infectious virus recovered was 0.5 to 1.5 $\log_{10}$ lower. Electron microscopy revealed the presence of typical virus particles in fractions having a density range of 1.158 to 1.216 g/cc, the greatest number being found in the two fractions of density 1.190 and 1.197 g/cc. A virus particle from this gradient is shown in Figure 38.

c) Caesium Chloride. As shown in Figure 39, the radioactive

profile in the caesium chloride gradient was characterised by a plateau covering the density range 1.238 to 1.262 g/cc. The infectivity titres of the non-radioactive virus from the gradient are shown in Table 5. Comparison of these with Figure 39 will show that the correlation between the radioactivity and the infectivity at densities of greater than 1.245 g/cc was poor. With the electron microscope, typical virus particles were demonstrated in fractions having a density range of 1.193 to 1.275 g/cc, the greatest number being found in two fractions of density 1.219 and 1.234 g/cc respectively.

Conclusions and Discussion. The radioactive profiles obtained from the potassium tartrate and sucrose gradients were very similar, both having peaks of radioactivity at around 1.2 g/cc. This was also where most of the infectious virus was found and where the greatest number of viral particles were seen with the electron microscope. This correlation, coupled with the fact that the material was RNase treated to eliminate as much cellular RNA as possible, suggests that the peaks of radioactivity were due to banded labelled virus. The peak in the potassium tartrate gradient differed from that in sucrose in being higher but less broadly based, indicating that the virus had been concentrated into a narrower band. The amount of infectious virus in the fraction of density 1.2 g/cc was also greater in potassium tartrate than in sucrose. The prozone phenomenon observed in the titrations of the fractions from the

potassium tartrate gradient will be discussed at the end of this section.

The radioactive profile obtained from the CsCl gradient differed considerably from those seen in the other two gradients, the highest CPM being in the density region 1.24 to 1.26 g/cc. Also, as noted earlier, the region of greatest radioactivity did not correspond either to the region where most of the infectivity was found or to that where most virus was seen with the electron microscope. Since the samples were partially purified and treated with RNase, and there was a close correlation between radioactivity and infectivity in the sucrose and potassium tartrate gradients, the radioactivity in the CsCl gradient must therefore have been due to viral material. The difference in the density of the radioactivity and infectivity in the CsCl is therefore difficult to understand. One possibility is that the virus was partially broken up and the radioactive peak was due to banded nucleocapsid with pieces of envelope attached. On investigation, it was found that the pH of the CsCl solutions was only 5.4, whereas that of the sucrose and potassium tartrate solutions was 7.4. Though it was obvious from the infectivity results that infectious virus was still present in the gradients after 16 hours at pH 5.4, it was thought that some break up of the virus might have occurred and hence produced the discrepancy between the radioactive and infectivity results.

EXPERIMENT 4.4

To Determine the Effect of pH on the Radioactive Profile of CDV in CsCl Gradients and Confirm the Results of the Sucrose and Potassium Tartrate Centrifugation in the Last Experiment.

The present experiment was designed to determine whether or not the radioactive profile obtained in CsCl was due to break up of the virus by the low pH. It also had the aims of confirming the radioactive profiles obtained from the sucrose and potassium tartrate gradients.

Procedure. CsCl solutions of density 1.1 and 1.3 g/cc were prepared in TS, TE buffer, and a mixture of TS and TE, to give solutions of pH 5.4, 7.4 and 8.1. These solutions were then used to make three gradients of density 1.1 to 1.3 g/cc, with pH 5.4, 7.4 and 8.1.

Five ampoules of radioactive virus were prepared from a pool as described in the previous experiment. Three of these were layered on top of the CsCl gradients, one per gradient. The fourth and fifth were layered onto gradients of potassium tartrate and sucrose respectively, these being identical to those used in the previous experiment. Centrifugation and fractionation of gradients and radioassay were carried out as described previously.

Results. The profiles obtained with potassium tartrate and sucrose were identical to those obtained in the

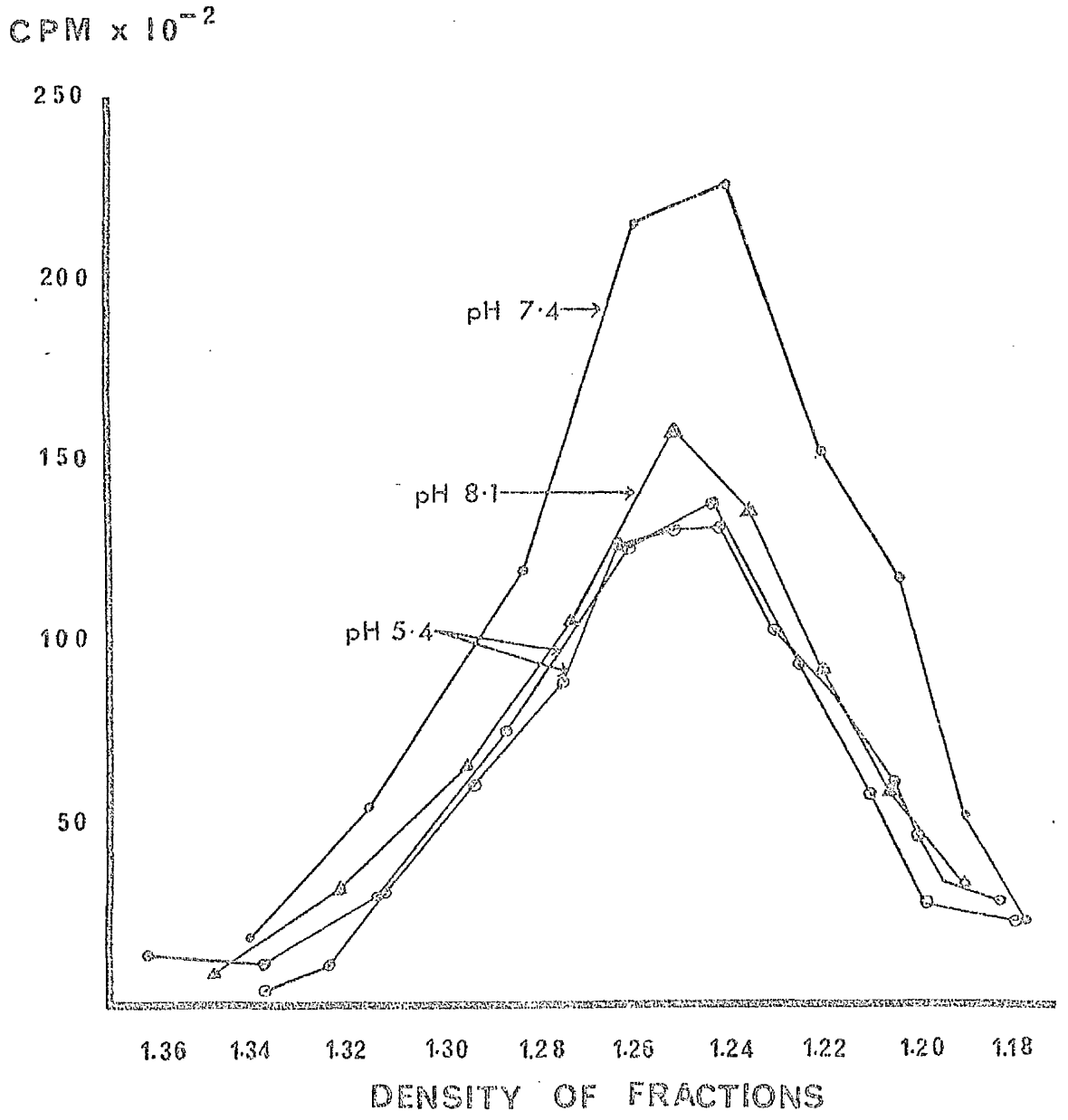


Figure 40. Profiles obtained from the centrifugation of ^{3}H-uridine labelled CDV in CsCl gradients of different pH values.

previous experiment, the potassium tartrate again producing tighter banding of the virus. The peaks occurred at much the same density as before, namely 1.193 g/cc in potassium tartrate and 1.196 g/cc in sucrose. The distribution of radioactivity in each gradient is shown in the following table.

Gradient	pH	Buoyant Density Range[a]	Buoyant Density At peak	% of recovered radioactivity At peak.
K.T.	7.4	1.205-1.182	1.193	35
Sucrose	7.4	1.201-1.182	1.196	21
CsCl	5.4	1.286-1.224	1.242	22
CsCl	7.4	1.282-1.202	1.239	21
CsCl	8.1	1.250-1.234	1.250	12

a - Density of fractions containing 10% or greater of the recovered radioactivity.

The results obtained with CsCl are shown in Figure 40. As can be seen, the pH value had little influence on the profile, although the total amount recovered was about 60% higher at pH 7.4 than at pH 5.4.

Conclusions and Discussion. The results described above confirmed that the radioactive profile of the virus in CsCl density gradients was quite distinct from that observed in potassium tartrate and sucrose gradients. Since the same profile was obtained at different pH values, it was clearly not the result of break-up due to low pH, as suggested by the results of the last experiment. Of the

three gradients tested, the tightest banding was again obtained with potassium tartrate.

EXPERIMENT 4.5

To Compare the Degree of Separation of Virus from Mycoplasma obtained by Centrifugation through Potassium Tartrate and Sucrose Gradients.

The results of the previous two experiments showed that sharper banding of virus was obtained in potassium tartrate than in sucrose. It seemed possible that this might have been due to the inability of potassium tartrate to resolve small differences in density. If this was true, separation of virions from non-viral particles of similar density would probably prove difficult; the value of potassium tartrate for the purification of the virus would therefore be limited. In order to compare the efficacy of potassium tartrate and sucrose for the separation of CDV virions from particles of similar size, structure and density, the decision was made to compare the distribution of the mycoplasma, Acholeplasma laidlawii in the two types of gradients. By so doing, it was hoped that tighter banding would again be obtained in potassium tartrate but at a density quite distinct from that of the virus. The buoyant density of mycoplasmata in sucrose gradients has been reported to lie in the range of 1.20 to 1.24 g/cc (Todaro et al., 1971).

Procedure. Some mycoplasma broth containing Acholeplasma laidlawii was mixed with tissue culture growth medium containing sufficient ^{3}H-uridine to give a final concentration of 20 µCi/ml, and the sample was incubated at 37°C for

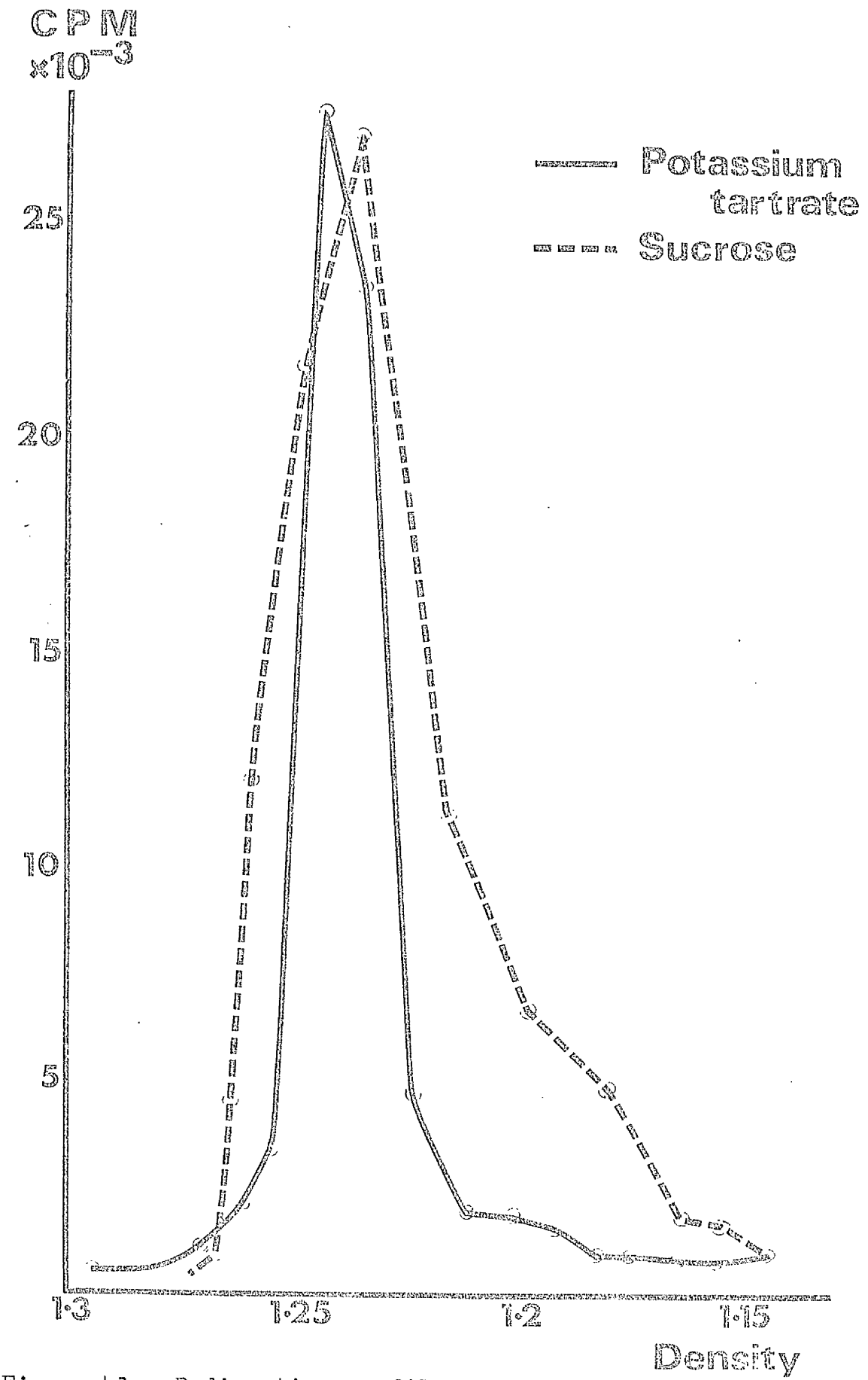


Figure 41. Radioactive profiles obtained from the centrifugation of Acholeplasma laidlawii in a sucrose and a potassium tartrate density gradient.

two days. Half of the sample was then layered on top of an 8 ml gradient of 20-60% sucrose, with a 1.5 ml buffer layer, in an SW 40 tube, and the other half was layered on top of an 8 ml gradient of 1.1 to 1.3 g/cc of potassium tartrate, with a 1.5 ml buffer layer, again in an SW 40 tube. The tubes were centrifuged at 37,000 for 16 hours. The gradients were then fractionated and radioassay carried out as described for the virus.

Results. The results are shown in Figure 41 from which it will be seen that tighter banding was again obtained in potassium tartrate. In both gradients, the mycoplasmas banded at a density of around 1.24 g/cc – considerably greater than that of the virus – but whereas the tight banding in potassium tartrate meant that few, if any, organisms were present at a density of less than 1.22 g/cc, the radioactive profile in sucrose indicated that they were still numerous at a density of 1.19 to 1.20 g/cc.

Conclusions and Discussion. Potassium tartrate permitted sharper banding of both virus and mycoplasma than did sucrose though in both types of gradient there was a difference of 0.04 g/cc in the density of the two peaks. The sharper banding in potassium tartrate thus provided better conditions for the separation of the two populations. In sucrose, on the other hand, the spread of each band was such as to produce overlap and hence preclude any possibility

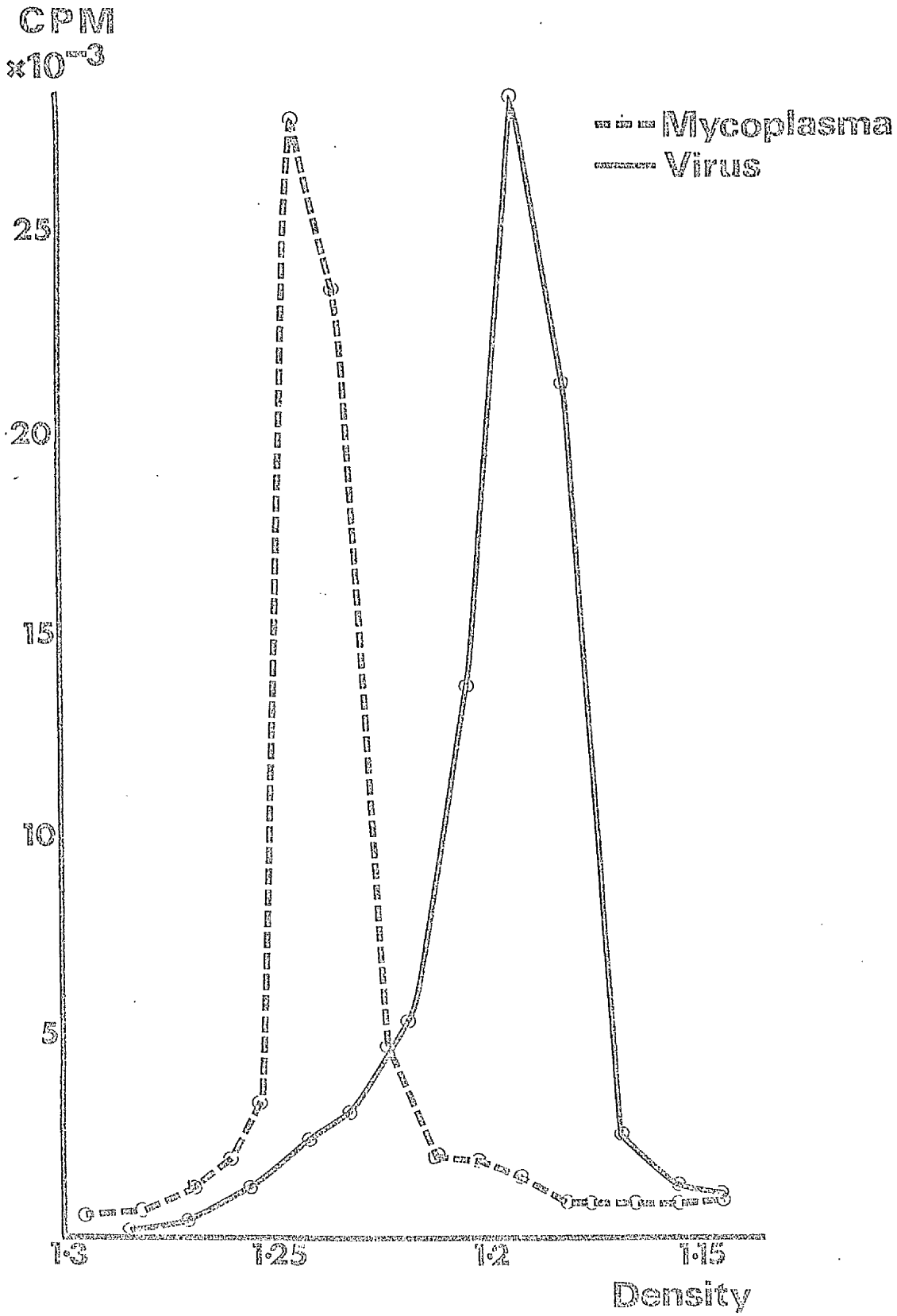


Figure 42. Comparison of the profiles obtained from the centrifugation of mycoplasma and CDV in potassium tartrate gradients.

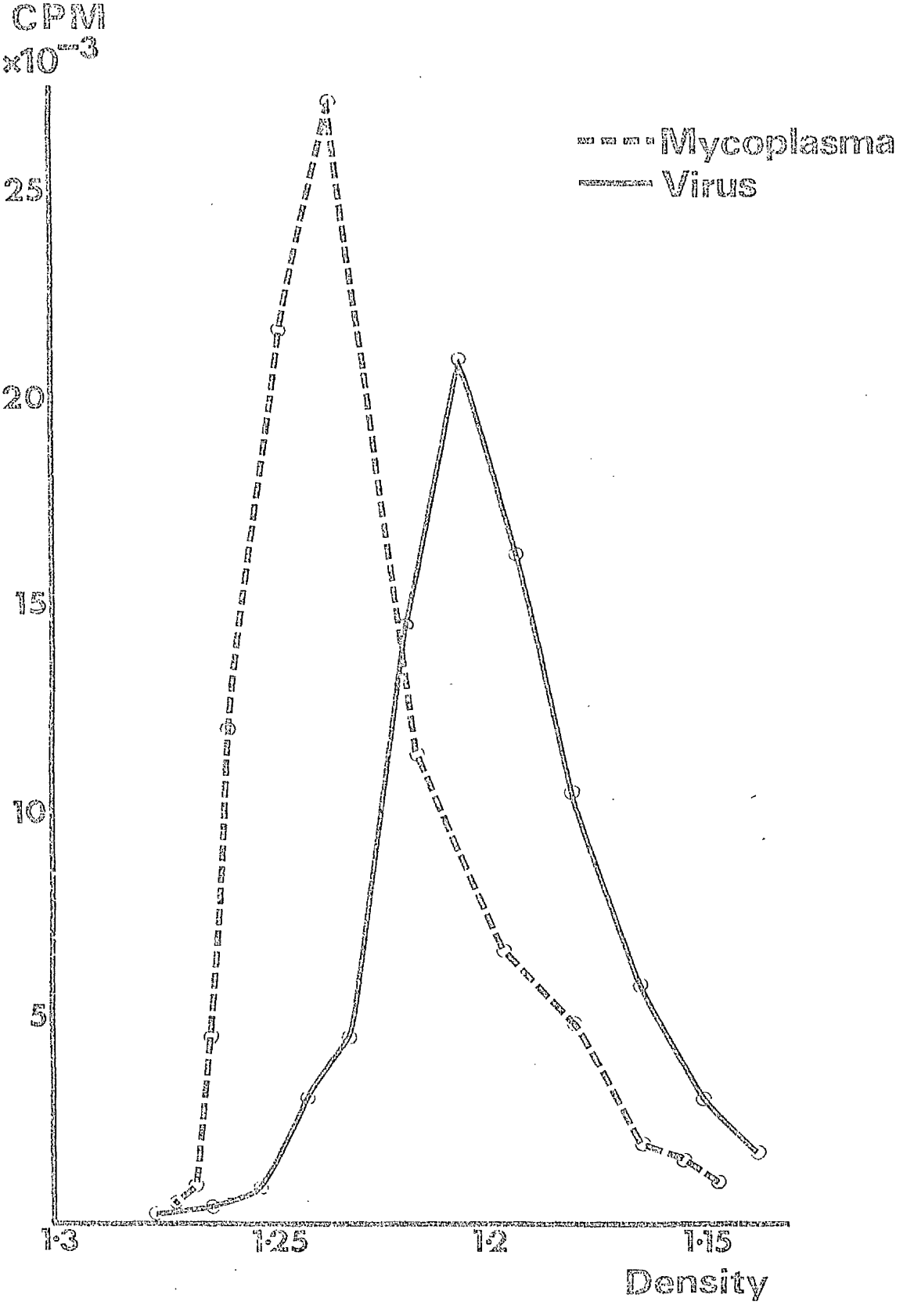


Figure 43. Comparison of the profiles obtained from the centrifugation of mycoplasma and CDV in sucrose gradients.

of achieving complete separation without sacrificing some of the virus. This is illustrated in Figures 42 & 43, drawn from the results of Experiment 4.3 and 4.5. On this basis, it was concluded that potassium tartrate provides better resolution of different populations and hence is more ideal for the purification of the virus.

DISCUSSION.

From the results of these four experiments, a system was evolved for the preparation of virus for biochemical studies.

Unconcentrated tissue culture fluid contained insufficient labelled virus to allow its detection in density gradients so a system of concentration and partial purification was introduced. This involved the production of a large pool of virus, precipitation of the virus with SAS, and its sedimentation through 20% sucrose onto a pad of 60% sucrose, a 64-fold concentration being achieved. Precipitation did not affect the virus adversely, but centrifugation through 20% sucrose resulted in a substantial loss of infectivity. It was not surprising, therefore, that when virus concentrated in this manner was centrifuged through linear density gradients, the infectivity titres of fractions from these gradients were relatively low. This loss of infectivity in sucrose may have been due to the high osmotic pressure exerted by the solution; since the viral envelope is derived from cellular membrane and cells can be disrupted by osmotic shock, it was likely that envelopedviruses can be similarly affected. There was no evidence, however, that the loss of infectivity was matched by a loss of particles; although particle morphology

was poor (again perhaps due to sucrose), the very fact that virions were found with the electron microscope indicated that a substantial number were present; Waterson (1964) stated that a concentration of 10^9 particles per ml is required for visualisation in the electron microscope. One other piece of evidence suggested that the particle/infectivity ratio was high. This was the prozone phenomenon which occurred when fractions from the potassium tartrate gradient were titrated for infectivity. The fact that this occurred mainly with fractions of the same density as those forming the radioactive peak suggests that blockade of cell receptors by non-infectious virus may have been largely responsible for the phenomenon.

When samples of labelled virus were centrifuged through potassium tartrate gradients, peaks of radioactivity were obtained at a density of approximately 1.20 g/cc. When unlabelled virus was centrifuged through identical gradients, it was found that infectious virus and virus particles were likewise present in greatest amount at this density. Since the virus had been treated with RNase, it was concluded that the radioactive peak represented banded labelled virus and that the buoyant density of the virus was therefore 1.20 g/cc. The peak in potassium tartrate was higher than that in sucrose, with a radioactive content of 42,000 CPM compared to 32,000 CPM, but had a narrower base. The titres of infectious virus recovered from the potassium tartrate were likewise greater than those obtained from the

sucrose. These results showed that tighter banding occurred in potassium tartrate, i.e., the virus was concentrated into a narrower density range. As mentioned previously, McCrea et al., (1961) found that potassium tartrate permitted tight banding of influenza and NDV virions, while Elliot and Ryan (1970) reported that, in sucrose, CDV occupied a fairly wide density range, namely 1.14 to 1.22 g/cc.

Centrifugation of samples through caesium chloride produced distributions of radioactivity and infectivity which differed not only from those in potassium tartrate and sucrose, but also from each other. The radioactive profile obtained was a plateau covering the density range 1.24 to 1.26 g/cc, whereas the results of the infectivity assay and electron microscopy indicated that infectious virus particles occurred at a lower density range, with most around 1.23 g/cc. Although the pH of the caesium chloride gradients was only 5.4, infectious virus was recovered from them, and when neutral and slightly alkaline gradients were used, the radioactive profile remained the same, indicating that the acid pH had not broken up some of the virus and hence had not been responsible for the difference between the profiles of radioactivity and infectivity. The recovery of infectious virus after 16 hours at pH 5.4 is in agreement with the report by Celiker and Gillespie (1954) that viability for CDV is retained between pH values of 4.4 and 10.4. The difference between the two profiles is difficult to interpret. Since all of

the radioactivity in the other two types of gradients was associated with virus particles, the radioactive material in the caesium chloride gradients must also therefore have been viral in nature. Although the acid pH did not apparently cause break-up of the virus, it is possible that some other factor had produced partial break-up, resulting in a band of density 1.24 to 1.26 g/cc, consisting of nucleocapsid with pieces of envelope attached.

From the results of the infectivity tests and electron microscopy, the buoyant density of CDV in caesium chloride was 1.23 g/cc. This is in accordance with the findings of Phillips and Bussell (1973) who reported that the peak of infectivity in caesium chloride gradients occurred at a density of 1.233 g/cc. However, the latter workers found that infectious virus banded at the same density in potassium tartrate as in caesium chloride, whereas in the present investigation, virus was found to band at a lower density in potassium tartrate and sucrose than in caesium chloride. This difference in density may have been due to the binding of caesium ions to the virions. However, if this did in fact occur, it might have been expected that potassium ions would have done likewise but, as the buoyant density of the virus was the same in potassium tartrate as in sucrose, this seems unlikely. The difference between the two gradients might have been due to the difference in the anions present, as it has been shown by Rowlands et al., (1971) that the buoyant densities of certain picornaviruses

are much lower in caesium sulphate than in caesium chloride. Dependence of buoyant density on gradient composition has been discussed by Manning et al., (1970) who found that mouse mammary tumour virus banded at the same density in potassium tartrate as in sucrose, but at a greater density in caesium chloride. Without making similar comparisons, other workers, however, have reported a buoyant density of 1.23 g/cc for both measles (Hall and Martin, 1973) and SV5 viruses (Klensk and Choppin, 1969), a figure very similar to that for their buoyant density in caesium chloride (Phillips and Bussell, 1973). On the other hand, McNulty (personal communication) found that the buoyant density of egg-grown NDV in potassium tartrate was 1.20 g/cc. Variation in the reported buoyant densities of viruses in potassium tartrate may be related to the absence of published tables giving refractive indices for a range of densities. Each laboratory must therefore produce its own set of figures and calibration curve. Standardisation of this would permit easier comparison of results.

From the work of Phillips and Bussell (1973), it is clear that the variation in the density of virions within a single gradient is due to real variation in their density; virions from a particular zone of the band were shown to sediment to the same position on recentrifugation. This variation in density is easy to understand since electron microscopy has shown variation in particle size, with resultant differences in the ratio of nucleocapsid to envelope. From the results of Experiments 4.3 and 4.4,

judgement could not be made as to which of the gradients tested had the greatest ability to resolve small differences in density. However, from the results of Experiment 4.5, it appeared that much better separation of viral and non-viral material could be achieved in potassium tartrate than in sucrose.

PART FIVE.

INTRODUCTION AND REVIEW OF THE LITERATURE.

Until 1973, no estimations of the number or size of the polypeptides of CDV or measles virus had been reported but, in that year, two papers were published on this subject. The first was by Hall and Martin who reported the finding of six polypeptides from sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of measles virus, the molecular weights of these being 75,600; 69,000; 60,000; 53,000; 51,000 and 45,700. The second was by Waters and Bussell who also obtained six polypeptides from both measles and CDV virions and reported the molecular weights of these to be 78,000; 71,000; 61,000; 53,000; 45,000 and 37,000, in both cases. As can be seen, there is some variation between these two sets of figures, especially with regard to the two smallest polypeptides.

Although fuller documentation exists for the polypeptides of certain other paramyxoviruses, not only are there variations in the reported size of these but also in the number present. For example, six polypeptides were separated from NDV by Scheid and Choppin, (1973) whereas Moore and Burke, (1974) found ten. It is generally accepted, however, that paramyxoviruses contain at least

six polypeptides. In the case of NDV, three major polypeptides are always found, though again, the estimations of the molecular weights of these vary. For example, the estimated molecular weights for the haemagglutinin range from 90,000 (Evans and Kingsbury, 1969) to 74,000 (Mountcastle et al., 1970). Some of the variation may be due to differences between the viral strains studied. Shapiro and Bratt (1971) reported that among the polypeptides obtained from four strains of NDV, the three major classes were always present but variation was seen in the proportion, and possibly the number, of other classes.

The different results obtained in the above investigations may also have been due to the differing techniques employed, e.g., the method of solubilising the viral protein, the composition and concentration of the gels, the time allowed for electrophoresis and the current passed. It is possible that variation may also be related to the different methods of calculating the molecular weights from the marker polypeptides. Some workers, (e.g. Haslam et al., 1969; Hall and Martin, 1973), have compared the distances migrated (DM) by the test and marker polypeptides while others (e.g. Evans and Kingsbury, 1969; and Waters and Bussell, 1973) have compared the relative migration (RM) values, these being the ratios of the distances migrated by the polypeptides to the distance migrated by the tracker dye. As will be seen from the results of the present investigation, the system has an inherent degree of

inaccuracy, variation occurring in the results obtained in a single experiment with a single virus preparation.

The aim of the subsequent experiments was to determine the number and size of the polypeptides obtained from purified CDV, to compare these with the polypeptides described by Waters and Bussell (1973) for the Onderstepoort strain of CDV and to investigate some of the technical factors governing the results obtained.

MATERIALS AND METHODS.

1. Purification of virus. Before storage at -70°C, all virus-infected tissue culture fluid used for this work was clarified by low-speed centrifugation at the time of harvesting in order to remove floating syncytia and cell debris, and thus reduce the amount of cellular protein to a minimum. A pool of 1,200 ml of virus-containing tissue culture fluid was prepared. Following clarification by centrifugation, the virus was precipitated with SAS, centrifuged through 20% sucrose onto a pad of 60% sucrose, and subjected to two consecutive bandings in potassium tartrate density gradients, the time and speed being as reported in Part Four. After the second banding, the virus was dialysed against PBS at 4°C for several hours and then pelleted in a siliconised SW 50.1 tube by centrifugation at 35,000 rpm for one hour. It was then resuspended in 3 ml PBS and dispensed in 0.5 ml volumes which were stored at -70°C. Estimation of the protein content of this preparation by the method described by Lowry et al. (1951) showed it to be 220 µg/ml.

2. Marker polypeptides. Rather than employ non-viral polypeptides as markers, it was decided to use the polypeptides of NDV since it was expected that these would be similar to those of CDV. The egg-grown virus preparation

was kindly supplied by Dr. M.S. McNulty. The protein content of this was 300 µg/ml. The three major polypeptides were used as the markers. The molecular weights of these were taken as 78,000; 59,000; and 42,000 by averaging the figures published by Evans and Kingsbury (1969), Haslam et al. (1969), Mountcastle et al. (1970) and Scheid and Choppin (1973), abnormally high or low estimations being ignored.

3. Solubilisation of polypeptides. The solutions used were;-
(a) Reducing solubiliser - 6 M urea, 1% SDS, 1% 2-mercapto-ethanol, 10% sucrose, a few grains of bromophenol blue (to colour, and form a tracker dye), in PBS.
(b) Non-reducing solubiliser - as for (a) but without mercaptoethanol.
The virus was thoroughly mixed with the solubiliser, incubated at 70°C for 30 minutes, followed by 100°C for 1 minute, and left to cool before being layered on top of the gels.

4. Preparation of gels. The tubes used for the gels were 70 mm long, 6.5 mm wide, and made of glass. They were stored in chromic acid until required, then boiled in detergent, left to cool, rinsed several times in water, rinsed in acetone to aid drying, and left to dry. Parafilm was placed over the bottom of the tubes, and they were then placed upright in the electrophoresis apparatus.

The solutions used for the preparation of the gels were:-

Solution 1 - 40 g acrylamide, 1.2 g N,N,methylenebisacrylamide, DW to 100 ml.

Solution 2 - 24 ml 1 N HCl, 18.15 g Tris, 0.3 ml TEMED, DW to 100 ml.

Solution 3 - 0.3 g ammonium persulphate, 60.06 g urea, DW to 100 ml.

Solutions 1 and 2 were stored in dark bottles at 4°C. Solution 3 was freshly prepared. Solutions 1, 2 and 3 were mixed in the proportions 1:1:2 and the gel tubes filled immediately with this mixture to within 20 mm of the top. Water was immediately layered on top of the gel mixture. The gels polymerised in approximately 15 minutes but for saftey were left undisturbed for at least 30 minutes. The parafilm was then removed and the tubes rolled to check that the top surface of the gel was completely flat.

5. Electrophoresis. The two solutions required for the apparatus were:-

a) "Upper tray buffer" - 3.16 g Tris, 1.97 g glycine, 0.5 g SDS, DW to 500 ml. The pH was adjusted to 8.9.

b) "Lower tray buffer" - 6.05 g Tris, 25 ml 1 N HCl, 0.5 g SDS, DW to 500 ml. The pH was adjusted to 8.1.

When the polypeptide preparation was ready, the lower tray of the electrophoresis apparatus was filled with "lower tray buffer", and the positive electrode was connected. The tops of the gels were rinsed with water, followed by "upper tray buffer". The gels were then set in position, and 200-300 µl of the polypeptide preparation was placed on

top of the gels in the tubes. "Upper tray buffer" was layered carefully on top of the samples to fill the tubes and the upper reservoir of the apparatus filled with this buffer. The negative electrode was connected up and a current of 2 mA per gel was applied, the voltage being constant. The current was passed through the system for approximately 2 hours, until the bromophenol blue tracker dye was near the base of the gels. The latter were then removed from the tubes by carefully introducing water into the bottom of the tube, between the glass and the gel, by means of a syringe and needle.

6. Slab gel electrophoresis. The apparatus used was the same as that described by Akroyd (1967). It consisted of two rectangular sheets of glass, approximately 10 by 15 cm, and two strips of glass (spacers) the thickness of the gel and 15 cm long. These two strips were placed between the two sheets of glass so as to form the side walls of a chamber and the glass sheets were held close to them by two strong clips. The bottom of the chamber thus formed was temporarily sealed with plasticine. The gel was prepared by filling the chamber with gel solution, prepared as described above, to about two-thirds of its depth. The DW was immediately added to the top of the solution and, when the latter had polymerised, the top part of the gel was rinsed with water, followed by "upper tray buffer". The chamber, above the gel, was then divided into the requisite number of compartments for the samples by inserting some

pieces of plastic tubing. The lower ends of the tubes touched the gel and the tops were 1 cm below the top of the glass sides. The plasticine was then removed and the lower end of the apparatus was placed in a tray containing "lower tray buffer". The samples were layered into the compartments and "upper tray buffer" was placed on top of these. A wick of filter paper soaked in "upper tray buffer" was set with one end in the "upper tray buffer" above the samples and the other in a separate tray containing "upper tray buffer". Electrodes of platinum wire were placed in the two trays and a current of 8 mA was passed until the samples had entered the top of the gel. The current was then switched off, the compartment dividers were removed and fresh "upper tray buffer" was placed above the gel. With voltage constant, a current of 15-20 mA was passed until the tracker dye reached the bottom of the gel.

The gel was taken out of the apparatus by removing the clips and the spacer strips of glass and submerging the gel and glass sheets in water, the glass then being eased off the surface of the gel.

7. Processing of the gels. Solutions required were:-
Stain solution - 0.25% Coomassie blue dissolved in 5 parts methanol: 1 part glacial acetic acid: 5 parts DW.
Destain solution - 5 parts methanol: 1 part glacial acetic acid: 5 parts DW.
Following electrophoresis, the gels were submerged in the stain solution for at least one hour. The rod gels were

then destained by puting them into test tubes filled with destain solution and rotating them for approximately 24 hours, the destain solution being changed at least once during this time. The gels were then put in water overnight and finally scanned at 580λ in a gel scanner attached to a pen-recorder. The trace thus produced was used to measure the distances migrated by the polypeptides. The slab gels were placed in a dish with destain solution which was changed twice during the succeeding 24 hours. After hydration, the gel was photographed and this was used for the measurement of the distances migrated by the polypeptides.

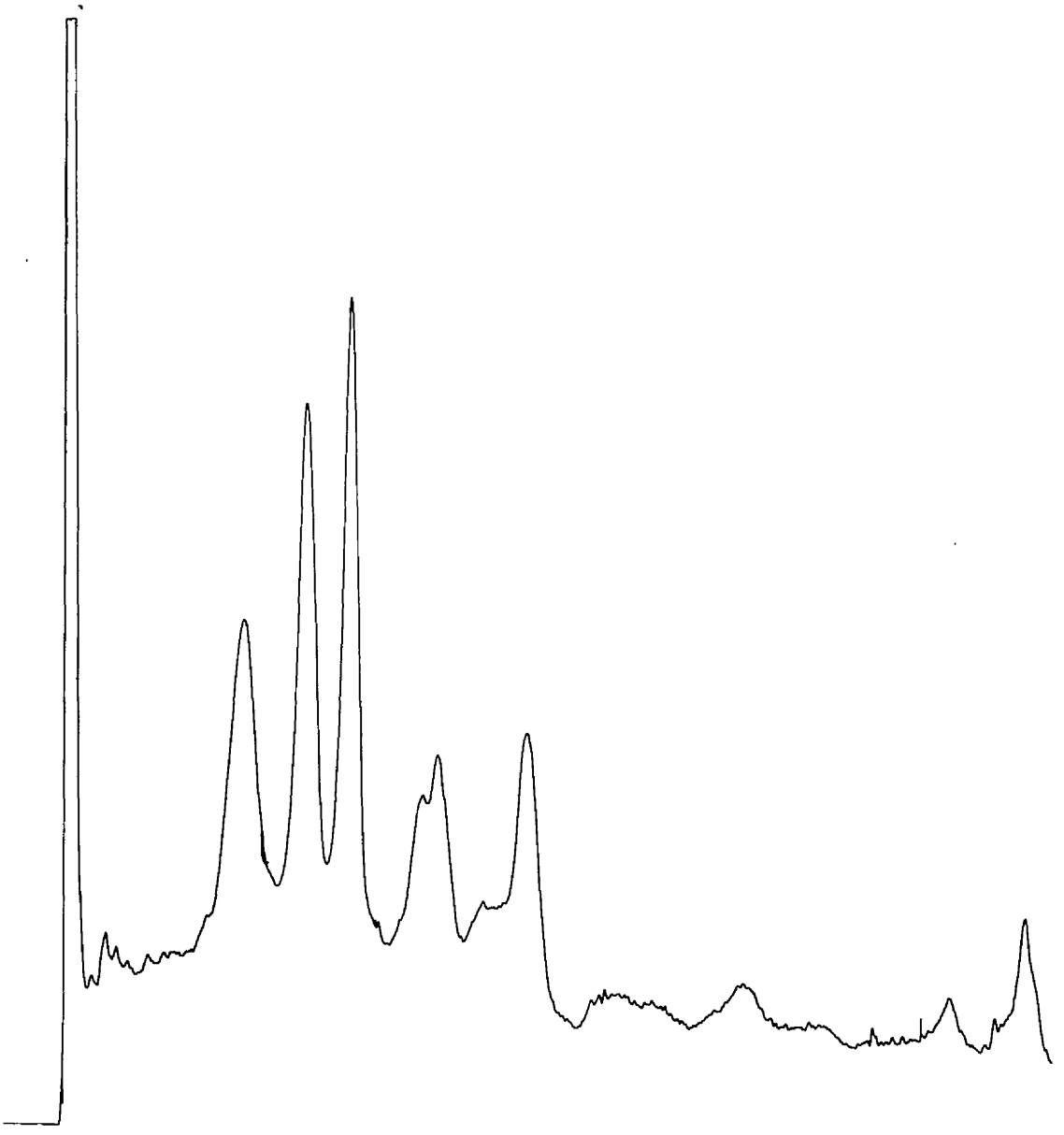


Figure 44. Trace from a gel containing the CDV polypeptides. Note the six peaks, corresponding to the six polypeptides. The origin is on the left hand side, and the tracker dye mark is on the end, on the right.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 5.1

To Determine the Number and Size of the Polypeptides in the Canine Distemper Virion.

Procedure. Two samples of 100 µl of NDV, two samples of 100 µl of CDV and two samples of 150 µl of CDV were treated with the reducing solubiliser. The six samples were layered on top of the gels, and the current passed as described in the Materials and Methods section.

Results. The patterns obtained from the gel scanner for each of the four CDV samples were very similar and one of these is shown in Figure 44. As can be seen, six polypeptides were found.

From each of the two traces of the NDV polypeptides, a graph was prepared by plotting the distances migrated by the three major polypeptides against the logarithms of the molecular weights. Two additional graphs were prepared by plotting the logarithm of the molecular weight against the RM values for each of the three polypeptides. In this way, four reference graphs were obtained. The distances migrated by the six polypeptides from each of the four CDV traces

Table 6 Estimation of the Molecular Weights of the CDV Polypeptides.

Polypeptide.	Molecular Weights. (a) From DM Values. Range	Average	(b) From RM Values. Range	Average	Average from (a) & (b)
1.	92,000-85,000	89,180	95,000-93,000	94,370	91,770
2	77,000-70,000	74,310	82,000-79,000	80,120	77,210
3	68,000-61,000	64,870	71,000-70,000	70,310	67,590
4	55,000-47,500	51,680	58,000-56,000	57,310	54,490
5	52,500-45,000	49,310	56,000-53,000	54,710	52,010
6	41,000-32,000	37,180	43,750-41,000	42,620	39,900

were measured and the RM values calculated. In this way, 16 estimations of each of the six CDV polypeptides were obtained. These are summarised in Table 6.

Conclusions and Discussion. The pattern of the gel scan was very similar to that obtained by Hall and Martin (1973) for measles virus, and resembled less closely the trace published by Waters and Bussell (1973) for distemper virus. In general, the estimated molecular weights of the six polypeptides were greater than those reported by Waters and Bussell (1973) for CDV.

The molecular weights were calculated from the DM and RM values. By using both methods, it was hoped to ascertain which produced the greater uniformity. It was found that less variation was present amongst the results from different gels when the estimations were based on the RM values.

Although all factors appeared to be the same in each gel, the distance travelled by the tracker dye varied. In three of the CDV traces, the distance migrated by the dye was 219 mm whereas, in the fourth, the dye travelled 226 mm. In one of the two NDV gels, the dye moved 193 mm whereas in the other one it reached 208 mm. The factors affecting the distance travelled by the dye presumably also affected the distance travelled by the polypeptides, though not necessarily to the same extent. The calculations of

molecular weight based on RM values might therefore be expected to give a more accurate result. The results presented above would appear to support that theory.

The question then arises as to what causes the variation observed from one gel to another. In discussing the advantages and disadvantages of rod and slab gel systems, Akroyd (1967) drew attention to the existence of slight differences in the DM value of a particular polypeptide in rods and suggested that this might be due to slight variation in the lengths of the gels. It is technically impossible to produce them all to exactly the same length. Akroyd also suggested that another cause of variation in the length migrated may be the existence of slight differences in composition at the top of the gel due to disturbances produced by overlaying with DW in the preparatory stages. Two of the samples of solubilised CDV were of larger volume than the other samples and the resultant difference in the number and concentration of ions may have altered the electrical conditions.

It seems unlikely that any of the above variations would cause more than marginal differences in the actual distances migrated but when the gels were hydrated and scanned and the results converted to a comparatively large scale on paper, such differences would become magnified.

EXPERIMENT 5.2

To Determine the Effect of Varying the Length of the Gel and the Sample Volume.

Since the distances migrated by the tracker dye varied somewhat, it was decided to conduct a short investigation into the effect of varying gel length and starting volume on the distance migrated by the dye.

Procedure. Two long gels (approximately 60 mm) two medium length gels (approximately 55 mm) and two short gels (approximately 50 mm) were prepared and placed in the electrophoresis apparatus. Reducing solubiliser was layered on top of each, the two long gels receiving 100 µl - the maximum volume which could be put on top of these. One of the medium length and one of the shorter gels also received 100 µl of solubiliser and the other two gels 150 µl of solubiliser each. The current was passed until the dye was close to the bottom of the gels. With the gels still in the glass tubes, the distances migrated by the dye were measured .

Results. These were as follows:-

Gel.	Distance travelled by the dye in mm.
Long gel with 100 µl	56
Long gel with 100 µl	55.5
Medium gel with 100 µl	53
Medium gel with 150 µl	50
Short gel with 100 µl	47.5
Short gel with 150 µl	46.5

Conclusions and Discussion. As can be seen, an increase in the length of the gel resulted in an increase in the distance migrated by the dye. In the previous experiment, the latter varied from 193 mm to 226 mm (a difference of 33 mm) for what were, presumably, very small differences in the lengths of the gels. In the present experiments, measurements made while the gels were still in the tubes showed a variation in the distance travelled by the dye of up to 8.5 mm for major differences in the lengths of the gels. This would be equivalent to a variation of approximately 34 mm in the traces produced by the scanner. It is clear, therefore, that the differences in the distance migrated by the dye in the previous experiment were not solely due to variation in the lengths of the gels.

The effect of differences in the sample volume was much less marked. In the previous experiment, an increase in sample volume apparently led to an increase in the length travelled by the dye. In the present experiment, however, an increase in volume actually led to a decrease in the distance travelled. It is therefore impossible to assess the likely effect of varying the sample volume.

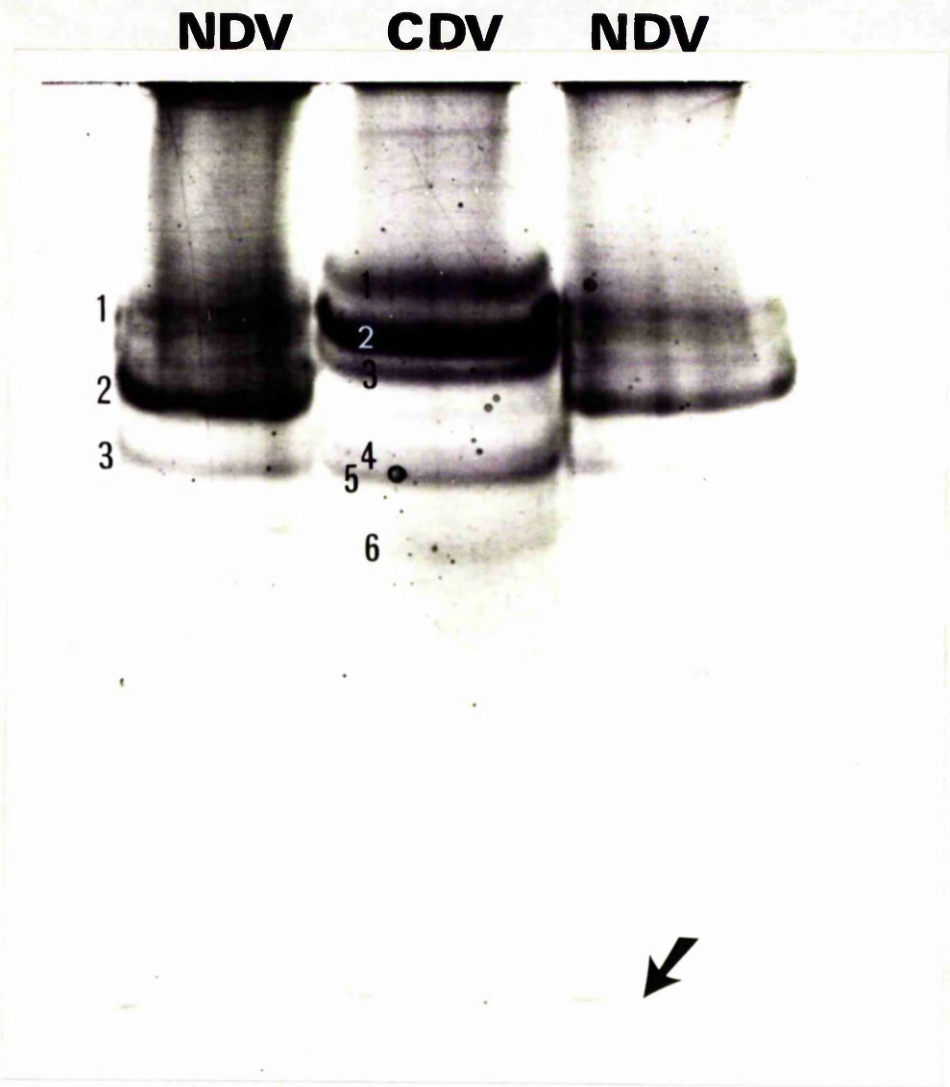


Figure 45. Stained slab gel showing the bands due to the six CDV polypeptides, the three major NDV polypeptides, and the tracker dye at the bottom (arrow).

EXPERIMENT 5.3

To Determine the Molecular Weights of the CDV Polypeptides by means of a Slab Gel System.

One solution to the problem of variation in the conditions occurring in rod gels is to use slab gels. In the latter system, both the test sample and the marker polypeptides travel through the same gel, hence any variation within the one gel must reflect true differences in the samples. The present experiment describes the results obtained using this technique.

Procedure. The apparatus used was described in the Materials and Methods section. Three compartments were set up in the slab gel apparatus. 150 µl of NDV, which had been treated with 150 µl of reducing solubiliser, was placed in each of the two outer compartments while the middle one received 150 µl of CDV, treated with 150 µl of reducing solubiliser. Electrophoresis was carried out as previously described.

Results. Figure 45 shows the slab gel after staining, destaining and hydration. From this it will be seen that the tracker dye in each sample had migrated at a uniform rate so as to form a straight line across the bottom of the gel. The distances migrated by the three major NDV polypeptides and the six CDV polypeptides were measured. From these figures, the molecular weights of the six CDV

polypeptides were estimated to be 86,000; 73,000; 65,000; 51,000; 48,000 and 39,000.

Conclusions and Discussion. Six polypeptides were again obtained from solubilised CDV. The estimated molecular weights from the slab gel system were lower than those obtained from the rod gels and were closer to the figures reported by Waters and Bussell (1973) for CDV, and those reported for measles by both Waters and Bussell (1973) and Hall and Martin (1973).

In the slab gel system, the tracker dye migrated at a uniform rate from all samples, thus indicating that the conditions were the same in each sample. This therefore would seem to be a much better system for estimating unknown molecular weights than the rod gel system. The results obtained in the present experiment are therefore probably more accurate than those obtained in Experiment 5.1. Unfortunately, however, it is more awkward to set up and operate the slab gel system and, should breakage of the gel occur during its removal from the apparatus, or mishap occur during staining, no results may be obtained at all. With rod gels on the other hand, it is highly improbable that all gels would be lost. For these reasons, the remainder of the work was carried out in rods, attempts being made to keep the conditions in the gels as uniform as possible.

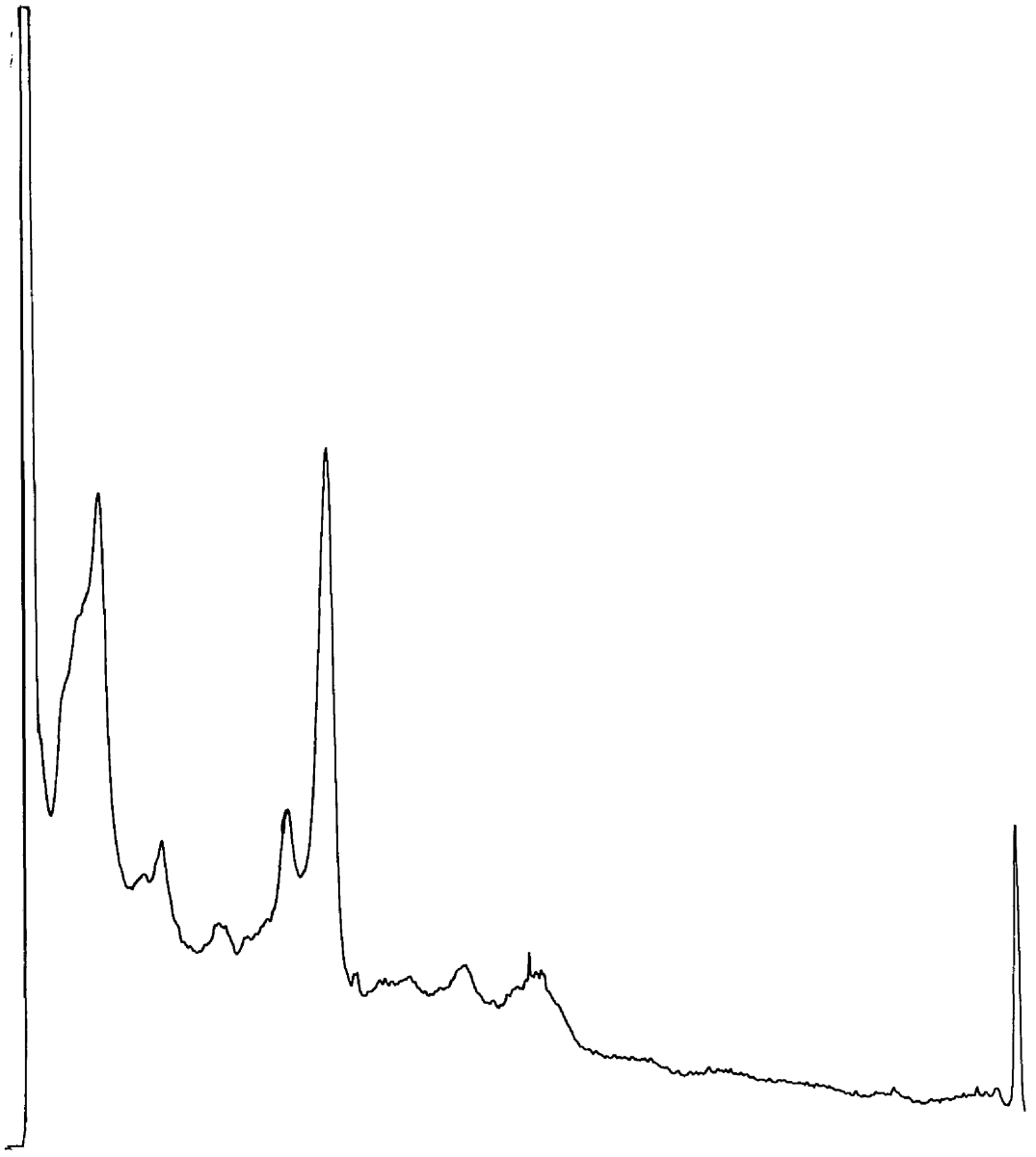


Figure 46. Trace from a gel containing the CDV polypeptides, solubilised under non-reducing conditions. Note the broad peak due to the high molecular weight material, close to the origin on the left, and the two peaks due to the two polypeptides of molecular weight less than 100,000.

EXPERIMENT 5.4

Gel Electrophoresis of CDV Treated with Non-Reducing Solubiliser.

In the previous experiments, complete disruption of viral protein into polypeptides was carried out by means of reducing solubiliser containing SDS, urea and mercaptoethanol. However, by solubilisation of the polypeptides in the absence of mercaptoethanol, Moore and Burke (1974) demonstrated that, in their natural state some of the polypeptides of NDV were linked by disulphide bridges. The present experiment was designed to investigate this for CDV.

Procedure. Apart from the absence of mercaptoethanol in the two gels with non-reducing solubiliser, the method of solubilisation was the same as before. The following five samples of solubilised virus were run through gels:- two samples of 100 µl of NDV, one sample of 100 µl of CDV, all with reducing solubiliser, and two samples of 100 µl of CDV with non-reducing solubiliser.

Results. The two traces from the CDV treated with non-reducing solubiliser were identical to each other but completely different from the other CDV traces. One of these two traces is shown in Figure 46. From this it will be seen that a fairly large amount of high molecular weight material was present and there were only two peaks indicative of polypeptides having molecular weights of less

than 100,000. By taking the DM and the RM values for both gels, the average molecular weights of the two polypeptides were found to be 71,700 and 64,100.

Conclusions and Discussion. The above results indicated that four of the polypeptides obtained from the solubilisation of the virus under reducing conditions were produced by the dissociation of disulphide bonds in protein units having a molecular weight of over 100,000. The two polypeptides of molecular weight less than 100,000 obtained with the non-reducing solubiliser may have been present, in this form in the virus or they may have been produced by the dissociation of larger molecules under the influence of the SDS and urea. Their molecular weights were similar to those of polypeptides 2 and 3 as determined in the slab gel experiment.

EXPERIMENT 5.5

To Determine the Molecular Weight of the Polypeptides from the Nucleocapsid.

Nucleocapsid extracted from the cytoplasm of cells infected with the Onderstepoort strain of CDV has been utilised for determination of the molecular weight of the nucleoprotein by Waters and Bussell (1974) but no studies of the nucleocapsid obtained from virions have been published. However, Mountcastle *et al.* (1970) found no differences between the molecular weights of NDV, Sendai, and SV5 nucleoproteins from nucleocapsids extracted from virions, and those extracted from infected cells which had not been dispersed with trypsin. The aim of the present experiment was to determine which of the six CDV polypeptides obtained by solubilising the whole virus was derived from the nucleocapsid. The method used for the preparation of the nucleocapsid was that described by Mountcastle *et al.*, (1970).

Procedure. The contents of one ampoule of the purified CDV were dialysed overnight in the cold against TS at pH 7.2. The resultant 0.6 ml was mixed with 0.2 ml of 5% Triton X-100. This was then incubated at 37°C for 20 minutes and 50 µl of 10% DOC were added. This mixture was then layered on top of a non-linear gradient consisting of 0.5 ml of 40%, 1 ml of 30% and 1 ml of 25% CsCl with a buffer zone of 0.8 ml of 5% sucrose between the gradient and the sample, the whole being contained in a 5 ml

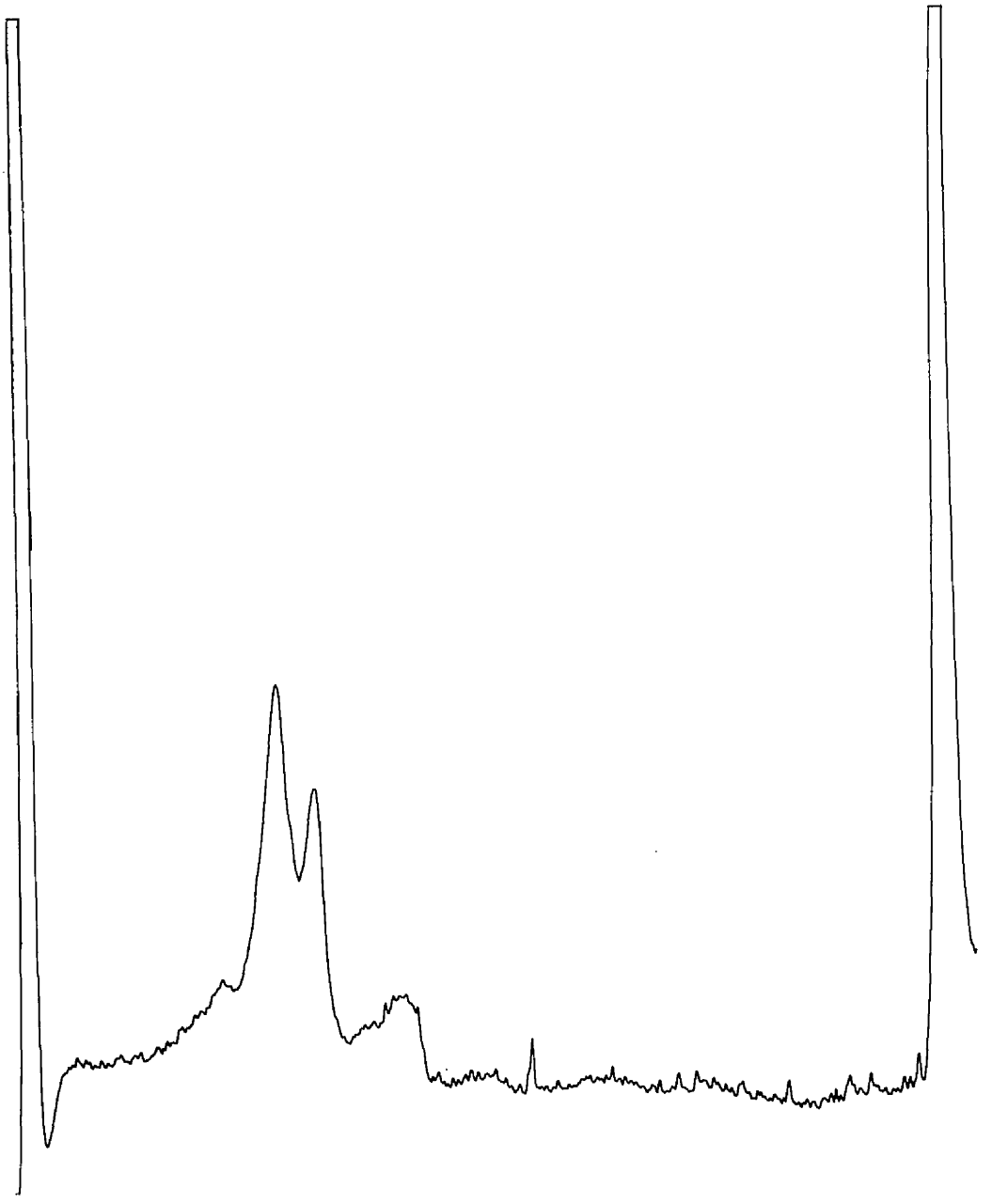


Figure 47. Trace from the gel containing the solubilised nucleocapsid, showing two peaks, indicative of the presence of two polypeptides.

ultracentrifuge tube. All solutions were prepared in TS. The tube was then centrifuged at 30,000 rpm for 90 minutes. At the end of this time, a faintly visible band was found in the 30% CsCl. This was collected and following determination of its refractive index, it was dialysed against cold DW for three hours. The volume was then reduced to about 150 µl by dialysis against sucrose powder. From the refractive index, the density of the band was found to be approximately 1.29 g/cc, which corresponds to the density of nucleocapsid (Experiment 3.6).

100 µl of NDV, 100 µl of CDV and the nucleocapsid preparation were treated with reducing solubiliser and placed on top of three rod gels for electrophoresis.

Results. The trace obtained from the nucleocapsid preparation is shown in Figure 47 from which it will be seen that two peaks were present. The distance migrated by the tracker dye in this gel was 11 mm less than that in the NDV gel. The molecular weights of the two polypeptides were therefore estimated from the RM values to be 73,000 and 66,000 respectively.

Six peaks were again obtained in the trace of whole CDV. In this experiment, the distances migrated by the tracker dye in the CDV and NDV gels were very similar and the estimates of the molecular weights from the DM and RM values were likewise very similar. These molecular weights

were 89,000; 75,000; 67,000; 52,000; 50,000; and 38,000.

Conclusions and Discussion. From the above results, it would seem that two polypeptides were present in the nucleocapsid-containing preparation. These were of approximately the same molecular weights as polypeptides 2 and 3 in the slab gel experiment, polypeptides 2 and 3 in the present experiment, and the two polypeptides of molecular weight less than 100,000 present under non-reducing conditions.

The discovery of two polypeptides in the nucleocapsid preparation was unexpected since, in studies of other paramyxoviruses, only one polypeptide — of molecular weight around 60,000 — has been found in the nucleocapsid (Haslam et al., 1969; Bikel and Duesberg, 1969; Evans and Kingsbury, 1969; Mountcastle et al., 1970; Hall and Martin, 1973; Waters and Bussell, 1974). Because of this, it was decided to repeat the experiment, but with nucleocapsid prepared in a different manner. By so doing, it was hoped that any adherence of envelope proteins to nucleocapsid which might have occurred in the present experiment would be eliminated.

EXPERIMENT 5.6

To Determine the Molecular Weight of the Polypeptide from the Nucleoprotein after Potassium Tartrate Banding of the Nucleocapsid.

In studies of the buoyant density of the nucleocapsid in potassium tartrate (Part Three), attempts to demonstrate nucleocapsid with the electron microscope had been unsuccessful and it was concluded that this may have been due to fragmentation. It was felt that small pieces of nucleocapsid would be less likely to remain adherent to viral envelope than large pieces which could, presumably, attach at numerous points. Hence, for studies of the nucleocapsid polypeptide, it seemed possible that centrifugation through potassium tartrate might provide a product containing less membranous material. The decision was therefore made to substitute potassium tartrate for caesium chloride in the preparation of the nucleocapsid.

Procedure. One ml of the purified virus was mixed with Sterox SL to give a final concentration of 1% and left in the dark, at room temperature, for 45 minutes. The sample was then layered on top of an 8 ml gradient of potassium tartrate, density 1.1 to 1.3 g/cc, in an SW 40 centrifuge tube, with a 3 ml buffer layer of 1.1 g/cc potassium tartrate between the sample and the gradient. This was then centrifuged at 37,000 rpm for 16 hours. The density region 1.235 to 1.255 g/cc was collected since the results of Experiment 3.7 had suggested that nucleocapsid would

be present in this range. The fractions were pooled and dialysed against PBS for four hours. The volume was made up to 5 ml and the nucleocapsid in the sample pelleted by centrifugation at 40,000 rpm for 1½ hours in a siliconised SW 50.1 centrifuge tube. The pelleted material was left overnight at 4°C to resuspend in 100 µl of PBS.

Two samples of 100 µl of CDV, and the nucleocapsid preparation were each treated with 100 µl of the reducing solubiliser and applied to gels as before.

Results. Two polypeptides were again obtained from the nucleocapsid preparation. Their estimated molecular weights were 76,000 and 68,000.

Conclusions and Discussion. The above results corresponded fairly closely to those of the previous experiment. Thus, with the system employed, it would seem that two polypeptides may be present in or be associated with the nucleocapsid.

DISCUSSION.

From the results of the above experiments, it was established that the CDV used in these studies contained six polypeptides of approximately the same molecular weights as those reported by other workers for measles and distemper viruses (Hall and Martin, 1973; Waters and Bussell, 1973). However, since two polypeptides of identical molecular weight may form one band in the gels, it is possible that more than six were actually present; it has been shown by Moore and Burke (1974) that one band obtained from NDV virions was composed of two polypeptides. The system formulated in the previous section for the purification and concentration of the virus efficiently removed contaminating host cell material from the virus, and produced a preparation that was sufficiently concentrated for this work.

A degree of variation in the estimated molecular weights of each polypeptide was obtained, less variation being present when RM values were employed than when D^M values were used. On investigation, it was found that the distance travelled by the tracker dye was influenced by the length of the gel and, to a lesser extent, by the volume of the sample. As judged by the uniform migration both of the tracker dye and of the three major NDV polypeptides,

Table 7 Molecular Weights of the polypeptides of CDV and Measles Virions.

Poly-peptide.	From Rods. Range	Average	Slab gel	Waters& Bussell	Hall & Martin
1	95,000-74,000	89,650	86,000	78,000	75,600
2	82,000-59,000	75,090	73,000	71,000	69,000
3	71,000-51,000	65,750	65,000	61,000	60,000
4	58,000-36,000	50,790	51,000	53,000	53,000
5	56,000-29,000	48,940	48,000	45,000	51,000
6	43,750-24,500	39,050	39,000	37,000	45,700

electrophoresis of NDV and CDV polypeptides in a slab gel system apparently provided more uniform conditions than occurred in rod gels.

Altogether 22 estimations of the molecular weights of the polypeptides were made from rod gels and one from the slab gel. The results of these, together with the figures published by Waters and Bussell (1973) for CDV and measles, and by Hall and Martin (1973) for measles are shown in Table 7. From this it will be seen that the average figures obtained by rod gel electrophoresis compare closely with the figures from the single slab gel experiment.

The traces obtained by treating the virus with the non-reducing solubiliser was completely different from that obtained in the presence of mercaptoethanol. Polypeptides of molecular weight greater than 100,000 were present but the system did not allow exact determination of their molecular weights. Only two polypeptides with molecular weights less than 100,000 were present. This indicated that the other four polypeptides found under reducing conditions were normally component parts of larger protein molecules in the virion. Moore and Burke (1974) found that, under non-reducing conditions, the molecular weight of the smaller glycoprotein of NDV was greater than under reducing conditions. In the present investigation, it is therefore impossible to conclude that the two polypeptides of less than 100,000 molecular weight found under non-reducing

conditions were identical to polypeptides 2 and 3 present under reducing conditions, though their molecular weights were similar.

When nucleocapsid was extracted from purified virus by the method of Mountcastle et al., (1970), polypeptides with molecular weights similar to those of polypeptides 2 and 3 were found. A similar result was obtained when a different method of extraction, based on the author's earlier studies, was employed. All published work on the molecular weight of the nucleoprotein extracted from virus particles has indicated that this corresponds to a single polypeptide, of molecular weight around 60,000. Waters and Bussell, (1974) reported that the molecular weight of the nucleoprotein of the Onderstepoort strain of CDV was 54,000 and that of measles was 61,000, the nucleocapsids for this study having been extracted from infected cells. If trypsin was employed to disperse the cells prior to extraction, two polypeptides were obtained from each of the nucleocapsid preparations. The molecular weights of those from the CDV infected cells were 27,000 and 22,500 whereas those from cells infected with measles virus were 38,000 and 24,000. Mountcastle et al. (1970) reported similar findings for the nucleocapsids of SV5, Sendai and NDV viruses. When nucleocapsid was extracted from the virions or cells not treated with trypsin, the molecular weights of these were 61,000; 60,000; and 56,000 respectively. When the molecular weights of the nucleoprotein from nucleocapsids extracted

from trypsin dispersed cells were determined, the results were 43,000; 46,000; and 47,000. Both Waters and Bussell (1974) and Mountcastle et al. (1970) concluded that the trypsin had triggered an intracellular enzymatic reaction which had resulted in cleavage of nucleoprotein.

In the present investigation, the nucleocapsid was extracted from virions and exposure to trypsin was not involved. The fact that polypeptide 3 was present along with polypeptide 2 cannot therefore be due to the same mechanism.

Stone et al., (1972), and Zaides et al., (1974 and 1975) have shown that nucleocapsids isolated from Sendai virus infected cells are capable of carrying out transcription. Determination of the number and size of the polypeptides in these structures revealed that two main polypeptides were present. The molecular weight of the first – 60,000 – corresponded to that of the polypeptide from the nucleocapsid of virions whereas that of the second – 71,000-75,000 – corresponded to that of the largest major polypeptide present in the envelope. Stone et al. (1972) suggested that the polypeptide of molecular weight 75,000 might be from a transcriptase enzyme, but also pointed out that the superabundance of the polypeptide both in the virion and in the cytoplasmic nucleocapsid would seem to make this unlikely.

In the present study, the nucleocapsid preparations which contained the two polypeptides were prepared from virions rather than from cytoplasmic extracts of infected cells but it has been shown conclusively that transcription *in vitro* can be carried out using disrupted Sendai virions and that cellular material is not required (Stone *et al.*, 1971; Robinson, 1971). Robinson (1971) further showed that nucleocapsid extracted from virions and banded in sucrose was capable of carrying out transcription. Furthermore, Stone *et al.*, (1972) found that DOC and CsCl both eliminated the transcriptase function of their cytoplasmic nucleocapsid and speculated that the widespread use of these two compounds for the isolation of nucleocapsids may have accounted for the findings of only one polypeptide in nucleocapsid extracted from virions. However, in the present study, one of the methods employed for the preparation of the nucleocapsid involved the use of both DOC and caesium chloride though in the other, neither were used.

Another indication of linkage of nucleoprotein to another polypeptide was obtained by Moore and Burke (1974). In a study of 14 strains of NDV, these workers found that a large polypeptide was obtained when solubilisation of the virion proteins was carried out under non-reducing conditions, but only in eight of the strains. When this polypeptide was isolated and subjected to reducing conditions, it was found to consist mainly of the polypeptide from the haemagglutinin (molecular weight 75,000) but it

also contained a substantial amount of the polypeptide from the nucleocapsid, together with traces of four of the minor polypeptides. This indicated that, in the virions of these eight strains, these polypeptides were linked by disulphide bridges.

Though it is not clear why the isolated nucleocapsids in the present investigation contained two polypeptides, it would seem possible from the above mentioned work that the results obtained were not simply due to contamination of the preparations by envelope proteins, but reflected a stero-chemical relationship between the polypeptide of the nucleocapsid and one of the polypeptides from the envelope.

PART SIX.

INTRODUCTION AND REVIEW OF THE LITERATURE.

If "old-dog-encephalitis" is caused by a defective CDV infection analagous to that occurring in SSPE in man, it is possible that most of the virus RNA produced is of sub-genomic length. The subgenomic RNA would then be encapsidated in nucleocapsids which are smaller than those containing the complete genome. In order, therefore, to demonstrate that a given nucleocapsid RNA is indeed sub-genomic, knowledge of the size of the complete nucleocapsid and genome is obviously necessary. Since the size of these structures is most easily determined by measurement of their sedimentation coefficients (or S values), the aim of the following experiments was to determine these S values for the CDV used throughout this investigation.

As far as the author is aware, only one paper giving information on the S value of the CDV nucleocapsid and one paper on the S value of CDV RNA has so far been published. That concerning the nucleocapsid was by Waters and Bussell (1974) who estimated from sedimentation of ^{32}P-labelled CDV that the S value of the nucleocapsid was 276 S. From studies with an analytical ultracentrifuge, the S value was estimated as 300 S, but the nucleocapsid sometimes had a

Table 8 Reported S Value of the Nucleocapsid and the Viral RNA of some Paramyxoviruses.

a) Nucleocapsid

Virus	S Value	Reference
Measles	220-265	Norrby and Hammarskjold (1972)
Measles	195	Waters et al. (1972)
Measles	280	Hall and Martin (1973)
NDV	200	Kingsbury and Darlington (1968)
NDV	225	Bikel and Duesberg (1969)
HVJ	250	Hosaka (1968)
Sendai	280	Blair (1970)
SV5	300	Compans and Choppin (1968)

b) RNA

Virus	S Value	Reference
Measles	52-54	Hall and Martin (1973)
Measles	52	Carter et al. (1973)
Measles	52	Hammarskjold and Norrby (1974)
NDV	57	Duesberg and Robinson (1965)
NDV	45-50	Adams (1965)
NDV	49.2	Kingsbury (1966)
NDV	55	Nakajima and Obara (1967)
NDV	57	Bratt and Robinson (1967)
NDV	50	Kingsbury and Darlington (1968)
NDV	57	Blair and Robinson (1968)
NDV	54	Duesberg (1968)
Sendai	57	Blair and Robinson (1968)
Sendai	57	Barry and Bukrinskaya (1969)
Sendai	57	Blair (1970)
Sendai	50	Kingsbury et al. (1970)
SV5	50	Compans and Choppin (1968)

bimodal distribution indicating the presence of 210 & 273 S nucleocapsids. The publication on the CDV RNA was by Hammarskjold and Norrby (1974), who estimated its S value to be 50 S.

A large number of estimations of the S values of the nucleocapsids and RNA's from other paramyxoviruses have been published and some of these are summarised in Table 8, from which it will be seen that those for the nucleocapsids lie in the range 200 to 300 S, and those for the RNA's are in the range 50-57 S. The existence of defective paramyxovirus particles with sub-genomic RNA has also been documented. These particles, which contain RNA molecules of less than 50 S, have been found in preparations of Sendai virions (Kingsbury et al., 1970).and measles virions (Schluederberg, 1971; Carter et al., 1973; Hall and Martin, 1974a). In addition, nucleocapsids of 130-150 S, and containing 18 S RNA have been isolated from defective measles particles (Hall and Martin, 1974a).

MATERIALS AND METHODS.

1. Virus Preparation. Ampoules of purified virus, labelled with ^{3}H-uridine, were prepared by the same method as was used in the study of the viral polypeptides. Following the final pelleting, the virus was resuspended in TS saturated with diethylpyrocarbonate. The ampoules were then stored at -70°C.

2 Markers.

a) For the nucleocapsid. A commercial preparation of MS2 bacteriophage was used. The S value of this is 80 S. The preparation had an optical density (OD) of 56 units per ml and was obtained from Miles Laboratories Inc.

b) For the RNA. This marker was provided by a commercial preparation of a mixture of 70% 23 S and 30% 16 S E.coli ribosomal RNA, having an OD of 200 units per ml. This was obtained from Miles Laboratories Inc.

3. Extraction of Nucleocapsid. This was done by mixing a sample of the virus with sufficient 10% Sterox SL in TS to give a final concentration of 1%. This was then left at room temperature for 45 minutes prior to rate-zonal centrifugation.

4. Extraction of the RNA. Solutions used were :-

a) Stock solution of 10% sarkosyl in TS. This detergent is similar to SDS and was used in preference to SDS as it does not precipitate in the cold.

b) Phenol/Chloroform. This was prepared by thoroughly mixing phenol with an equal volume of TS and 1/5th volume of chloroform. This was left to settle, and the bottom layer (phenol) then collected.

Sufficient sarkosyl was added to the virus to give a final concentration of 1%. An equal volume of phenol was then added and the mixture shaken at room temperature for 5-10 minutes. The phases were then separated by centrifugation at 1,000 rpm for 5 minutes, and the top aqueous layer was collected. This was then treated with a volume of ether slightly greater than the sample volume in order to remove traces of phenol. After thorough shaking, this mixture was spun at 1,000 rpm for about 30 seconds and the ether was then removed from the top. Following a second treatment with ether, traces of the latter were then removed from the sample by bubbling nitrogen through it.

5. Gradient preparation and ultracentrifugation.

a) For the nucleocapsid. The gradients were prepared using the gradient mixer, as described in the Materials and Methods section of Part Three. They were prepared in 18 ml SW 27 ultracentrifuge tubes and consisted of an 0.5 ml pad of 60% sucrose in the bottom of the tube and a 16.6 ml

gradient of 15-30% sucrose with 0.5% Sterox in TS on top of this.

b) For the RNA. The gradients were prepared in SW 50.1 ultracentrifuge tubes which had been rinsed with DW containing 0.1% sarkosyl and dried in a hot air oven. The gradients were prepared by carefully layering 1.2 ml volumes of 20%, 15%, 10% and 5% sucrose plus 1% sarkosyl in TS on top of each other in the tubes, and leaving these overnight at 4°C. The speed and length of ultracentrifugation employed is described in the procedure section of the individual experiments.

6. Fractionation of Gradients. This was carried out as described in the Materials and Methods section of Part Three.

7. Optical density measurements. Fractions from each gradient were diluted with 0.5 ml of DW and their optical densities at 260 λ measured and recorded using a Unicam Spectrophotometer.

8. Radioassay. This was carried out as described in Part Three except that 1 ml volumes of BSA and 10% TCA was added to the fractions instead of 0.5 ml volumes.

9. Calculation of S values. The S values were calculated from the position of the peak in the gradient by means of the formula -

$$\frac{\text{Distance migrated by the marker}}{\text{Distance migrated by the unknown}} = \frac{\text{S value of marker}}{\text{S value of unknown.}}$$

(Martin and Ames, (1961).

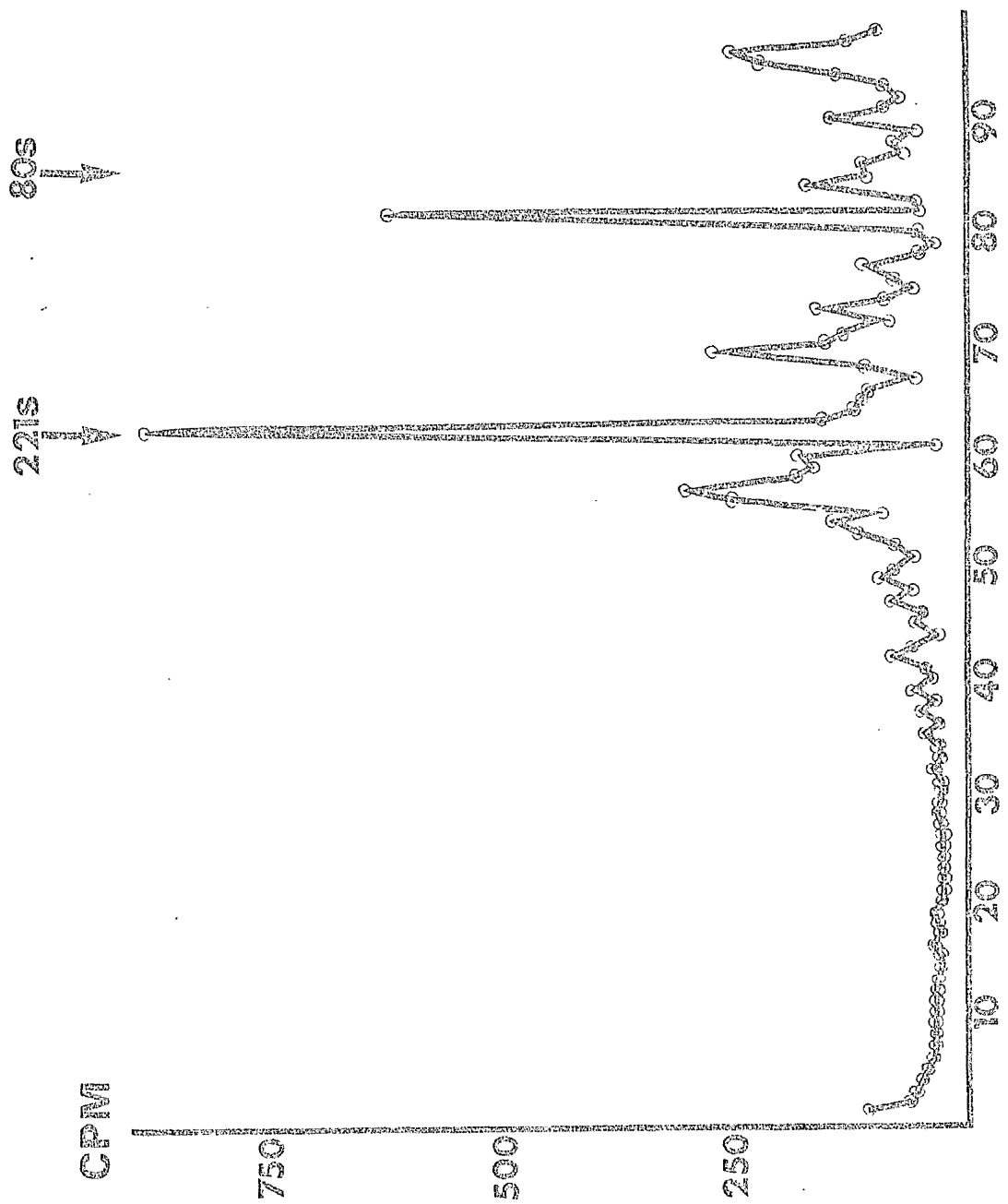


Figure 48. Profile obtained from rate zonal centrifugation of 3H-uridine labelled CDV nucleocapsids.

EXPERIMENTAL PROCEDURES AND RESULTS.

EXPERIMENT 6.1

To Determine the Sedimentation Coefficient of the Nucleocapsid of CDV.

Procedure. 0.2 ml of the concentrated virus was mixed with 20 λ of 10% Sterox and left for 45 minutes at room temperature. 50 λ of the MS2 bacteriophage virus suspension was then added and the sample was layered on top of a 15-30% sucrose gradient. The tube was centrifuged at 24,000 rpm for 1 hour 55 minutes. 98 six-drop fractions were then collected. The OD and radioactivity of each fraction were measured as previously described.

Results. These are shown in Figure 48, from which it can be seen that several peaks of radioactivity were obtained. Two major ones were present at 221 and 105 S and smaller but nevertheless distinct ones at 252, 178 and 115 S.

Conclusions and Discussion. From the above results, it seemed that most of the nucleocapsid existed in two different sizes, namely 221 S and 105 S, though smaller populations of the other sizes were also present. It seemed probable that the 221 S structure represented the

complete nucleocapsid with intact genome. The small peak at 252 S may have been analagous to the 273 S part of the bimodal peak described by Waters and Bussell (1974).

The 105 S peak may have been due either to fragmentation of nucleocapsid at a specific site or to the presence of short nucleocapsids in virus particles. In the latter instance, the short nucleocapsid may have been present along with the full nucleocapsid or alone. Kiley et al. (1974) obtained 110 and 200 S and occasionally 170 S nucleocapsids from the cytoplasm of measles virus infected cells. Kiley and Payne (1974) further showed that, in cells infected with measles virus containing a large number of defective particles, 110 S nucleocapsids were present in greater numbers than the 200 S nucleocapsids, whereas, when the cells were infected with virus containing few defective particles, 200 S nucleocapsids predominated. Nucleocapsids of 130-150 S have been isolated from defective measles virus particles (Hall and Martin, 1974a). In the present investigation, therefore, the 105 S and 170 S peaks of radioactivity may have been due to the banding of smaller nucleocapsids containing subgenomic RNA molecules.

The discovery of two main populations of nucleocapsids suggested that rate sedimentation studies of the extracted RNA would reveal the presence of two distinct populations of RNA molecules. In the present experiment, small peaks of radioactivity occurred at 50 S and 11 S and these may have been due to banding of free viral RNA.

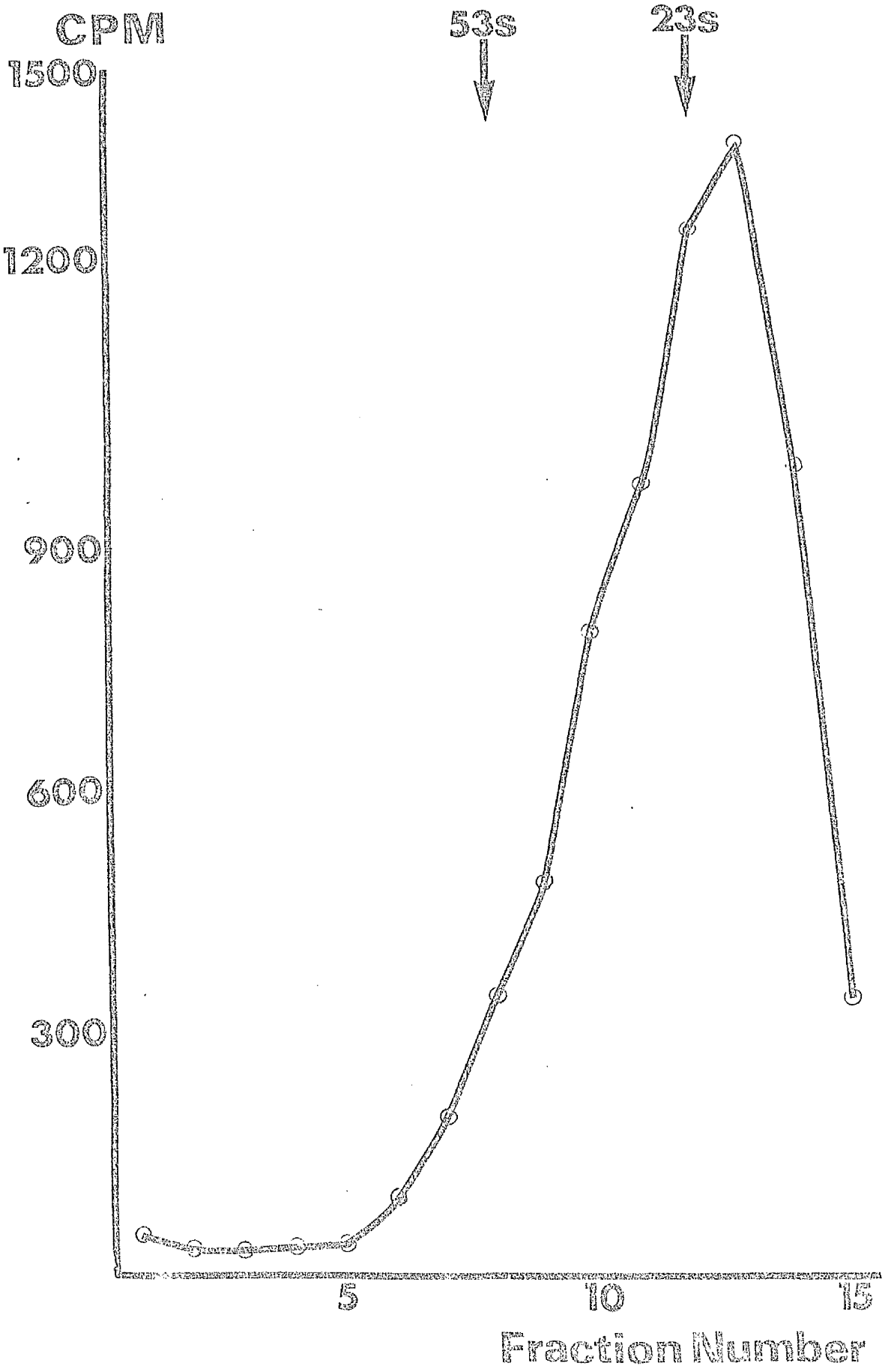


Figure 49. Profile obtained from rate zonal centrifugation of ^{3}H-uridine labelled CDV RNA.

EXPERIMENT 6.2

To Determine the S value of the CDV RNA Extracted with Sarkosyl and Phenol.

Procedure. 0.2 ml of stock virus was mixed with 20λ of the E.coli ribosome preparation and treated with phenol as described previously. The sample was layered on top of a 5 to 20% sucrose gradient and centrifuged at 50,000 rpm for 1 hour. Due to the low surface tension and small drop size resulting from the presence of detergent in the gradient, twenty-drop fractions were collected. The optical density and the radioactivity of each were measured as previously described.

Results. The results are shown in Figure 49, from which it will be seen that most of the radioactivity lay in the same region as the 23 S OD peak, and there was no peak of radioactivity in the 50 S region.

Conclusions and Discussion. From the above results, only RNA molecules of less than 50 S were apparently present. This may have been due to break-up of the 50 S RNA. The profile of the ^{3}H counts suggested that several populations of RNA molecules were present and, as these counts were still close to the top of the gradient, it was decided to increase the time of centrifugation in the next experiment and to collect smaller fractions in order to achieve better separation of the different populations.

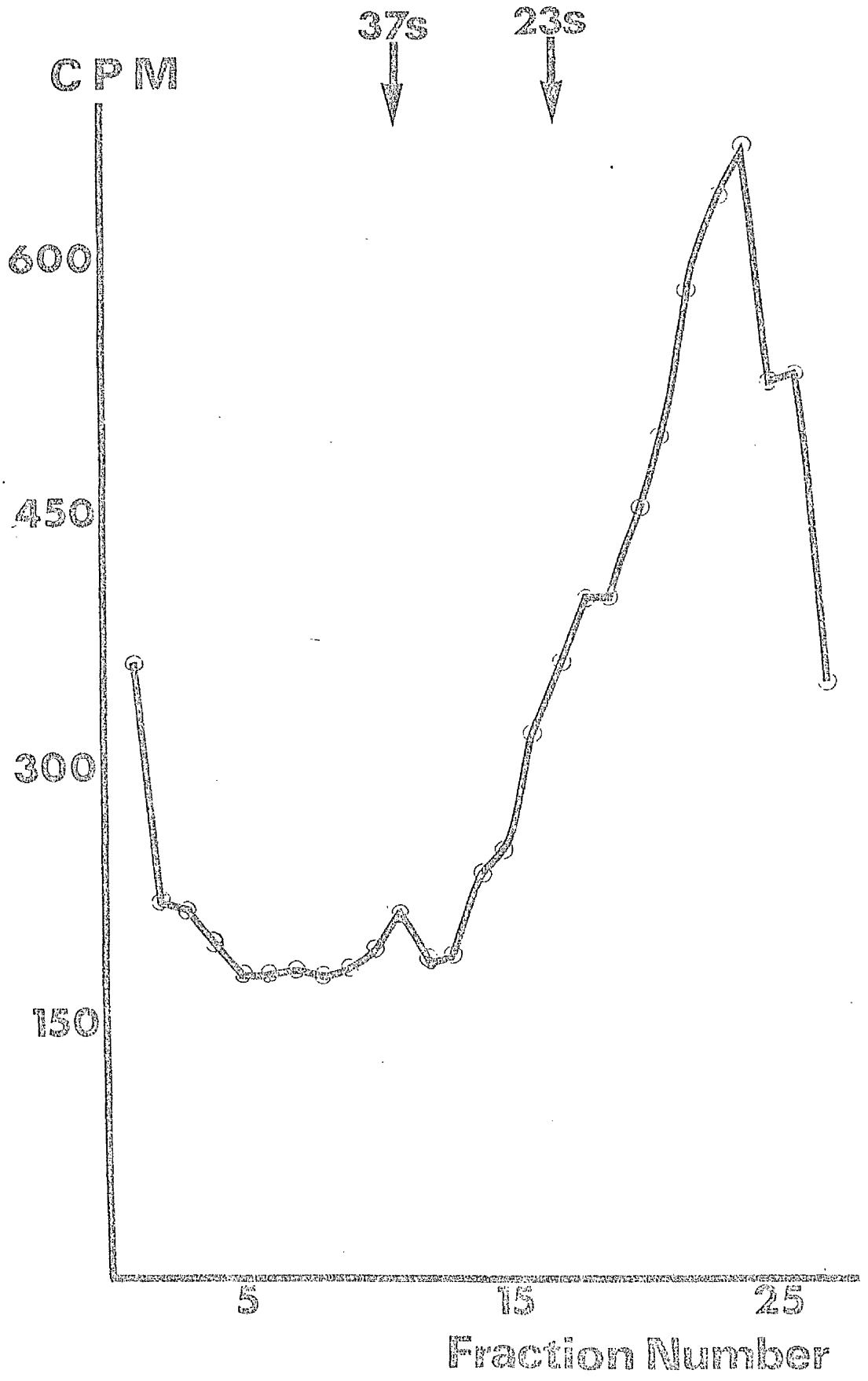


Figure 50. Profile obtained from rate zonal centrifugation of ^{3}H-uridine labelled CDV RNA after 1 hour 45 minutes of centrifugation at 50,000 rpm.

EXPERIMENT 6.3

To Determine the S Value of the CDV RNA Extracted with Sarkosyl alone.

Several workers (Barry and Bukrinskaya, 1968; Kingsbury et al., 1970) have reported that the same results are obtained when RNA is extracted with SDS alone as when SDS and phenol are employed together. In order to ensure that no break-up of RNA occurred due to the phenol/ether treatment, it was decided to repeat the last experiment but to omit the latter steps.

Procedure. 0.2 ml of stock virus was mixed with 20λ of the E.coli preparation and 20λ of sarkosyl and layered on top of a 5 to 20% sucrose gradient. Centrifugation at 50,000 rpm was carried out for 1 hour 45 minutes. At the end of this time, ten-drop fractions were collected from the gradient and the OD and the radioactivity of each fraction measured as previously described.

Results. The results are shown in Figure 50, from which it can be seen that once again no clear-cut peak of radioactivity indicative of the presence of 50 S RNA was obtained. There was a small peak of radioactivity at the 37 S position and a noticeable shoulder around 21 S. Most of the radioactivity, however, lay in the region of 16 S to 4 S, with a peak at 6 S.

Conclusions and Discussion. From the above results, it is obvious that most of the RNA recovered by the techniques described above consisted of relatively small molecules. Possible explanations for this are described in the following Discussion.

DISCUSSION.

Since sedimentation of virus disrupted with Sterox revealed the presence of two distinct populations of nucleocapsid, the one of 221 S being of sufficient size to contain the full genome, it was expected that sedimentation of the viral RNA would reveal the presence of genomic RNA. However, no RNA of this size (50 S) was recovered. Since the CDV nucleocapsid produced in GH cells was shown to be of the same length and density (Part Three) as that of other paramyxovirus nucleocapsids which are known to contain 50-57 S RNA, it seems highly unlikely that the genomic RNA of CDV is less than 50 S. It is possible that break-up of the 50 S RNA had occurred due to the action of RNase though the ultracentrifuge tubes had been washed with DW, and sarkosyl was included in the gradients to help eliminate any RNase. Also, in Experiment 6.3 only mild sarkosyl treatment was used in order to ensure that the nucleocapsid was broken up with minimum damage to its RNA. However, if only a small amount of fully genomic RNA was present in the first place, any traces of RNase may have been sufficient to reduce this to undetectable levels.

A large amount of RNA smaller than 50 S was recovered. Since the viral particles were RNase treated during purification and little material of less than 50 S was

obtained in Experiment 6.1, it is very unlikely that the small molecular weight RNA was cellular in origin. Furthermore, due to the precautions taken to eliminate as much RNase as possible, it is also unlikely that all of the small RNA resulted from a massive break-up of larger RNA molecules. Taking into consideration the result obtained from the sedimentation of the nucleocapsid, it seems more likely that some of this smaller RNA represented subgenomic RNA from virus particles.

All of the results obtained therefore suggested that some defective virus may have been present along with infectious virus. From the formula of Abelson and Thomas (1966),

$$\frac{D_1}{D_2} = \left(\frac{M_1}{M_2}\right)^{0.38}$$

where D_1 and D_2 are the distances migrated and M_1 and M_2 are the molecular weights,

the 221 S nucleocapsid must have been approximately 7 times (7.094) greater in size than the 105 S nucleocapsid. From the radioactive counts at each peak, there must therefore have been 5 times as many 105 S nucleocapsids as 221 S nucleocapsids, provided that the proportion of the ^3H label was the same in both. What is unknown, however, is whether the 105 S nucleocapsids were present in particles also containing the 221 S structures or whether they were present singly or in chains. In the electron microscopic studies recorded in Part Two, no particles were seen which appeared to be deficient in nucleocapsid. Thus, in those

sectioned equatorially, nucleocapsid was seen in cross-section along the whole length of the periphery. Similarly in virions sectioned tangentially, bands of nucleocapsid appeared throughout the entire length of the particle. Moreover, the amount and distribution of the aligned nucleocapsid seen in the detached syncytia indicated that it was present in fairly long pieces. On this basis, it is difficult to believe that particles containing only one or two 105 S nucleocapsids were produced under the conditions employed by the author. It is possible, therefore, that 105 S nucleocapsids may have united prior to alignment. However, if chains were produced in that way, it is clear that exposure of the virions to Sterox had broken them up into the individual 105 S units. Since Sterox did not apparently break up the 221 S nucleocapsid, it would seem unlikely that chains of 105 S did in fact occur, though it is possible that adhesion of the nucleocapsid to the envelope occurs in such a way as to give the impression of continuity between individual nucleocapsids. End-to-end linkage of extracted nucleocapsids has been reported by several workers including Compans and Choppin (1967a), Tajima et al. (1971) and Yeh and Iwasaki (1972). A virion containing only 105 S nucleocapsids would, of course, be defective but it is not known whether or not the presence of 105 S nucleocapsids in virions also containing 221 S structures would result in interference with the replication of the full genomic RNA.

It has been shown conclusively that defective viral particles occur in some preparations of measles and Sendai viruses and may also occur in suspensions of rinderpest (Underwood and Brown, 1974) and NDV viruses (Wilcox, 1959). These particles, which interfere with the replication of infectious virus, have been seen most frequently in stocks of virus prepared by infecting the cells under conditions of high multiplicity of infection (MOI) (Kingsbury et al., 1970; Chiarini and Norrby, 1970; Carter et al., 1973; Hall and Martin, 1974a; Kiley and Payne, 1974). However, Kingsbury and Portner (1970) concluded that these particles arose from spontaneous mistakes in the replication of the viral genome and that, although the conditions of high MOI greatly favoured their replication, it was not the cause of their occurrence. Huang and Baltimore (1970) put forward the following theory to account for the cyclical rise and fall in the proportion of defective particles produced during serial passage. When a defective particle is formed, it can replicate only if the cell which it infects is simultaneously infected with a fully infectious particle. If this occurs, more defective particles are produced, and the formation of new infectious particles is suppressed. Thus, over several cycles or passages of the virus, the number of defective particles increases. As this process continues, however, the chance of simultaneous infection of a cell by a defective and an infectious particle is reduced and so the production of defective particles diminishes again. This increases the probability of a cell being infected only with an infectious particle, and hence the

number of infectious particles again increases. This cyclical increase and decrease in infectivity titres during passage has been shown to occur by Huang and Baltimore (1970) and also Sokol et al., (1964). It can easily be seen, therefore, that if defective particles are present, high MOI will favour their replication. This has been shown to occur by Kingsbury et al., (1970); Chiarini and Norrby (1970); Kiley and Payne (1974); Hall and Martin (1974a). Similarly, decreasing the MOI has been shown to favour the production of infectious particles (Chiarini and Norrby, 1970; Underwood and Brown, 1974; Kiley and Payne, 1974).

The defective viral particles differ from the infectious particles in several ways. The sedimentation coefficient of the defective particles is approximately 400 S whereas that of infectious particles is at least 1,000 S (Kingsbury et al., 1970; Hall and Martin, 1974a), their buoyant density is less than that of infectious particles (Chiarini and Norrby, 1970; Underwood and Brown, 1974). Electron microscopic examination of the two types of particles shows that the infectious particles consist of envelopes packed with nucleocapsid, whereas the defective particles consist of envelope with little enclosed material (Chiarini and Norrby, 1970). The infectivity titres of virus suspensions containing a large number of these defective particles is low, though the haemagglutinin and haemolysin titres generally remain high. Hall and Martin (1974a) showed that, whereas the 1,000 S infectious measles

virus particles contained 250-280 S nucleocapsids with 50 S RNA, the slower sedimenting, non-infectious particles contained 130-150 S nucleocapsids with 18 S RNA. Hall and Martin (1974a) also showed that, while dilute passage virus contained 52 and 4 S RNA, three undilute passages resulted in the production of virus containing 52, 18 and 4 S RNA species and, after 8 undilute passages, 52, 30, 18, 11 and 4 S RNA species were present. Similarly, Underwood and Brown, (1974) reported that rinderpest virus passaged at an MOI of 1 contained 20 S and 5 S RNA but when passaged at an MOI of 0.001, a small amount of 50 S RNA was present.

The results of the three experiments described in this section were unexpected since all the previous work had suggested that most, if not all, of the virus produced under the conditions employed by the author was infectious. For example, the buoyant density of the virus was similar to that of other paramyxoviruses, electron microscopy showed full particles, not empty ones, 1 μ lengths of nucleocapsid were found in negatively-stained homogenates of infected cells, and virus passaged over 20 times at dilutions ranging from 1 in 1 to 1 in 4 was still capable of producing an extensive CPE within four days. Over this period, the titres of harvested virus generally reached $10^5 TCID_{50}$ per ml. On the few occasions when virus was passaged at a higher dilution, the period between infection and the development of the CPE was correspondingly prolonged. Therefore, by waiting for the CPE to become obvious before the virus was harvested, the same conditions were produced as were present

when cells were infected at a higher MOI but the virus harvested earlier, i.e. in both situations, the same opportunity for dual infection with infectious and defective particles must have existed just before the virus was harvested. It therefore had seemed that nothing was likely to be gained by infecting the cells at low MOI. It should also be emphasised that, even with virus passaged undilute, the MOI was never greater than approximately 0.1, so that several cycles must have occurred before dual infection was possible.

In support of the above argument, there is evidence that, in some systems, undilute passage does not lead to the formation of defective particles. A strain of Sendai virus studied by Kingsbury and Portner (1970) was passaged several times at high MOI without the production of defective particles. Also, Huang and Baltimore (1970) and Kingsbury and Portner (1970) both found that the cell system influenced the formation of defective particles, some cell systems producing fewer defective particles than others when infected with identical proportions of infectious and defective virus particles. It is possible that in all of the author's earlier work, little or no defective virus was present but that a population of defective particles was formed at the time that the radioisotopically-labelled virus was being prepared for this work. A density range of approximately 1.16 to 1.22 g/cc was collected from the density gradients and, therefore, if defective particles were

present, these would have been included in the purified virus. Another possibility is that the 105 S nucleocapsids occurred along with the 221 S nucleocapsids in the virions, but without the former producing any interference with the replication of the 50 S RNA and hence of the infectious virus. Further work would have been required to show whether the results obtained in this section were due to the presence of defective viral particles, or simply to massive break-up of 50 S genomic RNA. A search for 400 S virus particles might have provided an answer to this and a study of the effect on the virus RNA of serial passage of the virus at high dilution might have proved rewarding.

FINAL DISCUSSION.

The two principal aims of the author's investigation were to obtain data on the structure and replication of the virus. At its commencement, methods were not available for the study of viral replication at the molecular level but the electron microscopic study carried out revealed the full sequence of ultrastructural events from the earliest appearance of nucleocapsid in the cell to the point where virus production had fallen to an almost negligible level. Thus, for the first time, the eclipse phase of the virus in dog cells was established, information was gained on the method of virus maturation and morphological differences in the nucleocapsid at different times and in different situations was obtained.

Before replication could be studied at the molecular level, it was clearly necessary to characterise the individual macromolecules of the virus. Before this could be done, however, the necessary technology had to be developed. Since the virus grows relatively poorly in cell-culture, a method of obtaining the titre of virus required for biochemical studies had to be found. From the experiments described in Part One, a method was evolved for producing large quantities of virus which could then be concentrated by ultracentrifugation. The information thus gained should be of value not only to those interested in

the biochemistry of the virus but also to those who require virus for other purposes such as vaccine production.

Although density gradient centrifugation is widely used for the purification of paramyxoviruses, little has been published on the degree of purification obtainable in different types of gradient, i.e. caesium chloride, sucrose etc., and on the proportion of virus recoverable from each. By preparing a single stock of virus labelled with ^{3}H-uridine, it was found that potassium tartrate was superior to sucrose and markedly superior to caesium chloride for the concentration and purification of the virus and provided a higher recovery rate. Again this information should be of importance, not only to those engaged in distemper research but also to those interested in other paramyxoviruses. Furthermore, it was found that better separation of distemper virus from mycoplasma could be obtained in potassium tartrate than in sucrose and this information may be of value in attempting to eliminate mycoplasma contamination from stocks of paramyxoviruses.

Although infected cells contain a large amount of nucleocapsid, it proved difficult to recover large amounts of this in completely intact form. Based on the results of electron microscopy of nucleocapsid banded in density gradients following extraction with various detergents, Sterox SL appeared to be the most satisfactory of the detergents tested in this respect. However, since quantitative methods of comparison were not available at this

stage of the investigation, this could not be confirmed with certainty. Some fragmentation of nucleocapsid always occurred, but this was reduced when BSA was incorporated into both the detergent solution and the density gradient.

Difficulty was experienced in incorporating ^{3}H-uridine into viral RNA, the amount of labelled virus RNA being very small compared to that of labelled cellular RNA. In the case of virus which had been concentrated and purified there was no problem in demonstrating that the radioactivity was specific to the virus, but problems of specificity arose in the case of nucleocapsid extracted from cells. Although Actinomycin D prevented cellular RNA synthesis, it proved too toxic to the cells to be of any real value in the solution of this problem. However, treatment of the extracted material with RNase was found to remove a very large amount of the cellular RNA and thus facilitated the detection of viral RNA. In density gradients of caesium chloride solution, two bands were present, each corresponding to a separate peak of radioactivity, and electron microscopy showed that only the less dense of these contained recognisable nucleocapsid. In potassium tartrate, a single peak of radioactivity was obtained but electron microscopy failed to reveal nucleocapsid at that density. Further work is therefore required to assess the best method for extracting and purifying the nucleocapsid from infected cells.

Although the method employed for the production of virus stocks was satisfactory for studies of the viral proteins, it is possible that it resulted in the formation of large amounts of defective virus. This possibility will obviously have to be investigated before studies on the viral RNA can be completed. The six polypeptides extracted from the virus seemed to correspond quite closely to those described by Waters and Bussell (1973) but, for the first time with CDV, it was shown that four of them were derived from larger protein molecules in the virion. Nucleocapsid extracted from virus in two different ways and banded in two different ways was found to contain two polypeptides. Since most previous work on paramyxovirus nucleocapsid has shown that it consists of only one polypeptide, confirmation of this discovery is required. Further work will also have to be done to identify the other polypeptides, i.e., to show which are derived from the envelope and which are the glycoproteins. Procedures for the isolation and purification of the various polypeptides will have to be developed, perhaps involving some of the techniques described by Scheid and Choppin (1973) and Hall and Martin (1974b) for the isolation of some of the polypeptides of NDV and measles viruses respectively. In both these latter studies, the biological activities of the polypeptides, i.e. haemagglutinating and haemolytic activity, were not destroyed by the procedures employed. It may be possible therefore to obtain separate CDV proteins retaining their antigenic and biological activities. Production of antisera to these would then permit a more detailed study of CDV

replication to be carried out by immuno-electron microscopy. This could also be applied to the study of virus infection in sections of brains of dogs with encephalitis, and also to cell cultures derived from these animals. In this way valuable information may eventually be gained on the mechanisms involved in defective CDV infection of the central nervous system. It is hoped that this thesis will have helped pave the way to that goal.

REFERENCES.

Abelson, J., Thomas, C.A. (1966): The anatomy of the T5 bacteriophage DNA molecule. J. molec. Biol. 18, 262.

Adams, W.R., (1965): Extraction of a rapidly sedimenting ribonucleic acid fraction from purified Newcastle disease virus. Fedn Proc. 24, 159.

Akroyd, P. (1967): Acrylamide gel slab electrophoresis in a simple glass cell for improved resolution and comparison of serum proteins. Analyt. Biochem. 19, 399.

Appel, M.J.G., Gillespie, J.H. (1972): Canine distemper virus. Virological Monographs 11. Edited by Gard, S., Hallauer, C., Meyer, K.F. Springer-Verlag, Vienna, New York.

Appel, M., Jones, O.R. (1967): Use of alveolar macrophages for cultivation of canine distemper virus. Proc. Soc. exp. Biol. Med. 126, 571.

Appel, M., Robson, D.S. (1973): A microneutralisation test for canine distemper virus. Am. J. vet. Res. 34, 1459.

Barry, R.D., Bukrinskaya, A.G. (1968): The nucleic acid of Sendai virus and ribonucleic acid synthesis in cells infected with Sendai virus. Jnl gen. Virol. 2, 71.

Bikel, I., Duesberg, P.H. (1969): Proteins of Newcastle

disease virus and of the viral nucleocapsid. Jnl Virol. 4, 388.

Bittle, J.L., York, C.J., Newberne, J.W. (1961): Adaption and modification of a strain of canine distemper virus in tissue culture. Cornell Vet. 51, 359.

Black, F.L. (1959): Growth and stability of measles virus. Virology 7, 184.

Blair, C.D. (1970): Intracellular components of Sendai virus. In 'The Biology of Large RNA Viruses'. Edited by Barry, R.D., Mahy, B.W.J. Academic Press, London, New York.

Blair, C.D., Robinson, W.S. (1968): Replication of Sendai virus. 1. Comparison of the viral RNA and virus-specific RNA synthesis with Newcastle disease virus. Virology 35, 537.

Bratt, M.A., Robinson, W.S. (1967): Ribonucleic acid synthesis in cells infected with Newcastle disease virus. J. molec. Biol. 23, 1.

Breese, S.S., de Boer, C.J. (1973): Ferritin-tagged antibody cross-reactions among rinderpest, canine distemper, and measles viruses. Jnl gen. Virol. 20, 121.

Bukrinskaya, A.G. (1973): Transcriptive functions of Sendai virus nucleocapsid in infected cells. Virology 52, 344.

Bussell, R.H., Karzon, D.T. (1962): Canine distemper virus in chick embryo cell culture. Virology 18, 589.

Bussell, R.H., Karzon, D.T. (1965a): Canine distemper virus in ferret, dog, and bovine kidney cell cultures. Arch. ges. Virusforsch. 17, 163.

Bussell, R.H., Karzon, D.T. (1965b): Canine distemper virus in primary and continuous cell lines of human and monkey origin. Arch. ges. Virusforsch. 17, 183.

Cabasso, V.J., Kiser, K., Stebbins, M.R. (1959): Propagation of canine distemper (CD) virus in tissue culture. Proc. Soc. exp. Biol. Med. 100, 551.

Carré, H. (1905): Sur la maladie des jeunes chiens. C. r. hebd. Séanc. Acad. Sci. Paris 140, 689 and 1489.

Carter, C., Schluederberg, A., Black, F.L. (1973): Viral RNA synthesis in measles virus-infected cells. Virology 53, 379.

Cascardo, M.R., Karzon, D.T. (1965): Measles virus giant cell inducing factor (fusion factor). Virology 26, 311.

Celiker, A., Gillespie, J.H. (1954): The effect of temperature, pH and certain chemicals on egg-cultivated distemper virus. Cornell Vet. 44, 276.

Chiarini, A., Norrby, E. (1970): Separation and characterisation of products of two measles virus variants. Arch. ges. Virusforsch. 29, 205.

Compans, R.W., Choppin, P.W. (1967a): The length of the helical nucleocapsid of Newcastle disease virus. Virology 33, 344.

Compans, R.W. Choppin, P.W. (1967b): Isolation and properties of the helical nucleocapsid of the parainfluenza virus SV5. Proc. natn Acad. Sci. U.S.A. 57, 949.

Compans, R.W., Choppin, P.W. (1968): The nucleic acid of the parainfluenza virus SV5. Virology 35, 280.

Confer, A.W., Kahn, D.E., Koestner, A., Krakowka, S. (1975a): Biological properties of a canine distemper virus isolate associated with demyelinating encephalomyelitis. Infec. Immunity 11, 835.

Confer, A.W., Kahn, D.E., Koestner, A., Krakowka, S. (1975b): Comparison of canine distemper viral strains: an electron microscopic study. Am. J. vet. Res. 36, 741.

Cornwell, H.J.C., Vantsis, J.T., Campbell, R.S.F., Penny, W. (1965): Studies in experimental canine distemper. 2. Virology, inclusion body studies and haematology. J. comp. Path. Ther. 75, 19.

Cornwell, H.J.C., Weir, A.R., Wright, N.G., Thompson, H. (1970): The susceptibility of a dog kidney cell-line (MDCK) to canine distemper, infectious canine hepatitis and canine herpes viruses. Res. vet. Sci. 11, 580.

Cornwell, H.J.C., Laird, H.M., Wright, N.G., Thompson, H. (1971): Ultrastructural features of canine distemper virus

infection in a dog kidney cell line. Jnl gen. Virol. 12, 281.

Cruikshank, J.G., Waterson, A.P., Kanarek, A.D., Berry, D.M. (1962): The structure of canine distemper virus. Res. vet. Sci. 3, 485.

Dubois-Dalcq, M., Barbosa, L.H., Hamilton, R., Sever, J.L. (1974): Comparison between productive and latent subacute sclerosing panencephalitis viral infection in vitro. An electron microscopic and immunoperoxidase study. Lab. Invest. 30, 241.

Duesberg, P.H. (1968): Physical properties of rous sarcoma virus RNA. Proc. natn Acad. Sci. U.S.A. 60, 1511.

Duesberg, P.H., Robinson, W.S.(1965): Isolation of the nucleic acid of Newcastle disease virus (NDV). Proc. natn Acad. Sci. U.S.A. 54, 794.

Dunkin, G.W., Laidlaw, P.P. (1926): Studies in dog-distemper. 1. Dog-distemper in the ferret. J. comp. Path. Ther. 39, 201.

Dunkin, G.W., Laidlaw, P.P. (1926): Studies in dog-distemper. 2. Experimental distemper in the dog. J. comp. Path. Ther. 39, 213.

Elliot, J.A., Ryan, W.L. (1970): Continuous-flow ultracentrifugation of canine distemper virus and infectious canine hepatitis virus. Appl. Microbiol. 20, 667.

Evans, M.J., Kingsbury, D.W. (1969): Separation of Newcastle disease virus proteins by polyacrylamide gel electrophoresis. Virology 37, 597.

Finch, J.T., Gibbs, A.J. (1970): Observations on the structure of the nucleocapsids of some paramyxoviruses. Jnl gen. Virol. 6, 141.

Hall, W.W., Martin, S.J. (1973): Purification and characterisation of measles virus. Jnl gen. Virol. 19, 175.

Hall, W.W., Martin, S.J. (1974a): Defective interfering particles produced during the replication of measles virus. Med. Microbiol. Immunol. 160, 155.

Hall, W.W., Martin, S.J. (1974b): The biochemical and biological characteristics of the surface components of measles virus. Jnl. gen. Virol. 22, 363.

Hamilton, R., Barbosa, L., Dubois, M. (1973): Subacute sclerosing panencephalitis measles virus: study of biological markers. Jnl Virol. 12, 632.

Hammarskjold, B., Norrby, E. (1974): A comparison between virion RNA of measles virus and some other paramyxoviruses. Med. Microbiol. Immunol. 160, 99.

Harrison, M.J., Smith, F.A., Graydon, J.J. (1968): The virus of canine distemper virus in cell culture. 1. Adaption of canine distemper virus to growth and serial passage in ferret kidney cell cultures and in BS-C-1 cell cultures. J. comp. Path. 78, 121.

Haslam, E.A., Cheyne, I.M., White, D.O. (1969): The structural proteins of Newcastle disease virus. Virology 39, 118.

Hopper, P.K. (1959): The propagation of canine distemper virus in tissue culture. J. comp. Path. Ther. 69, 78.

Hosaka, Y. (1968): Isolation and structure of the nucleocapsid of HVJ. Virology 35, 445.

Hosaka, Y., Shimizu, K. (1968): Lengths of the nucleocapsids of Newcastle disease and mumps viruses. J. mol. Biol. 35, 369.

Hosaka, Y., Kitano, H., Ikeguchi, S. (1966): Studies on the pleomorphism of HVJ virions. Virology 29, 205.

Huang, A.S., Baltimore, D. (1970): Defective viral particles and viral disease process. Nature 226, 325.

Imagawa, D.T., Baird, C.D., Adams, J.M. (1963): Measles and distemper viruses in continuously cultured dog kidney cells. Fedn. Proc. 22, 488.

Jenner, E. (1809): Canine distemper, its complications, sequelae and treatment. First Edition, Balliere, Tindall and Cox, London, 1922. Cited by Appel, M.J.G. and Gillespie, J.H. (1972) in Virological Monographs 11.

Kallman, F., Adams, J.M., Williams, R.C., Imagawa, D.T. (1959): Fine structure of cellular inclusions in measles virus infections. J. biophysic. biochem. Cytol. 6, 379.

Karber, G. (1931): Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch. exp. Path. Pharmak. 162, 480.

Karzon, D.T., Bussell, R.H. (1959): Cytopathic effect of canine distemper virus in tissue culture. Science 130, 1708.

Kasza, L. (1968): Recovery of canine distemper virus from subcultured primary dog kidney cell culture. Res. vet. Sci. 9, 187.

Kiley, M.P., Payne, F.E. (1974): Evidence of precursors of defective measles virus. Med. Microbiol. Immunol. 160, 91.

Kiley, M.P., Gray, R.H., Payne, F.E. (1974): Replication of measles virus: distinct species of short nucleocapsids in cytoplasmic extracts of infected cells. Jnl Virol. 13, 721.

Kingsbury, D.W. (1966): Newcastle disease virus RNA. 1. Isolation and preliminary characterisation of RNA from virus particles. J. mol. Biol. 18, 195.

Kingsbury, D.W., Darlington, R.W.(1968): Isolation and properties of Newcastle disease virus nucleocapsid. Jnl Virol. 2, 248.

Kingsbury, D.W., Portner, A. (1970): On the genesis of incomplete Sendai virions. Virology 42, 872.

Kingsbury, D.W., Portner, A., Darlington R.W. (1970): Properties of incomplete Sendai virions and subgenomic viral RNAs. Virology 42, 857.

Klensk, H.D., Choppin, P.W. (1969): Chemical composition of the parainfluenza virus SV5. Virology, 37, 155.

Koestner, A., Long, J.F. (1970): Ultrastructure of canine distemper virus in explant tissue cultures of canine cerebellum. Lab. Invest. 23, 196.

Laidlaw, P.P., Dunkin, G.W. (1926): Studies in dog-distemper. 3. The nature of the virus. J. comp. Path. Ther. 39, 222.

Lavender, J.F., Bewsey, B.J. (1973): Immunogenicity of canine distemper virus vaccine produced in a continuous cell line (LLC-MK_2 cells). Am. J. vet. Res. 34, 1189.

Lawn, A.M. (1970): Ultrastructural features of canine distemper virus infection of the chorioallantoic membrane of the hen's egg. Jnl gen. Virol. 8, 157,

Lincoln, S.D., Gorham, J.R., Ott, R.L., Hegreberg, G.A. (1971): Aetiologic studies of old dog encephalitis. 1. Demonstration on canine distemper viral antigen in the brain in two cases. Vet. Path. 8, 1.

Lincoln, S.D., Gorham, J.R., Davis, W.C., Ott, R.L. (1973): Studies of old dog encephalitis. 2. Electron microscopic and immunohistologic findings. Vet. Path. 10, 124.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951): Protein measurement with the Folin phenol reagent. J. biol. Chem. 193, 265.

Manning, J.S., Hackett, A.J., Cardiff, R.D., Mel, H.C., Blair, P.B. (1970): Isopycnic zonal centrifugation and characterisation of the mouse mammary tumour virus (MTV) in different gradient solutions. Virology 40, 912.

Martin, R.G., Ames, B.N. (1961): A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J. biol. Chem. 236, 1372.

McCrea, J.F., Epstein, R.S., Barry, W.H. (1961): Use of potassium tartrate for equilibrium density-gradient centrifugation of animal viruses. Nature 189, 220.

Meselson, M., Stahl, F.W., Vinograd, J. (1957): Equilibrium sedimentation of macromolecules in density gradients. Proc. natn Acad. Sci. U.S.A. 43, 581.

Moore, N.F., Burke, D.C. (1974): Characterisation of the structural proteins of different strains of Newcastle disease virus. Jnl gen. Virol. 25, 275.

Mountcastle, W.E., Compans, R.W., Caliguri, L.A., Choppin, P.W. (1970): Nucleocapsid protein subunits of simian virus 5, Newcastle disease virus and Sendai virus. Jnl Virol 6, 677.

Nakai, M., Imagawa, D.T. (1969): Electron microscopy of measles virus replication. Jnl Virol. 3, 187.

Nakai, T., Shand, F.L., Howatson, A.F. (1969): Development of measles virus *in vitro*. Virology 38, 50.

Nakajimi, H., Obara, J. (1967): Physicochemical studies of Newcastle disease virus. 3. The content of virus nucleic acid and its sedimentation pattern. Arch. ges. Virusforsch. 20, 287.

Norrby, E. (1964): Separation of measles virus components by equilibrium centrifugation in CsCl gradients. 1. Crude and Tween and ether treated concentrated tissue culture material. Arch. ges. Virusforsch. 14, 306.

Norrby, E., Hammarskjold, B. (1972): Structural components of measles virus. Microbios 5, 17.

Norrby, E., Friding, B., Rockborn, G., Gard, S. (1963): The ultrastructure of canine distemper virus. Arch. ges. Virusforsch. 13, 335.

Norrby, E.C.J., Magnusson, P., Falksveden, L. -G., Gronberg, M. (1964): Separation of measles virus components by equilibrium centrifugation in CsCl gradients. 2. Studies on the large and the small haemagglutinin. Arch. ges. Virusforsch. 14, 462.

Numazaki, Y., Karzon, D.T. (1966): Density separable fractions during growth of measles virus. J. Immun. 97, 458.

Palm, C.R., Black, F.L. (1961): A comparison of canine distemper and measles viruses. Proc. Soc. exp. Biol. Med. 107, 588.

Phillips, L.A., Bussell, R.H. (1973): The buoyant density of canine distemper virus. Arch. ges. Virusforsch. 41, 310.

Plowright, W., Herniman, K.A.J., Rampton, C.S. (1969): Studies on rinderpest culture vaccine. 1. Some factors affecting virus production. Res. vet. Sci. 10, 373.

Poste, G. (1970): Increased growth of viruses in lymphocytes treated with heterologous antilymphocyte serum. Transplantation 10, 106.

Poste, G. (1971): The growth and cytopathogenicity of virulent and attenuated strains of canine distemper virus in dog and ferret macrophages. J. comp. Path. 81, 49.

Poste, G. (1972): Electron microscopic study of nuclear and nucleolar changes in cells infected with canine distemper virus. Arch. ges. Virusforsch. 37, 183.

Raine, C.S., Feldman, L.A., Sheppard, R.D., Bornstein, M.B. (1969): Ultrastructure of measles virus in cultures of hamster cerebellum. Jnl Virol. 4, 169.

Rankin, A.M., Fisher, L.E., Bussell, R.H. (1972): Cell fusion by canine distemper virus-infected cells. Jnl Virol. 10, 1179.

Reynolds, E.S. (1963): The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. cell. Biol. 17, 208.

Richter, W.R., Moize, S.M. (1970): Ultrastructural nature of canine distemper inclusions in the urinary bladder. Path. vet. 7, 346.

Robinson, W.S. (1971): Ribonucleic acid polymerase activity in Sendai virions and nucleocapsid. Jnl Virol. 8, 81.

Rockborn, G. (1958): Canine distemper virus in tissue culture. Arch. ges. Virusforsch. 8, 485.

Rowlands, D.J., Sangar, D.V., Brown, F. (1971): Buoyant density of picornaviruses in caesium salts. Jnl gen. Virol. 13, 141.

Scheid, A., Choppin, P.W. (1973): Isolation and purification of the envelope proteins of Newcastle disease virus. Jnl Virol. 11, 263.

Schluederberg, A. (1971): Measles virus RNA. Biochem. biophys. Res. Commun. 42, 1012.

Schluederberg, A.E., Roizman, B. (1962): Separation of multiple antigenic components of measles virus by equilibrium sedimentation in caesium chloride. Virology 16, 80.

Shapiro, S.C., Bratt, M.A. (1971): Proteins of four biologically distinct strains of Newcastle disease virus. Proc. Soc. exp. Biol. Med. 136, 834.

Shaver, D.N., Bussell, R.H., Barron, A.L. (1964): Comparative cytopathology of canine distemper and measles viruses in ferret kidney cell cultures. Arch. ges. Virusforsch. 14, 487.

Shishido, A., Yamanouchi, K., Hikita, M., Sato, T., Fukuda, A., Kobune, F. (1967): Development of a cell culture system susceptible to measles, canine distemper, and rinderpest viruses. Arch. ges. Virusforsch. 22, 364.

Smith, O.K., Dunlop, R.C., Thiel, J.F., Newman, J.T., Palmer, A.E. (1970): Isolation of viruses from primary dog cell cultures and the occurrence of viral antibody in donor animals. Proc. Soc. exp. Biol. Med. 133, 560.

Sokol, F., Neurath, A.R., Vilcek, J. (1964): Formation of incomplete Sendai virus in embryonated eggs. Acta Virol. 8, 59.

Stenback, W.A., Durand, D.P. (1963): Host influence on the density of Newcastle disease virus (NDV). Virology 20, 545.

Stone, H.O., Portner, A., Kingsbury, D.W. (1971): Ribonucleic acid transcriptase in Sendai virions and infected cells. Jnl Virol. 8, 174.

Stone, H.O., Kingsbury, D.W., Darlington, R.W. (1972): Sendai virus-induced transcriptase from infected cells: polypeptides in the transcriptive complex. Jnl Virol. 10, 1037.

Stromberg, K. (1972): Surface-active agents for isolation of the core component of avian myeloblastosis virus. Jnl Virol. 9, 684.

Tajima, M., Motohashi, T., Kishi, S., Nakamura, J. (1971):

A comparative electron microscopic study on the morphogenesis of canine distemper and rinderpest viruses. Jap. J. vet. Sci. 33, 1.

de Thé, G., O'Connor, T.E. (1966): Structure of a murine leukaemia virus after disruption with Tween-ether and comparison with two myxoviruses. Virology 28, 713.

Thiel, J.K., Smith, K.O., Newman, J., Dunn, J., Trousdale, M.D. (1968): Isolation and identification of adventitious agents from primary cell cultures of dogs. Bact. Proc. 97, 161.

Todaro, G.J., Aaronson, S.A., Rands, E. (1971): Rapid detection of mycoplasma-infected cell cultures. Expl Cell Res. 65, 256.

Underwood, B., Brown, F. (1974): Physico-chemical characterisation of rinderpest virus. Med. Microbiol. Immunol. 160, 125.

Vantsis, J.T. (1959): Preliminary note on the propagation of canine distemper virus in different tissue-culture systems. Vet. Rec. 71, 99.

Warren, J. (1960): The relationships of the viruses of measles, canine distemper and rinderpest. Adv. Virus. Res. 7, 27.

Waters, D.J. Bussell, R.H. (1973): Polypeptide composition of measles and canine distemper viruses. Virology 55, 554.

Waters, D.J., Bussell, R.H. (1974): Isolation and comparative study of the nucleocapsids of measles and canine distemper viruses from infected cells. Virology 61, 64.

Waters, D.J., Hersh, R.T., Bussell, R.H. (1972): Isolation and characterisation of measles nucleocapsid from infected cells. Virology 48, 278.

Waterson, A.P. (1964): In 'Techniques in Experimental Virology'. Academic Press, London. Cited by Gregory, D.W., Pirie, B.J.S. (1972): Wetting agents for electron microscopy of biological specimens. Proc. Fifth European Congress on Electron Microscopy, 234.

Waterson, A.P. (1965): Measles virus. Arch. ges. Virusforsch. 16, 57.

Waterson, A.P., Rott, R., Ruckle-Enders, G. (1963): The components of measles virus and their relation to rinderpest and distemper. Z. Naturforsch. B. 18, 377.

Watson, M.L. (1958): Staining of tissue sections for electron microscopy with heavy metals. J. biophys. biochem. Cytol. 4, 475.

Watson, A.D.J., Wright, R.G. (1974): The ultrastructure of cytoplasmic inclusions in circulating lymphocytes in canine distemper. Res. vet. Sci. 17, 188.

Weast, R.C. (1972): Handbook of Chemistry and Physics, 53rd Edition, 1972-1973. The Chemical Rubber Co. Press.

Wilcox, W.C. (1959): Quantitative aspects of an *in vitro* virus-induced toxic reaction. 1. General aspects of the reaction of Newcastle disease virus with L cells. Virology 9, 30.

Wright, N.G., Cornwell, H.J.C., Thompson, H., Lauder, I.M. (1974): Canine distemper: current concepts in laboratory and clinical diagnosis. Vet. Rec. 94, 86.

Yeh, J., Iwasaki, Y. (1972): Isolation and characterisation of subacute sclerosing panencephalitis virus nucleocapsids. Jnl Virol. 10, 1220.

Zaides, V.M., Nicolaeva, O.G., Selimova, L.M., Zhirnov, O.P., Bukrinskaya, A.G. (1974): The properties of the Sendai virus ribonucleoproteins involved in genome transcription in infected cells. Jnl gen. Virol. 24, 409.

Zaides, V.M., Selimova, L.M., Zhirnov, O.P., Bukrinskaya, A.G. (1975): Protein synthesis in Sendai virus-infected cells. Jnl gen. Virol. 27, 319.