PERSISTENT INFECTION OF CONTINUOUS CELL CULTURES

WITH NEWCASTLE DISEASE VIRUS

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SUMMARY

Continuous cultures of pig, ox and calf kidney cells consistently infected with Newcastle disease virus (NDV) had the characteristics of a regulated-type of infection.

Most of the cells were infected but did not release infectious virions. The cultures could not be cured by specific antiserum and resisted superinfection with homologous virus but not with unrelated viruses. Antibody or other neutralizing factors were not necessary for maintaining the equilibrium state and the infected cells continued to divide and gave without detrimental effects.

Virus was present in the fluid phase of all three types of carrier cultures and the haemagglutinin did not suffice spontaneously from application to fowl erythrocytes.

The extracellular virus was deficient in neuraminidase and electron micrographs showed that the released virions were morphologically similar to those of NDV although they lacked the fringes of spikes normally associated with intact, infectious virus. The internal nucleoprotein component was ill-defined and disrupted virions tended to show strands of entangled nucleoprotein.

Unsuccessful attempts were made to activate and transmit the defective agent but the carrier state appeared to be associated with non-infectious virus.
Continuous cultures of pig, ox and calf kidney cells persistently infected with Newcastle disease virus (NDV) had the characteristics of a regulated-type of infection.

Most of the cells were infected but did not release infectious virions. The cultures could not be cured by specific antisera and resisted superinfection with homologous virus but not with unrelated viruses. Antibody or other antiviral factors were not necessary for maintaining the equilibrium state and the infected cells continued to divide and grow without detrimental effects.

Virus was present in the fluid phase of all three types of carrier cultures and the haemagglutinins did not elute spontaneously from agglutinated fowl erythrocytes.

The extracellular virus was deficient in neuraminidase and electron micrographs showed that the released virions were morphologically similar to those of NDV although they lacked the 'fringe' of spikes normally associated with intact, infectious virus. The internal nucleoprotein component was ill-defined and disrupted virions seldom showed strands of extruding nucleoprotein.

Unsuccessful attempts were made to activate and transmit the defective agent but the carrier state appeared to be associated with non-infectious virus.

Heterokaryons /
Heterokaryons artificially induced with mixed populations of healthy and persistently infected cells stimulated the formation of inclusions in the healthy nuclei but this was not associated with activation of the latent infection.

There was evidence that the defective virus was capable of inducing cellular transformation.
INTRODUCTION

The introduction of antibiotics in microbiology, together with the use of enzymes for dispersing cells from whole tissue fragments, led to the widespread use of animal cell cultures in virology. However, the comparatively slow rate of progress in animal virology, which was dependent on experiments using animals or embryonated eggs, was quickly overcome and cell culture methods were quickly introduced and adopted as standard methods for the isolation, identification and purification of viruses.

Yet, there is little doubt that the use of antibiotics has largely overcome the problem of bacterial contamination in cell cultures; it was only their continued use was associated with a marked increase in the incidence of resistant strains of bacteria and mycoplasmas. In many instances the presence of these contaminating organisms was not suspected since they rarely produced visible changes in the affected cells. For example, Batlle, Malasse and Higgins (1963) isolated mycoplasma-like organisms from 10 out of 11 cell cultures obtained from commercial sources and showed that their presence was directly related to the use of antibiotics in the cell culture medium.

Fortunately, however, the difficulties associated with the contamination of cell lines by mycoplasmas has largely been overcome following the introduction of a new antibiotic, tylan*, which will eradicate, or at least control, most strains of PML when it is incorporated in the culture media (Cross, Goodman and Shaw, 1967).

* Clinton Products Limited, London.
CELL CULTURES

Development:

The introduction of antibiotics in microbiology, together with the use of trypsin for dispersing cells from whole tissue fragments, led to the widespread use of animal cell cultures in virology. Thus, the comparatively slow rate of progress in animal virology, which was dependent on experiments using animals or embryonated eggs, was quickly overcome and cell culture methods were quickly introduced and adapted as standard methods for the isolation, identification and purification of viruses.

Whilst there is little doubt that the use of antibiotics largely overcame the problem of bacterial contamination in cell cultures it was quickly realised that their continued use was associated with a marked increase in the incidence of resistant L forms of bacteria and mycoplasmas. In many instances the presence of these contaminating organisms was not suspected since they rarely produced visible changes in the affected cells. For example, Barile, Malizia and Riggs (1962) isolated pleuropneumonia-like organisms (PPLO) from 10 out of 11 cell cultures obtained from commercial sources and showed that their presence was directly related to the use of antibiotics in the cell culture medium.

Fortunately, however, the difficulties associated with the contamination of cell lines by mycoplasmas has largely been overcome following the introduction of a new antibiotic, Tylan*, which will eradicate, or at least control, most strains of PPLO when it is incorporated in the culture medium (Cross, Goodman and Shaw, 1967).

The /

* Elanco Products Limited, London.
The practical importance of this discovery is readily appreciated when it is realised that cell culture methods are now widely used for the isolation of PPLO from cases of rheumatoid arthritis and various other disease conditions of unknown aetiology (Bartholomew, 1965). In such experiments the validity and significance of the findings rests largely on the knowledge that the cell culture systems used are free from contaminating mycoplasmas, prior to inoculation. The use of "clean" cultures is also of importance in virology since many forms of PPLO organisms bear a close resemblance to some myxoviruses, not only in their size and shape but also in their ability to haemagglutinate animal red cells. Despite these disadvantages the use of cell cultures, under carefully controlled conditions undoubtedly provides one of the most convenient and reliable methods for propagating animal viruses.

**Virus Growth:**

Virus activity in infected monolayer cultures may be characterised by a wide range of cytopathic effects (CPE). These include complete cellular destruction, as shown by poliomyelitis virus and other enteroviruses; multinucleated cells or syncytial formation, as produced by many species of myxoviruses; and cell transformation, with excessive cell proliferation, due to loss of contact inhibition induced by many tumour viruses. Despite these different manifestations of virus activity many viruses, especially myxoviruses and other membrane forming viruses, are capable of growth in susceptible host cell systems without the /
the production of an overt cytopathic effect. The presence of these inapparent infections may be detected by the phenomenon of haemadsorption which is produced by the adsorption of healthy red blood corpuscles to the surface of the infected cells (Vogel and Shelokov, 1957).

Latent Infections:

As cell culture techniques became more sophisticated and a greater range of cell culture types became available, it was quickly realised that many apparently healthy tissues were latently infected with viruses which only manifested themselves under conditions of artificial culture. Indeed, the name adenovirus was derived from the fact that members of this group were first isolated from cell cultures of apparently healthy adenoid tissue. The ever increasing use of primary monkey kidney cell cultures in medical virology also revealed the presence of a latent simian tumour virus which was originally referred to as the "foamy agent" and is now designated SV40. Many other latent simian viruses have come to light under conditions of artificial culture and, during the course of a single year's survey, Anderson and Doane (1970) detected latent simian viruses by means of electron microscopy in 23 out of 65 batches of primary monkey kidney cell cultures.
4.

CARRIER CULTURES

Definition:

This new awareness of the problems of latency and masking of viruses in cell cultures led to the discovery that animal viruses of almost all virus groups were capable of establishing persistent non-cytocidal infections in cultures, not only of basically resistant cells but also in populations of genetically susceptible cells. The phenomenon is generally described as the "carrier state". The name was "borrowed" from the work of Lwoff (1953), who first used it to describe a mixed population of bacteria and bacteriophages in a more or less stable equilibrium. Although the mechanisms of persistent viral infections of animal cell cultures appear to be more complex than those involved in Lwoff's original carrier cultures of bacterial cells, the term has survived and is now in common usage.

Review of Carrier Culture Systems:

Reports on the "carrier state" which have appeared over the past decade have implicated a wide range of animal viruses and host cell types, and various suggestions have been made as to the nature of the mechanisms that are most likely to be involved in maintaining the equilibrium state. For example, Takemoto and Habel (1959) recorded a Coxsackie A9 persistent infection in a resistant HeLa cell population in which the state of equilibrium was maintained by the relative resistance of the host cells. Similarly, Ackermann and Kurtz (1955) described a carrier state in /
in HeLa cells with poliomyelitis virus type 3 which was maintained by the addition of a specific immune serum to the medium, while Ginsberg and Boyer (1956) described an adenovirus infection in which active cell growth was an essential prerequisite for maintaining the carrier state. Other examples include a persistent infection of human embryonic fibroblast cells with a strain of reovirus type 3 isolated from a patient with Burkitt's lymphoma (Bell and Ross, 1966).

However, there is little doubt that the most numerous and most fully investigated examples of carrier state cultures are those associated with paramyxoviruses. For example, Cole and Hetrick (1965) described a carrier culture of human conjunctival cells, persistently infected with para-influenza type 3 virus, in which active cell growth was a requirement of the system, and Ishida, Homma, Osato, Hinuma and Miyamoto (1964) reported on a carrier culture of HeLa cells involving Sendai virus, in which there was no requirement for antibody, interferon or other inhibitors. Many other paramyxoviruses have been incriminated and the reports range from persistent infections of a line of Earles' L cells with mumps and Newcastle disease virus, respectively, (Henle, Deinhardt, Bergs and Henle, 1958), to a persistent, non-cytocidal infection of BHK 21 cells with a human strain of para-influenza type 2 (Fraser and Anderson, 1966). The last report is of particular interest because it describes one of the few carrier cultures which permits superinfection with the homologous /
homologous virus. Membrane viruses with a myxovirus-like structure are also frequently involved in latent infections of cell cultures and Rustigian (1962) reported on a carrier culture of HeLa cells involving measles virus. Rustigian's model is of particular interest to this present work since it is apparently associated with the release of completely non-infectious virus. It is emphasised, however, that the system he describes was obtained only after prolonged exposure of the culture to measles antiserum. Other membrane viruses capable of producing a carrier state include rabies virus, which may do so on rabbit endothelial cells or neonatal hamster kidney fibroblasts (Fernandes, Wiktor and Koprowski, 1964), and rubella virus on monkey kidney cells (Maassab and Veronelli, 1966). It is of interest that interferon production was not considered to be a feature of the rabies virus system although it was present in detectable amounts in the cultures infected with rubella virus.

Scheme of Classification:

Certain aspects of the systems involved in the maintenance of carrier state cultures were carefully reviewed by Ginsberg (1958) and, in the light of more recent developments, by Walker (1964) who attempted a more comprehensive review and included a proposed system of classification. Despite the variety of viruses and the wide divergence of host cell types involved, present knowledge suggests that only a limited number of mechanisms are concerned in maintaining the persistent state. Although Walker included four categories in his scheme of classification, he stipulated, as did Ginsberg, that in many carrier-cell systems more than one mechanism /
7.

mechanism might be involved in maintaining equilibrium and that multiple mechanisms might operate simultaneously or in series. The first of Walker's categories was one in which the carrier state was established in basically resistant cells in the absence of antibody. This type, which is mainly associated with enteroviruses, is well illustrated by the work of Pácsa (1961) who succeeded in establishing a persistent infection of HeLa cells with poliovirus types 2 and 3 that could be subcultured indefinitely without the addition of immune serum to the culture medium. His second category included those systems occurring in genetically susceptible cells that were protected by antiserum or other antiviral factors supplied in the medium. Carrier cultures of Herpes simplex virus in various cell lines of serially cultivated human cells provide the best example of this type of carrier state. In the system described by Fernandez (1960) in which HeLa cells were persistently infected with Herpes simplex virus, infectivity was restored when the herpes antiserum was withdrawn from the medium. It is of interest, that in these first two categories of persistent infection, only a small proportion of the total cell population is infected when the carrier state is stable. The third category in Walker's scheme of classification occurs most readily with viruses of the myxovirus group where persistent infections are maintained by interferons or interfering particles produced in demonstrable amounts in the medium. In the system described by Henle, Henle, Deinhardt and Bergs (1959) interferon was produced in detectable amounts in a line of Earle's L cells persistently infected with Newcastle disease /
disease virus although only a minority of the cells were infected when the virus and cells were in equilibrium. In this latter respect the system was similar to those of Walker's first two categories. The fourth category, which Walker refers to as a 'regulated infection' appears to be restricted to myxoviruses and other viruses of the membrane type. Although this class of persistent infection occurs in a susceptible cell population, it does not require the presence of antiviral factors in the medium to maintain the carrier-state and, unlike the other three categories, the great majority of the cells are infected when the culture is in equilibrium. The measles and rabies carrier cell systems described by Rustigian (1962) and Fernandes et al (1964), respectively, belong in this category. In both of these examples, 80 - 100 percent of cells were infected but, despite this, the cells were capable of repeated subculture in the absence of immune serum. The HeLa cell carrier culture involving Sendai virus, which has also been described previously (Ishida et al, 1964) is another good example of a regulated type of infection.

In a later series of investigations, Walker attempted to classify his carrier cultures according to the following criteria. The requirement for antibody or other antiviral factors in the medium, the ability to 'cure' persistently-infected cultures with immune serum, the ability of the culture to resist superinfection with the homologous virus, the ability of the culture to divide and grow at the same rate as uninfected cells and, finally, by an estimation of the percentage of infected cells in the culture.
He also stipulated that in cases where the percentage of infected cells is low, the culture should be examined for the presence of interferons or other interfering factors. Walker's method of classifying carrier cultures is of considerable value because it serves as a useful means of distinguishing the different types of carrier cultures and provides a better understanding of the mechanisms involved in maintaining equilibrium in virus-cell interactions.

**Virus-Host Cell Interactions:**

When a virus is introduced into a susceptible host cell system, the normal pattern of infectivity is one of adsorption and penetration, followed by a replicative cycle which results in assembly and release of infectious progeny virus. However, under certain circumstances, the normal sequence of events may be inhibited or altered. For example, the growth cycle may be temporarily blocked by abnormal environmental conditions, such as a deficiency of the medium, and may proceed normally only when the missing factor or factors are supplied. An example of this is the latent infection of chick embryo fibroblasts by psittacosis which can be activated by the addition of the amino acids, phenylalanine and tryptophane, to the medium (Heggie and Morgan, 1956).

Interference of virus growth may occur if the culture is inoculated with high multiplicities of virus. This procedure often results in the production of comparatively low yields of infectious virus and high yields of non-infectious virus, similar to the phenomenon described by von Magnus with influenza virus /
virus (von Magnus, 1954). In other instances, a high multiplicity of infection may initiate an abortive infection, resulting in an incomplete growth cycle with the production of completely non-infectious virus. This abortive type of infection has also been described with influenza virus whereby infected HeLa cell cultures were shown to be capable of supporting only a single cycle of incomplete infection with the release of non-transmissible haemagglutinins (Henle, Girardi and Henle, 1955).

Virus infected cell cultures may also exhibit a delayed but non-defective growth cycle with the production of infectious virus only after a prolonged period of growth; as is the case with many tumour viruses. Finally, when the virus enters a cell it may come under some form of intracellular regulation or control, resulting in a defective viral growth cycle which is characterised by the formation of mainly non-infectious virus. Such defective infections are not aborted and may persist indefinitely. This last type of defective growth cycle may give rise to the persistent, defective type of carrier state described by Fraser (1967), and corresponds to the regulated infection of Walker (1964).

It seems likely, therefore, that a variety of different virus-host cell interactions may occur following entry of a virus into a susceptible host cell.

Mechanisms of Virus-Host Cell Interaction:

Although the mechanisms of viral persistence are not fully understood, various theories have been postulated. These include
The effects of antibodies, interferons or other inhibiting substances in the environment which may prevent or seriously disrupt the transfer of extracellular virus to neighbouring susceptible cells. It is also possible that incomplete or defective viral genomes may be a contributory factor as is sometimes the case with strains of Rous sarcoma virus which can only be activated in the presence of a Rous associated virus (Rubin and Vogt, 1962).

Other workers have shown that incompetence of the host cell, with failure to assemble viral components, may give rise to a form of persistent infection and Horta-Barbosa, Fuccillo and Sever (1969) found that active measles virus could be released from persistently infected brain cells by a method of co-cultivation with susceptible HeLa cells.

These and many other observations have stimulated a great deal of interest in the problems of persistent defective viral infections and it is reasonable to suppose that the *in vitro* carrier state may serve as a useful model for the better understanding of many latent and chronic viral infections of man and animals.

**Persistent Infections in Man and Animals:**

Latent or recurrent infections, such as "cold sores" in human subjects, due to *Herpes simplex* virus (Anderson and Hamilton, 1949) and viral pneumonias in mice (Horsfall and Hahn, 1940) are prime examples of situations in which the causative agent is detectable during the initial infection but is subsequently masked and the disease does not recur until the virus is reactivated by such predisposing factors as stress, strain or ultra-violet light.
light. Another example of viral persistence is that illustrated by a chronic infection of mice caused by lymphocyticchorio-
meningitis virus. In this infection, mice infected in utero, or in very early life, do not show the clinical disease but develop a form of tolerance due to the presence of latent virus which apparently survives in the immune environment (Traub, 1936). In many cases, the oncogenic or tumour forming viruses also assume a latent or persistent form. For example, the Bittner agent of mouse mammary carcinoma occurs in the latent form in adult female mice and may give rise to tumour formation in her offspring due to ingestion of the so-called milk-factor (virus) in her milk (Bittner, 1936). The Riley virus or lactic dehydrogenase agent of mice described by Riley, Lilly, Huerto and Bardell (1960) is the classical example of a latent agent whose presence can be detected only by increased production of the enzyme, lactic dehydrogenase, in the circulating blood of the affected host. Other biochemical methods for detecting latent viral infections will undoubtedly be developed in the future but, at present, the detection of defective viruses presents virologists with many interesting and perplexing problems.

Investigations of laboratory carrier cultures should increase our knowledge of the mechanisms that enable infectious virus particles to survive in an immune environment without obvious cell damage. It has recently been suggested that the interaction of virus and neutralizing antibody and the protection of suscept-
able cells by defective interfering virus may be possible mechanisms in cell-virus equilibrium (Huang and Baltimore, 1970).
RELEASE OF DEFECTIVE VIRUS

Many attempts have been made to release defective virus from persistently infected host cells and a variety of techniques, both old and new, have been employed.

Ultra-violet Irradiation:

Ultra-violet irradiation, which has long been known to activate prophage from lysogenic bacterial cells, is one of the techniques commonly applied in rescue experiments, and Sompolinsky, Yiflah and Aboud (1968) have suggested that ultra-violet irradiation acts by inhibiting the synthesis of a newly formed protein ("repressor") which blocks the vegetative cycle of phage development. Unfortunately, very little success has been achieved when this method has been applied to studies of tissue-culture carrier states and it is possible that the mechanisms involved are quite different in both systems.

Co-cultivation:

Attempts to rescue virus from persistently infected cells have also been made using the methods of co-cultivation. For example, Horta-Barbosa and his colleagues (1969) showed that measles virus could be released from the brain cells of human patients affected with subacute sclerosing panencephalitis by co-cultivating the brain fibroblasts with HeLa cells. This method of rescuing virus by the formation of multinucleated heterokaryons was an exciting breakthrough because previous attempts to release the virus by other methods had proved unsuccessful. The technique of co-cultivation, with the addition /
addition of inactivated Sendai virus to enhance cell fusion has also been used successfully to reactivate a latent defective simian tumour virus, SV40, with the release of infectious virus from the resulting heterokaryons (Watkins and Dulbecco, 1967; Koprowski, Jensen and Steplewski, 1967). However, the technique is not always successful and Koprowski and his co-workers failed to recover SV40 from a number of carrier lines. On the other hand, while Watkins and Dulbecco succeeded in recovering infectious SV40 from carrier cell systems they failed to release a co-latent polyoma virus.

"Helper" Virus:

It is now known that so-called "helper" viruses play an important part in the activation of defective Rous sarcoma virus which is capable of transforming cells and producing tumours with the release of minimal amounts of infectious virus, or none at all. Rous activator virus (RAV) acts by providing an additional viral component which appears to be essential for the completion of infectious Rous sarcoma virus (Hanafusa, Hanafusa and Rubin, 1963). These avian tumour inducing viruses are of particular interest since they are RNA membrane-associated viruses which may give rise to infections of the regulated type as described by Walker (1964).

Recombination:

Another in vitro method used to reactivate virus is that of recombination which has proved especially useful in investigations with orthomyxoviruses. Tumova and Pereira (1965) showed /
showed genetic interaction between influenza-A viruses of human and avian origin and Easterday, Laver, Pereira and Schild (1969) activated an inactive influenza virus by transfer of neuraminidase from the participating active influenza virus.

These techniques of reactivation, co-cultivation, use of "helper" virus and recombination have all been used in recent years in attempts to release incomplete latent virus from carrier cells and it is reasonable to assume that failure to demonstrate other relationships between viruses and tumours may well be due to inadequate knowledge of the virus-cell relationships and the insensitivity of the methods currently available for detecting latent virus infections.

Macrophage Cultures:

In the veterinary field studies by Mackay (1969) on Jaagsiekte in sheep have provided evidence of a possible method of serial transmission of latent viruses both in vitro and in vivo. This interesting work clearly demonstrated the presence of a herpes-like agent in the lung macrophages of affected sheep which could only be serially transmitted in cell cultures of sheep lung macrophages. It is unlikely that the phagocytosing capacity of the macrophages is the only factor involved in this system and some other property of the macrophage may be important for virus growth.

It is evident, from this review of the recent literature, that the carrier state is providing research workers with an important and convenient model for in vitro studies of the virus-host /
virus-host cell relationships which occur in many latent virus infections and oncogenic diseases of man and animals.

Avian or Newcastle disease virus is a paramyxovirus and is classified as a member of the parainfluenza virus main group largely on the basis of the absence of the internal nucleocapsid, which is larger (100nm) than that of orthomyxoviruses (65nm). It is further characterized by the fact that it produces an hemorrhage and that it is incapable of multiplication, neutralization or genetic recombination.

Studies of the morphology of Newcastle disease virus by electron microscopy indicate that the mature virions are roughly spherical in shape with an average diameter of 120-300nm (Salter and Crambahn, 1963) or particles extending to 500 or even 1000nm in length may occasionally be seen. The intact virion possesses an outer membrane or envelope consisting of proteins, lipid and carbohydrates which serves an external surface of short, clubby process radiating spikes or projections, each about 100nm in length. These ruffles, which probably represent the inner surface of the virion contain the hemagglutinating and neuraminidase components of the virus. Thus, three of the tests virological reactions of this envelope are, the agglutination of erythrocytes, the neuraminidase enzymatic activity and the stimulation of hemagglutination-inhibiting and neutralizing antibodies.

Electron microscopy of the outer envelope in not uncommon and in electron micrographs the contours of disrupted virions,
Properties of the Virus:

Newcastle disease virus is a myxovirus and is classified as a member of the paramyxovirus subgroup largely on the basis of the diameter of its internal helical component, which is larger (18nm) than that of orthomyxoviruses (9nm). It is further characterised by the fact that it produces an haemolysin and that it is incapable of multiplicity reactivation or genetic recombination.

Studies of the morphology of Newcastle disease virus by electron microscopy indicate that the mature virions are roughly spherical in shape with an average diameter of 120-300nm (Waterson and Cruikshank, 1963) but particles extending to 500 or even 1000nm in length may occasionally be seen.

The intact virion possesses an outer membrane or envelope consisting of proteins, lipid and carbohydrate which carries an external fringe of short, closely packed, radiating spikes or projections; each about 8nm in length. These rods, which probably penetrate the inner depths of the virion contain the haemagglutinating and neuraminidase components of the virion. Thus, three of the main biological functions of this envelope are, the agglutination of erythrocytes, the neuraminidase enzymatic activity and the stimulation of haemagglutination-inhibiting and neutralizing antibodies.

Spontaneous disruption of the outer envelope is not uncommon and in electron micrographs the contents of disrupted virions, which /
which are frequently released during preparation, appear as herringbone filaments protruding from the virus particle. The herringbone structure constitutes the inner helical ribonucleoprotein component which has the antigenic properties of the S or soluble antigen (Lief and Henle, 1956).

Disruption of the outer envelope of the virion is readily achieved by treatment with ether and this enables the haemagglutinating component of the envelope to be distinguished from the inner nucleoprotein or S antigen component. The haemagglutinating particles produced by disruption of the outer envelope are called rosettes because they consist of star-like aggregates of short, rigid rods. The size and shape of these rods is very similar to the projections seen on the surface fringe of the intact virion in that they have a drumstick form with the spherical ends in close contact with each other. The outer ends of the rods often appear to be swollen or, possibly, forked. These particles which are derived from the envelope of the virus carry the specific V antigen and show strain specificity. The haemagglutinating component of the V antigen is the site of attachment of the virus to cell receptors.

The neuraminidase protein is also a component of the V antigen and is, to a large extent, virus specific within the myxovirus group and this specificity is used to place the virus within a scale, known as the Receptor Gradient (Burnet, McCrea and Stone, 1946). The function of the neuraminidase is somewhat obscure but it has been suggested that the enzyme may facilitate the
the entry of the virus into the host cell. However, other workers have suggested that it may be more intimately involved with the release of newly-formed virus from infected cells (Seto and Rott, 1966; Brown and Laver, 1968).

The soluble or S antigen obtained by fractionation of Newcastle disease virus is in the form of elongated structures about 18nm in diameter and up to 600nm in length, but recent observations have suggested that the length may extend up to 1000nm. Electron micrographs of the ultrastructure of the S component suggest that it probably consists of small subunits arranged in the form of a double helix with, perhaps, 5 or 6 protein units to each turn of the helix. Nevertheless, typical coiled inner components are only visible in a comparatively small number of virions and in most preparations, even where the phosphotungstate acid (PTA) has penetrated to the interior of the virus particle, the inner component appears as little more than an amorphous structure. In some particles, e.g. incomplete virus, the internal helix may be absent, whereas in others the inner nucleoprotein component, which is deeply embedded in a haemagglutinin gel, can only be seen in virions containing less haemagglutinin than normal.

Studies on the chemical composition of the virus indicate that it contains about one percent single-stranded RNA with a molecular weight of $4 - 8 \times 10^6$ daltons. The nucleocapsid accounts for 45 percent of the total virus protein and the haemagglutinating and /
and neuraminidase components each contribute 20 percent to the protein complement. Remaining polypeptides account for a further 10 percent (Haslam, Cheyne and White, 1969) part of which has been attributed to an RNA-polymerase and the rest to an unidentified protein. It has been postulated that the unidentified protein may be a maturation protein involved in the viral assembly process (Iinuma, Nagai, Maeno, Yoshida and Matsumoto, 1971).

**Carrier State Cultures:**

Thus, Newcastle disease virus (NDV) is an RNA membrane-associated virus of the type which readily establishes a regulated infection in tissue culture and the ease with which it does so constitutes a major hazard in tissue culture laboratories. Despite this, there are few reports in the literature of persistent infections of cell cultures with NDV.

One such report is that of Puck and Cieciura (1957) who successfully induced a persistent infection in HeLa cells by repeated challenge with NDV and maintained the culture for over two years. During this time, there was no evidence of CPE, and the culture characteristics were similar to those of the original parental stock. Nevertheless, a CPE was obtained when the carrier culture was plated on a 'feeder' layer of X-irradiated giant cells and the effect was specifically neutralized by NDV antiserum. By this method, the authors demonstrated that 99.9 percent of the virus was associated with the cellular fraction and that the majority of cells appeared to be infected.
Another interesting example is that reported by Henle et al (1958) who found that initiation of an NDV persistent infection in MCN cells (bone marrow cells from a patient with leukaemia) and Lung-To cells (human embryo lung cells) was accomplished with ease and was readily reproducible. Although their system was similar to that of Puck and Cieciura, in that the virus was mainly cell associated, it is of interest that Henle and his co-workers could demonstrate 1-10 percent infectious virus in the cell culture medium, in the absence of detectable haemagglutinins. They also noted differences between carrier and control cultures with regard to growth rate and aerobic glycolysis and, contrary to the results obtained by Puck and Cieciura they were able, in one instance, to 'cure' a culture with homologous antiserum. Although the MCN cells were described as bone marrow cells from a patient suffering from leukaemia, they were later found to be of murine origin and to be identical with Earle's strain of L cells (Rodriguez and Henle, 1964).

A transient persistent infection of L cells by NDV was reported by Wilcox (1959) which proved to be resistant to super-infection with the homologous virus for short periods of 5-7 days. In due course, the cultures gradually regained their susceptibility to NDV although small amounts of virus persisted in the cells for extended periods of up to 30 days.

Persistent infections with NDV in L cells and U₁₂ cells (human uterus cells) were described by Mason and Kaufman (1961) who showed that small amounts of infectious virus were detectable in/
in both systems. Although the most consistent results were obtained with the cultures of L cells, they suggested that this was probably due to inherent differences in the ability of different cell types to maintain a persistent infection. They also noted that both carrier states could readily be superinfected with large multiplicities of the homologous virus.

Despite these early reports of persistent infections of cell cultures by Newcastle disease virus, it is surprising that these interesting phenomena have received such scant attention during the past decade.
OBJECTIVE

A persistent infection by Newcastle disease virus of three continuous cell lines of pig, ox and sheep kidney has existed in this laboratory for the past ten years. The origin of the persistent infection is unknown and the cultures have been maintained routinely throughout this period, without obvious signs of cell destruction.

The purpose of this present investigation was to confirm the identity of the latent virus and to study the characteristics of the carrier cell cultures. An attempt was also made to investigate the defective nature of the virus and the mechanisms involved in maintaining the equilibrium state.
MATERIALS AND METHODS

Primary Cell Cultures

These were prepared from tips of cultures stained. The vein, iliac and visceral were removed surgically and dissected and the remaining biceps were thoroughly rinsed in sterile phosphate-buffered saline (PBS). The tissues were minced and then subjected to 5-s treatments with 0.25% test-tube trypsin solution at 37°C for 20 minutes periods. The test current was disassembled and subsequent trypsinizations were placed in cold sterile calf serum at 4°C until the whole series of homogenates had been collected. The cells were then centrifuged at 1,000 rpm for 10 minutes and resuspended in growth medium.
CELL CULTURES

Continuous Cell Cultures:

Carrier Cultures: Three carrier cultures were studied. A bovine kidney cell culture, originally MDBK (Madin and Darby, 1958) and now designated Line '4'; an ovine kidney culture, originally MDOK (Madin and Darby, 1958) now designated Line '5' and a swine kidney culture, originally the PK2a Stice line (Stice, 1955) and now referred to as PK(2D).

Control Cultures: These included the bovine kidney line, MDBK, purchased from Flow Laboratories and the swine kidney culture, PK/W/K6, obtained from the Moredun Animal Research Institute. The latter was originally obtained from the Ministry of Agriculture Laboratories, Weybridge, as the PK15 strain of pig kidney cells. Later in the study a second line of PK15 was obtained from Flow Laboratories. This line was derived from the fifteenth clone of the original PK2a Stice line.

Primary Cell Cultures:

Chick Embryo Fibroblasts: These were prepared from 10-day old chicken embryos. The head, limbs and viscera were removed aseptically and discarded and the remaining tissue was thoroughly rinsed in warm phosphate buffered saline (PBS). The tissue was minced and then subjected to 3-4 treatments with 0.25 per cent trypsin (Difco) at 37°C for 20 minute periods. The first harvest was discarded and subsequent trypsinisations were stored in cold sterile calf serum at 4°C until the whole series of harvests had been collected. The cells were then centrifuged at 1,000 RPM for 10 minutes and resuspended in growth medium.
Viable cell counts were performed using 0.1 per cent Trypan Blue in a Fuchs Rosenthal counting-chamber. Cultures were set up at $5 \times 10^5$ viable cells per ml. Chick embryo kidney cultures were prepared in a similar manner.

**Chick Embryo Lung:** The lung culture cells were prepared according to the method of Darlington, Portner and Kingsbury (1970) except that 0.25 per cent trypsin was used in place of 0.25 per cent pronase to digest the tissue. This provided individual cells rather than the large aggregates obtained by Darlington's method. Cultures prepared with $10^6$ cells per ml produced satisfactory monolayers after 48 hours of incubation.

**Chicken Macrophages:** Macrophage cultures were prepared from the "buffy coat" of chicken blood freshly drawn from the brachial vein or by cardiac puncture and collected in sterile 3.8 per cent sodium citrate solution. After removal of the citrate solution by centrifugation, the whole blood was centrifuged in sterile capillary tubes at 2,500 RPM for 15 minutes. The "buffy coat" was then transferred to a capillary tube by means of a Pasteur pipette, washed twice in PBS and finally resuspended in growth medium. Ten to fifteen coverslip macrophage cultures were generally obtained from each 30ml of whole blood. After 2 hours of incubation at 37°C, the medium and unattached cells were discarded from each tube and the remaining attached macrophages were refed with fresh growth medium.

**Growth Medium:**

This consisted of Earle's salt solution with added 0.5 per cent /
cent lactalbumin hydrolysate (Micro-Bio Laboratories), 0.1 per cent yeast extract (Difco), 0.1 per cent glucose and 0.088gm per cent sodium bicarbonate as buffer. The medium, designated EYL, was sterilised by filtration through sintered glass filters, porosity size 5, at a low negative pressure of approximately 5 inches of mercury. The medium was completed by the addition of 10 per cent inactivated pooled calf serum and antibiotics (vide infra). The growth medium for macrophages consisted of EYL balanced with 0.11gm per cent sodium bicarbonate and supplemented with 10 per cent non-inactivated fresh fowl serum. Antibiotics were incorporated in all media in the following concentrations: penicillin, 100 units per ml; streptomycin, 100μg per ml; and fungizone (Squibb), 5μg per ml. Tylan (Elanco Products Ltd) was used in concentrations of 167μg per ml or 100μg per ml to eradicate mycoplasmas. The cell cultures were refed every 3-4 days, subcultured at weekly intervals by means of a saline-trypsin-versene mixture (STV), and reseeded at 10^5 viable cells per ml in sterile 4oz medical flats.

**Isolation of PPLO from Tissue Cultures:**

The method used was similar to that described by Hayflick (1965). Tissue culture fluid removed from 3-day-old cell cultures was centrifuged at 1000 rpm in order to sediment free cells and the supernatant was retained as the inoculum. The cells from these cultures were dislodged from the glass with versene. The dispersed cells were then washed in saline to remove traces of residual versene, centrifuged at 1000 rpm and the deposited cells used as the inoculum.
The two samples were inoculated separately into PPLO sloppy agar and also onto PPLO agar plates. The cultures were then incubated aerobically and anaerobically. The agar plates were examined at 48 hours and every 24 hours thereafter. Any colonies resembling those of PPLO were marked and subcultured into fresh medium. The sloppy agar cultures were subcultured on agar plates after 6 days of incubation and examined as before.

Positive colonies were identified and typed by growing them in PPLO broth and by inoculating agar plates to obtain a confluent growth. Discs, soaked in specific antisera, were then placed on the surface of the medium and, after incubation, the plates were examined for inhibition of growth around the discs. The species of mycoplasma was confirmed by immunofluorescence staining, with type specific antisera.

**Tylan Treatment:** Overnight monolayer cultures were refed with growth medium containing 167µg per ml Tylan and with added penicillin, streptomycin and fungizone, at the usual concentrations. After 96 hours of incubation the cells were removed from the glass and reseeded at $10^5$ cells per ml in growth medium containing Tylan at 100µg per ml. The cell lines were maintained on this medium for four weeks in the usual manner. Thereafter the Tylan was withdrawn and the cultures were maintained on routine growth medium with strict aseptic precautions. The cultures were checked every month for three months and were found to be free from mycoplasmas.

**Growth Rates:**

Growth rates were performed on 7-day-old monolayer cultures and /
and the discarded medium was collected and estimated for pH by means of a Pye electric pH meter. The monolayer was then washed with STV and the cells were stripped from the glass using fresh STV. The dispersed cells were centrifuged at 1000 RPM for two minutes and resuspended in growth medium. Viable cell counts were performed with 0.1 per cent Trypan Blue in a Fuchs Rosenthal counting chamber. Fresh cultures were set up at $10^5$ viable cells per ml using media containing four different batches of inactivated calf serum, and growth rates were estimated at weekly intervals over a ten week period.

**TCID**\(_{50}\) **Estimations:**

All titrations were performed on two-day-old monolayer cultures grown in 6 x 5/8" pyrex test tubes. Maintenance medium consisted of 0.8 ml of EYL with an added two per cent calf serum. The inocula consisted of 0.2 ml of the appropriate virus dilution prepared in maintenance medium. The cultures were examined daily for four days and the fifty per cent tissue culture infective dose (TCID\(_{50}\)) was calculated by the method of Kärber (1931).

**Detection of Transformed Cells:**

This was investigated by the agar suspension culture technique for the assay of transformed cells as described by MacPherson and Montagnier (1964). This method, which relies on the ability of transformed cells to grow in semi-solid agar, was carried out by adding 7 ml amounts of 0.5 per cent Difco agar in growth medium to each disposable tissue culture petri dish and allowing it to set at room temperature. This agar base was overlaid with 1.5 ml of /
of 0.3 per cent agar in growth medium containing $5 \times 10^5$ cells per ml and the plates were then incubated in an atmosphere of air plus 5 per cent CO$_2$ for 7-10 days at 37°C.

Inhibition of Oxygen Uptake:

Inhibition of oxygen uptake by the cells was achieved by incorporating sodium malonate at concentrations of 0.01M to 0.03M (Ackermann, 1951) in the final agar overlay as used in the plaque technique of Dulbecco (1952). Cells were seeded at $3 \times 10^5$ per ml in sterile disposable tissue culture petri dishes (Sterilin) and allowed to monolayer at 37°C in an atmosphere of 5 per cent CO$_2$ for 2 days. The growth medium was then removed and the monolayers were overlaid with one per cent Difco agar in EYL incorporating sodium malonate at the recommended concentrations. The agar medium was allowed to set at room temperature for 30 minutes before being incubated in the inverted position in 5 per cent CO$_2$ at 37°C for a further four days. At the end of the incubation period the agar medium was removed and the underlying cell sheet was stained with a dilute solution of carbol fuchsin.
STAINING TECHNIQUES

Light Microscopy:

Haematoxylin and Eosin: Coverslip preparations were washed in PBS, fixed in methyl alcohol and passed through a series of graded alcohols to water. They were then carefully washed in water, stained with Harris' haematoxylin and rinsed in water. The preparations were differentiated in a saturated solution of lithium carbonate, thoroughly washed, and counterstained with Putt's eosin. The stained monolayers were then dehydrated through a series of acetone and acetone-xylol mixtures; and finally mounted from xylol in DePex.

Giemsa: Coverslip cultures were prepared as for haematoxylin and eosin staining. They were then stained for 15 minutes in 1:5 Giemsa stain (Hopkins and Williams Ltd) and differentiated in buffer at pH 7.0, for a similar period. The preparation was dehydrated and mounted from xylol in DePex, in the usual manner.

Fluorescent Microscopy:

Fluorescent Antibody: Both the direct and indirect methods of fluorescent antibody staining were used to demonstrate the presence of viral antigen (Coons, Snyder, Cheever and Murray, 1950).

Cultures on "flying coverslips" were washed three times in PBS (pH 7.1) and dried at 37°C for approximately 30 minutes. The coverslips were then fixed in acetone for 10 minutes at room temperature. The fixed monolayers were allowed to dry for a further 10 minutes at 37°C and were either stained immediately or stored /
stored in tightly-sealed containers at -70°C. The staining technique was performed, according to standard methods in a moist atmosphere at 37°C for 30 minutes. After thoroughly washing the cultures in three changes of PBS, they were mounted in buffered glycerol and finally sealed with nail varnish to prevent movement during photography. The preparations were examined under a Zeiss fluorescence microscope and photographs were taken on high speed daylight Ektachrome film, ASA 150.

Conjugation of Antibody: Antibody prepared against NDV was precipitated with saturated ammonium sulphate. This precipitate, which was first dialysed against running tap water and then against PBS, contained 0.69gm globulin per 100ml. The antibody was then conjugated with fluorescein isothiocyanate (FITC) by standard techniques (Nairn, 1964). Unconjugated fluorescein was adsorbed on charcoal and non-specific fluorescence was removed with pig liver powder.

Acridine Orange: The sites of single-stranded and double-stranded nucleic acid production in cells were detected using the technique of Anderson, Armstrong and Niven (1959).

Coverslip cultures were rinsed once in PBS (pH 7.1) and fixed, without delay, in 3 per cent acid alcohol for 5 minutes. They were then rinsed in two changes of citrate-phosphate buffer (pH 3.8) for 2 minutes and stained in freshly prepared 0.01 per cent acridine orange for 10 minutes. This was followed by two further 2 minute washes in citrate-phosphate buffer whereupon the coverslips were mounted in buffer and ringed with nail varnish to prevent movement, and evaporation.
Electron Microscopy:

**Negative Staining**: A small drop of the specimen to be examined was placed on a 'Formvar' carbon-coated copper grid and a drop of two per cent potassium phosphotungstate (PTA), pH 6.5, was added as the negative stain. After an interval of thirty seconds, to allow the stain to penetrate, excess fluid was removed by touching the grid side with a piece of torn filter paper, and the preparation was allowed to dry. Specimens were examined with an EM 6B electron microscope (Associated Electrical Industries) and photographs were taken on Ilford electron microscope plates, EM4.

**Positive Staining**: Sections were prepared according to the sandwich-embedding technique for monolayers of cells cultured on araldite surfaces, developed by Smith, Gray and Mackay (1969). The plates were "conditioned" with EYL for twelve hours at 37°C and, after removal of the salt solution, were sterilised by exposure to an Hanovia ultra violet unit for one hour, at a distance of 25cms. Cells were seeded on the araldite surface at 5 x 10^5 cells per ml and the cultures were held in a laboratory incubator at 37°C. An atmosphere containing increased carbon dioxide was not required because the polythene containers, in which the araldite had been allowed to polymerise, had tight sealing lids. The methods of processing and cutting the specimens were those recommended by Smith et al. The sections were stained with lead citrate and examined in a Siemens Elmiskop I.
HAEMADSORPTION AND HAEMAGGLUTINATION TECHNIQUES

Haemadsorption Test: The method used was that of Vogel and Shelokov (1957).

Washed 24 hour old coverslip cultures overlaid with 1 ml of 0.05 per cent guinea-pig red cells were held at 37°C for 15 minutes, gently washed in PBS and examined by light microscopy. Permanent preparations were obtained by fixing the haemadsorbed coverslip cultures in methyl alcohol for 5 minutes and staining them with haematoxylin and eosin.

Haemagglutination Tests: These were performed in WHO perspex plates by standard techniques (WHO Expert Committee on Influenza, 1953). Doubling dilutions of test material were prepared in veronal buffer and a one per cent fowl red cell suspension in buffer was added to detect the presence of viral haemagglutination. A standard volume of 0.25 ml was used throughout. The tests were read after 60 minutes at room temperature. On occasions a one per cent horse red cell suspension was used.

Haemagglutination Inhibition Test: A standard volume of four haemagglutinating units (4HAU) of test virus was added to doubling dilutions of antisera. The same volume of one per cent fowl cells was added and the test was examined for inhibition of haemagglutination after 60 minutes at room temperature. In certain instances, the sensitivity of the test was increased by leaving the virus-serum mixtures for 30 minutes at room temperature before adding the fowl cells. All sera were pretreated with receptor destroying enzyme (RDE) to remove non-specific inhibitors.
Adsorption of Haemagglutinin: Tests for the identification of lentogenic strains of NDV were carried out according to the method first described by Piraino and Hanson (1960).

Brains removed from 12-day-old chicken embryos were macerated in saline and centrifuged at 5,000 RPM for 15 minutes. A 10 per cent brain cell suspension was prepared from the top layer of the brain deposit and 2ml of this suspension were allowed to adsorb 0.5ml of NDV for 15 minutes at 4°C. The mixture was then centrifuged and the supernatant fluid was examined for haemagglutinins, since the haemagglutinating activity of lentogenic strains, unlike that of virulent strains, is adsorbed by chicken brain.

Receptor Gradient: Newcastle disease virus in allantoic fluid was adsorbed on one per cent washed fowl red cells at 4°C. The virus was then eluted from the agglutinated cells at 37°C. The sensitised red cells were washed three times in PBS, reconstituted as a one per cent suspension in fresh buffer and finally tested against NDV, line '4' agent and influenza-A virus.

Heat Inactivation of Haemagglutinin: Samples of line '4' tissue culture fluid of known haemagglutinating titre (4HAU per ml) were held at 56°C and 41°C, respectively, until haemagglutinating activity was no longer detectable. The samples were checked at intervals for their ability to haemagglutinate fowl cells. A sample of Herts strain of NDV of similar titre was tested under the same conditions.
SERUM NEUTRALIZATION TESTS IN FERTILE EGGS

Standard virus neutralization tests were carried out using 100EID₅₀ of the virus (50 per cent egg infectious doses). Virus-serum mixtures were incubated at 4°C for 2 hours prior to inoculation into the allantoic cavity of 10-day-old fertile white Leghorn hen's eggs. The eggs were incubated at 35-36°C and candled daily for death of the embryo. A final reading was taken on the fourth day after inoculation and the eggs were then reincubated for a further twenty-four hours to ensure that no more deaths had occurred. Although death of the embryos, due to infection with the Herts strain of NDV, was directly related to lack of neutralization of the virus, the results were confirmed by spot haemagglutination tests of the allantoic fluids.
Ultrafiltration of Virus:

Serum-free tissue culture fluid, harvested at 7-day intervals from line '4' Roux flasks, was collected and stored at 4°C. This pooled fluid was centrifuged at 3,000 RPM for 15 minutes to deposit all cells and cell debris and the supernatant was concentrated through visking tubing under negative pressure. When the volume of the fluid in the visking tubing was sufficiently reduced the vacuum was released and the tubing system introduced into a fresh flask containing 10 times the volume of PBS and ultrafiltered by the process of simultaneous dialysis against PBS. By this method, the titre of the line '4' agent was concentrated approximately 200 times.

Serum-free tissue culture fluid from MDBK cultures infected with the Herts strain of NDV and Sendai virus, respectively, were concentrated by the same method; as was the control tissue culture fluid from uninoculated MDBK cultures.
NEURAMINIDASE ESTIMATIONS

The method was based on the neuraminidase assay technique described by Aminoff (1959). Test materials prepared and concentrated by the ultrafiltration technique were held on ice until ready for use.

A mixture of 0.2ml of the enzyme preparation to be tested and 0.3ml acetate buffer containing 0.1 per cent CaCl₂ (pH 5.1) was added to 0.5ml of human glycoprotein substrate (HGP5) and incubated for thirty minutes at 37°C. The mixture, in 0.5ml amounts, was assayed directly by Aminoff's procedure for the detection of sialic acid by thiobarbituric acid assay.

A baseline control of 0.2ml of the test material, plus 0.8ml acetate buffer, was examined at the same time in order to obtain an estimate of the amount of sialic acid that was present before the addition of the substrate and the action of the enzyme on this substrate. Controls consisting of 0.5ml substrate plus 0.5ml acetate buffer were also tested for the same reason. A blank control, in which the test material was replaced with distilled water, was also examined.

The results were read on a spectrophotometer, at a wave length of 549μ, with a blue filter and were expressed as optical density readings. The corrected result was obtained by subtracting the sum of the baseline and substrate controls from the test reading.
ATTEMPTS TO ACTIVATE AND TRANSMIT THE DEFECTIVE AGENT

**Tissue Culture:** Primary chick fibroblast cultures were set up in tubes with flying coverslips and allowed to monolayer. These monolayers were overlaid with 0.2ml of line '4' tissue culture fluid for 1-2 hours at 37°C. The inoculum was then removed and the cultures were refed with maintenance medium. The cultures were checked at regular intervals for haemadsorption, cytopathic changes and haemagglutinating activity.

**Eggs:** Ten-day old fertile white Leghorn hen's eggs were inoculated by the allantoic and amniotic routes with 0.2ml of line '4' tissue culture fluid. The eggs were candled daily and, on the fourth day, the allantoic and amniotic fluids were 'spot-checked' for haemagglutinating activity. The embryos were later removed and examined for abnormalities.

**Chickens:** Day-old chicks were inoculated intracerebrally with fluids harvested from line '4' cultures and were observed over a period of seven days for evidence of clinical illness.

**Sephadex Fractionation:** Equal volumes (1.5ml) of serum-free line '4' fluid and Herts NDV allantoic fluid, each at a titre of 1:160, were passed through Sephadex G-200 columns, 60cm in length and 2.8cm in diameter, at room temperature. The fractions were collected automatically at timed intervals and tested for haemagglutinating activity. Line '4' fractions which showed agglutination of one per cent fowl cells were inoculated into the allantoic sac of ten-day-old eggs. The titre of the line '4' fluid /
fluid was concentrated to 1:160 by the process of ultrafiltration and the sample of active NDV was inactivated with 0.01 per cent formalin before being introduced into the column.

**Growth in Serum-free Medium:** Young monolayer cultures of carrier lines were washed with serum-free medium and held in the same medium for 4 days at 37°C. At this stage, the serum-free medium was harvested, checked for haemagglutinating activity and inoculated allantoically into ten-day-old embryonated eggs. After four days, the allantoic fluid was 'spot-checked' with one per cent fowl cells for haemagglutinating activity.

**Ultra-violet Light Activation:** Aliquots of $10^5$ cells per ml of each of the carrier lines were exposed to UV irradiation, from a Hanovia light at a distance of 25cm. The cell suspensions were held in shallow layers of serum-free medium in petri dishes and were treated for periods of 15, 30 and 60 seconds, respectively. Calf serum, to a concentration of ten per cent, was then added to the treated suspensions and tube cultures were set up from each sample. After three days, the tissue culture fluids were tested for haemagglutinating activity and were also inoculated allantoically into ten-day-old eggs.

**Neuraminidase Experiment**

**Toxicity of Receptor Destroying Enzyme (RDE):** Receptor destroying enzyme (Vibrio cholerae filtrate, Burroughs Wellcome) was used as a source of neuraminidase, and its toxicity for tissue cultures was examined by overlaying young monolayer cultures with 0.2ml of doubling dilutions of RDE in serum-free EYL (1:10 - 1:160) /
40.

(1:10 - 1:160) for one hour at 37°C. The cell sheets were rinsed twice in growth medium, refed and examined at daily intervals for toxic effects. Monolayers of secondary chick fibroblasts were washed twice in EYL to remove the serum. A 10⁻¹ dilution of ultrafiltered serum-free, line '4' tissue culture fluid (titre 1:160) was prepared in 1:100 RDE in EYL, giving a final haemagglutinating titre of 1:16. Serum-free line '4' fluid showing specific haemagglutinating activity was also used as diluent for RDE (1:100).

The washed tube cultures, containing coverslips, were overlaid with 0.2ml volumes of these preparations for one hour at 37°C. Control tubes incorporating 0.2ml volumes of EYL and 1:100 RDE in EYL were also prepared. After adsorption, the cultures were washed twice in growth medium, refed with 1ml of growth medium and incubated at 37°C. Haemadsorption tests were performed daily and coverslips were removed at regular intervals and stained either with haematoxylin and eosin or by fluorescent antibody methods.

"Helper" Virus Experiment: A laboratory stock strain of Sendai virus was grown in MDBK and line '4' respectively. A high multiplicity of infection was used and the virus was harvested on the third day and stored at -70°C. These harvests were inoculated into the allantoic sac of ten-day-old embryonated eggs and into coverslip cultures of chick fibroblasts. The allantoic fluids were harvested after five days of incubation and tested for haemagglutinating /
haemagglutinating activity with one per cent fowl cells and one per cent horse cells. The coverslip cultures of chick fibroblasts were removed at intervals and stained with haematoxylin and eosin and fluorescent antibody. Tissue culture fluids, harvested on the eighth day, from cultures infected with Sendai virus were inoculated into eggs and these were examined by standard methods.

Infection of Chicken Macrophages: Medium from carrier cultures of line '4' agent was passed through sterile membrane filters (Millipore, MF50), tested for haemagglutinating activity and inoculated, in 0.3ml amounts, onto forty-eight hour old coverslip cultures of chicken macrophages. After an adsorption period of one hour at 37°C the cultures were fed, without removing the inoculum, with medium containing five per cent fowl serum, and were refed every two days.

Co-cultivation: Trypsinised line '4' cells at 2.5 x 10^5 cells per ml and trypsinised chick fibroblasts at 5 x 10^5 cells per ml were held in growth medium for two hours at 4°C in an attempt to synchronise cell growth. The cultures were mixed in equal volumes and inoculated in 1 ml amounts into a number of coverslip culture tubes. Cultures of line '4' and chick fibroblast cells respectively were set up as controls.

Similar cultures were prepared from line '5' and PK(2D). The methods were identical except that line PK(2D) was used at 1 x 10^5 cells per ml because of its faster rate of growth compared with other lines. Duplicate cultures were also prepared using primary /
primary chicken kidney in place of chick fibroblasts.

Coverslip cultures were stained at two day intervals. Tissue culture fluids harvested at the fifth day were inoculated into the allantoic cavity of ten-day-old eggs and into day-old chickens by the intra-cerebral route.

**Co-cultivation plus 'Helper' Virus:** Co-cultures prepared with $1.5 \times 10^5$ line '4' cells per ml and $3 \times 10^5$ chick fibroblast cells per ml were allowed to monolayer for five days at $37^\circ$C. They were then inoculated with $0.2\text{ml}$ of $10^{-1} - 10^{-3}$ dilutions of Sendai virus and incubated at $37^\circ$C for one hour. After this period of adsorption, the co-cultures were fed with $0.8\text{ml}$ of growth medium, without removing the inoculum. Cultures of chick fibroblasts, chick fibroblasts plus MDBK, MDBK and line '4' cells infected with Sendai virus were included as controls.

After three days of incubation, the tissue culture fluids were removed from each group of cultures and tested for haemagglutinating activity and egg infectivity. The monolayers were also checked for haemadsorption.
Defective Line '4' Antigen: Blood was withdrawn from the wing veins of white Leghorn chickens, collected in sterile 3.8 per cent sodium citrate solution, lightly centrifuged and washed three times in sterile PBS. Each sample was then aseptically prepared as a one per cent suspension in tissue culture fluid harvested from line '4' cultures. Adsorption of the defective virus to fowl red cells was allowed to proceed for 2-3 hours at 4°C, or until all detectable haemagglutinin has disappeared from the fluid. The red blood cells were centrifuged, washed three times in sterile PBS and resuspended in sterile buffer as a 1:2 suspension.

Antibody Production in White Leghorns: These sensitised blood cells were inoculated intravenously into the same chicken from which they had been removed. Each bird received three injections of approximately 1000 HA units at four weekly intervals. The number of HA units in each inoculum was estimated as follows:

\[
\text{Titre of Line '4' fluid (0.25ml)} = 1:2
\]
\[
\therefore \text{No. of HA units per ml} = 8
\]

Therefore, one per cent fowl cells in 100ml of line '4' medium adsorbs 800 HA units.

Active Herts NDV Antigen: The method of preparing the virus antigen and the inoculation schedules employed were similar to those recommended by Fazekas de St. Groth, Webster and Davenport (1969) in their studies of the homologous antibody response.
response in rabbits to influenza viruses.

Virus from infected allantoic fluid was adsorbed on to one per cent human group 'O' cells at 4°C for thirty minutes, lightly centrifuged in chilled tubes and the supernatant fluid discarded. The agglutinated red blood corpuscles were washed twice in chilled PBS and the virus was eluted into one tenth of the original volume of PBS, at 37°C for thirty minutes. After centrifugation, to deposit the cells, the virus was harvested, and this constituted the purified antigen.

**Antibody Production in New Zealand White Rabbits:** The primary inoculation schedule consisted of three intravenous injections of 0.5ml antigen at two day intervals. Since the antibody titres at one, two and three weeks did not exceed 1:160 a fourth injection of 0.75ml of antigen was given intraperitoneally at week three and was followed two days later by a further 1ml, given by the intravenous route. This raised the antibody response to a titre of 1:2560.

**Herts NDV Nucleoprotein ('S') Antigen:** The viral envelope of the purified, concentrated NDV antigen was disrupted with Tween 80 and ether, according to the method used by Ramachandran (1970) for the preparation of measles virus haemagglutinin. The aqueous phase was freed from ether by gently bubbling nitrogen through the solution and the disrupted haemagglutinin was removed by four adsorptions with two per cent human group 'O' cells for fifteen minute periods at 4°C. After final adsorption the solution was centrifuged in chilled centrifuge tubes /
tubes to deposit the agglutinated cells and the resulting supernatant constituted the 'S' antigen. This antigen contained no haemagglutinating activity at a dilution of 1:10 and was stored at -70°C before use.

Antibody Production in New Zealand White Rabbits: In an attempt to elicit the maximum antibody response, the antigen used in the initial intramuscular injections was mixed with Freund's adjuvant. The initial intramuscular injection of 0.25ml of antigen was followed a fortnight later with a second intramuscular injection of 0.6ml of antigen. One week later 0.7ml was given intraperitoneally followed two days later by intravenous injections of 0.2ml and 0.3ml respectively, at two day intervals. The total inoculum was therefore of the order of 2ml. The rabbit was bled, from the ear vein, five days after the final inoculation.

The globulin fraction of the serum was precipitated by means of saturated ammonium sulphate solution and after dialysis against tap water and PBS, was concentrated by ultrafiltration.
INTRODUCTION

The three continuous lines of pig kidney (Stiles, 1962), porcine kidney (MOX) and avian kidney (MDXK) used in this present study were received in 1967 by courtesy of Doctor Radin, Berkeley, California (Fayed and Barry, 1967) and are designated lines P11K, MOX and MDXK, respectively.

The cultures were received in good condition and showed no visible abnormalities. However, in the autumn of 1962, line 11K showed mitotic syncytial formation and the absence of normal antipolyploid abnormalities were not present in the pig kidney line MOX in antipolyploid preparations. A derivative of the original strain, pig kidney culture, hereina referred to as MDXK, showed rounding of the cells at the edge of the monolayer to form small and stationary cultures which could be delayed until the second day by incubation in millipore drums and disappeared completely following treatment of the culture with tyrian. Further examination showed that all three continuous cell cultures have developed patent-ary syncytial and agglutinated tests and other species of red blood cell line that both effects were inhibited by antipolyploid antisera but not by antisera prepared against influenza A, B, or Rh adsorbed type 2 and 3 of antipolyploid (Freese, 1962).

It is widely known that monolayer and synchronous anti-
INTRODUCTION

The three continuous lines of pig kidney (Stice, PK2), bovine kidney (MDBK) and sheep kidney (MDOK) used in this present study were received in 1957 by courtesy of Doctor Madin, Berkeley, California (Madin and Darby, 1957) and are designated lines PK(2D), '4' and '5', respectively.

The cultures were received in good condition and showed no visible abnormalities. However, in the Autumn of 1962, lines '4' and '5' showed multiple syncytial formation and the presence of numerous eosinophilic inclusions. These abnormalities were not present in the pig kidney line nor in earlier stained coverslip preparations of the ox and sheep kidney cells. A derivative of the original Stice, pig kidney culture, herein referred to as PK(2D), showed rounding of the cells at the edge of the monolayer in four-day old stationary cultures which could be delayed until the seventh day by incubation in roller-drums and disappeared completely following treatment of the culture with Tylan. Further examination showed that all three continuous cell cultures haemadsorbed guinea-pig erythrocytes and agglutinated fowl and other species of red blood cells; and that both effects were inhibited by Newcastle disease antiserum but not by antisera prepared against influenza (A₂), parainfluenza types 1 and 3 or mumps viruses (Fraser, 1969).

It is widely known that mycoplasmas and myxoviruses may resemble /
resemble each other in size, morphology and filterability and may haemadsorb and haemagglutinate healthy erythrocytes. In view of this, it was necessary to examine the three continuous cell cultures for the presence of mycoplasmas and, if necessary, to eliminate persistent PPLO infection as a source of error.

A mycoplasma, which was identified as *Mycoplasma hominis* was isolated from all three cultures, by the culture technique of Hayflick (1965) but mycoplasmas were not isolated after treating the cultures with Tylan. Cell lines of pig kidney (PK/W/K6) and ox kidney (MDBK), which were obtained by normal purchase and were not persistently infected with NDV, were also treated with Tylan.
RESULTS

Haemadsorption:

The ability of the three continuous cultures of pig (PK(2D)), ox (line '4') and sheep (line '5') kidney cells to haemadsorb 0.05 per cent guinea-pig red blood cells was confirmed (Fig 1). Haemadsorption counts were carried out on coverslip preparations of 24 hour-old lightly seeded cultures. The coverslips were stained with haematoxylin and eosin and a total of 400-700 cells were counted to evaluate the proportion of affected cells in each culture (Fig 2). Cells showing no haemadsorption but exhibiting intracytoplasmic eosinophilic inclusions of the myxovirus type, were also counted. A third count, on duplicate cultures, was made using 0.5 per cent guinea-pig cells in place of the usual 0.05 per cent suspension. The results (Table 1) indicate that a large proportion of the cells in all three carrier cultures are infected. Counts on tests done with the more concentrated red cell suspension and counts of haemadsorption negative cells showing typical intracytoplasmic inclusions suggest that over 80 per cent of the cells are infected in each of the three continuous cultures.

The results also confirm that intracytoplasmic inclusions are not a feature of the carrier pig kidney culture, PK(2D).

Haemagglutination:

Haemagglutinins were detectable in tissue culture fluids from all three carrier cultures. These were present in minimal amounts /
Fig. 1. - Two-day old monolayer culture of pig kidney cell line, PK(2D), showing haemadsorption of guinea-pig red cells. x 300 (Haematoxylin eosin).

Fig. 2. - Lightly seeded PK(2D) culture showing haemadsorption of guinea-pig red cells. x 300 (Haematoxylin eosin).
amounts, seldom showing titres greater than 1 in 4 or 16 haem-
agglutinating units (HAU) per ml (Table 2). The pig kidney
culture PK(2D) showed a slightly higher yield of haemagglutinins
when refed on the third day of incubation and this is probably
related to the faster rate of growth of this cell line (Table 8B).
Agglutination tests were carried out with concentrated line '4'
haemagglutinin and the Herts 33 strain of Newcastle disease virus
(Herts NDV) grown in MDBK, against one per cent guinea-pig cells,
one per cent fowl cells and one per cent horse cells, respect-
ively (Table 3). The line '4' agent showed haemagglutination
with all three species of red cells but the reaction was four
times more sensitive with fowl and horse erythrocytes than with
guinea-pig red cells. Similarly, the reaction with the Herts NDV
strain was four times more sensitive with fowl and horse red
blood cells than with guinea-pig cells.

Haemagglutination Inhibition:

A standard dose (4HAU) of line '4' agent was tested against
NDV, Sendai and Influenza A_2 antisera. No inhibition was obtained
with the Sendai or influenza antisera but the NDV antiserum
showed inhibition at a dilution of 1:320. This titre was four-
fold lower than that obtained with a standard dose of Herts NDV
(Table 4).

Receptor Gradient:

A one per cent fowl red cell suspension, prepared from cells
agglutinated by Herts NDV at 4°C and subsequently eluted at 37°C,
failed to show agglutination, when tested, with either NDV or line '4' agent but was agglutinated, to titre, by influenza virus. An attempt to repeat the experiment in reverse, using fowl cells sensitised with line '4' agent, suggested that the defective virus might be deficient in neuraminidase, because spontaneous elution did not occur following incubation at 37°C (Table 5).

Fluorescent Antibody:

The direct staining technique was used on lightly seeded twenty-four hour-old coverslip cultures similar to those used for haemadsorption counts. Distinct foci of fluorescence were observed in the cytoplasm and in the perinuclear zones of affected cells (Fig 3). In line '4' cultures the fluorescent particles were variable in size but readily identifiable whereas in cultures of line '5' cells the foci varied from small, evenly dispersed particles to large plaques of antigen resembling the 'corona' described by Reda, Rott and Schäfer (1964) with NDV infected cells (Fig 4). The fluorescing foci in PK(2D) were generally tiny and more difficult to distinguish. Counts of fluorescing cells and counts on duplicate cultures grown in the presence of NDV immune serum to prevent transfer of the virus via the medium (Wheelock and Tamm, 1961) were very similar and were closely correlated with the haemadsorption counts (Tables 1 and 6).

Serum Inhibitors:

There is some evidence (Walker, 1964) that persistent virus infections might be under the control of antibodies or non-specific inhibitors /
Fig. 3. - Line 15 sheep kidney cell persistently infected with Newcastle disease virus stained by FITC conjugated rabbit immune serum to show discrete cytoplasmic foci of virus antigen.

x 3,600.

Fig. 4. - A similar experiment to Fig. 3.
To show perinuclear 'corona' of specific Newcastle disease virus fluorescence. Notice the absence of intranuclear staining.

x 3,600.
### TABLE 1

PERCENTAGE HAEMADSORPTION COUNTS ON CARRIER LINES AT 24hrs USING 0.05% AND 0.5% GUINEA PIG CELLS.

<table>
<thead>
<tr>
<th>Line</th>
<th>Haemadsorption Count 0.05% Cells</th>
<th>Haemadsorption Count 0.5% Cells</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>76% (80%)</td>
<td>86% (89%)</td>
<td>Sept. '69</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>-</td>
<td>Oct. '70</td>
</tr>
<tr>
<td>5</td>
<td>78% (90%)</td>
<td>85% (91%)</td>
<td>Sept. '69</td>
</tr>
<tr>
<td></td>
<td>88%</td>
<td>-</td>
<td>Oct. '70</td>
</tr>
<tr>
<td>PK(2D)</td>
<td>89% (89%)</td>
<td>99% (99%)</td>
<td>Sept. '69</td>
</tr>
<tr>
<td></td>
<td>90%</td>
<td>-</td>
<td>Oct. '70</td>
</tr>
</tbody>
</table>

( ) = Count inclusive of cells showing no haemadsorption but exhibiting intracytoplasmic inclusions of the myxovirus type by H & E
<table>
<thead>
<tr>
<th>Line</th>
<th>Haemagglutinating Units per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>'4'</td>
<td>0</td>
</tr>
<tr>
<td>'5'</td>
<td>0</td>
</tr>
<tr>
<td>PK(2D)</td>
<td>0</td>
</tr>
<tr>
<td>VIRUS</td>
<td>1/10</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
</tr>
<tr>
<td>Ultra-</td>
<td></td>
</tr>
<tr>
<td>filtered</td>
<td></td>
</tr>
<tr>
<td>Line '4' fluid</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Herts NDV</td>
<td>+</td>
</tr>
<tr>
<td>grown in</td>
<td></td>
</tr>
<tr>
<td>MDBK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ = Agglutination  - = No agglutination
<table>
<thead>
<tr>
<th>ANTISERUM</th>
<th>1/10</th>
<th>1/20</th>
<th>1/40</th>
<th>1/80</th>
<th>1/160</th>
<th>1/320</th>
<th>1/640</th>
<th>1/1280</th>
<th>1/2560</th>
<th>CONTROL</th>
<th>4HAU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herts</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>NDV</td>
</tr>
<tr>
<td>NDV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>'4'</td>
</tr>
<tr>
<td>Sendai</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Sendai</td>
</tr>
<tr>
<td>Influenza A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>'4'</td>
</tr>
</tbody>
</table>

Virus/antibody mixture held at room temperature for 15 minutes before addition of 1% fowl cells.

+ = Agglutination  - = No Agglutination.
<table>
<thead>
<tr>
<th>VIRUS</th>
<th>1/10</th>
<th>1/20</th>
<th>1/40</th>
<th>1/80</th>
<th>1/160</th>
<th>1/320</th>
<th>1/640</th>
<th>1/1280</th>
<th>1/2560</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>'4'</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl</td>
</tr>
<tr>
<td>NDV</td>
<td>+E</td>
<td>+E</td>
<td>+E</td>
<td>+E</td>
<td>+E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl</td>
</tr>
<tr>
<td>Influenza A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl</td>
</tr>
<tr>
<td>'4'</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl after treatment with NDV</td>
</tr>
<tr>
<td>NDV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl after treatment with NDV</td>
</tr>
<tr>
<td>Influenza A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1% Fowl after treatment with Line '4'</td>
</tr>
<tr>
<td>'4'</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1% Fowl after treatment with Line '4'</td>
</tr>
<tr>
<td>NDV</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1% Fowl after treatment with Line '4'</td>
</tr>
</tbody>
</table>

+ = Agglutination  
- = No Agglutination  
E = Elution after incubation at 37°C
<table>
<thead>
<tr>
<th>Line</th>
<th>No Immune Serum</th>
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<tbody>
<tr>
<td>S4</td>
<td>93%</td>
<td>88%</td>
<td>Oct. '70</td>
</tr>
<tr>
<td>'4'</td>
<td>94%</td>
<td>95%</td>
<td>Oct. '70</td>
</tr>
<tr>
<td>PK(2D)</td>
<td>85%</td>
<td></td>
<td>Oct. '70</td>
</tr>
</tbody>
</table>

**TABLE 6**

PERCENTAGE FLUORESCENT ANTIBODY COUNTS ON CARRIER LINES IN THE PRESENCE AND ABSENCE OF IMMUNE SERUM

Inhibitors in the series of the growth medium. In view of this, the batches of pooled inactivated cell sera were heated at a dilution of 1:10 by non-agglutination inhibition against E. coli No. 154. The sera were not treated with peroxide destroying enzymes (NAD) to remove non-specific inhibitors and no NAD-like antibody or non-specific inhibition was detected in any of the sera. The cell cultures were held in growth medium containing 20% fetal calf serum at 37°C. All cultures were subcultured at the end of this period. The fresh cultures were held at a temperature of 4°C, fed weekly and subcultured at fortnightly intervals, by the second subculture, hemadsorption, hemagglutination activity and intracytoplasmic inclusions had disappeared but returned 2-4 days after the cultures were returned to the 37°C incubator. By the fifth subculture, at a temperature of 4°C, the ability to hemadsorb and hemagglutinate red blood cells was restored and intracytoplasmic inclusions were formed despite the higher temperature of incubation.
inhibitors in the serum of the growth medium. In view of this, ten batches of pooled inactivated calf serum were tested at a dilution of 1:10 by haemagglutination inhibition against 4 HAU of NDV. The sera were not treated with receptor destroying enzyme (RDE) to remove non-specific inhibitors and no NDV-like antibody or non-specific inhibition was detected in any of the sera tested.

Attempts to Cure the Carrier Cultures:

Growth in Antiserum:

Line '4' cells were held, in growth medium containing 1:40 NDV immune serum (HI titre 1:1240), for four weeks at 37°C. The ability of the carrier culture to haemadsorb guinea-pig red blood cells disappeared within 24 hours in the presence of the immune serum but reappeared 24 hours after it was replaced with ten per cent normal calf serum.

Growth at High Temperature:

Cultures of line '4' were held at 41°C and were fed daily, for fourteen days. The cultures remained healthy and were subcultured at the end of this period. The fresh cultures were held at a temperature of 41°C, fed weekly and subcultured at fortnightly intervals. By the second subculture, haemadsorption, haemagglutinating activity and intracytoplasmic inclusions had disappeared but returned 2-4 days after the cultures were returned to the 37°C incubator. By the fifth subculture, at a temperature of 41°C, the ability to haemadsorb and haemagglutinate red blood cells was restored and intracytoplasmic inclusions were formed despite the higher temperature of incubation.
Superinfection of Carrier Cultures:

Control and carrier cultures of pig kidney and calf kidney, respectively, were infected with ten-fold dilutions of the Herts strain of NDV as indicated in Materials and Methods for the estimation of virus TCID$_{50}$ calculations. Virus activity was detected by cellular changes and haemagglutination. The results (Table 7) indicate resistance on the part of the carrier cultures to superinfection with NDV while control cultures show no such resistance. Sendai virus, a member of the same paramyxovirus group, was shown to be capable of infecting line '4' but not to the same extent as on healthy MDBK cultures (Table 15, Section 11).

Growth Rates:

Growth rates were carried out on carrier and control cultures both contaminated with and free from mycoplasma infection.

Haemadsorption counts were performed on PPLO positive and PPLO negative cultures as proof that no selection of cell population had taken place following the course of treatment with Tylan. The growth rates are shown in Tables 8A and 8B. The pH estimations show that cellular metabolism improved in the absence of PPLO infection and that pig kidney cultures were especially sensitive to infection with mycoplasmas since their growth rates increased 3-5 times in cultures freed from PPLO. The growth rates of the calf kidney cultures were relatively unaffected.
unaffected by the presence of PPLO but the sheep kidney culture benefited, to some extent, from treatment with Tylan. Growth rates of control and carrier cultures free from PPLO infection showed no significant differences (Table 8B).

Heat Inactivation:

The haemagglutinin in line '4' tissue culture fluid held at 56°C failed to agglutinate fowl cells after five minutes at this temperature whereas the NDV haemagglutinin continued to agglutinate fowl cells after twenty minutes (Table 9). The tests on the samples held at 41°C were carried out at weekly intervals and the line '4' haemagglutinin retained its agglutinating capacity over a four week period while the NDV haemagglutinin continued to function until the ninth week (Table 10).

Adsorption of Haemagglutinins:

The haemagglutinins of lentogenic strains of NDV are inactivated within 5 minutes at 56°C and are adsorbed by suspensions of chicken brain cells (Hanson, Spalatin, Estupinan and Schloer, 1966). The line '4' agent treated with ten per cent chick brain suspension failed to show agglutinating activity when tested against one per cent fowl cells. On the other hand, a Herts NDV sample was reduced in titre only one fold (Table 11).

Transformation:

It is known that persistent infections of cell cultures are capable of causing cell transformation and MacPherson and Montagnier (1964) developed an agar suspension culture technique for the selective assay of cells transformed by polyoma virus.

Examination /
### TABLE 7

GROWTH OF ACTIVE HERTS NDV IN CONTROL AND CARRIER CULTURES AT FOUR DAYS

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PK(2D)</th>
<th>PK/W/K6</th>
<th>(^<em>4^</em>)</th>
<th>MDBK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>CPE</td>
<td>HAU/ml</td>
<td>CPE</td>
<td>HAU/ml</td>
</tr>
<tr>
<td>10(^{-1})</td>
<td>-</td>
<td>12</td>
<td>+++</td>
<td>&gt;32</td>
</tr>
<tr>
<td>10(^{-2})</td>
<td>-</td>
<td>0</td>
<td>++</td>
<td>&gt;32</td>
</tr>
<tr>
<td>10(^{-3})</td>
<td>-</td>
<td>0</td>
<td>++</td>
<td>8</td>
</tr>
<tr>
<td>10(^{-4})</td>
<td>-</td>
<td>0</td>
<td>+</td>
<td>&lt;2</td>
</tr>
<tr>
<td>10(^{-5})</td>
<td>-</td>
<td>0</td>
<td>Tr</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

+++ = complete shattering       ++ = 50% CPE

+ = 25% CPE                   + = 10% CPE

Tr = Trace CPE
### TABLE 8

**GROWTH RATES OF CONTROL AND CARRIER CULTURES IN THE PRESENCE AND ABSENCE OF PPLO.**

<table>
<thead>
<tr>
<th>A</th>
<th>PPLO +ve CULTURES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
<td><strong>Average pH</strong></td>
</tr>
<tr>
<td>MDBK</td>
<td>7.2</td>
</tr>
<tr>
<td>'4'</td>
<td>6.95</td>
</tr>
<tr>
<td>PK/W/K6</td>
<td>7.2</td>
</tr>
<tr>
<td>PK(2D)</td>
<td>7.0</td>
</tr>
<tr>
<td>'5'</td>
<td>7.1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>B</th>
<th>PPLO -ve CULTURES</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Line</strong></td>
<td><strong>Average pH</strong></td>
</tr>
<tr>
<td>MDBK</td>
<td>6.95</td>
</tr>
<tr>
<td>'4'</td>
<td>6.85</td>
</tr>
<tr>
<td>PK/W/K6</td>
<td>6.8</td>
</tr>
<tr>
<td>PK(2D)</td>
<td>6.9</td>
</tr>
<tr>
<td>'5'</td>
<td>6.85</td>
</tr>
</tbody>
</table>
**TABLE 9**

EFFECT OF TEMPERATURE (56°C) ON HAEMAGGLUTININS OF HERTS NDV AND LINE '4' AGENT

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>Time (Minutes)</th>
<th>Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Line '4'</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Herts NDV</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**TABLE 10**

EFFECT OF TEMPERATURE (41°C) ON HAEMAGGLUTININS OF HERTS NDV AND LINE '4' AGENT

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>Time (Weeks)</th>
<th>Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Line '4'</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Herts NDV</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = Agglutination  - = No Agglutination  
Tr = Trace Agglutination
### TABLE 11

**ADSORPTION OF HERTS NDV AND LINE '4' HAEMAGGLUTININ BY A 10% CHICK BRAIN SUSPENSION**

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>1/10</th>
<th>1/20</th>
<th>1/40</th>
<th>1/80</th>
<th>1/160</th>
<th>1/320</th>
<th>1/640</th>
<th>1/1280</th>
<th>1/2560</th>
<th>CONTROL</th>
<th>TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herts NDV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5ml Virus +2ml Buffer</td>
<td></td>
</tr>
<tr>
<td>Line '4' Agent</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5ml Agent +2ml Buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5ml Agent +2ml 10% Chick Brain</td>
<td></td>
</tr>
</tbody>
</table>

+ = Agglutination  
- = No Agglutination
Examination of control and carrier pig kidney and calf kidney lines by this technique, showed some evidence of transformation in the carrier lines. The extent of transformation, as detected by morphologically distinct colonies of cells piled in disarray, was particularly striking in PK(2D) but evidence of transformation was also evident with line '4' (Figs 5 - 9).

Inhibition of Oxygen Uptake:

An experiment, unrelated to this present work, suggested that the pig kidney carrier culture, PK(2D), had a higher requirement for oxygen than the control culture, PK/W/K6, and it has been reported (Broadfoot, Walker, Paul, MacPherson and Stoker, 1964) that BHK21 cells transformed by polyoma virus showed a slightly higher oxygen uptake as measured by the Cartesian diver technique. The control and carrier pig kidney and calf kidney cultures were therefore tested to ascertain the effect of inhibition of oxygen uptake. Inhibition of oxygen uptake was achieved by incorporating low concentrations of sodium malonate in the medium (Ackermann, 1951). All the cell lines, with the exception of PK(2D), were unaffected at the concentrations of sodium malonate used. However, PK(2D), as had been suspected, showed a greater dependence on oxygen availability in that complete cellular destruction resulted at every concentration of sodium malonate (Fig 10).
Fig. 5. - Uninfected bovine kidney cell line cultured in sloppy agar. There is no evidence of cellular transformation. x 6.

Fig. 6. - Persistently infected bovine kidney cell line, Line '4', cultured in sloppy agar showing distinct colonies of cells piled in disarray. x 6.
Fig. 7. - Uninfected pig kidney cell line, PK/W/K6, grown in sloppy agar showing some signs of morphological conversion or transformation. x 6.

Fig. 8. - Infected pig kidney cell line, PK(2D), grown in sloppy agar showing extensive transformation. x 4.

Fig. 9. - Carrier culture of pig kidney cells, PK(2D), showing two colonies composed of transformed cells. x 6.
Fig. 10. - The effect of sodium malonate on healthy cell culture, PK/W/K6, and persistently infected cell culture, PK(2D).

There is no growth of the carrier cells in the presence of sodium malonate whereas the healthy cells are unaffected.
Effect of Sodium Malonate on PK(2D) and PK(W)K6

Top row: PK(2D)
Bottom row: PK(W)K6

C  0.03M  0.025M  0.02M  0.015M  0.01M
INTRODUCTION

The viral carrier state is a complex system that is frequently characterized by the release of minimal amounts of infectious virus. Although such infections are mainly associated with adenovirus or other adenoid forming viruses, there is a report by Ruskin (1962) of a cell system persistently infected with measles virus, but without the production of infectious virus.

Since preliminary observations suggested that the carrier state described in this present study was similar to that induced by Ruskin with measles virus, it was decided to investigate the infectious nature for the properties of the 'latent' MV.

RESULTS (Section II)

Attempts to Activate and Transmit the Defective Agent
INTRODUCTION

The viral carrier state is a complex system that is frequently characterised by the release of minimal amounts of infectious virus. Although such infections are mainly associated with myxoviruses or other membrane forming viruses, there is a report by Rustigian (1962) of a cell system persistently infected with measles virus, but without the production of infectious virus.

Since preliminary observations suggested that the carrier state described in this present study was similar to that induced by Rustigian with measles virus, it was decided to investigate the infectious nature and other properties of the 'latent' NDV.
RESULTS

Electron Microscopic Evidence of Virus Release:

Haemagglutinins were detectable, in minimal amounts, in the tissue culture fluid of all three carrier cultures. Since this was not sufficient evidence that a virus-like agent was being released, the agent, whose HA titre seldom exceeded 1:4, was concentrated and examined by electron microscopy. The methods used were those of ultracentrifugation and of adsorption and elution from fowl red cells. The Herts 33 strain of NDV was examined by similar methods.

Electron micrographs prepared from serum-free, line '4' tissue culture fluid by ultracentrifugation showed groups of virus-like particles morphologically similar to those of NDV (Fig 11). They differ, however, from the Herts strain of NDV which usually shows extraviral strands of RNP and a clearer fringe of outer projections on the surrounding envelope (Fig 12).

Since the line '4' agent did not elute at 37°C from agglutinated fowl red cells, preparations for electron microscopy were also obtained by treating the sensitised cells with RDE. The released particles were clarified at 6,000 RPM for thirty minutes and finally concentrated at 40,000 RPM for two hours.

The electron micrographs (Figs 13 and 14) confirm that the defective agent differs from typical NDV in that there is no clearly defined outer 'fringe' of radiating projections nor a distinct /
Fig. 11. - Electron micrograph of virions of 'defective' Newcastle disease virus produced by line '4' cells, showing the ill-defined fringe of haemagglutinin spikes and the apparent lack of an inner ribonucleoprotein component. The bar represents 100 nm (Negative staining).

Fig. 12. - Electron micrograph of a virion of the Herts 33 strain of Newcastle disease virus, showing the typical fringe of projections and the ribonucleoprotein component extruding from the virus particle (Negative staining).
distinct centre zone of internal RNP component. The electron micrograph (Fig 13) also shows a defective virus particle attached to a remnant of a 'ghost' red cell. It is stressed that there was no evidence in any of these preparations of typical 'herring-bone' strands of RNP extruding from the defective virions nor of a morphologically identifiable inner component.

Inoculation of Chick Embryo Fibroblast Cultures:

Because electron micrographs of the agent from line '4' indicated that the virus might be deficient in some respects, attempts were made to transmit the defective agent from the fluid phase of the carrier cultures to healthy susceptible cells.

Chick fibroblast cultures inoculated with tissue culture fluid from line '4' showed no cellular changes or haemagglutinating activity over a seven day period. However, weak haemadsorption was detected on the sixth day but this effect was not detectable on subsequent subculture. Further observation suggested that the cells showing haemadsorption were epithelial in origin and were probably introduced with the inoculum.

Egg Inoculation:

Allantoic and amniotic fluids from eggs inoculated with line '4' supernatants showed no haemagglutinating activity and pooled fluids from these eggs, passaged on five occasions, yielded negative results. Embryos harvested and examined at the third passage showed no dwarfing or other teratogenic effects.

Intracerebral Inoculation of Day-Old Chicks:

Day-old chicks inoculated intracerebrally with tissue culture fluid /
fluid from line '4' were observed over a seven day period. No
deaths occurred and the birds were sacrificed on the seventh day.
The brains were removed aseptically and tissue suspensions
inoculated intracerebrally into a second group of day-old chicks
produced no deaths during a further seven day period of
observation.

**Inoculation of Six-Month-Old Susceptible Chickens:**

Since there was no evidence of clinical symptoms of NDV in
day-old chicks inoculated with line '4' agent, it was decided to
study the virus response of older chickens to this infection.
Accordingly, a group of four six-month-old susceptible White
Leghorn chickens (B3, B13, B18 and B99) inoculated intravenously
with much larger doses of line '4' agent was observed over a
twelve week period. No symptoms of clinical illness developed
but their sera contained antibodies to both line '4' agent and the
Herts strain of NDV. In Figure 15, the haemagglutination inhibit-
ion antibody response (HI) was measured against 4HAU of line '4'
agent and Herts NDV and showed that higher titres were obtained
with the homologous antigen. Serum neutralizing antibodies (SN)
were estimated against 100 egg infectious dose 50 (EID₅₀) of the
Herts 33 strain of NDV and the results show a good neutralizing
antibody response in three chickens (B13, B18 and B99). Serum
from the fourth bird (B3) was not examined.

**Sephadex G-200 Fractionation of Line '4' Agent:**

In an attempt to separate complete from incomplete virus
particles, a sample of line '4' haemagglutinins (HA titre 1:160)
was /
Antibody Response of Young Susceptible Chickens to Line '4' Agent.
was passed through a Sephadex G-200 column and the individual fractions were collected and tested for haemagglutinating activity (HA) and egg infectivity. As a control, inactivated Herts NDV allantoic fluid, having the same titre as that of the line '4' sample, was used and the individual fractions were collected and compared with those of line '4' agent. The results (Table 12) show that the HA activity of both line '4' and NDV had developed by fraction 9. In the case of the Herts NDV sample the activity increased, particularly through fractions 13 - 30 with a peak between fractions 27 - 29. The effect did not continue beyond the 39th collection. Although the line '4' agent also showed initial HA activity at fraction 9, there was no definite peak and haemagglutinins were not detectable beyond fraction 30. Nor was the HA titre of line '4' agent (1:2) as great as that of NDV (1:8).

In order to test the various fractions for the presence of active virus, fractions 8 to 31 of the line '4' agent (i.e. below and beyond the points of detectable HA activity) were treated with antibiotics, held for one week at 4°C, and inoculated individually into the allantoic cavity of fertile eggs with negative results.

There was no evidence that the sodium azide, used in the Sephadex as a bactericidal agent, possessed antiviral activity, since aliquots of NDV suspended in the presence or absence of sodium azide (1:10,000) had similar infectivity titres when tested in the developing chick embryo.
Growth in Serum-Free Medium:

Since the above attempts to demonstrate the presence of infectious virus in cell-free tissue culture fluids proved unsuccessful, the possibility remained that antibodies or other antiviral substances in the calf serum might be responsible for maintaining the carrier state. It was decided, therefore, to transfer the three persistently infected cell lines to EYL medium without added serum. After four days of incubation at 37°C no difference was observed in the HA titres of the cultures grown in the presence or absence of ten per cent calf serum; and the supernatant fluids inoculated intra-allantoically in ten-day-old fertile eggs did not produce deaths of the embryos or detectable amounts of haemagglutinin.

Activation by Ultra-Violet Light (UV):

Attempts to activate the intracellular virus were also made by irradiating thin layers of carrier cell suspensions with UV light for periods of 15, 30 and 60 seconds respectively. There were no visible cellular changes in cultures exposed to UV Light for periods of 15 and 30 seconds and cells treated for 60 seconds failed to divide and grow. No differences were detected between the haemagglutination titres of untreated and irradiated carrier cultures after three days of incubation. Frozen and thawed cell suspensions of irradiated cultures failed to show evidence of viral activity when inoculated intra-allantoically in fertile eggs.

Neuraminidase Experiments:

The earlier demonstration that the line '4' agent failed to elute spontaneously from agglutinated fowl red cells and the
failure to demonstrate infectious virus in the carrier cell systems suggested that the persisting NDV might be deficient in viral neuraminidase. It was decided, therefore, to test suspensions of extracellular line '4' agent for neuraminidase activity, since this enzyme is believed to play an important role in the mechanisms of entry or release of myxoviruses.

**Neuraminidase Estimations:**

Estimations of viral neuraminidase were carried out on line '4' agent maintained in a serum-free medium and concentrated by ultrafiltration from a titre of 1:4 to 1:640. As a control, the Herts strain of NDV was grown in MDBK (NDV/MDBK) and was concentrated by the same procedure from 1:32 to 1:320. Tissue culture fluid from uninfected MDBK cells on serum-free medium was concentrated tenfold by ultrafiltration and served as a second control. Since the results, as shown in Table 13, indicated that the latent agent in line '4' was deficient in neuraminidase, a second series of samples was prepared to confirm this observation. In this experiment, line '4' agent was concentrated, as before, to a final titre of 1:160. The Herts 33 strain of NDV grown on MDBK was concentrated fivefold from 1:32 to 1:160 and was designated NDV/MDBK. As additional controls, Sendai virus grown on MDBK was concentrated fivefold from 1:32 to 1:160, and was designated Sendai/MDBK while a second sample grown on line '4' with an HA titre of 1:64 was concentrated by ultrafiltration to a titre of 1:160 and was designated Sendai/line '4'.

The /
The results of this second experiment (Table 14) indicate that the line '4' agent is deficient in neuraminidase activity. Although comparison of the results in Tables 13 and 14 show that the line '4' sample has a higher baseline reading (0.065) compared with those of the other samples, the reason for this difference is probably due to the greater concentration of fluid that was necessary to raise the HA titre of the line '4' sample to the desired level.

Receptor Destroying Enzyme as a Neuraminidase Source:
Since the defective virus seems to lack neuraminidase activity, an attempt was made to supply the missing enzyme in the form of receptor destroying enzyme (RDE).

Receptor destroying enzyme at the non-toxic dilution of 1:100 showed neuraminidase activity of 0.143 or + when tested by Aminoff's method.

Healthy chicken fibroblast cultures, previously washed in PBS to remove serum, were overlaid with 0.2ml amounts of RDE diluted 1:100 in cell-free line '4' tissue culture fluid. After adsorption, the cultures were washed twice with ten per cent serum growth medium and then refed with 1ml of growth medium. Cultures were examined daily and haemadsorption tests were performed at regular intervals. On the fourth day coverslip cultures stained with haematoxylin and eosin showed no haemadsorption and no intracytoplasmic inclusions. Coverslip preparations stained with fluorescein conjugated NDV antiserum showed no fluorescence. By the ninth day the cultures showed slight /
slight deterioration due to the action of the enzyme but there was no evidence of haemadsorption and no indication that RDE as an extracellular source of neuraminidase had assisted entry of the defective line '4' agent into the cell.

'Helper' Virus as a Source of Neuraminidase:

Although Sendai virus grew well in line '4' cultures at a high multiplicity of infection, the titres obtained with higher dilutions of virus compared less favourably with those on healthy MDBK (Table 15) due perhaps to a slight interference effect by the line '4' agent.

Sendai virus inoculated at a high multiplicity of infection into MEBK and line '4' cultures was harvested three days post infection. The HA titre of both samples was adjusted to 1:160 and the neuraminidase content was estimated by the method of Aminoff. The results (Table 14) showed not only that Sendai virus was an excellent source of neuraminidase but that it appeared to have a higher neuraminidase content (1:293) when grown in line '4' cells. This is not fully understood but it may be due to variations in the virus content of samples with similar HA titres or it may reflect the ability of different cell types to manufacture and accumulate certain specific viral proteins.

On the assumption that Sendai virus, as an intracellular source of neuraminidase or simply as a 'helper' virus of unknown action, might assist release of the latent NDV nucleoprotein in the form of Sendai-NDV heterozygotes, an experiment was performed on the Sendai harvests from line '4' to ascertain whether any NDV /
TABLE 13
NEURAMINIDASE ESTIMATIONS ON TISSUE CULTURE SAMPLES OF NERTS NDV AND LINE '4' AGENT

<table>
<thead>
<tr>
<th>Sample</th>
<th>H.A. Titre</th>
<th>Test Reading</th>
<th>Controls and Baseline Readings</th>
<th>Corrected Result</th>
<th>Relative Neuraminidase Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line '4' Agent</td>
<td>1:640</td>
<td>0.185</td>
<td>0.065</td>
<td>0.076</td>
<td>Trace or -ve</td>
</tr>
<tr>
<td>NDV/MDBK</td>
<td>1:320</td>
<td>0.915</td>
<td>0.000</td>
<td>0.871</td>
<td>++++</td>
</tr>
<tr>
<td>MDBK Control</td>
<td>-ve</td>
<td>0.044</td>
<td>0.002</td>
<td>0.000</td>
<td>-ve</td>
</tr>
<tr>
<td>Water Blank</td>
<td></td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Substrate HGP₅</td>
<td></td>
<td>0.044</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 14

NEURAMINIDASE ESTIMATIONS ON SAMPLES OF LINE '4' AGENT, HERTS NDV AND SENDAI VIRUS

<table>
<thead>
<tr>
<th>Sample</th>
<th>H.A. Titre</th>
<th>Test Reading</th>
<th>Controls and baseline Readings</th>
<th>Corrected Result</th>
<th>Relative Neuraminidase Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Line '4' Agent</td>
<td>1:160</td>
<td>0.144</td>
<td>0.065</td>
<td>0.032</td>
<td>-ve</td>
</tr>
<tr>
<td>NDV/MDBK</td>
<td>1:160</td>
<td>0.900</td>
<td>0.002</td>
<td>0.851</td>
<td>++++</td>
</tr>
<tr>
<td>Sendai/MDBK</td>
<td>1:160</td>
<td>1.159</td>
<td>0.014</td>
<td>1.098</td>
<td>++++</td>
</tr>
<tr>
<td>Sendai/Line '4'</td>
<td>1:160</td>
<td>1.350</td>
<td>0.010</td>
<td>1.293</td>
<td>+++++</td>
</tr>
<tr>
<td>MDBK Control</td>
<td>-ve</td>
<td>0.074</td>
<td>0.022</td>
<td>0.005</td>
<td>-ve</td>
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<tr>
<td>Water Blank</td>
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<td></td>
</tr>
<tr>
<td>Substrate HGP&lt;sub&gt;5&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>Sendai Dilution</td>
<td>MDBK</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td>----------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>1/2</td>
<td>1/4</td>
<td>1/8</td>
<td>1/16</td>
<td>1/32</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>$10^{-3}$</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>$10^{-4}$</td>
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<td>-</td>
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<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

+ = Haemagglutination  
- = No Haemagglutination  
Table represents H.A. titration on tissue culture fluids from 4 day old infected cultures. No CPE was recorded.
NDV nucleoprotein had been released in Sendai virus capsids during this mixed infection. Chick fibroblast coverslip cultures were overlaid with 0.2ml volumes of Sendai/line '4' and Sendai/MDBK respectively, for one hour at 37°C. After removal of the inoculum the cultures were refed with EYL containing two per cent normal calf serum. The cultures were examined daily for cytopathic changes and haemadsorption. Haemadsorption became evident on the second day but specific NDV fluorescence was not observed during an eight day observation period in the cultures inoculated with Sendai/line '4'. Many multinucleated cells were seen and these were attributed along with the haemadsorption to the action of Sendai virus, as control cultures inoculated with Sendai/MDBK produced similar effects.

Sendai/line '4' culture fluids inoculated into the allantoic cavity of ten-day-old eggs were harvested after four days of incubation and tested for the presence of haemagglutinins against one per cent fowl cells and one per cent horse cells. All fluids agglutinated fowl but not horse cells and, as the defective line '4' agent is capable of agglutinating both species of red blood cells it suggested that Sendai virus had failed to rescue the defective NDV.

**Infection of Chicken Macrophages:**

On the assumption that failure to transmit line '4' agent to other cells might be due to lack of neuraminidase and inability to penetrate host cells, it was decided to study the phagocytic action of chicken macrophages as a means of actively engulfing /
engulfing the latent agent.

Chicken macrophage cultures were prepared and inoculated with line '4' material as described previously (p.41). Four days after inoculation the cultures were tested for the presence of NDV by haemadsorption with guinea-pig red cells. Stained coverslip preparations showed occasional macrophages with several adherent guinea-pig cells (Fig 16). This was not considered to be due to erythrophagocytosis since none of the control macrophage cultures showed more than an occasional adherent or ingested red blood cell. These tests were performed with freshly taken guinea-pig erythrocytes as red cells stored for several days at 4°C were more readily phagocytosed by the chicken macrophages. Phagocytosis of effete red blood corpuscles by homologous and heterologous macrophages has been reported by other workers (Lee and Cooper, 1966). Tests performed two days later gave similar results, whereas lightly seeded chick fibroblast coverslip cultures treated with line '4' agent showed no haemadsorption. Material from the line '4' treated macrophages inoculated intracerebrally into day-old chicks failed to produce clinical signs of NDV infection over a two week observation period and the same material passaged twice in the allantoic sac of fertile eggs produced no evidence of virus growth. Immunofluorescence studies of coverslip cultures of line '4' treated macrophages were inconclusive due to the numerous yellow granules present in the cytoplasm of the macrophages.

In view of these negative findings further attempts were made to establish the nature of the haemadsorption phenomenon and its possible /
Fig. 16. - Chicken macrophages inoculated four days previously with line '4' agent showing haemadsorption of guinea-pig erythrocytes. X 500. (Giemsa).
possible relationship with NDV. Since spontaneous agglutination of chicken macrophages occurs in the presence of line '4' agent it seemed possible that the cytoplasm membrane lining the macrophage might be especially sensitive to the action of the defective virus. In order to test this hypothesis the following experiment was designed.

Two-day-old macrophage coverslip cultures, seeded at three times the usual concentration, were overlaid with 0.2ml volumes of pooled, filtered fluids from all three carrier cultures. The pooled fluid had an HA content of 16 HAU per ml. The cultures were left in contact with this material for one hour at 37°C and, after a gentle but thorough washing, were tested immediately for haemadsorption and then stained with haematoxylin-eosin. It was evident from the results of this experiment that the macrophage membrane was highly sensitive to line '4' agglutinin since eighty per cent of the macrophages had phagocytosed guinea-pig red cells (Fig 17). Numerous red blood cells were seen adherent to the macrophage membrane and some were also ingested into cytoplasmic vacuoles. In many instances no fewer than 10-20 guinea-pig red blood cells were engulfed by a single macrophage (Fig 18).

Lightly seeded chick fibroblast cultures, sensitised with line '4' agent in an identical manner, showed no haemadsorption. Control cultures of untreated macrophages showed only an occasional macrophage actively phagocytosing healthy guinea-pig red blood cells and very few contained more than five or six ingested erythrocytes. Phagocytic action by control macrophages involving these numbers of /
Fig. 17. - Culture of chicken macrophages sensitised with line '4' agent showing extensive haemadsorption and phagocytosis. 
\[ x \times 400. \] (Giemsa).

Fig. 18. - The phagocytic action of chick macrophages sensitised with line '4' agent to show the presence of several guinea-pig red blood cells within cytoplasmic vacuoles. 
\[ x \times 1,600. \] (Giemsa).
of red cells had not been encountered in the initial experiments. However, the original haemadsorption tests had been carried out after the macrophages had been under conditions of artificial culture for six days or more and it is probable that the phagocytosing activity of the macrophages diminished with age. Prior treatment of sensitised line '4' macrophages with 1:40 NDV antiserum reduced the extent of phagocytosis to that of the control cultures.

Co-Cultivation:

The work of Horta-Barbosa et al (1969) and other workers suggests that host cell incompetence might be a reason for failure to demonstrate active virus in carrier cells, and that co-cultivation techniques, which allow "latent" virus to gain entry into a susceptible cell, are useful in activating these persistent infections. In view of these observations attempts were made to co-cultivate carrier cells with primary chick fibroblasts or chick kidney cells.

Co-cultivation methods were used with all three carrier cultures and stained coverslip preparations showed that marked syncytial formation had occurred after forty-eight hours incubation. The number of syncytia in the co-culture increased with further incubation and eventually formed a large part of the monolayer (Figs 19 and 20). By the 5th-7th day nuclear inclusions appeared in the multinucleated cells and numerous intracytoplasmic inclusions were also evident (Figs 21 and 22). Giemsa stained coverslip preparations showed differential staining of the two types /
Fig. 19. - Mixed culture of chick fibroblasts and healthy MDBK cells after three days of incubation, showing fibroblastic and epithelial cells growing independently. x 80. (Haematoxylin eosin).

Fig. 20. - Co-culture of chick fibroblasts and line '4' cells showing numerous multinucleated syncytia. x 80. (Haematoxylin eosin).
types of nuclei involved and this suggested that the multiao:ucleate cells were heterokaryons produced by fusion of the
carrier cells and chick embryo cells (Figs 23, 24 and 25). Counts
of both species of nuclei involved in line '4'/chick fibroblast
heterokaryons are shown in Table 16. The results show that there
is a wide variation in the numbers of nuclei involved in each
heterokaryon (5-56) and in the percentage of chick fibroblast
nuclei incorporated in the multinucleated cells (12-88 per cent).
However, they also show that over fifty per cent of the hetero-
karyons (19/33) contain forty-sixty per cent of chick fibroblast
nuclei, which indicate a reasonable balance between the two species
of nuclei involved. In order to confirm that cell fusion is an
inrate quality of the carrier cultures, a second experiment was
carried out with mixed populations of MDBK and chick fibroblast
cells. At no time was cell fusion observed and the two cell types
continued to divide and grow well independently (Fig 19). Further
proof that cell fusion was associated with the defective NDV in
the carrier cells was obtained by growing mixed populations of
line '4' cells and chick fibroblasts in a medium containing 1:12
anti-NDV serum (HI titre 1:640). There was no evidence of cell
fusion by the third day of incubation but, twenty-four hours after
replacing the NDV antiserum with normal calf serum, numerous
multinucleated cells appeared (Figs 26 and 27).

There was no difference in the HA titres of the supernatant
fluids of the control and co-culture tubes. Tissue culture fluid
and cells from six-day-old mixed cultures of line '4' and chick
fibroblast /
<table>
<thead>
<tr>
<th>Chick (CF) Fibroblast Nuclei</th>
<th>Line '4' Nuclei</th>
<th>Total Nuclei</th>
<th>CF%</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>8</td>
<td>12%</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>8</td>
<td>25%</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>11</td>
<td>27%</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>11</td>
<td>36%</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>18</td>
<td>33%</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>10</td>
<td>40%</td>
</tr>
<tr>
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<td>35</td>
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</tr>
<tr>
<td>26</td>
<td>32</td>
<td>56</td>
<td>43%</td>
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</tr>
<tr>
<td>7</td>
<td>1</td>
<td>8</td>
<td>88%</td>
</tr>
</tbody>
</table>
Figs. 23 - 24. - Control monolayer cultures of line '4' cells and chicken fibroblasts.

Fig. 25. - Heterokaryon produced by line '4' and chicken fibroblast cells, showing differential staining of the nuclei by Giemsa.
Fig. 26. - Mixed culture of line '4' and chicken fibroblast cells grown in the presence of anti-NDV serum. The presence of the antiserum has prevented cell fusion taking place.

x 250. (Haematoxylin eosin).

Fig. 27. - The same experiment as in Fig. 26 after removal of the antiserum. There is evidence of heterokaryon formation 24 hours after removal of the antiserum.

x 200. (Haematoxylin eosin).
fibroblast co-cultures were inoculated intracerebrally in day-old chicks and intra-allantoically in ten-day-old fertile eggs; but no deaths occurred in either host system and no active NDV was recovered.

There are a number of reports that Newcastle disease virus has an intranuclear stage of development and Bukrinskaya, Burducea and Vorkunova (1966) showed by autoradiographic techniques that the ribonucleic acid (RNA) of NDV replicates in the nucleoli and migrates into the cytoplasm where it functions as a template for S and V antigen synthesis. It was decided, therefore, to investigate the specific nature of the intranuclear inclusions stimulated by co-cultivation. Coverslip cultures of nine-day-old co-cultures of line '4' and chick fibroblast cells, showing numerous multinucleates with intranuclear inclusions, were stained with acridine orange and examined by fluorescent microscopy. The mammalian line '4' nuclei presented a stippled appearance and the avian fibroblast nuclei showed more uniform staining. Both species of nuclei within the syncytia showed numerous structures resembling RNA containing inclusions (Fig 28) but the staining reaction was somewhat variable and the inclusions frequently stained a light khaki colour. Duplicate coverslip preparations stained with fluorescein conjugated hyperimmune NDV serum were difficult to evaluate since the multinucleated cells were friable and were readily detached from the coverslip. However, careful examination failed to reveal specific NDV fluorescence in the nuclei of the syncytia although extensive areas of fluorescence were observed in the /
Fig. 28. - Heterokaryon of line '4' and chick fibroblast cells stained with acridine orange. Notice that the avian nuclei contain multiple inclusions.

x 1,500.
the cytoplasm of the affected cells. The intensity of the staining reaction in the cytoplasmic material was less intense and more diffuse than that usually seen in younger carrier cultures. Since these inconclusive results might have been due to the insensitivity of the direct fluorescent antibody technique or to the fact that the intranuclear inclusion contained specific NDV nucleoprotein (S) antigen, the experiment was repeated by the indirect staining method and the serum used was prepared by inoculating rabbits with NDV nucleoprotein antigen. The results of this second experiment also failed to show specific fluorescence in the nuclei of the artificially induced heterokaryons although extensive diffuse fluorescence was observed in the cytoplasm.

Electron microscopic examination of co-cultures was attempted in order to study the ultrastructure of the intranuclear inclusions and the mechanisms of cell fusion. The araldite sandwich embedding method of Smith et al (1969) was preferred because it permits critical examination of adjacent cell surfaces. Unfortunately, however, it was necessary to process the monolayer preparations by the fifth day since the cell sheet became detached from the araldite surface after further incubation.

Examination of ultra-thin sections showed few multinucleated cells, none of which appeared to contain any definite intracytoplasmic or intranuclear inclusions (Fig 29). In other regions, the monolayer culture showed evidence of early cell fusion where the plasma membrane was characterised by diffuse thickening along the points of contiguity between the cells (Fig 30). In this preparation /
Fig. 29. - A multinucleated cell involving three nuclei. There is no evidence of inclusion material in this specimen. x 15,000.
Fig. 30. - Thin section of 5-day-old co-culture of line '4' and chicken fibroblast cells, showing intracytoplasmic inclusions. Note the early evidence of cell fusion as shown by thickening of the plasma membranes between some of the cells. x 6,000.
preparation a number of inclusions can be seen in the cytoplasm of the cells which appear to be undergoing fusion. In other preparations fusion of the cells was associated with the presence of numerous microvilli and the formation of cytoplasmic bridges (Fig 31) or at a later stage, with intermeshed microvilli (Fig 32). This electron micrograph also shows fusion between the cytoplasmic membranes of contiguous cells and, in areas where fusion is not complete, what appear to be virus particles are seen in the intercellular space. In an electron micrograph taken at higher magnification (Fig 33) rows of spherical, virus-like particles are seen accumulating between cell surfaces due, perhaps, to a process of budding from modified cell membranes. Stages of the development and maturation of the virus by the process of budding from microvilli (Figs 34 and 35) are similar to that described for other membrane-associated viruses (Morgan, Hsu and Rose, 1962; Duc-Ngugen, Rosenblum and Zeigel, 1966).

Detailed examination of the intracytoplasmic inclusion material, present in cells undergoing fusion, revealed a closely woven network of fine fibrillar tubules, somewhat similar to that reported by Nakai, Shand and Howatson (1969) with measles virus (Fig 36). However, there was little evidence of the typical nucleoprotein strands described by these workers. One of the few intranuclear inclusions observed in the preparations (Fig 37) was also examined in greater detail. These inclusions were generally smaller and more diffuse than intracytoplasmic inclusions, with an amorphous structure in no way resembling the tubule formation characteristic /
Fig. 31. - Electron micrograph of 5-day-old co-culture showing numerous microvilli between adjacent cells.

x 30,000.
Fig. 33. - The same experiment as Fig. 32, showing several virus-like particles in the intercellular space. x 60,000.
Figs. 34 - 35. - Surface microvilli showing some evidence of viral development. x 60,000.
Fig. 36. - Section of infected cell in a mixed culture of line '41' and chicken fibroblast cells, showing an intracytoplasmic inclusion composed of a closely woven network of fine fibrillar tubules. x 45,000.
characteristic of cytoplasmic inclusions.

Thus, little information is available regarding the nature of these inclusions and there is no evidence as to whether they are of viral aetiology or not.

**Co-Cultivation plus 'Helper' Virus:**

In a further effort to release infectious NDV from the carrier cells, Sendai virus was inoculated as a 'helper' virus into five-day-old co-cultures of CF + 4 showing signs of intranuclear activity. The sensitivity of this system for Sendai virus replication was compared with control cultures of CF, Line '4', MDBK and CF + MDBK, using haemadsorption, haemagglutination and egg infectivity estimations. The results (Table 17) showed that a $10^{-1}$ dilution resulted in the maximum recovery of Sendai virus in all systems and this dilution was selected as the optimum dilution for demonstrating whether the released Sendai virions were incorporating defective NDV nucleocapsid into the Sendai envelope in the form of heterozygotes. The virus demonstrated by egg inoculation of the $10^{-3}$ dilutions, in the absence of haemadsorption (Table 17), corresponded to the 'half-life' of this dose of Sendai virus, which was shown by Darlington et al. (1970) to be six hours at 37°C. Tissue culture fluids harvested three days post infection from CF + 4 and MDBK cultures respectively were inoculated intracerebrally into day-old chicks. All chicks behaved normally over a ten day observation period. However, it was not clear from this experiment whether in fact Sendai virus was capable of penetrating chicken brain cells. For this reason
a second experiment was designed to allow Sendai virus, grown in a CF + 4 co-culture system, to enter susceptible cells in which it could be demonstrated that all three stages of penetration, replication and assembly of virus took place. A normal growth cycle of this nature would thus result in NDV nucleocapsid, if present in the Sendai virion, being introduced into the cytoplasm of indicator cells. The cells chosen for susceptibility were chick embryo lung cells (CEL) as Darlington et al (1970) have shown that these cells are one hundred times more sensitive than chick embryo fibroblasts (CEF) to the growth of Sendai virus. Before this experiment was carried out the sensitivity of CEL cultures to the growth of NDV was ascertained. Although CPE in CEL cultures was less extensive than in CEF cultures, haemadsorption and haemagglutination indicated that both lines were equally sensitive and had a sensitivity similar to that obtained by egg inoculation (Table 18).

Having thus determined the sensitivity of CEL cultures for NDV replication, 0.2ml Sendai virus, harvested three days post infection from CF + 4 cultures, was overlayed for one hour at 37°C on coverslip preparations of these cultures. After the adsorption period the cultures were refed and fifty per cent of the cultures were fed with medium incorporating 1:20 Sendai antiserum (HI titre 1:320). Line '4' cultures, with and without antiserum were set up as controls. Five days post infection haemadsorption and fluorescent antibody tests were carried out on these cultures.

The /
The haemadsorption test demonstrated that the Sendai virus had penetrated and set up a replication cycle in the CEL cultures and an in ovo titration of the tissue culture fluid from these cells showed that multiplication had taken place. It was also demonstrated that the presence of 1:20 homologous antiserum neutralized this growth cycle (Table 19). Haemadsorption and fluorescent antibody tests with conjugated NDV antiserum on line '4' cultures grown in the presence of 1:20 Sendai antiserum showed that this dilution of antiserum had no effect on NDV haemadsorption or fluorescence (Table 19).

This being the case, the negative results obtained in attempts to demonstrate NDV haemadsorption and fluorescence in the CEL cultures, where release of Sendai virus was neutralized by the homologous antiserum, (Table 19) suggest that no NDV nucleocapsid had been introduced into the cultures in the form of heterozygotes.
<table>
<thead>
<tr>
<th>Tissue Culture System</th>
<th>10^{-1} Sendai</th>
<th>10^{-2} Sendai</th>
<th>10^{-3} Sendai</th>
<th>Infectivity of 10^{-1} T.C. for Day Old Chickens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H_{A_{*}} Titre</td>
<td>H_{ADS_{*}} In Ovo Infectivity</td>
<td>H_{A_{*}} Titre</td>
<td>H_{ADS_{*}} In Ovo Infectivity</td>
</tr>
<tr>
<td>'4'</td>
<td>1:8 +++</td>
<td>10^{-5}</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>MDBK</td>
<td>1:4 +++</td>
<td>50%</td>
<td>10^{-6}</td>
<td>-</td>
</tr>
<tr>
<td>CF</td>
<td>1:8 +++</td>
<td>10^{-5}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CF+4</td>
<td>1:4 +++</td>
<td>50%</td>
<td>10^{-7}</td>
<td>-</td>
</tr>
<tr>
<td>CF+MDBK</td>
<td>1:8 +++</td>
<td>50%</td>
<td>10^{-6}</td>
<td>-</td>
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H_{A_{*}}ADS = Haemadsorption

ND = NOT DONE
# TABLE 18

**GROWTH OF NDV (EID$_{50}$ $10^{-8.75}$) IN CHICK EMBRYO CELLS**

**RESULTS 4 DAYS POST INFECTION**

<table>
<thead>
<tr>
<th>Tissue Culture System</th>
<th>Test</th>
<th>$10^{-1}$</th>
<th>$10^{-2}$</th>
<th>$10^{-3}$</th>
<th>$10^{-4}$</th>
<th>$10^{-5}$</th>
<th>$10^{-6}$</th>
<th>$10^{-7}$</th>
<th>$10^{-8}$</th>
<th>$10^{-9}$</th>
<th>C</th>
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<tr>
<td>Chick</td>
<td>C CPE</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>Embryo</td>
<td>E HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>F H$<em>2$ADS$</em>*$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chick</td>
<td>C CPE</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Embryo</td>
<td>E HA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>33%</td>
<td>+</td>
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<tr>
<td>Lung</td>
<td>L H$<em>2$ADS$</em>*$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>33%</td>
<td>+</td>
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</tr>
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</table>

CPE = Cytopathogenic Effect:  HA = Haemagglutination:  H$_2$ADS$_*$ = Haemadsorption
<table>
<thead>
<tr>
<th>Tissue Culture System</th>
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<th>F•A⁺ (NDV-) (ANTISERUM)</th>
<th>In Ovo Infectivity</th>
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<tbody>
<tr>
<td>CEL</td>
<td>+</td>
<td>-</td>
<td>+ 10⁻⁵</td>
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<tr>
<td>CEL 1:20 Sendai antiserum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*¹⁴⁺</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>*¹⁴⁺ 1:20 Sendai antiserum</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

H•ADS⁺ = Haemadsorption, F•A⁺ = Fluorescent Antibody
ND = NOT DONE

TABLE 19
GROWTH OF SENDAI VIRUS
(3 DAY HARVEST CF + *¹⁴⁺ EID₅₀ 10⁻⁶)
IN CHICK EMBRYO LUNG CULTURES
RESULTS 5 DAYS POST INFECTION
The precise circumstances in which the rig was used were not known. The kidney cell cultures first became infected with the non-infecting agent and the non-infecting agent are not known nor, unfortunately, are there any means by which this can be determined. There seems little doubt from the evidence presented that the agent is, in fact, a strain of Newcastle disease virus and, since only one strain, the Harts 33 virus, has been maintained in this laboratory during the past ten years, it is possible that this was the source of the infective virus or that some other unknown strain of NDV accidentally contaminated the cultures during routine procedures. It is also interesting to note that the infection first occurred in the carrier lines soon after the Virus Unit was transferred to accommodation formerly occupied by the Diagnostic Rabies Department of this School.

The possibility that the infective agent was some other paramyxovirus or even a previously unknown virus was excluded by the fact that the typical intracytoplasmic inclusions in the carrier cultures were shown to fluoresce specifically with fluorescein conjugated hyperimmune NDV serum and the monoclonal antibodies produced by the carrier cultures were specifically inhibited by NDV antisera. Further evidence, that the agent was a strain of NDV, was obtained from the appropriate position on the Receptor Gradient (Barnes, McCrea and Itano, 1946). Not only do these features suggest that the infective agent is NDV.
The precise circumstances in which the pig, ox and sheep kidney cell cultures first became infected with the haemadsorbing agent are not known nor, unfortunately, are there any means by which this can be ascertained. There seems little doubt from the evidence presented in this thesis that the agent is, in fact, a strain of Newcastle disease virus and, since only one strain, the Herts 33 virus, has been maintained in this laboratory during the past ten years, it is possible either that this was the source of the defective virus or that some other unknown strain of NDV accidentally contaminated the cultures during routine maintenance procedures. It is also interesting to note that the infection first occurred in the carrier lines soon after the Virus Unit was transferred to accommodation formerly occupied by the Diagnostic Poultry Department of this School.

The possibility that the defective agent was some other paramyxovirus or even a previously unknown virus was excluded by the fact that the typical intracytoplasmic inclusions in the carrier cultures were shown to fluoresce specifically with fluorescein conjugated hyperimmune NDV serum and the haemagglutinins produced by the carrier cultures were specifically inhibited by NDV antiserum. Further evidence, that the agent was a strain of NDV, was obtained from its approximate position on the Receptor Gradient (Burnet, McCrea and Stone, 1946). Not only do these features suggest that the defective agent is NDV /
NDV but its ability to agglutinate horse red blood corpuscles, the sensitivity of its haemagglutinins to a temperature of 56°C, its adsorption by chick brain cells and its inability to produce clinical symptoms when inoculated intracerebrally in day-old-chicks, indicate that the defective agent has most of the properties of a lentogenic strain of NDV. These criteria for classifying lentogenic strains of NDV were first proposed by Hanson, Spalatin, Estupinan and Schloer (1967) and are now generally accepted by most workers in this field.

One of the most interesting features of the present study was the absence of infectious virus in the carrier cultures despite the fact that the majority of the cells in the monolayers appeared to be infected when examined by haemadsorption and fluorescent techniques.

Attempts to cure the persistently infected cultures, by growing them at an elevated temperature or by treating them with specific antiserum, failed to cure the infection and the affected cells continued to divide and grow in the presence of the agent. Since further evidence showed that the carrier cultures could not be superinfected with the homologous virus and as antibody or other antiviral factors were not necessary in maintaining the state of equilibrium, it seems likely that the carrier cultures are similar to Walker's (1964) type of 'regulated infection'. The present carrier cultures are of particular interest because they failed to release even minimal amounts /
amounts of infectious virus and are, therefore, rare examples of a latent, membrane-associated RNA virus carrier state in which failure to demonstrate infectious virus has been recorded. The uniqueness of this present system is underlined by the fact that it was not developed in the presence of immune serum, as was the case in the measles-cell system described by Rustigian (1962).

Although biological experiments adequately confirmed the absence of infectious virus particles in the supernatant culture fluids, electron microscopy of negatively stained, tissue culture fluids showed not only that morphologically identifiable particles were present in the extracellular environment but that they differed from normal NDV particles in that they lacked a well defined 'fringe', the central core of ribonucleoprotein was indistinct and the preparations did not contain extruded 'herring-bone' nucleoprotein strands which are usually present in specimens of paramyxoviruses.

The viral 'fringe' of spikes on the envelope of myxoviruses has long been assumed to be associated with the haemagglutinating and neuraminidase activities of these viruses and this has recently been confirmed by Laver and Valentine (1969). The Sephadex G-200 fractionation of line '4' agent indicated a deficiency in the haemagglutinating activity of the various fractions as compared with the Herts NDV fractions and the morphology of the defective line '4' agent, together with its inability to elute from agglutinated fowl erythrocytes, suggested /
suggested that the latent virus might also be deficient in neuraminidase. Chemical tests using the thiobarbituric acid assay method of Aminoff (1959) confirmed the absence of this enzyme.

The functional significance of neuraminidase is not well understood but its absence from the viral envelope may partly explain how the defective agent is unable to enter and replicate in susceptible cells. Unfortunately, this hypothesis could not be confirmed since experiments designed to supply either an extracellular or intracellular source of neuraminidase, in the form of receptor destroying enzyme or Sendai virus, yielded negative results. Moreover, it seems unlikely that neuraminidase is essential for successful entry of the virus into susceptible cells because antiserum prepared against the defective virus, and thus containing no neuraminidase antibody, was no less efficient than a standard NDV antiserum in protecting chick embryos infected with 100EID\textsubscript{50} of virulent Herts NDV.

The neutralization titre of the serum prepared against the neuraminidase deficient virus was approximately fifteen times higher than the haemagglutination inhibiting (HI) titre obtained against 4HAU Herts NDV, whereas an immune anti-NDV serum showed a neutralizing titre only four times greater than the HI titre. It was also of interest that the SN titre induced by line '4' agent was only four times greater than the HI titre when tested against 4HAU of the homologous line '4' agent. But, as Fazekas /
Fazekas de St. Groth (1962) has pointed out, such differences could be accounted for by the quality as well as the quantity of antibody in the serum. Allowing for such differences in antibody avidity it is possible that the lack of neuraminidase antibody is in no way detrimental to the protective quality of the serum. These observations are in agreement with the findings of other workers (Seto and Rott, 1966; Webster and Laver, 1967; Brown and Laver, 1968) who showed that antibody directed against neuraminidase neither inhibited haemagglutination nor neutralized the infectivity of influenza virus.

However, these workers successfully demonstrated that neuraminidase antibody had an adverse effect on the release of virus from infected cells and Seto and Rott (1966) suggested that the functional significance of neuraminidase might be to facilitate release of myxoviruses from host cells. This suggestion is given additional support by the work of Laver and his colleagues who have shown in recombination experiments with different strains of influenza virus that reactivation of inactivated parent virus was accompanied by transfer of neuraminidase activity from the active parent virus (Laver and Kilbourne, 1966; Easterday, Laver, Pereira and Schild, 1969). It seems likely, therefore, that viral-coded neuraminidase may be more closely involved in the mechanisms of viral release than in the process of cellular entry.

In this present study negative results were obtained in experiments with Sendai virus as a 'helper' virus and as a source of /
of intracellular neuraminidase despite the fact that Sendai has a similar base composition to NDV (Blair and Robinson, 1968). Although it is well known (Granoff, 1959) that recombination does not occur with NDV the experiment was attempted because NDV is capable of forming complementing heterozygotes with other strains of NDV (Granoff, 1959; Dahlberg and Simon, 1969).

Because all attempts to demonstrate the presence of even small numbers of infective particles by various in vitro and in vivo methods proved unsuccessful, an attempt was made to introduce the defective particles, or portions of the defective virus, into susceptible cells by the technique of co-cultivation.

Fusion between the carrier cultures and healthy chicken fibroblasts readily occurred in the absence of inactivated Sendai virus due, presumably to alterations of the plasma membranes of the carrier cells by the persisting virus. The heterokaryons produced in this way contained large numbers of avian and mammalian nuclei, as demonstrated by Giemsa and acridine orange staining methods. The formation of these heterokaryons was specifically inhibited by NDV antiserum, which clearly proved that cell fusion resulted from changes of the cell membrane associated with the latent Newcastle disease virus.

Suspensions of co-cultivated cells containing large numbers of heterokaryons did not produce infectious virus by in vitro or in vivo methods. An interesting feature of these heterokaryons was the presence not only of many well defined intracytoplasmic inclusions but also of numerous, deeply staining, intranuclear /
intranuclear inclusions.

The nature of these intranuclear inclusions was investigated further since it was thought that their presence might indicate enhanced activity of the latent agent or perhaps the transfer of the agent to a new species of host cell. There has been much debate over the years as to whether nuclear involvement occurs in the growth cycle of Newcastle disease virus and a nuclear stage of development has been reported by a number of workers using a variety of techniques, including autoradiography and immunofluorescence (Bukrinskaya, Burducea and Vorkunova, 1966; Johnson and Scott, 1964). However, other workers are of the opinion that the growth cycle of NDV is confined to the cytoplasm (Barry, Ives and Cruikshank, 1962; Wheelock, 1963; Reda et al, 1964). In this present study intranuclear inclusions could not be demonstrated by specific immunofluorescent staining although some evidence of nuclear activity was obtained by the presence of diffuse khaki-coloured inclusions in the nuclei of heterokaryons stained with acridine orange. These intranuclear structures were not shown to be specific for NDV but it is possible that their presence might represent an excess of cellular nucleic acids produced under the conditions of co-cultivation. This suggestion is not unreasonable since it has been reported that hen erythrocytes, introduced into the cytoplasm of human or mouse macrophages by inactivated Sendai virus, may develop nucleoli, in the previously dormant nucleated cells, and that synthesis of specific hen antigens is restored after a few days /
days in the presence of the active macrophage nuclei (Harris, Sidebottom, Grace and Bramwell, 1969; Harris, 1970). Further evidence that the intranuclear activity may be cellular rather than viral in origin was obtained from electron micrographs of the intranuclear inclusions. These showed that the inclusion material was amorphous in appearance, and unlike the fibrillar cytoplasmic inclusion material seen in the carrier cells, which closely resembled that described by Yunis and Donnelly (1969) in chicken cells infected with NDV. The amorphous nuclear material was also distinct from the fibrillar nucleoprotein material described in the nuclei of cells infected with measles virus by Nakai, Shand and Howatson (1969), and may correspond to the nuclear activity seen in the artificially induced heterokaryons stained with acridine orange. The fact that intranuclear inclusions were seldom found in carrier cultures is in agreement with the findings of Butler (1960) and Brandt (1961) who noted the absence of intranuclear inclusions in a variety of cell lines infected with NDV. Since it was not possible to confirm that the intranuclear inclusion material had been induced by co-cultivation, attempts were made, using the procedure of Harris and Watkins (1965), to stimulate the formation of intranuclear inclusions by co-cultivating healthy MDBK cells and primary chick fibroblasts in the presence of inactivated Sendai virus. However, the results of this experiment showed that cell fusion did not take place with mixed populations of healthy cells even in the presence of inactivated Sendai virus. This observation
was not unexpected since Poste (1970), who obtained similar findings, suggested that the phenomenon of cell fusion is probably a variable reaction.

The production of multinucleated heterokaryons by mixed populations of carrier cells and chicken fibroblasts is of interest and studies of electron micrographs suggest that cell fusion occurs in a manner similar to that described by Hosaka and Koshi (1968) with Ehrlich ascites tumour cells infected with active Sendai virus. These workers suggested that cytoplasmic bridges were formed at the area of contact between adjacent cells in the neighbourhood of the adsorbed Sendai virions; and that this was quickly followed by dissolution of the contiguous membranes. The cell fusing capabilities of the carrier cultures are also of interest in that the phenomenon appears to support the suggestion that an enzymatic reaction involving neuraminidase is not an essential mechanism of cell fusion. Although this does not agree with the theory advanced by Ho Yun-de (1962) and Zhdanov and Bukrinskaya (1962) of an enzyme action in the formation of syncytia by myxoviruses, it is interesting to note that Harris (1970) did not consider that neuraminidase was necessary for cell fusion.

The increased phagocytic activity of macrophages sensitised by the defective agent is possibly a further manifestation of cell fusion but the relationship between the process of cell fusion by virions and phagocytosis is still unknown (Hosaka and Koshi, 1968). The studies by Harris et al (1969) on cell fusion of /
of hen erythrocytes with mammalian macrophages suggest that the phenomenon is one of phagocytosis, followed by dissolution of the erythrocyte cytoplasm and incorporation of the avian nucleus into the foreign macrophage. A similar type of fusion appears to have taken place between the chick macrophages sensitised with defective NDV and the guinea-pig erythrocytes used for haemadsorption, and it is likely that these erythrocytes, being anucleated, would have been removed from the chick macrophage cytoplasm by enzymatic action had incubation at 37°C been prolonged. Phagocytosis by chick macrophages of erythrocytes sensitised with line '4' agent was the first indication that defective, non-infectious virus had been introduced into an independant host cell system.

The relationship between defective latent viral infections and oncogenesis is now well known and many of the causal viruses are capable of inducing transformation of the affected cells. Earlier studies of chick embryo cell cultures infected with Rous sarcoma virus revealed that persistently infected cells underwent cellular change including transformation (Temin and Rubin, 1958) as did mouse or hamster embryo cells infected with polyoma virus (Vogt and Dulbecco, 1960). The present carrier cell cultures, which are persistently infected with a defective Newcastle disease virus, show varying degrees of transformation as demonstrated by their growth characteristics under sloppy agar (MacPherson and Montagnier, 1964). This observation is of interest in view of a recent report that RNA oncogenic viruses possess /
possess an RNA-dependant DNA polymerase (Baltimore, 1970; Temin and Mizutani, 1970) which supports the provirus concept proposed by Temin (1964). Although the results of a more recent experiment suggest that NDV does not possess this polymerase (Spiegelman, Burny, Das, Keydar, Schom, Travnicek and Watson, 1970), it is possible that the defective NDV may utilise a host cell enzyme to accomplish the same function. The possibility that the latent NDV virus persists in a provirus state is supported by the fact that prolonged growth at 41°C failed to eradicate the infection. Since it has been demonstrated by Di Gioia, Licciardello, Nickerson and Goldblith (1970) that degradation of NDV ribonucleic acid occurs rapidly at 43.3°C, it is reasonable to suppose that loss of infectivity would have resulted from prolonged incubation at 41°C had the latent virus not been protected in an integrated state with the cell genome.
The present study did not reveal the reasons for failure of the carrier cultures to produce active virus. Electron micrographs indicated that the particles released from the carrier lines were probably non-infectious virions as the electron dense central core suggested the absence of a nucleo-protein component. The inability of the carrier cultures to release even minimal amounts of infectious virus is of particular interest since it is one of the few latent RNA membrane-associated virus carrier states, produced in the absence of an immune serum, in which there is complete failure to produce infectious virus.

It is not known whether the absence of the protein component, neuraminidase, from the viral capsid is due to lack of the required gene or simply to its malfunction or masking on the viral genome, but the deficiency might well be an important contributory factor to the non-infectious nature of the virus. In the field of viral oncogenesis, carrier cultures of this type are useful models of a system in which a defective viral genome is capable of inducing cellular transformation without the release of infectious virus. If the virus exists in a provirus state, the detection of an enzyme which is capable of forming complementary DNA strands to the viral RNA genome or, attempts to excise the viral genome from the integrated state, using chemicals /
chemicals such as 5'-bromo-2'-deoxyuridine (Dubbs, Kit, De Torres and Anken, 1967) might prove rewarding.
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